MECHANISTIC REGULATION AND NUTRIENT MODULATION OF SKELETAL MUSCLE PROTEIN TURNOVER: FROM CELLS TO HUMANS

By

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ABSTRACT

Skeletal muscle plays a key role in healthy ageing. Skeletal muscle mass is controlled by net protein balance, the difference between muscle protein synthesis and muscle protein breakdown rates, which is regulated by nutrient availability and contractile activity. This thesis identifies and addresses the knowledge gaps in the literature, if filled, may advance the knowledge to regulate skeletal muscle mass. For healthy older adults, it is continuously discussed that dietary protein recommendation should be increased to counteract age-related loss of muscle mass, sarcopenia. Herein, our evidence does not support the growing hypothesis when individuals already adhere to the current internationally recommended dietary protein intake level established by WHO/FAO/UNU. To increase muscle protein synthesis, Cluster Dextrin carbohydrate was co-ingested with meat protein hydrolysate after an acute bout of a whole-body resistance exercise in moderately trained younger individuals. Co-ingestion of Cluster Dextrin activates mTORC1 signalling pathway, but it does not increase postprandial amino acid availability or myofibrillar protein synthesis rate. While muscle protein synthesis and its signaling mechanisms are well established, our knowledge of muscle protein breakdown and its regulatory molecular mechanisms remains in its infancy. The final experimental chapter in this thesis shows that muscle-specific ubiquitin E3 ligases Atrogin-1 and MuRF1 protein, makers of skeletal muscle atrophy, are regulated by different mechanisms in the rapamycin-sensitive mTOR-S6K1-dependent signalling pathway in C2C12 myotubes. Altogether, this thesis improves the understanding of mechanistic regulation and nutrient modulation of skeletal muscle protein turnover and mass.

KEYWORDS: sarcopenia, skeletal muscle mass, dietary protein, mTORC1

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AUTHORS DECLARATION

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that thesis is of my own composition. This thesis has not, in whole or part, been previously presented for a higher degree. Work other than my own was clearly indicated in the text by reference to the relevant researchers or publications.

Declaration of experimental work presented in this thesis but carried out without my involvement:

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E-binding protein
aBW	adjusted body weight
Akt	protein kinase B
AMPK	adenosine monophosphate activated protein kinase
ANOVA	analysis of variance
ASMI	appendicular skeletal muscle index
BCAA	branched-chain amino acid
BMI	body mass index
BMR	basal metabolic rate
BV	biological value
BW	body weight
cAMP	cyclic adenosine monophosphate
CDX	Cluster Dextrin
CT	computed tomography
CV	coefficient of variation
D_2O	deuterium oxide
DIAAS	Digestible Indispensable Amino Acid Score
DUB	deubiquitylating enzyme
DXA	dual-energy X-ray absorptiometry
EAA	essential amino acid
eEF2	eukaryotic elongation factor 2
eEF2K	eukaryotic elongation factor 2 kinase
EI	energy intake

eIF4E	eukaryotic translation initiation factor 4E
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
FBR	fractional breakdown rate
FFM	fat free mass
FoxO	forkhead box
FSR	fractional synthesis rate
GC-C-IRMS	gas chromatography combustion isotope ratio mass spectrometry
GDP	guanosine diphosphate
GLC	glucose
GTP	guanosine triphosphate
iAUC	incremental area under the curve
IGF-1	insulin-like growth factor-1
IRS	insulin receptor substrate
IU	international unit
LBM	lean body mass
LC-MS/MS	liquid chromatography tandem mass-spectrometer
LKB1	liver kinase B1
MET	metabolic equivalent of tasks
MPB	muscle protein breakdown
MPE	mole percent excess
MPS	muscle protein synthesis
mTOR	mechanistic target of rapamycin
MVC	maximal voluntary isometric contraction

NAP	N-acetyl-propyl
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PDE	phosphodiesterase
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PTC	phenylthiocarbamyl
PVDF	polyvinylidene fluoride
RDA	Recommended Dietary Allowance
REE	resting energy expenditure
Rheb	Ras homolog enriched in brain
RM	repetition maximum
rps6	ribosomal protein S6
S6K	the ribosomal protein S6 kinase
SDS-PAGE	sodium dodecyl sulfate– polyacrylamide gel electrophoresis
TA	transamination
TBS-T	Tris-buffered saline Tween-20
TCA cycle	tricarboxylic acid cycle
T_{max}	time to reach maximum concentration
TSC	tuberous sclerosis
TTR	tracer to tracee ratio
UNU	United Nations University
UPS	the ubiquitin proteasome system
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Portions of the general introduction of this thesis have been utilized in review articles published in the peer-reviewed journals under Creative Commons Attribution 4.0 International,

Nishimura, Y., Højfeldt, G., Breen, L., Tetens, I., and Holm, L. (2021). Nutrition Research Reviews.

Nishimura, Y., Musa, I., Holm, L., and Lai, YC. (2021). American Journal of Physiology-Cell Physiology.

1.1 Introduction

1.1.1 The importance of skeletal muscle mass – with a focus on healthy ageing

Skeletal muscle has a critical role in whole-body metabolism given that it accounts for 40% of the total body mass (1). For example, skeletal muscle is the primary site for glucose uptake and skeletal muscle regulates blood glucose concentrations by supplying amino acids via gluconeogenesis in the post-absorptive state (2). Hence, greater maintenance of skeletal muscle mass is associated with the reduced risks of the onset of metabolic diseases, such as type II diabetes and obesity (3). Further, the body cannot store amino acids as an inactive form unlike glucose/glycogen. Thus, skeletal muscle practically plays an essential role as a reservoir of amino acids. Despite the significant advances in the field of skeletal muscle protein turnover in physiology, our understanding of the factors and signalling pathways responsible for controlling skeletal muscle mass still grows exponentially.

Maintenance of skeletal muscle mass is an important factor for healthy ageing. Importantly, maintenance of greater muscle mass and strength is associated with lower mortality (4-7), and lower skeletal muscle mass prior to hospitalization predicts a higher risk of falls and mortality following discharge (8). Alarmingly, progressive population ageing has been recognized as a widespread global phenomenon. The proportion of people aged 60 or over is predicted to be ～22% of the population across the world by 2050 (9, 10). Ageing is associated with involuntary loss of muscle mass, which is originally termed sarcopenia (11-13). The average prevalence of sarcopenia has been reported to be 5-13% in older people aged 60-70 years old and 11-60% over the age of 80, indicating the progressive increase of the prevalence of sarcopenia over the course

of ageing (14). The development of sarcopenia leads to a decline in physical function and disability (15, 16), an increase in the risk of falls (17, 18), fractures (19), which ultimately result in a lower quality of life in older individuals. Despite the accumulated evidence that has shown an association of sarcopenia with multiple negative health consequences, it has only recently been acknowledged by World Health Organization's International Classification of Disease as an independent condition (20). However, sarcopenia does not appear to be evident until the later life stage (i.e., the fourth or fifth decade of life) due to a gradual reduction in muscle mass, which is approximately 1-2 % of muscle loss per year (21). Conversely, the rates of decline in muscle strength are faster than muscle mass (22), which is approximately 2-4 % per year (22, 23). This discrete response between muscle mass and muscle strength could be explained by the age-associated muscle morphological changes. A decline in both the number of fibres and cross-sectional area have been reported with ageing, which has predominantly been observed in type II fibres (24). Therefore, developing strategies to preserve skeletal muscle throughout lifespan are important to enhance both longevity and quality of life.

1.1.2 Regulation of skeletal muscle by protein turnover

Skeletal muscle is a highly plastic and adaptive organ. From a cellular perspective, skeletal muscle protein turnover is a key mechanism for modulating skeletal muscle mass (25-28). Skeletal muscle mass is modulated by various physiological factors, such as hormones, nutrient and energy availability and contractile activity/physical activity (29, 30). The constant kinetics of protein synthesis and breakdown of body proteins were originally demonstrated by Schoenheimer and colleagues in 1939 (31) and were termed protein turnover, which is a modifiable and adaptable process. Thus, any

changes in skeletal muscle mass are dependent on acute and chronic net balance of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) rates (i.e., MPS minus MPB) (32). Therefore, achievement of positive net balance (i.e., anabolic state) is theoretically necessary to increase muscle mass (i.e., hypertrophy), whereas negative net balance (i.e., catabolic state) leads to loss of muscle mass (i.e., atrophy). Indeed, resistance exercise in combination with dietary protein intake is the potent stimuli to induce anabolism and increase skeletal muscle mass (33, 34). In contrast, physical inactivity leads to catabolism and loss of skeletal muscle mass (35, 36). Since the magnitude of MPS response to resistance exercise stimulus is greater than that of MPB (37), this led to building the hypothesis that MPS is a key driver for skeletal muscle adaptation to resistance exercise training (33, 38). Thus, many studies have aimed to manipulate MPS by performing dietary protein and/or exercise interventions to augment skeletal muscle mass. However, given that a post-resistance exercise response of MPS and MPB is strongly associated (27), it is important to highlight that both MPS and MPB are likely important for skeletal muscle adaptation (39).

In addition to the regulation of skeletal muscle mass, protein turnover is likely important to repair and remodel damaged and/or misfolded protein in skeletal muscle, and thereby maintaining protein quality control (39). The process of MPS and MPB is highly associated (27), which is required to maintain protein quality control. For example, breakdown of misfolded proteins during the process of new protein synthesis is essential (40). Furthermore, protein breakdown determines the half-life of protein, and it is required in skeletal muscle to replace damaged protein to remodel sarcomeric proteins because of changes in skeletal muscle activity. MPB is also a way to provide

energy substrates for the cell under the condition of catabolic state (e.g., energy stress) (41), and thereby maintaining internal homeostasis. Finally, an increase in MPB provides amino acids in the circulation, which are used by other tissues as precursors for protein synthesis in post-absorptive state or oxidized in the case of essential amino acids (EAAs) (41, 42).

Although we still do not fully understand the mechanisms, previous studies indicated that protein turnover rate decreased under ageing and/or physical inactivity conditions (35, 36, 43), resulting in accumulated misfolded/damaged proteins and impaired protein quality control (44, 45). In contrast, exercise promotes protein turnover (26-28, 43), which facilitates the removal of misfolded/damaged proteins, thereby improving protein quality control in skeletal muscle (46). Thus, in addition to the measurement of protein turnover kinetics, understanding signalling events that regulate protein turnover may provide therapeutic targets for healthy ageing (**Figure 1.1**).

Therefore, the following introductory paragraphs focus on MPS, MPB, and their intracellular signalling mechanisms regulated by exercise and dietary protein intake where relevant, as the modifications of MPS and MPB are the fundamental drivers for skeletal muscles mass. Then, the roles of dietary protein intake on the maintenance of skeletal muscle mass in healthy older adults are discussed. Finally, the knowledge gaps in the current literature are identified, and the specific objectives of the thesis and each Chapter are presented.

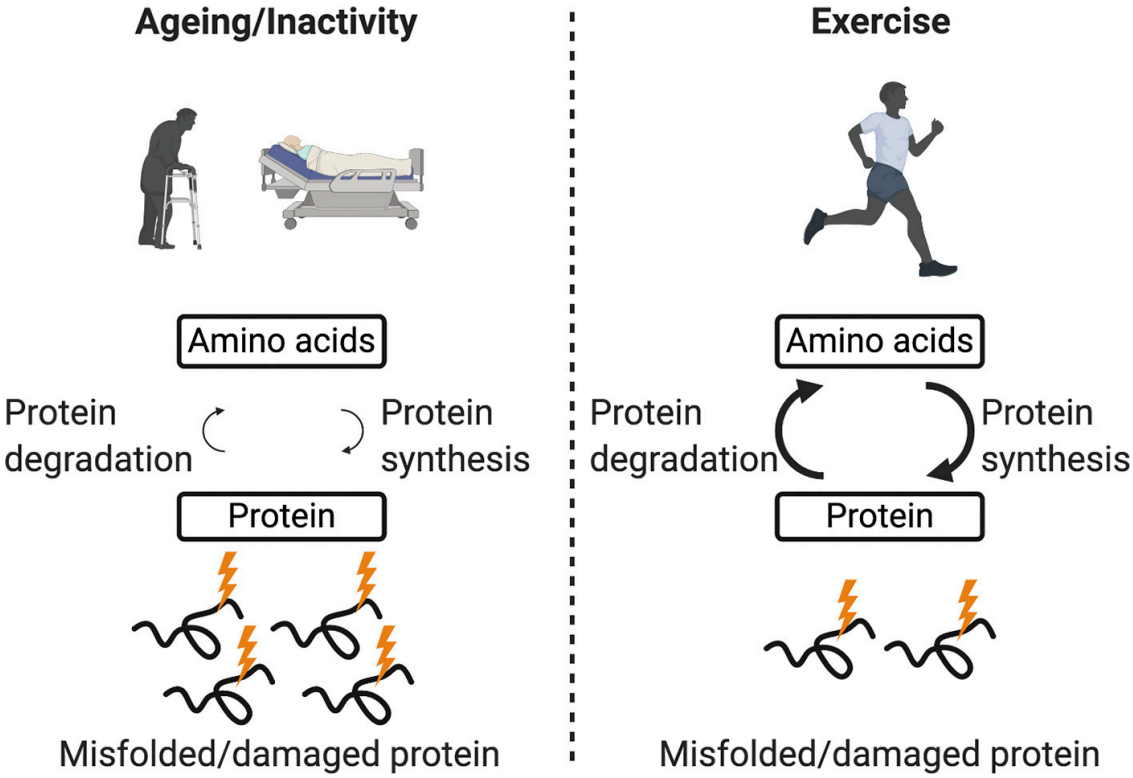

Figure 1.1 Proposed concept of the maintenance of protein turnover and protein quality control. Ageing and inactivity result in an inevitable decline in protein turnover rate. This leads to the accumulation of damaged and misfolded proteins due to the inability to remove damaged and misfolded proteins through protein degradation. In contrast, exercise is known to facilitate protein turnover through the increased rates of both protein synthesis and degradation, thus better clearance of damaged and misfolded protein. Understanding how exercise regulates rates of protein turnover and signalling events will advance our knowledge on how exercise improves health.

1.2 Protein synthesis and its molecular regulations in skeletal muscle

It is now clear that intricate signalling pathways regulate the size of skeletal muscle. Importantly, there is often a cross-talk between different signalling pathways, meaning that protein synthesis and protein breakdown are simultaneously controlled at different levels of signalling mechanisms.

1.2.1 Akt/mTOR signalling pathway and the regulation of skeletal muscle growth

Insulin and insulin-like growth factor-1 (IGF-1) and amino acid have been well known to activate the Akt/mTORC1 signalling pathway and increase protein synthesis, leading to skeletal muscle hypertrophy (47, 48) (**Figure 1.2**). Many studies have shown that stimulation of insulin/IGF1 and amino acid-mediated activation of the Akt/mTORC1 pathway is critical for skeletal muscle growth.

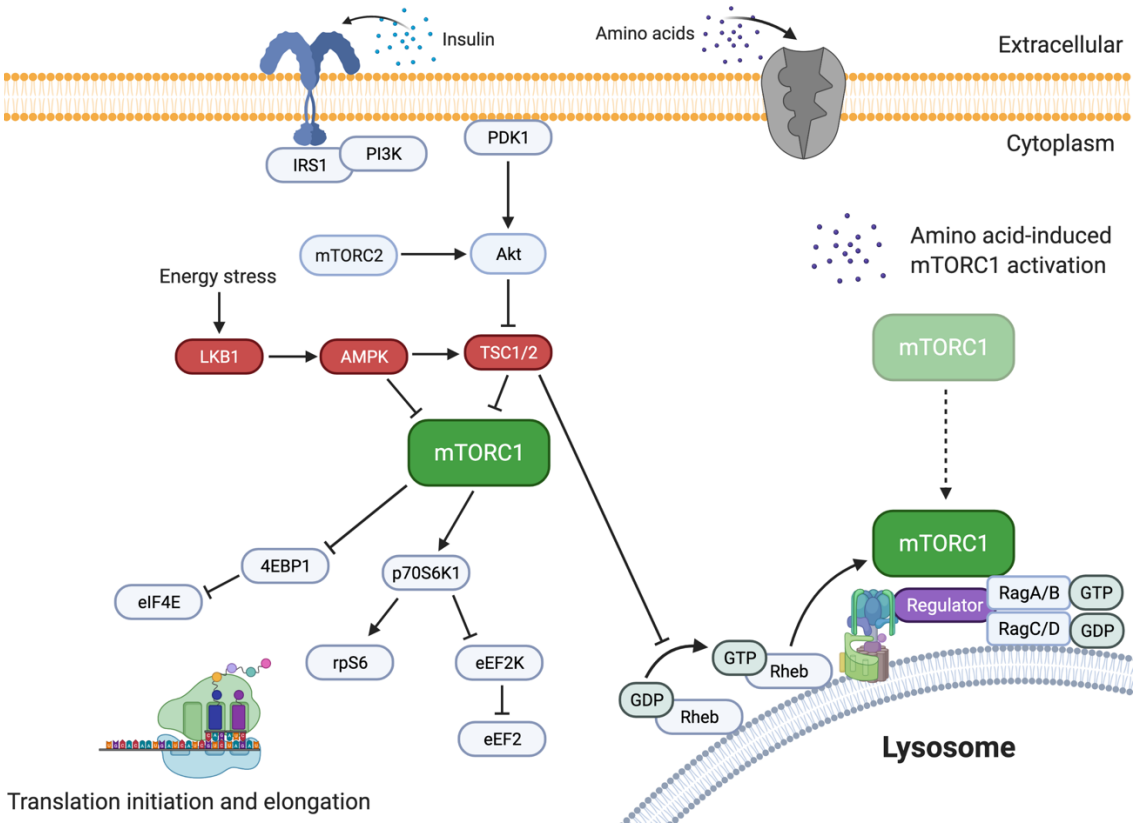

Figure 1.2 Insulin/IGF-1 and amino acid-mediated activation of Akt/mTORC1 increases protein synthesis.

Upon the binding of insulin or IGF-1 to their respective receptors, IRS1 is activated and PI3K is recruited. These signalling events trigger the phosphorylation of Akt by PDK1. Akt then activates the downstream targets of mTORC1 and S6K1, leading to increased protein synthesis. Amino acids facilitate the formation of the active configuration of the Rag GTPase complex at the lysosome. When amino acids are available, a guanine exchange factor acts as regulator for RagA/B, which increases the conversion of RagA/B•GDP to RagA/B•GTP. Amino acids also increase RagC/D•GDP to rise active Rag complex. mTORC1 binds to the Rag complex and is translocated to the lysosome and activated. Downstream of insulin/IGF-1 signaling, GTP-bound Rheb activates mTORC1. IGF-1, insulin-like growth factor 1; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol-3-kinase; PDK1, phosphoinositide-dependent kinase-1; LKB1, liver kinase B1; AMPK, 5' adenosine monophosphate-activated protein kinase; TSC, tuberous sclerosis; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; eIF4E, eukaryotic translation initiation factor 4E; eEF2K, eukaryotic elongation factor 2 kinase; eEF2, eukaryotic elongation factor 2; rpS6, ribosomal protein S6; Rheb, Ras homolog enriched in brain; GDP, guanosine diphosphate; GTP, guanosine triphosphate. Red shape indicates an upstream negative regulator of mTORC1.

1.2.1.1 Akt

Akt/mTORC1 signalling pathway has been implicated in the regulation of cell growth and more specifically anabolism in skeletal muscle in mammals. Akt is a central node of insulin action (30, 49, 50). Upon the binding to the insulin receptor, insulin triggers trans autophosphorylation and activation of insulin receptor substrate (IRS) proteins. IRS proteins recruit phosphoinositide-3-kinase (PI3K), which phosphorylates PIP2 to PIP3, leading to the recruitment of Akt to the plasma membrane. At the membrane, Akt is phosphorylated by at least two different kinases, PDK1 and mTOR-rictor complex (also known as mTORC2) on different amino acid residues, leading to

the activation of Akt kinase activity. The role of Akt in skeletal muscle growth was demonstrated by Bodine et al. (51) and Pallafacchina et al. (52). These studies confirmed that the overexpression of the constitutively activated form of Akt is sufficient to induce skeletal muscle growth. The same finding was also reported by generating conditional transgenic mice, in which Akt is expressed only after a treatment of tamoxifen (53) or doxycycline (54). Overall, Akt is a major regulator of skeletal muscle growth.

1.2.1.2 mTOR

One of the major downstream signalling pathways of Akt is mechanistic target of rapamycin complex (mTOR) (55). mTOR is a highly conserved serine/threonine protein kinase, which is a master regulator of cell growth and metabolism (**Figure 1.3a**) (56). mTOR is sensitive to environmental cues, such as growth factors, oxygen, nutrients, and energy availability (56-58). mTOR is part of two distinctive multiprotein kinase complexes. mTORC1 (**Figure 1.3b**) and mTORC2 (**Figure 1.3c**) are characterized by the key unique components Raptor and Rictor, respectively (59). mTORC1 is sensitive to rapamycin. Rapamycin binds to members of the FK binding protein (FKBP) family and the complex of rapamycin/FKBP binds to FRB domain of mTOR and inhibits the mTOR activity (**Figure 1.3a**) (59). In contrast, mTORC2 is not directly inhibited by rapamycin though mTORC2 is also known to be inhibited by longer exposure to rapamycin (60).

mTOR1 plays a critical role in skeletal muscle growth. The activation of mTOR1 by Akt is mediated through phosphorylation and inhibition of tuberous sclerosis 2 (TSC2)

(50). TSC2 works together with TSC1 as GTPase-activating protein towards Ras homolog enriched in brain (Rheb) (61). Akt-mediated inhibition of TSC2 activity allows to accumulate Rheb in its active GTP-bound form, resulting in the activation of mTORC1. Transgenic mice overexpressed with TSC1 showed atrophic phenotype in mice (62). In contrast, activation of mTORC1 via short-term TSC1 knockdown induced skeletal muscle growth in mice (62). Consistent with TSC1 knockdown, overexpression of Rheb, a direct mTORC1 activator (61), was shown to increase mTORC1 activity and induce skeletal muscle hypertrophy in mice (63).

mTORC1 is also involved in regulating protein breakdown. The regulation of protein breakdown via autophagy lysosome system and the ubiquitin proteasome system (UPS) is another critical role of mTORC1 to induce skeletal muscle growth (64, 65). Under a nutrients unavailable condition, mTORC1 activates both autophagy lysosome system and UPS to increase protein breakdown and provide energy substrates to cells (59, 66). In contrast, both autophagy lysosome system and UPS are suppressed when nutrients are available (59, 66). Altogether, these findings supported that mTORC1 plays an important role in skeletal muscle growth.

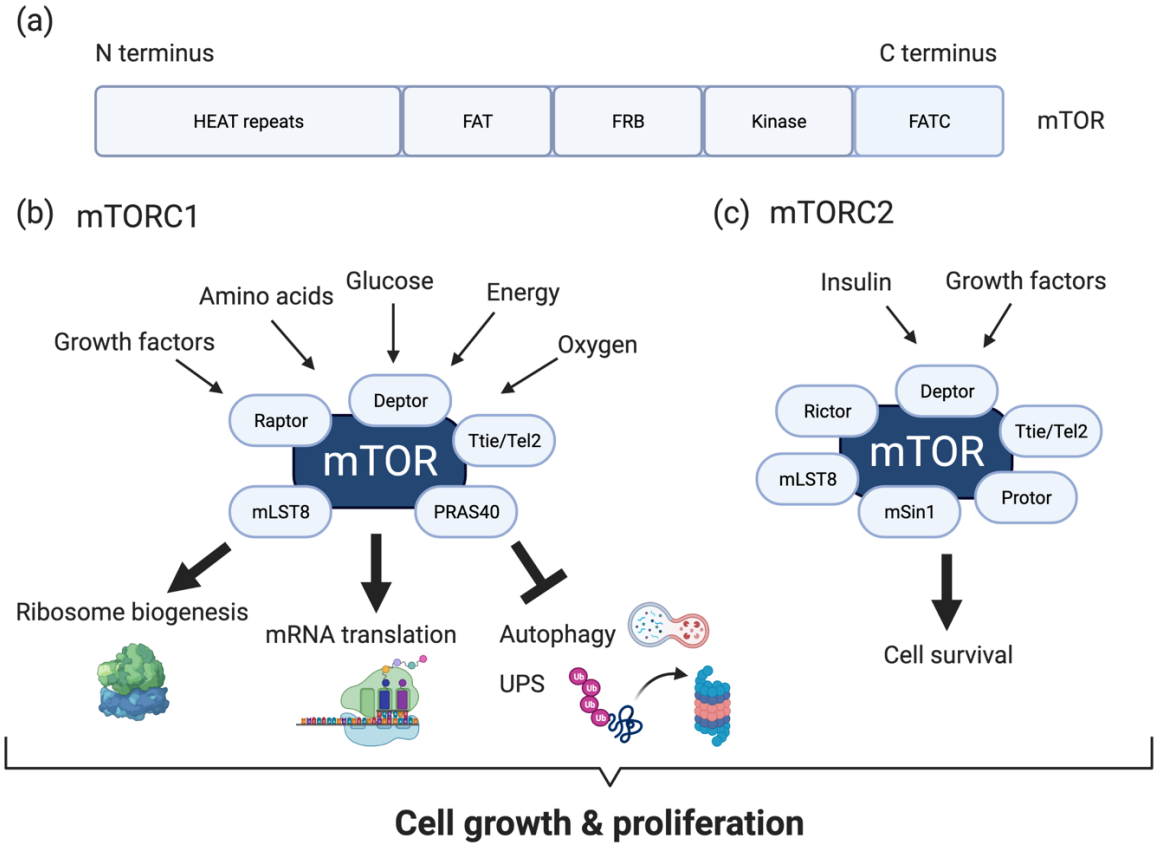

Figure 1.3 mTOR as a master regulator of cell growth.

Mammalian target of rapamycin (mTOR) complex1 (mTORC1) and mTORC2 are composed of similar, but distinct components. (a) both mTORC1 and mTORC2 have mTOR, which contains multiple domains. At the N terminus of mTOR, there are repeats of HEAT (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1), which mediates protein-protein interactions. These are followed by a FRAP, ATM and TRRAP (FAT) domain; the FKBP12-rapamycin binding (FRB) domain, where the FKBP12-rapamycin complex binds to induce the inhibitory action of mTOR; the Ser/Thr kinase catalytic domain; and the FATC domain at the C terminus. (b) mTORC1 is comprised of Raptor, Deptor, Ttie/Tel2, mLST8, and PRAS40. mTORC1 integrates external factors to control cell growth and proliferation via ribosome biogenesis, mRNA translation, autophagy, and the ubiquitin proteasome system (UPS). (c) mTORC2 is comprised of Rictor, Deptor, Ttie/Tel2, mLST8, mSin1, and Protor. mTORC2 integrates insulin and growth factors to regulate cell survival.

1.2.1.3 S6K

The ribosomal protein S6 kinases (S6K1 and S6K2), serine/threonine kinases, are a downstream signalling pathway of mTORC1 that transduces anabolic signal (30, 50). mTORC1-mediated phosphorylation of S6K1 is critical in activating the translation machinery and protein synthesis. The importance of S6K1 in skeletal muscle growth is evident by the loss of function studies. For example, the deletion of S6K1 reduced the body weight approximately by 20% as compared to wild type mice (67) and decreased muscle cross sectional area (68). In contrast, constitutively active S6K1 increased myotube diameter in C2C12 myotubes (47). These findings support the importance of S6K1 in skeletal muscle growth.

1.2.2 Skeletal muscle protein synthesis in response to acute resistance exercise

Resistance exercise is a form of exercise that exercising muscles are contracted against an external weight or force. This kind of exercise has been shown to increase skeletal muscle size (69-71) and strength (72, 73) in humans. Resistance exercise is by far the most potent intervention strategy to induce skeletal muscle protein anabolism. It is well accepted that such phenotypical and functional adaptations are due in part to an increase in the rates of protein synthesis following a bout of resistance exercise (33, 38). For example, evidence has shown that the rates of mixed muscle protein synthesis are elevated over the basal condition for up to 24-48 hours following an acute bout of resistance exercise (27, 74).

1.2.3 Activation of mTORC1 signalling in response to acute resistance exercise

Mechanical stress by skeletal muscle contraction activates mTORC1 signalling pathway, resulting in the upregulation of translation initiation and elongation. In a pioneering study exploring molecular signalling events in muscle hypertrophy in 1999, Baar and Esser et al. (75) identified a strong correlation between the mTORC1 signalling and an increase in muscle mass following a period of resistance training in rats. This study inspired a substantial growth in skeletal muscle research, which generated extensive findings on the molecular mechanisms and key signalling pathways for muscle protein synthesis (76, 77). In support of this notion, Drummond et al. (78) and Gundermann et al. (79) demonstrated that resistance exercise-induced increase in the rates of mixed muscle protein synthesis is inhibited by rapamycin (an mTORC1 inhibitor) administration in healthy younger adults. Moreover, You et al. (80) generated skeletal muscle-specific and tamoxifen-inducible raptor knockout mice to eliminate mTORC1 signalling and showed that mTORC1 is necessarily to induce mechanical load-induced hypertrophy. Overall, these studies demonstrated the importance of mTORC1 signalling in resistance exercise-mediated increase in MPS and subsequent skeletal muscle hypertrophy.

More recently, a rapamycin-insensitive mTORC1-dependent or mTORC1-independent mechanisms are suggested to regulate the mechanical load-induced increase in MPS. In a rat model, rapamycin inhibited MPS at 1 h following percutaneous electrical stimulation, but not at 3 h and 6 h. Interestingly, the increase in MPS was inhibited by the active site mTOR kinase inhibitor, AZD8055 in all time points (1, 3, and 6 h) following percutaneous electrical stimulation. Furthermore, You et al. (80) showed that

skeletal muscle-specific and tamoxifen-inducible raptor knockout mice did not impair MPS measured by puromycin incorporation in response to mechanical loading as compared to wild type. These results suggest that either a mTORC1-dependent, but not rapamycin-insensitive or mTORC2-dependent mechanisms are responsible for MPS regulation.

Resistance exercise training enhances the transcription of genes and accretion of proteins within skeletal muscle (81, 82). These processes in turn elicit an increase in MPS following the acute bout of resistance exercise. However, Greenhaff et al. (83) reported that there is a discordance between intracellular signalling events and acute MPS rates. Neinast et al. (84)also showed that metabolic flux cannot be predicted by intracellular signalling events. Furthermore, Phillips (32) discussed that the changes in MPS rates are dependent on the availability of substrates (i.e., amino acids). Thus, skeletal muscle contraction itself can alter mTORC1 signalling, but substrates availability (amino acids) might be the key determinant for MPS.

1.2.4 Skeletal muscle protein synthesis after acute post-resistance exercise protein feeding

Resistance exercise enhances the sensitivity of skeletal muscle to amino acid/protein feeding to increase MPS up to 24 h (85) and likely 48 h (86). Although an acute bout of resistance exercise stimulates MPS, MPB is increased greater than MPS (27, 28), creating a negative protein balance. However, ingestion of protein intake following resistance exercise can result in a positive protein balance and net protein accretion in skeletal muscle (87). Importantly, resistance exercise and amino acids/protein feeding

have a synergistic effect on MPS (88). Thus, protein ingestion following resistance exercise is important for skeletal muscle adaptations (34).

The dose of protein is one factor that can influence MPS response. Studies demonstrated that the ingestion of 20 g of high-quality protein maximally stimulates MPS following resistance exercise both in younger (89) and older (90) healthy individuals. The ingestion of protein greater than 20 g is likely oxidized at whole-body metabolism (91), which indicates that the excess amount of protein might not be utilized for tissue remodelling. It is also important to highlight that a unilateral leg study design is often used when measuring MPS in response to resistance exercise and/or protein nutrition. Thus, an argument could be made that the amount of protein maximally increases MPS following an acute bout of whole-body resistance exercise might be greater. Consistent with this notion, Macnaughton et al. (92) suggested that 40 g of protein ingestion might be required when whole-body resistance exercise is performed in younger individuals, which indicates that post-exercise protein feeding might be dependent on the amount of muscle recruited during exercise.

In addition to the importance of protein dose, Trommelen et al. (93) discussed several factors that influence protein feeding-induced MPS response, which includes digestion and absorption kinetics. Since the greater peak of aminoacidemia (94) is associated with an increase in the rate of muscle protein synthesis, quick absorption and rise in amino acid availability in the circulation is important to increase muscle protein synthesis (94, 95). Digestion and absorption are known to be impacted by protein sources. For example, whey and soy protein are digested quickly, which rapidly provides amino

acids in the circulation (95, 96). Accordingly, whey and soy protein induce rapid, but a transient increase in MPS as compared to casein protein (95, 96). Thus, manipulating amino acid availability from orally consumed protein can be an intervention target to augment MPS.

1.3 Protein breakdown and its molecular regulations in skeletal muscle

1.3.1 The role of the ubiquitin proteasome system in skeletal muscle

Skeletal muscle protein breakdown is mediated by multiple pathways, including UPS (97), autophagy lysosomal (98), calpain (99), and caspase (100) pathways. UPS appears to be the main system responsible for degrading damaged and misfolded proteins (40, 101, 102). By using bortezomib (proteasome inhibitor) and concanamycin A (inhibitor of lysosomal acidification) to study proteasomal and lysosomal protein degradation, respectively, Zhao et al. (64) showed that proteasome-mediated protein degradation was responsible for at least two-thirds of the total protein degradation in both C2C12 myoblasts and myotubes (64).

1.3.1.1 The 26S proteasome

The eukaryotic 26S proteasome plays a major role in ubiquitin-mediated protein degradation. The 26S proteasome consists of two or three particles (one or two terminal 19S regulatory particles, plus one barrel-shaped 20S catalytic core particle) (97, 102, 103). The 19S regulatory particle serves as a gatekeeper that recognizes substrate for degradation. The 19S regulatory particle is formed by two subcomplexes: a base neighbouring the 20S and a lid sitting on the base. The base contains AAA ATPases unfoldases (Rpt1-6) and three non-ATPases subunits (Rpn1-2 and 13) (102, 103), while

the lid has eight subunits (Rpn3, Rpn5-9 and Rpn12, and the deubiquitylating enzyme, DUB Rpn11) (102, 103). Rpn10 ties the base and lid subcomplexes. Importantly, Rpn1, Rpn10 and Rpn13 are responsible for the recognition of ubiquitylated protein. Once ubiquitylated proteins are recognized, the ubiquitin chains are removed by DUBs (Usp6/Usp14, Uch37 and Rpn11). Such a reaction is known as deubiqutylation (97, 103, 104). Substrates are then unfolded within the 19S regulatory particle before translocating to the 20S core particle for peptide hydrolysis, which is driven by Rpt1-6 in an ATP-dependent process (97, 102, 103). The 20S core particle is responsible for peptide hydrolysis because it contains β1, β2, and β5 subunits, which possess caspase-, trypsin- and chymotrypsin-like peptidase activity, respectively (97).

1.3.1.2 Protein ubiquitylation and ubiquitin codes

Since the discovery of the critical role of ubiquitin in proteasome-mediated protein degradation, protein ubiquitylation has been widely regarded as a key signal for proteasome-mediated protein degradation. However, the progress of recent research revealed that protein ubiquitylation can regulate all aspects of biological functions (105, 106) and that these multi-functional roles of ubiquitylation have not widely been recognized in the skeletal muscle research field.

Protein ubiquitylation consists of a coordinated process that involves ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes (107) (**Figure 1.4a**). In addition to labelling substrate proteins, ubiquitin can be ubiquitylated on any of its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the first methionine residue (M1) to form eight different homotypic ubiquitin chain types (**Figure 1.4b**). Protein

ubiquitylation can appear as mono- or poly-ubiquitin chains on a substrate protein. Due to structural and topological differences, different chain types have been referred to as "ubiquitin code" to elicit distinctive biological functions (105). In addition to homotypic poly-ubiquitin chain types, the emerging roles of mixed or branched heterotypic poly-ubiquitin chain types add complexity to the ubiquitin code (106, 108, 109). Among the eight different homotypic poly-ubiquitin chain types, the K11 and K48 poly-ubiquitin chains are known as a signal for proteasome-mediated degradation (110), whereas other chain types (K6, K27, K29, K33, K63, and M1) may have non-degradative roles (105, 106). The emerging mixed or branched heterotypic poly-ubiquitin chain types, such as K29/K48 and K63/K48 are shown to direct protein substrates to proteasome-mediated degradation (111, 112) and K11/K48 branched ubiquitin chains increase proteasome-mediated degradation compared to homotypic K11 poly-ubiquitin chains (113). In contrast, poly-ubiquitin chains can be cleaved by DUBs (104).

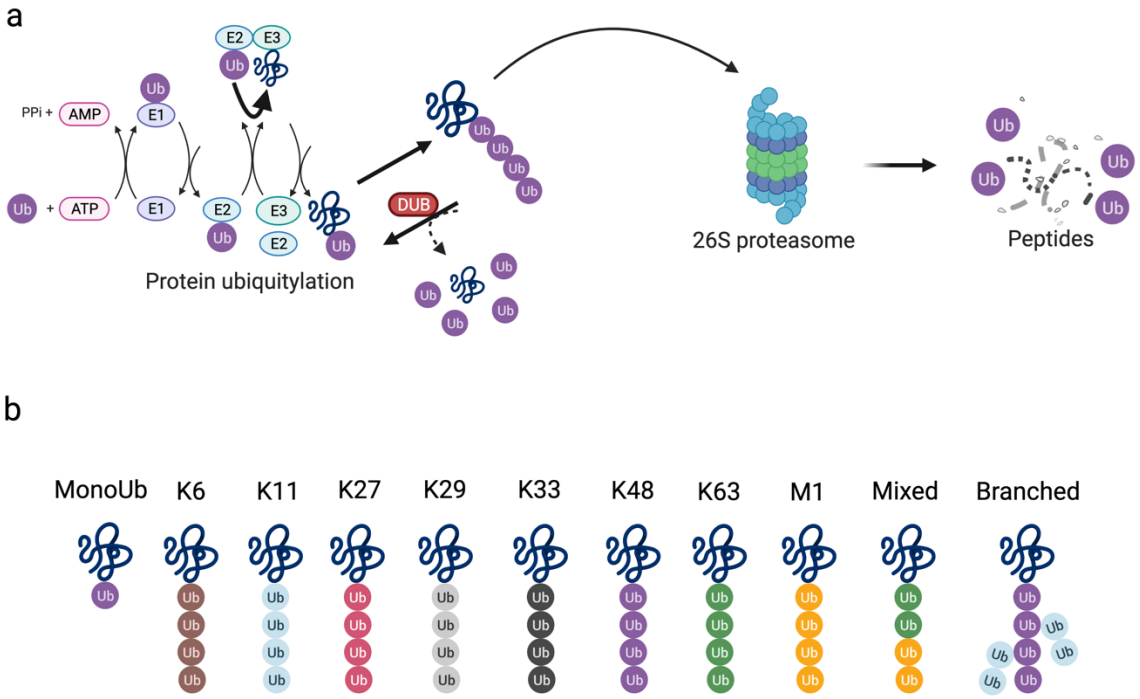

Figure 1.4 Schematic diagram of the ubiquitin proteasome system and the ubiquitin code.

(a) In the ubiquitin proteasome system, substrate proteins are targeted by protein ubiquitylation through sequential reactions, involving ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes. E3 ligase determines a substrate and attaches ubiquitin in conjunction with E2. Deubiquitylation is executed by deubiquitylating enzymes (DUBs) to eliminate ubiquitin modifications from protein substrates. K48-linked polyubiquitin chains are a known signal for protein degradation at the 26S proteasome. Once ubiquitylated protein is recognized by the 26S proteasome, polyubiquitin chain is removed by DUBs and free ubiquitin is recycled for subsequent protein ubiquitylation processes. Then, protein is degraded to peptides at the 20S core particle of the 26S proteasome. (b) Schematic representation of the ubiquitin modifications in the ubiquitin code to regulate various biological functions. AMP, adenosine monophosphate; ATP, adenosine triphosphate; PPi, inorganic pyrophosphate; DUB, deubiquitylating enzyme; Ub, ubiquitin.

The most recent evidence suggest that protein ubiquitylation not only has a role in proteasome-mediated protein degradation, but also plays a key role in regulating autophagy lysosome-mediated protein degradation (40). This was evidenced by the findings that K63 poly-ubiquitin chain is involved in autophagy lysosomal protein degradation (40, 114). This was also evidenced by the fact that most of autophagy receptors (e.g., p62/SQSTM1, OPTN, TAX1BP1, NBR1, and NDP52) have ubiquitin-binding domain, which recognizes ubiquitylated proteins and links them to the autophagosomal membrane (115). In support of the above, Zhao et al. also showed that lysosomal-mediated protein degradation accounted for 20-30% of total protein degradation in C2C12 skeletal muscle myoblasts and myotubes (64). Thus, protein ubiquitylation is considered to regulate both proteasome-mediated and autophagy lysosome-mediated protein degradation.

1.3.1.3 E3 ligases in skeletal muscle

Skeletal muscle has drawn much attention in the ubiquitin field because corresponding ubiquitin signalling was reported to be abnormal in muscle atrophy. This is highlighted by the identification of muscle-specific E3 ligases, MuRF1 (*TRIM63*) and Atrogin-1 (*FBXO32*), whose mRNA expressions are increased in various atrophic rodent models, including immobilization, denervation, and hind limb suspension (107, 116, 117). Furthermore, knockout of either MuRF1 or Atrogin-1 attenuated denervation-induced muscle loss (116). These seminal works clearly indicated that the increased expression of MuRF1 and Atrogin-1 is a valid biomarker of skeletal muscle atrophy (118).

1.3.1.4 Insulin/IGF1-Akt/FoxO signalling to regulate MuRF1 and Atrogin-1 mRNA expression

Identifying the signalling pathways that regulate the mRNA expression of ubiquitin E3 ligases is important to develop effective therapeutic targets. In addition to the stimulation of protein synthesis, IGF-1/insulin signalling plays an important role in regulating Atrogin-1 and MuRF1 mRNA expression (119-121). IGF-1 or insulin suppresses the mRNA expression of both Atrogin-1 and MuRF1 (**Figure 1.5**) (120). In line, overexpression of constitutively active Akt prevented dexamethasone-induced mRNA transcriptional upregulation of Atrogin-1 (119) and denervation-induced muscle atrophy (51). The suppressive effect of insulin or IGF-1 on Atrogin-1 and MuRF1 mRNA expression is mediated via the forkhead box (FoxO) family of transcription factors (122).

The FoxO family of transcription factors consists of three different isoforms: FoxO1, FoxO3, and FoxO4 in skeletal muscle. The localization of FoxOs is determined by their phosphorylation status. FoxOs are exported to the cytoplasm when they are phosphorylated, whereas FoxOs are localized in the nucleus when they are less phosphorylated (122), which promotes the mRNA expression of MuRF1 and Atrogin-1. Evidence showed that FoxO can directly bind to Atrogin-1 and MuRF1 promotor (123). Moreover, over expression of FoxO3a is sufficient to induce the mRNA expression of Atrogin-1 and subsequent atrophic phenotype in mouse skeletal muscle (119), whereas the siRNA knockdown of FoxO1-3 blocks the mRNA expression of Atrogin-1 (119). Not surprisingly, the decline in the Akt signalling pathway is often observed in various

muscle atrophy conditions, leading to the accumulation of nuclear FoxOs (124). Thus, Akt-FoxO axis is critical for regulating MuRF1 and Atrogin-1 mRNA expressions.

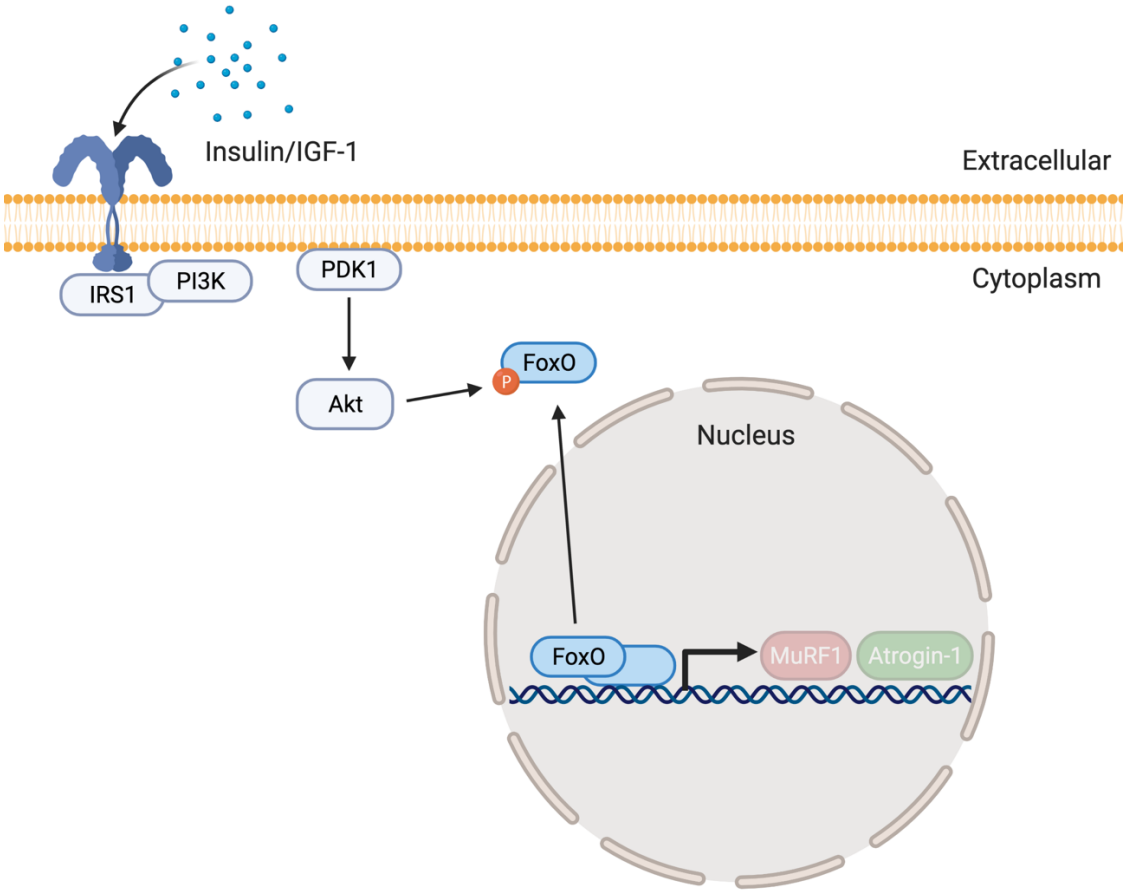

Figure 1.5 Insulin/IGF1-mediated activation of Akt decreases FoxO-mediated MuRF1 and Atrogin-1 mRNA transcription.

Upon the binding of insulin or IGF-1 to their respective receptors, IRS1 is activated and PI3K is recruited. These signalling events trigger the phosphorylation of Akt by PDK1, leading to the phosphorylation FoxO. The phosphorylation of FoxO prevents FoxO from translocation to the nucleus, and thus transcription of MuRF1 and Atrogin-1 is attenuated. IGF-1, insulin-like growth factor 1; IRS1, insulin receptor substrate 1; PI3K, phosphatidylinositol-3-kinase; PDK1, phosphoinositide-dependent kinase-1; FoxO, forkhead box O.

1.3.2 Skeletal muscle protein breakdown in response to acute resistance exercise

Resistance exercise also increases rates of MPB. Unfortunately, in stark contrast to the wealth of findings from studies on MPS, there has been a meager understanding of or substantially less research on MPB (39, 125). The lack of studies on protein breakdown is partially due to methodological limitations, including the recycling of stable isotopically labelled amino acids released from muscle protein breakdown for protein synthesis (39). To advance our understanding of protein turnover, it is crucial to improve the accuracy and reliability of the methodology for studying the molecular mechanisms in MPB. Nonetheless, Phillips et al. (27) demonstrated that the rates of mixed muscle protein fractional protein breakdown are upregulated at 3 h (31 %) and 24 h (18 %) following the acute bout of resistance exercise and returned to the basal levels by 48 h in healthy young adults.

1.3.3 Ubiquitin signalling in human skeletal muscle is dynamically modified by high-intensity exercise

Earlier studies showed that an acute bout of exercise increased ubiquitin conjugation to proteins and increased the expression of components associated with the ubiquitylation processes (e.g., expressions of ubiquitin, ubiquitin conjugates, components of proteasome, E2) in skeletal muscle (126), indicating that protein ubiquitylation event is activated by exercise. Consistently, recent studies showed that both acute exercise (127, 128) and functional overload (129) increase proteasome activity in both human and rodent skeletal muscle. These studies highlight an essential role of UPS in protein quality control, muscle remodelling, and muscle adaptation to exercise.

While protein ubiquitylation is known as one of the key determinants of controlling protein degradation (64, 97), our current challenge to study the event of protein ubiquitylation is the lack of valid and commonly applicable tools to measure substrate ubiquitylation (130). In recent years, a high-throughput proteomic approach emerged as a useful tool for identifying ubiquitylation at the whole proteome level (131). Parker et al. (132) recently applied this approach and made the first publication to document exercise-mediated ubiquitylome in human skeletal muscle. They found that an acute bout of high-intensity exercise altered the landscape of protein ubiquitylation in skeletal muscle proteome. While the abundance of many proteins decreased immediately after exercise, the total amount of K11, K48, and K63 ubiquitin chains decreased in the same fashion. The latter observation, particularly the decreased K11, K48, and K63 ubiquitin chains, suggests that both proteasome- and lysosome-mediated protein degradation increased during or immediately after exercise (40, 114). Interestingly, the authors also reported that the protein abundance and the reduced ubiquitin chains (K11, K48 and, K63) were returned to pre-exercise levels after only 2 hours of recovery from exercise, indicating that the effect of exercise on ubiquitin signalling is transient. Such evidence of the rapid alternations in ubiquitin signalling supports the idea that exercise facilitates the removal of misfolded/damaged proteins, thereby improving the protein quality control in skeletal muscle (46). Although these data clearly indicate that exercise dramatically affects protein ubiquitylation status, it remains unclear what biological functions are governed by these rapid ubiquitylation alterations. It will be particularly interesting to know whether any of these alterations account for the beneficial effects of exercise. While methods of proteomic approach for detecting protein ubiquitylation are

improving (133), more studies are required to identify and confirm new exercise-mediated ubiquitin signalling in skeletal muscle.

Parker et al. (132) also showed that the MuRF1 ubiquitylation status (at site of MuRF1 K152, K123, and K116) and protein abundance are transiently reduced immediately after exercise but restored rapidly during the recovery from exercise. This suggests that MuRF1 may be autoubiquitylated and its activity is possibly regulated by exercise. Moreover, Parker et al. (132) reported changes in the overall ubiquitylation status in some of the myofibrillar proteins after exercise. Even though previous studies have reported that myofibrillar proteins, such as myosin heavy chain (134), myosin light chain (135), and actin (136) are ubiquitylated by MuRF1. In the study led by Parker et al. (132), however, it is unclear if the changes of ubiquitylation in myofibrillar proteins are mediated by MuRF1 or other E3 ligases, and whether these ubiquitylated proteins undergo degradative or non-degradative pathways are also not clear. Although their intention was to understand the role of ubiquitylation in exercise-mediated muscle protein degradation, protein degradation rate was not measured in the study. We therefore cannot extrapolate the results of ubiquitin signalling into either protein degradation or other physiological function during and after exercise. Despite all these, Parker et al. (132) have provided an important first-step toward establishing exercise-mediated ubiquitin signalling in skeletal muscle.

1.3.4 The 26S proteasome activity is activated by exercise-modulated phosphorylation of the 19S proteasome subunit

The rate of protein ubiquitylation has long been regarded as the sole determinant of UPS-mediated protein degradation. However, recent studies indicate that phosphorylation is also required for UPS-mediated protein degradation (127, 137). Particularly, protein kinase A (PKA)-mediated phosphorylation of 19S proteasome subunit Rpn6 was reported to be one of the mediating mechanisms for exercise-induced proteasome activation. This finding was elicited from the finding by Lokireddy et al. (137) showing that elevation of cyclic adenosine monophosphate (cAMP) and PKA signalling can lead to an increase in proteasome activity in C2C12 myotubes via the phosphorylation of 19S proteasome subunit Rpn6 at Ser 14. Although the study by Lokireddy et al. (137) did not use an exercise model, cAMP-PKA signalling is well known to be activated during exercise (138). The identification of Rpn6 phosphorylation is particularly important because this phosphorylation also facilitates the ATP-dependent processes of substrate unfolding, deubiquitylation, and the translocation of the substrates into the 20S proteasome subunit where peptide hydrolysis occurs (137). The same study also used a pulse-chase technique (radioactive tracer, ^{3}H-phenylalanine) to show that an increase of cAMP induced by rolipram promoted protein degradation in C2C12 myotubes (137). Their study also indicated that cAMP-mediated protein degradation mainly degrades misfolded and fast-turnover protein degradation, but not structural proteins and myofibrillar proteins (127, 137), suggesting cAMP-mediated protein degradation contributes to protein quality control. Following the findings discussed above, Goldberg's group further demonstrated that an acute bout of high intensity exercise also increased PKA-dependent phosphorylation of Rpn6 at Ser

14 and proteasome activity in human skeletal muscle (127). However, protein degradation rate was not measured in this human study (127), and the assumption of increased protein degradation was referred to the results obtained from cell culture experiments (137).

Altogether, these *in vitro* (137) and *in vivo* (127) studies show that an acute bout of high intensity exercise activates PKA signalling through the elevation of circulating adrenaline (**Figure 1.6**). The activation of cAMP-PKA signalling induces the phosphorylation of the 19S proteasome subunit Rpn6 at Ser14 to stimulate the processes of protein degradation in the proteasome. Meanwhile, exercise also decreases the amount of K48-linked polyubiquitin chain (132), which plays a key signal for recognizing substrate degradation in the proteasome (110). These findings highlight that both protein phosphorylation and ubiquitylation have to work in concert to regulate exercise-mediated proteasome activation in skeletal muscle. Furthermore, as reported by Parker et al. (132), MuRF1 abundance and its ubiquitylation status are also altered following a bout of high intensity exercise. However, it is unclear what makes the changes of MuRF1 abundance, and how the ubiquitylation of MuRF1 affects the biological functions. These new and important findings show that the protein ubiquitylation status is altered rapidly by exercise, which raises an important question of identifying what E3 ligases and/or DUBs contribute to the changes of protein ubiquitylation in exercising skeletal muscle.

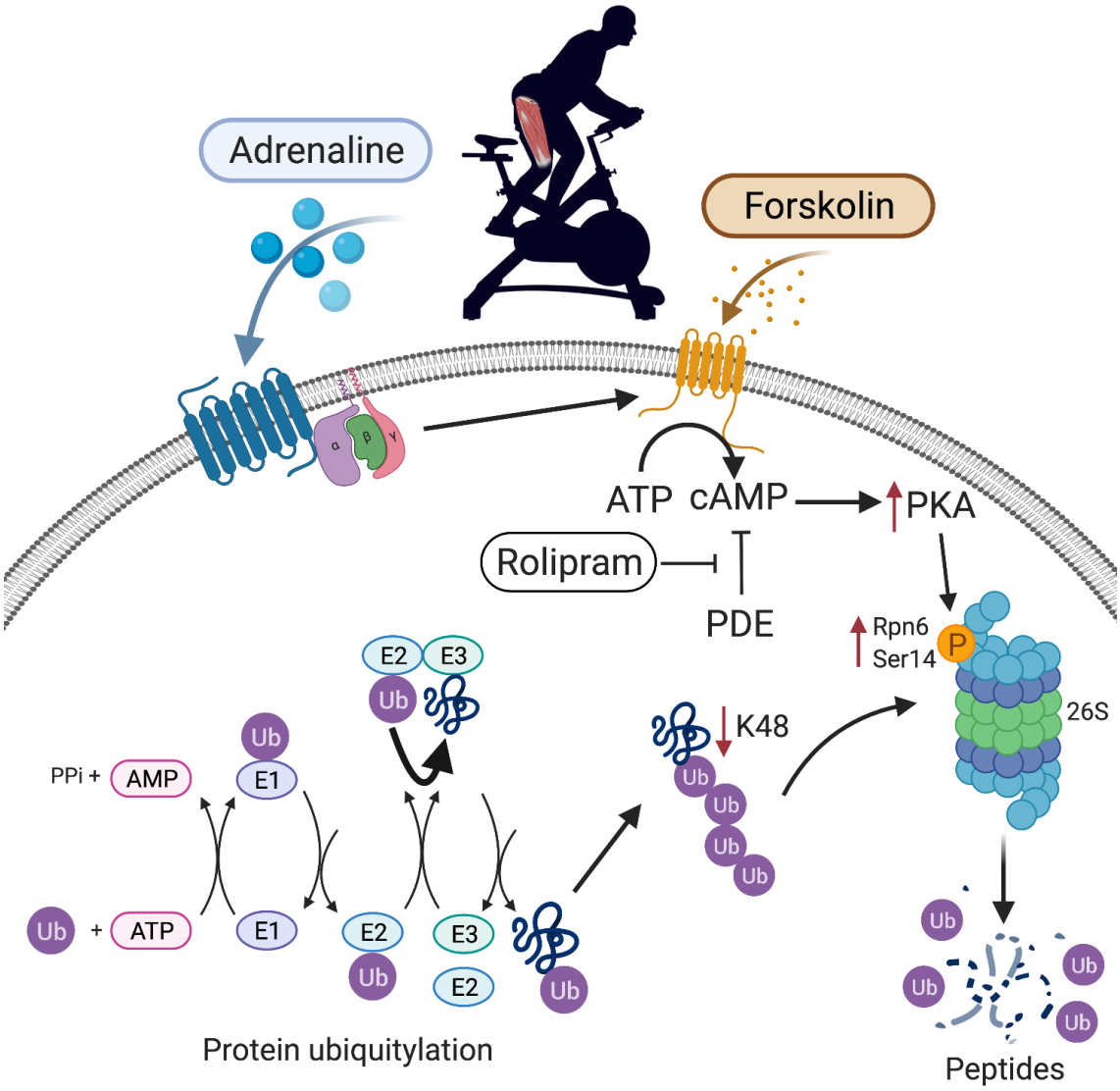

Figure 1.6 Schematic diagram of the crosstalk between phosphorylation and ubiquitylation in the 26S proteasome-mediated protein degradation in exercising skeletal muscle.

Phosphorylation is involved in enhancing the 26S proteasome activity after exercise. Exercise increases adrenaline in circulation, which then binds to a G protein-coupled receptor and activates adenylyl cyclase. The activation of adenylyl cyclase increases cyclic adenosine monophosphate (cAMP) production that activates protein kinase A (PKA). This cAMP-PKA activation in turn phosphorylates Rpn6 at Ser 14 of the 19S regulatory particle. The phosphorylation of Rpn6 at Ser 14 has been shown to be a critical signal that enhances the 26S proteasome activity in exercised human skeletal muscle. On the other hand, protein ubiquitylation aided by sequential reactions by ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes also plays a key role in determining the fate of ubiquitylated protein for the 26S

proteasome-mediated degradation. E3 ligase determines a substrate and attaches ubiquitin in conjunction with E2. Exercise decreases the amount of K48-linked polyubiquitin chain due to increased 26S proteasome-mediated degradation because K48-linked polyubiquitin chain is known as a signal for proteasome degradation. Thus, both protein ubiquitylation and phosphorylation modifications are involved in the 26S proteasome-mediated protein degradation in skeletal muscle. Red arrows indicate changes of abundance or activity. AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; PPi, inorganic pyrophosphate; Ub, ubiquitin.

1.4 Resistance exercise training exerts skeletal muscle protein anabolic response

Chronic resistance exercise training can induce skeletal muscle hypertrophy through the cumulated increase in MPS, which in turn accumulates contractile protein and increases the size of individual muscle fibres (139, 140). Reidy et al. (141) reported that pre-post changes of MPS are correlated with the change in vastus lateralis muscle thickness in younger males. In line, Brook et al. (142) reported that MPS in the early period of resistance exercise training is associated with skeletal muscle hypertrophy. Contrasting findings were also reported previously. For example, Mitchell et al. (143) reported that there is no correlation between acute MPS and skeletal muscle hypertrophy, which suggests that acute increase in MPS might not contribute to hypertrophy. This notion was supported by a study led by Damas et al. (144) that the increased MPS at the earlier period of resistance exercise training contributes to the remodelling of damaged proteins in skeletal muscle, but not skeletal muscle hypertrophy. Witard et al. (145) discussed that a training status and methodologies used to measure muscle mass could affect the predictability of acute MPS on skeletal muscle hypertrophy. Thus, further studies are required to conclude whether MPS is reflective of skeletal muscle hypertrophy after days or weeks of resistance exercise training, and how MPB is involved in skeletal

muscle adaptation to resistance exercise training. For example, the role of MPB beyond the control of skeletal muscle mass, such as protein quality control within skeletal muscle is still unclear. In summary, the current evidence suggests that MPS is a plausible intervention target to achieve skeletal muscle remodelling and subsequent hypertrophy. Accordingly, strategies to increase MPS after resistance exercise are required to augment skeletal muscle anabolic response.

1.5 The roles dietary protein on the maintenance of body protein

1.5.1 Dietary protein requirements and recommendations

Adequate protein intake is required for the maintenance of whole-body protein mass. The protein mass in a 70 kg adult is about 11 kg. Whole-body protein mass maintenance relies on equal rates of protein synthesis and breakdown, resulting in a zero net balance. In adult humans, the daily whole-body protein turnover rate is 5.7 g/kg body weight (146), meaning that approximately 400 g mixed proteins are turned over every day for a 70 kg adult individual. Behind that number, a large proportion of amino acids are recycled and reutilized for protein synthesis (39, 42, 147), whereas some are lost via oxidation for energy production and the formation of urea to scavenge nitrogen (42). Further, nitrogen-containing substances, such as skin, hair, sweat, urine, and feces are lost from the body. To maintain whole-body protein mass, irreversibly loss of essential amino acids and nitrogen needs to be reconstituted via dietary protein, which founds the basis for the recommended dietary protein intake.

1.5.2 Historical development of determining protein requirements and recommendations

The first official recommendation for protein intake of 1.0 g/kg/day, published in 1936 by the League of Nations (148), was set based on observations from practice rather than relying on data from a strict scientific approach.

Several joint FAO/WHO expert committees faced challenges in defining "protein requirements" (149). In the 1957 report, the expert committee decided that mean minimum requirements to maintain nitrogen balance in adults should be used as the guideline, using a "reference protein" of high nutritional value and a protein requirement and recommendation for adults at 0.53 g/kg/day and 0.66 g/kg/day, respectively (150).

During the 1960s, protein received increased attention with the finding of kwashiorkor in West Africa, a serious disease occurring especially in malnourished children (151). At this time, the FAO advocated that protein malnutrition is a major nutritional problem among children in developing countries (152). The Joint FAO/WHO Expert Committee decided to use the 'factorial approach', where a safe level of intake is calculated based on the obligatory nitrogen losses in urine, feces, sweat, hairs when the diet contains no protein and addition for the formation of new tissue was formed for infants, children, pregnant and lactating women.

In 1965, the Joint FAO/WHO Expert Committee assessed the protein requirement of adults to be 0.71 g/kg/day with the recommendation of 0.89 g/kg/day (152), using an intake of a high-quality reference protein. In 1973, expert members of the FAO/WHO

Expert Committee continued to employ a factorial approach and arrived at a protein requirement of 0.57 g /kg/day for men and 0.52 g /kg/day for women and a safe level of protein intake of 0.75 g/kg/day for both sexes (153).

After reviewing the evidence on protein requirements, the FAO/WHO/UNU expert consultation in 1981, concluded that adult protein requirements should be based on nitrogen balance studies (154). Using results from short- and long-term nitrogen balance studies, the FAO/WHO/UNU in 1985 determined a population average requirement of 0.6 g/kg/day, and a safe level of intake at 0.75 g/kg/day was established (154). In their report, the expert committee concluded that unfortunately, the information on which to base more accurate estimates related to age was too scanty.

In 2007, the WHO/FAO/UNU updated the protein requirements (155). Based on a meta-analysis of nitrogen balance studies in humans (mainly younger adults) by Rand et al. (156), the average requirement was set at 0.66 g/kg/day, resulting in the safe level of intake at 0.83 g/kg/day of high quality protein (155). While using the definition of protein requirement as "*the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity…*", it is uncertain that this amount is sufficient to counteract protein loss in catabolic conditions (41). Even though the Expert Committee rigorously reassessed all available data on protein balance in older people, it was concluded that no studies unequivocally demonstrate that the protein requirement would be higher in older adults when expressed as protein

requirement per kg body weight. **Table 1.1a** provides an extensive overview of the WHO/FAO/UNU recommendations over the years.

Table 1.1a Successive protein requirements and recommendations by international groups to ensure nitrogen balance in adults.

Report	Age	Methodological approach	Biological value of dietary protein (%)	Average protein requirements (g/kg/day)	Recommendation/ safe level of intake (g/kg/day)
League of Nations 1936 (148)	Adults	-	-	-	1.0
FAO 1957 (149)	Adults	N-balance	80	0.53	0.66
FAO/WHO 1965 (152)	Adults	Factorial	80	0.71	0.89
FAO/WHO 1973 (153)	Adults (20-39 yrs.)	Factorial	75	0.57	0.75
FAO/WHO/UNU 1985 (154)	Adults	N-balance	100	0.6	0.75
FAO/WHO/UNU 2007 (155)	Adults (≥ 18 yrs.)	Meta-analysis (N-balance studies) (156)	100	0.66	0.83

Adopted from NS Scrimshaw (150) and updated.

Protein recommendation of older adults determined by WHO/FAO/UNU has been challenged by other expert bodies. **Table 1.1b** summarizes major guidelines from external expert bodies, which are not, though, official recommendations. In the Australia and New Zealand recommendations published in 2006 (157), the factorial method was used as a basis for the recommendation of 1.07 g/kg/day and 0.94 g/kg/day for men and women, respectively. In the Nordic Nutrition Recommendations in 2014 (158), the PROT-AGE Study Group in 2013 (159) and the ESPEN Expert Group in 2014 (160) justified their recommended protein intakes for older adults by considering muscle mass, muscle strength, and physical function are more relevant outcomes than whole-body protein balance and recommended a protein intake at 1.0-1.2 g/kg/day for healthy older adults with a higher intake for those who are malnourished or at risk of malnutrition.

Table 1.1b Protein recommendations by expert bodies to maintain muscle mass and strength in older adults.

Report	Age	Methodological Approach/study design	Functional outcomes	Recommendation (g/kg/day)	Rationale
Nutrient Reference Values for Australia and New Zealand 2006 (157)	> 70 yrs.	Factorial	Estimation of the amount needed for growth and maintenance on a fat-free mass basis	Men, 1.07 (81 g/day) Women, 0.94 (57 g/day)	Based on Rand et al. (156) and additional studies (156, 161, 162), the average requirement was increased by 25% over that of younger adults.
The Nordic Nutrition Recommendations 2012 (158)	> 65 yrs.	Systematic review (163) (prospective cohort, case control, and intervention studies)	N-balance Muscle mass Muscle strength Bone mass Morbidity Mortality	1.1-1.3 (15-20 E% and the protein E% should be increased with decreasing energy intake)	The grade of evidence was assessed as *convincing*, *probable*, *suggestive*, and *inconclusive* (163). The evidence of protein requirement (0.66 g/kg/day) and recommendation (0.83 g/kg/day) based on N-balance studies was assessed as *probable*. The evidence from prospective cohort studies in relation to functional outcomes (e.g., muscle mass and muscle strength) with a safe intake of at least 1.2-1.5 g/kg/day was assessed as suggestive to inconclusive.

The PROT-AGE Study Group 2013 (159)	> 65 yrs.	Epidemiological studies Clinical trials	Muscle mass Muscle strength Physical function Muscle protein synthesis	1.0-1.2	Decrease in anabolic response to protein intake in older adults; higher protein intake is required to offset inflammatory and catabolic conditions often observed in older adults. Both endurance and resistance exercise were recommended, and even higher protein intake (> 1.2 g/kg/day) was recommended those who engage in exercise.
The ESPEN Expert Group 2014 (160)	> 65 yrs.	Epidemiological studies Clinical trials	Muscle mass Muscle strength Physical function Muscle protein synthesis	1.0-1.2	Older adults require higher protein due to anabolic resistance, low postprandial amino acid availability, decreased muscle perfusion, sarcopenia, and disease-related protein catabolism. Both endurance and resistance exercise were recommended.

It is well established how much dietary protein should be ingested to account for obligatory nitrogen loss in healthy adults using nitrogen balance methodology (155, 156). However, it has continuously been debated (164-170) whether increasing dietary protein intake in older age is an effective strategy to counteract the age-dependent loss of muscle mass (1, 171) and strength (22, 172), a phenomenon termed sarcopenia (11, 13, 173). Although our understanding of protein requirements and recommendations have improved over these 60 years, the main reason for changing the absolute values of the recommended intake of protein is the outcome measure and the methodology on which the measure is founded. Historically, protein recommendations have been derived from various methodologies, which impact the absolute values of the recommended intake of protein. In the current debate of protein recommendations, even other study designs and methodological approaches that have not been used in the WHO/FAO/UNU protein recommendation were included to provide scientific evidence (see **Table 1.1b**). Essentially, the recommended amount of protein is defined as the amount of dietary protein needed to maintain whole-body protein mass (155, 156). Three principles can be used to determine the value of protein requirements. Firstly, the protein mass can be assessed either cross-sectionally or longitudinally in large cohorts. Individuals ingest various amounts of protein and the minimum intake that results in steady whole-body protein mass and/or muscle mass is what is required. Secondly, the protein balance can be measured over days and the intake resulting in a zero balance (intake equals loss) is the amount that is needed. Finally, the kinetics of protein turnover (synthesis and breakdown rates) as well as the irreversible loss of amino acids can be measured. Once the balance is achieved, it corresponds to the required amount of protein intake. Discussion of protein requirements without definitive criteria may lead to an erroneous

conclusion, which will be discussed in the next section. In theory, all approaches are conceptually alike. However, protein requirements determined by different outcome measurements do not necessarily match, partly due to various important factors that impact the requirement.

As shown in **Table 1.1b**, independent authorities and expert groups have adopted different criteria and evidence from distinct methodological approaches from WHO/FAO/UNU (**Table 1.1a**), resulting in differences in published recommended values of protein recommendation. Consequently, there is currently confusion about the requirement of dietary protein for healthy older adults both in the scientific community and amongst the general older population, who wish to follow the recommendations. Thus, study and methodological approaches that underlie the generation of the evidence for protein requirements in healthy older adults will be explored in the following section 1.6.

1.6 Generation of evidence for protein requirements and recommendations for healthy older adults

1.6.1 Protein/nitrogen balance studies

The nitrogen balance methodology has most widely been used and is considered 'gold standard methodology' to establish the protein requirement (155, 156). Whole-body nitrogen balance is determined by measuring or estimating all nitrogen intake (protein intake) and all excretion and loss (skin, hair, sweat, urine, and feces). A positive balance means excess nitrogen intake relative to losses (i.e., protein accretion within the body). Two crucial prerequisites for a valid measurement are: 1) metabolic adaptation to any

given amount of daily protein intake before conducting the measurements (174-176), and 2) the achievement of energy balance during the period of measurement (156). However, the nitrogen balance methodology has its inherent shortcomings: it has been criticized for insufficient sensitivity (i.e., inability to detect a small difference between nitrogen intake and excretion) (177). Further, the overestimation of nitrogen intake through food and underestimation of nitrogen loss can lead to erroneous balance measurements.

While there is a limited mechanistic insight of the nitrogen balance data (177, 178), Waterlow and colleagues proposed in 1977 (179) that a relationship between nitrogen balance and protein balance could be expressed at a steady state as: Flux (Q) = protein synthesis (S) + nitrogen oxidations/excretion (O) = protein breakdown (B) + nitrogen input (I) $\rightarrow$ $Q = I - O = S - B$. Protein balance calculated as the difference between protein synthesis and breakdown rates provides information about the underlying dynamics of protein kinetics. Protein balance is a rate of either gain or loss of protein per unit time and indicates whether protein mass in the body will quantitatively change over time. Therefore, the prolonged readout of the protein balance data is whole-body protein mass and/or changes herein. Whole-body protein mass, often determined by the surrogate measure of muscle mass, is determined in another set of scientific studies, namely the epidemiological studies.

1.6.2 Epidemiological studies of protein intake

Epidemiological studies, such as cross-sectional and prospective cohort studies can be used for assessing the association between habitual dietary intake patterns and whole-

body/muscle mass (180-190). Epidemiological studies have the strength that adaptation to a given protein intake level would be inherent, yet they often suffer from several confounding factors. It limits the translation into other settings and makes it impossible to use the results for assessing protein requirements. Longitudinal intervention studies can be applied to examine the cause-effect relationship and/or dose-response relationship between protein intake and most often physiological and functional outcomes, such as muscle mass, muscle strength, and physical function which is particularly relevant to older adults (191-196).

1.6.2.1 Cross-sectional studies

The role of protein intake on the maintenance of muscle mass has been investigated across the lifespan in numerous studies (see **Table 1.2**). A cross-sectional study from the Framingham Offspring Cohort showed a positive association between the total daily and total animal protein intake and muscle mass after adjustment of physical activity and energy intake (190). The association between protein intake and muscle mass became apparent when dividing the cohort into quartiles of protein intake. In men, the difference appeared between the highest (101.1 g/day) and the lowest quartile (64.9 g/day) for total protein intake. In women, muscle mass differed between the highest and the second-lowest quartile for total protein and animal protein intake (93.4 g/day vs. 63.1 g/day) (190). Mangano et al. (188) examined the third-generation offspring of the original Framingham Heart Study. They divided the cohort into four quartiles based on their protein intake from the lowest intake (quartile 1) 59 g/day (0.8 g/kg/day) to the highest intake (quartile 4) 129 g/day (1.8 g/kg/day). After adjustment for various confounders including physical activity and energy intake, they found a positive

association between protein intake and appendicular lean mass index. A difference was found between the first and the second quartile (80 g/day or 1.1 g/kg/day). However, there was no further difference between the first quartile and the third (99 g/day or 1.3 g/kg/day) and the fourth quartile (129 g/day or 1.8 g/kg/day) (188). Collectively, cross-sectional observational studies suggest that protein intake around the international recommendation (0.8 g/kg/day, 60 - 65 g/day in average-sized individuals, 75-80 kg) is sub-optimal for the maintenance of muscle mass throughout adult life. They also suggest that muscle mass may be better maintained when protein intake is higher (80 g/day or 1.1 g/kg/day) than the international recommendation (0.83 g/kg/day), and that no further beneficial effects on muscle mass are observed beyond this amount, suggesting a non-linear association above this level of intake.

Cross-sectional observational studies including healthy older adults have provided an insight into the role of dietary protein intake in the maintenance of muscle mass (180, 184, 187) (see **Table 1.2**). A positive association was observed between protein intake and muscle mass (180, 184). Geirsdottir et al. (184) reported data adjusted for sex, body mass index (BMI), age, and physical activity level and found a higher lean body mass in the quartile with the highest protein intake (1.15 - 1.92 g/kg/day, the fourth quartile) compared to only the first quartile (0.41 - 0.75 g protein/kg/day, $P = 0.04$) and the second quartile (0.76 - 0.92 g/kg/day, $P = 0.05$) but not the third quartile. Interestingly, the differences disappeared when correcting for total energy intake, which emphasizes the crucial importance of considering energy intake when evaluating the impact of protein intake on the regulation of body composition and especially lean body mass in older adults. Similarly, data by Asp et al. (180) emphasize the dependency between

protein and energy intake on muscle and body size as they found that protein intake correlated positively with nutritional status and BMI even when accounting for age, sex, and activity level. In support of protein intake as a determining factor for a lean body composition, Gregorio et al. (187) divided a total of 387 older women into those ingesting ≥ 0.8 g/kg/day (n = 290) or < 0.8 g/kg/day (n = 97) and found that although the women in the ≥ 0.8 g/kg/day group had lower lean mass than those in the < 0.8 g/kg/day group, they also had a lower fat mass, resulting in a lower fat to a fat-free ratio in the high-protein group. Collectively, some cross-sectional observational studies have reported positive associations between protein intake and muscle mass in older adults. However, it seems that when accounting for nutritional status, energy intake, and physical activity, there is limited evidence to recommend that protein intake greater than the international recommendation is beneficial for muscle mass preservation.

Table 1.2 Selected cross-sectional observational studies assessing the association between protein intake and muscle mass.

Author, location	Subjects Men (M), Women (W)	Age, years	Dietary assessment	Body composition measurement	Average protein intake	Protein intake	Outcomes	Additional information
Sahni et al. 2015 (190), USA	1139 M, 1497 W	29-86	FFQ	DXA	M: 80 g/day W: 76 g/day	g/day Q1: M 64.9, W 57.8 Q2: M 70.8, W 63.1 Q3: M 79.2, W 73.5 Q4: M 101.1, W 93.4	A positive association between protein intake and leg lean mass	
Mangano et al. 2017 (188), USA	2905 M&W	19-72	FFQ	DXA	93 g/day	g/day (g/kg/day) Q1: 59 (0.8) Q2: 80 (1.1) Q3: 99 (1.3) Q4: 129 (1.8)	A positive association between protein intake and ALM	
Geirsdottir et al. 2013 (184), Iceland	99 M, 138 W	65-92	3-d food record	DXA	M: 90.3 g/day, 19.6 E% W: 69.6 g/day, 18.8 E%	g/kg/day Q1: 0.63 Q2: 0.85 Q3: 1.01 Q4: 1.36	A positive association between protein intake and LM. Higher LM in Q4 vs Q1 (2.3 kg LM difference)	Significance was not evident after the correction with energy intake
Asp et al. 2012 (180), USA	47 M, 95 W	60-88	DHQ	CC	15.5 E%	Protein E%	A positive association between protein intake and CC	A positive association between beef protein intake and CC
Gregorio et al. 2014 (187), USA	387 W	60-90	4-d food record	DXA	72.2 g/day, 1.1 g/kg/day	g/kg/day < 0.8 ≥ 0.8	LM was lower in ≥ 0.8 compared to < 0.8. Fat-to-Lean Ratio was lower in ≥ 0.8 g/kg/day	

FFQ, food-frequency questionnaire; E, energy; DXA, Dual-energy X-ray absorptiometry; Q, quartile; DHQ, diet history questionnaire; CC, calf circumference; LM, lean body mass; ALM, appendicular lean body mass; ASMI, appendicular skeletal muscle index.

1.6.2.2 Observational studies

Association between protein intake and muscle mass was investigated in an observational study design in healthy older adults (181-183, 185, 186, 189) (see **Table 1.3**). A positive association between protein intake and muscle mass was observed in some studies (181-183, 185), and these studies concluded that protein intake above the current international recommendation is beneficial in preserving muscle mass in older adults. In all these studies, baseline average protein intake was higher (~1.2 g/kg/day) than the current international recommendation (0.83 g/kg/day) (181-183, 185). In contrast, other studies found that higher protein intake than the current international recommendation does not preserve muscle mass in older adults (186, 189). Chan et al. (186) discussed that the null association between protein intake and muscle mass was due in part to the relatively higher protein intake in this cohort (1.3 and 1.1 g/kg/day in men and women). More recently, Verreijen et al. (189) reported that protein intake was not associated with the 5-year changes in muscle mass. The contradicting results from Houston et al. (181) were explained by the methodological approaches used to assess muscle mass. Whilst Houston et al. (181) employed dual-energy X-ray absorptiometry (DXA), Verreijen et al. (189) used computed tomography (CT), which is regarded as a more accurate methodology to examine muscle mass. In summary, inconsistent results have been reported in observational studies regarding the association between protein intake above the international recommendation (0.83 g/kg/day) (155). Thus, the newly suggested protein recommendations for healthy older adults of 0.94-1.3 g/kg/day from different authorities and expert groups (158-160) can still be questioned.

Table 1.3 Selected observational studies assessing the association between protein intake and muscle mass.

Author, location	Subjects Men (M), Women (W)	Age, years	Dietary assessment	Body composition measurement	Follow-up duration	Baseline average protein intake	Baseline protein intake	Energy intake/balance	Outcomes
Houston et al. 2008 (181), USA	967 M, 1099 W	70-79	FFQ	DXA	3 years		% of energy (g/kg/day) Q1: 11.2 (0.7) Q2: 12.7 (0.7) Q3: 14.1 (0.8) Q4: 15.8 (0.9) Q5: 18.2 (1.1)		Higher protein intake was associated with higher preservation of LM and aLM
Meng et al. 2009 (182), Australia	862 W	70-85	FFQ	DXA	5 years	81 g/day, 1.2 g/kg/day, 19 E%	g/day, g/kg/day (E%) Q1: <66, 0.84 (17.7) Q2: 66-87, 1.17 (19.0) Q3: >87, 1.64 (20.4)		Higher baseline protein intake was associated with higher LM and aLM at the 5-y follow-up
Scott et al. 2010 (183), Australia	370 M, 370 W	50-79	FFQ	DXA	1.4-4.8 years (mean 2.6 years)	87.6/day, 1.13 g/kg/day	< RDI ≥ RDI	Energy intake and BW were maintained between baseline and follow-up. Step counts were decreased in follow-up compared to baseline.	Higher protein intake was associated with aLM at baseline and the follow-up
McDonald et al. 2016 (185), Denmark	39 M, 40 W	>65	Interview	Bioelectrical impedance	6 years	74.6g/day, 1.07 g/kg/day	g/kg/day Q1: 0.61 Q2: 0.92 Q3: 0.97 Q4: 1.26		Higher protein intake was associated with higher preservation of LM
Chan et al. 2014 (186), China	1411 M, 1315W	≥65	FFQ	DXA	4 years	1.3 and 1.1 g/kg/day for M and W	g/kg/day Q1: ≤0.9 Q2: 0.91-1.2 Q3: 1.1-1.6 Q4: ≥1.61		No association was found between protein intake and ASM

Verreijen et al. 2019 (189), USA	749 M, 812 W	70-79	FFQ	CT	5 years	66.0g/day, 0.90 g/kg/day	g/kg/day Q1: 0.50 Q2: 0.68 Q3: 0.85 Q4: 1.03 Q5: 1.39		Protein intake was not associated with 5-year change in muscle cross-sectional area

FFQ, food-frequency questionnaire; E, energy; DXA, Dual-energy X-ray absorptiometry; Q, quartile; CT, computed tomography; RDI, recommended dietary intake; BW, body weight; LM, lean body mass; ASM, appendicular skeletal muscle mass; aLM, appendicular lean body mass.

1.6.2.3 Intervention studies

Several randomized controlled intervention studies have investigated whether higher protein intake increases muscle mass in older adults (191-196) (see **Table 1.4**). Some studies were unable to show a beneficial effect of increasing dietary protein intake above their average intake of ~1.2 g/kg/day on muscle accretion or maintenance in older adults (191, 192, 196). Some studies showing no effect of higher protein intake (≥ 0.8 g/kg/day) tended to have a longer study period (≥ 1 year) compared to studies in which an increase in muscle mass was demonstrated (~ 24 weeks) (191-195), suggesting a possible adaptation effect. Mitchell et al. (194) showed that protein intake at 2 recommended dietary allowance (RDA) increased muscle mass compared to RDA in a group of men aged >70 yrs over a period of 10 weeks. However, the individuals in this study consumed a habitual protein intake of 1.1-1.2 g/kg/day on average and the individuals in the RDA group consumed less protein than the habitual intake during the intervention, which may explain the loss of appendicular lean mass and caused a group difference between RDA and 2RDA. For this study, several limitations can be listed, including that energy balance was not maintained in the RDA group; that lack of a steady metabolic state condition as adaptation was not present for the entire intervention period of 10 weeks; and finally, that no habitual protein intake group was included, which therefore does not allow to conclude on changes of lean mass without altered protein intake. In summary, null effects of longer-term intervention of elevated dietary protein intake may indicate that metabolic adaptation may level out acute benefits, which was exemplified by a recent study by Højfeldt et al. (197).

Table 1.4 Selected randomized controlled trials investigating the impact of protein intake on muscle mass in healthy older adults.

Author, location	Study design	Subjects Men (M), Women (W)	Age, years	Follow-up duration	Dietary assessment	Body composition measurement	Baseline average protein intake	Energy intake/balance	Intervention	Outcomes
Mitchell et al. 2017 (194), New Zealand	Parallel	29 M	70-81	10 weeks	3-d food record	DXA	88-101 g/day, ~1.2 g/kg/day, 14-17 E%	Energy intake was decreased in post from pre in RDA (-440 kcal) and increased in 2RDA (+555 kcal). BW was decreased in RDA (-2.1 kg) and 2RDA (-0.5 kg).	Protein intake at RDA (0.8 g/kg/day) vs 2RDA (1.6 g/kg/day)	2RDA increased LM (+1.49 kg) compared with RDA (-0.55 kg)
Ten Haaf et al. 2019 (195), Netherlands	Parallel	93 M, 21 W	67-73	12 weeks	Two 24h recalls	DXA	0.89 g/kg/day, 16 E%	Energy intake was reduced in post from pre in both protein (-78 kcal) and placebo (-10 kcal) groups. BW was decreased in protein (-0.59 kg) and placebo (-0.15 kg) groups.	A total of 31 g of protein in 500 ml milk protein concentrate drink per day or placebo	Protein supplementation increased LM (+0.93 %) compared to placebo (+0.44%)
Norton et al. 2016 (193), Ireland	Parallel	14 M, 46 W	50-70	24 weeks	4-d food record	DXA	83-86 g/day, 1.2 g/kg/day, 16-19 E%	Energy intake was increased in post from pre in protein (+111 kcal) and placebo (+116 kcal) groups.	A total of 145.4 g of protein per day or placebo	Protein supplementation increased LM (+0.45 kg) compared to placebo (-0.16 kg)

Mertz et al. 2021 (196), Denmark	Parallel	184 M&W	65-82	1 year	3-d food record	MRI/DXA	82.8 g/day, 1.1 g/kg/day, 17.6 E%	Energy intake was increased in post from pre in whey (+518 kcal), collagen (+408 kcal), and placebo (+949kcal). BW was increased in whey (+0.4 kg), collagen (+0.7 kg), and placebo (+1.2 kg).	A total of 1) 40 g whey protein, 2) 40 g collagen protein, or 3) placebo	Protein supplementation did not increase LM and CSA
Kerstetter et al. 2015 (191), USA	Parallel	30 M, 178 W	75 (mean)	18 months	3-d food record	DXA	72.9 g/day, ~1.07 g/kg/day	Energy intake was decreased in post from pre in protein (-120 kcal) and placebo (-4 kcal) groups. BW was maintained in both groups.	A total of 45 g whey protein or placebo	Protein supplementation did not increase LM
Zhu et al. 2015 (192), Australia	Parallel	181 W	70-80	2 years	3-d food record	DXA	76 g/day, 1.1-1.2 g/kg/day	BW was increased in protein (+0.5 kg) and placebo (+0.4 kg).	A total of 30 g of skim milk-based protein supplement or placebo	Protein supplementation did not increase LM

DXA, Dual-energy X-ray absorptiometry; E, energy; BW, body weight; LM, lean body mass; RDA, recommended dietary allowance; MRI, magnetic resonance imaging; BW, body weight; CSA, cross sectional area.

1.6.3 Protein turnover kinetic measurement

Protein turnover kinetic studies are used as more exploratory and mechanistic measurements of the underlying protein turnover kinetic rates. Since the 1970s (198), experimental settings with the results of muscle protein synthesis (MPS) based on the stable isotope tracer direct incorporation measurement using precursor-product methods (199, 200), have established an important basis of knowledge in protein nutrition. In particular, stable isotope tracer and mass spectrometer methodology have advanced our knowledge on MPS in response to amino acids or protein intake (201-203). Experiments with an infusion of stable isotope amino acid tracers are usually conducted for <24 hours and responses to ingestion of amino acids/proteins or mixed meals are measured in a controlled laboratory setting. Experiments are usually designed to measure postprandial MPS (~ 6 hours) in response to protein intake while participants are kept in artificial conditions such as fasting and bedridden, often with standardized prior dietary intake. The tissue of interest is often skeletal muscle with an emphasis on MPS rate. As muscle protein turnover contributes only approximately 25-35% of whole-body protein turnover in humans (204, 205), it is important to take this into consideration if the results from these studies are to be used for estimating protein requirement.

1.6.3.1 Skeletal muscle protein

Amino acid availability in the circulation is a determinant of MPS stimulation (206); a response that is dose-dependent and saturable even in the presence of sustained elevated circulating amino acids (90, 91, 207-211). Although originally hypothesized (212-214), recent accumulated evidence has concluded that postabsorptive MPS rates do not differ

between older and younger individuals (215, 216). However, older individuals exhibit a blunted postprandial MPS response to amino acid/protein intake when compared to younger individuals (208, 217-219), which has been termed age-related “anabolic resistance”. Moore and colleagues synthesized post-prandial MPS data generated in the lab previously (207) and suggested that younger and older individuals are required to consume 0.24 g/kg/meal and 0.4 g/kg/meal, respectively, to maximally stimulate MPS, meaning that approximately 70% greater protein is required to maximally stimulate MPS in older individuals compared to younger individuals.

A blunted MPS, and hence sub-optimal net balance in response to protein feeding and meals could be a plausible driver in the development of sarcopenia. This indicates that older adults need more dietary protein. However, it is important to note that this evidence is derived from acute postprandial MPS studies in response to high quality, rapidly digested, mostly animal-based proteins (e.g., egg, whey, or casein protein) (207, 219).

In addition to the limitations involved in this experimental design, the potential beneficial effects of higher protein intake for muscle and/or whole-body net protein balance or anabolic response are unknown without the simultaneous measurement of muscle or whole-body protein breakdown. Further, the protein recommendation refers to whole-body protein balance (155, 156), which is not easily comparable to the responsiveness of skeletal muscle. Hence, we argue that data originating from experimental settings on MPS responsiveness to a single dose of protein may lead to misinterpretation and erroneous conclusions on dietary protein requirement in humans

at the whole-body level, and therefore cannot be extrapolated to whole-body protein requirement in daily living.

In summary, studies measuring the acute postprandial response to protein feeding with the stable isotope tracer technique have led to the hypothesis that age-related muscle anabolic resistance. It is suggested as one of the underlying mechanisms of the development of sarcopenia (207, 208, 219). Such quantitative data suggests that protein intake of 0.4 to 0.6 g/kg/meal at 3 main meals and a snack per day is required to preserve muscle mass in healthy older individuals (see **Table 1.5**), which equates to the protein intake of <1.2-1.8 g/kg/day (220).

Table 1.5 Protein recommendation derived from acute stable isotope tracer incorporation studies in younger and older adults.

	Younger	Older
Dietary protein	(g/kg BM)	
Single meal	0.24	0.40
Day (3-4 meals)	0.72 - 0.96	1.2 - 1.6
Dietary protein	(g/ kg LBM)	
Single meal	0.25	0.61
Day (3-4 meals)	0.75 - 1.0	1.83 - 2.44
Dietary protein	(g)[1]	
Single meal	18	30
Day (3-4 meals)	54 - 72	90 - 120

[1] An individual with 75 kg of body mass (BM).

LBM, lean body mass.

Values were adopted from Moore et al. (207) determined by Biphasic Linear Regression Model from 6 independent studies (35, 85, 90, 91, 221, 222).

1.6.3.2 Whole-body protein

Previous studies investigating whole-body protein turnover in the context of mixed macronutrient meal intake demonstrated that anabolic response is not limited by protein synthesis (223-225). Deutz and Wolfe (226) advocated that there is no limit to *in vivo* whole-body protein anabolism when protein is consumed as a part of a mixed macronutrient meal (97; 101). More recently, Park et al. (225) showed in healthy older adults (69.3 ± 1.8 yrs.) that there is a higher MPS response following the consumption of a higher protein intake (70 g) compared to moderate (35 g) as part of a mixed macronutrient meal, and a greater whole-body net protein balance in the higher protein intake group due to the suppression of protein breakdown as well as increased protein synthesis (**Figure 1.7**). Importantly, the doses of protein used in this study (35 or 70 g) were beyond the amount suggested to maximally stimulate MPS in healthy older men (207) (see **Table 1.5**). Similar findings were also found by the same research group in healthy younger adults (223).

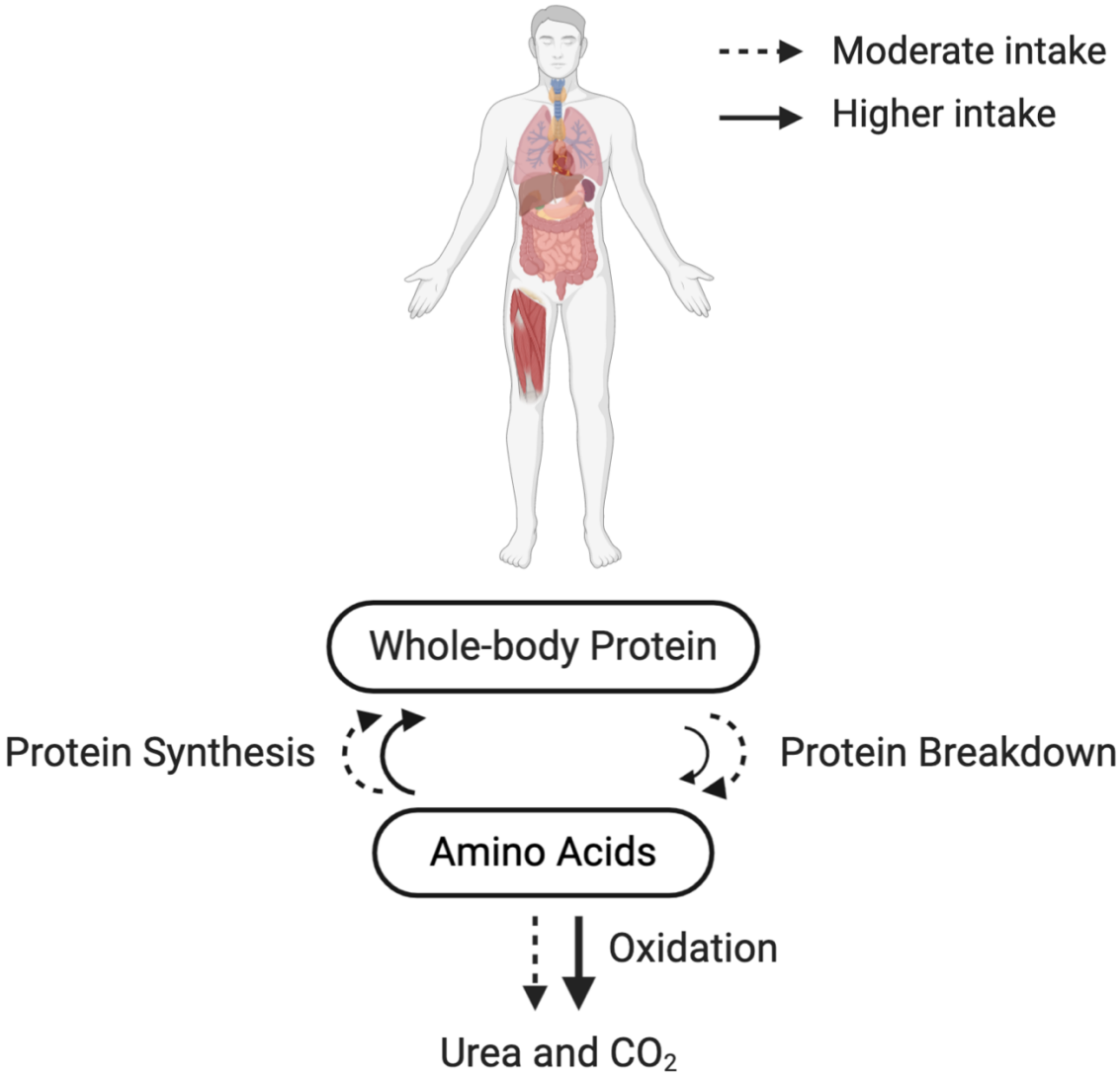

Figure 1.7 Whole-body protein turnover in response to the recommended or higher protein intake in a mixed macronutrient meal.

Dashed (- -) and solid (–) lines indicate a moderate (~35 g/meal) and a higher protein intake (~70 g/meal) in a mixed macronutrient meal, respectively. Protein synthesis is saturable at the given amounts of protein intake (223) – illustrated by the similar-sized arrows for protein synthesis between the moderate and higher protein intake. Protein breakdown is suppressed by the higher protein intake in a mixed macronutrient meal (illustrated by a smaller solid arrow), resulting in a greater whole-body net protein balance (223, 225-227). Be aware that a higher protein intake (i.e., a surplus of amino acids) also inevitably increases amino acid oxidation and urea excretion (197, 228). The results depicted in this figure were originated from collective data based on stable isotope tracer studies.

The suppression of protein breakdown may also be explained by insulin secretion from non-protein energy sources (229, 230). Higher insulin concentration in circulation is observed when protein is consumed in a mixed macronutrient meal (231). A blunted protein breakdown suppression (232) and a failure to achieve positive phenylalanine whole-body net balance (233, 234) in response to insulin have been reported in older individuals, which may suggest a difficulty in achieving net positive whole-body protein balance in older individuals in response to mixed macronutrient meal intake. A systematic review and meta-analysis concluded that insulin has a permissive role in MPS, whereas insulin attenuates MPB independent of amino acid availability (235, 236). These studies highlight the importance of protein breakdown measurement in the context of a mixed meal intake due to the suppression of protein breakdown in response to higher protein intake and insulin from non-protein energy sources, resulting in increased whole-body protein anabolism. Moving forward to the justification of the necessity to increase the current international safe level of intake for protein in healthy older adults, the upper limit of protein intake that suppresses protein breakdown, and thus better preserves whole-body protein, should be defined, as highlighted recently by Phillips et al. (170). We argue that whole-body protein turnover data and real-life interventions are more appropriate to extrapolate to protein requirements.

1.7 Knowledge gaps in the current literature

Over the past decades, extensive efforts have been made to uncover the regulatory mechanisms of skeletal muscle protein turnover and skeletal muscle mass control. Despite the progressive understanding in nutrition and exercise physiology, many

knowledge gaps still exist to fully understand the underlying mechanisms of skeletal muscle mass control for potential therapeutic targets.

Firstly, dietary protein plays an important role in the maintenance of skeletal muscle mass and physical function in healthy older individuals. However, it has long been discussed by many expert groups whether healthy older adults require more dietary protein than what is currently recommended by WHO/FAO/UNU to maintain skeletal muscle mass. It is currently unclear if other factors (e.g., protein distribution pattern, whole-body metabolic adaptation) in addition to the total amount of dietary protein intake are responsible for the maintenance of skeletal muscle mass and physical function in healthy older individuals.

Secondly, resistance exercise in combination with dietary protein intake has been well established to augment MPS response. Especially, amino acid availability in the circulation is the key factor to modulate MPS response after exercise. However, a strategy to enhance the absorption of orally consumed protein is currently unavailable. Such a strategy may facilitate skeletal muscle adaptations to resistance exercise.

Finally, the regulatory mechanisms of skeletal muscle protein breakdown are not fully understood. To date, muscle-specific ubiquitin E3 ligases Atrogin-1 and MuRF1 have been identified as useful markers of skeletal muscle atrophy. However, upstream signalling mechanisms that regulate Atrogin-1 and MuRF1 protein in skeletal muscle are not yet clearly defined, which essentially execute the enzymatic ubiquitin E3 ligase activity. mTORC1 signalling pathway is well characterized as a master regulator of

protein synthesis, but it is unclear whether the mTORC1 signalling pathway plays a role in regulating Atrogin-1 and MuRF1 protein in skeletal muscle.

1.8 Specific objectives of the thesis

The overarching aim of this thesis is to provide novel data in skeletal muscle physiology to develop our current understanding of the mechanistic regulation and nutritional modulation of skeletal muscle protein turnover from a cellular level to humans.

In **CHAPTER 2**, we investigated daily protein intake and its distribution throughout the day and how they are associated with muscle mass and physical function in healthy older individuals in a cross-sectional study.

In **CHAPTER 3,** we aimed to elucidate the impact of Cluster Dextrin carbohydrate on the absorption of orally consumed intrinsically labelled meat protein hydrolysate, myofibrillar protein fractional synthetic rate, and Akt/mTORC1 signalling following a whole-body resistance exercise in moderately trained younger individuals.

In **CHAPTER 4**, we used small molecule inhibitors to investigate if the mTORC1 signalling pathway plays a role in regulating Atrogin-1 and/or MuRF1 protein in C2C12 myotubes.

Finally, **CHAPTER 5** integrates the results obtained from this thesis into the current literature and provides the implications and future directions to advance the knowledge within skeletal muscle physiology.

CHAPTER 2

DAILY PROTEIN AND ENERGY INTAKE ARE NOT ASSOCIATED WITH MUSCLE MASS AND PHYSICAL FUNCTION IN HEALTHY OLDER INDIVIDULAS – THE CALM STUDY

2 Daily protein and energy intake are not associated with muscle mass and physical function in healthy older individuals – The CALM study

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2.1 Abstract

Dietary protein has a pivotal role in muscle mass maintenance with advancing age. However, an optimal dose and distribution of protein intake across the day as well as the interaction with energy intake for the maintenance of muscle mass and physical function in healthy older adults remain to be fully elucidated. The purpose of this study was to examine the association between muscle mass, strength, and physical function, and the total amount and distribution of protein and energy intake across the day in healthy older individuals. The research question was addressed in a cross-sectional study including 184 Danish men and woman (age: 70.2 ± 3.9 years, body mass: 74.9 ± 12.1 kg, Body Mass Index (BMI): 25.4 ± 3.7 kg/m^2) where a 3-day dietary registration, muscle mass, strength, and functional measurements were collected. We found that neither daily total protein intake nor distribution throughout the day were associated with muscle mass, strength, or physical function. Consequently, we do not provide an incentive for healthy older Danish individuals who already adhere to the current internationally accepted recommended dietary protein intake (0.83 g/kg/day) to change dietary protein intake or its distribution pattern throughout the day.

KEYWORDS: sarcopenia; ageing; dietary protein; protein distribution; elderly; muscle mass

2.2 Introduction

Dietary protein continues to receive attention in an effort to combat sarcopenia (164, 165, 169, 237, 238). The most recent recommendation from the Nordic Council of Ministers is 1.1–1.3 g protein/kg Body Weight (BW)/day (15–20 energy %) for older adults above the age of 65 years (158). Meanwhile, globally, the recommended intake for all adults remains at 0.83 g protein/kg BW/day (155, 239). The global recommendation was developed based on a meta-analysis of nitrogen balance measurements from various dietary protein intake levels (156). However, the recommended value was based on nitrogen balance methodology that, with currently available data, neither distinguishes age nor accounts for factors such as sex and meal distribution patterns across the day. While some studies report similar protein requirements in healthy younger and older adults using the nitrogen balance methodology (166, 240), a growing number of epidemiological studies assessing the associations between protein intake and muscle mass suggest that muscle mass and/or function in older individuals can be maintained with protein intake levels matching the recommendation made by the Nordic Council of Ministers (181, 190, 241-243).

Protein distribution throughout the day is considered an important factor in muscle mass maintenance (244-247). This notion has grown from the findings of a marked, transient stimulation of Muscle Protein Synthesis (MPS) in response to hyperaminoacidemia (209, 248) by the bolus intake of fast absorptive proteins (207). The dose–response relationship between protein intake and MPS plateaus at ~30–45 g protein per serving (0.4–0.6 g/kg BW) for a 75 kg older adult (>65 years) (207) with no cumulative effect of protein intake over a period of time and rather oxidized beyond this amount (91).

This has led to the concept of an optimal dose of ~30 g of protein per meal to maximally stimulate MPS (207) which is required at all three main meals and snacks in a day (1.2–1.8 g/kg BW/day) (220, 249). However, some challenges exist when daily protein recommendations are extrapolated from MPS, which have been determined in acute experimental studies in response to various doses of protein ingestion. Among these, the interaction between protein and energy intake (250) as the intake of other macronutrients and/or total energy is known to markedly affect postprandial aminoacidemia (231, 251, 252) and whole-body net protein balance (253). In addition, the type of studies investigating the stimulatory effect of a bolus ingestion of protein rarely account for the habituated level of dietary intake (i.e., the protein intake the participant is accustomed to). We and others have demonstrated that adaptation to higher protein intakes alters amino acid utilization via an increase in amino acid oxidation and urea production (197, 254-256).

A cross-sectional study allows for an investigation of the association between habitual protein and energy intake in a matrix of normal foods and physiological outcomes, such as muscle mass, strength, and functional capabilities. In westernized and industrialized cultures, the common dietary pattern consists of three main meals throughout the day with smaller in-between snacks. A recent cross-sectional analysis including 38 older UK citizens above the age of 70 years found that more than half of total protein intake was consumed in one meal (257). Similar dietary patterns have been reported in studies from westernized countries, showing that >43% of protein is ingested in one single meal (258, 259). If we accept the premise that there is a dose–response relationship between protein intake and net postprandial protein anabolism, the protein content per meal is

important. Additionally, total daily protein intake and per meal protein requirement for the maintenance of whole-body protein are interrelated with energy intake (250, 260, 261) and energy balance (262). Thus, optimal protein intake for the maintenance of muscle mass should be assessed in combination with energy intake (263).

The purpose of this cross-sectional study was to examine how daily protein and energy intake as well as distribution are associated with muscle mass, strength and physical function in healthy older, and well-functioning Danish men and women.

2.3 Materials and Methods

The results are based on a cross-sectional study of 184 older Danish home-dwelling men and women from the CALM (Counteracting Age-related Loss of skeletal Muscle mass) cohort, which is described in detail elsewhere (264). Methods with relevance for this article are explained below.

2.3.1 Participants

A total of 184 men and women older than 65 years of age were included in this cross-sectional study. The participants were recruited through local newspapers, magazines, radio programs, social media, presentations at senior centers and public events. All participants were within the exclusion criteria listed in the method paper (264) and deemed healthy, as assessed by a medical doctor based on blood samples and an oral interview. Briefly, exclusion criteria include care dependency, disabilities in lower extremities, arthritis, diagnosed or suspected knee osteoarthritis, bilateral knee alloplastic and hip alloplastic material, connective tissue disorders, severe chronic obstructive pulmonary disease, unstable cardiac arrhythmias or decreased left

ventricular ejection fraction, gut diseases affecting food absorption, surgical diseases affecting ability to conduct heavy load strength exercise, embodied magnetic metal, endocrinological diseases potentially affecting muscles, alcohol consumption >21 U/week for men and 14 U/week for women (1U = 15.2 ml of alcohol), medications including systemic corticosteroids, sex hormone therapy, anti-sex hormone therapy, anticoagulants, >1 h of weekly heavy strength training, dementia or other severe cognitive impairment, and not holding Danish citizenship or not fluent in Danish. Participant data are presented in **Table 2.1**. Participants were informed of the study design, risks, and exclusion criteria prior to obtaining written consent. The study complied with the latest Declaration of Helsinki (7th version). Ethical approval was obtained through The Danish Regional Committees of the Capital Region on 4 July 2013 (number H-4-2013-070). The CALM intervention study was registered at Clinicaltrials.org as NCT02034760.

Table 2.1 Participant characteristics for all, and divided into sex for higher (≥1.1 g/kg aBW/day) and lower protein intakes (<0.83 g/kg aBW/day).

	All (*n* = 184)	Women Lower (*n* = 13	Women Higher (*n* = 48)	Men Lower (*n* = 12)	Men Higher (*n* = 50)	*p* Women/ Men
Age (years)	70 2 ± 3 9	71 7 ± 4 1	71 0 ± 4 0	71 8 ± 5 7	68 9 ± 3 5	0 52/0 0 4
Age range (years)	65 – 82	65 – 80	65 – 81	66 – 82	65 – 78	
Height (m)	1 72 ± 0 10	1 65 ± 0 07	1 66 ± 0 06	1 79 ± 0 06	1 76 ± 0 06	0 93/0 3 0
Body Weight (kg)	74 9 ± 12 1	69 6 ± 7 7	65 4 ± 11 4	78 7 ± 7 2	79 0 ± 11 8	0 19/1 0 0
BMI (kg/m^2)	25 4 ± 3 7	25 6 ± 4 0	23 8 ± 3 9	24 6 ± 2 2	25 4 ± 3 5	0 13/0 6 1
aBW (kg)	73 ± 8 7	67 5 ± 3 1	65 2 ± 7 0	78 4 ± 6 5	76 6 ± 7 6	0 23/0 4 4
WB LBM (kg)	48 5 ± 8 6	39 9 ± 2 6	40 2 ± 4 2	54 5 ± 4 3	55 0 ± 5 3	0 76/0 8 1
App LBM (kg)	22 4 ± 4 6	18 2 ± 3 0	18 3 ± 2 0	25 4 ± 2 1	26 1 ± 3 3	0 84/0 4 3
EI (MJ/day)	8 2 ± 2 1	6 0 ± 1 3	8 5 ± 1 8	6 3 ± 1 6	9 7 ± 2 0	<0 001/ <0 001
Protein (Energy %)	17 6 ± 4 0	14 3 ± 2 6	18 7 ± 4 8	15 9 ± 5 4	18 6 ± 3 1	<0 01/0 03
Protein (g/day)	82 8 ± 22 2	49 0 ± 8 5	90 6 ± 16 7	55 2 ± 10 2	104 3 ± 17 9	<0 001/ <0 001
Protein (g/kg BW/day)	1 13 ± 0 34	0 70 ± 0 11	1 41 ± 0 30	0 70 ± 0 11	1 34 ± 0 25	<0 001/ <0 001
Protein (g/kg aBW/day)	1 15 ± 0 31	0 73 ± 0 12	1 39 ± 0 25	0 70 ± 0 11	1 37 ± 0 23	
Goldberg Score						
EI/BMR	0 96 ± 0 24	0 77 ± 0 14	1 10 ± 0 24	0 65 ± 0 17	1 00 ± 0 19	<0 001/ <0 001
Underreporters, *n*	41 (22%)	8 (53%)	4 (8%)	11 (85%)	5 (11%)	
Overreporters, *n*	18 (10%)	0 (0%)	9 (19%)	0 (0%)	6 (13%)	
Physical activity Step counts (Steps/day)	9740 ± 4358	9598 ± 3600	10,723 ± 4232	10,059 ± 5771	9297 ± 3392	0 036/0 30

aBW: adjusted body weight, BMI: body mass index, WB LBM: whole body lean body mass, App LBM: appendicular lean body mass, EI: energy intake, BMR: basal metabolic rate. The higher and lower protein intake differences of participant characteristics were compared using an unpaired t test. Significance was set at $P < 0.05$.

2.3.2. Dietary Records

A 3-day consecutive weighed dietary and liquid registration (Wednesday to Friday) was collected by following instructions from trained staff as described by Schacht et al. (265). Trained staff entered the dietary records into the VITAKOSTTM (MADLOG

Aps, Kolding, Denmark) program which uses the Danish Food Composition Databank (version 7.01; Søborg; Denmark) for calculating the nutrient intakes.

2.3.3. Physical Activity Level

The activity level of the participant was estimated by taping an activPal (activPal 3TM, activPal 3cTM, or activPal micro; PAL Technologies, Glasgow, UK) to the anterior surface of the thigh, which monitoring the steps and body (thigh) position over a 4-day period. Weekend days were always included.

Energy expenditure reflecting the average daily Metabolic Equivalent of Tasks (MET) was calculated based on the algorithms provided in the activPal software. Data are reported as the average daily step count and average daily METs.

2.3.4. Identification of Under- and Overreporters

Under- and overreporters of energy intake were identified based on the Goldberg cut-off, which is determined by the ratio between the Energy Intake and Basal Metabolic Rate (EI:BMR) (266). The total energy expenditure was estimated via the Cunningham equation (Resting Energy Expenditure (REE, kJ/day) = 370 + 21.6 × fat free mass (Lean Body Mass; LBM) × 4.184) (267) multiplied by the physical activity level in each participant (METs) assessed by an activPal (268). As described by Black (268) a cut-off <0.76 was defined as an underreporter and >1.24 as an overreporter. All statistical analyses were performed including and excluding under- and overreporters.

2.3.5. Coefficient of Variation for Protein Distribution

The evenness of protein distribution between main meals in the day was determined by calculating the Coefficient of Variation (CV) (Standard Deviation (SD) divided by the mean) of the protein in grams per main meal, covering breakfast, lunch and dinner for each participant as previously reported (245, 257, 269). Thus, a lower CV indicates that protein was consumed more evenly across main meals compared to a higher CV.

2.3.6. Appendicular Lean Mass

As a marker of muscle mass, the Appendicular Skeletal Muscle Index (ASMI) was assessed by whole body Dual-energy X-ray Absorptiometry (DXA) scans, using the encore v.16 software (Lunar iDXA; GE Medical Systems, Pewaukee, WI, USA). The scans were performed in an overnight fasted and euhydrated state, with the participants refraining from strenuous physical activity for 48 h prior to the scans. Regions of interest were set based on the default definitions provided by the scanner software. The same examiner controlled the default positioning of all regions, which were adjusted slightly when appropriate to take into account the interindividual differences in body placement and body size. Appendicular lean mass was assessed by the sum of lean mass in the arm and leg regions, and the ASMI was calculated by dividing the appendicular lean mass by the height squared (270).

2.3.7. Muscle Strength and Functional Capability

The functional capacities of the participants were assessed by applied functional measures and strength measures. Detailed methods for these measurements can be found elsewhere (264, 271). The strength measures included: the dominant hand grip

strength using a grip strength dynamometer (DHD-1 (SH1001); SAEHAN Corporation, Changwon City, Korea), Maximal Voluntary isometric Contraction (MVC) of the dominant m. quadricep muscle strength, measured at 70-degree flexion in a Kinetic Communicator (model 500-11, Kinetic Communicator; Isokinetic International, Chattanooga, TN, USA). The applied functional measures included a 30 s chair stand test and a 400 m gait test. In the 30 s chair stand test (272), the number of stands completed in 30 s from a seated position (seat height: 44.5 cm) with hands crossed across the chest were counted. The 400 m gait test was performed on a 20 m course with no helping remedies, instructing the participants to walk the 400 m as fast as possible without running (273).

2.3.8. Food Questionnaire

At baseline, the participants received a questionnaire containing a range of questions related to their food preferences and habits as well as lifestyle and dietary changes throughout life. The questionnaires combined basic socioeducational data, quantitative questions and quantifiable qualitative questions. For this article, 149 questionnaires have been screened for information on dietary changes in relation to retirement.

2.3.9. Groups Division

Adjusted Body Weight (aBW) was determined to give a BMI of either 22 or 27 for participants exhibiting a BMI below 22 or above 27, respectively (i.e., BMI >27: 27 × height in meters squared (m^2), BMI <22: 22× height in meters squared (m^2) (259, 274). This was done to ensure that the energy and nutritional requirements are expressed

relative to a body weight representing a body composition within a healthy range (275-277).

If participants did not report any intake at a meal, they received a zero in their energy intake, but they were excluded from the group mean for protein intake.

For data on total intake and distribution of dietary protein and energy, participants were divided into two groups: a lower (<0.83 g/kg aBW/day) and a higher (≥1.1 g/kg aBW/day) protein intake group. These were based on the current Recommended Daily Allowance (RDA) by the European Food Safety Authority (EFSA) (239) and World Health Organization (WHO)/Food and Agriculture Organization of the United Nations (FAO)/United Nations University (UNU) (155) at 0.83 g/kg/day for adults and the current fifth edition of Nordic Nutrition Recommendations 2012 (158) at 1.1–1.3 g/kg/day for older adults above the age of 65 years in men and women. Further, we also converted the absolute protein intake in g/kg/day to the energy % expression, as the relative energy contribution from protein intake of total energy intake.

For the comparison between total protein intakes and physical parameters, participants were divided into 3 different total daily protein intake groups as follows: <0.83 g/kg aBW/day, ≥0.83–<1.1 g/kg aBW/day, ≥1.1 g/kg aBW/day in men and women.

Associations between the distribution of protein intake and functional abilities were examined only within the groups ingesting a lower protein (<0.83 g/kg aBW/day) and those ingesting a higher protein (≥1.1 g/kg aBW/day) in order to investigate 'extremes' and exclude the middle group, where inherent variation of the food recording methodology and participants eating behavior may make the group-affiliation unreliable.

2.3.10. Statistical Analysis

The higher and lower protein intake differences of participant characteristics were assessed using an unpaired t test. A three-way mixed effects model (average daily protein intake levels × sexes × main meals) was used for energy and protein intake and energy% from protein intake. A two-way ANOVA (sexes × average daily protein intake levels) was used for the ASMI, grip strength, MVC, 400 m gate time, 30 s chair stand test and CV of protein intake. Turkey's multiple comparisons test was used as a post hoc test. Person's correlation coefficient was used to identify associations between protein and energy intake and associations between the CV of protein distribution and ASMI, grip strength, MVC, 400 m gait time, and 30 s chair stand test. Statistical significance was set at $P < 0.05$ (two-tailed). The statistical analyses were performed using Prism version 8.1.2 (GraphPad Software, San Diego, CA, USA).

2.4 Results

2.4.1 Participant Characteristics

A total of 184 participants (53% men and 47% women) with a 3-day weighed dietary record with muscle mass, muscle strength, and muscle function measurements at the time of study entry of the CALM study (264) were included in the present study. **Table 2.1** shows the participant characteristics for all 184 participants included, as well as divides men and women into lower (<0.83 g/kg aBW/day) and higher (≥1.1 g/kg aBW/day) protein intake groups. Individuals with a protein intake that does not fit in the category 'lower' or 'higher' (i.e., ≥0.83–<1.1 g/kg aBW/day) were included into the 'all' group only.

The age of the participants ranged from 65 to 82 years with no significant difference between men (69.8 ± 3.9 years) and women (70.5 ± 4.0 years), and no significant difference between women with a higher and a lower protein intake. However, the men with a higher protein intake were significantly younger than the men ingesting a lower protein intake ($P < 0.05$). Within groups of men and women, there was no difference between groups with higher and lower protein intakes with regards to body weight, adjusted body weight, lean body mass (LBM), appendicular LBM and step counts. Based on the Goldberg cut-offs (266), underreporters (22% (n = 41)) and overreporters (10% (n = 18)) were identified based on their reported energy intake. However, all statistical outcomes were identical irrespective of the inclusion or exclusion of under- and overreporters. Thus, all figures and statistical outcomes were illustrated including all participants (i.e., both underreporters and overreporters).

2.4.2 Total Daily Energy and Protein Intake and Distribution per Main Meal

In our cohort, participants consumed on average 82.8 ± 22.2 g protein/day corresponding to 1.13 ± 0.34 g protein/kg/day (men; 1.10 ± 0.31 g/kg/day, women; 1.16 ± 0.38 g/kg/day) (**Table 2.1**). The total daily energy intake and protein intake were greater in the higher protein intake group than the lower protein intake group for both men and women (right panel of **Fig. 2.1a,b**). For both energy and protein intakes, there was a main effect between main meals ($P < 0.0001$). A post hoc test showed that in the higher protein intake group there was a higher protein intake at dinner compared to both at breakfast ($P < 0.05$) and at lunch ($P < 0.05$) for both men and women. In the lower protein intake group, energy and protein intakes at dinner were greater than at breakfast ($P < 0.05$) for women only. Main effects were also found for protein intake level

between meals ($P < 0.0001$), with post hoc differences showing that women with a high protein intake had a higher total energy intake at breakfast, and a higher protein intake at all three main meals ($P < 0.05$). The men who had a higher protein intake only ingested more protein at lunch and dinner, compared to the men with a lower protein intake. Finally, there was main effect of sex ($P = 0.01$) for energy and protein intake. The distribution of energy (**Fig. 2.1c**) and protein (**Fig. 2.1d**) expressed as the coefficient of variation (CV) between main meals did not differ between men and women nor between higher (42 ± 22 in men and 46 ± 20 in women) and lower (60 ± 34 in men and 59 ± 22 in women) protein intakes.

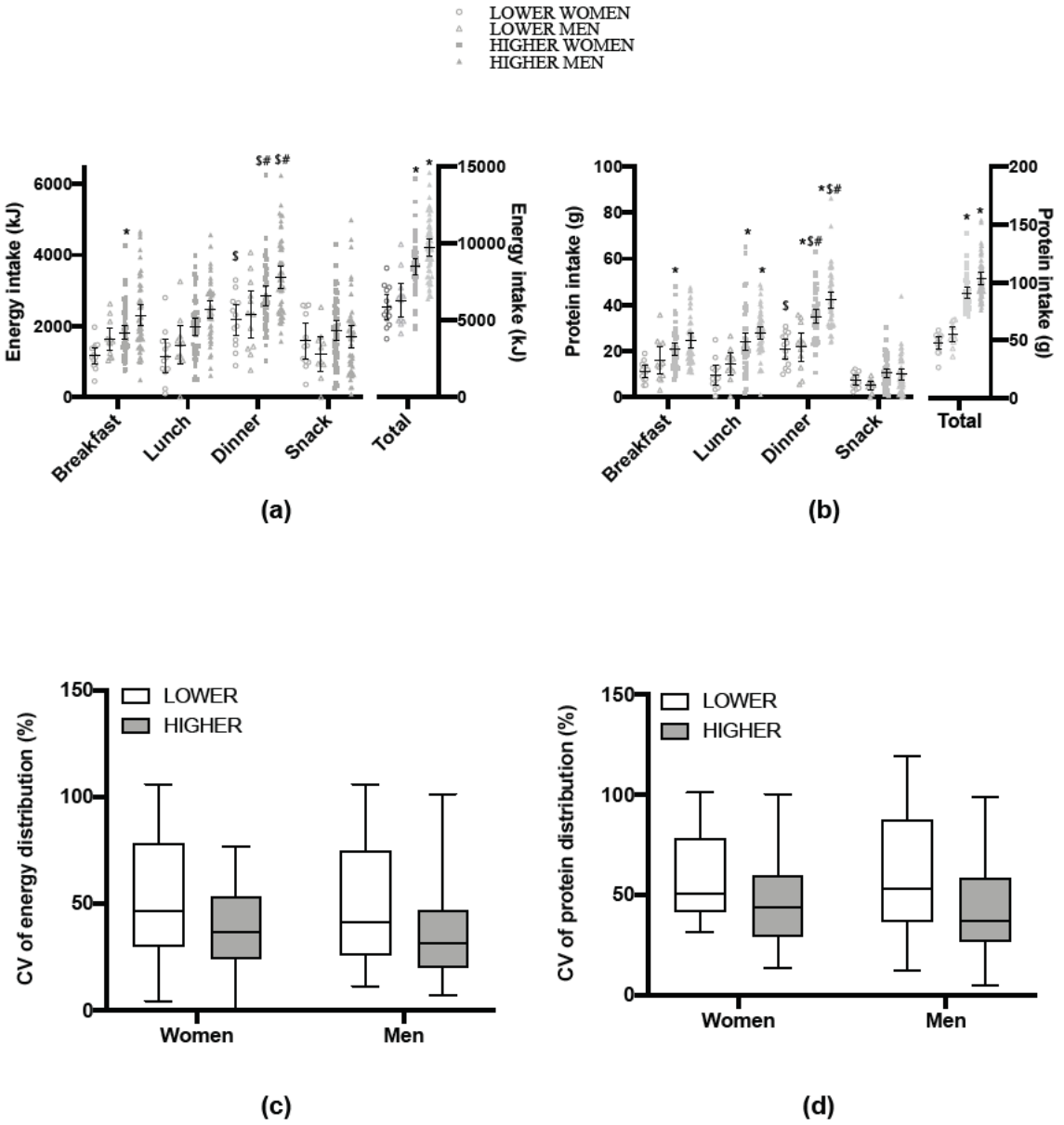

Figure 2.1 Baseline average energy (a) and protein (b) intake and distribution per meal, snacks during the day, and total daily intake. The Coefficient of Variation (CV), as a measure of the distribution between the three main meals was shown for energy intake (c) and protein intake (d). For (a) and (b), Turkey's multiple comparison test (comparing between main meals and sexes). Values are means with 95% Confidence Interval (CI). Significance was set at $P < 0.05$. * indicates significant difference between the lower and higher protein intake in the same meal, $ indicates significant difference between breakfast and dinner, # indicates significant difference between lunch and dinner. For (c) and (d), the participants were divided a lower (<0.83 g/kg Adjusted Body Weight (aBW)/day; n = 25, 12 men and 13 women) or higher (≥1.1 g/kg aBW/day; n = 98, 50 men and 48 women) protein intake. The boxes

include the 25th, 50th, and 75th quartiles and whiskers represent the maximum and minimum values. Significance was set at $P < 0.05$. No main effect for intake amount or sex was found ($P > 0.05$).

2.4.3. Daily Protein Intakes and the ASMI, Grip Strength, MVC, 400 m Gait Time, 30 s Chair Stand

Overall, the ASMI, grip strength, MVC, 400 m gait time, 30 s chair stand did not differ between the three different protein intakes (<0.83 g/kg aBW/day, ≥0.83–<1.1 g/kg aBW/day, ≥1.1 g/kg aBW/day) in both men and women (**Fig. 2.2**). However, sex differences were noted in the ASMI, grip strength, and MVC at <0.83 g/kg aBW/day, ≥0.83–<1.1 g/kg aBW/day, ≥1.1 g/kg aBW/day protein intake levels. The main effect of sex was identified in the ASMI ($P < 0.0001$), grip strength ($P < 0.0001$), and MVC ($P < 0.01$).

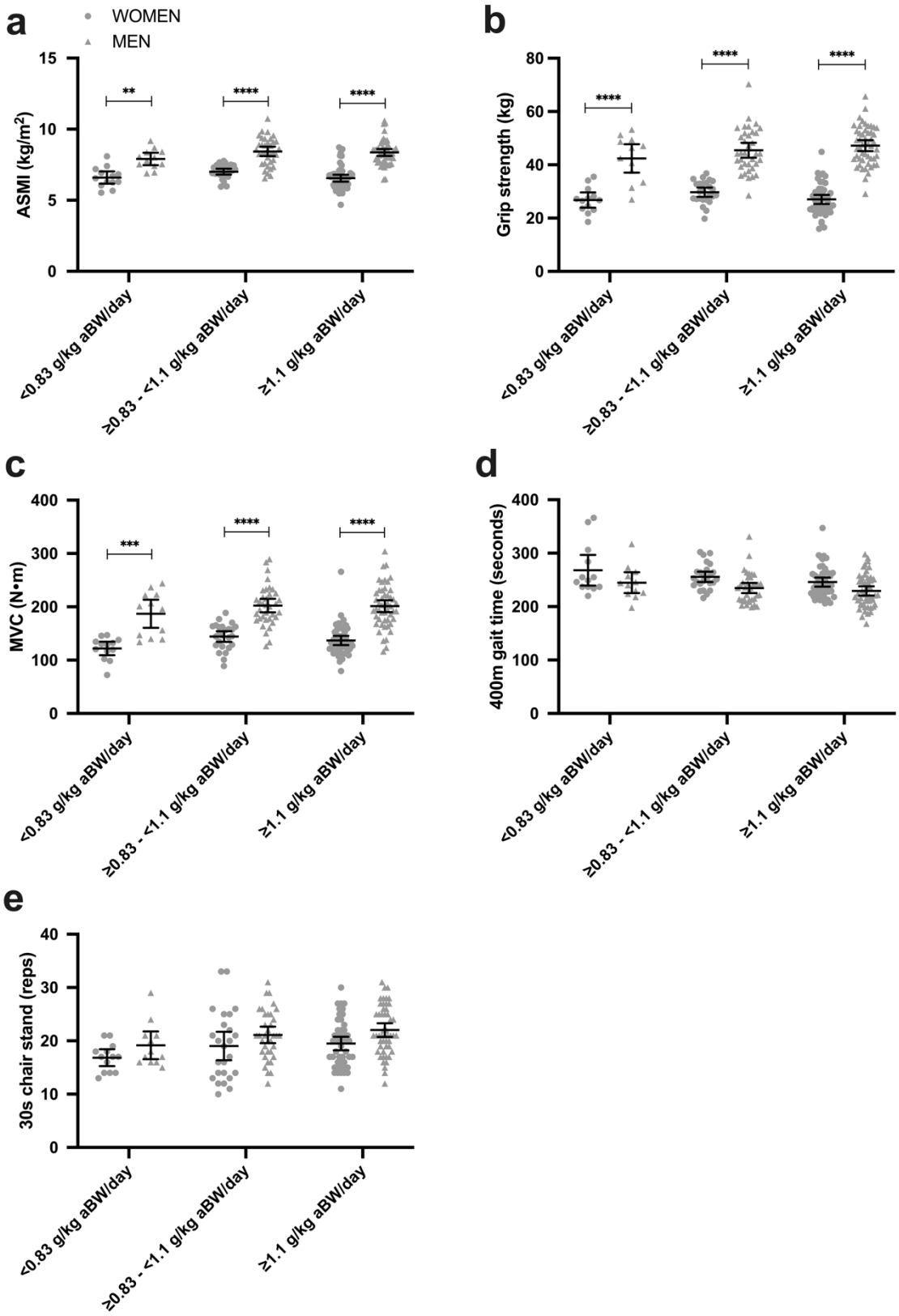
a
WOMEN
MEN
ASMI (kg/m²)
15
10
5
0
**
****
****
<0.83 g/kg aBW/day
≥0.83 - <1.1 g/kg aBW/day
≥1.1 g/kg aBW/day
b
Grip strength (kg)
80
60
40
20
0
****
****
****
<0.83 g/kg aBW/day
≥0.83 - <1.1 g/kg aBW/day
≥1.1 g/kg aBW/day
c
MVC (N•m)
400
300
200
100
0
***
****
****
<0.83 g/kg aBW/day
≥0.83 - <1.1 g/kg aBW/day
≥1.1 g/kg aBW/day
d
400m gait time (seconds)
400
300
200
100
0
<0.83 g/kg aBW/day
≥0.83 - <1.1 g/kg aBW/day
≥1.1 g/kg aBW/day
e
30s chair stand (reps)
40
30
20
10
0
<0.83 g/kg aBW/day
≥0.83 - <1.1 g/kg aBW/day
≥1.1 g/kg aBW/day

Figure 2.2 Appendicular Skeletal Muscle Index (ASMI) (a), grip strength (b), knee extension Maximal Voluntary isometric Contraction (MVC) (c), 400 m gait time (d), 30 s chair stand (e) divided into individuals with a protein intake of <0.83 g/kg aBW/day (n = 25, 12 men and 13 women), ≥0.83–<1.1 g/kg aBW/day (n = 61, 36 men and 25 women), ≥1.1 g/kg aBW/day (n = 98, 50 men and 48 women (n = 47 for MVC due to one missing value).

Values are means with 95% CI. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Turkey's multiple comparison test (comparing between average daily protein intake levels and sexes). Significance was set at $P < 0.05$.

2.4.4. Associations between the Protein Distribution and ASMI, Grip Strength, MVC, 400 m Gait Time and 30 s Chair Stand

The association between the distribution of protein intake and functional abilities was examined separately for the participants ingesting lower amounts of protein (<0.83 g/kg aBW/day) and those ingesting higher amounts of protein (≥1.1 g/kg aBW/day) (**Fig. 2.3**). Independent of the level of protein intake, the distribution was not associated with any of the functional measures ($P > 0.05$), except for MVC, where for women ingesting the highest level of protein there was a significantly higher MVC at the lowest CV values ($P = 0.03$, $r = -0.32$) for women ingesting the lowest level of protein, there was a significantly faster 400 m gait speed at the highest CV values ($P = 0.02$, $r = 0.62$).

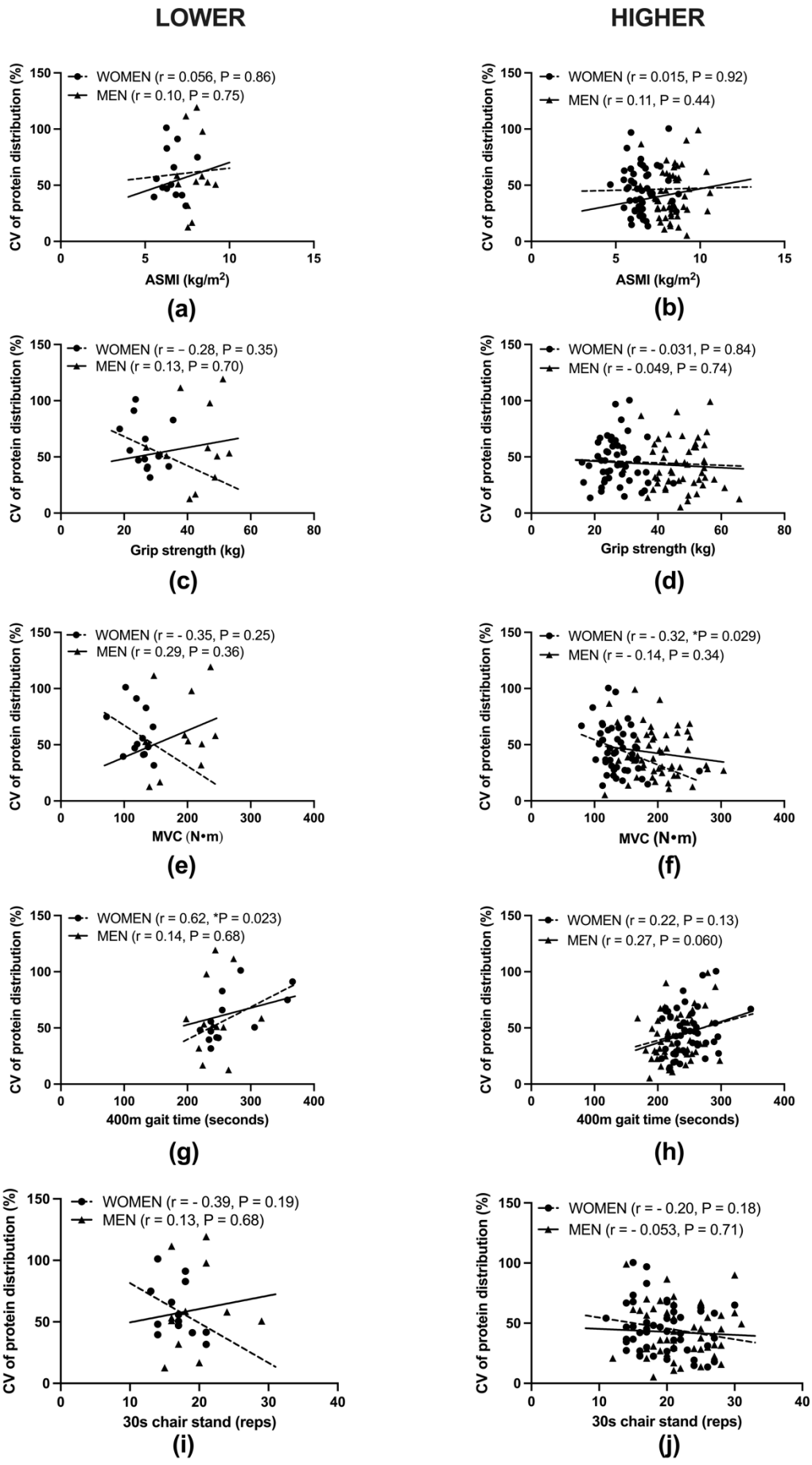
LOWER
HIGHER
WOMEN (r = 0.056, P = 0.86)
MEN (r = 0.10, P = 0.75)
CV of protein distribution (%)
ASMI (kg/m²)
(a)
WOMEN (r = 0.015, P = 0.92)
MEN (r = 0.11, P = 0.44)
ASMI (kg/m²)
(b)
WOMEN (r = – 0.28, P = 0.35)
MEN (r = 0.13, P = 0.70)
Grip strength (kg)
(c)
WOMEN (r = - 0.031, P = 0.84)
MEN (r = - 0.049, P = 0.74)
Grip strength (kg)
(d)
WOMEN (r = - 0.35, P = 0.25)
MEN (r = 0.29, P = 0.36)
MVC (N•m)
(e)
WOMEN (r = - 0.32, *P = 0.029)
MEN (r = - 0.14, P = 0.34)
MVC (N•m)
(f)
WOMEN (r = 0.62, *P = 0.023)
MEN (r = 0.14, P = 0.68)
400m gait time (seconds)
(g)
WOMEN (r = 0.22, P = 0.13)
MEN (r = 0.27, P = 0.060)
400m gait time (seconds)
(h)
WOMEN (r = - 0.39, P = 0.19)
MEN (r = 0.13, P = 0.68)
30s chair stand (reps)
(i)
WOMEN (r = - 0.20, P = 0.18)
MEN (r = - 0.053, P = 0.71)
30s chair stand (reps)
(j)

Figure 2.3 The association between the protein distribution for participants with a lower protein intake (*n* = 25, 12 men and 13 women) and a higher protein intake (*n* = 98, 50 men and 48 women (*n* = 47 for Maximal Voluntary isometric Contraction (MVC) due to one missing value)) and Appendicular Skeletal Muscle Index (ASMI) ((a) /Lower, (b) /Higher), grip strength ((c) /Lower, (d) /Higher), knee extension MVC ((e) /Lower, (f) /Higher), 400 m gait time ((g) /Lower, (h) /Higher), and 30 s chair stand ((i) /Lower, (j) /Higher).

*$P < 0.05$, Pearson's correlation coefficient was used to identify the associations. Significance was set at $P < 0.05$.

2.4.5. Associations between Protein (g/kg aBW) and Energy (kJ/kg aBW) Intake

For total daily intakes and individual main meals at breakfast, lunch, and dinner, protein intakes were positively associated with energy intakes irrespective of sex ($P < 0.0001$) (**Table 2.2**).

Table 2.2 Associations between protein (g/kg aBW) and energy (kJ/kg aBW) intake.

	Total		Breakfast		Lunch		Dinner	
	r	R^2	r	R^2	r	R^2	r	R^2
Women	0.69	0.48	0.72	0.52	0.82	0.68	0.56	0.32
Men	0.70	0.49	0.89	0.79	0.74	0.55	0.61	0.37

For all associations *P*-values are <0.0001.

2.5 Discussion

In the present study, we investigated the association between the total and meal distribution of protein and energy intake during the day, and the muscle mass, strength and physical function in healthy and well-functioning older Danish men and women. No association with the ASMI, grip strength, knee extension MVC or 400 m gait time appeared when dividing participants into three groups based on their protein intake (<0.83; ≥0.83–<1.1; ≥1.1 g/kg aBW), comparing the lowest and the highest of these

three groups, or when correlating the protein meal distribution pattern. This suggests that within a population of healthy Danish older adults, neither the total protein intake nor meal distribution appear to be associated with muscle mass, strength, or physical functions. Our data do not strengthen the emerging hypothesis that older adults need more protein than what is currently recommended to maintain muscle mass and physical function (159, 160, 278).

On average, the participants ingested more protein than 0.83 g/kg BW/day as recommended by the EFSA (239) and WHO/FAO/UNU (155). However, there were no differences between those ingesting more than the recommendations and those ingesting less protein (**Fig. 2.2**) when the total protein intake (<0.83; 0.83–<1.1; ≥1.1 g/kg aBW) and ASMI (**Fig. 2.2a**) and various functional measures (**Fig. 2.2b–e**) were compared. Similarly, Bollwein and colleagues found that among German seniors above the age of 75 years, daily protein intake was relatively high and did not make up any risk factor for frailty, and it was not evident even when they compared the highest and lowest quantile of protein intakes (269).

Protein intake pattern across the day, in addition to the total protein intake, has been suggested as an important factor for protein turnover and muscle mass (246). A possible link between meal distribution pattern and benefits for muscle mass maintenance was demonstrated in an acute experimental study in healthy adults (36.9 ± 3.1 years) by Mamerow et al. (244). They reported that the 24 h fractional synthesis rate is higher in an even protein distribution pattern (breakfast: lunch: dinner = 30: 30: 30 g) compared to a skewed distribution pattern (breakfast: lunch: dinner = 10: 15: 65 g). In a cross-

sectional study, Bollwein and colleagues observed that the individuals in the frailest group (75–96 years) had a more skewed protein distribution pattern (less at breakfast and more at lunch) than those in the least frail group (76–91 years). It should be noted that neither group had an even distribution (269). Supporting this notion, an observational study in adults (50–85 years) reported by Loenneke et al. (247) showed that those ingesting two meals or more containing above 30 g protein had a higher lean mass than those ingesting one meal or less containing 30 g protein. Similarly, in a two year follow up study in a 67–84 year old Canadian population by Farsijani et al. (243), those categorized as having the most even protein intake distribution pattern determined as the CV had a higher lean mass throughout the study regardless of total protein intake (~1.1 g/kg BW/day). In contrast, the decline in lean mass (243) and physical function (279) over the two-year period did not differ between protein intake patterns. Likewise, Kim and colleagues reported that total protein intake, but not the intake pattern, is responsible for the achievement of greater whole-body protein net balance in a study where a mixed macronutrient diet was provided in healthy older adults (52–75 years) (253). They further supported the finding in an 8-week randomized controlled study that protein intake patterns in the context of a mixed macronutrients meal across the day is not a determinant of whole-body protein anabolism, MPS, muscle mass, and muscle function when an average amount of protein is consumed (i.e., 1.1 g/kg/day) in healthy older adults (51–69 years) (224).

In the present study, the participants were divided into higher and lower protein intake groups in order to investigate the association between the protein distribution (CV) and ASMI and physical performance parameters (**Fig 2.1** and **Fig. 2.3**). The use of the CV

was adopted to isolate the distribution of protein, and not the amount of protein, as a variable. For example, it would be difficult to compare a skewed protein intake over the three main meals of 30, 30 and 60 g (120 g in total) with an even intake of 15, 15 and 15 g (45 g in total), as recently discussed by Hudson et al. (280). It is evident, despite the limited number of participants, especially in the lower protein/energy intake groups, that in this population the protein distribution is not associated with the ASMI nor physical function. This is in line with a cross-sectional study conducted by Gingrich et al. (281), in which 97 community-dwelling older individuals were included (a mean age of 77 years). The divergent findings between our results, the study by Gingrich et al. (281), and other studies (243, 247) could at least in part be explained by the different study populations that were investigated. Ten Haaf et al. (245) showed in a group of older individuals (≥65 years) that those having an even distribution of protein throughout the day were also the individuals who stayed the most active. This might explain why a population consuming protein evenly throughout the day, at least in some studies, had a higher lean mass.

Whole-body protein metabolism is influenced by energy intake (250, 260, 261) and energy balance (262). Thus, the fate of utilization of amino acids for protein synthesis or energy production depends partly on total energy intake and energy balance. We found that protein and energy intake are positively correlated (**Table 2.2**), revealing that the participants ingesting high amounts of protein consume more energy in general, including protein and other macronutrients as part of their habitual dietary patterns. This is in accordance with observations in community-dwelling, frail and institutionalized elderly people as reported by Tieland et al. (282) and Smeuninx et al. (283). The

causality here cannot be determined, but we speculate that people with a high LBM and body mass may just eat more, leading to a concomitant increase in protein intake.

The phenotype observed in cross-sectional studies (lean body mass underlying the ASMI, muscle and grip strength, gait speed, etc.) is supposedly formed as a consequence of living conditions over several years. An underlying assumption for associating these phenotypic characteristics with food intake is, therefore, that the food recordings are representative of the long-term retrospective habitual food intake. The food questionnaires allowed us to explore this assumption. We found that 85 (46%) out of the total cohort mentioned that their food intake had changed after being retired. Of those, 21 (11%) directly stated that they had become more aware of eating a healthier diet, whereas nine (5%) stated that they were eating less and consumed easier foods (i.e., quick snacks, ready meals, and fast food), snacked more and had become more relaxed towards healthy food choices. Twelve (7%) said that they went from being used to catering foods in excess at their workplace to more moderate and simple foods at home. In support for a transition in food intake occurring at retirement, a change in daily energy intake patterns was found among middle-aged British adults over a 17-year period, directed away from lunch and toward the evening meal (284). Although it is not directly comparable to our population, this finding combined with our food questionnaires suggest that a change in food choices and intake pattern could be expected. Hence, longitudinal intervention studies should be conducted to account for such unavoidable uncertainty in a cross-sectional study design and investigate whether different protein intakes and dietary protein distributions preserve muscle mass and physical function in healthy older individuals.

A cross-sectional study design brings some limitations—as discussed above—and the sample size was relatively small in our study, especially in the lowest protein intake group (<0.83 g/kg/day). Further, the representative nature of a 3-day dietary registration and a 4-day physical activity registration may be questionable and thus creates some degree of uncertainly.

2.6 Conclusions

In conclusion, we found no associations between the protein and energy intake or distribution and ASMI and physical parameters in a cohort of healthy Danish older individuals. These data do not provide an incentive to recommend healthy, well-functioning older individuals who already adhere to the current internationally recommended dietary protein intake (0.83 g/kg/day) to change their dietary protein intake or distribution pattern throughout the day.

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CHAPTER 3

CO-INGESTION OF CLUSTER DEXTRIN CARBOHYDRATE DOES NOT INCREASE EXOGENOUS PROTEIN-DERIVED AMINO ACID RELEASE OR MYOFIBRILLAR PROTEIN SYNTHESIS FOLLOWING A WHOLE-BODY RESISTANCE EXERCISE IN MODERATELY TRAINED YOUNGER MALES: A DOUBLE-BLINDED RANDOMIZED CONTROLLED CROSS OVER TRIAL

3 Co-ingestion of Cluster Dextrin carbohydrate does not increase exogenous protein-derived amino acid release or myofibrillar protein synthesis following a whole-body resistance exercise in moderately trained younger males: a double-blinded randomized controlled crossover trial

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3.1 Abstract

Purpose: This study investigates if co-ingestion of Cluster Dextrin (CDX) augments the appearance of intrinsically labeled meat protein hydrolysate-derived amino acid (D_5-phenylalanine), Akt/mTORC1 signaling, and myofibrillar protein fractional synthetic rate **(**FSR).

Methods: Ten moderately trained healthy males (age: 21.5 ± 2.1 years, body mass: 75.7 ± 7.6 kg, body mass index (BMI): 22.9 ± 2.1 kg/m^2) were included for a double-blinded randomized controlled crossover trial. Either 75 g of CDX or glucose (GLC) was given in conjunction with meat protein hydrolysate (0.6 g protein * FFM^{-1}) following a whole-body resistance exercise. A primed-continuous intravenous infusion of L-[^{15}N]-phenylalanine with serial muscle biopsies and venous blood sampling was performed.

Results: A time × group interaction effect was found for serum D_5-phenylalanine enrichment ($P < 0.01$). Serum EAA and BCAA concentrations showed a main effect for group ($P < 0.05$). T_{max} serum BCAA was greater in CDX as compared to GLC ($P < 0.05$). However, iAUC of all serum parameters did not differ between CDX and GLC ($P > 0.05$). T_{max} serum EAA showed a trend towards a statistical significance favoring CDX over GLC. The phosphorylation of p70S6K^{Thr389}, rpS6$^{Ser240/244}$, ERK1/2$^{Thr202/Tyr204}$ was greater in CDX compared to GLC ($P < 0.05$). However, postprandial myofibrillar FSR did not differ between CDX and GLC ($P = 0.17$).

Conclusion: In moderately trained younger males, co-ingestion of CDX with meat protein hydrolysate does not augment the postprandial amino acid availability or myofibrillar FSR as compared to co-ingestion of GLC during the recovery from a whole-body resistance exercise despite an increased mTORC1 signaling.

Clinical trial registration number: ClinicalTrials.gov ID: NCT03303729 (registered on October 3, 2017)

KEYWORDS: resistance exercise, muscle protein synthesis, stable isotope tracer, amino acids, intrinsically labeled protein, mTORC1

3.2 Introduction

Protein intake is essential for skeletal muscle protein adaptation to resistance exercise training (34). Muscle protein synthesis is maximally stimulated when exercise is combined with protein ingestion (89, 91, 285). Essential amino acids (EAA) have been shown to be potent in stimulating muscle protein synthesis (87, 286). Mechanistically, leucine, one of the branched-chain amino acids (BCAA), is a potent stimulator of mammalian target of rapamycin complex 1 (mTORC1), which is a serine/threonine kinase regulating the translation and initiation (248, 287, 288). Further, the availability of leucine and other essential amino acids (206, 289) and the achievement of higher peak aminoacidemia (94, 96) are associated with an increase in the rate of muscle protein synthesis, suggesting the importance of quick absorption and rise in essential amino acid availability in the circulation to induce muscle protein synthesis (94, 95).

Carbohydrate is often added to protein supplementations for various reasons, such as increasing energy intake, providing readily available substrate for energy metabolism, and improving taste. However, the uptake rate of exogenous amino acids and their availability in circulation is attenuated when consumed with carbohydrate (290-292) or a mixed macronutrients meal (231, 252). This is due to a decrease in the rate of digestion and absorption (291) and an increase in the retention of amino acids in the portal drained viscera (293). In addition, amino acid availability in the circulation is reduced by suppression of protein breakdown (227, 294). Although plasma insulin concentration was greater when protein was ingested with carbohydrate (290-292) or a mixed meal (231), muscle protein synthesis was not augmented as compared to protein intake alone (290-292). A systematic review concluded that a systemic administration

of insulin does not have a stimulatory or inhibitory effect for muscle protein synthesis and that amino acid availability dictates muscle protein synthesis in healthy younger individuals (236). Thus, the current evidence does not provide the interactive effect of carbohydrate co-ingestion with protein to augment muscle protein synthesis. However, if the addition of carbohydrate delays the availability of amino acids in the circulation, it could be speculated that carbohydrate would result in a delayed increase in muscle FSR, as observed in slower digestible proteins (96).

Cluster Dextrin (CDX) is a branched carbohydrate produced from waxy maize starch by the cyclization of a branching enzyme (295). CDX is highly soluble in water, has low viscosity, and has a relatively low tendency for retrogradation (296) compared to commercial dextrin (297). CDX has also been shown to increase the rate of gastric emptying compared to glucose (GLC) and standard dextrin due to a lower osmotic pressure (298). Accordingly, the rapid gastric emptying of CDX might alleviate the lower amino acid availability when co-ingested with protein as compared to GLC. Thus, a measurement of exogenous protein-derived amino acid availability (i.e., a downstream measurement of digestion and absorption) is required to determine whether co-ingestion of CDX increases amino acid availability for the periphery.

Therefore, we hypothesized that the appearance of amino acids from orally ingested meat protein hydrolysate into the circulation would be faster when it is co-ingested with CDX than GLC. Accordingly, we further hypothesized that the ingestion of the meat protein hydrolysate with CDX would result in a greater Akt/mTORC1 signaling

response and myofibrillar FSR following an acute bout of whole-body resistance exercise as compared to GLC in moderately trained younger males.

3.3 Methods

3.3.1 Ethical approval

This study was approved by the Ethics Committee of the Capital Region (H-17017363) and adhered to the Helsinki II declaration. Before inclusion, each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to obtaining written informed consent. This trial was registered at clinicaltrails.gov as NCT03303729.

3.3.2 Subjects

Ten moderately trained healthy males (21.5 ± 2.1 years, 22.9 ± 2.1 kg/m^2; values are mean ± SD) volunteered to participate in a double-blinded, randomized controlled crossover study. Inclusion criteria were as follows: healthy men who conduct structured whole-body resistance training between 1 and 3 times (1-3 hours) per week on average over the last 3 months. Exclusion criteria were as follows: subjects younger than 18 years or above 30 years of age, BMI > 30, smoking, active cancer, renal diseases, diabetes mellitus, vegetarian, physical inactivity (i.e., no systematized exercise), and perform systematized resistance exercise more than 3 times per week. Baseline subject characteristics are presented in **Table 3.1**.

Table 3.1 Baseline subject characteristics.

Age, y	21.5 ± 2.1
Height, m	1.82 ± 0.54
Body mass, kg	75.7 ± 7.6
FFM, kg	60.4 ± 5.4
BMI, kg/m^2	22.9 ± 2.1
1-RM leg press, kg	283.0 ± 50.6
1-RM leg extension, kg	116.5 ± 12.1
10-RM shoulder press, kg	50.6 ± 4.5
10-RM pulldown, kg	56.5 ± 9.0

All values are presented as means ± SD. *n* = 10. FFM; fat free mass, BMI; body mass index.

3.3.3 Study overview

This was a double-blinded randomized controlled crossover trial conducted at The Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital. The overall timeline of the study is shown in **Fig. 3.1a**, and a CONSORT flow chart diagram is displayed in **Fig. 3.2**. Briefly, at least two weeks prior to the first trial, subjects underwent preliminary assessments. The two experimental trials were separated by at least 2 weeks to minimize any interaction on the second trial from the previous trial. On both trial days, the participants arrived in an overnight fasted state at 08:00 hours at The Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital. Personnel with no direct involvement in the experiment rolled dice and created a scheme where the order of the interventions (CDX or GLC) was in code for each subject. On the day of an experiment, the personnel prepared the designated intervention and handed it over to the investigator (MJ). Thus, the allocation of interventions was concealed from the

participants and the study investigator until the completion of data analysis. In each experimental visit, muscle biopsies and blood samples were obtained during a primed-continuous stable isotope amino acid infusion (^{15}N-phenylalanine) to determine amino acid availability from orally ingested intrinsically labelled meat protein hydrolysate (D_5-phenylalanine), myofibrillar muscle protein synthesis, and intracellular signaling in response to a whole-body resistance exercise and the intake of meat protein hydrolysate with either GLC or CDX.

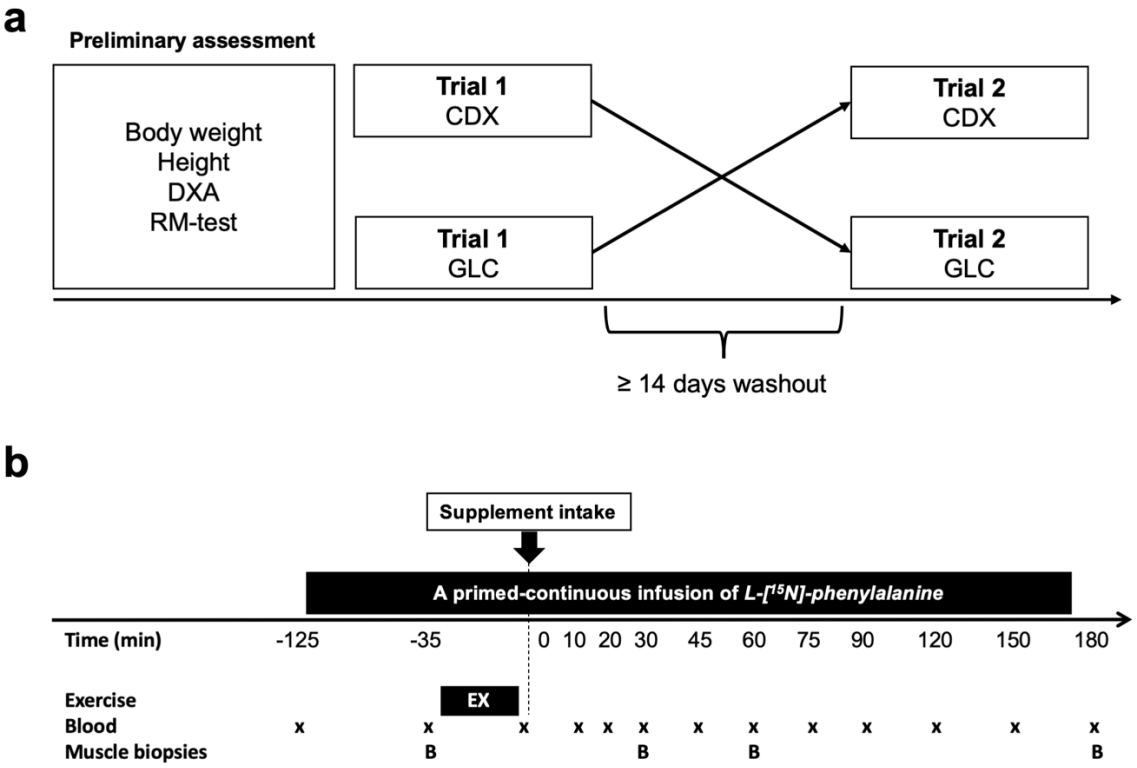

Figure 3.1 Schematic overview of crossover study design (a) and experimental protocol (b).

DXA, Dual X-ray Absorptiometry; RM, Repetition Maximum; EX, exercise; B, muscle biopsy; GLC, glucose; CDX, cluster dextrin.

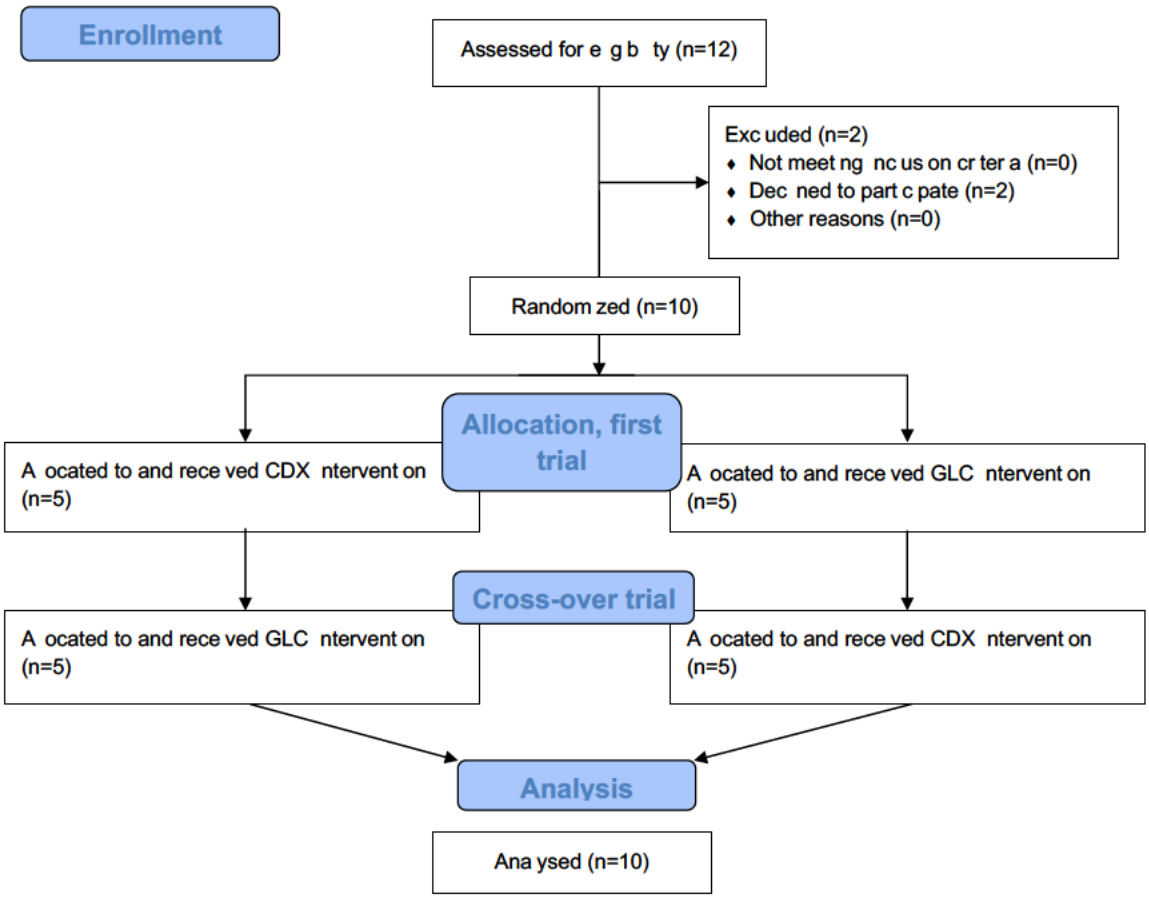

Figure 3.2 A CONSORT flowchart diagram.

3.4 Preliminary assessments

3.4.1 Body mass and height

Height was measured in the upright position without shoes against a wall and body weight was measured on a digital scale (Seca 719, Seca gmbh & co., Hamburg, Germany) in light clothing.

3.4.2 Body composition

Dual x-ray absorptiometry (DXA) was performed to determine the whole-body fat free mass (FFM) by using the enCORE v.16 software (Lunar iDXA; GE Medical Systems,

Pewaukee, WI, USA) after having emptied their bladder. DXA scans were performed after at least 12 h overnight fast.

3.4.3 Strength tests

After the scanning, strength was assessed by leg press, knee extension (Super Executive Line, TechnoGym, Gambettola, Cesana, Italy), shoulder press (TR Equipment model 9025, Tranås, Sweden) and shoulder pull/pull-down (Lat Mach, TechnoGym, Gambettola, Cesana, Italy) strength exercise machines. The strength tests were 1 repetition maximum (RM) and 10RM for leg exercises and upper body exercises, respectively. The 10RM test for the upper body exercises was chosen to minimize any risk of injury by unaccustomed exercises during testing.

3.5 Experimental Protocol

The experimental protocol is shown in **Fig. 3.1b**. The overall experimental protocol consisted of a primed (4.0 µmol * kg FFM^{-1}) continuous (3.8 µmol * kg FFM^{-1} * $hour^{-1}$) infusion of L-[^{15}N]-phenylalanine, which was applied over the course of the experimental trial. The participants rested in the supine position for the remainder of the trial day, only interrupted by the training program and toilet visits. Two antecubital catheters were inserted into each arm of the participant, one for the infusion of a stable isotope amino acid tracer (^{15}N-phenylalanine) and the other for serial blood sampling. A background blood sample was drawn after which the primed continuous infusion was started. The subject rested for approximately 1.5 hours after which the first biopsy was taken, and a blood sample was drawn. The leg and site of the biopsies were randomized using dice. Hereafter, the participant walked a small distance of 100 m to the training

facility where they conducted the resistance exercise program under full guidance and supervision (see 3.5.1 resistance exercise protocol). Upon completion, the participant walked back to the trial room where a post-exercise blood sample was drawn. Hereafter, an independent research assistant prepared the drink containing the intrinsically labeled (D_5-phenylalanine) meat protein hydrolysate (0.6 g protein * FFM^{-1}) mixed with either 75g of GLC or CDX in 350 mL of cold tap water. The drink was consumed in less than 5 min by the participant. Furthermore, the post-exercise drink was enriched with 5% ^{15}N-phenylalanine to minimize any fluctuations in the serum enrichment after consumption of the protein-rich drink (unlabeled phenylalanine). The amount of meat protein hydrolysate was decided to provide sufficient amino acids following a whole-body resistance exercise. A previous study showed that a higher protein intake is required when a whole-body resistance exercise is performed as compared to a unilateral leg resistance exercise (92). Nutritional composition of meat protein hydrolysate, CDX, GLC is presented in **Table 3.2**. Once the drink was consumed, blood was sampled in a vacutainer coated with Z serum clot activator (Vacuette tube, Greiner Bio-One GhmB, Austria) at 10, 20, 30, 45, 60, 75, 90, 120, 150, and 180 minutes following the post-exercise drink. Muscle biopsies were taken from the vastus lateralis at 30, 60, and 180 minutes post drink under local anesthesia (1% lidocaine) using the Bergström technique (299). Muscle samples were rinsed in ice-cold saline (9 mg/ml) and freed from any visible blood and connective tissues before being snap frozen in liquid nitrogen and stored at -80°C for future analysis. After the last biopsy, the infusion was stopped, catheters were removed, and the participant was given a sandwich before being sent home.

Table 3.2 Nutritional composition of meat protein hydrolysate, cluster dextrin (CDX), and glucose (GLC).

	Meat protein hydrolysate (0.6 g protein/ FFM kg)[1]	CDX	GLC
Total served weight, g	300	75	75
Energy, kcal	162.05	300	300
Protein, g	36	-	-
Protein, kcal	144	-	-
Fat, g	1.8	-	-
Fat, kcal	16.2	-	-
Carbohydrate, g	0	75	75
Carbohydrate, kcal	0	300	300
Alanine, g	2.04	-	-
Arginine, g	2.06	-	-
Aspartic acid, g	3.27	-	-
Cysteine, g	0.28	-	-
Glutamic acid, g	5.46	-	-
Glycine, g	1.46	-	-
Histidine, g	1.36	-	-
Isoleucine, g	1.61	-	-
Leucine, g	2.86	-	-
Lysine, g	3.21	-	-
Methionine, g	0.8	-	-
Phenylalanine, g	1.54	-	-
Proline, g	1.27	-	-
Serine, g	1.37	-	-
Threonine, g	1.61	-	-
Tryptophan, g	0.42	-	-
Tyrosine, g	1.17	-	-
Valine, g	1.74	-	-
Total essential amino acids, g	15.16	-	-
Total amino acids, g	33.55	-	-
D_5-phenylalanine enrichment[2], MPE ± SD	0.73 ± 0.01	-	-

[1]For an individual with 60 fat free mass (FFM) kg.

[2]Value was reported by Reitelseder et al. (231).

CDX; Cluster Dextrin, GLC; glucose, MPE; mole percent excess, SD; standard deviation.

3.5.1 Resistance exercise protocol

The resistance exercise session consisted of four different exercises for both the lower and upper body and was the same as those used in the preliminary assessment: leg press, knee extension, shoulder press and shoulder pull/pull down. The subjects completed 3 sets of 8 repetitions at 70% of 1RM in each of the two leg exercises and 3 sets of 10 reps at 10RM in the two upper body exercises. Three sets of each exercise were completed before conducting the next exercise. Rest periods of 2 min were allowed between sets and exercises.

3.5.2 Meat protein hydrolysate

The meat, from which the hydrolysate has been produced, had been intrinsically labeled with ring-D_5-phenylalanine by infusing Holstein cows with the ring-D_5-phenylalanine tracer for 72 hours before slaughter (231). The meat protein hydrolysate is quickly absorbed and induces an immediate and high availability of amino acids in the circulation after a bolus intake (231). This allows to directly measure the availability of the orally consumed meat protein hydrolysate-derived amino acids in the circulation.

3.6 Blood analysis

Blood was sampled with vials coated with Z serum clot activator and left at room temperature for 30 minutes before being centrifuged (3,970 x g, 10 min, 4°C) in an Eppendorf 5810R (Eppendorf AG, Hamburg, Germany) to obtain serum. Serum samples were stored at -80 °C for further analysis.

Serum amino acid concentrations were determined as described in detail elsewhere (300). We used 100 µL of the serum, which was added internal standards for all amino

acids and were acidified with the addition of 120 µL of 50% acetic acid before being poured over columns (Medium HDPE Open tip column CC07, Intertech Medical Inc., Denver, CO) containing acidified cation exchange resin (Dowex AG 50W-X8 resin 100-200 mesh, BioRad, Copenhagen, Denmark). Purified amino acids were converted to their phenylthiocarbamyl (PTC) derivatives, by adding a coupling buffer (methanol: Milli-Q® water:triethylamin (2:2:1, %, v/v)), drying at 70°C under a flow of N_2, adding the derivatization solution (triethylamin: Milli-Q® water:PITC:methanol (1:1:1:7, %, v/v)). Then, the sample was vortexed and incubated at room temperature for 30 minutes. Hereafter, the solution was dried at 70°C under a flow of N_2 and acetonitrile, methanol, and Milli-Q® purified water (44:10:46, %, v/v) with 0.1 M ammonium acetate was added. The isotope ratios of D_5- and ^{15}N-phenylalanine and a full amino acid concentration profile were determined on a liquid chromatography tandem mass-spectrometer (LC-MS/MS; triple stage quadrupole mass spectrometer, TSQ Vantage, Thermo Fischer Scientific, San Jose, CA, USA).

Serum insulin concentrations were measured using a high-sensitivity human insulin enzyme-linked immunosorbent assay (ELISA) kit (DRG Instrument GmbH) at the time points -35, 0, 10, 20, 30, 45, 60, 90, 120, and 180 minutes.

3.7 Muscle tissue analyses

3.7.1 Intramuscular amino acid concentration

From 10 mg of wet weight muscle, BCAA concentrations were determined by using the same protocol as for serum amino acids as described above (300). Briefly, the frozen muscle specimens were homogenized in 1 ml of 6% perchloric acid with an added internal standard for the determination of BCAA concentrations. The samples were

spun down and the supernatant containing the tissue free amino acids was extracted. The samples were then poured over acidified cation exchange columns with resin (AG 50W-X8 resin, Bio-Rad laboratories, Hercules, Ca, USA). The amino acids were eluted with 2 × 2 ml 4M NH_4OH and derivatized into their phenylthiocabamyl (PTC) derivative. Derivatized samples were loaded and analyzed on LC-MS/MS (Thermo Fischer Scientific, San Jose, CA, USA). Each intramuscular BCAA concentration was normalized to the wet weight of muscle used to prepare for the analysis. The intramuscular water fraction was set as 0.77 of the muscle wet weight.

3.7.2 Myofibrillar protein bound tracer enrichments

From 20 mg of wet weight muscle, the abundance of myofibrillar protein bound ^{15}N-phenylalanine was measured according to our lab's standard protocol. Briefly, the frozen muscle specimen was homogenized in 1 mL of buffer (Tris 0.02 M [pH 7.4], NaCl 0.15 M, EDTA 2 mM, EGTA 2 mM, TritonX-100 0.5%, sucrose 0.25 M) for 4 × 45 sec, speed 5.5 (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA) and left at 5°C for 3 hours. Hereafter, samples were centrifuged at 800 x g for 20 minutes at 5°C. The supernatant was discarded, and 1 mL of homogenization buffer was added, homogenized for 45 sec at speed 5.5, incubated at 5°C for 30 min, and centrifuged. The supernatant was discarded, and 1.5 mL buffer (KCl 0.7 M, pyrophosphate ($Na_4P_2O_7$) 0.1 M) was added to the pellet, vortexed, and left overnight at 5°C. The day after, the samples were spun at 1600 x g for 20 minutes at 5°C and the supernatant was transferred to glass vials suitable for hydrolysis and added 2.3 × vol ethanol 99%, vortexed and left for 2 hours at 5°C, and subsequently spun at 1,600 x g for 20 minutes. The supernatant was then discarded, and 1 mL of 70% ethanol was added to the pellet,

after which the solvent was vortexed and centrifuged at 1,600 x g for 20 minutes. The supernatant was discarded and 1 mL of 1 M HCl and 1 mL of resin slurry was added to the pellet and left overnight at 110°C. The solvent was diluted with water and the amino acids were purified over cation exchange resin columns. The purified amino acids were derivatized as their N-acetyl-propyl (NAP) derivate and analyzed on gas chromatograph-combustion-isotope ratio mass spectrometer by following standard procedure described thoroughly by Bornø et al. (301).

3.7.3 Intracellular signaling

Western blotting was performed as reported previously with a slight modification (302). Briefly, approximately ~30 mg of frozen muscle tissue samples was homogenized in 10-fold volumes of RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease and phosphatase inhibitor cocktail (Roche Life Science, Indianapolis, IN, USA) per 10 mL of homogenization buffer. The resulting homogenates were centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was transferred to a new vial and total protein concentrations were determined by the Protein Assay Rapid kit (WAKO, Osaka, Japan). The samples were standardized to 2 μg protein per 1 μL by dilution with 3 × SDS sample buffer containing 15% β-mercaptoethanol, 6% SDS, 187.5 mM Tris–HCl (pH 6.8), 15% sucrose, and 0.015% bromophenol blue and boiled at 95°C for 5 min. An equal amount of protein (10 μg) was loaded into each lane and the samples were separated by electrophoresis on a 10 or 15% SDS-polyacrylamide gel for 45 min at 250 V. Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 1 h at 20 V via a semi-dry transfer. Membranes were subsequently blocked in 5% milk for 1 h at room

temperature. After blocking, membranes were washed 3 times for 5 min in Tris-buffered saline with 0.1% Tween (TBST) before being incubated overnight at 4°C with the appropriate primary antibodies (**Table 3.3**). Membranes were then washed again 3 times for 5 min in TBST and incubated for 1 h in their respective horseradish peroxidase-conjugated secondary antibody (**Table 3.3**) at room temperature and washed again 3 times for 5 min in TBST. Chemiluminescence (Luminata 200 Forte Western HRP Substrate; Merck Millipore, Temecula, CA, USA) was applied to each blot. Images were developed using an ImageQuant LAS 4000 (GE Healthcare, Amersham, UK). Band intensities were quantified using Image Studio Lite (Li-Cor, Lincoln, Nebraska, USA). Phosphorylation levels were determined by the expression of phosphorylated protein divided by the expression of non-phosphorylated total protein. The membranes were stained with Ponceau-S to verify equal loading and used as the normalization control.

Table 3.3 Antibodies for western blot.

Antibodies	Dilution	Source	Identifier
phospho-Ser473 Akt	1:1000	Cell Signaling Technology	Cat# 9271
Akt	1:1000	Cell Signaling Technology	Cat# 2920
phospho-Thr389 p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 9234
p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 2708
phospho-Ser240/244 rpS6	1:1000	Cell Signaling Technology	Cat# 2215
rpS6	1:1000	Cell Signaling Technology	Cat# 2217
phospho-Thr37/46 4E-BP1	1:1000	Cell Signaling Technology	Cat# 9459
4E-BP1	1:1000	Cell Signaling Technology	Cat# 9452
phospho-Thr172 AMPKα	1:1000	Cell Signaling Technology	Cat# 2535
AMPKα	1:1000	Cell Signaling Technology	Cat# 2793
phospho-Thr56 eEF2	1:1000	Cell Signaling Technology	Cat# 2331
eEF2	1:1000	Cell Signaling Technology	Cat# 2332
Phospho-Thr202/Tyr204 ERK1/2	1:1000	Cell Signaling Technology	Cat# 4376
ERK1/2	1:1000	Cell Signaling Technology	Cat# 4696
Anti-mouse IgG, HRP-linked Antibody	1:5000	Cell Signaling Technology	Cat# 7076
Anti-rabbit IgG, HRP-linked Antibody	1:5000	Cell Signaling Technology	Cat# 7074

3.8 Calculations

FSR was calculated using the precursor-product method (199):

$$FSR = \frac{\mathrm{E}_2 - E_1}{\acute{\mathrm{e}}_{t1-t2} * t_{1-2}} * 100\%$$

Where E is the protein bound enrichment, é is precursor enrichment between two samples estimated from venous serum samples and t is the time between two samples. The FSR will be calculated from 30 to 180 minutes post exercise.

3.9 Statistical analysis

To compare the effect of time within each of the two trials and the two dependent trials (GLC versus CDX), we applied a 2-factor [time × group (GLC compared with CDX)] repeated measures ANOVA when no missing data appeared and a mixed-effects model when data points were missing (insulin concentration measures; one data point was missing (Subject#1, CDX trial, time point 180 min). Turkey's multiple comparisons test was used as a post hoc test to identify the individual differences when there was a time × group interaction effect, a main effect of time, or a main effect of group. Paired student two-tailed t-test was used to compare postprandial (0.5-3 h) FSR between CDX and GLC trials, incremental area under the curve (iAUC), and time to reach maximum concentration (T_{max}) for serum D_5-phenylalanine enrichments and serum phenylalanine, EAA, BCAA, and insulin concentrations. iAUC is the definite integral of a curve that depicts the serum parameters as a function of time during the postprandial period using the value for the parameter at time point zero as the baseline value. iAUC was computed using the trapezoid rule. A straight line is connected between adjunct points, and the beneath area was calculated as ΔX*([(Y1+Y2)/2]-Baseline]. This was repeated for each region, and the sum of the areas was defined as iAUC. T_{max} was obtained from the concentration-time data, where the time of the highest concentration of serum parameters was observed during the postprandial period. A priori power analysis was performed for a matched paired t-test (two tails) with an α error probability = 0.05, power (1-β error probability) = 0.8, and Cohen's effect size dz = 1.0 using G*Power version 3.1 analysis software (Heinrich Hein University). Cohen's effect size dz = 1.0 was calculated based on the least detectable difference of 0.01%/h FSR between groups and the within subject standard deviation of 0.01%/h. This produced a minimum sample

size of n = 10. Data are expressed as means ± SD or SEM. An alpha level of 0.05 was used to determine statistical significance. All statistical analysis was performed using GraphPad Prism version 8.4.3 for Mac (GraphPad Software, La Jolla California USA).

3.10 Results

3.10.1 Participants

Baseline subject characteristics are shown in **Table 3.1**. **Fig. 3.2** shows a CONSORT flowchart diagram describing the progress from recruitment through completion of the study.

3.10.2 Phenylalanine amino acid tracer enrichment

Serum D_5-phenylalanine (**Fig. 3.3a**) enrichment originated from intrinsically labeled meat protein hydrolysate was increased following the ingestion of post-exercise drink at $t = 0$ (a main effect of time, $P < 0.0001$) between 20 and 180 mins ($P < 0.0001$). There was no main effect of group for serum D_5-phenylalanine enrichment ($P = 0.46$). There was a time × group interaction effect ($P = 0.0072$). The enrichment of the infused tracer, L-[ring-^{15}N]-phenylalanine (**Fig. 3.4a**), was elevated above basal value ($t = 0$) following the tracer infusion 20-180 mins (a main effect of time, $P < 0.0001$). There was no main effect of group ($P = 0.27$) and time × group interaction effect ($P = 0.067$).

3.10.3 Serum amino acid concentrations

Serum phenylalanine concentrations (**Fig. 3.3b**) were increased compared to basal value ($t = 0$) from 20-120 mins ($P < 0.01$) following the intake of post-exercise drink (a main effect of time, $P < 0.0001$). There was no main effect of group for serum phenylalanine

($P = 0.086$). Serum EAA concentrations (**Fig. 3.3c**) were increased compared to basal value ($t = 0$) from 20-150 mins ($P < 0.0001$) following the intake of post-exercise drink (a main effect of time, $P < 0.0001$). There was a main effect of group for serum EAA ($P = 0.021$). Likewise, serum BCAA concentrations (**Fig. 3.3e**) were increased above basal value ($t = 0$) between 20 and 120 min ($P < 0.01$) with an overall time effect ($P < 0.0001$). There was a main effect of group for serum BCAA concentrations ($P = 0.02$). In contrast, there was a main effect of time ($P < 0.05$) for muscle BCAA concentrations (**Fig. 3.3f**), and it was decreased at 180 min ($P < 0.05$) following the intake of post-exercise drink compared to basal value (t = - 35 min). There was no main effect of group for muscle BCAA ($P = 0.45$). There was no time × group interaction effect for serum EAA ($P = 0.58$), phenylalanine ($P = 0.82$), BCAA concentrations ($P = 0.67$), and muscle BCAA concentrations ($P = 0.91$).

3.10.4 Serum insulin concentrations

Serum insulin concentrations (**Fig. 3.3d**) were increased above basal value ($t = 0$) between 20 and 90 mins ($P < 0.05$) following the intake of post-exercise drink (an overall time effect, $P < 0.0001$). There was no main effect of group (GLC compared with CDX) for serum insulin concentrations ($P = 0.48$). There was no time × group interaction effect for insulin concentrations ($P = 0.13$).

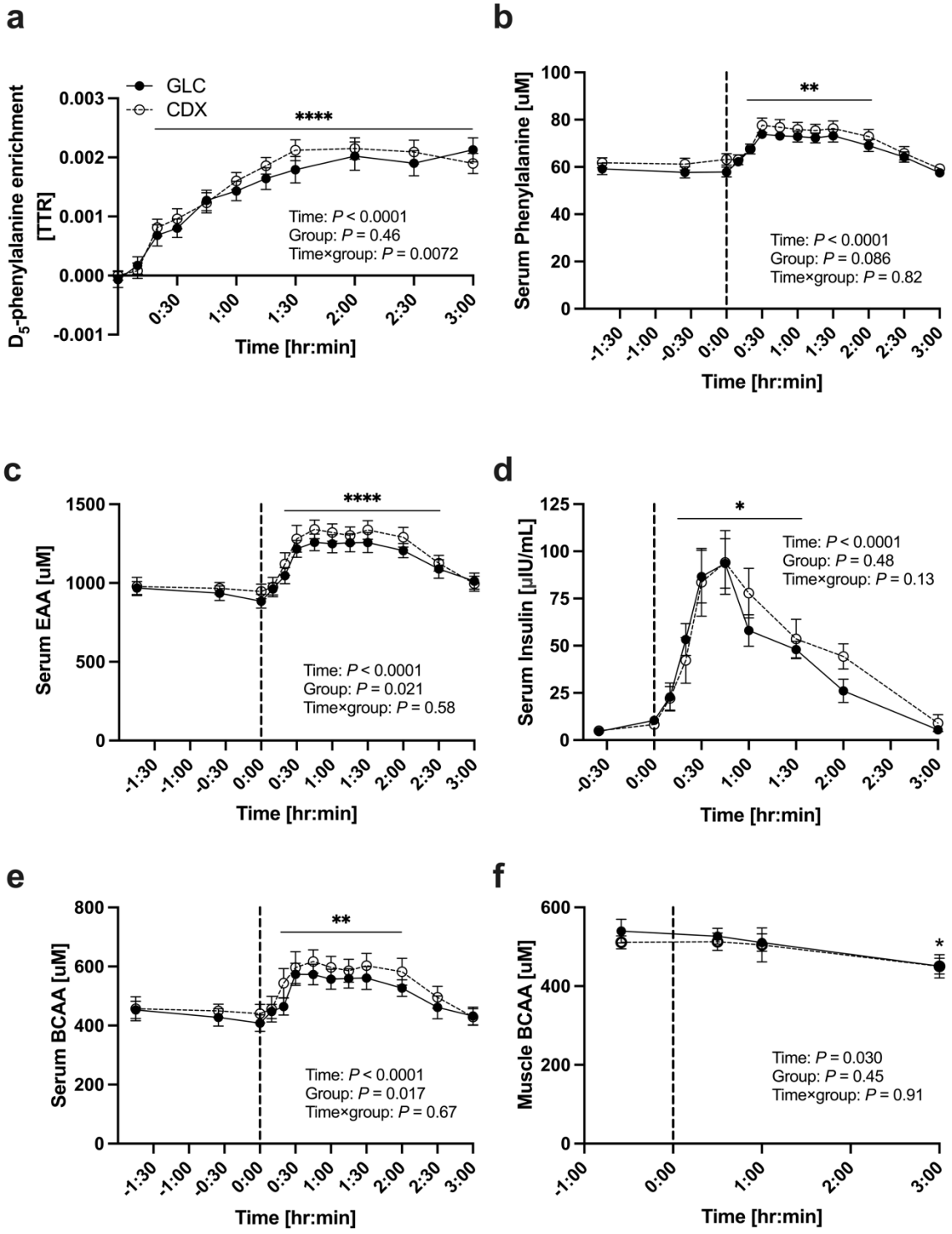

Figure 3.3 The time course of serum D_5-phenylalanine enrichment (a), phenylalanine (b), EAA (c) insulin (d) BCAA (e) muscle BCAA (f) concentrations.

The vertical dot line on each graph (at $t = 0$) indicates the transition from postabsorptive to postprandial conditions via the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either 75 g of GLC ($n = 10$) or CDX ($n = 10$) following a whole-body resistance exercise. Data were analyzed with the use of

a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. Values are means ± SEM. Significance was set at $P < 0.05$. There was a main effect of time for serum D_5-phenylalanine enrichment, phenylalanine, EAA, BCAA, insulin concentrations and ($P < 0.0001$) and muscle BCAA ($P < 0.05$). There was a main effect of group (GLC compared with CDX) for serum EAA and BCAA concentrations ($P < 0.05$). There was a time × group interaction effect for D_5-phenylalanine enrichment ($P < 0.05$). *, **, **** denotes significant difference from basal ($P < 0.05$, $P < 0.01$, $P < 0.0001$, respectively). TTR, tracer to tracee ratio; GLC, glucose; CDX, cluster dextrin; BCAA, Branched Chain Amino Acid.

3.10.5 iAUC and T_{max} in serum parameters

iAUC and T_{max} serum D_5-phenylalanine, phenylalanine, EAA, BCAA, and insulin are displayed in **Table 3.3**. T_{max} serum BCAA was higher in CDX compared to GLC ($P < 0.05$). T_{max} serum EAA showed a trend towards statistical significance favoring CDX over GLC ($P = 0.051$).

Table 3.4 iAUC and T_{max} serum D_5-phenylalanine, phenylalanine, EAA, BCAA, and insulin in response to meat protein hydrolysate intake with either GLC or CDX.

	GLC	CDX	*P* value
D_5-phenylalanine enrichment			
iAUC, TTR · min	0.0048 ± 0.00033	0.0050 ± 0.00029	0.46
T_{max}, min	133.5 ± 13.5	120 ± 11.0	0.51
Phenylalanine			
iAUC, μM · min	32.1 ± 2.3	27.0 ± 2.9	0.099
T_{max}, min	75.0 ± 7.7	69.0 ± 11.7	0.68
EAA			
iAUC, μM · min	825.2 ± 54.6	810.1 ± 64.2	0.84
T_{max}, min	84.0 ± 7.8	64.5 ± 9.8	0.051
BCAA			
iAUC, μM · min	335.8 ± 24.7	342.4 ± 37.4	0.87
T_{max}, min	70.5 ± 11.0	47.0 ± 8.0	0.049*
Insulin			
iAUC, μIU/mL · min	94.6 ± 10.8	119.0 ± 17.4	0.098
T_{max}, min	51.0 ± 8.4	39.5 ± 3.7	0.29

All values are presented as means ± SEM. n = 10. Paired student two-tailed t-test was used to compare between GLC and CDX. Significance was set at $P < 0.05$. * denotes significant difference between groups ($P < 0.05$). iAUC; incremental area under the curve, T_{max}; time to reach maximum concentration, EAA; essential amino acids, BCAA; branched-chain amino acids, CDX; Cluster Dextrin, GLC; glucose, TTR; tracer to tracee ratio, SEM; standard error of mean, IU; international unit.

3.10.6 Myofibrillar protein fractional synthesis rate

Postprandial myofibrillar FSR was calculated using the average serum L-[ring-^{15}N]-phenylalanine enrichments as the precursor pool (**Fig. 3.4b**). Myofibrillar protein FSR between 30 and 180 mins was not different between GLC and CDX (0.0862 ± 0.0137 and 0.1026 ± 0.0093 %•h^{-1}, respectively, $P = 0.17$).

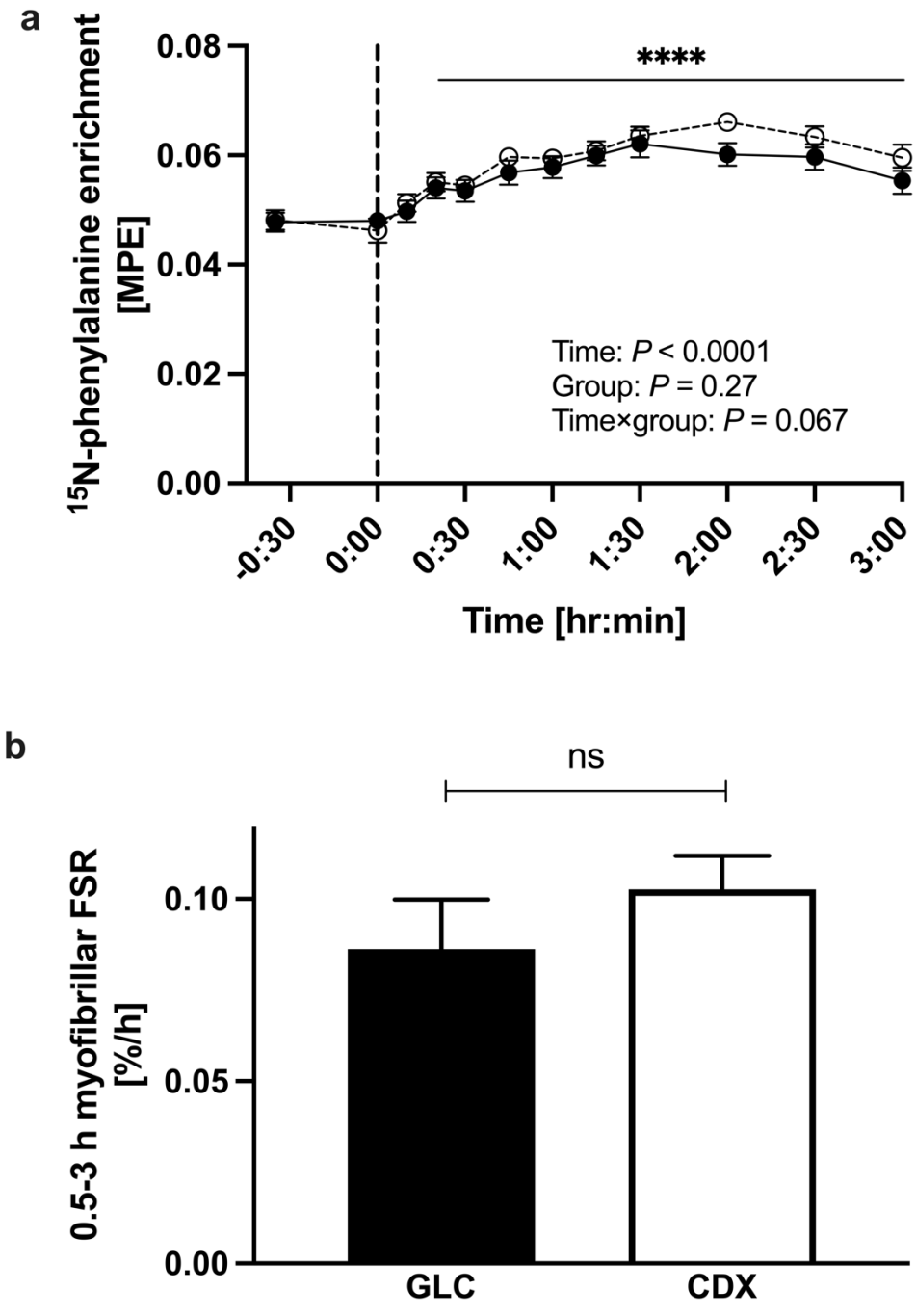

Figure 3.4 The time course of serum ^{15}N-phenylalanine enrichment (a) and myofibrillar FSR over a 2.5-hour postprandial period (b).

The vertical dot line on each graph (at $t = 0$) indicates the transition from postabsorptive to postprandial conditions via the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either 75 g of GLC ($n = 10$) or CDX ($n = 10$) following a whole-body resistance exercise. Serum ^{15}N-phenylalanine enrichment was analyzed with the use of a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. Values are means ± SEM. Significance was set at $P < 0.05$. There was a main effect of time for serum ^{15}N-phenylalanine enrichment ($P < 0.0001$). Myofibrillar FSR was analyzed with the use of a paired t-test (two-tailed). $n = 10$/group. Values are means ± SEM. Significance was set at $P < 0.05$. Analysis revealed no statistical difference between GLC and CDX ($P = 0.17$). MPE, mole percent excess; FSR, fractional synthesis rate; GLC, glucose; CDX, cluster dextrin.

3.10.7 Intracellular signaling

The time-dependent changes of intracellular signaling are displayed in **Fig. 3.5** and representative western blot images are shown in **Fig. 3.6**. The phosphorylation of p70S6K^{Thr389} (**Fig. 3.5b**), rpS6$^{Ser240/244}$ (**Fig. 3.5d**), ERK1/2$^{Thr202/\ Tyr204}$ (**Fig. 3.5f**), AMPKα^{Thr172} (**Fig. 3.5g**) showed a time × group interaction effect ($P < 0.05$). There was a main effect of time ($P < 0.05$) for Akt^{Ser473} (**Fig. 3.5a**), p70S6K^{Thr389} (**Fig. 3.5b**), 4E-BP1$^{Thr37/46}$ (**Fig. 3.5c),** rpS6$^{Ser240/244}$ (**Fig. 3.5d**), eEF2^{Thr56} (**Fig. 3.5e**), ERK1/2$^{Thr202/Tyr204}$ (**Fig. 3.5f**), AMPKα^{Thr172} (**Fig. 3.5g**). There was a main effect of group ($P < 0.05$) for Akt^{Ser473} (**Fig. 3.5a**), p70S6K^{Thr389} (**Fig. 3.5b**), rpS6$^{Ser240/244}$ (**Fig. 3.5d**), ERK1/2$^{Thr202/Tyr204}$ (**Fig. 3.5f**). The phosphorylation of Akt^{Ser473} (**Fig. 3.5a)** was increased from baseline at all time points ($P < 0.05$). The phosphorylation of p70S6K^{Thr389} (**Fig. 3.5b**) was increased from baseline at 30 min and 60 min in CDX ($P < 0.05$), and it was greater in CDX (83.8-fold) than GLC (18.3-fold) at 60 min ($P <$

0.05). The phosphorylation of 4E-BP1$^{Thr37/46}$ (**Fig. 3.5c)** was increased from baseline at 30 min and 60 min ($P < 0.05$ and $P < 0.0001$, respectively). The phosphorylation of rpS6$^{Ser240/244}$ (**Fig. 3.5d**) was increased from baseline at all time points in CDX group ($P < 0.05$), and it was greater in CDX than GLC ($P < 0.05$) at 60 min (6.6- vs 16.7-fold) and 180 min (5.6- vs 16.2-fold). The phosphorylation of eEF2^{Thr56} (**Fig. 3.5e**) was decreased from baseline at 60 min ($P < 0.01$). The phosphorylation of ERK1/2$^{Thr202/Tyr204}$ (**Fig. 3.5f**) was increased from baseline at all time points ($P < 0.05$), and CDX was greater as compared to GLC at all time points (1.1- vs 1.7-fold, 1.2- vs 1.6-fold, 0.9- vs 1.6-fold for 30, 60, 180 min, respectively, $P < 0.05$). The phosphorylation of AMPKα^{Thr172} (**Fig. 3.5g**) was greater in CDX than GLC at 180 min (0.9- vs 1.3-fold, $P < 0.05$).

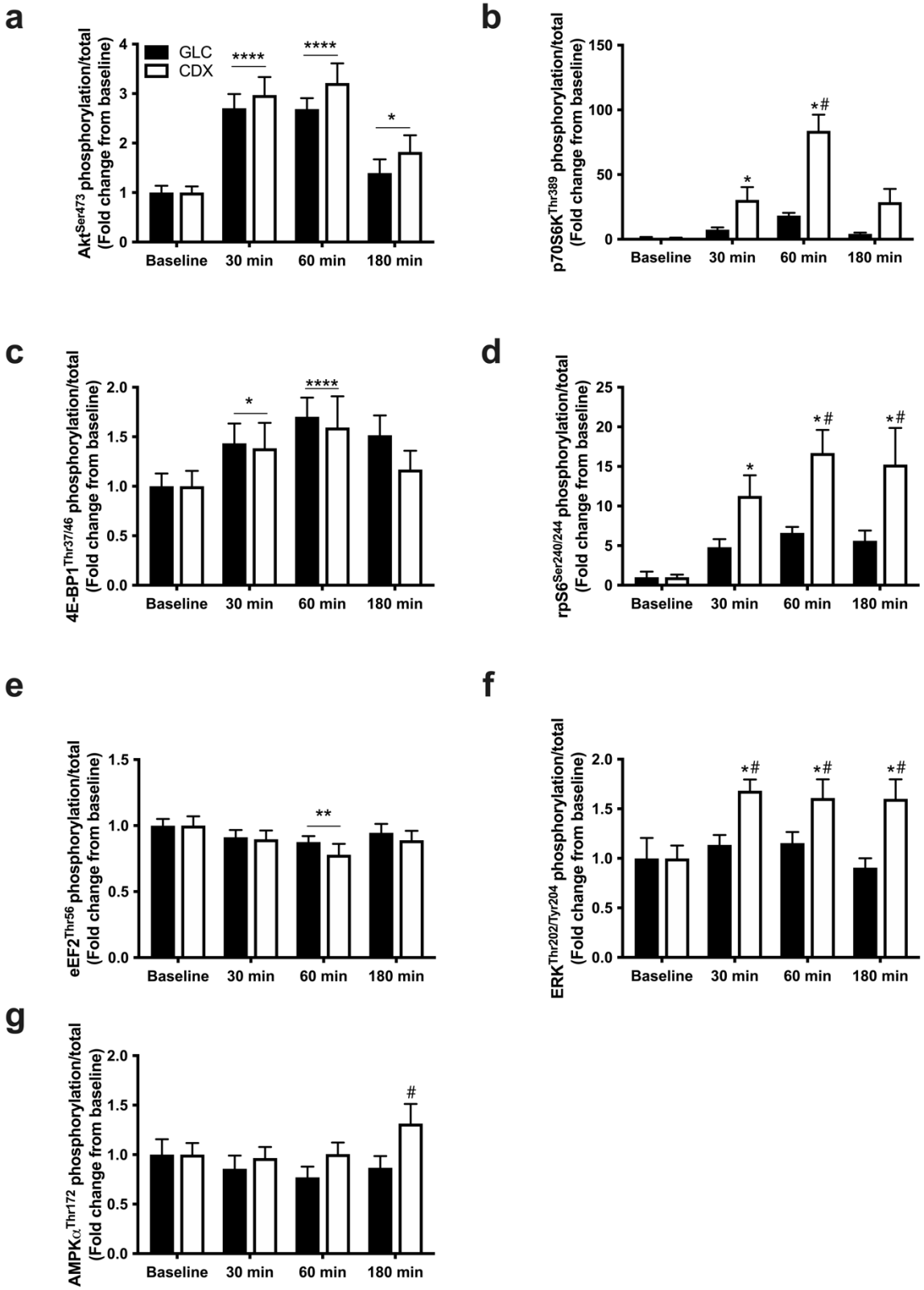
a
GLC
CDX
Akt^Ser473 phosphorylation/total (Fold change from baseline)
****
****
*
Baseline
30 min
60 min
180 min
b
p70S6K^Thr389 phosphorylation/total (Fold change from baseline)
*
*#
c
4E-BP1^Thr37/46 phosphorylation/total (Fold change from baseline)
*
****
d
rpS6^Ser240/244 phosphorylation/total (Fold change from baseline)
*
*#
*#
e
eEF2^Thr56 phosphorylation/total (Fold change from baseline)
**
f
ERK^Thr202/Tyr204 phosphorylation/total (Fold change from baseline)
*#
*#
*#
g
AMPKα^Thr172 phosphorylation/total (Fold change from baseline)
#

Figure 3.5 The phosphorylation of Akt^{Ser473} (a), p70S6K^{Thr389} (b), 4E-BP1$^{Thr37/46}$ (c), rpS6$^{Ser240/244}$ (d), eEF2^{Thr56} (e), ERK1/2$^{Thr202/Tyr204}$ (f), AMPKα^{Thr172} (g) at 30, 60, 180 min after the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either GLC (n = 10) or CDX (n = 10).

Data were analyzed with the use of a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. The data were expressed relative to baseline. Values are means ± SEM. There was a main effect of time for Akt^{Ser473}, p70S6K^{Thr389}, rpS6$^{Ser240/244}$, 4E-BP1$^{Thr37/46}$, eEF2^{Thr56}, ERK1/2$^{Thr202/ Tyr204}$, and AMPKα^{Thr172} ($P < 0.05$). There was a main effect of group (GLC compared with CDX) for Akt^{Ser473} p70S6K^{Thr389}, rpS6$^{Ser240/244}$, ERK1/2$^{Thr202/ Tyr204}$ ($P < 0.05$). There was a time × group interaction effect for p70S6K^{Thr389}, rpS6$^{Ser240/244}$, ERK1/2$^{Thr202/ Tyr204}$, AMPKα^{Thr172} ($P < 0.05$). Significance was set at $P < 0.05$. *, **, **** denotes significant difference from baseline in respective group ($P < 0.05$, $P < 0.01$, $P < 0.0001$). $^{\#}$ indicates significant difference between CDX and GLC at the same time point ($P < 0.05$). GLC, glucose; CDX, cluster dextrin.

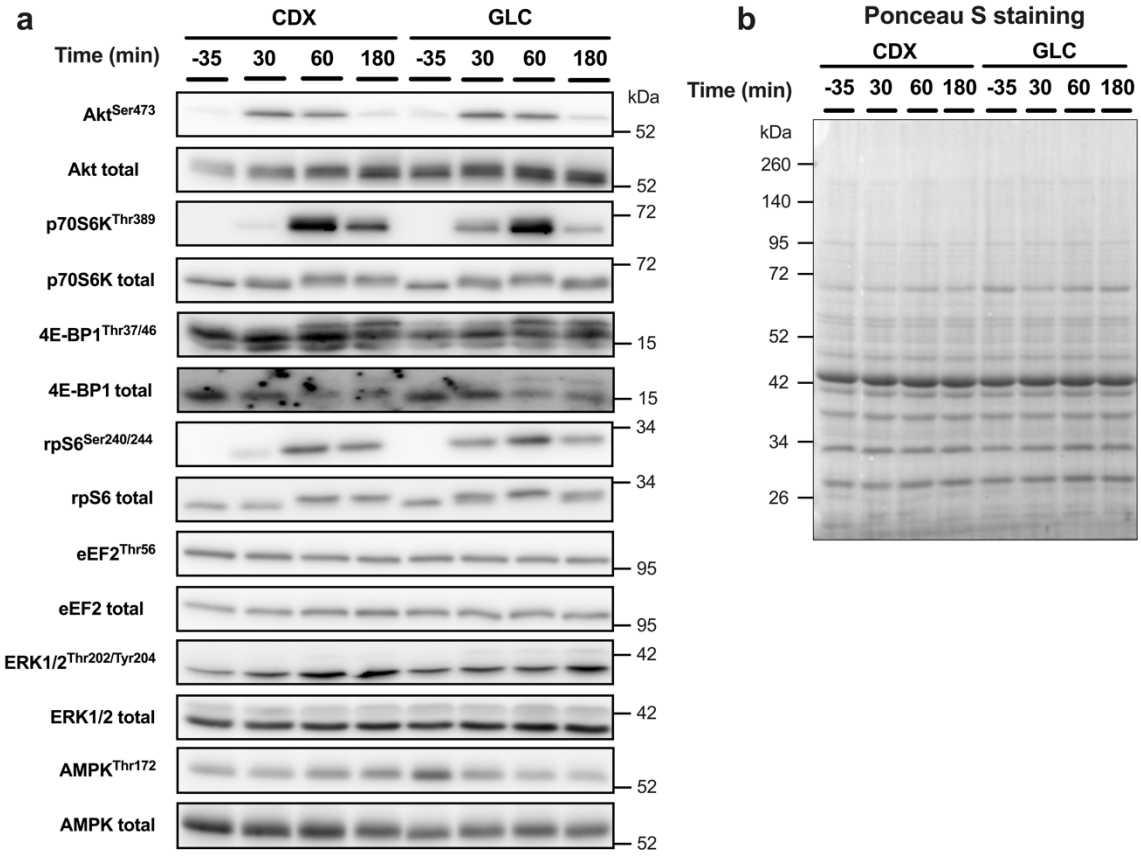

Figure 3.6 Representative western blot images for intracellular signaling (a) and Ponceau-S (b).

CDX, cluster dextrin; GLC, glucose.

3.11 Discussion

In the present study, we simultaneously assessed orally ingested protein-derived amino acid availability in the circulation using intrinsically labelled meat protein hydrolysate (D_5-phenylalanine) and myofibrillar protein synthesis of the vastus lateralis muscle after a whole-body resistance exercise in moderately trained younger males. Co-ingestion of CDX with meat protein hydrolysate did not enhance the total availability of protein-derived amino acids in the circulation as determined by the enrichment of serum D_5-phenylalanine derived from intrinsically labelled meat-protein, serum phenylalanine, EAA, BCAA, and muscle BCAA concentrations compared with GLC. Interestingly, the activation of mTORC1 signaling was higher in CDX than GLC following a whole-body resistance exercise. However, the enhanced activation mTORC1 signaling did not increase postprandial myofibrillar FSR in CDX as compared to GLC.

Amino acid availability in the circulation is a determinant of muscle protein synthesis (206, 289). It has been well established that protein/amino acids ingestion stimulate muscle protein synthesis following the recovery from resistance exercise (90, 91, 94, 95, 303, 304). In the previous studies, carbohydrate co-ingestion with protein was hypothesized to augment insulin secretion and enhance muscle protein synthesis following an acute bout of resistance exercise (290-292). However, we and others previously demonstrated that amino acid availability is decreased when protein is ingested with carbohydrate (290-292) or other macronutrients (231, 252). The nature of rapid gastric emptying of CDX (298) could be used as an approach to attenuate lower amino acid availability when protein is consumed with GLC. To our knowledge, this is the first study to investigate whether CDX ingestion together with the intake of protein

hydrolysate (here meat protein-derived) enhanced the availability of constituent amino acids in the circulation as compared to GLC ingestion. We made use of a previously produced intrinsically labelled meat protein hydrolysate (231) to directly measure exogenous protein-derived amino acid availability (with D_5-phenylalanine as a tracer) in the present study.

For the main outcome D_5-phenylalanine enrichment tracer, it appeared in serum from 20 min post-exercise and was maintained until 180 min post-exercise in both CDX and GLC groups **(Fig. 3.3a)** and iAUC (total availability) of the tracer did not differ between CDX and GLC (**Table 3.3**). Further, T_{max} for the tracer was not affected by CDX as compared to GLC. The group means for EAA and BCAA at t = -0:30 and t = 0 (**Fig. 3.3**) appeared different and were likely to cause the main effect of group in the two-way ANOVA test. Therefore, we further explored if there were any postprandial differences by calculating iAUC from t = 0, which revealed no difference for the postprandial rise in EAA and BCAA concentrations between CDX and GLC (**Table 3.3**). With these data in mind, the time × group interaction effect for D_5-phenylalanine enrichment (**Fig. 3.3a**, P = 0.0072) is hard to explain. Visually, it seems though that the D_5-phenylalanine enrichment peaks and starts to decrease within the 3-hour postprandial period in CDX, whereas it remains high in GLC. However, this is speculative, and more investigations are needed to enlighten this further. Of interest is that the T_{max} serum BCAA showed a statistical significance (P = 0.049) and T_{max} serum EAA showed a trend towards a statistical significance (P = 0.051, **Table 3.3**) although this is not the case for D_5-phenylalanine. However, a flux of different amino acids is regulated by different amino acid transport mechanisms (305), and hence, phenylalanine

may not be a representative tracer for the clearance of all amino acids. Possible reasons why we did not observe a between-group difference of amino acid availability consistently are that gastric emptying and/or the osmolarity of experimental drinks were not different between CDX and GLC. However, gastric emptying and the osmolarity of experimental drinks were not directly measured in this study, and these assumptions cannot be validated. Overall, we conclude that co-ingestion of CDX with meat protein hydrolysate did not markedly affect serum amino acid availability during the three hours of recovery from a whole-body resistance exercise.

In the present study, insulin was increased above baseline between 30 min and 90 min post-exercise drink with no difference between CDX and GLC (**Fig. 3.3d**). Previously, studies have shown that CDX intake alone does not affect glucose concentrations as compared to glucose (306) or maltodextrin (307). Although some amino acids are insulinotropic (308, 309) and a higher insulin secretion is observed when protein is consumed in combination with carbohydrate (290-292) compared to protein alone, the even serum amino acid concentrations in CDX and GLC groups in this study reject the expectations for the insulin concentrations to be different. However, due to the lack of a "meat protein hydrolysate alone" group in the present study, we cannot conclude whether the co-ingestion of CDX or GLC increased insulin concentration above levels induced by the meat protein hydrolysate alone, although this must be anticipated. The roles of insulin on muscle protein turnover have long been debated. Systematic reviews have concluded that insulin does not have a stimulatory or inhibitory effect on muscle protein synthesis (236), but instead plays an important role in attenuating muscle protein breakdown independent of amino acid availability (235). The absence of

increased muscle BCAA concentrations at 30 and 60 min (where serum concentrations are elevating and peaking, respectively) reveals that the intramuscular disappearance rate of amino acids equals the influx. We did not follow the amino acid tracers further in the intramuscular metabolic pathways, but we suggest that muscle protein synthesis and energy metabolism are responsible for the utilization of excess amino acids. This is due to the concomitant intramuscular availability of glucose, which would meet the major requirement for energy production, dampening the anaplerotic processes. The drop at 180 min in the muscle BCAA concentrations (**Fig. 3.3f**) agrees with serum EAA (**Fig. 3.3c**), phenylalanine (**Fig. 3.3b**), BCAA (**Fig. 3.3e**), and insulin concentrations (**Fig. 3.3d**) as these substrates had all returned to basal levels. Hence, it is likely that participants were in the postabsorptive period around 180 min post-exercise drink, and that the muscle BCAA pool at that time point was drained by either a net outflux into the circulation or by a request from translation processes or both (42).

It is generally agreed that 20 g of high-quality protein intake is required following resistance exercise (e.g., leg press and knee extension) to maximally stimulate muscle protein synthesis in younger individuals (89, 91). However, Macnaughton et al. (92) reported findings that may suggest the amount of protein required to stimulate muscle protein synthesis may depend on the amount of muscle recruited during resistance exercise. This is because the demand for exogenous amino acids might be increased when more muscles are used during resistance exercise (whole-body vs unilateral leg) although the amount of lean body mass (LBM) itself does not affect muscle protein synthesis (92). Macnaughton et al. (92) provided either 0.34 g or 0.68 g protein * LBM^{-1} whey protein in the lower LBM group (59 kg LBM on average), and either 0.26 g

protein or 0.52 g protein * LBM^{-1} in the higher LBM group (77 kg LBM on average). In the present study, participants performed a whole-body resistance exercise followed by meat protein hydrolysate (0.6 g * FFM^{-1}) intake, which ended up as a mean of 36.3 g of meat protein hydrolysate (range 32.0 to 41.6 g) to our participants, which should provide a stimulus to maximally stimulate myofibrillar FSR following a whole-body resistance exercise. However, as we did not measure the baseline myofibrillar FSR we cannot say whether the myofibrillar FSR was enhanced by an acute bout of whole-body resistance exercise.

Akt/mTORC1 signaling pathway is crucial for muscle protein synthesis and skeletal muscle hypertrophy (51, 75, 248). Previous studies showed that protein/amino acid feeding (208, 210, 248, 310), resistance exercise (303, 311-314), or a combination of both (94, 95, 303, 304, 315) activate Akt/mTORC1 signaling in younger individuals. In line, our whole-body resistance exercise protocol with post-exercise meat protein hydrolysate ingestion increased the phosphorylation of Akt^{Ser473}, $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, 4E-$BP1^{Thr37/46}$, and $ERK1/2^{Thr202/Tyr204}$ and decreased the phosphorylation of $eEF2^{Thr56}$ over the course of 180 min post-exercise period (a main effect for time, $P < 0.05$). Interestingly, the phosphorylation of $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, and $ERK1/2^{Thr202/Tyr204}$ was greater in CDX compared to GLC during the recovery from a whole-body resistance exercise (a time × group interaction effect, $P < 0.05$), indicating an enhanced translation initiation and elongation in CDX. However, the enhanced mTORC1 signaling did not result in an increased postprandial myofibrillar FSR in CDX (**Fig. 3.4**), which is in line with previous studies that demonstrated that co-ingestion of carbohydrate does not further increase FSR compared to protein intake alone (290-292).

The absence of an enhanced postprandial myofibrillar FSR in CDX despite the increased mTORC1 signaling could be explained by no changes of amino acids availability in the circulation as well as muscle BCAA concentrations (**Fig. 3.3**). Previous studies have shown that metabolic flux in vivo cannot be predicted by intracellular signaling (316) or mRNA expression level (84). In support, dissociation between Akt/mTORC1 signaling and muscle protein synthesis in response to amino acids and insulin was previously reported by Greenhaff et al. (83).

The present randomized controlled crossover trial is a robust study design with high statistical power. However, the absence of a meat protein hydrolysate group alone makes it impossible to reveal any effects of CDX or GLC per se, which could have been interesting now that the hypothesized beneficial effects of CDX could not be verified.

3.12 Conclusions

In moderately trained younger males, co-ingestion of CDX with meat protein hydrolysate does not enhance the availability of protein-derived amino acids and myofibrillar FSR as compared to GLC with meat protein hydrolysate during the recovery from a whole-body resistance exercise despite an increased Akt/mTORC1 signaling.

Declarations

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Conflicts of interest: The authors declare no conflicts of interest.

Ethics approval: This study was approved by the Ethics Committee of the Capital Region (H-17017363) and adhered with the Helsinki II declaration.

Consent to participate: Before inclusion, each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to obtaining written informed consent.

Consent for publication: All authors read the final version of the manuscript and approved for publication.

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Curation, Visualization, Supervision, Project administration, Funding acquisition. Writing - Original Draft, Writing - Review & Editing.

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CHAPTER 4

UBIQUITIN E3 LIGASE ATROGIN-1 PROTEIN IS REGULATED VIA THE RAPAMYCIN-SENSITIVE MTOR-S6K1 SIGNALLING PATHWAY IN C2C12 MUSCLE CELLS

4 Ubiquitin E3 ligase Atrogin-1 protein is regulated via the rapamycin-sensitive mTOR-S6K1 signalling pathway in C2C12 muscle cells

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4.1 Abstract

Muscle-specific ubiquitin E3 ligases, Atrogin-1 and MuRF1, are highly expressed in multiple conditions of skeletal muscle atrophy. The PI3K/Akt/FoxO signaling pathway is well known to regulate Atrogin-1 and MuRF1 gene expressions. Evidence supporting this is largely based on stimuli by insulin and IGF-1, that activate anabolic signaling, including Akt and Akt-dependent transcription factors. However, Akt activation also activates the mammalian target of rapamycin complex 1 (mTORC1) which induces skeletal muscle hypertrophy. However, whether mTORC1-dependent signaling has a role in regulating Atrogin-1 and/or MuRF1 gene and protein expression is currently unclear. In this study, we confirmed that activation of insulin-mediated Akt signaling suppresses both Atrogin-1 and MuRF1 protein content and that inhibition of Akt increases both Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Interestingly, inhibition of mTORC1 using a specific mTORC1 inhibitor, rapamycin, increased Atrogin-1, but not MuRF1, protein content. Furthermore, activation of AMP-activated protein kinase (AMPK), a negative regulator of the mTORC1 signaling pathway, also showed distinct time-dependent changes between Atrogin-1 and MuRF1 protein content, suggesting differential regulatory mechanisms between Atrogin-1 and MuRF1 protein content. To further explore the downstream of mTORC1 signaling, we employed a specific S6K1 inhibitor, PF-4708671, and found that Atrogin-1 protein content was dose-dependently increased with PF-4708671 treatment, whereas MuRF1 protein content was not significantly altered. Overall, our results indicate that Atrogin-1 and MuRF1 protein contents are regulated by different mechanisms, the downstream of Akt, and that Atrogin-1 protein content can be regulated by rapamycin-sensitive mTOR-S6K1 dependent signaling pathway.

4.2 Introduction

Atrogin-1 (also known as Muscle atrophy F-box protein: MAFbx or *FBXO32*) and Muscle-specific RING finger protein 1 (MuRF1 or *TRIM63*) are muscle specific E3 ligases and their expression is highly associated with various skeletal muscle atrophic models (116, 117). In agreement with the above, a plethora of studies has confirmed that Atrogin-1 and MuRF1 mRNA expression are useful molecular biomarkers of skeletal muscle atrophy (118). Although both Atrogin-1 and MuRF1 gene expressions increase in almost all atrophic models, various muscle atrophic conditions (e.g., fasting, immobilization, diabetes, insulin resistance) are likely to alter multiple signaling pathways to control Atrogin-1 and MuRF1 gene and protein expression (107). While the PI3K-Akt signaling pathway is known to regulate Atrogin-1 and MuRF1 gene expression, the mechanisms that regulate protein content of these two E3 ligases remain to be elucidated. Many studies have assumed that mRNA expressions implicitly reflect the corresponding changes of protein content, but in reality, the expression levels of individual mRNA and its corresponding protein are indeed poorly correlated (317, 318). The poor correlation can be explained by multiple processes, including transcription and degradation of mRNAs, translation, folding, and degradation of proteins (319, 320). As protein is the final product executing gene function, direct measurement of protein content should be more relevant to biological functions (320, 321). However, in the cases of Atrogin-1 and MuRF1, poor quality of antibodies is often a major obstacle to reveal protein content in biological samples (39, 118, 322).

PI3K/Akt/forkhead box (FoxO) signaling is one of the most well studied pathways known to regulate Atrogin-1 and MuRF1 mRNA transcription expression (119-121).

Studies have shown that treatment with IGF-1 or the introduction of constitutively active Akt prevents both Atrogin-1 and MuRF1 mRNA transcription expression in C2C12 myotubes (119, 120). In addition, denervation-induced skeletal muscle atrophy was prevented by IGF-1 treatment (120). IGF-1 increases Akt phosphorylation and suppresses Atrogin-1 and MuRF1 mRNA transcription expression in mouse skeletal muscle (120), indicating a link between Akt and Atrogin-1/MuRF1 axis in skeletal muscle atrophy. Mechanistically, Akt phosphorylates the transcription factor FoxO to induce FoxO nuclear exclusion, which downregulates FoxO-dependent gene transcription (122). A study has also confirmed that overexpression of FoxO3a in mouse skeletal muscle is able to induce Atrogin-1 mRNA expression and an atrophic phenotype (119). In contrast, siRNA knockdown of FoxO1-3 inhibits Atrogin-1 promoter activity measured by Atrogin-1 luciferase reporter constructs during fasting-induced muscle atrophy (119). All these findings have evidenced that Akt-FoxO axis is critical for regulating Atrogin-1 and MuRF1 mRNA transcriptional expression. However, some contradictory results have also been reported. For example, a study showed that deletion of Akt1 or Akt2 did not alter Atrogin-1 mRNA and protein expressions in mouse skeletal muscle (323). Atrogin-1 and MuRF1 mRNA expression, including Atrogin-1 protein content, were shown to be unchanged in ageing-induced muscle atrophy, where Akt activity and FoxO3a phosphorylation were elevated, compared to young control skeletal muscles (324). These contradictory findings raise the question of whether Akt signaling pathway solely regulates Atrogin-1 and MuRF1 expression.

mTORC1 plays an important role in regulating protein synthesis and the autophagy-lysosome system (59), and its activation has been well associated with skeletal muscle hypertrophy (75, 76). Surprisingly, the involvement of mTORC1 in regulating muscle protein degradation has not been well investigated. A recent study led by Zhao et al. (64) suggested that mTOR (including mTORC1 and mTORC2) may be involved in the regulation of protein degradation in C2C12 myotubes. Their previous study has shown that treatment of rapamycin, a specific mTORC1 inhibitor, can increase protein degradation in C2C12 myotubes (325), which led the authors to suggest that mTORC1 may contribute to control protein degradation via the ubiquitin proteasome system. Furthermore, there is also evidence suggesting that Atrogin-1 and MuRF1 mRNA expressions are regulated by distinct signaling mechanisms. Sacheck et al. (325) showed that rapamycin treatment increases Atrogin-1, but not MuRF1, mRNA expression. However, the proof at protein level is currently lacking and such information is needed to better understand what the signaling mechanisms are controlling Atrogin-1 and MuRF1 protein content, which essentially execute the enzymatic ubiquitin E3 ligase activity.

The present study, therefore, aims to investigate whether the downstream targets of Akt, such as the mTORC1 and S6K1 signaling pathway, is involved in controlling Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Using small molecules inhibiting mTORC1 or S6K1 activity, we demonstrated that Atrogin-1, but not MuRF1, protein content is regulated in the rapamycin-sensitive mTOR and S6K1-dependent signaling pathways. Our results suggest that the role of Akt-FoxO is not the only signaling pathway regulating Atrogin-1 protein content and that the downstream of Akt, such as

the rapamycin-sensitive mTOR and S6K1-dependent signaling pathways, are involved in regulating Atrogin-1 protein content in skeletal muscle.

4.3 MATERIALS AND METHODS

4.3.1 C2C12 cell culture

Mouse skeletal muscle C2C12 myoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were seeded and maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Loughborough, UK, 31966021) containing GlutaMAX, 25 mM of glucose, and 1 mM of sodium pyruvate, supplemented with 10% (v/v) of Hyclone fetal bovine serum (FBS, Fisher Scientific, Loughborough, UK, SV30180.03), 1% (v/v) of Penicillin-Streptomycin (10 000 Units/mL-ug/mL, Thermo Fisher Scientific, Loughborough, UK, 15140122). Myoblasts were seeded onto six-well multidishes (greiner bio-one, 657 160) and when confluency reached at 90%, myoblasts were differentiated into myotubes for six days in DMEM supplemented with 2% (v/v) of horse serum (Sigma-Aldrich, Cambridgeshire, UK, H1270), 1% (v/v) of Penicillin-Streptomycin. The media was changed every 48 h. Cultures were maintained in a humified incubator at 37 ℃ with an atmosphere of 5% of CO2 and 95% of air.

4.3.2 Drug reconstitution and cell treatment

Akt1/2/3 inhibitor MK-2206 dihydrochloride (ApexBio, A3010), Rapamycin (Sigma-Aldrich, 553211), adenosine monophosphate (AMP)–activated protein kinase (AMPK) activator 991 (AOBIOUS, MA, USA, AOB8150), S6K1 Inhibitor, PF-4708671 (Sigma-Aldrich, Dorset, UK, 559278) were prepared in DMSO and treatment conditions were

described in the figure legend. Insulin solution human was obtained from Sigma (Sigma Aldrich, Dorset, UK, I9278).

4.3.3 Cell lysis

Cells were lysed in ice-cold sucrose lysis buffer containing: 250 mM of sucrose, 50 mM of Tris-base (pH 7.5), 50 mM of sodium fluoride, 10 mM of sodium β-glycerophosphate, 5 mM of sodium pyrophosphate, 1 mM of EDTA, 1 mM of EGTA, 1 mM of benzamidine, 1 mM of sodium orthovanadate, 1 x complete Mini EDTA-free protease inhibitor cocktail (Roche), 1% of Triton X-100, and 100 mM of 2-chloroacetamide. Cell lysates were centrifuged for 15 minutes at 13 000 x g at 4°C and the supernatant was stored at -80°C before analysis for total protein concentrations using the Bradford protein assay (Thermo Fisher Scientific, Leicestershire, UK, 23200). Protein in each sample was quantified from a standard curve using BSA standards (Thermo Fisher Scientific, Leicestershire, UK, 23209).

4.3.4 Western blot

Cell lysates were prepared in 1x NuPAGE LDS sample buffer (Invitrogen, NP0008) containing 2-mercaptoethanol (final concentration 1.5%) and left to denature overnight at room temperature. Prepared cell lysates (10-15 μg of total protein) were loaded into 8% or 10% Bis/Tris gels prior to sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE). Gels were run in 1x MOPS buffer for approximately 60 minutes at 140V. Proteins were transferred onto 0.2 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Hertfordshire, UK) for 1 hour at 100V. Membranes were blocked in 5% of milk diluted in Tris-buffered saline Tween-20 (TBS-T): 137 mM of

sodium chloride, 20 mM of Tris-base 7.5 pH, 0.1% of Tween-20 for 1 hour. After blocking, membranes were washed 3 times for 5 min in TBS-T before being incubated overnight at 4°C with the appropriate primary antibodies (**Table 4.1**). Membranes were washed 3 times for 5 min in TBS-T prior to incubation in horseradish peroxidase-conjugated secondary antibodies (**Table 4.1**) at room temperature for 1 h. Membranes were washed a further three times in TBS-T prior to antibody detection using enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Hertfordshire, UK). Imaging was undertaken using a G:BOX Chemi-XR5 (Syngene, Cambridgeshire, UK). Band intensities were quantified using ImageJ/Fiji (NIH, Bethesda, MD, USA). Phosphorylation levels were determined by the expression of phosphorylated protein divided by the expression of non-phosphorylated total protein. Vinculin was used as the loading control.

Table 4.1 Antibodies for western blot.

Antibodies	Dilution	Source	Identifier
phospho-Ser473 Akt	1:1000	Cell Signaling Technology	Cat# 4060
phospho-Thr308 Akt	1:1000	Cell Signaling Technology	Cat# 2965
Akt	1:1000	Cell Signaling Technology	Cat# 4691
phospho-Thr389 p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 9234
p70 S6 Kinase	1:1000	Cell Signaling Technology	Cat# 2708
phospho-Ser240/244 S6 Ribosomal Protein	1:8000	Cell Signaling Technology	Cat# 5364
S6 Ribosomal Protein	1:8000	Cell Signaling Technology	Cat# 2217
phospho-Thr172 AMPKα	1:1000	Cell Signaling Technology	Cat# 2535
AMPKα	1:1000	Cell Signaling Technology	Cat# 2532
Atrogin-1	1:1000	ECM Biosciences	Cat# AM3141
MuRF1	1:1000	Santa Cruz	Cat# SC-398608
phospho-Ser555 ULK1	1:1000	Cell Signaling Technology	Cat# 5869
ULK1	1:1000	Cell Signaling Technology	Cat# 4773
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	1:750	Cell Signaling Technology	Cat# 9464
Vinculin	1:2000	Abcam	Cat# Ab129002
Anti-mouse IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat# 7076
Anti-rabbit IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat# 7074
Anti-Rat IgG, HRP-linked Antibody	1:10000	Cell Signaling Technology	Cat#7077

4.3.5 Statistical analysis

The statistical analyses were performed using Prism version 8.1.2 (GraphPad Software, San Diego, California USA, www.graphpad.com). Values of $P < 0.05$ (*) were considered statistically significant. For time course and dose-response experiments, a one-way analysis of variance (ANOVA) was performed with Dunnett's post-hoc test

compared to control (CON). Data are presented as mean ± SD. All experiments were performed in duplication and repeated at least twice.

4.4 Results

4.4.1 Evidence of Insulin/Akt/FoxO signaling pathway modulating Atrogin-1 and MuRF1 protein content

We first confirmed if insulin/Akt/FoxO signaling pathway is sufficient to modulate both Atrogin-1 and MuRF1 protein content in C2C12 myotubes. Using an allosteric Akt inhibitor (MK-2206), we showed that Atrogin-1 protein content was significantly increased at 3 h, 6 h, and 9 h after the treatment of 10 μM MK-2206 (**Fig. 4.1b**). MuRF1 protein content was also significantly increased at 6 h after the treatment of MK-2206 (**Fig. 4.1c**). In line with a previous study (326), Akt phosphorylation at Ser^{473} and Thr^{308} was completely abolished over the course of 9 h treatment with MK-2206 (**Fig. 4.1a**). We also confirmed that inhibition of Akt activity prevents FoxO1 and FoxO3a phosphorylation and reduces S6K1 and rpS6 phosphorylation (**Fig. 4.1a**).

Atrogin-1 protein content was significantly decreased at 3 h, 6 h, and 9 h following the treatment of 100 nM insulin stimulation (**Fig. 4.1b**). MuRF1 protein content was also significantly decreased at 6 h and 9 h after insulin treatment (**Fig. 4.1c**). As expected, insulin stimulated Akt phosphorylation at both Ser^{473} and Thr^{308} sites. The enhanced Akt activity was also confirmed by the increases in its downstream targets, such as FoxO1, FoxO3a, S6K1, and rpS6 phosphorylation (**Fig. 4.1a**).

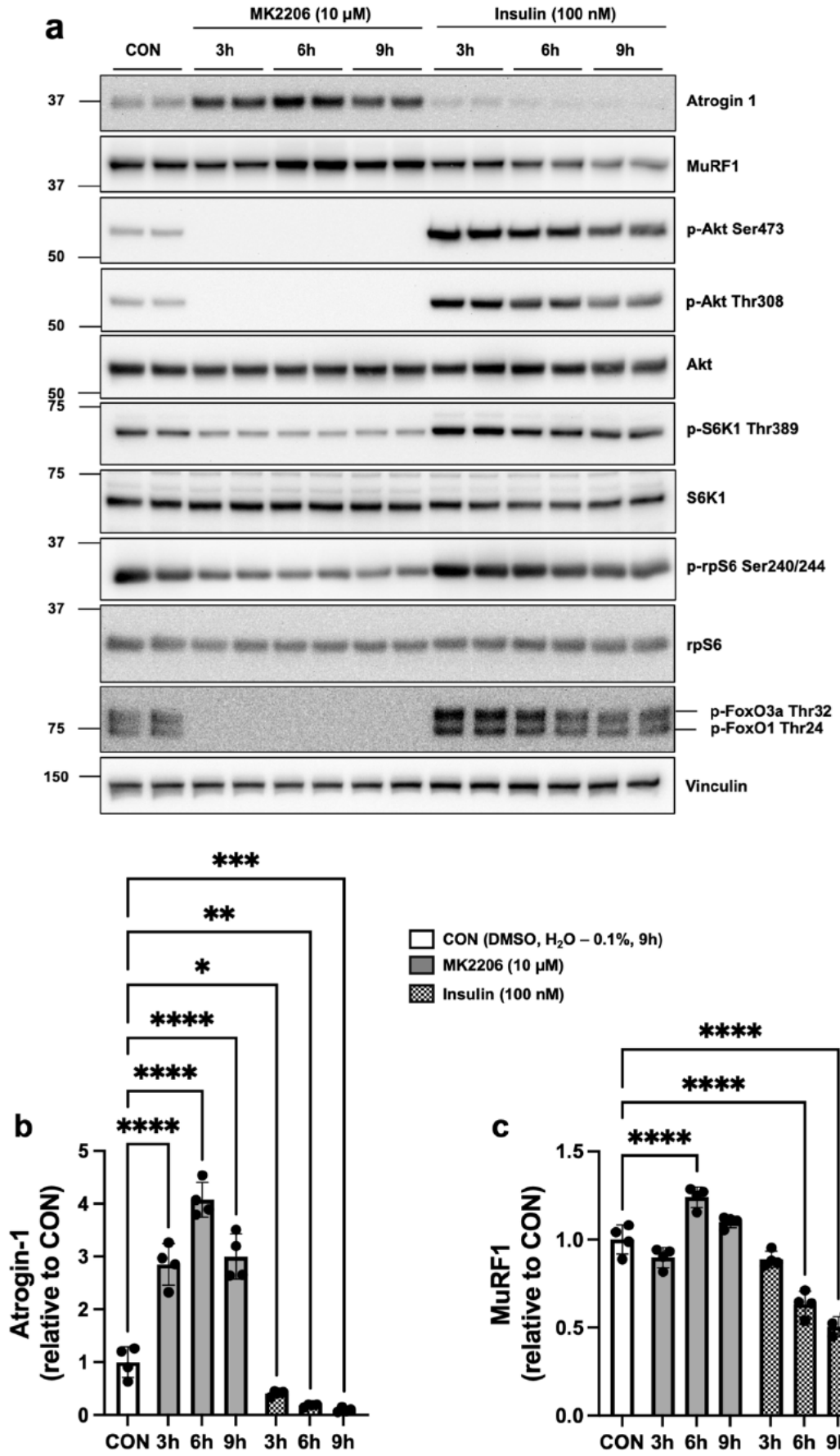
a
MK2206 (10 μM)
Insulin (100 nM)
CON
3h
6h
9h
3h
6h
9h
37
Atrogin 1
37
MuRF1
50
p-Akt Ser473
50
p-Akt Thr308
50
Akt
75
p-S6K1 Thr389
75
S6K1
37
p-rpS6 Ser240/244
37
rpS6
75
p-FoxO3a Thr32
p-FoxO1 Thr24
150
Vinculin
CON (DMSO, H2O – 0.1%, 9h)
MK2206 (10 μM)
Insulin (100 nM)
b
Atrogin-1 (relative to CON)
c
MuRF1 (relative to CON)
CON 3h 6h 9h 3h 6h 9h

Figure 4.1 Insulin/Akt signaling pathway is sufficient to modulate Atrogin-1 and MuRF1 protein contents in C2C12 myotubes.

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON), MK2206 (10 μM), or insulin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (a) Representative images from one of two independent experiments. (b) Quantification of Atrogin-1. (c) Quantification of MuRF1. Data are expressed as means ± SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ compared to CON.

4.4.2 Atrogin-1, but not MuRF1, protein content is increased by the rapamycin-sensitive mTOR inhibition

Acute treatment with Rapamycin can specifically inhibit mTORC1 activity without directly affecting mTORC2 activity, but a long-term treatment (≥ 24 h) is known to inhibit mTORC2 activity (60). Therefore, we have limited the treatment time of small molecules to not more than 9 h. Interestingly, Atrogin-1 protein content was increased at 3 h, 6 h, and 9 h following the treatment of 100 nM rapamycin (**Fig. 4.2b**). Despite that Atrogin-1 protein content was increased, MuRF1 protein content remained unchanged (**Fig. 4.2c**). As anticipated, rapamycin treatment completely inhibited S6K1 and rpS6 phosphorylation (**Fig. 4.2a**) without inducing a significant change in Akt phosphorylation ($P = 0.38$).

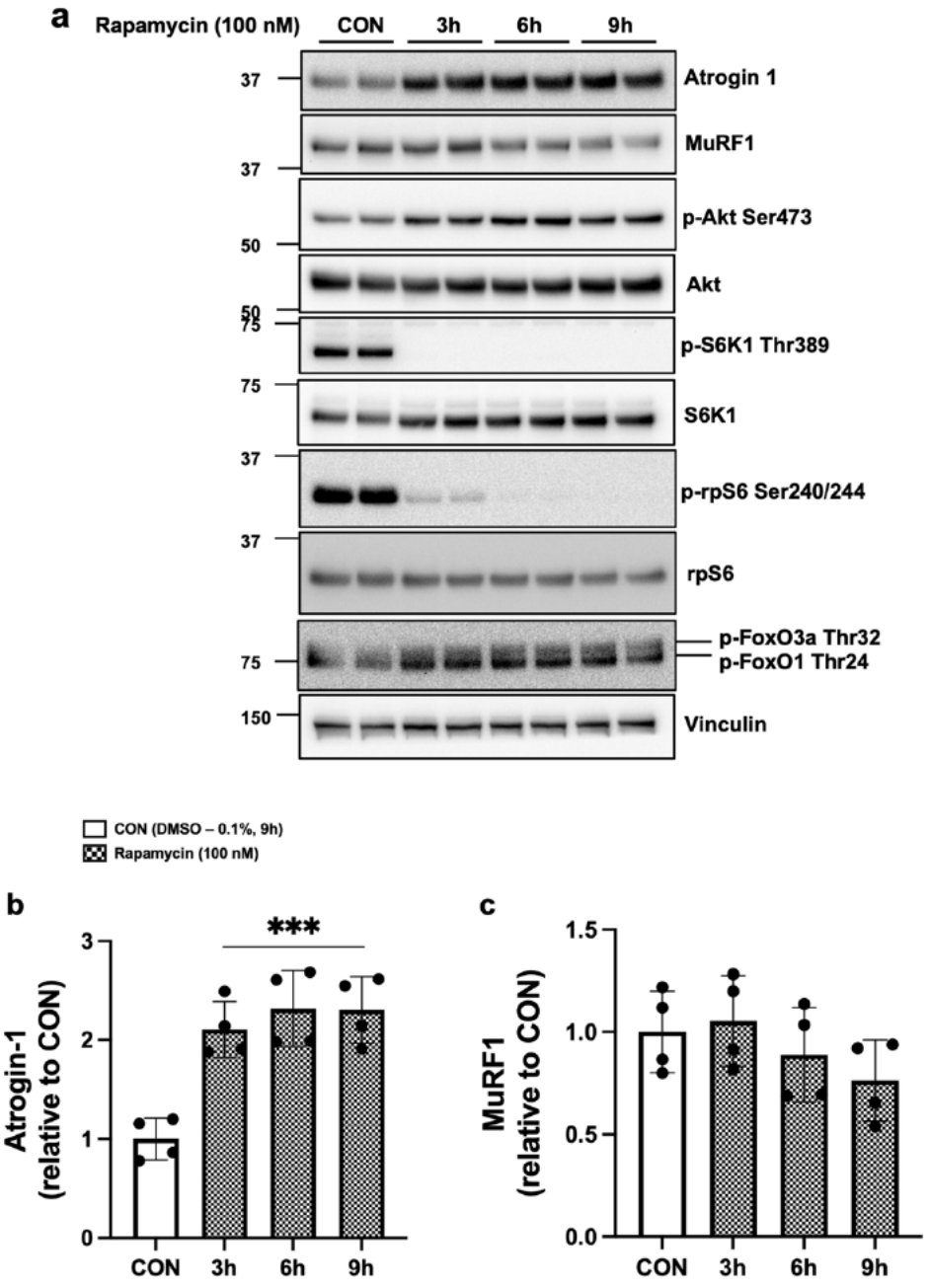

Figure 4.2 Rapamycin-sensitive mTOR inhibition increases Atrogin-1, but not MuRF1 protein contents in C2C12 myotubes.

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or Rapamycin (100 nM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (a) Representative images from one of two independent experiments. (b) Quantification of Atrogin-1. (c) Quantification of MuRF1. Data are expressed as means ± SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, ***$P < 0.001$ compared to CON.

4.4.3 Distinct time-dependent changes of Atrogin-1 and MuRF1 protein content following AMPK activation

AMPK activation is known to inhibit mTORC1 activity (327) via the phosphorylation of tuberous sclerosis complex 2 (TSC2) (328) and Raptor (329). To further investigate the role of mTORC1 on the regulation of Atrogin-1 and MuRF1 protein content, we used a direct AMPK activator, 991, to increase AMPK activity in C2C12 myotubes (327, 330). Interestingly, Atrogin-1 protein content was increased rapidly at 3 h and 6 h, despite returning to the basal level after 9 h of treatment (**Fig. 4.3b**). In contrast, MuRF1 protein content had obviously delayed increases at 6 h and 9 h after 991 treatment (**Fig. 4.3c**). These results again suggest that Atrogin-1 and MuRF1 protein content are regulated by distinct signaling mechanisms. As expected, ULK1 phosphorylation at Ser^{555} was increased by the treatment of 991 (**Fig. 4.3a**) (331) and the inhibition of mTORC1 activity was confirmed by showing a decrease in S6K1 and rpS6 phosphorylation (**Fig. 4.3a**).

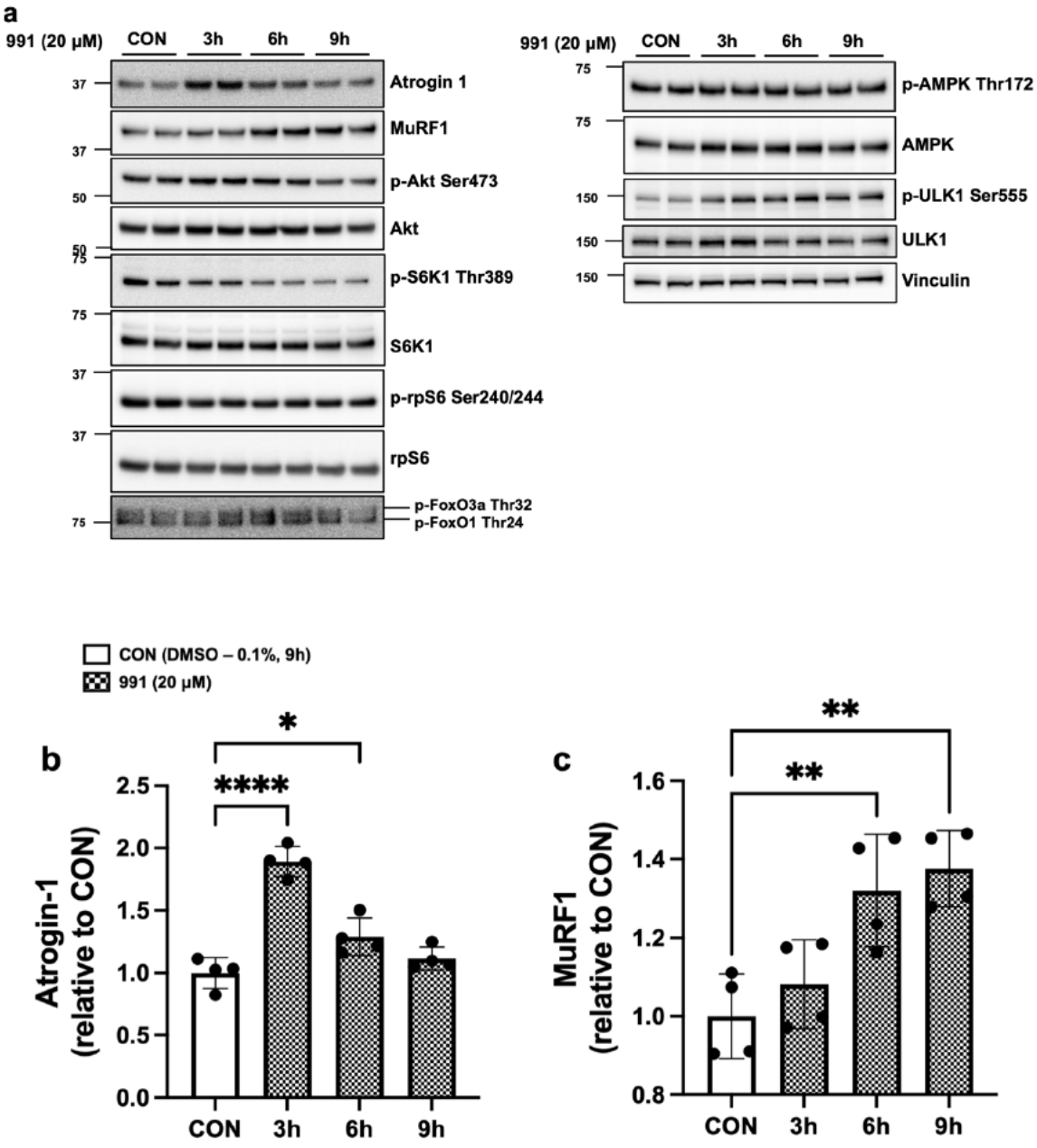

Figure 4.3 Inhibition of the mTORC1 pathway by AMPK activator 991 on Atrogin-1 and MuRF1 protein contents in C2C12 myotubes.

C2C12 myotubes were treated with DMSO (0.1%, 9 h) as a vehicle control (CON) or 991 (20 μM) for 3, 6, or 9 h. Lysates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (a) Representative images from one of two independent experiments. (b) Quantification of Atrogin-1. (c) Quantification of MuRF1. Data are expressed as means ± SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ compared to CON.

4.4.4 Atrogin-1 protein content is increased by S6K1 inhibition

To further explore the distinct mechanisms that regulate Atrogin-1 and MuRF1 protein content, we asked whether mTORC1 downstream, such as S6K1, is involved in regulating Atrogin-1 or MuRF1 protein content. Using a specific S6K1 inhibitor (332), we showed that Atrogin-1 (**Fig. 4.4b**) protein content was increased in a dose-response manner, where significant increases were seen with the treatment of 40 μM and 50 μM PF-4708671. Instead of increasing, MuRF1 protein content was indeed decreased at 50 μM (**Fig. 4.4c**). Inhibition of S6K1 was confirmed by the observation of reduced rpS6 phosphorylation (**Fig. 4.4a**). As expected, the phosphorylation of S6K1 was increased by the treatment of PF-4708671 (332) (**Fig. 4.4a**). Next, we performed Pearson's correlation coefficient to identify the relationship between p-rpS6$^{Ser240/244}$/rpS6 and Atrogin-1 or MuRF1 by plotting the dose-response data (**Fig. 4.4d**). Interestingly, a strong negative correlation was observed between p-rpS6$^{Ser240/244}$/rpS6 and Atrogin-1 ($r = -0.90$, $P < 0.0001$), whereas no significant association was observed between p-rpS6$^{Ser240/244}$/rpS6 and MuRF1 ($r = 0.17$, $P = 0.44$).

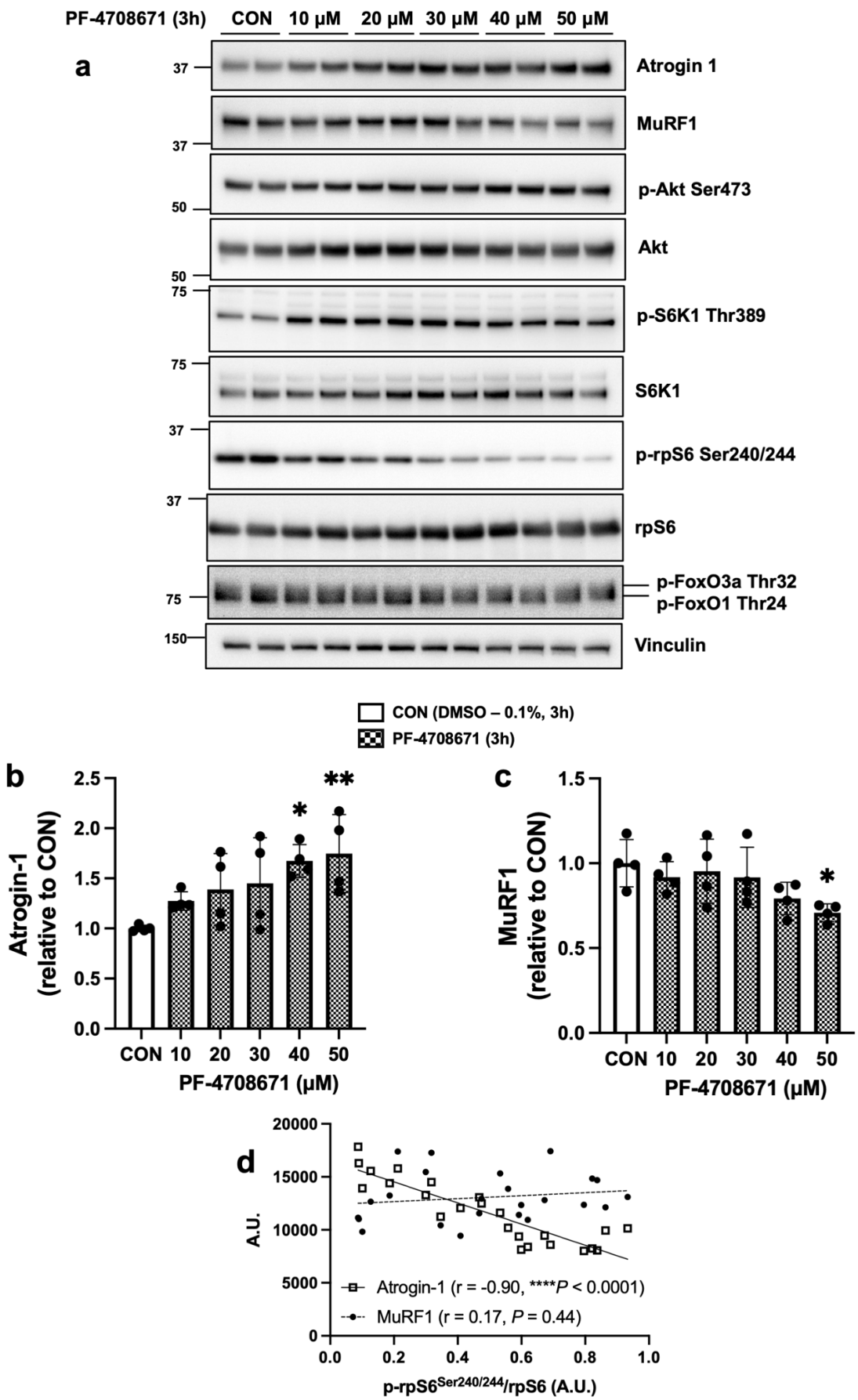
PF-4708671 (3h)
CON
10 μM
20 μM
30 μM
40 μM
50 μM
a
Atrogin 1
MuRF1
p-Akt Ser473
Akt
p-S6K1 Thr389
S6K1
p-rpS6 Ser240/244
rpS6
p-FoxO3a Thr32
p-FoxO1 Thr24
Vinculin
CON (DMSO – 0.1%, 3h)
PF-4708671 (3h)
b
Atrogin-1 (relative to CON)
PF-4708671 (μM)
c
MuRF1 (relative to CON)
PF-4708671 (μM)
d
A.U.
Atrogin-1 (r = -0.90, ****P < 0.0001)
MuRF1 (r = 0.17, P = 0.44)
p-rpS6Ser240/244/rpS6 (A.U.)

Figure 4.4 A dose-response effect of S6K1 inhibitor on Atrogin-1 and MuRF1 protein contents in C2C12 myotubes.

C2C12 myotubes were treated with DMSO (0.1%, 3 h) as a vehicle control (CON) or PF-4708671 at the indicated doses for 3 h. Lysates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (a) Representative images of 2 independent experiments. (b) Quantification of Atrogin-1. (c) Quantification of MuRF1. Data are expressed as means ± SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ compared to CON. (d) Pearson's correlation coefficient to identify the association between p-rpS6$^{Ser240/244}$/rpS6 and Atrogin-1 or MuRF1.

To confirm that S6K1 inhibition increases Atrogin-1, but not MuRF1, protein content, we performed a time course experiment using 30 μM of PF-4708671 for up to 24 h (**Fig. 4.5a**). As anticipated, the protein content of Atrogin-1 was increased over the course of PF-4708671 treatment at 3 h, 6 h, and 24 h (**Fig. 4.5b**). Although MuRF1 protein content (**Fig. 4.5c**) remained unchanged over the majority of the time points, there was still an unexpected increase that occurred at 6 h after PF-4708671 treatment based on PF-4708671 dose-response data in **Fig. 4.4c**.

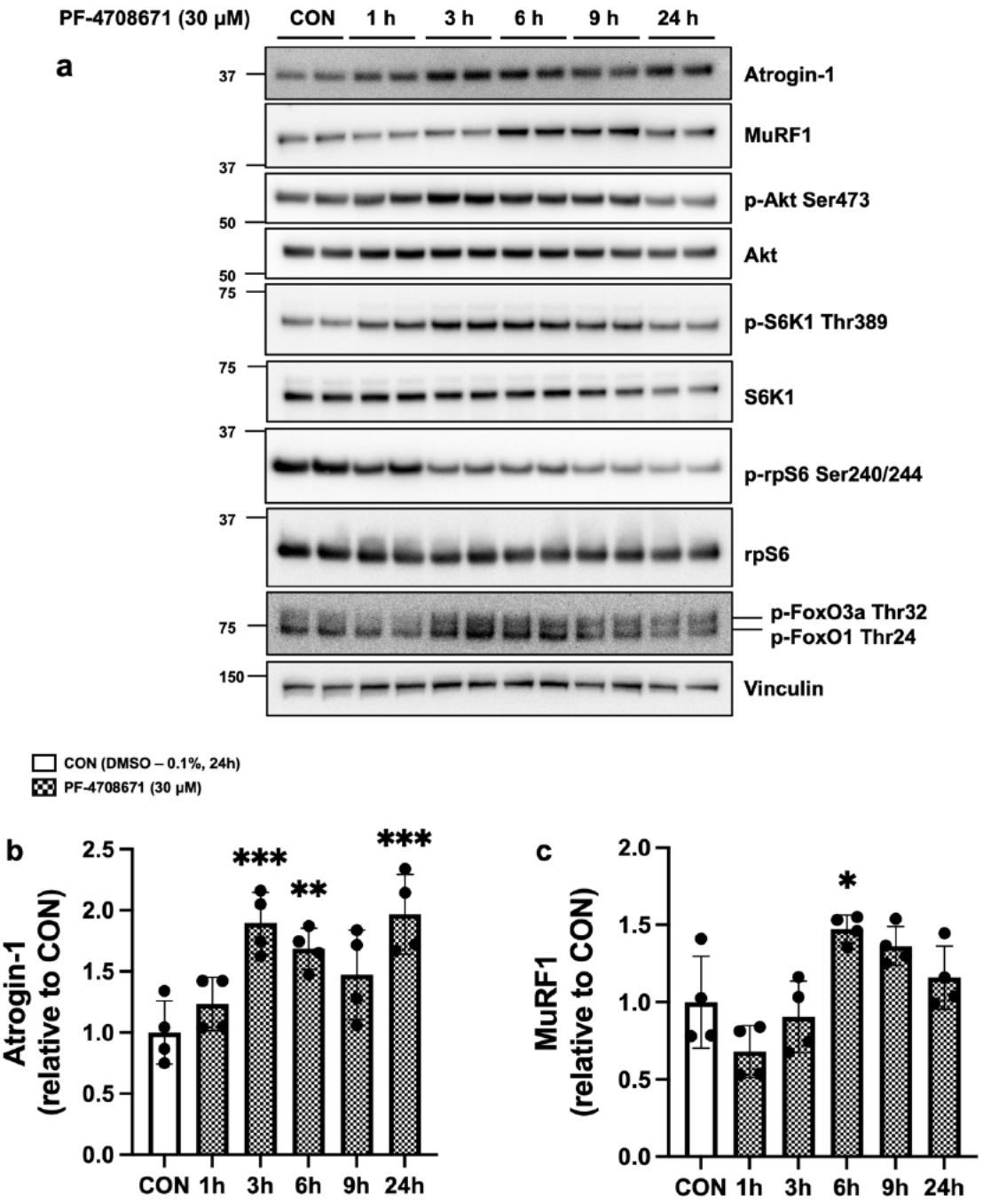

Figure 4.5 A time course effect of S6K1 inhibitor on Atrogin-1 and MuRF1 protein contents in C2C12 myotubes.

C2C12 myotubes were treated with DMSO (0.1%, 24 h) as a vehicle control (CON) or PF-4708671 (30 μM) for up to 24 h. Lysates were analyzed by SDS-PAGE and western blotting with the indicated antibodies. (a) Representative images from one of two experiments. (b) Quantification of Atrogin-1. (c) Quantification of MuRF1. Data are expressed as means ± SD (n = 4) fold changes relative to CON. One-way ANOVA with Dunnett's post-hoc test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to CON.

4.5 Discussion

The gene expression of Atrogin-1 and MuRF1 are highly associated with almost all kinds of skeletal muscle atrophy (116-118). Genetic studies have also shown that knockout of Atrogin-1 or MuRF1 partially rescues denervation-induced skeletal muscle atrophy (116). However, the molecular mechanisms of how Atrogin-1 and MuRF1 contribute to skeletal muscle atrophy are still unclear. The most recent study has indicated that the enzymatic activity of these ubiquitin E3 ligases is particularly important in controlling skeletal muscle mass (333). Therefore, obtaining information relevant to the regulation of Atrogin-1 and MuRF1 protein content will provide an alternative opportunity to manipulate their functional E3 ligase activity. This information will also help identify new therapeutic targets to treat and/or prevent skeletal muscle atrophy. Here, we have made use of small molecules to evaluate some key signaling pathways that modulate Atrogin-1 and MuRF1 protein contents in C2C12 myotubes. In accordance with previous studies, we confirmed that insulin/Akt/FoxO pathway is sufficient to modulate both Atrogin-1 and MuRF1 protein contents, which is in agreement with the tendency of measuring mRNA transcriptional expression (119-121, 325). Further investigation revealed that Atrogin-1, but not MuRF1, protein content is predominantly increased when rapamycin-sensitive signaling pathway is inhibited. These findings show that Atrogin-1 protein content is also regulated via Akt-FoxO independent mechanisms. More interestingly, our studies also revealed that Atrogin-1 protein content can be regulated by S6K1 dependent signaling pathway.

Inactivation of PI3K/Akt/FoxO signaling pathway is well known as an “atrophic signal” that increases both MuRF1 and Atrogin-1 mRNA expression (50). However, few

studies have investigated whether MuRF1 and Atrogin-1 protein contents are regulated in accordance with their gene/mRNA expressions. The current study confirmed that protein content of both Atrogin-1 and MuRF1 were suppressed by insulin, whereas Atrogin-1 and MuRF1 protein contents were upregulated by the treatment of MK-2206. These findings are consistent with the mRNA expressions investigated by previous studies (119-121, 325).

In the present study, we showed that Atrogin-1 protein content was increased after 3 h treatment of rapamycin, whereas MuRF1 protein content was not changed throughout the time course (**Fig. 4.2**). This data indicates that inhibition of mTORC1 signaling can enhance Atrogin-1, but not MuRF1, protein content. This is indeed consistent with a previous study that reported that inhibition of rapamycin-sensitive signaling pathway increases Atrogin-1, but not MuRF1, mRNA expression (325). Our findings strengthened the previous evidence of mRNA data (325) by showing that inhibition of the rapamycin-sensitive mTOR-S6K1 signaling pathway also induces an increase in Atrogin-1 protein content. We also used AMPK activator, 991 to reduce mTORC1 activity, as AMPK activation is known to inhibit mTORC1 activity (327) via the phosphorylation of tuberous sclerosis complex 2 (TSC2) (328) and Raptor (329). However, AMPK possibly modulated Atrogin-1 and/or MuRF1 protein content via mTORC1-independent mechanisms. For example, AMPK is known to phosphorylate FoxO3 at different amino acid residues and regulate FoxO3 transcriptional activity without affecting cellular localization (334). Thus, AMPK-FoxO axis may play a role in modulating Atrogin-1 and MuRF1 protein content after 991 treatment. However, the

distinct time-course changes of Atrogin-1 and MuRF1 protein content indicate that these proteins are controlled by distinct signaling mechanisms.

The most interesting findings in the present study are that Atrogin-1 and MuRF1 protein contents can be regulated differently, and that Atrogin-1 protein content is regulated by rapamycin-sensitive and S6K1 dependent signaling pathways. In the present study, the phosphorylation of Akt at Ser473, FoxO3a at Thr^{32} and FoxO1 at Thr^{24} were increased after rapamycin or PF-4708671 treatment. We reason that this observation is due to the suppression of a negative feedback loop to IRS1. S6K1 has been known to control a feedback loop that inhibits the PI3K/Akt/mTOR pathway (**Fig. 4.6**). It was reported that when PI3K/Akt/mTOR pathway is hyperactivated, S6K1 phosphorylates insulin receptor substrate-1 (IRS-1) on multiple serine residues, which induces its degradation (335, 336). In our study, Atrogin-1 was increased following rapamycin or PF-4708671 treatment while phosphorylation of Akt at Ser473, FoxO3a at Thr^{32} and FoxO1 at Thr^{24} were increased, suggesting that mTORC1 might be dominant over Akt in regulating Atrogin-1 protein content. This may also suggest FoxOs are not the most critical factor regulating Atrogin-1 (as well as MuRF1) protein content. Multiple transcription factors, including the NF-κB transcription factors CCAAT/enhancer-binding protein-β (C/EBPβ) and Smad3, can work cooperatively to regulate Atrogin-1 mRNA transcription Atrogin-1 (118, 337). Thus, complex cooperative mechanisms of transcription factors might have been involved in the distinct protein expression patterns between Atrogin-1 and MuRF1 protein content. In supporting our finding that Atrogin-1 protein content is regulated by S6K1 dependent signaling, previous studies have also shown that the absence of S6K1 causes skeletal muscle atrophy in mice (338). In

addition, Marabita et al. (339) reported that S6K1 is required for the prevention of protein aggregation during skeletal muscle hypertrophy in mice. These observations led us to hypothesize that protein quality control, mainly protein degradation, is the mechanism inducing the increased Atrogin-1 protein content when rapamycin-sensitive mTOR-S6K1 signaling is inhibited. However, future studies should confirm this hypothesis by investigating the process of Atrogin-1 protein turnover rate, and subsequent protein content.

The mTORC1 signaling pathway has been shown as a positive regulator of skeletal muscle mass in several models of hypertrophy (51, 75, 76). In support of age-related muscle loss, studies have demonstrated that muscle contraction-induced activation of mTORC1 signaling is impaired with ageing (313, 314). In contrast, constant activation of mTORC1 is known to cause myopathy, but not hypertrophy (340). Moreover, a most recent study led by Joseph et al. (341) showed that mTORC1 signaling pathway is indeed hyperactivated in age-related muscle loss with a concomitant increase in both Atrogin-1 and MuRF1 mRNA expression in basal rat skeletal muscle. More interestingly, partial inhibition of mTORC1 via RAD001 restored age-related skeletal muscle loss (341). RAD001 treatment also decreased MuRF1 mRNA expression while Atrogin-1 mRNA was not altered in ageing muscle. We cannot directly compare our findings to their results as they did not report Akt activity and information of MuRF1 and Atrogin-1 protein contents was not available. Nonetheless, these findings indicate the importance of fine tuning the mTORC1 activity in maintaining skeletal muscle mass, and Atrogin-1 and/or MuRF1 may be responsible for this.

Protein content is determined by protein turnover, which is a continuous process of protein synthesis and protein degradation (25, 39). In this study, Atrogin-1 and MuRF1 protein contents were investigated following time-course and/or dose-dependent small molecule treatments, which is a snapshot in time of the impact of the protein turnover kinetics on protein balance. mTORC1 is a well-known signaling pathway to control protein synthesis. Thus, after the treatment of rapamycin or PF-4708671, a decrease in protein synthesis would be expected and a greater decrease in protein degradation would, in theory, contribute to the observed increase in Atrogin-1 protein content. Post-translational modifications and the subsequent degradation make it more complicated to understand how protein content is regulated. For example, many ubiquitin E3 ligases have been implicated to regulate their own protein abundance (342) because most E3 ligases have the ability to ubiquitylate themselves (known as autoubiquitylation) and trigger self-degradation processes (either via proteasome or autophagic lysosome). For example, the greater autoubiquitylation usually demonstrates greater E3 ligase activity (322), which was observed in MuRF1 via in vitro reaction (116). However, the degree of autoubiquitylation on MuRF1 and Atrogin-1 is currently not clear in any muscle atrophy conditions. Although it is not clear from the present study, autoubiquitylation might have been involved in the regulation of Atrogin-1 and MuRF1 protein contents.

While our findings suggest that Atrogin-1 and MuRF1 protein contents are regulated by different signaling mechanisms, future studies should determine which molecules in the rapamycin-sensitive mTOR-S6K1 signaling cascade are responsible for regulating Atrogin-1 protein content. With the use of our protein content data, other studies should also investigate whether E3 ligase activity of MuRF1 and/or Atrogin-1 is associated

with their protein content, and thus a measurement of protein content can be used as a biomarker for E3 ligase activity or vice versa. Additionally, protein degradation contributes half of the equation to determine protein content (i.e., protein synthesis – protein degradation = protein content). Thus, determining degradation mechanisms of Atrogin-1 and MuRF1 protein contents is also important to modulate protein half-life. Thus, understanding the degradation mechanisms of Atrogin-1 and MuRF1 is required as an important step towards understanding the underlying mechanisms of skeletal muscle atrophy and manipulating their functional E3 ligase activity.

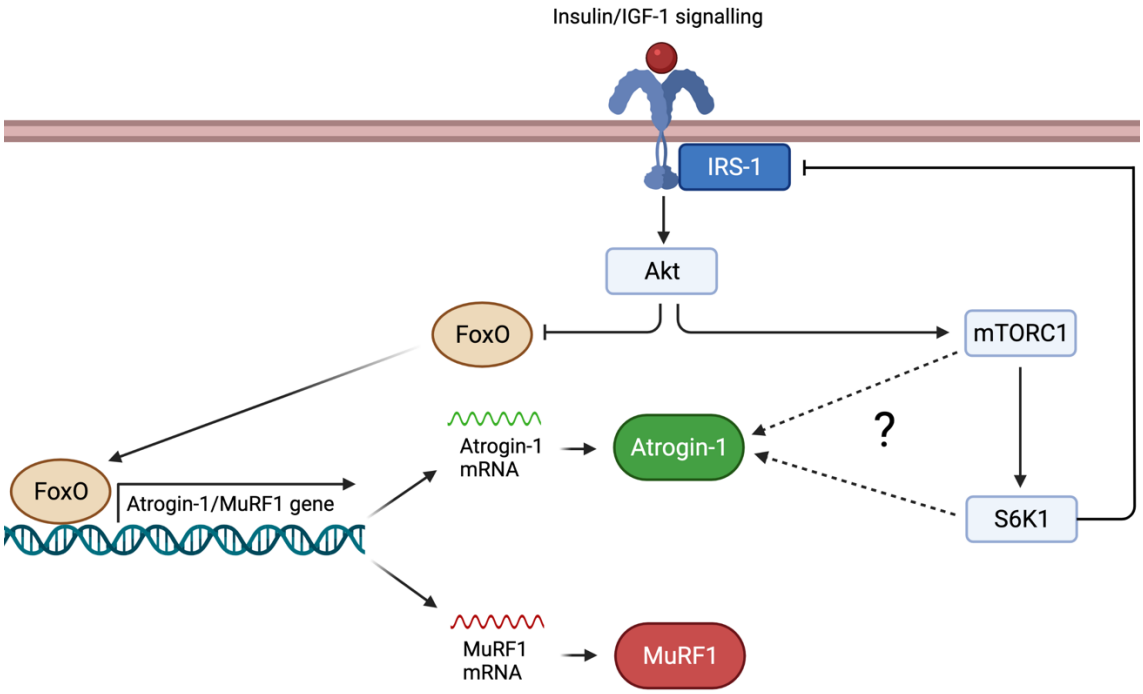

Figure 4.6 Atrogin-1 and MuRF1 protein contents are differentially regulated in Akt and the rapamycin-sensitive mTOR-S6K1 signaling pathway.

Insulin/IGF-1/Akt/FoxO signaling pathway is a predominant mechanism regulating Atrogin-1 and MuRF1 expression at both mRNA transcription and protein levels in skeletal muscle. Upon insulin or IGF-1 stimulation, the binding of their respective receptors triggers a signaling cascade to activate Akt. Akt phosphorylates and inhibits FoxO by preventing their localization to the nuclei, and thus FoxO remains in the cytoplasm. In catabolic conditions, FoxO is less phosphorylated and remains in the nuclei

to promote Atrogin-1 and MuRF1 mRNA transcription and thereby increasing their protein content. Inhibition of mTORC1 or S6K1, one of the Akt downstream signaling, can promote Atrogin-1, but not MuRF1, protein content without altering Akt and FoxO phosphorylation. The evidence indicates that Atrogin-1 and MuRF1 protein content are regulated by at least two different mechanisms. How rapamycin-sensitive mTOR and S6K1 dependent signaling pathways regulate Atrogin-1 protein content remains undetermined. S6K1 has been known to control a feedback loop that inhibits the PI3K/Akt/mTOR pathway when its signaling pathway is hyperactivated. Insulin receptor substrate-1 (IRS-1) on multiple serine residues are phosphorylated, which promotes its degradation.

4.6 Conclusions

Based on the findings from the present study and the existing literature, we propose potential signaling mechanisms that may be involved in the regulation of Atrogin-1 and MuRF1 protein contents in skeletal muscle (**Fig. 4.6**). The anabolic Akt signaling, which can be activated by Insulin/IGF-1, is a critical upstream signal to modulate MuRF1 and Atrogin-1 at both gene and protein expression levels. However, Atrogin-1, but not MuRF1, protein content is increased when the rapamycin-sensitive and S6K1 dependent signaling pathways are inhibited. Thus, the regulatory mechanisms of protein content are distinct between Atrogin-1 and MuRF1. Our study provides evidence that Atrogin-1 protein content can be regulated by the rapamycin-sensitive mTOR-S6K dependent signaling pathway. Future studies should determine the underlying mechanisms by which the rapamycin-sensitive mTOR-S6K1 signaling regulates Atrogin-1 protein content.

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Fig. 6 was created with BioRender.com.

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CRediT authorship contribution statement

Yusuke Nishimura: Conceptualization, Investigation, Visualization, Writing - original draft, Writing - review & editing. Ibrahim Musa: Investigation, Writing - review & editing. Peter Dawson: Writing - review & editing. Lars Holm: Writing - review & editing. Yu-Chiang Lai: Conceptualization, Writing - review & editing.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

CHAPTER 5

GENERAL DISCUSSION

Portions of the general discussion of this thesis have been utilised in review articles published in the following peer-reviewed journals under Creative Commons Attribution 4.0 International,

Nishimura, Y., Højfeldt, G., Breen, L., Tetens, I., and Holm, L. (2021). Nutrition Research Reviews.

Nishimura, Y., Musa, I., Holm, L., and Lai, YC. (2021). American Journal of Physiology-Cell Physiology.

Net balance between MPS and MPB (muscle protein turnover) rates determines skeletal muscle mass. MPS is more responsive to resistance exercise stimulus as compared to MPB (37), which built the current hypothesis that MPS is a key driver for skeletal muscle adaptation (33, 38). As a result, many studies have adopted dietary protein and/or exercise interventions to manipulate MPS and augment skeletal muscle mass. It is continuously discussed within the scientific community that dietary protein recommendation should be increased to counteract the age-related loss of muscle mass, sarcopenia (164-170). However, there is currently no consensus on the growing suggestions to increase the international recommendation of dietary protein intake in healthy older individuals. Amino acid availability in the circulation is one factor that plays a key role in increasing MPS (94, 95). Thus, strategies to increase amino acids availability in the circulation are still an ongoing area of investigation to augment MPS. MPS and its signalling mechanisms in response to nutrition and muscle contraction are well established. However, our knowledge of myofibrillar protein breakdown and its regulatory molecular mechanisms remains in its infancy (39). To date, muscle-specific ubiquitin E3 ligases Atrogin-1 and MuRF1 are demonstrated as useful biomarkers of skeletal muscle atrophy (118). Despite the accumulated evidence showing the importance of mTORC1 in protein synthesis and hypertrophy (51, 75, 76), whether the mTORC1 signalling pathway plays a role in regulating Atrigin-1 and MuRF1 protein is unclear. Therefore, the purpose of this thesis was three-fold:

i) To determine daily protein intake and its distribution throughout the day and how they are associated with muscle mass and physical function in healthy older individuals.

ii) To determine the impact of Cluster Dextrin carbohydrate on the absorption of orally consumed intrinsically labelled meat protein hydrolysate and myofibrillar protein fractional synthetic rate following a whole-body resistance exercise.

iii) To understand whether the mTORC1 signalling pathway plays a role in regulating Atrogin-1 and/or MuRF1 protein content in C2C12 myotubes.

The present chapter summarizes the key findings from the studies described in **CHAPTER 2-4** of this thesis and how they improve the current knowledge within skeletal muscle physiology. Moreover, the limitations of the current research and recommendation for future directions will be discussed. Firstly, based on the findings shown in **CHAPTER 2**, this section will provide recommendations for future studies to reach an international consensus on dietary protein recommendation in healthy older adults. Secondly, based on the findings shown in **CHAPTER 3**, this section will provide recommendations for future studies investigating nutritional interventions to augment MPS. Finally, based on the findings of **CHAPTER 4**, this section will discuss recent advances in the roles of MuRF1 and Atrogin-1 and measurement and understanding of the regulation of exercise-mediated protein breakdown in skeletal muscle. Then, recommendations for future studies will be discussed to improve our knowledge of the roles of skeletal muscle protein breakdown with a focus on UPS.

5.1 Dietary protein requirements and recommendations for healthy older individuals

It is well established how much dietary protein should be ingested to account for obligatory nitrogen loss in healthy adults using nitrogen balance methodology (155, 156). Evidence from the nitrogen balance methodology is the basis of the current internationally recommended dietary protein intake level (0.83 g/kg/day) by WHO/FAO/UNU (155, 156). However, it has continuously been debated (164-170) whether increasing dietary protein intake in older age is an effective strategy to counteract the age-dependent loss of muscle mass, sarcopenia (11, 13, 173). Independent authorities and expert groups have adopted different criteria and evidence from distinct methodological approaches (157-160), resulting in differences in published recommended values of protein recommendation (**Table 1.1a,b**). Consequently, there is currently confusion about the requirement of dietary protein for healthy older adults both in the scientific community and amongst the general older population, who wish to follow the recommendations.

In our study (**CHAPTER 2**), we provided evidence that muscle mass and physical function do not differ when healthy Danish older individuals consumed higher (0.83 – <1.1 g/kg/day) or lower (<0.83 g/kg/day) than the current internationally recommended dietary protein intake level (0.83 g/kg/day). Moreover, individuals consuming dietary protein (≥ 1.1 g/kg/day) around what is currently suggested by independent authorities and expert groups (0.94 - 1.3 g/kg/day) do not show changes in muscle mass and physical function as compared to individuals who consume dietary protein higher or lower than the current international recommended level. Indeed, our findings are in line

with the most recent systematic review and meta-analysis that concluded that increased dietary protein intake augments appendicular lean mass and strength only when resistance exercise is combined in sarcopenic older adults (343). This may also highlight the importance of physical activity in the maintenance of skeletal muscle mass (344).

Dietary protein distribution pattern within a day has been suggested as an important factor. By equally splitting the amount of protein into each main meal, better maintenance of amino acid availability in the circulation and muscle protein turnover have been hypothesized. However, the total amount of protein intake in a day affects the dose of protein per meal, which may result in a suboptimal dose of protein. Thus, we have first divided individuals into either higher (1.1 g/kg/day) or lower (0.83 g/kg/day) and examined whether dietary protein distribution pattern is associated with muscle mass and physical function. We showed that dietary protein distribution pattern does not play a role in maintaining muscle mass and physical function in healthy Danish older individuals. These data do not support the ongoing debate regarding the suggestions to modify the international protein recommendation and distribution pattern in healthy older individuals to maintain muscle mass and physical function.

5.2 Future directions of protein requirements and recommendations for healthy older adults

In the study described in **CHAPTER 2**, we demonstrated that neither the total nor distribution pattern of dietary protein intake are not associated with better maintenance of muscle mass and physical function in healthy older individuals. Although our

understanding of nutritional protein requirement and recommendation have improved over these 60 years, the outcome measurements, and the methodologies on which the outcome measurement is founded have not evolved as we discussed in section 1.6. We also identified that the discrepancy in protein recommendations, is partly due to the lack of definitive criterion for "adequate protein", as well as differences in outcome measurements. As energy balance, metabolic adaptation to dietary protein intake, and protein quality can impact protein requirement, those factors will be discussed to accelerate the agreement on dietary protein requirement, and thus dietary protein recommendation for healthy older individuals.

5.2.1 Energy balance

The importance of energy intake in relation to the determination of protein requirement was raised already by the Joint FAO/WHO ad hoc Expert Committee on Energy and Protein Requirements in 1971 (153), and later in 1981 (154). Notably, Vernon R. Young (178) and Joe D. Millward (250) explored the important concept of protein and energy interrelation to determine protein requirement. The interaction between energy and nitrogen intake on the maintenance of body nitrogen was well documented by studies on the role of non-protein energy sources (carbohydrate and fat) (260, 345, 346) using nitrogen balance methodology. For example, Colloway et al. (260) showed back in 1954 that exogenous nitrogen-bearing sources contribute to energy production rather than deposition in the body until adequate energy is consumed. Further, increased non-protein energy intake can also mitigate nitrogen loss in a dose-dependent manner (260). However, the provision of energy attenuates the recruitment of amino acid metabolism for energy turnover and hence retains nitrogen, leading to improvement in nitrogen

balance (260). Accordingly, the achievement of energy balance is an underlying assumption for the current internationally recommended protein intake (155).

In recent years, protein metabolism and turnover rate have been studied during a condition of negative energy balance (262, 347, 348) in healthy adults and with an attempt to preserve muscle mass or attenuate muscle loss during negative energy balance by increasing protein intake (349-354). The effect of protein intake above the current recommended safe level of intake (0.83 g/kg/day) on muscle mass maintenance during negative energy balance has been well documented (262, 347, 355). Evidence indicates that whilst amino acids contribute more to energy production at negative energy balance (351, 356), they are utilized more to de novo protein synthesis when energy balance is achieved. However, evidence is scarce in healthy older individuals, and it is unclear whether increased protein intake during negative energy balance is sufficient to maintain whole-body and muscle protein mass in healthy older adults.

In contrast, limited knowledge is currently available on the impact of positive energy balance on the regulation of protein turnover rates and muscle mass in healthy older adults. Nonetheless, based on knowledge from energy deficit studies (351, 356) as well as studies by Woolfson (357) and Calloway and Spector (260), it can be assumed that positive energy balance reduces amino acid oxidation. Accordingly, exogenous amino acids (dietary protein) under such conditions are more efficiently utilized to achieve net positive protein balance. The impact of protein content relative to total energy intake during overfeeding on body composition in younger healthy individuals (18 -35 yrs.) was investigated by Bray et al. (358). Participants were divided into 5% (low protein),

15% (normal protein), or 25% (high protein) of total energy intake from protein after a weight-stabilizing diet period. Following an 8-week intervention, fat mass was similarly increased in all groups because of overfeeding (40% excess energy from a weight-stabilizing diet). However, lean body mass was significantly increased with normal and high protein groups, along with a concomitant increase in resting energy expenditure. These results suggest that energy intake from protein is a sole determinant to increase lean body mass, but not the accretion of fat mass during overfeeding in healthy younger individuals. In this cohort, additional protein may not be required when energy balance is maintained since no impact on lean body mass changes was detected between normal and high protein intake groups (358). Since these findings are limited to healthy younger individuals, further studies are required to investigate in healthy older adults. The concept of interaction between energy balance and protein intake on amino acid oxidation, urea excretion, and whole-body net protein and nitrogen balance at a steady state is illustrated in **Fig. 5.1**.

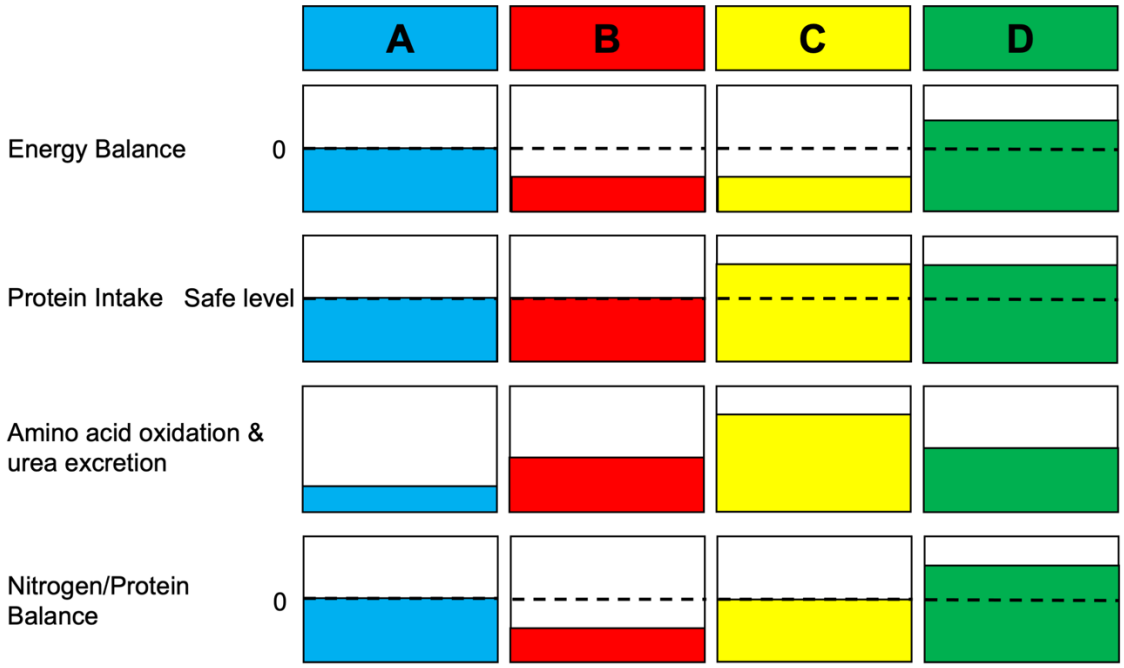

Figure 5.1 Interaction between energy and protein intake on amino acid oxidation, urea excretion, and whole-body net nitrogen and protein balance during the condition of adaptation.
In each column, relative energy balance, protein intake, amino acid oxidation & urea excretion, nitrogen/protein balance are expressed. For energy balance and nitrogen/protein balance, 0 (dashed line) indicates that a balance is maintained. Safe intake (dashed line) in protein intake shows a protein intake recommended by WHO/FAO/UNU (0.83 g/kg/day). (**A**) the column indicates zero whole-body net nitrogen and protein balance at the safe level of intake for protein recommended by WHO/FAO/UNU under an energy balance condition (155). (**B**) and (**C**) indicate a negative energy balance condition. (**B**) the column shows the protein intake at the safe level of intake, but amino acid oxidation and urea excretion are increased under a negative energy balance condition, leading to negative whole-body net protein and nitrogen balance during negative energy balance (351, 356). (**C**) the column demonstrates that an increased protein intake (> 0.83g/kg/day) preserves whole-body net protein and nitrogen balance whilst increasing amino acid oxidation and urea excretion under a negative energy balance condition (350, 353, 354). (**D**) the column denotes that amino acid oxidation and urea excretion are reduced under a positive energy balance condition with an increased protein intake (> 0.83g/kg/day), resulting in positive whole-body net protein and nitrogen balance (358).

In summary, energy balance in addition to protein intake is a key determinant of protein turnover rates and net protein balance at the whole-body level. However, energy surplus itself does not seem to increase muscle mass although it reduces amino acid oxidation. Thus, as repeatedly stated in the international reports from WHO/FAO/UNU, energy balance needs to be considered when determining protein requirements. In addition, it is important to highlight that evidence of the interaction between energy balance and protein intake is currently limited to younger individuals, and further evidence is required in healthy older individuals.

5.2.2 Metabolic adaptation to dietary protein intake

The consideration of metabolic adaptation to any given amount of protein intake is required for a valid measure of protein requirement. Protein turnover in the splanchnic area as well as in the periphery is adaptable to a given amount of protein intake (174-

176). Metabolic adaptation covers processes affecting the utilization and fate of amino acids, primarily in the splanchnic area. A prolonged exposure to a given amount of protein intake forces enzyme and transporter levels to change accordingly to handle the amino acid availabilities. Hence, metabolic adaptation to a given protein intake level is a fundamental prerequisite when estimating protein requirements. In physiology, *adaptation* covers conditions where achievement of a steady state can be obtained after adjustments of metabolism and physiological function (359, 360). In contrast, when conditions are too extreme for metabolic pathways to adjust sufficiently, but rather continuously lag and the changes are beyond the range of adaptation, the condition is defined as *accommodation* (361).

Recent emerging evidence highlight the mechanisms of *adaptation* to a higher protein intake than the current safe level of dietary protein intake. For example, Gorissen et al. (362) measured the availability of dietary protein using intrinsically labeled whey protein. In this study, older individuals (62 ± 1 yrs.) were habituated to a protein intake of either 0.7 g/kg/day (LOW) or 1.5 g/kg/day (HIGH) for 2 weeks from a habitual intake of protein at 1.0 g/kg/day. Interestingly, no group differences were noted in either postabsorptive or postprandial MPS, and more intrinsically labeled whey protein was available in the circulation in LOW (61%) in comparison with HIGH (56%). This was in agreement with a study by Højfeldt et al. (197), where responses in amino acid and protein metabolism were investigated after habituation to a protein intake of > 2.1 g /kg LBM/day (0.82 g/kg/day) and a protein intake of 1.1 g/kg LBM/day (1.76 g/kg/day) for 20 days in older men (65-70 yrs.). These findings suggest that exogenous amino acids are directed less effectively into the circulation when habituated to a high protein intake.

Habituation to a high protein intake also resulted in a diminished postprandial synthesis rate of plasma proteins and a more negative overnight fasted whole-body net protein balance, suggesting a less effective utilization of exogenous amino acids for protein synthesis. In accordance, Walrand et al. (228) demonstrated that higher protein intake showed a postabsorptive catabolic state, as demonstrated by higher amino acid oxidation and whole-body protein turnover, without changing MPS in both younger (24 ± 1 yrs.) and older individuals (70 ± 2 yrs.) when higher protein intake (3.0 g/kg fat-free mass) was compared to 'usual' protein intake (1.5 g/kg fat-free mass) over 10 days. However, nitrogen balance was improved in the higher protein intake group, which may suggest insufficient time to adapt to the new protein intake level in this study. Evidence emphasizes the necessity of allowing time for metabolism to *adapt* to increased protein intake. Further evidence adopting study designs and methodological approaches that can account for metabolic adaptation is required to obtain a meaningful value for protein requirement, which can then be translated into recommendations.

Metabolic adaptation to changes in protein intake may explain the null findings when an intervention was performed long enough to achieve adaptation (175, 197, 363, 364). In other words, sustained increases in muscle mass would not be achieved when individuals chronically consume dietary protein greater than habitual protein intake level. Baseline protein intake level might be a key determinant of the beneficial effect of dietary protein intervention on muscle mass gain in healthy older adults in a randomized controlled intervention study. This notion was highlighted previously (365) and is supported by a meta-analysis conducted by Ten Haaf et al. (366), where they assessed the impact of protein supplementation on lean body mass, muscle strength, and physical

performance in community-dwelling older individuals. Protein supplementation is not beneficial for those outcome measures when sufficient habitual protein intakes are already consumed in non-frail older individuals.

A recent meta-analysis including a total of 8107 community-dwelling older individuals from cohorts in the Netherlands, UK, Canada, and the USA showed that the prevalence of protein intake lower than the currently recommended level of 0.8 g/kg adjusted body weight/day is 14 - 30% (367). Thus, this low-habitual protein intake group may be a more relevant target group for protein supplementation intervention with a concomitant focus on ensuring energy requirements. However, when the purpose is to determine whether a higher than currently recommended protein intake is favorable for muscle and whole-body protein mass, healthy older individuals habitually consuming protein around the current safe level of intake should be the target group and *not* the low-habitual intake group. Collectively, more randomized controlled intervention studies are required to conclude whether the newly suggested higher protein intakes from several expert groups above the safe level of intake at 0.83 g/kg/d has a favorable impact on muscle mass in healthy older adults consuming protein at the current international recommendation.

5.2.3 Protein quality

Protein quality is an overall measure of the ability of a protein source to meet the metabolic demand and is defined in terms of biological value (BV) (i.e., the fraction of amino acids absorbed by the gut from food that is subsequently retained by the body).

BV is expressed in terms of indispensable amino acid patterns relative to the requirement (155).

The concept of protein quality can be learned from the Ideal Protein Concept developed in the late 1950s by H. H. Mitchell (368) and H. M. Scott (369). The Ideal Protein Concept is defined as the exact amounts of amino acids needed for optimal growth, meaning that it does neither cause amino acid deficiency nor surplus availability. Thus, the Ideal Protein Concept is an effective way to define minimum protein in the diet to meet amino acid requirements for metabolic demands in animals. For example, the Ideal Protein Concept initially attempted to provide diets containing the exact balance of essential amino acids based on the composition of eggs and casein for the maximal growth and production performance of chickens. However, non-essential amino acids were not considered in the concept. Reevaluations in the area of the optimal animal feed for growth have found that the provision of non-essential amino acids is also required for development, growth, survival, reproduction, and health (370, 371). Consequently, the provision of non-essential amino acids will lower the required amounts of some essential amino acids as they will not be needed as precursors for de novo synthesis of non-essential amino acids. Therefore, protein quality must also be considered when discussing protein requirements and should be reflected in the overall recommendations (372), including the consideration of non-essential amino acids (373).

Digestibility of amino acids is an integrated part of the protein quality measure. FAO developed the Protein Digestibility Corrected Amino Acid Score (PDCAAS) as a measure of overall nitrogen digestibility considering the loss in fecal matter. This

concept had some inherent limitations (374) that were attempted later to be overcome by replacing it by the new term Digestible Indispensable Amino Acid Score (DIAAS), which includes 1) the adoption of ileal amino acid digestibility, 2) abrogation of truncation of scores, 3) taking the influence of food processing into account, and 4) the use of individual amino acid digestibility.

The earlier protein requirements and recommendations by the WHO/FAO/UNU specified protein quality, whereas the current recommendations refer to 'high-quality protein' with a biological value of 100, usually an animal source protein (see **Table 1.1a**). Animal-based proteins are generally accepted as high-quality protein due to better essential amino acid profile and higher digestibility (around 100) as compared to plant-based proteins (ranging 80-85) (372, 375). Plant-based proteins are less digestible due to the existence of dietary fibre and compounds that inhibit enzymatic protein digestion.

Despite the accumulated evidence that supports muscle protein anabolic response by the ingestion of animal-based proteins, plant-based proteins have attracted more attentions due to a sustainable point of view and population health benefits (376). Recently, Burd et al. (377) discussed that consideration of protein quality is critical when protein recommendation is determined in relation to environmental considerations (e.g., managing greenhouse gas emissions to land, water use, and loss of biodiversity). Evidence showed that greenhouse gas emission is lower in plant-based proteins as compared with animal-based proteins (376). Growing evidence has shown that the health benefits of vegetarian and vegan diets, including lower incidence and mortality from ischemic heart disease and lower incidence of cancer (378). However, the

beneficial effects of vegetarian and vegan diets for overall mortality rates are currently unclear (379).

The impact of vegan and vegetarian diets on muscle mass maintenance in healthy older adults is an important consideration. Cross-sectional studies have shown that total protein and animal protein intake, but not plant protein intake, are positively associated with muscle mass index in older women (380). Results from a longitudinal observational study show that higher intakes of total protein and animal-based protein are associated with a reduced loss of lean mass over 3 years of follow-up, whereas plant-based protein intake is not associated with lean mass with the fully adjusted models in older adults (181). These data suggest that a plant-based diet may not be favorable for muscle mass maintenance in older adults. However, we argue that more studies are required to fully elucidate this association.

Several strategies have been proposed by Gorissen and Witard (381) to overcome the perceived inferior anabolic properties of plant-based proteins. Firstly, the doses of plant-based protein intake can be increased; secondly, several plant-based protein sources can be mixed in a meal and in the whole diet to overcome any deficiency of a single essential amino acid; thirdly, co-ingestion of leucine can be added as an anabolic stimulant; or fourthly, enhancement of the muscle anabolic sensitivity by physical activity or by other means (e.g., by providing fish oil-derived n-3 PUFA in the diet).

Considering the potential metabolic roles of individual amino acids is also important. Amongst the nine essential amino acids, leucine, isoleucine, and valine are called

branched-chain amino acids (BCAAs). In particular, leucine plays an important role in the upregulation of translation initiation and subsequent increase in MPS independent of a precursor to MPS (382-384). Thus, the leucine trigger hypothesis, minimum leucine availability required to stimulate MPS following feeding, has been proposed (385), which was also supported by a systematic review (386). This notion has been exemplified by a study demonstrating that increasing the proportion of leucine to mixed amino acids without altering total amino acids content overcomes anabolic resistance in older individuals (387). Furthermore, an addition of leucine to a suboptimal amount of protein is hypothesized to optimize anabolic response. For example, Wall et al. (388) demonstrated a greater post-prandial MPS following the consumption of 20 g casein protein with 2.5 g of crystalline leucine compared with no leucine in older men (74±1 yrs), indicating that modifying and/or supplementing specific amino acid content might be an effective and practical strategy to improve anabolic response. This is particularly relevant to older individuals, where energy requirement and appetite are decreasing with advancing age (389). Protein requirement that maintains whole-body and muscle protein mass can be achieved with less total protein intake by optimizing protein quality, which is also an important consideration for environmental issues. However, evidence is required whether the modification of protein quality is a feasible approach to maintain whole-body protein balance in healthy older adults.

5.2.4 Concluding remarks

Considering the historical development of protein requirements and the use of various research methodologies to create scientific evidence for assessing protein requirements to establish recommendations, we found that the previously used whole-body nitrogen

balance methodology is challenged by methods evaluating whole body and/or muscle mass and whole body and/or muscle protein turnover rate as criteria. The newly suggested protein recommendations (0.94 - 1.3 g/kg/day) formulated by some authorities and expert groups target muscle mass maintenance instead of whole-body protein mass as their primary outcome criterion.

To close the knowledge gap between protein requirements for maintaining both muscle mass and whole-body mass, we identified that future research should assess the degree of agreement between those different though related outcome measures based on distinct methodologies. We claim a strong need for prospective longitudinal studies with frequent monitoring of reliable dietary intake and concurrent measurements of whole-body protein mass and muscle mass with multiple methodologies. These would include short-term measurements (e.g., muscle and whole-body protein balance, kinetic rates, and nitrogen fluxes) and longer-term measurements (ensuring metabolic adaptation and energy balance) with consideration of protein quality. Preferably also evaluating clinically relevant outcomes, such as muscle strength and function, physical function, body composition, and metabolic health parameters would be relevant information. Such a holistic experimental approach would support establishing an agreement between muscle and whole-body protein mass maintenance, and thereby reveal the “true” and healthy dietary protein requirements and recommendations for healthy older adults.

5.3 Utilization of exogenous protein in acute resistance exercise

Resistance exercise stimulates an increase in MPS. It has widely been shown that consumption of amino acids and/or protein after exercise provides synergistic effects for MPS, as muscle contraction sensitizes skeletal muscle to amino acids and/or protein. This is exemplified by Biolo et al. (88) and Tipton et al. (390), where a positive net protein balance is achieved when amino acids are consumed after resistance exercise. Thus, dietary protein plays an essential role in supporting skeletal muscle protein turnover following resistance exercise.

Amino acids concentrations in the circulation reflect the sum of influx (i.e., protein breakdown and exogenous amino acids) and outflux (i.e., protein synthesis and amino acid oxidation) of amino acids, and thus it is not merely from orally consumed amino acids/protein. Stable isotope tracer was added to a mixed meal to overcome this and investigate an absorption kinetic of in a previous study (391). However, peptides or proteins undergo digestion and absorption in the gastro-intestinal system, whereas amino acids do not, meaning that the addition of stable isotope tracer to a mixed meal does not provide digestion and absorption kinetics. Fortunately, intrinsically labelled meat protein hydrolysate (D_5-phenylalanine) was available in our hand (231), which allowed us to specifically monitor how orally consumed protein is absorbed (e.g., appearance in the circulation) while stable isotope tracer (L-[^{15}N]-phenylalanine) was infused to examine MPS.

Taking advantage of intrinsically labelled meat protein hydrolysate, our study (**CHAPTER 3**) examined whether co-ingestion of Cluster Dextrin (CDX) carbohydrate

facilitates absorption of orally consumed meat protein hydrolysate and increases MPS as compared to co-ingestion of glucose (GLC) after whole-body resistance exercise in moderately trained younger individuals. We report that co-ingestion of CDX with meat protein hydrolysate does not increase postprandial amino acid availability as compared to co-ingestion of GLC. mTORC1 signalling was activated greater in CDX as compared to GLC, but postprandial MPS was not different between CDX and GLC. Our data agree with previous studies reporting null effects of co-ingestion of carbohydrate with protein to stimulate MPS (290-292) and postprandial amino acid availability has a critical role in MPS (94, 95). Thus, these findings suggest that co-ingestion of CDX enhances mTORC1 signalling, but not postprandial amino acid availability and MPS after whole-body resistance exercise in moderately trained younger individuals.

5.3.1 Future studies

Over the past decades, substantial research using stable isotope tracers has been performed to understand muscle protein turnover kinetics in response to exercise, amino acids/protein, or a combination of both (199-203). When it comes to protein, the use of isolated protein sources has been the mainstream approach. However, more recent investigations have shifted from the use of isolated protein sources to whole foods or a mixed macronutrient meal, which represents the form of protein consumption in everyday life. This shift is due to the interactive effects of protein contained in whole foods with other nutrients and bioactive compounds on skeletal muscle and whole-body protein turnover. To date, studies have primarily focused on MPS in response to beef (392, 393), eggs (251, 394), and mixed macronutrient meals (223, 225). These studies

highlighted the importance of considering complex food matrices on the regulation of muscle protein turnover in future studies.

Our study (**CHAPTER 3**) used a whole-body resistance exercise protocol. Most studies have used a unilateral leg exercise when investigating muscle protein turnover kinetics after resistance exercise in combination with protein ingestion. Based on the current evidence using a unilateral resistance exercise, it is recommended that 20 g of high-quality protein is required to maximally increase MPS. However, a study showed that the amount of protein required to maximally increase MPS in response to resistance exercise depends on the amount of muscle recruited during resistance exercise (92). For example, a higher protein dose (~40 g) than what is currently recommended (~20 g) induced greater MPS when whole-body resistance exercise was performed in younger adults (92). Thus, it would be prudent to investigate whether the dose of protein required to maximally stimulate MPS is greater when a whole-body resistance exercise (i.e., more muscles are exercised) is performed in older adults, as shown by Macnaughton et al. (92) in younger adults.

Ageing is accompanied by "anabolic resistance" to amino acids/protein intake (207, 208, 219, 387), insulin (232, 233), and acute (313) and chronic (395) resistance exercise. Thus, intervention approaches to overcome "anabolic resistance" have been the research focus for the successful maintenance of skeletal muscle mass during ageing. Given that co-ingestion of CDX with meat protein hydrolysate enhanced mTORC1 signalling following a whole-body resistance exercise in younger adults,

investigating the effect of CDX in healthy older individuals would be an interesting route for future studies to examine whether anabolic resistance is alleviated.

5.3.2 Concluding remarks

Co-ingestion of CDX activates mTORC1 signalling after a whole-body resistance exercise in younger males. However, postprandial amino acid availability myofibrillar protein synthesis did not differ between CDX and GLC. Isolated proteins are useful to provide high quality protein. However, there is a growing number of studies showing the beneficial effects of whole foods on skeletal muscle protein turnover, suggesting a potential interaction between nutrients and bioactive compounds. Thus, it is important for future studies to explore not only amino acids/protein, but also their interaction with other nutrients to support skeletal muscle protein turnover, as the research field has shifted a focus more on the roles of whole foods on skeletal muscle protein turnover. An optimal dose of protein should also be determined when a whole-body resistance exercise is performed in older adults. Finally, future studies should investigate nutritional strategies to overcome anabolic resistance in healthy older adults to counteract sarcopenia.

5.4 Intracellular signalling regulating E3 ubiquitin ligases protein content and recent advances in measuring and understanding the regulation of exercise-mediated protein degradation in skeletal muscle

5.4.1 Intracellular signalling mechanisms regulating E3 ubiquitin ligases Atrogin-1 and MuRF1 protein content in C2C12 myotubes

Ubiquitin E3 ligases Atrogin-1 and MuRF1 are well studied in relation to skeletal muscle atrophy. The PI3K/Akt/FoxO signalling pathway has been well known to control Atrogin-1 and MuRF1 mRNA expressions. Akt also controls mTORC1 signalling pathway, which is responsible for skeletal muscle hypertrophy. However, the roles of mTORC1 in regulating Atrogin-1 and MuRF1 protein content are currently not clear in skeletal muscle.

Our study (**CHAPTER 4**) investigated whether the mTORC1 signalling pathway is involved in regulating Atrogin-1 and MuRF1 protein content in C2C12 myotubes while Akt activity is not altered. Using small molecule inhibitors, we first identified that Atrogin-1, but not MuRF1, protein content was increased when rapamycin (a mTORC1 specific inhibitor) was treated. Moreover, activation of AMPK using a small-molecule activator, 991 (a well-known negative regulator of mTORC1) showed distinct time-dependent changes between Atrogin-1 and MuRF1 protein content. These findings led us to further investigate the downstream of the mTORC1 signalling pathway, S6K1. Interestingly, an S6K1 specific inhibitor (PF-4708671) showed that Atrogin-1 protein content was increased in a dose- and time-dependent manner while MuRF1 protein content was unchanged. Overall, these findings show that Atrogin-1 and MuRF1 protein

content are regulated by different mechanisms, the downstream of Akt, and that Atrogin-1 protein content can be regulated by the rapamycin-sensitive mTOR-S6K1 dependent signalling pathway via undetermined mechanisms. Although downstream mechanisms of action by Atrogin-1 and MuRF1 need to be determined, our data indicates therapeutic possibilities to specifically control Atrogin-1 or MuRF1 protein content, which essential execute the enzymatic ubiquitin E3 ligase activity.

The following sections will discuss the most recent evidence of the roles of Atrogin-1 and MuRF1 in skeletal muscle mass control and discuss how molecular mechanisms can be combined with protein degradation kinetic measurement.

5.4.2 Roles of MuRF1 and Atrogin-1 in skeletal muscle mass control

It is common to extrapolate findings based on inaccurate assumptions through indirect evidence. Because E3 ligase is the key determinant of substrate identification and the majority of ubiquitylated proteins undergo proteasome-mediated degradation, the finding of increased MuRF1 and/or Atrogin-1 expressions in atrophy muscle has widely been regarded as a direct indicator of muscle protein degradation. This assumption is based on the prediction that all ubiquitylated proteins will undergo protein degradation. As more evidence becomes available in the literature, it is now clear that protein ubiquitylation also has non-degradative roles (106). Furthermore, researchers often rely on changes in mRNA abundance without measuring protein level, due largely to, the poor quality of commercially available antibodies. However, Sandri (322) pointed out that atrophy-induced increase in mRNA abundance of E3 ligase does not always align with protein level. In catabolic conditions, it is hypothesized that an increased ligase

activity of skeletal muscle-specific ubiquitin E3 ligase would inevitably increase autoubiquitylation (116), which is then degraded by proteasome- or lysosome-mediated degradation (322). Hence, the upregulation of gene transcription is important to counteract the loss of E3 ligase protein due to the increased autoubiquitylation (322). Therefore, it is not appropriate to extrapolate the results of MuRF1 or Atrogin-1 protein/gene expression directly to muscle protein degradation before their regulatory roles in muscle protein degradation are properly understood. Furthermore, evidence suggests that MuRF1 gene expression is independent of proteasome-mediated protein degradation in skeletal muscle. For example, proteasome activity was reported to be increased after 14 days of functional overload in MuRF1-deficient mice (396). Even though MuRF1 expression is a useful marker for muscle atrophy, it should not be used as a marker of muscle protein breakdown.

More recently, Baehr et al. (333) demonstrated that overexpression of MuRF1, but not Atrogin-1, is sufficient to induce muscle atrophy in mice. In an attempt to understand how MuRF1 regulates myofibrillar protein degradation, Baehr et al. (333) identified MuRF1 overexpression-dependent ubiquitylation sites on 56 proteins. Surprisingly, their validation showed that the majority of these MuRF1 substrates do not undergo degradation, which contrasts with Clarke et al.'s findings (134) that MuRF1 physically associates with and degrades slow and fast myosin heavy chains under dexamethasone treatment in C2C12 myotubes. One possible reason that MuRF1 substrates did not undergo degradation despite increased ubiquitylation might be that MuRF1 recruits other E3 ligases (e.g., MuRF2, MuRF3, and TRIM25), which regulates the fate of ubiquitylated protein substrates for non-proteasomal degradation. This notion was also

supported by a recent study led by Goodman et al. (397) that overexpression of ASB2β not only induced atrophy but also increased expression of ubiquitin E1 and E2 enzymes and other E3 ligases (e.g., MUSA1, the muscle-specific Fbxo40, and MuRF2). Interestingly, Baehr et al. (333) also showed that muscle atrophy was prevented when the RING domain of MuRF1 is mutated at C44S/C47S. The RING domain is required for binding with E2 conjugating enzymes and catalyzing the transfer of ubiquitin from E2 to substrates (398). Their finding indicates that MuRF1 ligase activity is important to cause muscle atrophy. However, there is currently no studies measuring protein degradation rates when E3 ligases are overexpressed, and hence it is unclear if overexpression of any particular E3 ligases is sufficient to increase protein degradation rates. Thus, future studies should focus more on the measurement of protein degradation when studying potential E3 ligases relevant to muscle atrophy to improve our understanding of the mechanisms.

There are more than 600 E3 ligases (399) and around 100 DUBs (400) encoded in the human genome. It is not surprising to see that these key ubiquitin modifiers are increasingly recognized as key regulators of muscle mass and functions. As such, more E3 ligases (e.g. MUSA1 (401), Cbl-b (402), TRIM28 (403), TRIM32 (404), TRIM72 (405), UBR4 (406, 407), UBR5 (408, 409), and ASB2β (397)) are emerging as important regulators of skeletal muscle mass and metabolism. Similar to our understanding of MuRF1 and Atrogin-1, we still know very little about how other E3 ligases' activities are regulated, and what their downstream events and consequences are. For example, future studies should aim to identify the complete list of substrates,

clarify what ubiquitin chain types can be made by E3 ligases, and how this modification affects the fate of protein substrates.

5.4.3 Methodological advances in measuring myofibrillar protein degradation rate

Developing an accurate and reliable methodology to measure myofibrillar protein degradation is critical for understanding and explaining relevant molecular mechanisms. Stable isotopically labelled amino acid tracers in combination with mass spectrometry analyses (i.e., determination of relative tracer abundance) have been used for studying muscle protein turnover (200, 201, 203). While the tracer-based measurement of protein synthesis via the *direct incorporation* model is considered the gold standard for myofibrillar protein synthetic rate (also known as fractional synthetic rate, FSR) (199), the measurement of protein degradation with tracer methodologies appears much more complicated (125). The *tracer-dilution* principle is the most frequently used approach (27, 28, 125, 199, 229, 410).

In 1987, Gelfand and Barrett introduced the 2-pool arterio-venous model of the *tracer-dilution* principle, where the measurement of tracer enrichment in both artery and vein across a limb (or an organ) is performed while infusing stable isotopically labelled amino acid tracer(s) (229). Tracer enrichment is the abundance of tracer (administered labelled amino acid) relative to tracee (unlabelled amino acid), which is determined based on mass spectrometry analysis (199, 200). In principle, protein degradation rate is calculated as the dilution of relative abundance of a stable isotopically labelled amino acid at the venous site compared to the arterial site, which is anticipated to be a consequence of the release of tracee from the intracellular pool into the venous site due

to intracellular protein degradation (199, 229, 410). However, this 2-pool arterio-venous model of the *tracer-dilution* principle is very simplistic. To gain accuracy, the model was subsequently extended by including the intracellular pool of the targeted tissue (e.g. skeletal muscle) (411) and/or by sampling the interstitial fluid compartment (412). Despite these improvements, none of these *tracer-dilution* approaches contain information about where the traced amino acids are originated from (e.g., myofibrillar protein). Therefore, protein-specific approaches to measure protein degradation are warranted.

A methodology was developed that directly measures protein degradation in a comparable manner as the myofibrillar protein-specific *direct-incorporation* model for FSR. The approach measures a fractional breakdown rate (FBR), and it is based on the principle of determining the rate at which protein-bound amino acid tracers are disappearing from the protein pool. We originally used deuterium oxide (D_2O) for labelling proteins, hence we here abbreviate this methodology as FBR_{D2O} (413, 414). This approach is practically rather demanding (414). Briefly, D_2O firstly needs to be provided (orally or injection) to allow pre-labelling of (myofibrillar) proteins through *de novo* synthesized D-labelled amino acids. Alanine is a commonly used amino acid due to two reasons: i) it exchanges hydrogen/deuterium through transamination (TA) and through metabolic precursors for alanine in tricarboxylic acid cycle (TCA cycle) reactions at four possible exchange sites (C-H bonds), which improves analytical sensitivity with mass spectrometry and (ii) the metabolic exchange of hydrogen/deuterium occurs quickly and hence equilibration with body water enrichment appears very quickly (415, 416). After pre-labelling, at least two muscle samples are

collected to measure the enrichment of deuterium (D)-labelled amino acids in the myofibrillar protein pool and calculate the rate of loss, which can be expressed as myofibrillar protein degradation rate (413, 414). However, when measuring the disappearance of D-labelled amino acids from the myofibrillar protein, the availability of D_2O in the body pool has to be zero. This ensures that deuterium is not transferred to amino acids in *de novo* metabolism, and hence no further incorporation of D-labelled amino acids into myofibrillar protein (147, 413, 414). Further, using D_2O as the label-donor to amino acids has subsequently been found advantageous in regard to avoiding recycling of D-labelled amino acids into myofibrillar proteins during the period of the actual FBR measurement (147, 413, 414) (see **Fig. 5.2**). The reason for this is that D-label is both added and removed from C-H bonds of amino acids (415, 416). Thus, the recycling of D-labelled amino acids back to myofibrillar proteins is very unlikely when body D_2O enrichment is zero as free amino acids carrying the D-label will lose the label once released from protein degradation by reacting with H_2O in the cytoplasm (414). In contrast, when classic stable isotopically labelled amino acids have stable isotopic labels (deuterium, carbon and/or nitrogen atoms) at positions where they are only released by the irreversible metabolism of the amino acid, these stable isotopically labelled amino acids will be recycled for myofibrillar protein synthesis (413, 414). This was further explored experimentally in humans using both D_2O and ^{15}N-phenylalanine stable isotope tracer (147). It was observed that high ^{15}N-phenylalanine tracer enrichment was present in circulation after 10 days (TTR: 4%) and 24 days (TTR: 1%) of the exposure (147). Further, a high variation of tracer abundances in the myofibrillar protein fraction was observed when the same individuals were exposed to four acute ring-$^{13}C_6$-phenylalanine infusion trials and myofibrillar protein still carries the infused

phenylalanine stable isotope tracer after a year. Both findings emphasize the continuous and prolonged recycling of phenylalanine (amino acids) for muscle protein synthesis.

Fig. 5.2 illustrates the principle of determining myofibrillar protein degradation using classical stable isotopically labelled amino acid tracers (**Fig. 5.2a,b**) and D_2O (**Fig. 5.2C,d**). **Fig. 5.2** also describes the fate of D-labelled amino acids (e.g., alanine) and classic stable isotopically labelled amino acid tracers derived from intracellular protein degradation. Recycling at the time of FBR measurement will underestimate the disappearance rate of stable isotopically labelled amino acids present in the myofibrillar protein pool, and thus the myofibrillar protein degradation rate. Recycling must therefore be avoided for a valid approach. FBR_{D2O} approach is currently the only valid method used to determine myofibrillar protein degradation rate, which is a comparable approach to the *direct-incorporation* model of myofibrillar protein FSR. A recent study by Dideriksen et al. (43) has taken this advantage to demonstrate that myofibrillar protein degradation rate is higher during a 14-day period of resistance training ($2.12 \pm 0.34\%\cdot d^{-1}$) compared to a 14-day limb immobilization period ($1.61 \pm 0.14\%\cdot d^{-1}$) in older adults.

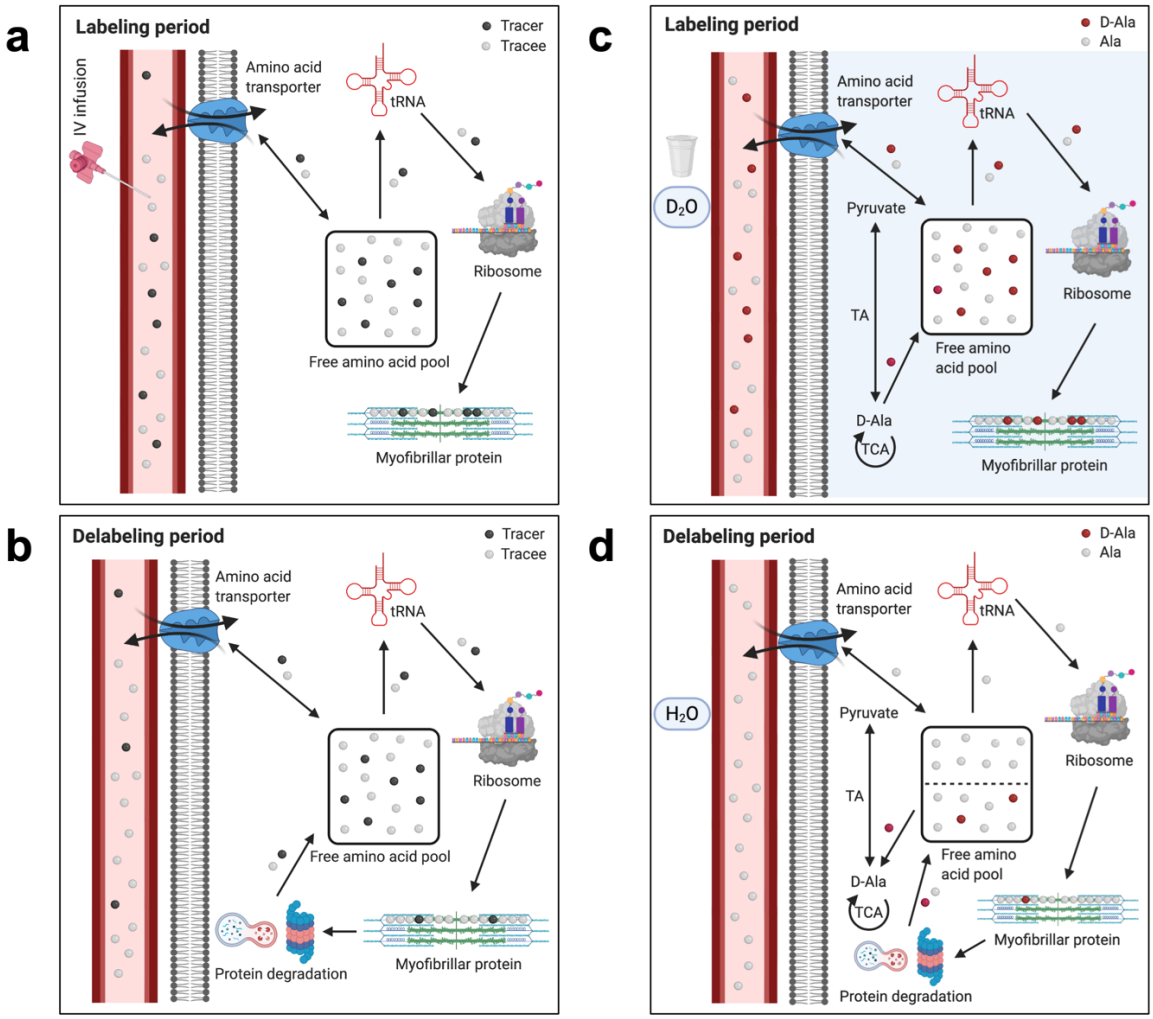

Figure 5.2 Principle of determining myofibrillar protein degradation using classical stable isotopically labelled amino acid tracers and D_2O.

In principle, myofibrillar protein degradation rate can be measured in a two-step manner regardless of the use of classic stable isotopically labelled amino acid tracers or deuterium oxide (D_2O). Firstly, myofibrillar proteins should be labelled through the incorporation of labelled amino acid tracers (labelling period). Then, the disappearance of myofibrillar protein labelled with stable isotopically labelled amino acid tracers can be determined (delabelling period), which can then be calculated as myofibrillar protein degradation rate. a: traditionally, stable isotopically labelled amino acids (black circles) are introduced via intravenous (iv) infusion. Extracellular stable isotopically labelled tracers and unlabelled amino acids (tracee, white circles) are transported into the cytoplasm and make up a large pool of free amino acids (free amino acid pool). Amino acids (tracer and tracee) charged with tRNA (aminoacyl-tRNA) are delivered to the ribosome for incorporation into the polypeptide and myofibrillar proteins (labelling

period). b: during the delabelling period, free amino acids (tracer and tracee) derived from myofibrillar protein degradation (e.g., the ubiquitin proteasome system and the autophagy-lysosome pathway) are recycled into free amino acid pool in the cytoplasm and a fraction of the stable isotopically labelled amino acid tracers is reutilized for myofibrillar protein synthesis. When stable isotopically labelled amino acid tracers have their stable isotopic label(s) (deuterium, carbon, and/or nitrogen atoms) at positions where they are released only by the irreversible metabolism of the amino acid, these stable isotopically labelled amino acids are likely to be recycled. Recycling of tracer underestimates the measured myofibrillar protein degradation rate calculated with this approach. c: D_2O can be orally consumed, and deuterium rapidly equilibrates within the body water (light blue background). Water serves as a hydrogen donor and exchanger in in vivo metabolism. Hence, deuterium (D) is incorporated into amino acids through transamination (TA) and tricarboxylic acid cycle (TCA cycle) reactions. D-labelled amino acids can then be incorporated into myofibrillar proteins prior to determining myofibrillar degradation (labelling period). At this stage, D-labelled amino acids stay in proteins until they are released by protein degradation (e.g., the ubiquitin proteasome system and autophagy lysosome system) as a free amino acid. Alanine is a commonly used amino acid as it has four possible exchange sites (C-H bonds) with deuterium through TA and through metabolic precursors for alanine in TCA reactions, improving analytical sensitivity with mass spectrometry. d: during the delabelling period, myofibrillar proteins labelled with D-Ala are degraded. Once D-Ala is released by protein degradation, D-Ala will undergo reactions (TA or TCA cycle) and exchange D with hydrogen from the unlabelled body water pool and thereby lose the D-label. Thus, the recycling of D-labelled amino acids via D_2O is very unlikely when body D_2O enrichment is zero, which may provide a more accurate measurement of myofibrillar protein degradation rate. Based on this theory, D_2O is a preferential tracer to study myofibrillar protein degradation. D_2O, deuterium oxide; D-Ala, deuterium-labelled alanine; TA, transamination; TCA cycle, tricarboxylic acid cycle; tRNA, transfer ribonucleic acid.

It is important to highlight that FBR_{D2O} approach is not without the limitations. Firstly, this approach is time demanding as aforementioned. Removal of D_2O from the circulation following the pre-labelling would take time due to the slow turnover of the body water pool (half-life ~9-11 days), and hence the practical application of the

method is challenging (414). This also suggests that this approach is only applicable to slow turnover protein, such as myofibrillar protein (~1-2 % day^{-1}). Secondly, FBR_{D2O} determines gross average of protein degradation rate (over several days) as the time window between skeletal muscle samplings needs to be extensive in order to detect the small difference of tracer enrichment in myofibrillar protein by mass spectrometry (414). This point challenges the usefulness of this approach to study acute responses (hours) of FBR to any interventions. If classic stable isotopically labelled amino acid tracers should be used for accurate measurement of myofibrillar degradation rate, a novel method that allows to account for recycling of amino acids needs to be developed.

Alternative non-stable isotope tracer method was developed by Crossland et al. (417) to measure global changes of muscle protein degradation in C2C12 myotubes. This method takes advantage of puromycin, which is an antibiotic that is a structural analogue of tyrosyl-tRNA (418, 419). Then, puromycin-labelled peptides can be detected by an antibody using western blot. Briefly, muscle proteins are initially labelled by puromycin followed by the determination of the loss of puromycin, which indicates muscle protein degradation. Crossland et al. (417) showed that known catabolic stimuli, such as dexamethasone, tumour necrosis factor alpha, and serum starvation all induced greater loss of puromycin than untreated control after 48 hours. This method would be a cost-effective approach to understand global changes of muscle protein degradation in cell culture. However, the nature of western blot data (i.e., semi quantitative) and the sensitivity to time resolution (i.e., can the changes of puromycin be detected within short-time period?) should be considered when combining this method with cellular signalling data.

5.4.4 Future studies

Linking the myofibrillar protein degradation rate with the preceding and underlying molecular mechanisms (e.g., UPS) remains to be an experimental challenge (see **Fig. 5.3**). Of importance is the temporal distinction between the *tracer-dilution* approach and the FBR_{D2O} approach in measuring protein degradation rate. The FBR_{D2O} approach detects the 'disappearance' of proteins carrying labelled amino acids at the 'early' stage of protein degradation, where labelled proteins are removed from the matrix pool. In contrast, the *tracer-dilution* approach assesses at the 'final' stage, where the proteins' constituent amino acids are released and appearing into the free amino acid pool and venous blood. This inherent distinction in the two tracer methodologies should in theory translate into different time-dependent associations with concomitant molecular signalling responses. Thus, future studies should investigate molecular mechanisms concomitant with different tracer approaches to assess the temporal changes in protein degradation rate in various physiological conditions.

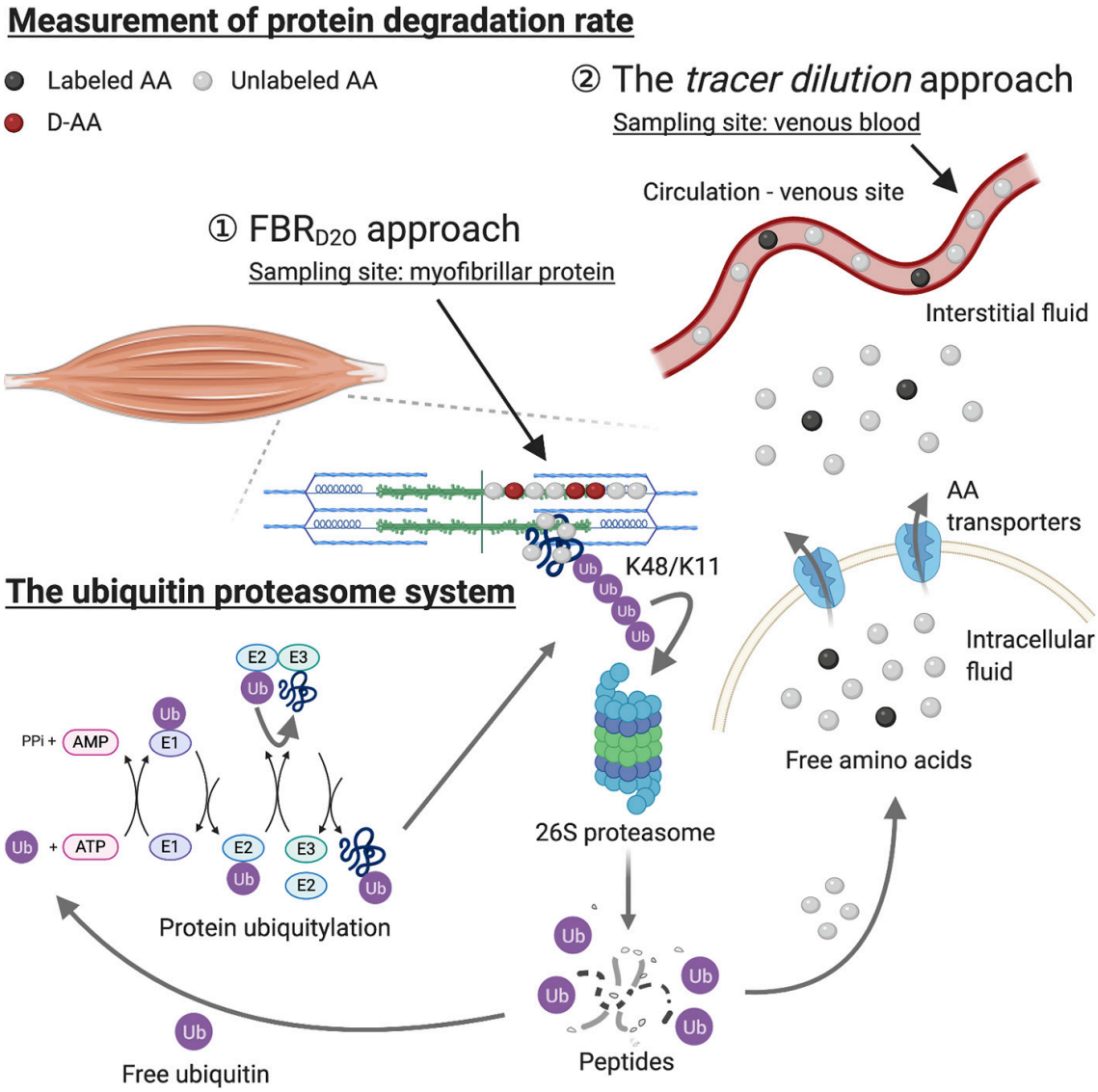

Figure 5.3. The tracer-based approaches to measure protein degradation rate and the link to the ubiquitin proteasome system.

Different tracer-based approaches inhere a temporal distinction of the measurement of protein degradation. Myofibrillar protein degradation rate can be measured by oral consumption of deuterium oxide (D_2O) to prelabell myofibrillar protein through de novo synthesized deuterium (D)-labelled amino acids (red circles), and the subsequent disappearance of myofibrillar proteins labelled with D-labelled amino acids can then be converted to a myofibrillar protein degradation rate (FBRD_2O approach). This FBRD_2O approach targets the "early" process of myofibrillar protein degradation, as the measurement of label abundance is at the level of myofibrillar proteins. In contrast, the tracer-dilution approach provides a measure at the "final" stage of protein degradation, as the determination of label abundance is at the level

of free amino acids. The time-dependent association of the underlying molecular regulations with myofibrillar protein degradation is suggested to be distinct depending on the use of FBRD_2O ("early" stage) or the tracer dilution ("final" stage) approach. In the ubiquitin proteasome system (UPS), myofibrillar proteins are targeted by protein ubiquitylation through sequential reactions, involving ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes. E3 ligase determines a substrate and attaches ubiquitin in conjunction with E2. K48- and K11-linked polyubiquitin chains are a known signal for protein degradation at the 26S proteasome. Once ubiquitylated protein is recognized by the 26S proteasome, polyubiquitin chain is removed by deubiquitylating enzymes (DUBs) and free ubiquitin is recycled for subsequent protein ubiquitylation processes. Then, protein is degraded to peptides at the 20S core particle of the 26S proteasome, which are then released as free amino acids into circulation. AA, amino acid; D2O, deuterium oxide; D-AA, deuterium-labelled amino acid; DUBs, deubiquitylating enzymes; FBR, fractional breakdown rate; Ub, ubiquitin.

5.4.5 Concluding remarks

We showed that Atrogin-1 and MuRF1 protein, valid makers of skeletal muscle atrophy, are regulated by different mechanisms in the rapamycin-sensitive mTOR-S6K1-dependent signalling pathway in C2C12 myotubes. A recent study demonstrated that the enzymatic activity of these ubiquitin E3 ligases plays a key role in controlling skeletal muscle mass. Thus, understanding signalling mechanisms that control these E3 ligases protein provides an alternative opportunity to manipulate their functional E3 ligase activity. However, the current challenge for the field is the lack of easily accessible tools for studying protein ubiquitylation and degradation. Unfortunately, the lack of solid stable isotope methods to measure myofibrillar protein degradation makes us unable to interpret these novel findings of signalling mechanisms accurately. We recommend and highlight the use of D_2O as the most appropriate tracer to measure myofibrillar protein degradation rate. Future studies should aim to integrate the results of both protein degradation and signalling events (e.g., phosphorylation and ubiquitylation) to gain a better understanding of how protein degradation is regulated at a molecular level in various physiological conditions. Knowledge of this can then be used to interpret a complete overview of protein turnover when combining our current understanding of protein synthesis mechanisms.

5.5 Reflections

Completing my PhD training at the University of Birmingham, School of Sport, Exercise and Rehabilitation Sciences has given me invaluable experiences to become an independent researcher. Those include knowledge, experimental techniques, building a collaborative network, which will all be essential in my future career. Nevertheless, there are some aspects that were beyond my control, and thus resulted in different research outcomes/directions from what I anticipated when I started my PhD. Firstly, I was originally going to conduct a human clinical trial involving stable isotope tracer infusion and skeletal muscle biopsy. I developed the study design and prepared documents for ethical approval. However, the research ethics committee at the University of Birmingham could not approve the stable isotope infusion protocol at the time, which resulted in the unavoidable change of my PhD direction when I was about to start the second year of my PhD. Although I played a major role in analysing and writing up manuscripts presented in **CHAPTER 2** and **3** (human trials), I was not able to drive a human trial on my own. Secondly, a positive aspect of changing the PhD direction was that I gained and developed some skills in molecular biology that I would not have otherwise had access to. However, unfortunately, the research questions I developed have not been able to be answered due to the disruptions of lab access and the damage to LC-MS/MS by the COVID-19 pandemic, which led to the further modification of research direction during the third year of my PhD. These unexpected challenges during my PhD have also given me the opportunities to adapt to any situations, where time and resources are limited. Nevertheless, I believe that being able to develop research questions and complete experiments under these unexpected challenges will be valuable assets for my future research career.

5.6 Final conclusions

In this thesis, we have provided new knowledge and insights into the mechanistic regulation and nutrient modulation of skeletal muscle protein turnover and mass. Firstly, we have shown that higher dietary protein intake than what is recommended by WHO/FAO/UNU is not associated with muscle mass or physical function in healthy older individuals. Protein distribution pattern within a day is also not associated with muscle mass and physical function. Hence, our data do not support growing suggestions to increase dietary protein recommendation in healthy older individuals. The discussion within this thesis identifies and provides considerations for future studies to accelerate an international agreement on dietary protein requirement and recommendation for healthy older adults. Secondly, we demonstrated that co-ingestion of Cluster Dextrin carbohydrate with meat protein hydrolysate activates mTORC1 signalling though postprandial amino acid availability and MPS did not differ between CDX and GLC. It would be interesting to investigate if CDX alleviates anabolic resistance in older adults and it is hoped that the findings from this thesis will encourage future studies in this area. Intriguingly, we identified for the first time that Atrogin-1 and MuRF1 protein are differentially regulated by the downstream of Akt and that Atrogin-1 protein is regulated by the rapamycin-sensitive mTOR-S6K1 signalling pathway in C2C12 myotubes. The discussion within the thesis provides directions for future studies to understand the complicated mechanisms of skeletal muscle protein breakdown. Overall, these data shown in this thesis extend the knowledge of mechanistic and nutritional modulation of skeletal muscle protein turnover and mass.

CHAPTER 6

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APPENDICES

Appendix 1.

Title: Dietary protein requirements and recommendations for healthy older adults – A critical narrative review of the scientific evidence

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Dietary protein requirements and recommendations for healthy older adults: a critical narrative review of the scientific evidence

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Abstract
Adequate protein intake is essential for the maintenance of whole-body protein mass. Different methodological approaches are used to substantiate the evidence for the current protein recommendations, and it is continuously debated whether older adults require more protein to counteract the age-dependent loss of muscle mass, sarcopenia. Thus, the purpose of this critical narrative review is to outline and discuss differences in the approaches and methodologies assessing the protein requirements and, hence, resulting in controversies in current protein recommendations for healthy older adults. Through a literature search, this narrative review first summarises the historical development of the Food and Agriculture Organization/World Health Organization/United Nations University setting of protein requirements and recommendations for healthy older adults. Hereafter, we describe the various types of studies (epidemiological studies and protein turnover kinetic measurements) and applied methodological approaches founding the basis and the different recommendations with focus on healthy older adults. Finally, we discuss important factors to be considered in future studies to obtain evidence for international agreement on protein requirements and recommendations for healthy older adults. We conclude by proposing future directions to determine 'true' protein requirements and recommendations for healthy older adults.

Key words: **Ageing: Sarcopenia: Amino acid: Protein synthesis: Protein breakdown: Net protein balance: Protein turnover: Metabolic adaptation: Energy balance: Protein intake**

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Introduction

Adequate protein intake is required for the maintenance of whole-body protein mass. The protein mass in a 70 kg adult is about 11 kg. Whole-body protein mass maintenance relies on equal rates of protein synthesis and breakdown, resulting in a zero net balance. The constant kinetics of protein synthesis and breakdown of body proteins were originally demonstrated by Schoenheimer and colleagues in 1939[1] and were termed protein turnover, which is a modifiable and adaptable process. In adult humans, the daily whole-body protein turnover rate is 5·7 g/kg body weight[2], meaning that approximately 400 g mixed proteins are turned over every day for a 70 kg adult individual. Behind that number, a large proportion of amino acids are recycled and reutilised for protein synthesis[3–5], whereas some are lost via oxidation for energy production and the formation of urea to scavenge nitrogen[3]. Further, nitrogen-containing substances, such as skin, hair, sweat, urine and faeces, are lost from the body. To maintain whole-body protein mass, irreversible loss of amino acids and nitrogen needs to be reconstituted via dietary protein, which founds the basis for the recommended dietary protein intake.

It is well established how much dietary protein should be ingested to account for obligatory nitrogen loss in healthy adults using nitrogen balance methodology[6,7]. However, it has continuously been debated[8–14] whether increasing dietary protein intake in older age is an effective strategy to counteract the age-dependent loss of muscle mass[15,16] and strength[17,18], a phenomenon termed sarcopenia[19–21]. However, an increase in the dietary protein recommendations for older adults would categorise numerous people as being protein malnourished and thereby increase the incidence of protein malnutrition worldwide. Such a change would challenge nutritional guidelines and nutritional societies and force governments to act with enormous socio-economic consequences. Thus, it is of utmost importance that we adopt a critical approach to the evidence underlying the dietary protein recommendations.

Independent authorities and expert groups have adopted different criteria and evidence from distinct methodological

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Table 1. Successive protein requirements and recommendations by international groups to ensure nitrogen balance in adults

Report	Age	Methodological approach	Biological value of dietary protein (%)	Average protein requirements (g/kg/d)	Recommendation/safe level of intake (g/kg/d)
League of Nations 1936[22]	Adults	–	–	–	1·0
FAO 1957[23]	Adults	N balance	80	0·53	0·66
FAO/WHO 1965[140]	Adults	Factorial	80	0·71	0·89
FAO/WHO 1973[94]	Adults (20–39 years)	Factorial	75	0·57	0·75
FAO/WHO/UNU 1985[95]	Adults	N balance	100	0·75	0·75
FAO/WHO/UNU 2007[6]	Adults (≥18 years)	Meta-analysis (N-balance studies)[7]	100	0·66	0·83

Adopted from NS Scrimshaw[141] and updated.

approaches, resulting in differences in published recommended values of protein recommendations. Consequently, there is currently confusion about the requirements of dietary protein for healthy older adults, both in the scientific community and amongst the general older population, who wish to follow the recommendations. Thus, the purpose of this narrative review is to critically discuss the existing evidence for protein requirements founding the basis for protein recommendations for older adults.

Methodology

This narrative review was undertaken by: (1) searching PubMed and Google Scholar using keywords related to each topic; (2) screening reference lists for relevant papers; and (3) searching nutritional societies' and authorities' guidelines and references. We specifically included studies conducted in older individuals and only included studies on younger adults when studies in older adults were not available. All articles had undergone peer review and were available in English.

The first part of this review is a summary of the historical development of the protein recommendation with a brief mention of the approaches used and methodologies applied. In the second part, we present and describe in more detail the methodological approaches that found the evidence for determining protein requirements and present the key studies providing data for older adults. Finally, we discuss major factors influencing the requirement for dietary protein and that thus need to be considered in future study designs striving to determine protein requirements. With such consensus, we can accelerate international agreement on protein recommendations for healthy older adults.

Historical development of determining protein requirements and recommendations

The first official recommendation for protein intake of 1·0 g/kg/d, published in 1936 by the League of Nations[22], was set based on observations from practice rather than relying on data from a strict scientific approach. Several joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) expert committees have faced challenges in defining 'protein requirements'[23]. In 1957, FAO/WHO[23] reported their first official protein recommendation, and the latest was reported in 2007[6], which thereafter has been challenged by other expert bodies. Table 1 provides an extensive overview of the WHO/FAO/United Nations University (UNU) recommendations over the years. While the 2007 recommendation defined the protein requirement as being '*the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity*…', it is uncertain whether this amount is sufficient to counteract protein loss in catabolic conditions[24]. Even though the Expert Committee rigorously reassessed all available data on protein balance in older people, it was concluded that no studies unequivocally demonstrate that the protein requirement would be higher in older adults when expressed as protein requirement per kg body weight when the purpose was to maintain whole-body protein balance.

Recently, this has led to some expert bodies challenging the protein recommendations for older adults. Table 2 summarises major guidelines from external expert bodies, which, however, are not official recommendations. Although our understanding of protein requirements and recommendations has improved over these 60 years, the outcome measurements, and the methodologies on which the outcome measurement is founded, have not evolved. The discrepancy in protein recommendations is partly due to the lack of a definitive criterion for 'adequate protein', as well as differences in outcome measurements will be discussed. Then, important factors that can impact protein requirements will be reviewed.

Generation of evidence for protein recommendations for older adults

Protein/nitrogen balance studies

The nitrogen balance methodology has been used most widely and is considered 'gold standard methodology' to establish the protein requirement[6,7]. Whole-body nitrogen balance is determined by measuring or estimating all nitrogen intake (protein intake) and all excretion and loss (skin, hair, sweat, urine and faeces). Positive balance means excess nitrogen intake relative to losses (i.e. protein accretion within the body). Two crucial prerequisites

Table 2. Protein recommendations by expert bodies to maintain muscle mass and strength in healthy older adults

Report	Age	Methodological approach/study design	Functional outcomes	Recommendation (g/kg/d)	Rationale
Nutrient Reference Values for Australia and New Zealand 2006[142]	>70 years	Factorial	Estimation of the amount needed for growth and maintenance on a fat-free mass basis	Men 1·07 (81 g/d) Women 0·94 (57 g/d)	Based on Rand *et al.*[7] and additional studies[7,143,144] the average requirement was increased by 25 % over that of younger adults
The Nordic Nutrition Recommendations 2012[51]	>65 years	Systematic review[146] (prospective cohort case–control and intervention studies)	N balance Muscle mass Muscle strength Bone mass Morbidity Mortality	1·1–1·3 (15–20 E % and the protein E % should be increased with decreasing energy intake)	The grade of evidence was assessed as *convincing*, *probable*, *suggestive* and *inconclusive*[145]. The evidence of protein requirement (0·66 g/kg/d) and recommendation (0·83 g/kg/d) based on N-balance studies was assessed as *probable*. The evidence from prospective cohort studies in relation to functional outcomes (e.g. muscle mass and muscle strength) with a safe intake of at least 1·2–1·5 g/kg/d was assessed as *suggestive* to *inconclusive*
The PROT-AGE Study Group 2013[49]	>65 years	Epidemiological studies Clinical trials	Muscle mass Muscle strength Physical function Muscle protein synthesis	1·0–1·2	Decrease in anabolic response to protein intake in older adults; higher protein intake is required to offset inflammatory and catabolic conditions often observed in older adults. Both endurance and resistance exercise were recommended and even higher protein intake (>1·2 g/kg/d) was recommended those who engage in exercise
The ESPEN Expert Group 2014[50]	>65 years	Epidemiological studies Clinical trials	Muscle mass Muscle strength Physical function Muscle protein synthesis	1·0–1·2	Older adults require higher protein owing to anabolic resistance, low post-prandial amino acid availability, decreased muscle perfusion, sarcopenia and disease-related protein catabolism. Both endurance and resistance exercise were recommended

for a valid measurement are: (1) metabolic adaptation to any given amount of daily protein intake before conducting the measurements[25 27]; and (2) the achievement of energy balance during the period of measurement[7]. However, the nitrogen balance methodology has inherent shortcomings: it has been criticised for insufficient sensitivity (i.e. inability to detect small differences between nitrogen intake and excretion)[28]. Further, the overestimation of nitrogen intake through food and underestimation of nitrogen loss can lead to erroneous balance measurements.

While nitrogen balance data provide limited mechanistic insight[28,29], Waterlow and colleagues proposed in 1977[30] that a relationship between nitrogen balance and protein balance could be expressed at steady state as: flux (Q) = protein synthesis (S) + nitrogen oxidations/excretion (O) = protein breakdown (B) + nitrogen input (I) → $Q = I \quad O = S \quad B$.

Protein balance calculated as the difference between protein synthesis and breakdown rates provides information about the underlying dynamics of protein kinetics. Protein balance is the rate of either gain or loss of protein per unit time and indicates whether protein mass in the body will quantitatively change over time. Therefore, the prolonged readout of the protein balance data is whole-body protein mass and/or changes herein. Whole-body protein mass, often determined by the surrogate measure of muscle mass, is determined in another set of scientific studies, namely epidemiological studies.

Epidemiological studies of protein intake

Epidemiological studies, such as cross-sectional and prospective cohort studies, can be used for assessing the association between habitual dietary intake patterns and whole-body/muscle mass[31 42]. Epidemiological studies have the strength that adaptation to a given protein intake level would be inherent, yet they often suffer from several confounding factors, limiting the translation into other settings and making it impossible to use the results for assessing protein requirements. Longitudinal intervention studies can be applied to examine the cause–effect relationship and/or dose–response relationship between protein intake and most often physiological and functional outcomes, such as muscle mass, muscle strength and physical function, which is particularly relevant to older adults[43 48].

Cross sectional studies. The role of protein intake in the maintenance of muscle mass has been investigated across the lifespan in numerous studies (Table 3). A cross-sectional study from the Framingham Offspring Cohort showed a positive association between the total daily and total animal protein intake and muscle mass after adjustment of physical activity and energy intake[42]. The association between protein intake and muscle mass became apparent when dividing the cohort into quartiles of protein intake. In men, the difference appeared between the highest (101·1 g/d) and the lowest quartile (64·9 g/d) for total protein intake. In women, muscle mass differed between the highest and the second-lowest quartile for total protein and animal protein intake (93·4 g/d versus 63·1 g/d)[42]. Mangano *et al.*[39] examined the third-generation offspring of the original Framingham Heart Study. They divided the cohort into four quartiles based on their protein intake, from the lowest intake (quartile 1) 59 g/d (0·8 g/kg/d) to the highest intake (quartile 4) 129 g/d (1·8 g/kg/d). After adjustment for various confounders, including physical activity and energy intake, they found a positive association between protein intake and appendicular lean mass index. A difference was found between the first and the second quartile (80 g/d or 1·1 g/kg/d). However, there was no further difference between the first quartile and the third (99 g/d or 1·3 g/kg/d) and the fourth quartile (129 g/d or 1·8 g/kg/d)[39]. Collectively, cross-sectional observational studies suggest that protein intake around the international recommendation (0·8 g/kg/d, 60–65 g/d in average-sized individuals, 75–80 kg) is sub-optimal for the maintenance of muscle mass throughout adult life. They also suggest that muscle mass may be better maintained when protein intake is higher (80 g/d or 1·1 g/kg/d) than the international recommendation (0·83 g/kg/d), and that no further beneficial effects on muscle mass are observed beyond this amount, suggesting a non-linear association above this level of intake.

Cross-sectional observational studies including healthy older adults have provided insight into the role of dietary protein intake in the maintenance of muscle mass[31,35,38,41] (Table 3). A positive association was observed between protein intake and muscle mass[31,35]. In contrast, it was recently reported that muscle mass did not differ between total daily protein intakes < 0·83, ≥ 0·83 < 1·1, and ≥ 1·1 g/kg adjusted body weight in healthy Danish older individuals (≥65 years.), but this might be due to the small sample size ($n = 25$) in protein intake below 0·83 g/kg/d[41]. Geirsdottir *et al.*[35] reported data adjusted for sex, BMI, age and physical activity level and found higher lean body mass in the quartile with the highest protein intake (1·15–1·92 g/kg/d, the fourth quartile) compared with only the first quartile (0·41–0·75 g protein/kg/d, $P = 0·04$) and the second quartile (0·76–0·92 g/kg/d, $P = 0·05$), but not the third quartile. Interestingly, the differences disappeared when correcting for total energy intake, which emphasises the crucial importance of considering energy intake when evaluating the impact of protein intake on the regulation of body composition and especially lean body mass in older adults. Similarly, data by Asp *et al.*[31] emphasise the dependency between protein and energy intake on muscle and body size as they found that protein intake correlated positively with nutritional status and BMI even when accounting for age, sex and activity level. In support of protein intake as a determining factor for a lean body composition, Gregorio *et al.*[38] divided a total of 387 older women into those ingesting ≥ 0·8 g/kg/d ($n = 290$) and those ingesting < 0·8 g/kg/d ($n = 97$) and found that, although the women in the ≥ 0·8 g/kg/d group had lower lean mass than those in the < 0·8 g/kg/d group, they also had a lower fat mass, resulting in a lower ratio of fat to fat-free mass in the high-protein group. Collectively, some cross-sectional observational studies have reported positive associations between protein intake and muscle mass in older adults. However, it seems that, when accounting for nutritional status, energy intake and physical activity, there is limited evidence to recommend that protein intake greater than the international recommendation is beneficial for muscle mass preservation.

Observational studies. Association between protein intake and muscle mass was investigated in an observational study design in healthy older adults[32 34,36,37,40] (Table 4). A positive association

Table 3. Selected cross-sectional observational studies assessing the association between protein intake and muscle mass

Author location	Subjects men (M) women (W)	Age years	Dietary assessment	Body composition measurement	Average protein intake	Protein intake	Outcomes	Additional information
Sahni *et al* 2015[42] USA	1139 M 1497 W	29–86	FFQ	DXA	M 80 g/d W 76 g/d	g/d Q1 M 64·9 W 57·8 Q2 M 70·8 W 63·1 Q3 M 79·2 W 73·5 Q4 M 101·1 W 93·4	A positive association between protein intake and leg lean mass	
Mangano *et al* 2017[69] USA	2905 M&W	19–72	FFQ	DXA	93 g/d	g/d (g/kg/d) Q1 59 (0·8) Q2 80 (1·1) Q3 99 (1·3) Q4 129 (1·8)	A positive association between protein intake and ALM	
Geirsdottir *et al* 2013[65] celand	99 M 138 W	65–92	3-d food record	DXA	M 90·3 g/d 19·6 E % W 69·6 g/d 18·8 E % %	g/kg/d Q1 0·63 Q2 0·85 Q3 1·01 Q4 1·36	A positive association between protein intake and LM Higher LM in Q4 *vs* Q1 (2·3 kg LM difference)	Significance was not evident after the correction with energy intake
Asp *et al* 2012[61] USA	47 M 95 W	60–88	DHQ	CC	15·5 E %	Protein E %	A positive association between protein intake and CC	A positive association between beef protein intake and CC
Gregorio *et al* 2014[58] USA	387 W	60–90	4-d food record	DXA	72·2 g/d 1·1 g/kg/d	g/kg/d < 0·8 ≥ 0·8	LM was lower in ≥ 0·8 compared to < 0·8 Fat-to-Lean Ratio was lower in ≥ 0·8 g/kg/d	
Højfeldt *et al* 2020[41] Denmark	98 M 86 W	65–82	3-d food record	DXA	82·8 g/d 1·13 g/kg/d 17·6 E %	g/kg aBW/d < 0·83 ≥ 0·83– < 1·1 ≥ 1·1	ASM did not differ between different protein intakes	

FFQ, food-frequency questionnaire; E, energy; DXA, Dual-energy X-ray absorptiometry; Q, quartile; DHQ, diet history questionnaire; CC, calf circumference; aBW, adjusted body weight; LM, lean body mass; ALM, appendicular lean body mass; ASM, appendicular skeletal muscle index.

Table 4. Selected observational studies assessing the association between protein intake and muscle mass in healthy older adults

Author location	SubjectsMen (M) Women (W)	Age years	Dietary assessment	Body composition measurement	Follow-up duration	Baseline average protein intake	Baseline protein intake	Energy intake/balance	Outcomes
Houston *et al* 2008[32] USA	967 M 1099 W	70–79	FFQ	DXA	3 years		% of energy (g/kg/d) Q1 11·2 (0·7) Q2 12·7 (0·7) Q3 14·1 (0·8) Q4 15·8 (0·9) Q5 18·2 (1·1)		Higher protein intake was associated with higher preservation of LM and aLM
Meng *et al* 2009[33] Australia	862 W	70–85	FFQ	DXA	5 years	81 g/d 1·2 g/kg/d 19 E %	g/d g/kg/d (E %) Q1 <66 0·84 (17·7) Q2 66–87 1·17 (19·0) Q3 >87 1·64 (20·4)		Higher baseline protein intake was associated with higher LM and aLM at the 5-years follow-up
Scott *et al* 2010[34] Australia	370 M 370 W	50–79	FFQ	DXA	1·4–4·8 years (mean 2·6 years)	87·6/d 1·13 g/kg/d	<RD ≥RD	Energy intake and BW were maintained between baseline and follow-up Step counts were decreased at follow-up compared with baseline	Higher protein intake was associated with aLM at baseline and follow-up
McDonald *et al* 2016[36] Denmark	39 M 40 W	>65	nterview	Bioelectrical impedance	6 years	74·6 g/d 1·07 g/kg/d	g/kg/d Q1 0·61 Q2 0·92 Q3 0·97 Q4 1·26		Higher protein intake was associated with higher preservation of LM
Chan *et al* 2014[37] China	1411 M 1315W	≥65	FFQ	DXA	4 years	1·3 and 1·1 g/kg/d for M and W	g/kg/d Q1 ≤0·9 Q2 0·91–1·2 Q3 1·1–1·6 Q4 ≥1·61		No association was found between protein intake and ASM
Verreijen *et al* 2019[40] USA	749 M 812 W	70–79	FFQ	CT	5 years	66·0 g/d 0·90 g/kg/d	g/kg/d Q1 0·50 Q2 0·68 Q3 0·85 Q4 1·03 Q5 1·39		Protein intake was not associated with 5-year change in muscle cross-sectional area

FFQ, food-frequency questionnaire; E, energy; DXA, dual-energy X-ray absorptiometry; Q, quartile; CT, computed tomography; RD , recommended dietary intake; BW, body weight; LM, lean body mass; ASM, appendicular skeletal muscle mass; aLM, appendicular lean body mass.

between protein intake and muscle mass was observed in some studies[32 34,36], and these studies concluded that protein intake above the current international recommendation is beneficial for preserving muscle mass in older adults. In all of these studies, baseline average protein intake was higher (~1·2 g/kg/d) than the current international recommendation (0·83 g/kg/d)[32 34,36]. In contrast, other studies found that protein intake higher than the current international recommendation does not preserve muscle mass in older adults[37,40]. Chan *et al.*[37] discussed that the null association between protein intake and muscle mass was due in part to the relatively higher protein intake in this cohort (1·3 and 1·1 g/kg/d in men and women, respectively). More recently, Verreijen *et al.*[40] reported that protein intake was not associated with 5-year changes in muscle mass. The contradicting results from Houston *et al.*[32] were explained by the methodological approaches used to assess muscle mass. Whilst Houston *et al.*[32] employed dual-energy X-ray absorptiometry (DXA), Verreijen *et al.*[40] used computed tomography (CT), which is regarded as a more accurate methodology to examine muscle mass. In summary, inconsistent results have been reported in observational studies regarding the association between protein intake above the international recommendation (0·83 g/kg/d)[6]. Thus, the newly suggested protein recommendations for healthy older adults of 0·94–1·3 g/kg/d from different authorities and expert groups[49 51,142] can still be questioned.

Intervention studies. Several randomised controlled intervention studies have investigated whether higher protein intake increases muscle mass in older adults[43 48] (Table 5). Some studies were unable to show a beneficial effect of increasing dietary protein intake above their average intake of ~1·2 g/kg/d on muscle accretion or maintenance in older adults[43,44,48]. Some studies showing no effect of higher protein intake (≥0·8 g/kg/d) tended to have a longer study period (≥1 year) compared with studies in which an increase in muscle mass was demonstrated (~24 weeks)[43 47], suggesting a possible adaptation effect. Mitchell *et al.*[46] showed that protein intake at 2RDA increased muscle mass compared with RDA in a group of men aged >70 years. over a period of 10 weeks. However, the individuals in this study consumed a habitual protein intake of 1·1–1·2 g/kg/d on average, and the individuals in the RDA group consumed less protein than the habitual intake during the intervention, which may explain the loss of appendicular lean mass and caused a group difference between RDA and 2RDA. For this study, several limitations can be listed, including that energy balance was not maintained in the RDA group; that lack of a steady metabolic state condition as adaptation was not present for the entire intervention period of 10 weeks; and finally, that no habitual protein intake group was included, which therefore does not allow us to conclude on changes of lean mass without altered protein intake. In summary, null effects of longer-term intervention of elevated dietary protein intake may indicate that metabolic adaptation may level out acute benefits, which was exemplified by a recent study by Højfeldt *et al.*[52].

Protein turnover kinetic measurement

Protein turnover kinetic studies are used as more exploratory and mechanistic measurements of the underlying protein turnover kinetic rates. Since the 1970s[53], experimental settings in which the results of muscle protein synthesis (MPS) based on the stable isotope tracer direct incorporation measurement using precursor–product methods[54,55], have established an important basis of knowledge in protein nutrition. In particular, stable isotope tracer and mass spectrometer methodology have advanced our knowledge on MPS in response to amino acids or protein intake[56 58]. Experiments with infusion of stable isotope amino acid tracers are usually conducted for <24 h, and responses to ingestion of amino acids/proteins or mixed meals are measured in a controlled laboratory setting. Experiments are usually designed to measure post-prandial MPS (~6 h) in response to protein intake while participants are kept in artificial conditions such as fasting and bedridden, often with standardised prior dietary intake. The tissue of interest is often skeletal muscle, with an emphasis on MPS rate. As muscle protein turnover contributes only approximately 25–35 % of whole-body protein turnover in humans[59,60], it is important to take this into consideration if the results from these studies are to be used for estimating protein requirement.

Skeletal muscle protein. Amino acid availability in the circulation is a determinant of MPS stimulation[61], a response that is dose-dependent and saturable even in the presence of sustained elevated circulating amino acids[62 68]. Although originally hypothesised[69 71], recent accumulated evidence has concluded that post-absorptive MPS rates do not differ between older and younger individuals[72,73]. However, older individuals exhibit a blunted post-prandial MPS response to amino acid/protein intake when compared with younger individuals[63,74 76], which has been termed age-related 'anabolic resistance'. Moore and colleagues synthesised post-prandial MPS data generated in the lab previously[62] and suggested that younger and older individuals are required to consume 0·24 g/kg/meal and 0·4 g/kg/meal, respectively, to maximally stimulate MPS, meaning that approximately 70 % more protein is required to maximally stimulate MPS in older individuals compared with younger individuals.

A blunted MPS and, hence, sub-optimal net balance in response to protein feeding and meals could be a plausible driver in the development of sarcopenia. This indicates that older adults need more dietary protein. However, it is important to note that this evidence is derived from acute post-prandial MPS studies in response to high-quality, rapidly digested, mostly animal-based proteins (e.g. egg, whey or casein protein)[62,76].

In addition to the limitations involved in this experimental design, the potential beneficial effects of higher protein intake for muscle and/or whole-body net protein balance or anabolic response are unknown without the simultaneous measurement of muscle or whole-body protein breakdown. Further, the protein recommendation refers to whole-body protein balance[6,7], which is not easily comparable with the responsiveness of skeletal muscle. Hence, we argue that data originating from experimental settings on MPS responsiveness to single dose of protein may lead to misinterpretation and erroneous conclusions on dietary protein requirements in humans at the whole-body level, and therefore cannot be extrapolated to whole-body protein requirements in daily living.

Nutrition Research Reviews

Table 5. Selected randomised controlled trials investigating the impact of protein intake on muscle mass in healthy older adults

Author location	Study design	Subjects men (M) women (W)	Age years	Follow-up duration	Dietary assessment	Body composition measurement	Baseline average protein intake	Energy intake/balance	ntervention	Outcomes
Mitchell *et al.* 2017[46] New Zealand	Parallel	29 M	70–81	10 weeks	3-d food record	DXA	88–101 g/d ~1·2 g/kg/d 14–17 E %	Energy intake was decreased in post from pre in RDA (−440 kcal) and increased in 2RDA (+555 kcal) BW was decreased in RDA (−2·1 kg) and 2RDA (−0·5 kg)	Protein intake at RDA (0·8 g/kg/d) versus 2RDA (1·6 g/kg/d)	2RDA increased LM (+1·49 kg) compared with RDA (−0·55 kg)
Ten Haaf *et al.* 2019[47] Netherlands	Parallel	93 M 21 W	67–73	12 weeks	Two 24-h recalls	DXA	0·89 g/kg/d 16 E %	Energy intake was reduced in post from pre in both protein (−78 kcal) and placebo (−10 kcal) groups BW was decreased in protein (−0·59 kg) and placebo (−0·15 kg) groups	A total of 31 g of protein in 500 ml milk protein concentrate drink per day or placebo	Protein supplementation increased LM (+0·93 %) compared with placebo (+0·44 %)
Norton *et al.* 2016[45] reland	Parallel	14 M 46 W	50–70	24 weeks	4-d food record	DXA	83–86 g/d 1·2 g/kg/d 16–19 E %	Energy intake was increased in post from pre in protein (+111 kcal) and placebo (+116 kcal) groups	A total of 145·4 g of protein per day or placebo	Protein supplementation increased LM (+0·45 kg) compared with placebo (−0·16 kg)
Mertz *et al.* 2021[48] Denmark	Parallel	184 M and W	65–82	1 year	3-d food record	MR /DXA	82·8 g/d 1·1 g/kg/d 17·6 E %	Energy intake was increased in post from pre in whey (+518 kcal) collagen (+408 kcal) and placebo (+949 kcal) BW was increased in whey (+0·4 kg) collagen (+0·7 kg) and placebo (+1·2 kg)	A total of (1) 40 g whey protein (2) 40 g collagen protein or (3) placebo	Protein supplementation did not increase LM and CSA
Kerstetter *et al.* 2015[43] USA	Parallel	30 M 178 W	75 (mean)	18 months	3-d food record	DXA	72·9 g/d ~1·07 g/kg/d	Energy intake was decreased in post from pre in protein (−120 kcal) and placebo (−4 kcal) groups BW was maintained in both groups	A total of 45 g whey protein or placebo	Protein supplementation did not increase LM
Zhu *et al.* 2015[44] Australia	Parallel	181 W	70–80	2 years	3-d food record	DXA	76 g/d 1·1–1·2 g/kg/d	BW was increased in protein (+0·5 kg) and placebo (+0·4 kg)	A total of 30 g of skim milk-based protein supplement or placebo	Protein supplementation did not increase LM

DXA dual-energy X-ray absorptiometry E energy BW body weight LM lean body mass RDA recommended dietary allowance MR magnetic resonance imaging BW body weight CSA cross-sectional area

Table 6. Protein recommendations derived from acute stable isotope tracer incorporation studies in younger and older adults

	Younger		Older
Dietary protein		(g/kg BM)	
Single meal	0·24		0·40
Day (3–4 meals)	0·72–0·96		1·2–1·6
Dietary protein		(g/kg LBM)	
Single meal	0·25		0·61
Day (3–4 meals)	0·75–1·0		1·83–2·44
Dietary protein		(g)*	
Single meal	18		30
Day (3–4 meals)	54–72		90–120

LBM, lean body mass.
* An individual with 75 kg of body mass (BM).
Values were adopted from Moore *et al.*[62] determined by Biphasic Linear Regression Model from six independent studies[64,65,146 149].

In summary, studies measuring the acute post-prandial response to protein feeding with the stable isotope tracer technique have led to the hypothesis of age-related muscle anabolic resistance. It is suggested as one of the underlying mechanisms of the development of sarcopenia[62,63,76]. Such quantitative data suggest that protein intake of 0·4–0·6 g/kg/meal at three main meals and a snack per day is required to preserve muscle mass in healthy older individuals (Table 6), which equates to a protein intake of <1·2–1·8 g/kg/d[77].

Whole body protein. More recent investigations have shifted from the use of isolated protein sources to whole foods, the form of protein consumption in everyday life. This shift is due to the interactive effects of protein contained in whole foods with other nutrients and bioactive compounds on muscle and whole-body protein turnover. Studies have primarily focused on MPS in response to beef[78,79], eggs[80,81] and mixed macronutrient meals[82,83]. These studies highlighted the importance of considering complex food matrices in the regulation of muscle and whole-body protein turnover.

Previous studies investigating whole-body protein turnover in the context of mixed macronutrient meal intake demonstrated that anabolic response is not limited by protein synthesis[82 84]. Deutz and Wolfe[85] advocated that there is no limit to *in vivo* whole-body protein anabolism when protein is consumed as a part of a mixed macronutrient meal[97,101]. More recently, Park *et al.*[88] showed in healthy older adults (69·3 ± 1·8 years) that there is a higher MPS response following the consumption of a higher protein intake (70 g) compared with moderate (35 g) as part of a mixed macronutrient meal, and a greater whole-body net protein balance in the higher protein intake group due to the suppression of protein breakdown as well as increased protein synthesis (Fig. 1). Importantly, the doses of protein used in this study (35 or 70 g) were beyond the amount suggested to maximally stimulate MPS in healthy older men[62] (Table 6). Similar findings were also found by the same research group in healthy younger adults[82].

The suppression of protein breakdown may also be explained by insulin secretion from non-protein energy sources[86,87]. Higher insulin concentration in circulation is observed when protein is consumed in a mixed macronutrient meal[88]. A blunted protein breakdown suppression[89] and a failure to achieve positive phenylalanine whole-body net

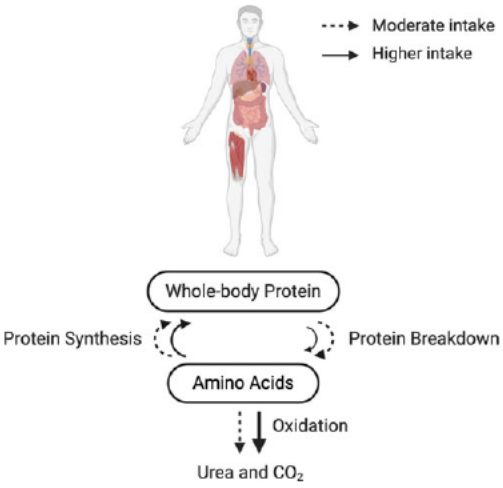

Fig. 1. Whole-body protein turnover in response to the recommended or higher protein intake in a mixed macronutrient meal. Dashed (--) and solid (–) lines indicate a moderate (~35 g/meal) and a higher protein intake (~70 g/meal) in a mixed macronutrient meal, respectively. Protein synthesis is saturable at the given amounts of protein intake[82], illustrated by the similar-sized arrows for protein synthesis between the moderate and higher protein intake. Protein breakdown is suppressed by the higher protein intake in a mixed macronutrient meal (illustrated by a smaller solid arrow), resulting in a greater whole-body net protein balance[82,83,85,150]. Be aware that a higher protein intake (i.e. surplus of amino acids) also inevitably increases amino acid oxidation and urea excretion[82,117]. The results depicted in this figure originate from collective data based on stable isotope tracer studies.

balance[90,91] in response to insulin have been reported in older individuals, which may suggest a difficulty in achieving net positive whole-body protein balance in older individuals in response to mixed macronutrient meal intake. A systematic review and meta-analysis concluded that insulin has a permissive role in MPS, whereas insulin attenuates muscle protein breakdown independently of amino acid availability[92,93]. These studies highlight the importance of protein breakdown measurement in the context of a mixed meal intake owing to the suppression of protein breakdown in response to higher protein intake and insulin from non-protein energy sources, resulting in increased whole-body protein anabolism. Moving forward to the justification of the necessity to increase the current international safe level of intake for protein in healthy older adults, the upper limit of protein intake that suppresses protein breakdown, and thus better preserves whole-body protein, should be defined, as highlighted recently by Phillips *et al.*[14]. We argue that whole-body protein turnover data and real-life interventions are more appropriate to extrapolate to protein requirements.

Future directions of protein requirements and recommendations for healthy older adults

Energy balance

The importance of energy intake in relation to the determination of protein requirement was raised already by the Joint FAO/

WHO Ad hoc Expert Committee on Energy and Protein Requirements in 1971[94], and later in 1981[95]. Notably, Young[29] and Millward[96] explored the important concept of protein and energy inter-relation to determine protein requirement. The interaction between energy and nitrogen intake on the maintenance of body nitrogen was well documented by studies on the role of non-protein energy sources (carbohydrate and fat)[97–99] using nitrogen balance methodology. For example, Colloway *et al.*[98] showed back in 1954 that exogenous nitrogen-bearing sources contribute to energy production rather than deposition in the body until adequate energy is consumed. Further, increased non-protein energy intake can also mitigate nitrogen loss in a dose-dependent manner[98]. However, the provision of energy attenuates the recruitment of amino acid metabolism for energy turnover and hence retains nitrogen, leading to improvement in nitrogen balance[98]. Accordingly, the achievement of energy balance is an underlying assumption for the current internationally recommended protein intake[6].

In recent years, protein metabolism and turnover rate have been studied under a condition of negative energy balance[100–102] in healthy adults and with an attempt to preserve muscle mass or attenuate muscle loss under negative energy balance by increasing protein intake[103–108]. The effect of protein intake above the current recommended safe level of intake (0·83 g/kg/d) on muscle mass maintenance under negative energy balance has been well documented[100,102,109]. Evidence indicates that, whilst amino acids contribute more to energy production at negative energy balance[105,110], they are utilised more for *de novo* protein synthesis when energy balance is achieved. However, evidence is scarce in healthy older individuals, and it is unclear whether increased protein intake during negative energy balance is sufficient to maintain whole-body and muscle protein mass in healthy older adults.

In contrast, limited knowledge is currently available on the impact of positive energy balance on the regulation of protein turnover rates and muscle mass in healthy older adults. Nonetheless, based on knowledge from energy deficit studies[105,110] as well as studies by Woolfson[111] and Calloway and Spector[98], it can be assumed that positive energy balance reduces amino acid oxidation. Accordingly, exogenous amino acids (dietary protein) under such conditions are more efficiently utilised to achieve net positive protein balance. The impact of protein content relative to total energy intake on body composition in younger healthy individuals during overfeeding (18–35 years) was investigated by Bray *et al.*[112]. Participants were divided into 5 % (low protein), 15 % (normal protein) and 25 % (high protein) of total energy intake from protein after a weight-stabilising diet period. Following an 8-week intervention, fat mass was similarly increased in all groups because of overfeeding (40 % excess energy from a weight-stabilising diet). However, lean body mass was significantly increased with normal and high protein groups, along with a concomitant increase in resting energy expenditure. These results suggest that energy intake from protein is a sole determinant to increase lean body mass, but not the accretion of fat mass during overfeeding in healthy younger individuals. In this cohort, additional protein may not be required when energy balance is maintained since no impact on lean body mass changes was detected between normal and high protein intake groups[112]. Since these findings are limited to healthy younger individuals, further studies are required in healthy older adults. The interaction between energy balance and protein intake in amino acid oxidation, urea excretion and whole-body net protein and nitrogen balance at steady state is illustrated in Fig. 2.

In summary, energy balance in addition to protein intake is a key determinant of protein turnover rates and net protein balance at the whole-body level. However, energy surplus itself does not seem to increase muscle mass, although it reduces amino acid oxidation. Thus, as repeatedly stated in the international reports from WHO/FAO/UNU, energy balance needs to be considered when determining protein requirements. In addition, it is important to highlight that evidence of the interaction between energy balance and protein intake is currently limited to younger individuals, and further evidence is required in healthy older individuals.

Metabolic adaptation to dietary protein intake

The consideration of metabolic adaptation to any given amount of protein intake is required for a valid measure of protein requirement. Protein turnover in the splanchnic area as well as in the periphery is adaptable to a given amount of protein intake[25–27]. Metabolic adaptation covers processes affecting the utilisation and fate of amino acids, primarily in the splanchnic area. A prolonged exposure to a given amount of protein intake forces enzyme and transporter levels to change accordingly to handle the amino acid availabilities. Hence, metabolic adaptation to a given protein intake level is a fundamental prerequisite when estimating protein requirements. In physiology, *adaptation* covers conditions where achievement of a steady state can be obtained after adjustments of metabolism and physiological function[113,114]. In contrast, when conditions are too extreme for metabolic pathways to adjust sufficiently, but rather continuously lag behind and the changes are beyond the range of adaptation, the condition is defined as *accommodation*[115].

Recent emerging evidence highlights the mechanisms of *adaptation* to a protein intake higher than the current safe level of dietary protein intake. For example, Gorissen *et al.*[116] measured the availability of dietary protein using intrinsically labelled whey protein. In this study, older individuals (62 ± 1 years) were habituated to a protein intake of either 0·7 g/kg/d (LOW) or 1·5 g/kg/d (HIGH) for 2 weeks from a habitual intake of protein at 1·0 g/kg/d. Interestingly, no group differences were noted in either post-absorptive or post-prandial MPS, and more intrinsically labelled whey protein was available in the circulation in LOW (61 %) in comparison with HIGH (56 %). This was in agreement with our recent study where responses in amino acid and protein metabolism were investigated after habituation to a protein intake of >2·1 g/kg lean body mass (LBM)/d (0·82 g/kg/d) and a protein intake of 1·1 g/kg LBM/d (1·76 g/kg/d) for 20 d in older men (65–70 years)[52]. These findings suggest that exogenous amino acids are directed less effectively into the circulation when habituated to a high protein intake. Habituation to a high protein intake also resulted in a diminished post-prandial synthesis rate of plasma proteins and a more negative overnight fasted whole-body net protein

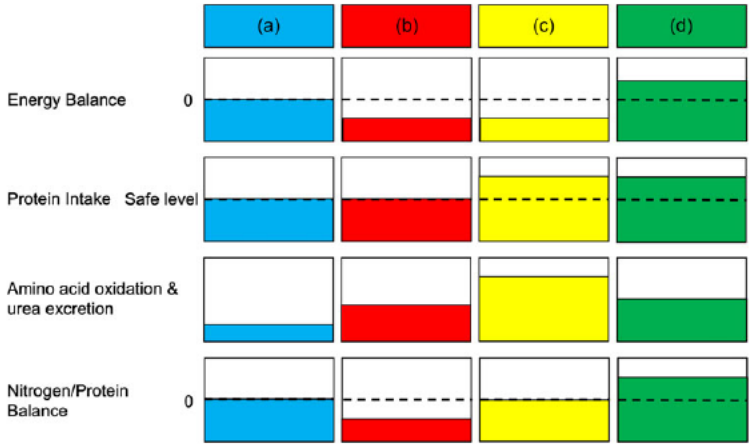

Fig. 2. Interaction between energy and protein intake on amino acid oxidation, urea excretion, and whole-body net nitrogen and protein balance during the condition of adaptation. In each column, relative energy balance, protein intake, amino acid oxidation and urea excretion, and nitrogen/protein balance are expressed. For energy balance and nitrogen/protein balance, 0 (dashed line) indicates that a balance is maintained. Safe intake (dashed line) in protein intake shows the protein intake recommended by WHO/FAO/UNU (0·83 g/kg/d). (a) The column indicates zero whole-body net nitrogen and protein balance at the safe level of intake for protein recommended by WHO/FAO/UNU under energy balance condition[6]. (b) and (c) indicate a negative energy balance condition. (b) The column shows the protein intake at the safe level of intake, but amino acid oxidation and urea excretion are increased under a negative energy balance condition, leading to negative whole-body net protein and nitrogen balance during negative energy balance[105,110]. (c) The column demonstrates that an increased protein intake (>0·83 g/kg/d) preserves whole-body net protein and nitrogen balance whilst increasing amino acid oxidation and urea excretion under a negative energy balance condition[104,107,108]. (d) The column denotes that amino acid oxidation and urea excretion are reduced under a positive energy balance condition with an increased protein intake (>0·83 g/kg/d), resulting in positive whole-body net protein and nitrogen balance[112].

balance, suggesting a less effective utilisation of exogenous amino acids for protein synthesis. In accordance, Walrand *et al.*[117] demonstrated that higher protein intake showed a post-absorptive catabolic state, as demonstrated by higher amino acid oxidation and whole-body protein turnover, without changing MPS in both younger (24 ± 1 years) and older individuals (70 ± 2 years) when higher protein intake (3·0 g/kg fat-free mass) was compared with 'usual' protein intake (1·5 g/kg fat-free mass) over 10 d. However, nitrogen balance was improved in the higher protein intake group, which may suggest insufficient time to adapt to the new protein intake level in this study. Evidence emphasises the necessity of allowing time for metabolism to *adapt* to increased protein intake. Further evidence adopting study designs and methodological approaches that can account for metabolic adaptation is required to obtain a meaningful value for protein requirement, which can then be translated into recommendations.

Metabolic adaptation to changes in protein intake may explain the null findings when an intervention was performed long enough to achieve adaptation[26,52,118,119]. In other words, sustained increases in muscle mass would not be achieved when individuals chronically consume dietary protein levels higher than habitual protein intake level. Baseline protein intake level might be a key determinant of the beneficial effect of dietary protein intervention on muscle mass gain in healthy older adults in a randomised controlled intervention study. This notion was highlighted previously[120] and is supported by a meta-analysis conducted by Ten Haaf *et al.*[121], where they assessed the impact of protein supplementation on lean body mass, muscle strength and physical performance in community-dwelling older individuals. Protein supplementation is not beneficial for those outcome measures when sufficient habitual protein intakes are already consumed in non-frail older individuals.

A recent meta-analysis including a total of 8107 community-dwelling older individuals from cohorts in the Netherlands, the UK, Canada and the United States showed that the prevalence of protein intake lower than the currently recommended level of 0·8 g/kg adjusted body weight/d is 14–30 %[122]. Thus, this low habitual protein intake group may be a more relevant target group for protein supplementation intervention with a concomitant focus on ensuring energy requirements. However, when the purpose is to determine whether a higher than currently recommended protein intake is favourable for muscle and whole-body protein mass, healthy older individuals habitually consuming protein around the current safe level of intake should be the target group and *not* the low-habitual intake group. Collectively, more randomised controlled intervention studies are required to conclude whether the newly suggested higher protein intakes from several expert groups above the safe level of intake at 0·83 g/kg/d have a favourable impact on muscle mass in healthy older adults consuming protein at the current international recommendation.

Protein quality

Protein quality is an overall measure of the ability of a protein source to meet the metabolic demand and is defined in terms of biological value (i.e. the fraction of amino acids absorbed by the gut from a food that is subsequently retained by the body). Biological value is

expressed in terms of indispensable amino acid patterns relative to the requirement[6].

The concept of protein quality can be learned from the Ideal Protein Concept developed in the late 1950s by Mitchell[123] and Scott[124]. The Ideal Protein Concept is defined as the exact amounts of amino acids needed for optimal growth, meaning that it causes neither amino acid deficiency nor surplus availability. Thus, the Ideal Protein Concept is an effective way to define minimum protein in the diet to meet amino acid requirements for metabolic demands in animals. For example, the Ideal Protein Concept initially attempted to provide diets containing the exact balance of essential amino acids based on the composition of eggs and casein for the maximal growth and production performance of chickens. However, non-essential amino acids were not considered in the concept. Re-evaluations in the area of optimal animal feed for growth have found that the provision of non-essential amino acids is also required for development, growth, survival, reproduction and health[125,126]. Consequently, the provision of non-essential amino acids will lower the required amounts of some essential amino acids as they will not be needed as precursors for *de novo* synthesis of non-essential amino acids. Therefore, protein quality must also be considered when discussing protein requirements and should be reflected in the overall recommendations[127], including the consideration of non-essential amino acids[128].

Digestibility of amino acids is an integrated part of the protein quality measure. FAO developed the Protein Digestibility Corrected Amino Acid Score (PDCAAS) as a measure of overall nitrogen digestibility considering loss in faecal matter. This concept had some inherent limitations[129] that were later attempted to be overcome by replacing it with the new term Digestible Indispensable Amino Acid Score (DIAAS), which includes: (1) the adoption of ileal amino acid digestibility; (2) abrogation of truncation of scores; (3) taking the influence of food processing into account; and (4) the use of individual amino acid digestibility.

The earlier protein requirements and recommendations by the WHO/FAO/UNU specified protein quality, whereas the current recommendations refer to 'high-quality protein' with a biological value of 100, usually an animal source protein (Table 1). Animal-based proteins are generally accepted as high-quality protein owing to better essential amino acid profile and higher digestibility (approximately 100) as compared with plant-based proteins (ranging from 80 to 85)[127,130]. Plant-based proteins are less digestible due to existence of dietary fibre and compounds that inhibit enzymatic protein digestion.

Despite the accumulated evidence that supports muscle protein anabolic response by the ingestion of animal-based proteins, plant-based proteins have attracted more attention due to their environmental sustainability and population health benefits[131]. Recently, Burd *et al.*[132] discussed that consideration of protein quality is critical when protein recommendations are determined in relation to environmental considerations (e.g. managing greenhouse gas emissions, land and water use, and loss of biodiversity). Evidence shows that greenhouse gas emission is lower in plant-based proteins as compared with animal-based proteins[131]. Growing evidence has shown health benefits of vegetarian and vegan diets, including lower incidence and mortality from ischemic heart disease and lower incidence of cancer[133]. However, the effects of vegetarian and vegan diets on overall mortality rates are currently unclear[134].

The impact of vegan and vegetarian diets on muscle mass maintenance in healthy older adults is an important consideration. Cross-sectional studies have shown that total protein and animal protein intakes, but not plant protein intake, are positively associated with muscle mass index in older women[135]. Results from a longitudinal observational study show that higher intakes of total protein and animal-based protein are associated with a reduced loss of lean mass over 3 years of follow-up, whereas plant-based protein intake is not associated with lean mass with the fully adjusted models in older adults[32]. These data suggest that plant-based diet may not be favourable for muscle mass maintenance in older adults. However, we argue that more studies are required to fully elucidate this association.

Several strategies have been proposed by Gorissen and Witard[136] to overcome the perceived inferior anabolic properties of plant-based proteins. Firstly, the doses of plant-based protein intake can be increased; secondly, several plant-based protein sources can be mixed in a meal and in the whole diet to overcome any deficiency of a single essential amino acid; thirdly, co-ingestion of leucine can be added as an anabolic stimulant; and fourthly, muscle anabolic sensitivity can be enhanced by physical activity or by other means (e.g. by providing fish oil-derived *n*-3 polyunsaturated fatty acids in the meal or diet).

Considering potential metabolic roles of individual amino acids is also important. This notion has been exemplified by a study demonstrating that increasing the proportion of leucine to mixed amino acids without altering total amino acids content overcomes anabolic resistance in older individuals[137]. Furthermore, addition of leucine to a sub-optimal amount of protein is hypothesised to optimise anabolic response. For example, Wall *et al.*[138] demonstrated a greater post-prandial MPS following the consumption of 20 g casein protein with 2·5 g of crystalline leucine compared with no leucine in older men (74 ± 1 years), indicating that modifying and/or supplementing specific amino acid content might be an effective and practical strategy to improve anabolic response. This is particularly relevant to older individuals, whose energy requirement and appetite are decreasing with advancing age[139]. Protein requirement that maintains whole-body and muscle protein mass can be achieved with lower total protein intake by optimising protein quality, which is also an important consideration for environmental issues. However, evidence is required on whether the modification of protein quality is a feasible approach to maintain whole-body protein balance in healthy older adults.

Conclusions

Considering the historical development of protein requirements and the use of various research methodologies to obtain scientific evidence for assessing protein requirements to create recommendations, we found that the previously used whole-body nitrogen balance methodology is challenged by methods

evaluating whole-body and/or muscle mass and whole-body and/or muscle protein turnover rate as criteria. The newly suggested protein recommendations (0·94–1·3 g/kg/d) formulated by some authorities and expert groups target muscle mass maintenance instead of whole-body protein mass as their primary outcome criterion.

To close the knowledge gap between protein requirements for maintaining both muscle mass and whole-body mass, we identified that future research should assess the degree of agreement between these different, though related, outcome measures based on distinct methodologies. There is a strong need for prospective longitudinal studies with frequent monitoring of reliable dietary intake and concurrent measurements of whole-body protein mass and muscle mass with multiple methodologies. These would include short-term measurements (e.g. muscle and whole-body protein balance, kinetic rates, and nitrogen fluxes) and longer-term measurements (ensuring metabolic adaptation and energy balance) with consideration of protein quality. Also evaluating clinically relevant outcomes, such as muscle strength and function, physical function, body composition and metabolic health parameters, would be preferable. Such a holistic experimental approach would support establishing an agreement between muscle and whole-body protein mass maintenance, and thereby reveal the 'true' and healthy dietary protein requirements and recommendations for healthy older adults.

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Nutrition Research Reviews, page 1 of 1 doi:10.1017/S0954422421000329

Dietary protein requirements and recommendations for healthy older adults: a critical narrative review of the scientific evidence – CORRIGENDUM

Yusuke Nishimura, Grith Højfeldt, Leigh Breen, Inge Tetens and Lars Holm

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The authors would like to apologise for an error in Table 1. The original version was:

Table 1. Successive protein requirements and recommendations by international groups to ensure nitrogen balance in adults

Report	Age	Methodological approach	Biological value of dietary protein (%)	Average protein requirements (g/kg/day)	Recommendation/ safe level of intake (g/kg/day)
League of Nations 1936[22]	Adults	–	–	–	1.0
FAO 1957[23]	Adults	N-balance	80	0.53	0.66
FAO/ WHO 1965[140]	Adults	Factorial	80	0.71	0.89
FAO/ WHO 1973[94]	Adults (20-39 yrs.)	Factorial	75	0.57	0.75
FAO/ WHO/ UNU 1985[95]	Adults	N-balance	100	0.75	0.75
FAO/ WHO/ UNU 2007[6]	Adults (≥ 18 yrs.)	Meta-analysis (N-balance studies)[7]	100	0.66	0.83

Adopted from NS Scrimshaw[141] and updated.

The corrected version is given below, with the corrected value in bold:

Table 1. Successive protein requirements and recommendations by international groups to ensure nitrogen balance in adults

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FAO/ WHO 1973[94]	Adults (20-39 yrs.)	Factorial	75	0.57	0.75
FAO/ WHO/ UNU 1985[95]	Adults	N-balance	100	**0.6**	0.75
FAO/ WHO/ UNU 2007[6]	Adults (≥ 18 yrs.)	Meta-analysis (N-balance studies)[7]	100	0.66	0.83

Adopted from NS Scrimshaw[141] and updated.

Reference:

Nishimura Y, Højfeldt G, Breen L, Tetens I and Holm L (2021). Dietary protein requirements and recommendations for healthy older adults: a critical narrative review of the scientific evidence. *Nutrition Research Reviews* FirstView, pp. 1–17.

Appendix 2.

Title: Recent advances in measuring and understanding the regulation of exercise-mediated protein degradation in skeletal muscle

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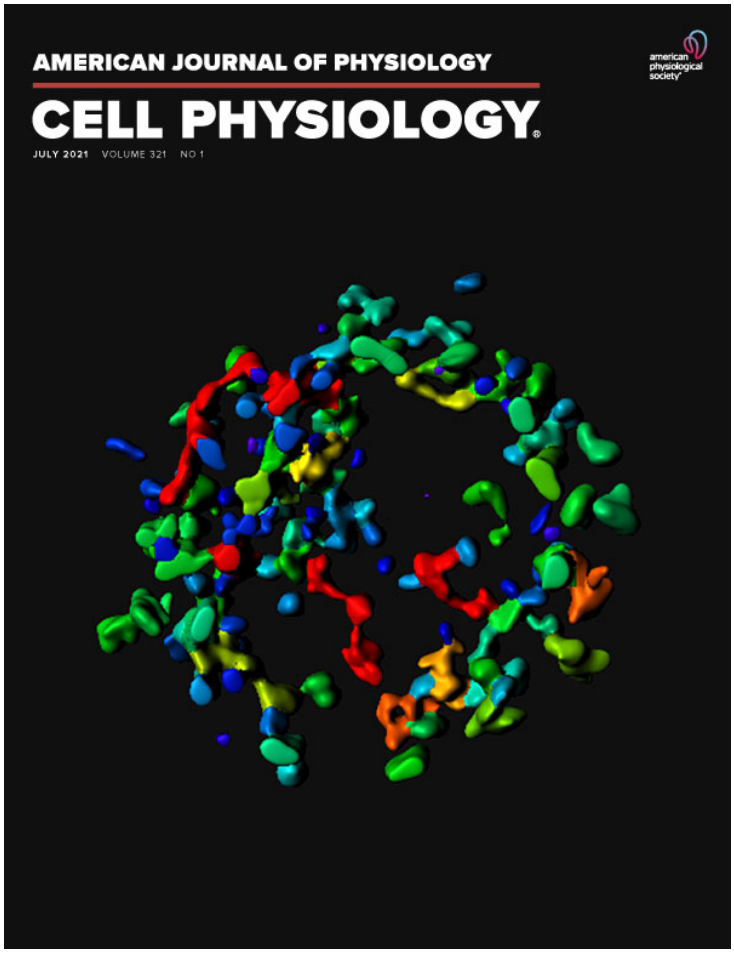

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REVIEW

Muscle Wasting: Cellular and Molecular Mechanisms

Recent advances in measuring and understanding the regulation of exercise-mediated protein degradation in skeletal muscle

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Abstract

Skeletal muscle protein turnover plays a crucial role in controlling muscle mass and protein quality control, including sarcomeric (structural and contractile) proteins. Protein turnover is a dynamic and continual process of protein synthesis and degradation. The ubiquitin proteasome system (UPS) is a key degradative system for protein degradation and protein quality control in skeletal muscle. UPS mediated protein quality control is known to be impaired in aging and diseases. Exercise is a well recognized, nonpharmacological approach to promote muscle protein turnover rates. Over the past decades, we have acquired substantial knowledge of molecular mechanisms of muscle protein synthesis after exercise. However, there have been considerable gaps in the mechanisms of how muscle protein degradation is regulated at the molecular level. The main challenge to understand muscle protein degradation is due in part to the lack of solid stable isotope tracer methodology to measure muscle protein degradation rate. Understanding the mechanisms of UPS with the concomitant measurement of protein degradation rate in skeletal muscle will help identify novel therapeutic strategies to ameliorate impaired protein turnover and protein quality control in aging and diseases. Thus, the goal of this present review was to highlight how recent advances in the field may help improve our understanding of exercise mediated protein degradation. We discuss *1*) the emerging roles of protein phosphorylation and ubiquitylation modifications in regulating proteasome mediated protein degradation after exercise and *2*) methodological advances to measure in vivo myofibrillar protein degradation rate using stable isotope tracer methods.

phosphorylation; protein turnover; stable isotope tracer; the ubiquitin proteasome system; ubiquitylation

INTRODUCTION

Skeletal muscle is a highly plastic and adaptive organ. Skeletal muscle mass and protein quality control can be modulated by various physiological factors, such as hormones, nutrient and energy availability, and contractile activity/physical activity (1). It is well established that exercise triggers the repair and remodeling of skeletal muscle, thereby inducing beneficial adaptations in skeletal muscle metabolism and improving overall health (2–4). Although it is widely acknowledged that exercise is a nonpharmacological therapeutic approach for preventing and treating metabolic diseases, the underlying mechanisms are incompletely understood (5). From a cellular perspective, muscle protein turnover is a key mechanism for modulating muscle mass and protein quality (6–9). There has been an emerging interest in exploring the molecular mechanisms responsible for exercise induced muscle protein turnover, as findings will help identify new therapeutic strategies and targets, and develop potential "exercise mimetics" to ameliorate impaired skeletal muscle protein metabolism in aging and diseases.

Protein turnover is a dynamic and continual process of protein synthesis and degradation. Compared with protein degradation, protein synthesis has been, arguably, easier to study from a technical/methodological point of view. In a pioneering study exploring molecular signaling events in muscle hypertrophy in 1999, Baar and Esser identified a strong correlation between the mammalian target of rapamycin complex 1 (mTORC1) signaling and an increase in muscle mass following a period of resistance training (10). This study inspired a substantial growth in skeletal muscle research, which generated extensive findings on the molecular mechanisms and key signaling pathways for muscle protein synthesis (11, 12). Within this context, protein synthesis was regarded as the major determinant of the net protein balance in skeletal muscle during recovery from exercise (13).

It is important to note that both protein synthesis and degradation are an integral part of the dynamic process of protein turnover. We will not understand protein turnover properly if we only measure protein synthesis while neglecting protein degradation. Unfortunately, in stark contrast to

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the wealth of findings from studies on protein synthesis, there has been a meager understanding of or substantially less research on protein degradation (14).

It is now generally accepted that the ubiquitin proteasome system (UPS), both protein ubiquitylation and proteasome mediated protein degradation, is one of the main degradative system responsible for protein quality control (15 17). Some evidence suggests that UPS mediated protein quality control is impaired in aging and disease. Although muscle atrophy, in general, is often associated with increased proteasome activ ity (18, 19), proteasome activity was not increased during hindlimb unloading or in aged skeletal muscle of rats (20). Furthermore, a decline, instead of an increase, in proteasome functioning was previously found in aged skeletal muscle (21). Thus, the current hypothesis is that, in aging and diseases, decreased proteasome activity negatively affects protein qual ity control, causing the accumulation of damaged and mis folded proteins (15), which ultimately causes malfunction of organelles (22 24). In support of this, overall impairment of growth, protein aggregation, and muscle atrophy was observed in a muscle specific proteasome dysfunctional mouse model (25, 26). Therefore, UPS dependent protein deg radation is crucial for regulating protein turnover and protein quality control in skeletal muscle.

The lack of studies on protein degradation from a molecu lar perspective is partially due to methodological limitations. To study muscle protein turnover kinetics, researchers make frequent use of stable isotopically labeled amino acid tracers in in vivo studies, aided by technical advances in mass spec trometry (27 29). However, stable isotopically labeled amino acids released from muscle protein degradation are recycled for protein synthesis, thus challenging a valid measure of protein degradation (30). To advance our understanding of protein turnover, it is crucial to improve the accuracy and reliability of methodology for studying the molecular mech anisms in protein degradation.

Recent studies began to unravel such a complex regulat ing mechanism by using exercise as a model to investigate the molecular signaling (31, 32), protein quality control (33), and protein degradation kinetic (9, 34). Although we still do not fully understand the mechanisms, previous studies indi cated that protein turnover rate decreased under aging and/ or physical inactivity conditions (34 36), resulting in accu mulated misfolded/damaged proteins and impaired protein quality control (37, 38). Exercise promotes protein turnover (6, 8, 9, 34), which facilitates the removal of misfolded/dam aged proteins, thereby improving protein quality control in skeletal muscle (33) (see Fig. 1). Because UPS is known as a key mechanism controlling proteasome mediated protein degradation, we need to understand exercise mediated ubiq uitin signaling in skeletal muscle. Knowledge of this can then be used to interpret protein degradation outcomes, thereby obtaining a complete overview of protein turnover and protein quality control when combining our current understanding of protein synthesis mechanisms.

In this review, we will first briefly introduce the role and the regulation of UPS in skeletal muscle. Second, we will dis cuss recent findings on how exercise modifies ubiquitin sig naling and phosphorylation of proteasome subunit, which contribute to proteasome activation. We will then discuss recent methodological advances in measuring myofibrillar protein degradation rates using deuterium oxide (D_2O). To accelerate our understanding of the above, we will propose potential directions and methodological improvements that are important and needed for future studies.

THE ROLE OF UBIQUITIN PROTEASOME SYSTEM AND ITS REGULATION IN SKELETAL MUSCLE

Muscle protein degradation is mediated by multiple path ways, including UPS (39), autophagy lysosomal (40), calpain (41), and caspase (42) pathways. UPS appears to be the main system responsible for degrading damaged and misfolded proteins (15 17). By using bortezomib (proteasome inhibitor)

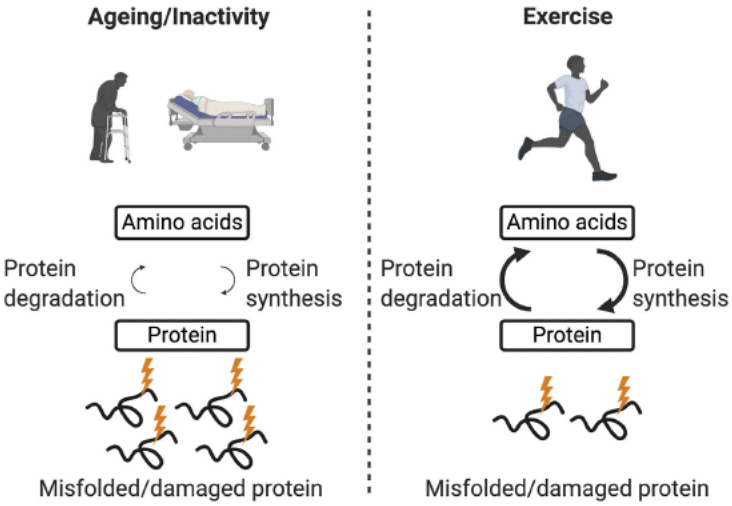

Figure 1. Proposed concept of the maintenance of protein turnover and protein quality control by exercise. Aging and inactivity result in an inevitable decline in protein turnover rate. This leads to the accumulation of damaged and misfolded proteins due to the inability to remove damaged and misfolded proteins through protein degradation. In contrast, exercise is known to facilitate protein turnover through the increased rates of both protein synthesis and degradation, thus better clearance of damaged and misfolded protein. Understanding how exercise regulates rates of protein degradation via ubiquitin signaling and proteasome activity will advance our knowledge on how exercise improves health.

and concanamycin A (inhibitor of lysosomal acidification) to study proteasomal and lysosomal protein degradation, respectively, Zhao et al. (43) showed that proteasome-mediated protein degradation was responsible for at least two-thirds of the total protein degradation in both C2C12 myoblasts and myotubes (43). Earlier studies showed that an acute bout of exercise increased ubiquitin conjugation to proteins and increased the expression of components associated with the ubiquitylation processes (e.g., expressions of ubiquitin, ubiquitin conjugates, components of proteasome, E2) in skeletal muscle (44), indicating that protein ubiquitylation event is activated by exercise. Consistently, recent studies showed that both acute exercise (31, 45) and the functional overload (46) increase proteasome activity in both human and rodent skeletal muscle. These studies highlight an essential role of UPS in protein quality control, muscle remodeling, and muscle adaptation to exercise.

The 26S Proteasome

The eukaryotic 26S proteasome plays a major role in ubiquitin-mediated protein degradation. The 26S proteasome consists of two or three particles (one or two terminal 19S regulatory particles, plus one barrel-shaped 20S catalytic core particle) (16, 39, 47). The 19S regulatory particle serves as a gatekeeper that recognizes substrate for degradation. The 19S regulatory particle is formed by two subcomplexes: a base neighboring the 20S and a lid sitting on the base. The base contains AAA ATPase unfoldases (Rpt1-6) and three non-ATPase subunits (Rpn1-2 and 13) (16, 47), whereas the lid has eight subunits (Rpn3, Rpn5-9 and Rpn12, and the DUB Rpn11) (16, 47). Rpn10 ties the base and lid subcomplexes. Importantly, Rpn1, Rpn10, and Rpn13 are responsible for the recognition of ubiquitylated protein. Once ubiquitylated proteins are recognized, the ubiquitin chains are removed by DUBs (Usp6/Usp14, Uch37, and Rpn11). Such a reaction is known as deubiquitylation (39, 47, 48). Substrates are then unfolded within the 19S regulatory particle before translocating to the 20S core particle for peptide hydrolysis, which is driven by Rpt1-6 in an ATP-dependent process (16, 39, 47). The 20S core particle is responsible for peptide hydrolysis because it contains β1, β2, and β5 subunits, which possess caspase-, trypsin-, and chymotrypsin-like peptidase activity, respectively (39). The structure of the 26S proteasome and the functions of each proteasome subunit have been discussed extensively elsewhere (47, 49, 50).

Protein Ubiquitylation and Ubiquitin Codes

Since the discovery of the critical role of ubiquitin in proteasome-mediated protein degradation, protein ubiquitylation has been widely regarded as a key signal for proteasome-mediated protein degradation. However, the progress of recent research revealed that protein ubiquitylation can regulate all aspects of biological functions (51, 52) and that these multifunctional roles of ubiquitylation have not widely been recognized in the skeletal muscle research field.

Protein ubiquitylation consists of a coordinated process that involves ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes (53) (see Fig. 2). In addition to labeling substrate proteins, ubiquitin can be ubiquitylated on any of its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the first methionine residue (M1) to form eight different homotypic ubiquitin chain types. Protein ubiquitylation can appear as mono- or poly-ubiquitin chains on a substrate protein. Due to structural and topological differences, different chain types have been referred to as "ubiquitin code" to elicit distinctive biological functions (51). In addition to homotypic poly-ubiquitin chain types, the emerging roles of mixed or branched heterotypic poly-ubiquitin chain types add the complexity to the ubiquitin code (52, 54, 55). Among the eight different homotypic poly-ubiquitin chain types, the K11 and K48 poly-ubiquitin chains are known as a signal for proteasome-mediated degradation (56), whereas other chain types (K6, K27, K29, K33, K63, and M1) may have non-degradative roles (51, 52). The emerging mixed or branched heterotypic poly-ubiquitin chain types, such as K29/K48 and K63/K48, are shown to direct protein substrates to proteasome-mediated degradation (57, 58), and K11/K48-branched ubiquitin chains increase proteasome-mediated degradation compared with homotypic K11 poly-ubiquitin chains (59). In contrast, poly-ubiquitin chains can be cleaved by deubiquitylating enzymes (deubiquitylases, DUBs) (48).

The most recent evidence suggests that protein ubiquitylation not only has a role in proteasome-mediated protein degradation but also plays a key role in regulating autophagy lysosome-mediated protein degradation (17). This was evidenced by the findings that K63 poly-ubiquitin chain is involved in autophagy lysosomal protein degradation (17, 60). This was also evidenced by the fact that most of autophagy receptors (e.g., p62/SQSTM1, OPTN, TAX1BP1, NBR1, and NDP52) have ubiquitin-binding domain, which recognizes ubiquitylated proteins and links them to the autophagosomal membrane (61). In support of the above, Zhao et al. (43) also showed that lysosomal-mediated protein degradation accounted for 20% 30% of total protein degradation in C2C12 skeletal muscle myoblasts and myotubes. Thus, protein ubiquitylation is considered to regulate both proteasome-mediated and autophagy lysosome-mediated protein degradation.

E3 Ligases in Skeletal Muscle

Skeletal muscle has drawn much attention in the ubiquitin field because corresponding ubiquitin signaling was reported to be abnormal in muscle atrophy. This is highlighted by the identification of muscle specific E3 ligases, MuRF1 (*TRIM63*) and MAFbx (*FBXO32*), whose mRNA expressions are increased in various atrophic rodent models, including immobilization, denervation, and hind limb suspension (53, 62, 63). Furthermore, knockout of either MuRF1 or MAFbx attenuated denervation-induced muscle loss (62). These seminal works clearly indicated that the increased expression of MuRF1 and MAFbx is a valid biomarker of skeletal muscle atrophy (18).

It is common to extrapolate findings based on inaccurate assumptions through indirect evidence. Because E3 ligase is the key determinant of substrate identification and the majority of ubiquitylated proteins undergo proteasome-mediated degradation, the finding of increased MuRF1 and/or MAFbx expressions in atrophy muscle has widely been regarded as a direct indicator of muscle protein degradation.

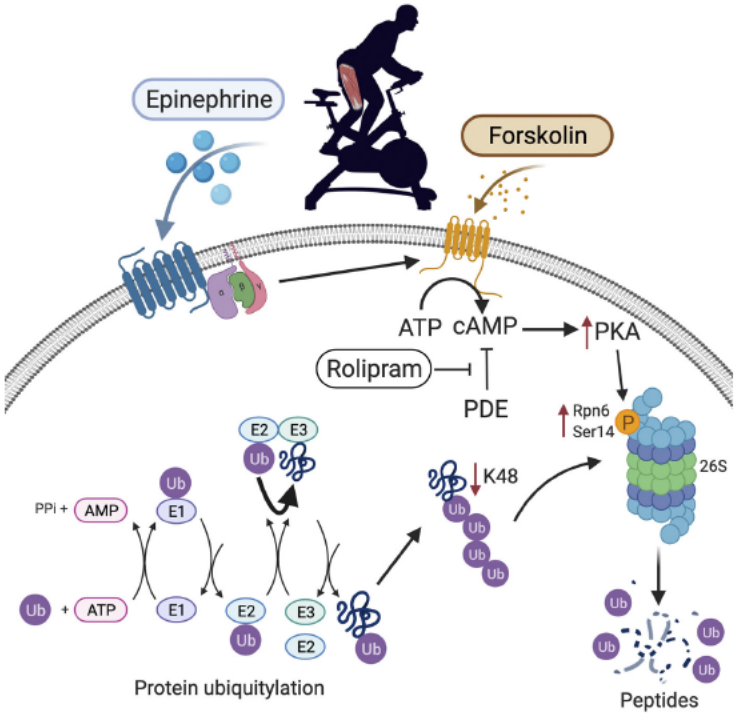

Figure 2. Schematic diagram of the crosstalk between phosphorylation and ubiquitylation in the 26S proteasome-mediated protein degradation in exercising skeletal muscle. Phosphorylation is involved in enhancing the 26S proteasome activity after exercise. Exercise increases epinephrine in circulation, which then binds to a G protein-coupled receptor and activates adenylyl cyclase. The activation of adenylyl cyclase increases cyclic adenosine monophosphate (cAMP) production that activates protein kinase A (PKA). This cAMP-PKA activation in turn phosphorylates Rpn6 at Ser 14 of the 19S regulatory particle. The phosphorylation of Rpn6 at Ser 14 has been shown to be a critical signal that enhances the 26S proteasome activity in exercised human skeletal muscle. On the other hand, protein ubiquitylation aided by sequential reactions by ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes also plays a key role in determining the fate of ubiquitylated protein for the 26S proteasome-mediated degradation. E3 ligase determines a substrate and attaches ubiquitin in conjunction with E2. Exercise decreases the amount of K48-linked polyubiquitin chain due to increased 26S proteasome-mediated degradation because K48-linked polyubiquitin chain is known as a signal for proteasome degradation. Thus, both protein ubiquitylation and phosphorylation modifications are involved in the 26S proteasome-mediated protein degradation in skeletal muscle. Red arrows indicate changes of abundance or activity. AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; PPi, inorganic pyrophosphate; Ub, ubiquitin.

This assumption is based on the prediction that all ubiquitylated proteins will undergo protein degradation. As more evidence becomes available in the literature, it is now clear that protein ubiquitylation also has nondegradative roles (52). Furthermore, researchers often rely on changes of mRNA abundance without measuring protein level due largely to the poor quality of commercially available antibodies. However, Sandri (64) pointed out that atrophy-induced increase in mRNA abundance of E3 ligase does not always align with protein level. In catabolic conditions, it is hypothesized that an increased ligase activity of skeletal muscle-specific ubiquitin E3 ligase would inevitably increase autoubiquitylation (62), which is then degraded by proteasome- or lysosome-mediated degradation (64). Hence, the upregulation of gene transcription is important to counteract the loss of E3 ligase protein due to the increased autoubiquitylation (64). Therefore, it is not appropriate to extrapolate the results of MuRF1 or MAFbx protein/gene expression directly to muscle protein degradation before their regulatory roles in muscle protein degradation are properly understood. Furthermore, evidence suggests that MuRF1 gene expression is independent of proteasome-mediated protein degradation in skeletal muscle. For example, proteasome activity was reported to be increased after 14 days of functional overload in MuRF1-deficient mice (65). Even though MuRF1 expression is a useful marker for muscle atrophy, it should not be used as a marker of muscle protein degradation.

More recently, Baehr et al. (66) demonstrated that overexpression of MuRF1, but not MAFbx, is sufficient to induce muscle atrophy in mice. In an attempt to understand how MuRF1 regulates myofibrillar protein degradation, Baehr et al. (66) identified MuRF1 overexpression-dependent ubiquitylation sites on 56 proteins. Surprisingly, their validation showed that the majority of these MuRF1 substrates do not undergo degradation, which is in contrast to Clarke et al.'s findings (67) that MuRF1 physically associates with and degrades slow and fast myosin heavy chains under

dexamethasone treatment in C2C12 myotubes. One possible reason that MuRF1 substrates did not undergo degradation despite an increased ubiquitylation might be that MuRF1 recruits other E3 ligases (e.g., MuRF2, MuRF3, and TRIM25), which regulates the fate of ubiquitylated protein substrates for nonproteasomal degradation. This notion was also supported by a recent study led by Goodman et al. (68) that overexpression of ASB2β not only induced atrophy but also increased expression of ubiquitin E1 and E2 enzymes and other E3 ligases (e.g., MUSA1, the muscle-specific Fbxo40, and MuRF2). Interestingly, Baehr et al. (66) also showed that muscle atrophy was prevented when the RING domain of MuRF1 is mutated at C44S/C47S. The RING domain is required for binding with E2 conjugating enzymes and catalyzing the transfer of ubiquitin from E2 to substrates (69). Their finding indicates that MuRF1 ligase activity is important to cause muscle atrophy. However, there are currently no studies measuring protein degradation rates when E3 ligases are overexpressed, and hence, it is unclear if overexpression of any particular E3 ligases is sufficient to increase protein degradation rates. Thus, future studies should focus more on the measurement of protein degradation when studying potential E3 ligases relevant to muscle atrophy to improve our understanding on the mechanisms.

There are more than 600 E3 ligases (70) and around 100 DUBs (71) encoded in the human genome. It is not surprising to see that these key ubiquitin modifiers are increasingly recognized as a key regulator of muscle mass and functions. As such, more E3 ligases [e.g., MUSA1 (72), Cbl-b (73), TRIM28 (74), TRIM32 (75), TRIM72 (76), UBR4 (77, 78), UBR5 (79, 80), and ASB2β (68)] are emerging as important regulators of skeletal muscle mass and metabolism. Similar to our understanding of MuRF1 and MAFbx, we still know very little about how other E3 ligases' activities are regulated, and what their downstream events and consequences are. For example, future studies should aim to identify the complete list of substrates, clarify what ubiquitin chain types can be made by E3 ligases, and how this modification affects the fate of protein substrates.

UBIQUITIN SIGNALING IN HUMAN SKELETAL MUSCLE IS DYNAMICALLY MODIFIED BY EXERCISE

Although protein ubiquitylation is known as one of the key determinants of controlling protein degradation (39, 43), our current challenge to study the event of protein ubiquitylation is the lack of valid and commonly applicable tools to measure substrate ubiquitylation (81). In recent years, a high-throughput proteomic approach emerged as a useful tool for identifying ubiquitylation at the whole proteome level (82). Parker et al. (32) recently applied this approach and made the first publication to document exercise-mediated ubiquitylome in human skeletal muscle. They found that an acute bout of high intensity exercise altered the landscape of protein ubiquitylation in skeletal muscle proteome. Although the abundance of many proteins decreased immediately after exercise, the total amount of K11, K48, and K63 ubiquitin chains decreased in the same fashion. The latter observation, particularly the decreased K11, K48, and K63 ubiquitin chains, suggests that both proteasome- and lysosome-mediated protein degradation increased during or immediately after exercise (17, 60). Interestingly, the authors also reported that the protein abundance and the reduced ubiquitin chains (K11, K48, and K63) were returned to pre-exercise levels after only 2 h of recovery from exercise, indicating that the effect of exercise on ubiquitin signaling is transient. Such evidence of the rapid alternations in ubiquitin signaling supports the idea that exercise facilitates the removal of misfolded/damaged proteins, thereby improving the protein quality control in skeletal muscle (33). Although these data clearly indicate that exercise dramatically affects protein ubiquitylation status, it remains unclear what biological functions are governed by these rapid ubiquitylation alterations. It will be particularly interesting to know whether any of these alterations account for the beneficial effects of exercise. Although methods of proteomic approach for detecting protein ubiquitylation are improving (83), more studies are required to identify and confirm new exercise-mediated ubiquitin signaling in skeletal muscle.

Parker et al. (32) also showed that the MuRF1 ubiquitylation status (at site of MuRF1 K152, K123, and K116) and protein abundance are transiently reduced immediately after exercise but restored rapidly during the recovery from exercise. This suggests that MuRF1 may be autoubiquitylated and its activity is possibly regulated by exercise. Moreover, Parker et al. (32) reported changes in the overall ubiquitylation status in some of the myofibrillar proteins after exercise. Even though previous studies have reported, myofibrillar proteins, such as myosin heavy chain (67), myosin light chain (84), and actin (85), are ubiquitylated by MuRF1. In the study led by Parker et al. (32), however, it is unclear if the changes of ubiquitylation in myofibrillar proteins are mediated by MuRF1 or other E3 ligases, and whether these ubiquitylated proteins undergo degradative or nondegradative pathways are also not clear. Although their intention was to understand the role of ubiquitylation in exercise-mediated muscle protein degradation, protein degradation rate was not measured in the study. We therefore cannot extrapolate the results of ubiquitin signaling into either protein degradation or other physiological function during and after exercise. Despite all these, Parker et al. (32) have provided an important first-step toward establishing exercise-mediated ubiquitin signaling in skeletal muscle.

THE 26S PROTEASOME ACTIVITY IS ACTIVATED BY EXERCISE-MODULATED PHOSPHORYLATION OF THE 19S PROTEASOME SUBUNIT

The rate of protein ubiquitylation has long been regarded as the sole determinant of UPS-mediated protein degradation. However, recent studies indicate that phosphorylation is also required for UPS-mediated protein degradation (31, 86). Particularly, protein kinase A (PKA)-mediated phosphorylation of 19S proteasome subunit Rpn6 was reported to be one of the mediating mechanisms for exercise-induced proteasome activation. This finding was elicited from the finding by Lokireddy et al. (86), showing that elevation of cyclic adenosine monophosphate (cAMP) and PKA signaling can

lead to an increase in proteasome activity in C2C12 myotubes via the phosphorylation of 19S proteasome subunit Rpn6 at Ser 14. Although the study by Lokireddy et al. (86) did not use an exercise model, cAMP-PKA signaling is well known to be activated during exercise (87). The identification of Rpn6 phosphorylation is particularly important because this phosphorylation also facilitates the ATP-dependent processes of substrate unfolding, deubiquitylation, and the translocation of the substrates into the 20S proteasome subunit where peptide hydrolysis occurs (86). The same study also used a pulse-chase technique (radioactive tracer, ^{3}H-phenylalanine) to show that an increase of cAMP induced by rolipram promoted protein degradation in C2C12 myotubes (86). Their study also indicated that cAMP-mediated protein degradation mainly degrades misfolded and fast-turnover protein degradation, but not structural proteins and myofibrillar proteins (31, 86), suggesting cAMP-mediated protein degradation contributes to protein quality control. Following the findings discussed above, Goldberg's group further demonstrated that an acute bout of high intensity exercise also increased PKA-dependent phosphorylation of Rpn6 at Ser 14 and proteasome activity in human skeletal muscle (31). However, protein degradation rate was not measured in this human study (31), and the assumption of increased protein degradation was referred to the results obtained from cell culture experiments (86).

Altogether, these in vitro (86) and in vivo (31) studies show that an acute bout of high-intensity exercise activates PKA signaling through the elevation of circulating epinephrine (Fig. 2). The activation of cAMP-PKA signaling induces the phosphorylation of the 19S proteasome subunit Rpn6 at Ser14 to stimulate the processes of protein degradation in the proteasome. Meanwhile, exercise also decreases the amount of K48-linked polyubiquitin chain (32), which plays a key signal for recognizing substrate degradation in the proteasome (56). These findings highlight that both protein phosphorylation and ubiquitylation have to work in concert to regulate exercise-mediated proteasome activation in skeletal muscle. Furthermore, as reported by Parker et al. (32), MuRF1 abundance and its ubiquitylation status are also altered following a bout of high-intensity exercise. However, it is unclear what makes the changes of MuRF1 abundance, and how the ubiquitylation of MuRF1 affects the biological functions. These new and important findings from aforementioned studies show that the protein ubiquitylation status is altered rapidly by exercise, which raises an important question of identifying what E3 ligases and/or DUBs contribute to the changes of protein ubiquitylation in exercising skeletal muscle.

METHODOLOGICAL ADVANCES IN MEASURING MYOFIBRILLAR PROTEIN DEGRADATION RATE

Developing an accurate and reliable methodology to measure myofibrillar protein degradation is critical for understanding and explaining relevant molecular mechanisms. Stable isotopically labeled amino acid tracers in combination with mass spectrometry analyses (i.e., determination of relative tracer abundance) have been used for studying muscle protein turnover (27 29). Although the tracer-based measurement of protein synthesis via the direct incorporation model is considered the gold standard for myofibrillar protein synthetic rate (also known as fractional synthetic rate, FSR) (88), the measurement of protein degradation with tracer methodologies appears much more complicated (14). The tracer-dilution principle is the most frequently used approach (6, 8, 14, 88 90).

In 1987, Gelfand and Barrett introduced the two-pool arterio-venous model of the *tracer-dilution* principle, where the measurement of tracer enrichment in both artery and vein across a limb (or an organ) is performed while infusing stable isotopically labeled amino acid tracer(s) (89). Tracer enrichment is the abundance of tracer (administered labeled amino acid) relative to tracee (unlabeled amino acid), which is determined based on mass spectrometry analysis (28, 88). In principle, protein degradation rate is calculated as the dilution of relative abundance of a stable isotopically labeled amino acid at the venous site compared with the arterial site, which is anticipated to be a consequence of the release of tracee from the intracellular pool into the venous site due to intracellular protein degradation (88 90). However, this 2-pool arterio-venous model of the tracer-dilution principle is very simplistic. To gain accuracy, the model was subsequently extended by including the intracellular pool of the targeted tissue (e.g., skeletal muscle) (91) and/or by sampling the interstitial fluid compartment (92). Despite these improvements, none of these tracer-dilution approaches contain information about where the traced amino acids are originated from (e.g., myofibrillar protein). Therefore, protein-specific approaches to measure protein degradation are warranted.

A methodology was developed that directly measures protein degradation in a comparable manner as the myofibrillar protein-specific direct-incorporation model for FSR.

The approach measures a fractional breakdown rate (FBR), and it is based on the principle of determining the rate at which protein-bound amino acid tracers are disappearing from the protein pool. We originally used deuterium oxide (D_2O) for labeling proteins, and hence, we here abbreviate this methodology as FBR_{D2O} (30, 93). This approach is practically rather demanding (30). Briefly, D_2O first needs to be provided (orally or injection) to allow prelabeling of (myofibrillar) proteins through de novo synthesized D-labeled amino acids. Alanine is a commonly used amino acid due to two reasons: *1*) It exchanges hydrogen/deuterium through transamination (TA) and through metabolic precursors for alanine in tricarboxylic acid cycle (TCA cycle) reactions at four possible exchange sites (C-H bonds), which improves analytical sensitivity with mass spectrometry, and *2*) the metabolic exchange of hydrogen/deuterium occurs quickly, and hence, equilibration with body water enrichment appears very quickly (94, 95). After prelabeling, at least two muscle samples are collected to measure the enrichment of deuterium (D)-labeled amino acids in the myofibrillar protein pool and calculate the rate of loss, which can be expressed as myofibrillar protein degradation rate (30, 93). However, when measuring the disappearance of D-labeled amino acids from the myofibrillar protein, the availability of D_2O in the body pool has to be zero. This ensures that deuterium is not transferred to amino acids in de novo

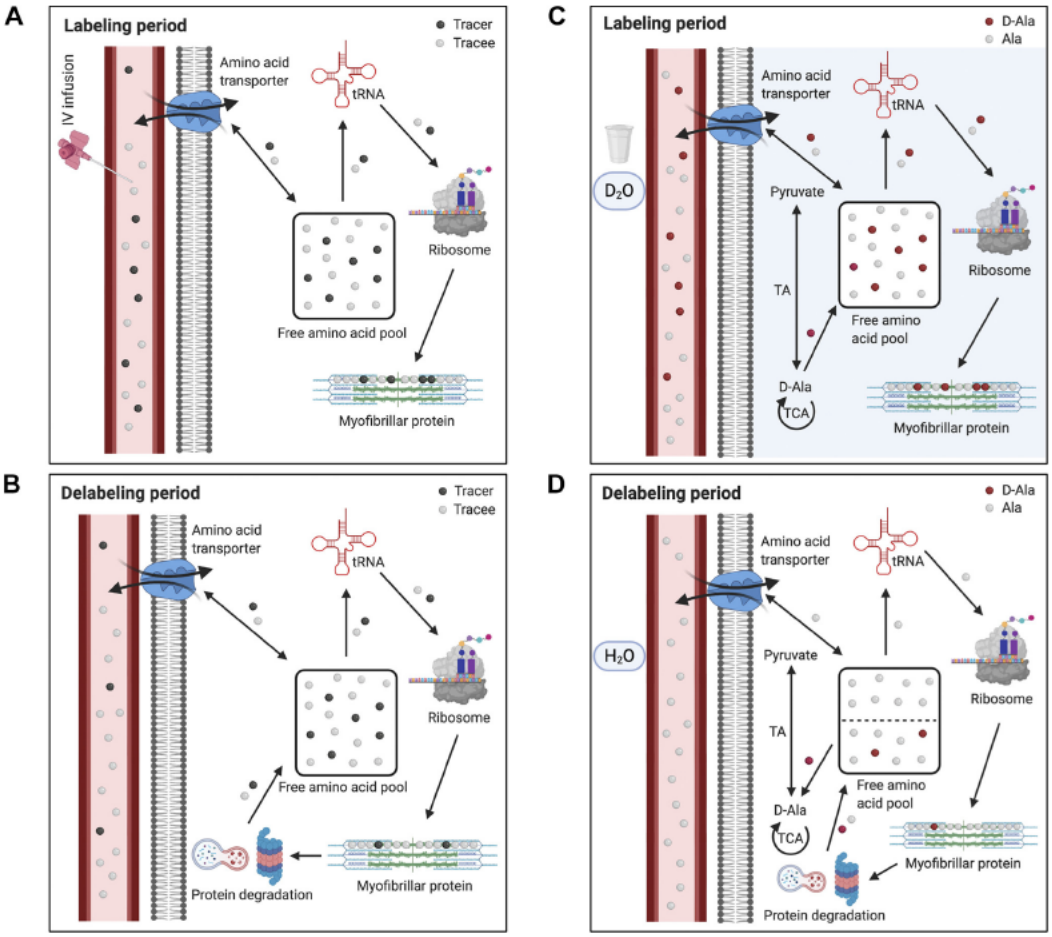

Figure 3. Principle of determining myofibrillar protein degradation using classical stable isotopically labeled amino acid tracers and D_2O. In principle, myofibrillar protein degradation rate can be measured in a two-step manner regardless of the use of classic stable isotopically labeled amino acid tracers or deuterium oxide (D_2O). Firstly, myofibrillar proteins should be labeled through the incorporation of labeled amino acid tracers (labeling period). Then, the disappearance of myofibrillar protein labeled with stable isotopically labeled amino acid tracers can be determined (delabeling period), which can then be calculated as myofibrillar protein degradation rate. *A*: traditionally, stable isotopically labeled amino acids (black circles) are introduced via intravenous (iv) infusion. Extracellular stable isotopically labeled tracers and unlabeled amino acids (tracee, white circles) are transported into cytoplasm and make up a large pool of free amino acids (free amino acid pool). Amino acids (tracer and tracee) charged with tRNA (aminoacyl-tRNA) are delivered to the ribosome for incorporation into the polypeptide and myofibrillar proteins (labeling period). *B*: during the delabeling period, free amino acids (tracer and tracee) derived from myofibrillar protein degradation (e.g., the ubiquitin proteasome system and the autophagy-lysosome pathway) are recycled into free amino acid pool in cytoplasm and a fraction of the stable isotopically labeled amino acid tracers is reutilized for myofibrillar protein synthesis. When stable isotopically labeled amino acid tracers have their stable isotopic label(s) (deuterium, carbon, and/or nitrogen atoms) at positions where they are released only by the irreversible metabolism of the amino acid, these stable isotopically labeled amino acids are likely to be recycled. Recycling of tracer underestimates the measured myofibrillar protein degradation rate calculated with this approach. *C*: D_2O can be orally consumed and deuterium rapidly equilibrates within the body water (light blue background). Water serves as a hydrogen donor and exchanger in in vivo metabolism. Hence, deuterium (D) is incorporated into amino acids through transamination (TA) and tricarboxylic acid cycle (TCA cycle) reactions. D-labeled amino acids can then be incorporated into myofibrillar proteins prior to determining myofibrillar degradation (labeling period). At this stage, D-labeled amino acids stay in proteins until they are released by protein degradation (e.g., the ubiquitin proteasome system and autophagy lysosome system) as a free amino acid. Alanine is a commonly used amino acid as it has four possible exchange sites (C-H bonds) with deuterium through TA and through metabolic precursors for alanine in TCA reactions, improving analytical sensitivity with mass spectrometry. *D:* during the delabeling period, myofibrillar proteins labeled with D-Ala are degraded. Once D-Ala is released by protein degradation, D-Ala will undergo reactions (TA or TCA cycle) and exchange D with hydrogen from the unlabeled body water pool and thereby lose the D-label. Thus, the recycling of D-labeled amino acids via D_2O is very unlikely when body D_2O enrichment is zero, which may provide more accurate measurement of myofibrillar protein degradation rate. Based on this theory, D_2O is a preferential tracer to study myofibrillar protein degradation. D_2O, deuterium oxide; D-Ala, deuterium-labeled alanine; TA, transamination; TCA cycle, tricarboxylic acid cycle; tRNA, transfer ribonucleic acid.

metabolism and hence no further incorporation of D-labeled amino acids into myofibrillar protein (30, 93, 96). Further, using D_2O as the label-donor to amino acids has subsequently been found advantageous in regard to avoiding recycling of D-labeled amino acids into myofibrillar proteins during the period of the actual FBR measurement (30, 93, 96) (see Fig. 3). The reason for this is that D-label is both added and removed from C-H bonds of amino acids (94, 95). Thus, the recycling of D-labeled amino acids back to myofibrillar proteins is very unlikely when body D_2O enrichment is zero as free amino acids carrying the D-label will lose the label once released from protein degradation by reacting with H_2O in cytoplasm (30). In contrast, when classic stable isotopically labeled amino acids have stable isotopic labels (deuterium, carbon, and/or nitrogen atoms) at positions where they are only released by the irreversible metabolism of the amino acid, these stable isotopically labeled amino acids will be recycled for myofibrillar protein synthesis (30, 93). This was further explored experimentally in humans using both D_2O and ^{15}N-phenylalanine stable isotope tracer (96). It was observed that high ^{15}N-phenylalanine tracer enrichment was present in circulation after 10 days (TTR: 4%) and 24 days (TTR: 1%) of the exposure (96). Further, a high variation of tracer abundances in the myofibrillar protein fraction was observed when the same individuals were exposed to four acute ring-$^{13}C_6$-phenylalanine infusion trials and myofibrillar protein still carries the infused phenylalanine stable isotope tracer after a year. Both of these findings emphasize the continuous and prolonged recycling of phenylalanine (amino acids) for muscle protein synthesis.

Figure 3 illustrates the principle of determining myofibrillar protein degradation using classical stable isotopically

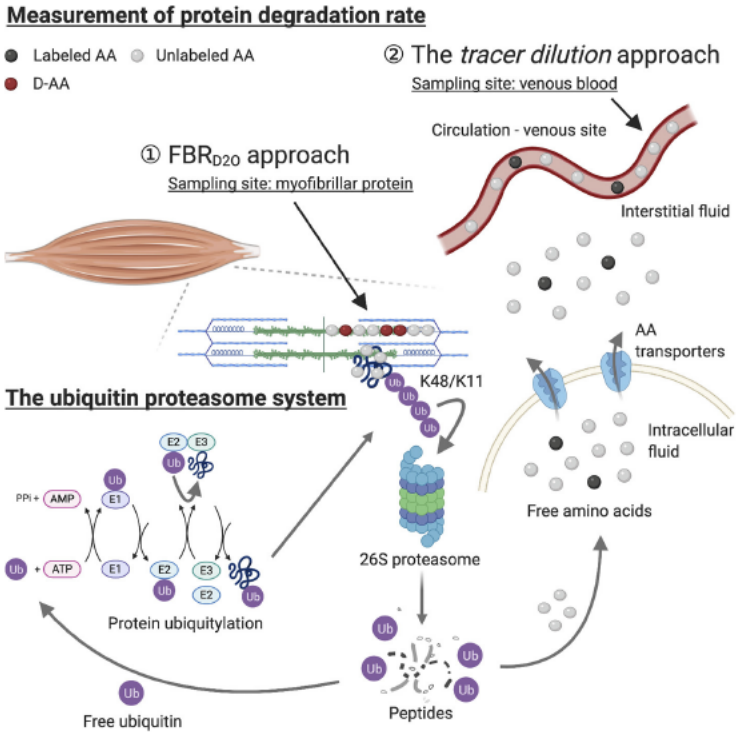

Figure. 4. The tracer-based approaches to measure protein degradation rate and the link to the ubiquitin proteasome system. Different tracer-based approaches inhere a temporal distinction of the measurement of protein degradation. Myofibrillar protein degradation rate can be measured by oral consumption of deuterium oxide (D_2O) to prelabel myofibrillar protein through de novo synthesized deuterium (D)-labeled amino acids (red circles), and the subsequent disappearance of myofibrillar proteins labeled with D-labeled amino acids can then be converted to a myofibrillar protein degradation rate (FBR$_{D2O}$ approach). This FBR$_{D2O}$ approach targets the "early" process of myofibrillar protein degradation, as the measurement of label abundance is at the level of myofibrillar proteins. In contrast, the tracer-dilution approach provides a measure at the "final" stage of protein degradation, as the determination of label abundance is at the level of free amino acids. The time-dependent association of the underlying molecular regulations with myofibrillar protein degradation is suggested to be distinct depending on the use of FBR$_{D2O}$ ("early" stage) or the tracer dilution ("final" stage) approach. In the ubiquitin proteasome system (UPS), myofibrillar proteins are targeted by protein ubiquitylation through sequential reactions, involving ubiquitin E1 (activating), E2 (conjugating), and E3 (ligase) enzymes. E3 ligase determines a substrate and attaches ubiquitin in conjunction with E2. K48- and K11-linked polyubiquitin chains are a known signal for protein degradation at the 26S proteasome. Once ubiquitylated protein is recognized by the 26S proteasome, polyubiquitin chain is removed by deubiquitylating enzymes (DUBs) and free ubiquitin is recycled for subsequent protein ubiquitylation processes. Then, protein is degraded to peptides at the 20S core particle of the 26S proteasome, which are then released as free amino acids into circulation. AA, amino acid; D_2O, deuterium oxide; D-AA, deuterium-labeled amino acid; DUBs, deubiquitylating enzymes; FBR, fractional breakdown rate; Ub, ubiquitin.

labeled amino acid tracers (Fig. 3, *A* and *B*) and D_2O (Fig. 3, *C* and *D*). Figure 3 also describes the fate of D-labeled amino acids (e.g., alanine) and classic stable isotopically labeled amino acid tracers derived from intracellular protein degradation. Recycling at the time of FBR measurement will underestimate the disappearance rate of stable isotopically labeled amino acids present in the myofibrillar protein pool and, thus, the myofibrillar protein degradation rate. Recycling must therefore be avoided for a valid approach. FBR_{D2O} approach is currently the only valid method used to determine myofibrillar protein degradation rate, which is a comparable approach to the direct-incorporation model of myofibrillar protein FSR. A recent study by Dideriksen et al. (34) has taken this advantage to demonstrate that myofibrillar protein degradation rate is higher during a 14-day period of resistance training ($2.12 \pm 0.34\%\cdot day^{-1}$) compared with a 14-day limb immobilization period ($1.61 \pm 0.14\%\cdot day^{-1}$) in older adults.

It is important to highlight that FBR_{D2O} approach is not without the limitations. Firstly, this approach is time demanding as aforementioned. Removal of D_2O from the circulation following the prelabeling would take time due to the slow turnover of body water pool (half-life ~9 11 days), and hence, the practical application of the method is challenging (30). This also suggests that this approach is only applicable to slow turnover protein, such as myofibrillar protein (~1% 2% day^{-1}). Secondly, FBR_{D2O} determines gross average of protein degradation rate (over several days) as the time window between skeletal muscle samplings need to be extensive in order to detect the small difference of tracer enrichment in myofibrillar protein by mass spectrometry (30). This point challenges the usefulness of this approach to study acute responses (hours) of FBR to any interventions. If classic stable isotopically labeled amino acid tracers should be used for accurate measurement of myofibrillar degradation rate, a novel method that allows to account for recycling of amino acids needs to be developed.

Linking the myofibrillar protein degradation rate with the preceding and underlying molecular mechanisms (e.g., UPS) remains to be an experimental challenge (see Fig. 4). Of importance is the temporal distinction between the tracer-dilution approach and the FBR_{D2O} approach in measuring protein degradation rate. The FBR_{D2O} approach detects the "disappearance" of proteins carrying labeled amino acids at the "early" stage of protein degradation, where labeled proteins are removed from the matrix pool. In contrast, the tracer-dilution approach assesses at the "final" stage, where the proteins' constituent amino acids are released and appearing into the free amino acid pool and venous blood. This inherent distinction in the two tracer methodologies should in theory translate into different time-dependent associations with concomitant molecular signaling responses. Thus, future studies should investigate molecular mechanisms concomitant with different tracer approaches to assess the temporal changes in protein degradation rate in various physiological conditions.

CONCLUDING REMARKS

There is an emerging interest in understanding the molecular mechanisms of UPS responsible for exercise-modulated protein degradation and protein quality control in skeletal muscle. As explained above, the current challenge for the field is the lack of easily accessible tools for studying protein ubiquitylation and degradation. Nevertheless, there have been some important methodological advances over the past few years. Although we still have little knowledge of how exercise-mediated ubiquitin signaling modulates specific physiological functions, a recent proteomic study showed that exercise dynamically modifies the landscape of protein ubiquitylation. Recent studies also reported that the phosphorylation of the 19S proteasome subunit Rpn6 activates the 26S proteasome following high-intensity exercise in human skeletal muscle. These studies indicate that protein ubiquitylation and phosphorylation must be regulated coordinatively, and that both signals must be converged at the 19S regulatory particle(s) of the 26S proteasome before executing protein degradation. Unfortunately, the lack of solid stable isotope methods to measure myofibrillar protein degradation makes us unable to interpret these novel findings of signaling mechanisms accurately. In this article, we recommend and highlight the use of D_2O as the most appropriate tracer to measure myofibrillar protein degradation rate. Future studies should aim to integrate the results of both protein degradation and signaling events (e.g., phosphorylation and ubiquitylation) to gain a better understanding of how exercise-mediated protein degradation is regulated at a molecular level. Apparently, this is not an easy task to achieve. Collaborative efforts are encouraged as multidisciplinary techniques and expertise are warranted.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Y.N. prepared figures; Y.N. drafted manuscript; Y.N., I.M., L.H., and Y. C.L. edited and revised manuscript; Y.N., I.M., L.H., and Y. C.L. approved final version of manuscript.

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Appendix 3.

Title: Daily protein and energy intake are not associated with muscle mass and physical function in healthy older individuals – The CALM study

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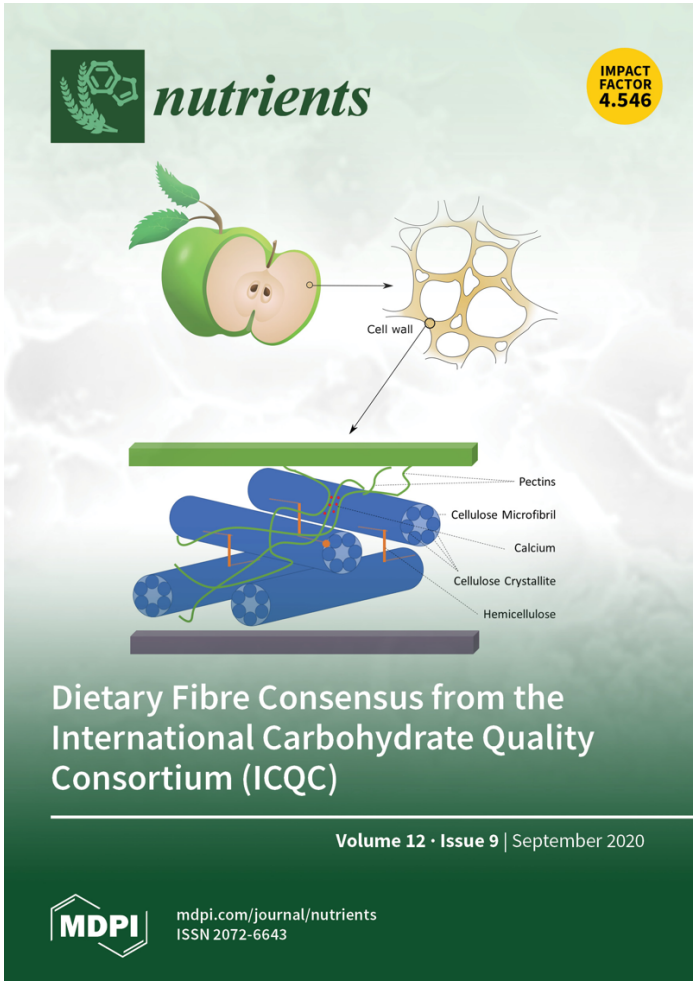

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Article

Daily Protein and Energy Intake Are Not Associated with Muscle Mass and Physical Function in Healthy Older Individuals—A Cross-Sectional Study

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Abstract: Dietary protein has a pivotal role in muscle mass maintenance with advancing age. However, an optimal dose and distribution of protein intake across the day as well as the interaction with energy intake for the maintenance of muscle mass and physical function in healthy older adults remain to be fully elucidated. The purpose of this study was to examine the association between muscle mass, strength, and physical function, and the total amount and distribution of protein and energy intake across the day in healthy older individuals. The research question was addressed in a cross-sectional study including 184 Danish men and woman (age: 70.2 ± 3.9 years, body mass: 74.9 ± 12.1 kg, Body Mass Index (BMI): 25.4 ± 3.7 kg/m^2) where a 3-day dietary registration, muscle mass, strength, and functional measurements were collected. We found that neither daily total protein intake nor distribution throughout the day were associated with muscle mass, strength, or physical function. Consequently, we do not provide an incentive for healthy older Danish individuals who already adhere to the current internationally accepted recommended dietary protein intake (0.83 g/kg/day) to change dietary protein intake or its distribution pattern throughout the day.

Keywords: sarcopenia; ageing; dietary protein; protein distribution; elderly; muscle mass

1. Introduction

Dietary protein continues to receive attention in an effort to combat sarcopenia [1–5]. The most recent recommendation from the Nordic Council of Ministers is 1.1–1.3 g protein/kg Body Weight (BW)/day (15–20 energy %) for older adults above the age of 65 years [6]. Meanwhile, globally, the recommended intake for all adults remains at 0.83 g protein/kg BW/day [7,8]. The global recommendation was developed based on a meta-analysis of nitrogen balance measurements

from various dietary protein intake levels [9]. However, the recommended value was based on nitrogen balance methodology that, with currently available data, neither distinguishes age nor accounts for factors such as sex and meal distribution patterns across the day. While some studies report similar protein requirements in healthy younger and older adults using the nitrogen balance methodology [10,11], a growing number of epidemiological studies assessing the associations between protein intake and muscle mass suggest that muscle mass and/or function in older individuals can be maintained with protein intake levels matching the recommendation made by the Nordic Council of Ministers [12–16].

Protein distribution throughout the day is considered an important factor in muscle mass maintenance [17–20]. This notion has grown from the findings of a marked, transient stimulation of Muscle Protein Synthesis (MPS) in response to hyperaminoacidemia [21,22] by the bolus intake of fast absorptive proteins [23]. The dose–response relationship between protein intake and MPS plateaus at ~30–45 g protein per serving (0.4–0.6 g/kg BW) for a 75 kg older adult (>65 years) [23] with no cumulative effect of protein intake over a period of time and rather oxidized beyond this amount [24]. This has led to the concept of an optimal dose of ~30 g of protein per meal to maximally stimulate MPS [23] which is required at all three main meals and snacks in a day (1.2–1.8 g/kg BW/day) [25,26]. However, some challenges exist when daily protein recommendations are extrapolated from MPS, which have been determined in acute experimental studies in response to various doses of protein ingestion. Among these, the interaction between protein and energy intake [27] as the intake of other macronutrients and/or total energy is known to markedly affect postprandial aminoacidemia [28–30] and whole-body net protein balance [31]. In addition, the type of studies investigating the stimulatory effect of a bolus ingestion of protein rarely account for the habituated level of dietary intake (i.e., the protein intake the participant is accustomed to). We and others have demonstrated that adaptation to higher protein intakes alters amino acid utilization via an increase in amino acid oxidation and urea production [32–35].

A cross-sectional study allows for an investigation of the association between habitual protein and energy intake in a matrix of normal foods and physiological outcomes, such as muscle mass, strength, and functional capabilities. In westernized and industrialized cultures, the common dietary pattern consists of three main meals throughout the day with smaller in-between snacks. A recent cross-sectional analysis including 38 older UK citizens above the age of 70 years found that more than half of total protein intake was consumed in one meal [36]. Similar dietary patterns have been reported in studies from westernized countries, showing that >43% of protein is ingested in one single meal [37,38]. If we accept the premise that there is a dose–response relationship between protein intake and net postprandial protein anabolism, the protein content per meal is important. Additionally, total daily protein intake and per meal protein requirement for the maintenance of whole-body protein are interrelated with energy intake [27,39,40] and energy balance [41]. Thus, optimal protein intake for the maintenance of muscle mass should be assessed in combination with energy intake [42].

The purpose of this cross-sectional study was to examine how daily protein and energy intake as well as distribution are associated with muscle mass, strength and physical function in healthy, older and well-functioning Danish men and women.

2. Materials and Methods

The results are based on a cross-sectional study of 184 older Danish home-dwelling men and women from the CALM (Counteracting Age-related Loss of skeletal Muscle mass) cohort, which is described in detail elsewhere [43]. Methods with relevance for this article are explained below.

2.1. Participants

A total of 184 men and women older than 65 years of age were included in this cross-sectional study. The participants were recruited through local newspapers, magazines, radio programs, social media, presentations at senior centers and public events. All participants were within the inclusion and exclusion criteria listed in the method paper [43] and deemed healthy, as assessed by a medical doctor

based on blood samples and an oral interview. Participant data are presented in Table 1. Participants were informed of the study design, risks, and exclusion criteria prior to obtaining written consent. The study complied with the latest Declaration of Helsinki (7th version). Ethical approval was obtained through The Danish Regional Committees of the Capital Region on 4 July 2013 (number H-4-2013-070). The CALM intervention study was registered at Clinicaltrials.org as NCT02034760.

Table 1. Participant characteristics for all, and divided into sex for higher (≥1.1 g/kg aBW/day) and lower protein intakes (<0.83 g/kg aBW/day).

	All (n = 184)	**Women Lower (n = 13)**	**Women Higher (n = 48)**	**Men Lower (n = 12)**	**Men Higher (n = 50)**	**p Women/Men**
Age (years)	70.2 ± 3.9	71.7 ± 4.1	71.0 ± 4.0	71.8 ± 5.7	68.9 ± 3.5	0.52/0.04
Age range (years)	65–82	65–80	65–81	66–82	65–78	
Height (m)	1.72 ± 0.10	1.65 ± 0.07	1.66 ± 0.06	1.79 ± 0.06	1.76 ± 0.06	0.93/0.30
Body Weight (kg)	74.9 ± 12.1	69.6 ± 7.7	65.4 ± 11.4	78.7 ± 7.2	79.0 ± 11.8	0.19/1.00
BMI (kg/m^2)	25.4 ± 3.7	25.6 ± 4.0	23.8 ± 3.9	24.6 ± 2.2	25.4 ± 3.5	0.13/0.61
aBW (kg)	73 ± 8.7	67.5 ± 3.1	65.2 ± 7.0	78.4 ± 6.5	76.6 ± 7.6	0.23/0.44
WB LBM (kg)	48.5 ± 8.6	39.9 ± 2.6	40.2 ± 4.2	54.5 ± 4.3	55.0 ± 5.3	0.76/0.81
App. LBM (kg)	22.4 ± 4.6	18.2 ± 3.0	18.3 ± 2.0	25.4 ± 2.1	26.1 ± 3.3	0.84/0.43
EI (MJ/day)	8.2 ± 2.1	6.0 ± 1.3	8.5 ± 1.8	6.3 ± 1.6	9.7 ± 2.0	<0.001/<0.001
Protein (Energy %)	17.6 ± 4.0	14.3 ± 2.6	18.7 ± 4.8	15.9 ± 5.4	18.6 ± 3.1	<0.01/0.03
Protein (g/day)	82.8 ± 22.2	49.0 ± 8.5	90.6 ± 16.7	55.2 ± 10.2	104.3 ± 17.9	<0.001/<0.001
Protein (g/kg BW/day)	1.13 ± 0.34	0.70 ± 0.11	1.41 ± 0.30	0.70 ± 0.11	1.34 ± 0.25	<0.001/<0.001
Protein (g/kg aBW/day)	1.15 ± 0.31	0.73 ± 0.12	1.39 ± 0.25	0.70 ± 0.11	1.37 ± 0.23	
Goldberg Score						
EI/BMR	0.96 ± 0.24	0.77 ± 0.14	1.10 ± 0.24	0.65 ± 0.17	1.00 ± 0.19	<0.001/<0.001
Underreporters, n	41,(22%)	8,(53%)	4,(8%)	11,(85%)	5,(11%)	
Overreporters, n	18,(10%)	0,(0%)	9,(19%)	0,(0%)	6,(13%)	
Physical activity						
Step counts (Steps/day)	9740 ± 4358	9598 ± 3600	10,723 ± 4232	10,059 ± 5771	9297 ± 3392	0.036/0.30

aBW: adjusted body weight, BMI: body mass index, WB LBM: whole body lean body mass, App LBM: appendicular lean body mass, EI: energy intake, BMR: basal metabolic rate. The higher and lower protein intake differences of participant characteristics were compared using an unpaired t test. Significance was set at $p < 0.05$.

2.2. Dietary Records

A 3-day consecutive weighed dietary and liquid registration (Wednesday to Friday) was collected by following instructions from trained staff as described by Schacht et al. [44]. Trained staff entered the dietary records into the VITAKOST™ (MADLOG Aps, Kolding, Denmark) program which uses the Danish Food Composition Databank (version 7.01; Søborg; Denmark) for calculating the nutrient intakes.

2.3. Physical Activity Level

The activity level of the participant was estimated by taping an activPal (activPal 3™, activPal 3c™, or activPal micro; PAL Technologies, Glasgow, UK) to the anterior surface of the thigh, which monitoring the steps and body (thigh) position over a 4-day period. Weekend days were always included.

Energy expenditure reflecting the average daily Metabolic Equivalent of Tasks (MET) was calculated based on the algorithms provided in the activPal software. Data are reported as the average daily step count and average daily METs.

2.4. Identification of Under- and Overreporters

Under- and overreporters of energy intake were identified based on the Goldberg cut-off, which is determined by the ratio between the Energy Intake and Basal Metabolic Rate (EI:BMR) [45]. The BMR was estimated via the Cunningham equation (Resting Energy Expenditure (REE, kJ/day) = 370 + 21.6 × fat free mass (Lean Body Mass; LBM) × 4.184) [46] multiplied by the physical activity level in each participant (METs) assessed by an activPal [47]. As described by Black [47] a cut-off <0.76 was defined as an underreporter and >1.24 as an overreporter. All statistical analyses were performed including and excluding under- and overreporters.

2.5. Coefficient of Variation for Protein Distribution

The evenness of protein distribution between main meals in the day was determined by calculating the Coefficient of Variation (CV) (Standard Deviation (SD) divided by the mean) of the protein in grams per main meal, covering breakfast, lunch and dinner for each participant as previously reported [18,36,48]. Thus, a lower CV indicates that protein was consumed more evenly across main meals compared to a higher CV.

2.6. Appendicular Lean Mass

As a marker of muscle mass, the Appendicular Skeletal Muscle Index (ASMI) was assessed by whole body Dual-energy X-ray Absorptiometry (DXA) scans, using the encore v.16 software (Lunar iDXA; GE Medical Systems, Pewaukee, WI, USA). The scans were performed in an overnight fasted and euhydrated state, with the participants refraining from strenuous physical activity for 48 h prior to the scans. Regions of interest were set based on the default definitions provided by the scanner software. The same examiner controlled the default positioning of all regions, which were adjusted slightly when appropriate to take into account the interindividual differences in body placement and body size. Appendicular lean mass was assessed by the sum of lean mass in the arm and leg regions, and the ASMI was calculated by dividing the appendicular lean mass by the height squared [49].

2.7. Muscle Strength and Functional Capability

The functional capacities of the participants were assessed by applied functional measures and strength measures. Detailed methods for these measurements can be found elsewhere [43,50]. The strength measures included: the dominant hand grip strength using a grip strength dynamometer (DHD-1 (SH1001); SAEHAN Corporation, Changwon City, Korea), Maximal Voluntary isometric Contraction (MVC) of the dominant m. quadricep muscle strength, measured at 70-degree flexion in a Kinetic Communicator (model 500-11, Kinetic Communicator; Isokinetic International, Chattanooga, TN, USA). The applied functional measures included a 30 s chair stand test and a 400 m gait test. In the 30 s chair stand test [51], the number of stands completed in 30 s from a seated position (seat height: 44.5 cm) with hands crossed across the chest were counted. The 400 m gait test was performed on a 20 m course with no helping remedies, instructing the participants to walk the 400 m as fast as possible without running [52].

2.8. Food Questionnaire

At baseline, the participants received a questionnaire containing a range of questions related to their food preferences and habits as well as lifestyle and dietary changes throughout life. The questionnaires combined basic socioeducational data, quantitative questions and quantifiable qualitative questions. For this article, 149 questionnaires have been screened for information on dietary changes in relation to retirement.

2.9. Groups Division

Adjusted Body Weight (aBW) was determined to give a BMI of either 22 or 27 for participants exhibiting a BMI below 22 or above 27, respectively (i.e., either 22 or 27 times height in meters squared) [38,53]. This was done to ensure that the energy and nutritional requirements are expressed relative to a body weight representing a body composition within a healthy range [54–56].

If participants did not report any intake at a meal, they received a zero in their energy intake, but they were excluded from the group mean for protein intake.

For data on total intake and distribution of dietary protein and energy, participants were divided into two groups: a lower (<0.83 g/kg aBW/day) and a higher (≥1.1 g/kg aBW/day) protein intake group. These were based on the current Recommended Daily Allowance (RDA) by the European Food Safety Authority (EFSA) [7] and World Health Organization (WHO)/Food and Agriculture Organization of

the United Nations (FAO)/United Nations University (UNU) [8] at 0.83 g/kg/day for adults and the current fifth edition of Nordic Nutrition Recommendations 2012 [6] at 1.1–1.3 g/kg/day for older adults above the age of 65 years in men and women. Further, we also converted the absolute protein intake in g/kg/day to the energy % expression, as the relative energy contribution from protein intake of total energy intake.

For the comparison between total protein intakes and physical parameters (), participants were divided into 3 different total daily protein intake groups as follows: <0.83 g/kg aBW/day, ≥0.83–<1.1 g/kg aBW/day, ≥1.1 g/kg aBW/day in men and women. Associations between the distribution of protein intake and functional abilities were examined only within the groups ingesting a lower protein (<0.83 g/kg aBW/day) and those ingesting a higher protein (≥1.1 g/kg aBW/day) in order to investigate 'extremes' and exclude the middle group, where inherent variation of the food recording methodology and participants eating behavior may make the group-affiliation unreliable.

2.10. Statistical Analysis

The higher and lower protein intake differences of participant characteristics were assessed using an unpaired t test. A three-way mixed effects model (average daily protein intake levels × sexes × main meals) was used for energy and protein intake and energy% from protein intake. A two-way ANOVA (sexes × average daily protein intake levels) was used for the ASMI, grip strength, MVC, 400 m gate time, 30 s chair stand test and CV of protein intake. Turkey's multiple comparisons test was used as a post hoc test. Person's correlation coefficient was used to identify associations between protein and energy intake and associations between the CV of protein distribution and ASMI, grip strength, MVC, 400 m gait time, and 30 s chair stand test. Statistical significance was set at $p < 0.05$ (two-tailed). The statistical analyses were performed using Prism version 8.1.2 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Participant Characteristics

A total of 184 participants (53% men and 47% women) with a 3-day weighed dietary record with muscle mass, muscle strength, and muscle function measurements at the time of study entry of the CALM study [43] were included in the present study. Table 1 shows the participant characteristics for all 184 participants included, as well as divides men and women into lower (<0.83 g/kg aBW/day) and higher (≥1.1 g/kg aBW/day) protein intake groups. Individuals with a protein intake that does not fit in the category 'lower' or 'higher' (i.e., ≥0.83–<1.1 g/kg aBW/day) were included into the 'all' group only.

The age of the participants ranged from 65 to 82 years with no significant difference between men (69.8 ± 3.9 years) and women (70.5 ± 4.0 years), and no significant difference between women with a higher and a lower protein intake. However, the men with a higher protein intake were significantly younger than the men ingesting a lower protein intake ($p < 0.05$). Within groups of men and women, there was no difference between groups with higher and lower protein intakes with regards to body weight, adjusted body weight, lean body mass (LBM), appendicular LBM and step counts.

Based on the Goldberg cut-offs [45], underreporters (22% ($n = 41$)) and overreporters (10% ($n = 18$)) were identified based on their reported energy intake. However, all statistical outcomes were identical irrespective of the inclusion or exclusion of under- and overreporters. Thus, all figures and statistical outcomes were illustrated including all participants (i.e., both underreporters and overreporters).

3.2. Total Daily Energy and Protein Intake and Distribution per Main Meal

In our cohort, participants consumed on average 82.8 ± 22.2 g protein/day corresponding to 1.13 ± 0.34 g protein/kg/day (men; 1.10 ± 0.31 g/kg/day, women; 1.16 ± 0.38 g/kg/day) (Table 1). The total daily energy intake and protein intake were greater in the higher protein intake group than the lower protein intake group for both men and women (right panel of Figure 1a,b). For both energy and protein intakes, there was a main effect between main meals ($p < 0.0001$). A post hoc test showed that in the higher protein intake group there was a higher protein intake at dinner compared to both at breakfast ($p < 0.05$) and at lunch ($p < 0.05$) for both men and women. In the lower protein intake group, energy and protein intakes at dinner were greater than at breakfast ($p < 0.05$) for women only. Main effects were also found for protein intake level between meals ($p < 0.0001$), with post hoc differences showing that women with a high protein intake had a higher total energy intake at breakfast, and a higher protein intake at all three main meals ($p < 0.05$) The men who had a higher protein intake only ingested more protein at lunch and dinner, compared to the men with a lower protein intake. Finally, there was main effect of sex ($p = 0.01$) for energy and protein intake.

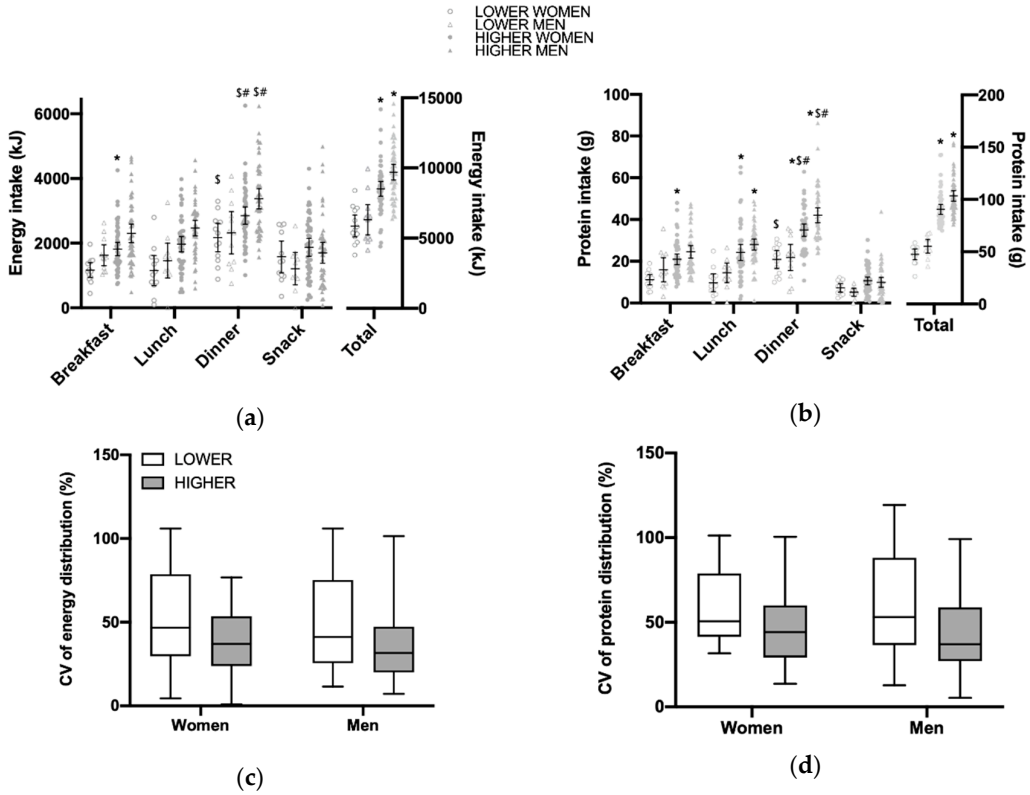


Figure 1. Baseline average energy (**a**) and protein (**b**) intake and distribution per meal, snacks during the day, and total daily intake. Turkey's multiple comparison test (comparing between main meals and sexes). Values are means with 95% Confidence Interval(CI). Significance was set at $p < 0.05$. * indicates significant difference between the lower and higher protein intake in the same meal, $ indicates significant difference between breakfast and dinner, # indicates significant difference between lunch and dinner. The Coefficient of Variation (CV), as a measure of the distribution between the three main meals was shown for energy intake (**c**) and protein intake (**d**). For both energy and protein intakes, the participants were divided a lower (<0.83 g/kg Adjusted Body Weight (aBW)/day; $n = 25$, 12 men and 13 women) or higher (≥1.1 g/kg aBW/day; $n = 98$, 50 men and 48 women) protein intake. The boxes include the 25th, 50th, and 75th quartiles and whiskers represent the maximum and minimum values. Significance was set at $p < 0.05$. No main effect for intake amount or sex was found ($p > 0.05$).

The distribution of energy (Figure 1c) and protein (Figure 1d) expressed as the coefficient of variation (CV) between main meals did not differ between men and women nor between higher (42 ± 22 in men and 46 ± 20 in women) and lower (60 ± 34 in men and 59 ± 22 in women) protein intakes.

3.3. Daily Protein Intakes and the ASMI, Grip Strength, MVC, 400 m Gait Time, 30 s Chair Stand

Overall, the ASMI, grip strength, MVC, 400 m gait time, 30 s chair stand did not differ between the three different protein intakes (<0.83 g/kg aBW/day ≥0.83–<1.1 g/kg aBW/day, ≥1.1 g/kg aBW/day) in both men and women (Figure 2). However, sex differences were noted in the ASMI, grip strength, and MVC at <0 83 g/kg aBW/day, ≥0.83–<1.1 g/kg aBW/day, ≥1 1 g/kg aBW/day protein intake levels. The main effect of sex was identified in the ASMI ($p < 0.0001$), grip strength ($p < 0.0001$), and MVC ($p < 0.01$).

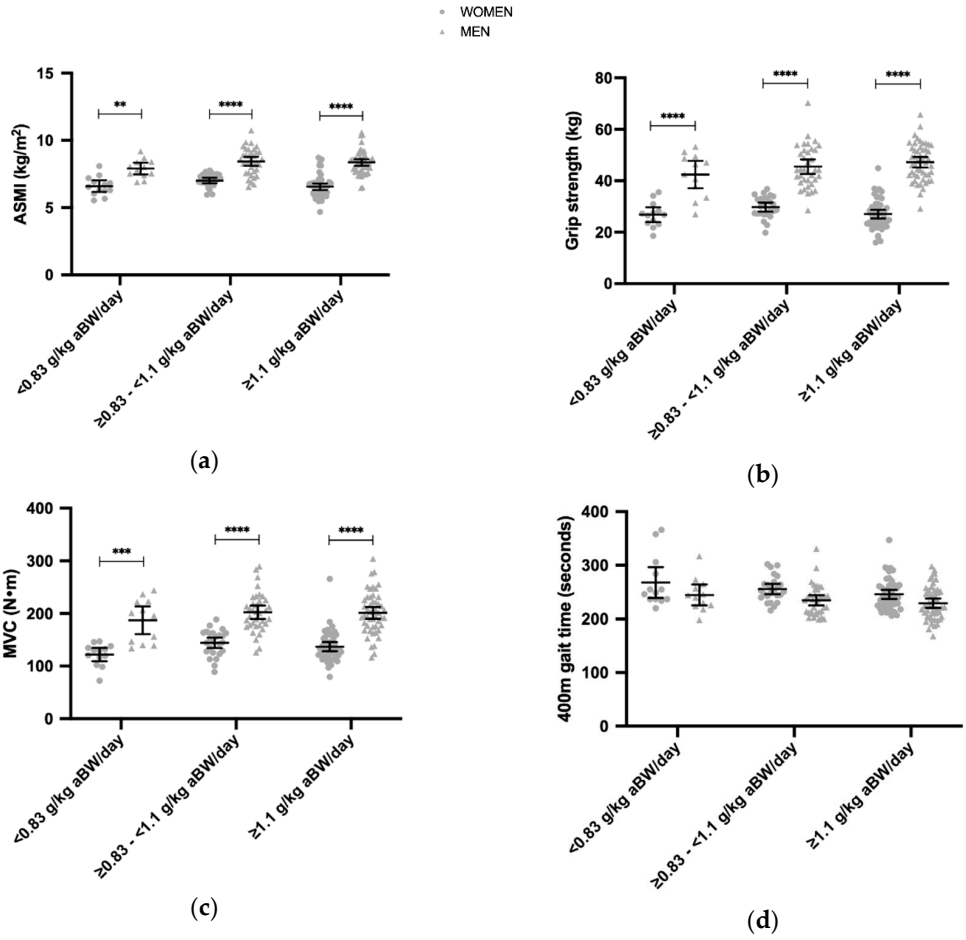
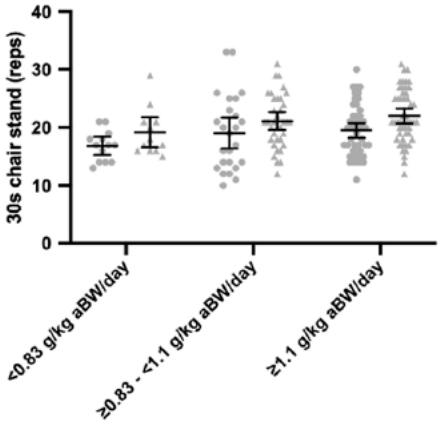


Figure 2. *Cont.*

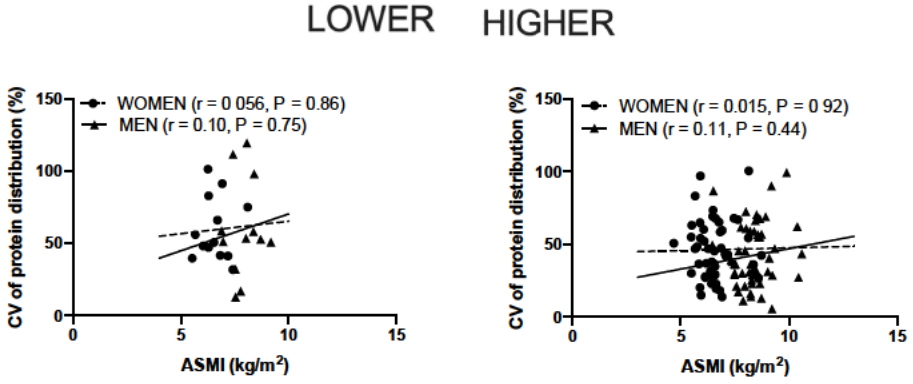


(e)

Figure 2. Appendicular Skeletal Muscle Index (ASMI) (**a**), grip strength (**b**), knee extension Maximal Voluntary isometric Contraction (MVC) (**c**), 400 m gait time (**d**), 30 s chair stand (**e**) divided into individuals with a protein intake of <0.83 g/kg aBW/day (n = 25, 12 men and 13 women), ≥0.83–<1.1 g/kg aBW/day (n = 61, 36 men and 25 women), ≥1.1 g/kg aBW/day (n = 98, 50 men and 48 women (n = 47 for MVC due to one missing value). Values are means with 95% CI. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Turkey's multiple comparison test (comparing between average daily protein intake levels and sexes). Significance was set at $p < 0.05$.

3.4. Associations between the Protein Distribution and ASMI, Grip Strength, MVC, 400 m Gait Time and 30 s Chair Stand

The association between the distribution of protein intake and functional abilities was examined separately for the participants ingesting lower amounts of protein (<0.83 g/kg aBW/day) and those ingesting higher amounts of protein (≥1.1 g/kg aBW/day) (Figure 3). Independent of the level of protein intake, the distribution was not associated with any of the functional measures ($p > 0.05$), except for MVC, where for women ingesting the highest level of protein there was a significantly higher MVC at the lowest CV values ($p = 0.03$, r = −0.32) for women ingesting the lowest level of protein, there was a significantly faster 400 m gait speed at the highest CV values ($p = 0.02$, r = 0.62).



(a) **(b)**

Figure 3. *Cont.*

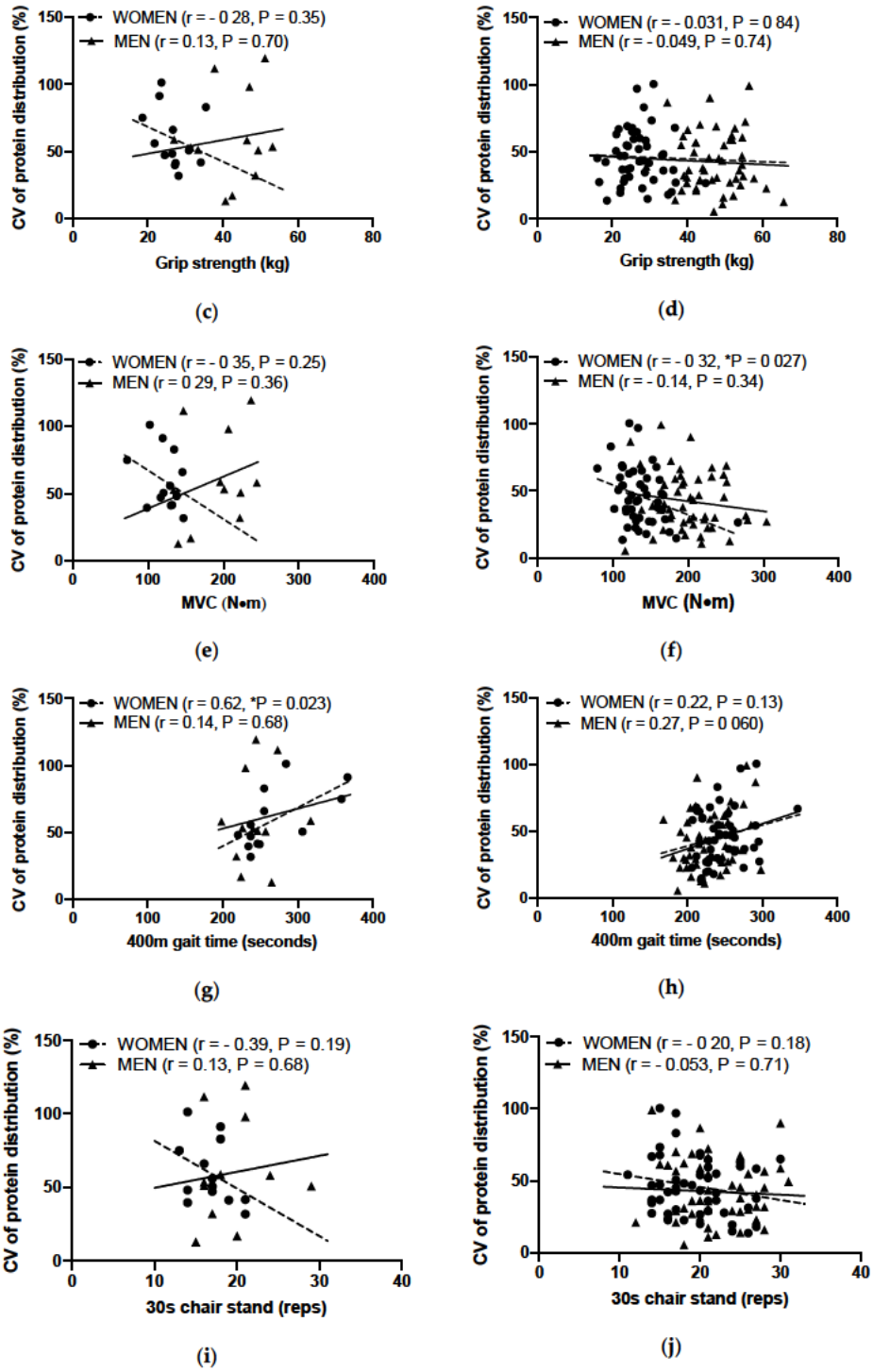


Figure 3. The association between the protein distribution for participants with a lower protein intake (n = 25, 12 men and 13 women) and a higher protein intake (n = 98, 50 men and 48 women (n = 47 for Maximal Voluntary isometric Contraction (MVC) due to one missing value)) and Appendicular Skeletal Muscle Index (ASMI) ((**a**) /Lower, (**b**) /Higher), grip strength ((**c**) /Lower, (**d**) /Higher), knee extension MVC ((**e**) /Lower, (**f**) /Higher), 400 m gait time ((**g**) /Lower, (**h**) /Higher), and 30 s chair stand ((**i**) /Lower, (**j**) /Higher). * $p < 0.05$, Pearson's correlation coefficient was used to identify the associations. Significance was set at $p < 0.05$.

3.5. Associations between Protein (g/kg aBW) and Energy (kJ/kg aBW) Intake

For total daily intakes and individual main meals at breakfast, lunch, and dinner, protein intakes were positively associated with energy intakes irrespective of sex ($p < 0.0001$) (Table 2).

Table 2. Associations between protein (g/kg aBW) and energy (kJ/kg aBW) intake.

	Total		Breakfast		Lunch		Dinner	
	r	R^2	r	R^2	r	R^2	r	R^2
Women	0.69	0.48	0.72	0.52	0.82	0.68	0.56	0.32
Men	0.70	0.49	0.89	0.79	0.74	0.55	0.61	0.37

For all associations *p*-values are <0.0001.

4. Discussion

In the present study, we investigated the association between the total and meal distribution of protein and energy intake during the day, and the muscle mass, strength and physical function in healthy and well-functioning older Danish men and women. No association with the ASMI, grip strength, knee extension MVC or 400 m gait time appeared when dividing participants into three groups based on their protein intake (<0.83; ≥0.83–<1.1; ≥1.1 g/kg aBW), comparing the lowest and the highest of these three groups, or when correlating the protein meal distribution pattern. This suggests that within a population of healthy Danish older adults, neither the total protein intake nor meal distribution appear to be associated with muscle mass, strength or physical functions. Our data do not strengthen the emerging hypothesis that older adults need more protein than what is currently recommended to maintain muscle mass and physical function [57–59].

On average, the participants ingested more protein than 0.83 g/kg BW/day as recommended by the EFSA [7] and WHO/FAO/UNU [8]. However, there were no differences between those ingesting more than the recommendations and those ingesting less protein (Figure 2) when the total protein intake (<0.83; 0.83–<1.1; ≥1.1 g/kg aBW) and ASMI (Figure 2a) and various functional measures (Figure 2b–e) were compared. Similarly, Bollwein and colleagues found that among German seniors above the age of 75 years, daily protein intake was relatively high and did not make up any risk factor for frailty, and it was not evident even when they compared the highest and lowest quantile of protein intakes [48].

Protein intake pattern across the day, in addition to the total protein intake, has been suggested as an important factor for protein turnover and muscle mass [19]. A possible link between meal distribution pattern and benefits for muscle mass maintenance was demonstrated in an acute experimental study in healthy adults (36.9 ± 3.1 years) by Mamerow et al. [17]. They reported that the 24 h fractional synthesis rate is higher in an even protein distribution pattern (breakfast: lunch: dinner = 30: 30: 30 g) compared to a skewed distribution pattern (breakfast: lunch: dinner = 10: 15: 65 g). In a cross-sectional study, Bollwein and colleagues observed that the individuals in the frailest group (75–96 years) had a more skewed protein distribution pattern (less at breakfast and more at lunch) than those in the least frail group (76–91 years). It should be noted that neither group had an even distribution [48]. Supporting this notion, an observational study in adults (50–85 years) reported by Loenneke et al. [20] showed that those ingesting two meals or more containing above 30 g protein had a higher lean mass than those ingesting one meal or less containing 30 g protein. Similarly, in a two year follow up study in a 67–84 year old Canadian population by Farsijani et al. [16], those categorized as having the most even protein intake distribution pattern determined as the CV had a higher lean mass throughout the study regardless of total protein intake (~1.1 g/kg BW/day). In contrast, the decline in lean mass [16] and physical function [60] over the two-year period did not differ between protein intake patterns. Likewise, Kim and colleagues reported that total protein intake, but not the intake pattern, is responsible for the achievement of greater whole-body protein net balance in a study where a mixed macronutrient diet was provided in healthy older adults (52–75 years) [31]. They further

supported the finding in an 8-week randomized controlled study that protein intake patterns in the context of a mixed macronutrients meal across the day is not a determinant of whole-body protein anabolism, MPS, muscle mass, and muscle function when an average amount of protein is consumed (i.e., 1.1 g/kg/day) in healthy older adults (51–69 years) [61].

In the present study, the participants were divided into higher and lower protein intake groups in order to investigate the association between the protein distribution (CV) and ASMI and physical performance parameters (Figures 1 and 3). The use of the CV was adopted to isolate the distribution of protein, and not the amount of protein, as a variable. For example, it would be difficult to compare a skewed protein intake over the three main meals of 30, 30 and 60 g (120 g in total) with an even intake of 15, 15 and 15 g (45 g in total), as recently discussed by Hudson et al. [62]. It is evident, despite the limited number of participants, especially in the lower protein/energy intake groups, that in this population the protein distribution is not associated with the ASMI nor physical function. This is in line with a cross-sectional study conducted by Gingrich et al. [63], in which 97 community-dwelling older individuals were included (a mean age of 77 years). The divergent findings between our results, the study by Gingrich et al. [63], and other studies [16,20] could at least in part be explained by the different study populations that were investigated. Ten Haaf et al. [18] showed in a group of older individuals (≥65 years) that those having an even distribution of protein throughout the day were also the individuals who stayed the most active. This might explain why a population consuming protein evenly throughout the day, at least in some studies, had a higher lean mass.

Whole-body protein metabolism is influenced by energy intake [27,39,40] and energy balance [41]. Thus, the fate of utilization of amino acids for protein synthesis or energy production depends partly on total energy intake and energy balance. We found that protein and energy intake are positively correlated (Table 2), revealing that the participants ingesting high amounts of protein consume more energy in general, including protein and other macronutrients as part of their habitual dietary patterns. This is in accordance with observations in community-dwelling, frail and institutionalized elderly people as reported by Tieland et al. [64] and Smeuninx et al. [65]. The causality here cannot be determined, but we speculate that people with a high LBM and body mass may just eat more, leading to a concomitant increase in protein intake.

The phenotype observed in cross-sectional studies (lean body mass underlying the ASMI, muscle and grip strength, gait speed, etc.) is supposedly formed as a consequence of living conditions over several years. An underlying assumption for associating these phenotypic characteristics with food intake is, therefore, that the food recordings are representative of the long-term retrospective habitual food intake. The food questionnaires allowed us to explore this assumption. We found that 85 (46%) out of the total cohort mentioned that their food intake had changed after being retired. Of those, 21 (11%) directly stated that they had become more aware of eating a healthier diet, whereas nine (5%) stated that they were eating less and consumed easier foods (i.e., quick snacks, ready meals and fast food), snacked more and had become more relaxed towards healthy food choices. Twelve (7%) said that they went from being used to catering foods in excess at their workplace to more moderate and simple foods at home. In support for a transition in food intake occurring at retirement, a change in daily energy intake patterns was found among middle-aged British adults over a 17-year period, directed away from lunch and toward the evening meal [66]. Although it is not directly comparable to our population, this finding combined with our food questionnaires suggest that a change in food choices and intake pattern could be expected. Hence, longitudinal intervention studies should be conducted to account for such unavoidable uncertainty in a cross-sectional study design and investigate whether different protein intakes and dietary protein distributions preserve muscle mass and physical function in healthy older individuals.

A cross-sectional study design brings some limitations—as discussed above—and the sample size was relatively small in our study, especially in the lowest protein intake group (<0.83 g/kg/day). Further, the representative nature of a 3-day dietary registration and a 4-day physical activity registration may be questionable and thus creates some degree of uncertainly.

5. Conclusions

In conclusion, we found no associations between the protein and energy intake or distribution and ASMI and physical parameters in a cohort of healthy Danish older individuals. These data do not provide an incentive to recommend healthy, well-functioning older individuals who already adhere to the current internationally recommended dietary protein intake (0.83 g/kg/day) to change their dietary protein intake or distribution pattern throughout the day.

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Appendix 4.

Title: Co-ingestion of Cluster Dextrin carbohydrate does not increase exogenous protein-derived amino acid release or myofibrillar protein synthesis following a whole-body resistance exercise in moderately trained younger males: a double-blinded randomized controlled crossover trial

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ORIGINAL CONTRIBUTION

Co-ingestion of cluster dextrin carbohydrate does not increase exogenous protein-derived amino acid release or myofibrillar protein synthesis following a whole-body resistance exercise in moderately trained younger males: a double-blinded randomized controlled crossover trial

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Abstract

Purpose This study investigates if co-ingestion of cluster dextrin (CDX) augments the appearance of intrinsically labeled meat protein hydrolysate-derived amino acid (D_5-phenylalanine), Akt/mTORC1 signaling, and myofibrillar protein fractional synthetic rate (FSR).

Methods Ten moderately trained healthy males (age: 21.5 ± 2.1 years, body mass: 75.7 ± 7.6 kg, body mass index (BMI): 22.9 ± 2.1 kg/m^2) were included for a double-blinded randomized controlled crossover trial. Either 75 g of CDX or glucose (GLC) was given in conjunction with meat protein hydrolysate (0.6 g protein * FFM^{-1}) following a whole-body resistance exercise. A primed-continuous intravenous infusion of L-[^{15}N]-phenylalanine with serial muscle biopsies and venous blood sampling was performed.

Results A time × group interaction effect was found for serum D_5-phenylalanine enrichment ($P < 0.01$). Serum EAA and BCAA concentrations showed a main effect for group ($P < 0.05$). T_{max} serum BCAA was greater in CDX as compared to GLC ($P < 0.05$). However, iAUC of all serum parameters did not differ between CDX and GLC ($P > 0.05$). T_{max} serum EAA showed a trend towards a statistical significance favoring CDX over GLC. The phosphorylation of p70S6K^{Thr389}, rpS6$^{Ser240/244}$, ERK1/2$^{Thr202/Tyr204}$ was greater in CDX compared to GLC ($P < 0.05$). However, postprandial myofibrillar FSR did not differ between CDX and GLC ($P = 0.17$).

Conclusion In moderately trained younger males, co-ingestion of CDX with meat protein hydrolysate does not augment the postprandial amino acid availability or myofibrillar FSR as compared to co-ingestion of GLC during the recovery from a whole-body resistance exercise despite an increased intramuscular signaling.

Trial registration ClinicalTrials.gov ID: NCT03303729 (registered on October 3, 2017).

Keywords Resistance exercise · Muscle protein synthesis · Stable isotope tracer · Amino acids · Intrinsically labeled protein · mTORC1

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Abbreviations

mTORC1	Mammalian target of rapamycin complex 1
CDX	Cluster dextrin
iAUC	Incremental area under the curve
T_{max}	Time to reach maximum concentration
DXA	Dual X-ray absorptiometry
FFM	Fat-free mass
BMI	Body mass index
RM	Repetition maximum
GLC	Glucose
PTC	Phenylthiocarbamyl
LC-MS/MS	Liquid chromatography tandem mass spectrometer
ELISA	Enzyme-linked immunosorbent assay
NAP	*N*-Acetyl-propyl
GC-C-IRMS	Gas chromatography combustion isotope ratio mass spectrometry
p70S6K	70 KDa S6 protein kinase
4E-BP1	Eukaryotic initiation factor 4E-binding protein
Akt	Protein kinase B
rps6	Ribosomal protein S6
eEF2	Eukaryotic elongation factor 2
FSR	Fractional synthetic rate
BCAA	Branched-chain amino acids
EAA	Essential amino acid
TTR	Tracer to tracee ratio
MPE	Mole percent excess
IU	International unit

Introduction

Protein intake is essential for skeletal muscle protein adaptation to resistance exercise training [1]. Muscle protein synthesis is maximally stimulated when exercise is combined with protein ingestion [2–4]. Essential amino acids (EAA) have been shown to be potent in stimulating muscle protein synthesis [5, 6]. Mechanistically, leucine, one of the branched-chain amino acids (BCAA), is a potent stimulator of mammalian target of rapamycin complex 1 (mTORC1), which is a serine/threonine kinase regulating the translation and initiation [7–9]. Further, the availability of leucine and other essential amino acids [10, 11] and the achievement of higher peak aminoacidemia [12, 13] are associated with an increase in the rate of muscle protein synthesis, suggesting the importance of quick absorption and rise in essential amino acid availability in the circulation to induce muscle protein synthesis [12, 14].

Carbohydrate is often added to protein supplementations for various reasons, such as increasing energy intake, providing readily available substrate for energy metabolism, and improving taste. However, the uptake rate of exogenous amino acids and their availability in circulation is attenuated when consumed with carbohydrate [15–17] or a mixed macronutrients meal [18, 19]. This is due to a decrease in the rate of digestion and absorption [16] and an increase in the retention of amino acids in the portal drained viscera [20]. In addition, amino acid availability in the circulation is reduced by suppression of protein breakdown [21, 22]. Although plasma insulin concentration was greater when protein was ingested with carbohydrate [15–17] or a mixed meal [18], muscle protein synthesis was not augmented as compared to protein intake alone [15–17]. A systematic review concluded that a systemic administration of insulin does not have a stimulatory or inhibitory effect for muscle protein synthesis and that amino acid availability dictates muscle protein synthesis in healthy younger individuals [23]. Thus, the current evidence does not provide the interactive effect of carbohydrate co-ingestion with protein to augment muscle protein synthesis. However, if the addition of carbohydrate delays the availability of amino acids in the circulation, it could be speculated that carbohydrate would result in a delayed increase in muscle FSR, as observed in slower digestible proteins [13].

Cluster dextrin (CDX) is a branched carbohydrate produced from waxy maize starch by the cyclization of a branching enzyme [24]. CDX is highly soluble in water, has low viscosity, and has a relatively low tendency for retrogradation [25] compared to commercial dextrin [26]. CDX has also been shown to increase the rate of gastric emptying compared to glucose (GLC) and standard dextrin due to a lower osmotic pressure [27]. Accordingly, the rapid gastric emptying of CDX might alleviate the lower amino acid availability when co-ingested with protein as compared to GLC. Thus, a measurement of exogenous protein-derived amino acid availability (i.e., a downstream measurement of digestion and absorption) is required to determine whether co-ingestion of CDX increases amino acid availability for the periphery.

Therefore, we hypothesized that the appearance of amino acids from orally ingested meat protein hydrolysate into the circulation would be faster when it is co-ingested with CDX than GLC. Accordingly, we further hypothesized that the ingestion of the meat protein hydrolysate with CDX would result in a greater Akt/mTORC1 signaling response and myofibrillar FSR following an acute bout of whole-body resistance exercise as compared to GLC in moderately trained younger males.

Methods

Ethical approval

This study was approved by the Ethics Committee of the Capital Region (H-17017363) and adhered to the Helsinki

II declaration. Before inclusion, each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to obtaining written informed consent. This trial was registered at clinicaltrails.gov as NCT03303729.

Subjects

Ten moderately trained healthy males (21.5 ± 2.1 years, 22.9 ± 2.1 kg/m^2; values are mean ± SD) volunteered to participate in a double-blinded, randomized controlled crossover study. Inclusion criteria were as follows: healthy men who conduct structured whole-body resistance training between 1 and 3 times (1–3 h) per week on average over the last 3 months. Exclusion criteria were as follows: subjects younger than 18 years or above 30 years of age, BMI > 30, smoking, active cancer, renal diseases, diabetes mellitus, vegetarian, physical inactivity (i.e., no systematized exercise), and perform systematized resistance exercise more than 3 times per week. Baseline subject characteristics are presented in Table 1.

Study overview

This was a double-blinded randomized controlled crossover trial conducted at The Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital. The overall timeline of the study is shown in Fig. 1a, and a CONSORT flowchart diagram is displayed in Fig. 2. Briefly, at least 2 weeks prior to the first trial, subjects underwent preliminary assessments. The two experimental trials were separated by at least 2 weeks to minimize any interaction on the second trial from the previous trial. On both trial days, the participants arrived in an overnight fasted state at 08:00 h at The Institute of Sports Medicine Copenhagen (ISMC), Bispebjerg Hospital. Personnel with no direct involvement in the experiment rolled dice and created a scheme where the order of the interventions (CDX or GLC) was in code for each subject. On the day of an experiment, the personnel prepared the designated intervention and handed it over to the investigator (MJ). Thus, the allocation of interventions was concealed from the participants and the study investigator until the completion of data analysis. In each experimental visit, muscle biopsies and blood samples were obtained during a primed-continuous stable isotope amino acid infusion (^{15}N-phenylalanine) to determine amino acid availability from orally ingested intrinsically labelled meat protein hydrolysate (D_5-phenylalanine), myofibrillar muscle protein synthesis, and intracellular signaling in response to a whole-body resistance exercise and the intake of meat protein hydrolysate with either GLC or CDX.

Table 1 Baseline subject characteristics

Age, y	21.5 ± 2.1
Height, m	1.82 ± 0.54
Body mass, kg	75.7 ± 7.6
FFM, kg	60.4 ± 5.4
BMI, kg/m^2	22.9 ± 2.1
1-RM leg press, kg	283.0 ± 50.6
1-RM leg extension, kg	116.5 ± 12.1
10-RM shoulder press, kg	50.6 ± 4.5
10-RM pulldown, kg	56.5 ± 9.0

All values are presented as means ± SD. $n = 10$

FFM fat free mass, *BMI* body mass index

Preliminary assessments

Body mass and height

Height was measured in the upright position without shoes against a wall and body weight was measured on a digital scale (Seca 719, Seca gmbh & co., Hamburg, Germany) in light clothing.

Body composition

Dual X-ray absorptiometry (DXA) was performed to determine the whole-body fat free mass (FFM) by using the enCORE v.16 software (Lunar iDXA; GE Medical Systems, Pewaukee, WI, USA) after having emptied their bladder. DXA scans were performed after at least 12 h overnight fast.

Strength tests

After the scanning, strength was assessed by leg press, knee extension (Super Executive Line, TechnoGym, Gambettola, Cesana, Italy), shoulder press (TR Equipment model 9025, Tranås, Sweden) and shoulder pull/pull-down (Lat Mach, TechnoGym, Gambettola, Cesana, Italy) strength exercise machines. The strength tests were 1 repetition maximum (RM) and 10RM for leg exercises and upper body exercises, respectively. The 10RM test for the upper body exercises was chosen to minimize any risk of injury by unaccustomed exercises during testing.

Experimental protocol

The experimental protocol is shown in Fig. 1b. The overall experimental protocol consisted of a primed (4.0 µmol * kg FFM^{-1}) continuous (3.8 µmol * kg FFM^{-1} * hour^{-1}) infusion of L-[^{15}N]-phenylalanine, which was applied over the course of the experimental trial. The participants rested in the supine position for the remainder of the trial day, only interrupted by the training program and toilet

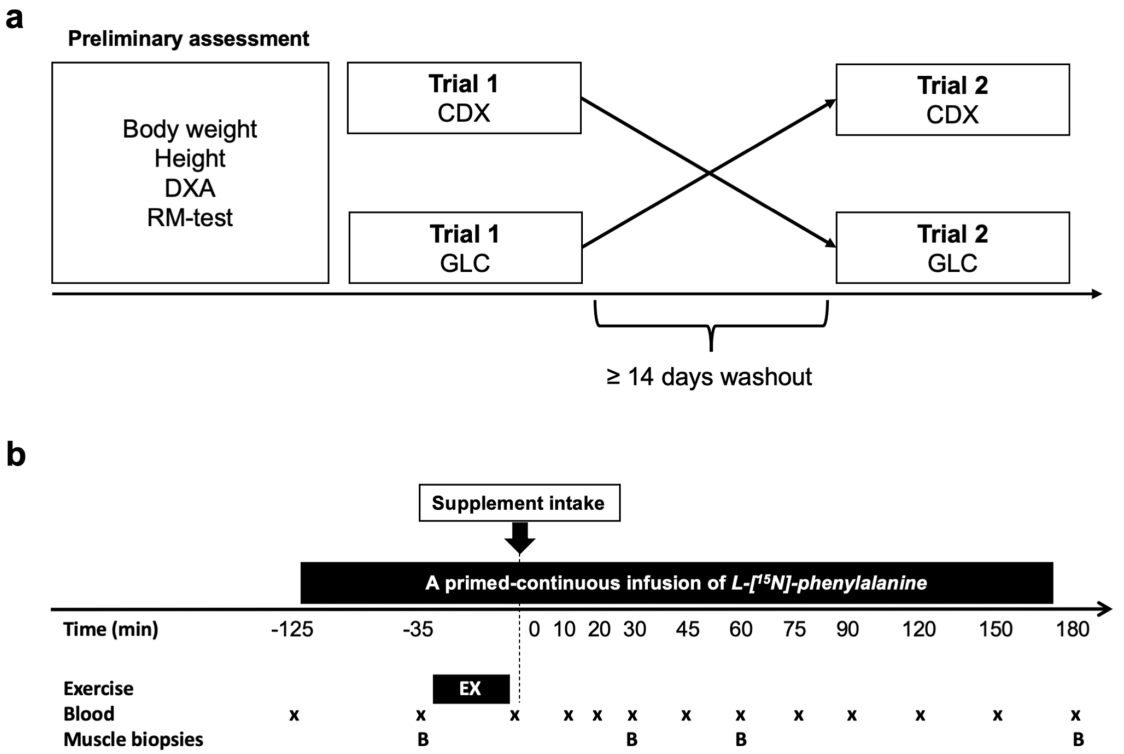


Fig. 1 Schematic overview of crossover study design (**a**) and experimental protocol (**b**). *DXA* dual X-ray absorptiometry; *RM* repetition maximum, *EX* exercise, *B* muscle biopsy, *GLC* glucose, CDX cluster dextrin

visits. Two antecubital catheters were inserted into each arm of the participant, one for the infusion of a stable isotope amino acid tracer (^{15}N-phenylalanine) and the other for serial blood sampling. A background blood sample was drawn after which the primed continuous infusion was started. The subject rested for approximately 1.5 h after which the first biopsy was taken, and a blood sample was drawn. The leg and site of the biopsies were randomized using dice. Hereafter, the participant walked a small distance of 100 m to the training facility where they conducted the resistance exercise program under full guidance and supervision (see Resistance exercise protocol). Upon completion, the participant walked back to the trial room where a post-exercise blood sample was drawn. Hereafter, an independent research assistant prepared the drink containing the intrinsically labeled (D_5-phenylalanine) meat protein hydrolysate (0.6 g protein * FFM^{-1}) mixed with either 75 g of GLC or CDX in 350 mL of cold tap water. The drink was consumed in less than 5 min by the participant. Furthermore, the post-exercise drink was enriched with 5% ^{15}N-phenylalanine to minimize any fluctuations in the serum enrichment after consumption of the protein-rich drink (unlabeled phenylalanine). The amount of meat protein hydrolysate was decided to provide sufficient amino acids following a whole-body resistance exercise. A previous study showed that a higher protein intake is required when a whole-body resistance exercise is performed as compared to a unilateral leg resistance exercise [28]. Nutritional composition of meat protein hydrolysate, CDX, GLC is presented in Table 2. Once the drink was consumed, blood was sampled in a vacutainer coated with Z serum clot activator (Vacuette tube, Greiner Bio-One GhmB, Austria) at 10, 20, 30, 45, 60, 75, 90, 120, 150, and 180 min following the post-exercise drink. Muscle biopsies were taken from the vastus lateralis at 30, 60, and 180 min post drink under local anesthesia (1% lidocaine) using the Bergström technique [29]. Muscle samples were rinsed in ice-cold saline (9 mg/ml) and freed from any visible blood and connective tissues before being snap frozen in liquid nitrogen and stored at -80 °C for future analysis. After the last biopsy, the infusion was stopped, catheters were removed, and the participant was given a sandwich before being sent home.

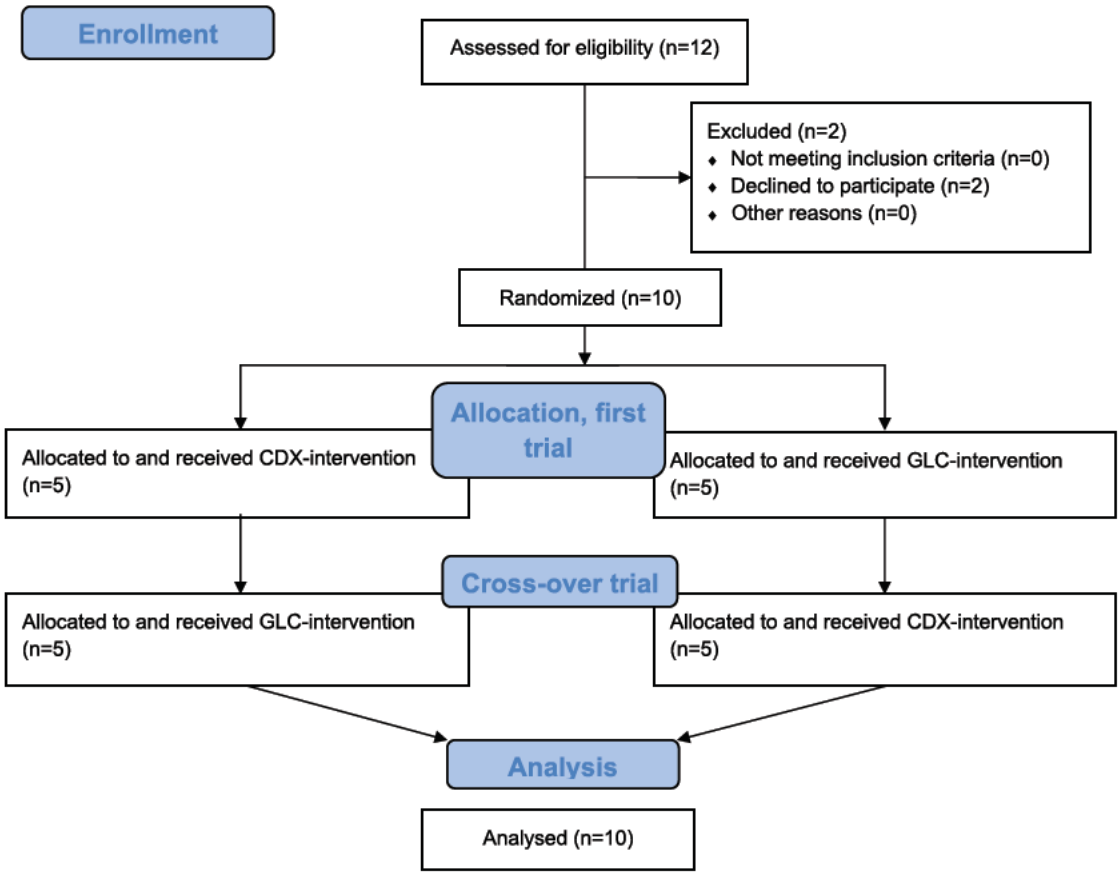


Fig. 2 A CONSORT flowchart diagram

Resistance exercise protocol

The resistance exercise session consisted of four different exercises for both the lower and upper body and was the same as those used in the preliminary assessment: leg press, knee extension, shoulder press and shoulder pull/pull down. The subjects completed 3 sets of 8 repetitions at 70% of 1RM in each of the two leg exercises and 3 sets of 10 reps at 10RM in the two upper body exercises. Three sets of each exercise were completed before conducting the next exercise. Rest periods of 2 min were allowed between sets and exercises.

Meat protein hydrolysate

The meat, from which the hydrolysate has been produced, had been intrinsically labeled with ring-D_5-phenylalanine by infusing Holstein cows with the ring-D_5-phenylalanine tracer for 72 h before slaughter [18]. The meat protein hydrolysate is quickly absorbed and induces an immediate and high availability of amino acids in the circulation after a bolus intake [18]. This allows to directly measure the availability of the orally consumed meat protein hydrolysate-derived amino acids in the circulation.

Blood analysis

Blood was sampled with vials coated with Z serum clot activator and left at room temperature for 30 min before being centrifuged (3,970 x g, 10 min, 4 °C) in an Eppendorf 5810R (Eppendorf AG, Hamburg, Germany) to obtain serum. Serum samples were stored at -80 °C for further analysis.

Serum amino acid concentrations were determined as described in detail elsewhere [30]. We used 100 µL of the serum, which was added internal standards for all amino acids and were acidified with the addition of 120 µL of 50% acetic acid before being poured over columns (Medium HDPE Open tip column CC07, Intertech Medical Inc.,

Table 2 Nutritional composition of meat protein hydrolysate, cluster dextrin (CDX), and glucose (GLC)

	Meat protein hydrolysate (0.6 g protein/ FFM kg)[1]	CDX	GLC
Total served weight, g	300	75	75
Energy, kcal	162	300	300
Protein, g	36	–	–
Protein, kcal	144	–	–
Fat, g	1.8	–	–
Fat, kcal	16.2	–	–
Carbohydrate, g	0	75	75
Carbohydrate, kcal	0	300	300
Alanine, g	2.04	–	–
Arginine, g	2.06	–	–
Aspartic acid, g	3.27	–	–
Cysteine, g	0.28	–	–
Glutamic acid, g	5.46	–	–
Glycine, g	1.46	–	–
Histidine, g	1.36	–	–
Isoleucine, g	1.61	–	–
Leucine, g	2.86	–	–
Lysine, g	3.21	–	–
Methionine, g	0.80	–	–
Phenylalanine, g	1.54	–	–
Proline, g	1.27	–	–
Serine, g	1.37	–	–
Threonine, g	1.61	–	–
Tryptophan, g	0.42	–	–
Tyrosine, g	1.17	–	–
Valine, g	1.74	–	–
Total essential amino acids, g	15.16	–	–
Total amino acids, g	33.55	–	–
D_5-phenylalanine enrichment[2], MPE ± SD	0.73 ± 0.01	–	–

CDX cluster dextrin, *GLC* glucose, *MPE* mole percent excess, *SD* standard deviation

[1]For an individual with 60 fat free mass (FFM) kg

[2]Value was reported by Reitelseder et al. [18]

Denver, CO) containing acidified cation exchange resin (Dowex AG 50 W-X8 resin 100–200 mesh, BioRad, Copenhagen, Denmark). Purified amino acids were converted to their phenylthiocarbamyl (PTC) derivatives, by adding a coupling buffer (methanol: Milli-Q® water:triethylamin (2:2:1, %, v/v)), drying at 70 °C under a flow of N_2, adding the derivatization solution (triethylamin: Milli-Q® water:PITC:methanol (1:1:1:7, %, v/v)). Then, the sample was vortexed and incubated at room temperature for 30 min. Hereafter, the solution was dried at 70 °C under a flow of N_2 and acetonitrile, methanol, and Milli-Q® purified water (44:10:46, %, v/v) with 0.1 M ammonium acetate was added. The isotope ratios of D_5- and ^{15}N-phenylalanine and a full amino acid concentration profile were determined on a liquid chromatography–tandem mass spectrometer (LC–MS/MS; triple stage quadrupole mass spectrometer, TSQ Vantage, Thermo Fischer Scientific, San Jose, CA, USA).

Serum insulin concentrations were measured using a high-sensitivity human insulin enzyme-linked immunosorbent assay (ELISA) kit (DRG Instrument GmbH) at the time points − 35, 0, 10, 20, 30, 45, 60, 90, 120, and 180 min.

Muscle tissue analyses

Intramuscular amino acid concentration

From 10 mg of wet weight muscle, BCAA concentrations were determined using the same protocol as for serum amino acids as described above [30]. Briefly, the frozen muscle specimens were homogenized in 1 ml of 6% perchloric acid with an added internal standard for the determination of BCAA concentrations. The samples were spun down and the supernatant containing the tissue free amino acids was extracted. The samples were then poured over acidified cation exchange columns with resin (AG 50 W-X8 resin, Bio-Rad laboratories, Hercules, Ca, USA). The amino acids were eluted with 2 × 2 ml 4 M NH_4OH and derivatized into their phenylthiocabamyl (PTC) derivative. Derivatized samples were loaded and analyzed on LC–MS/MS (Thermo Fischer Scientific, San Jose, CA, USA). Each intramuscular BCAA concentration was normalized to the wet weight of muscle used to prepare for the analysis. The intramuscular water fraction was set as 0.77 of the muscle wet weight.

Myofibrillar protein bound tracer enrichments

From 20 mg of wet weight muscle, the abundance of myofibrillar protein bound ^{15}N-phenylalanine was measured according to our lab's standard protocol. Briefly, the frozen muscle specimen was homogenized in 1 mL of buffer (Tris 0.02 M [pH 7.4], NaCl 0.15 M, EDTA 2 mM, EGTA 2 mM, TritonX-100 0.5%, sucrose 0.25 M) for 4 × 45 s, speed 5.5 (FastPrep 120A-230; Thermo Savant, Holbrook, NY, USA) and left at 5 °C for 3 h. Hereafter, samples were centrifuged at 800 g for 20 min at 5 °C. The supernatant was discarded, and 1 mL of homogenization buffer was added, homogenized for 45 s at speed 5.5, incubated at 5 °C for 30 min, and centrifuged. The supernatant was discarded, and 1.5 mL buffer (KCl 0.7 M, pyrophosphate ($Na_4P_2O_7$) 0.1 M) was added to the pellet, vortexed and left overnight at 5 °C. The day after, the samples were spun at 1,600 x g for 20 min at 5 °C and the supernatant transferred to glass vials suitable for hydrolysis and added 2.3 × vol ethanol 99%, vortexed and left for 2 h at 5 °C, and subsequently spun at 1,600 x g for 20 min. The supernatant was then discarded,

and 1 mL of 70% ethanol was added to the pellet, after which the solvent was vortexed and centrifuged at 1,600 x g for 20 min. The supernatant was discarded and 1 mL of 1 M HCl and 1 mL of resin slurry was added to the pellet and left overnight at 110 °C. The solvent was diluted with water and the amino acids were purified over cation exchange resin columns. The purified amino acids were derivatized as their N-acetyl-propyl (NAP) derivate and analyzed on gas chromatograph-combustion-isotope ratio mass spectrometer by following standard procedure described thoroughly by Bornø et al. [31].

Intracellular signaling

Western blotting was performed as reported previously with a slight modification [32]. Briefly, approximately ~ 30 mg of frozen muscle tissue samples was homogenized in ten-fold volumes of RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease and phosphatase inhibitor cocktail (Roche Life Science, Indianapolis, IN, USA) per 10 mL of homogenization buffer. The resulting homogenates were centrifuged at 14,000 x g for 10 min at 4 °C. The supernatant was transferred to a new vial and total protein concentrations were determined by the Protein Assay Rapid kit (WAKO, Osaka, Japan). The samples were standardized to 2 µg protein per 1 µL by dilution with 3 × SDS sample buffer containing 15% β-mercaptoethanol, 6% SDS, 187.5 mM Tris–HCl (pH 6.8), 15% sucrose, and 0.015% bromophenol blue and boiled at 95 °C for 5 min. An equal amount of protein (10 µg) was loaded into each lane and the samples were separated by electrophoresis on a 10 or 15% SDS–polyacrylamide gel for 45 min at 250 V. Following electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 1 h at 20 V via a semi-dry transfer. Membranes were subsequently blocked in 5% milk for 1 h at room temperature. After blocking, membranes were washed 3 times for 5 min in Tris-buffered saline with 0.1% Tween (TBST) before being incubated overnight at 4 °C in primary antibody against phospho-70 kDa S6 protein kinase (p70S6K) Thr389 (#9234), total p70S6K (#2708), phospho-eukaryotic initiation factor 4E-binding protein (4E-BP1) Thr37/46 (#9459), total 4E-BP1 (#9452), phospho-protein kinase B (Akt) Ser473 (#9271), total Akt (#2920), phospho-AMPKα Thr172 (#2535), total AMPKα (#2793), phospho-ribosomal protein S6 (rps6) Ser240/244 (#2215), total rps6 (#2217), phospho–eukaryotic elongation factor 2 (eEF2) Thr56 (#2331), total eEF2 (#2332) each purchased from Cell Signaling Technology (Danvers, MA, USA). Membranes were then washed again 3 times for 5 min in TBST and incubated for 1 h in their respective secondary antibody at room temperature and washed again 3 times for 5 min in TBST. Chemiluminescence (Luminata 200 Forte Western HRP Substrate; Merck Millipore, Temecula, CA, USA) was applied to each blot. Images were developed using an ImageQuant LAS 4000 (GE Healthcare, Amersham, UK). Band intensities were quantified using Image Studio Lite (Li-Cor, Lincoln, Nebraska, USA). Phosphorylation levels were determined by the expression of phosphorylated protein divided by the expression of non-phosphorylated total protein. The membranes were stained with Ponceau-S to verify equal loading and used as the normalization control.

Calculations

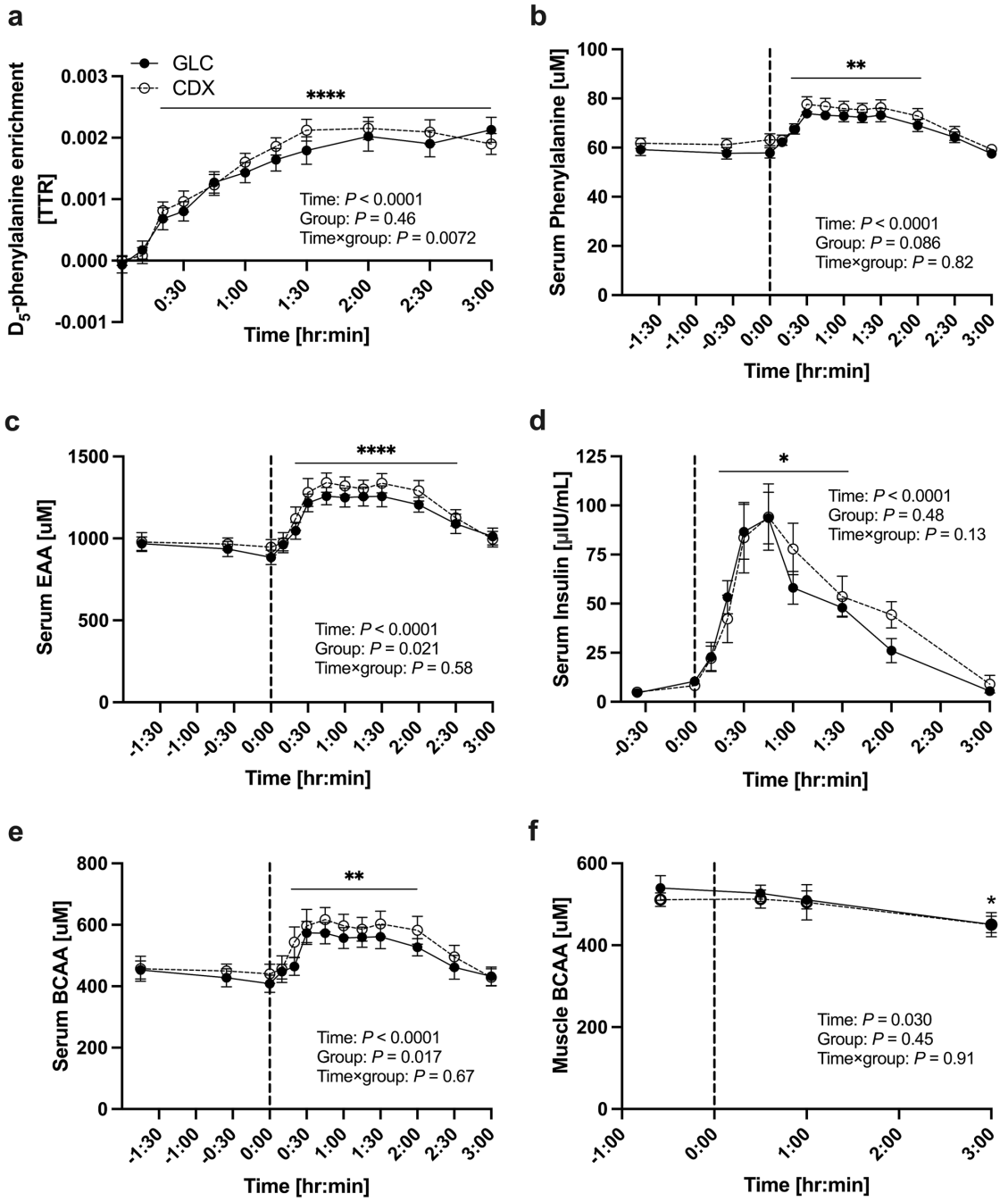
FSR was calculated using the precursor-product method [33]:

$$\mathrm{FSR} = \frac{\mathrm{E}_2 - E_1}{\acute{e}_{t1-t2} * t_{1-2}} * 100, \quad (1)$$

where E is the protein bound enrichment, é is precursor enrichment between two samples estimated from venous serum samples and t is the time between two samples. The FSR will be calculated from 30 to 180 min post exercise.

Statistical analysis

To compare the effect of time within each of the two trials and the two dependent trials (GLC versus CDX), we applied a 2-factor [time × group (GLC compared with CDX)] repeated measures ANOVA when no missing data appeared and a mixed-effects model when data points were missing (insulin concentration measures; one data point was missing (Subject#1, CDX trial, time point 180 min). Turkey's multiple comparisons test was used as a post hoc test to identify the individual differences when there was a time × group interaction effect, a main effect of time, or a main effect of group. Paired student two-tailed *t*-test was used to compare postprandial (0.5–3 h) FSR between CDX and GLC trials, incremental area under the curve (iAUC), and time to reach maximum concentration (T_{max}) for serum D_5-phenylalanine enrichments and serum phenylalanine, EAA, BCAA, and insulin concentrations. iAUC is the definite integral of a curve that depicts the serum parameters as a function of time during the postprandial period using the value for the parameter at time point zero as the baseline value. iAUC was computed using the trapezoid rule. A straight line is connected between adjunct points, and the beneath area was calculated as ΔX*([(Y1 + Y2)/2]-Baseline]. This was repeated for each region, and the sum of the areas was defined as iAUC. T_{max} was obtained from the concentration–time data, where the time of the highest concentration of serum parameters was observed during the postprandial period. A priori power analysis was performed for a matched paired *t*-test (two tails) with an α error probability = 0.05, power (1-β error probability) = 0.8, and Cohen's effect size dz = 1.0 using



G*Power version 3.1 analysis software (Heinrich Hein University). Cohen's effect size dz = 1.0 was calculated based on the least detectable difference of 0.01%/h FSR between groups and the within subject standard deviation of 0.01%/h. This produced a minimum sample size of n = 10. Data are expressed as means ± SD or SEM. An alpha level of 0.05 was used to determine statistical significance. All statistical

◂**Fig. 3** The time course of serum D_5-phenylalanine enrichment (**a**), phenylalanine (**b**), EAA (**c**) insulin (**d**) BCAA (**e**) muscle BCAA (**f**) concentrations. The vertical dot line on each graph (at $t=0$) indicates the transition from postabsorptive to postprandial conditions via the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either 75 g of GLC ($n=10$) or CDX ($n=10$) following a whole-body resistance exercise. Data were analyzed with the use of a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. Values are means ± SEM. Significance was set at $P<0.05$. There was a main effect of time for serum D_5-phenylalanine enrichment, phenylalanine, EAA, BCAA, insulin concentrations and ($P<0.0001$) and muscle BCAA ($P<0.05$). There was a main effect of group (GLC compared with CDX) for serum EAA and BCAA concentrations ($P<0.05$). There was a time × group interaction effect for D_5-phenylalanine enrichment ($P<0.05$). *, **, **** denotes significant difference from basal ($P<0.05$, $P<0.01$, $P<0.0001$, respectively). *TTR* tracer to tracee ratio, *GLC* glucose, *CDX* cluster dextrin, *BCAA* branched-chain amino acids

analysis was performed using GraphPad Prism version 8.4.3 for Mac (GraphPad Software, La Jolla California USA).

Results

Participants

Baseline subject characteristics are shown in Table 1. Figure 2 shows a CONSORT flow diagram describing the progress from recruitment through completion of the study.

Phenylalanine amino acid tracer enrichment

Serum D_5-phenylalanine (Fig. 3a) enrichment originated from intrinsically labeled meat protein hydrolysate was increased following the ingestion of post-exercise drink at $t=0$ (a main effect of time, $P<0.0001$) between 20 and 180 min ($P<0.0001$). There was no main effect of group for serum D_5-phenylalanine enrichment ($P=0.46$). There was a time × group interaction effect ($P=0.0072$). The enrichment of the infused tracer, L-[ring-^{15}N]-phenylalanine (Fig. 4a), was elevated above basal value ($t=0$) following the tracer infusion 20–180 min (a main effect of time, $P<0.0001$). There was no main effect of group ($P=0.27$) and time × group interaction effect ($P=0.067$).

Serum amino acid concentrations

Serum phenylalanine concentrations (Fig. 3b) were increased compared to basal value ($t=0$) from 20 to 120 min ($P<0.01$) following the intake of post-exercise drink (a main effect of time, $P<0.0001$). There was no main effect of group for serum phenylalanine ($P=0.086$). Serum EAA concentrations (Fig. 3c) were increased compared to basal value ($t=0$) from 20 to 150 min ($P<0.0001$) following the intake of post-exercise drink (a main effect of time, $P<0.0001$). There was a main effect of group for serum EAA ($P=0.021$). Likewise, serum BCAA concentrations (Fig. 3e) were increased above basal value ($t=0$) between 20 and 120 min ($P<0.01$) with an overall time effect ($P<0.0001$). There was a main effect of group for serum BCAA concentrations ($P=0.02$). In contrast, there was a main effect of time ($P<0.05$) for muscle BCAA concentrations (Fig. 3f), and it was decreased at 180 min ($P<0.05$) following the intake of post-exercise drink compared to basal value (t = − 35 min). There was no main effect of group for muscle BCAA ($P=0.45$). There was no time × group interaction effect for serum EAA ($P=0.58$), phenylalanine ($P=0.82$), BCAA concentrations ($P=0.67$), and muscle BCAA concentrations ($P=0.91$).

Serum insulin concentrations

Serum insulin concentrations (Fig. 3d) were increased above basal value ($t=0$) between 20 and 90 min ($P<0.05$) following the intake of post-exercise drink (an overall time effect, $P<0.0001$). There was no main effect of group (GLC compared with CDX) for serum insulin concentrations ($P=0.48$). There was no time × group interaction effect for insulin concentrations ($P=0.13$).

iAUC and T_{max} in serum parameters

iAUC and T_{max} serum D_5-phenylalanine, phenylalanine, EAA, BCAA, and insulin are displayed in Table 3. T_{max} serum BCAA was higher in CDX compared to GLC ($P<0.05$). T_{max} serum EAA showed a trend towards statistical significance favoring CDX over GLC ($P=0.051$).

Myofibrillar protein fractional synthesis rate

Postprandial myofibrillar FSR was calculated using the average serum L-[ring-^{15}N]-phenylalanine enrichments as the precursor pool (Fig. 4b). Myofibrillar protein FSR between 30 and 180 min was not different between GLC and CDX (0.0862 ± 0.0137 and $0.1026 \pm 0.0093\% \bullet h^{-1}$, respectively, $P=0.17$).

Intracellular signaling

The time-dependent changes of intracellular signaling are displayed in Fig. 5 and representative western blot images are shown in Fig. 6. The phosphorylation of p70S6K^{Thr389} (Fig. 5b), rpS6$^{Ser240/244}$ (Fig. 5d), ERK1/2$^{Thr202/\ Tyr204}$ (Fig. 5f), AMPKα^{Thr172} (Fig. 5g) showed a time × group interaction effect ($P<0.05$). There was a main effect of time ($P<0.05$) for Akt^{Ser473} (Fig. 5a), p70S6K^{Thr389} (Fig. 5b), 4E-BP1$^{Thr37/46}$ (Fig. 5c), rpS6$^{Ser240/244}$ (Fig. 5d), eEF2^{Thr56} (Fig. 5e), ERK1/2$^{Thr202/Tyr204}$ (Fig. 5f), AMPKα^{Thr172}

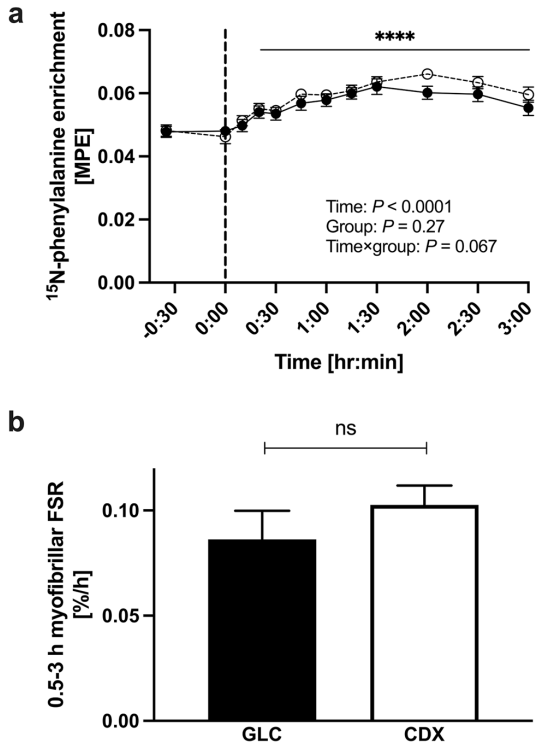


Fig. 4 The time course of serum ^{15}N-phenylalanine enrichment (**a**) and myofibrillar FSR over a 2.5-h postprandial period (**b**). The vertical dot line on each graph (at $t=0$) indicates the transition from post-absorptive to postprandial conditions via the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either 75 g of GLC ($n=10$) or CDX ($n=10$) following a whole-body resistance exercise. Serum ^{15}N-phenylalanine enrichment was analyzed with the use of a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. Values are means ± SEM. Significance was set at $P<0.05$. There was a main effect of time for serum ^{15}N-phenylalanine enrichment ($P<0.0001$). Myofibrillar FSR was analyzed with the use of a paired *t*-test (two-tailed). $n=10$/group. Values are means ± SEM. Significance was set at $P<0.05$. Analysis revealed no statistical difference between GLC and CDX ($P=0.17$). *MPE* mole percent excess, *FSR* fractional synthesis rate, *GLC* glucose, *CDX* cluster dextrin

Table 3 iAUC and T_{max} serum D_5-phenylalanine, phenylalanine, EAA, BCAA, and insulin in response to meat protein hydrolysate intake with either GLC or CDX

	GLC	CDX	*P* value
D_5-phenylalanine enrichment			
iAUC, TTR min	0.0048 ± 0.00033	0.0050 ± 0.00029	0.46
T_{max}, min	133.5 ± 13.5	120 ± 11.0	0.51
Phenylalanine			
iAUC, µM min	32.1 ± 2.3	27.0 ± 2.9	0.099
T_{max}, min	75.0 ± 7.7	69.0 ± 11.7	0.68
EAA			
iAUC, µM min	825.2 ± 54.6	810.1 ± 64.2	0.84
T_{max}, min	84.0 ± 7.8	64.5 ± 9.8	0.051
BCAA			
iAUC, µM min	335.8 ± 24.7	342.4 ± 37.4	0.87
T_{max}, min	70.5 ± 11.0	47.0 ± 8.0	0.049*
Insulin			
iAUC, µIU/mL min	94.6 ± 10.8	119.0 ± 17.4	0.098
T_{max}, min	51.0 ± 8.4	39.5 ± 3.7	0.29

All values are presented as means ± SEM. $n=10$. Paired student two-tailed *t* test was used to compare between GLC and CDX. Significance was set at $P<0.05$

iAUC incremental area under the curve, T_{max} time to reach maximum concentration, *EAA* essential amino acids, *BCAA* branched-chain amino acids, *CDX* cluster dextrin, *GLC* glucose, *TTR* tracer to tracee ratio, *SEM* standard error of mean, *IU* international unit

*Significant difference between groups ($P<0.05$)

(Fig. 5g). There was a main effect of group ($P<0.05$) for Akt^{Ser473} (Fig. 5a), p70S6K^{Thr389} (Fig. 5b), rpS6$^{Ser240/244}$ (Fig. 5d), ERK1/2$^{Thr202/Tyr204}$ (Fig. 5f). The phosphorylation of Akt^{Ser473} (Fig. 5a) was increased from baseline at all time points ($P<0.05$). The phosphorylation of p70S6K^{Thr389} (Fig. 5b) was increased from baseline at 30 min and 60 min in CDX ($P<0.05$), and it was greater in CDX (83.8-fold) than GLC (18.3-fold) at 60 min ($P<0.05$). The phosphorylation of 4E-BP1$^{Thr37/46}$ (Fig. 5c) was increased from baseline at 30 min and 60 min ($P<0.05$ and $P<0.0001$, respectively). The phosphorylation of rpS6$^{Ser240/244}$ (Fig. 5d) was increased from baseline at all time points in CDX group ($P<0.05$), and it was greater in CDX than GLC ($P<0.05$) at 60 min (6.6- vs 16.7-fold) and 180 min (5.6- vs 16.2-fold). The phosphorylation of eEF2^{Thr56} (Fig. 5e) was decreased from baseline at 60 min ($P<0.01$). The phosphorylation of ERK1/2$^{Thr202/Tyr204}$ (Fig. 5f) was increased from baseline at all time points ($P<0.05$), and CDX was greater as compared to GLC at all time points (1.1- vs 1.7-fold, 1.2- vs 1.6-fold, 0.9- vs 1.6-fold for 30, 60, 180 min, respectively, $P<0.05$). The phosphorylation of AMPKα^{Thr172} (Fig. 5g) was greater in CDX than GLC at 180 min (0.9- vs 1.3-fold, $P<0.05$).

Discussion

In the present study, we simultaneously assessed orally ingested protein-derived amino acid availability in the circulation using intrinsically labelled meat protein hydrolysate (D_5-phenylalanine) and myofibrillar protein synthesis of the vastus lateralis muscle after a whole-body resistance exercise in moderately trained younger males. Co-ingestion of CDX with meat protein hydrolysate did not enhance the total availability of protein-derived amino acids in the circulation as determined by the enrichment of serum D_5-phenylalanine

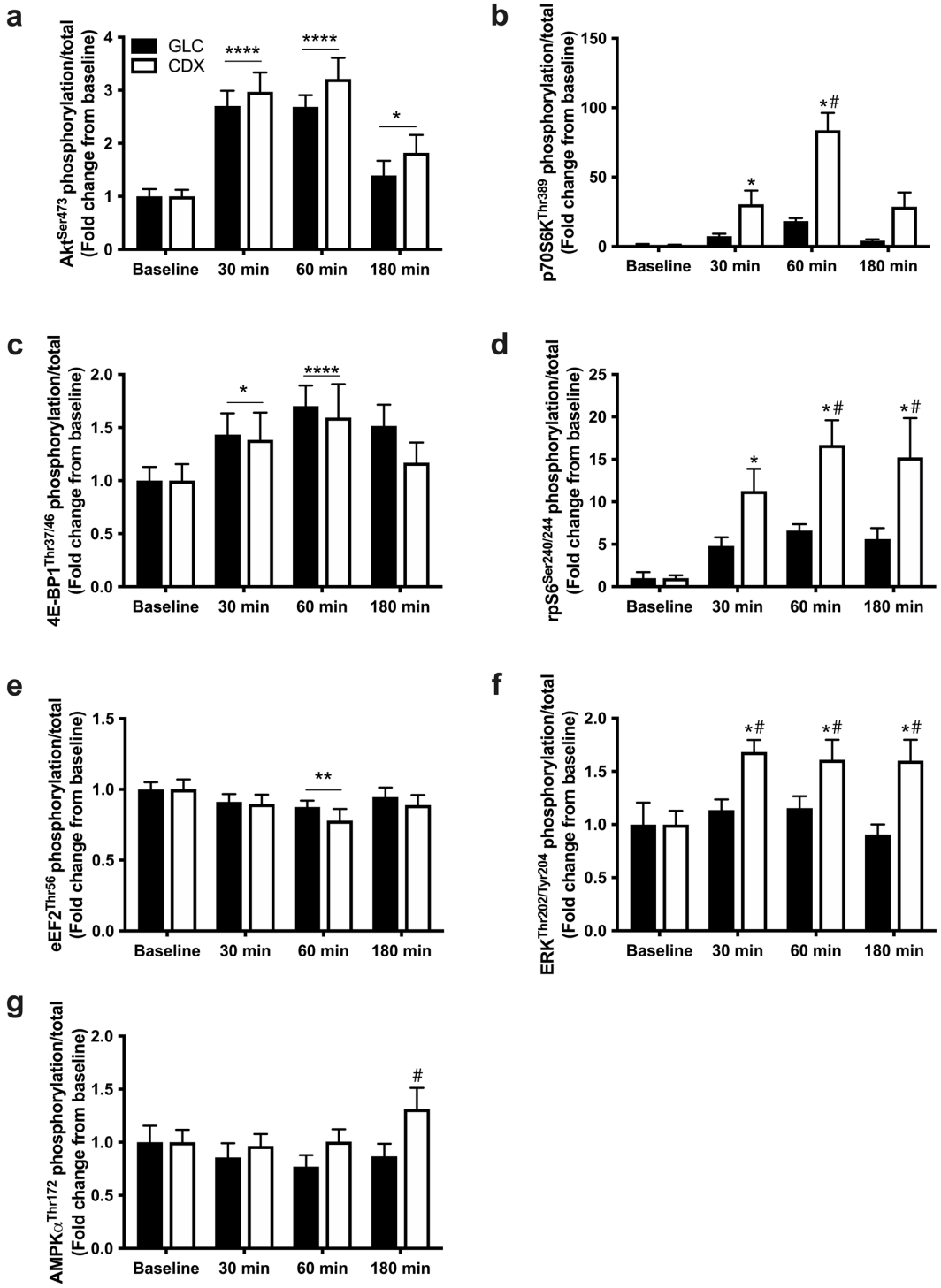
derived from intrinsically labelled meat-protein, serum phenylalanine, EAA, BCAA, and muscle BCAA concentrations compared with GLC. Interestingly, the activation of mTORC1 signaling was higher in CDX than GLC following a whole-body resistance exercise. However, the enhanced activation of mTORC1 signaling did not increase postprandial myofibrillar FSR in CDX as compared to GLC.

Amino acid availability in the circulation is a determinant of muscle protein synthesis [10, 11]. It has been well established that protein/amino acids ingestion stimulates muscle protein synthesis following the recovery from resistance exercise [2, 12, 14, 34–36]. In the previous studies, carbohydrate co-ingestion with protein was hypothesized to augment insulin secretion and enhance muscle protein synthesis following an acute bout of resistance exercise [15–17]. However, we and others previously demonstrated that amino acid availability is decreased when protein is ingested with carbohydrate [15–17] or other macronutrients [18, 19]. The nature of rapid gastric emptying of CDX [27] could be used as an approach to attenuate lower amino acid availability when protein is consumed with GLC. To our knowledge, this is the first study to investigate whether CDX ingestion together with the intake of protein hydrolysate (here meat protein-derived) enhanced the availability of constituent amino acids in the circulation as compared to GLC ingestion. We made use of a previously produced intrinsically labelled meat protein hydrolysate [18] to directly measure exogenous protein-derived amino acid availability (with D_5-phenylalanine as a tracer) in the present study.

For the main outcome D_5-phenylalanine enrichment tracer, it appeared in serum from 20 min post-exercise and was maintained until 180 min post-exercise in both CDX and GLC groups (Fig. 3a) and iAUC (total availability) of the tracer did not differ between CDX and GLC (Table 3). Further, T_{max} for the tracer was not affected by CDX as compared to GLC. The group means for EAA and BCAA at $t = -$ 0:30 and $t = 0$ (Fig. 3) appeared different and were likely to cause the main effect of group in the two-way ANOVA test. Therefore, we further explored if there were any postprandial differences by calculating iAUC from $t = 0$, which revealed no difference for the postprandial rise in EAA and BCAA concentrations between CDX and GLC (Table 3). With these data in mind, the time × group interaction effect for D_5-phenylalanine enrichment (Fig. 3a, $P = 0.0072$) is hard to explain. Visually, it seems though that the D_5-phenylalanine enrichment peaks and starts to decrease within the 3-h postprandial period in CDX, whereas it remains high in GLC. However, this is speculative, and more investigations are needed to enlighten this further. Of interest is though, that the T_{max} serum BCAA showed a statistical significance ($P = 0.049$) and T_{max} serum EAA showed a trend towards a statistical significance ($P = 0.051$, Table 3) although this is not the case for D_5-phenylalanine. However, a flux of different amino acids is regulated by different amino acid transport mechanisms [37], and hence, phenylalanine may not be a representative tracer for the clearance of all amino acids. Overall, we conclude that co-ingestion of CDX with meat protein hydrolysate did not markedly affect serum amino acid availability during the three hours of recovery from a whole-body resistance exercise.

In the present study, insulin was increased above baseline between 30 and 90 min post-exercise drink with no difference between CDX and GLC (Fig. 3d). Previously, studies have shown that CDX intake alone does not affect glucose concentrations as compared to glucose [38] or maltodextrin [39]. Although some amino acids are insulinotropic [40, 41] and a higher insulin secretion is observed when protein is consumed in combination with carbohydrate [15–17] compared to protein alone, the similar serum amino acid concentrations in CDX and GLC groups in this study reject the expectations for the insulin concentrations to be different. However, due to the lack of a "meat protein hydrolysate alone" group in the present study, we cannot conclude whether the co-ingestion of CDX or GLC increased insulin concentration above levels induced by the meat protein hydrolysate alone, although this must be anticipated. The roles of insulin on muscle protein turnover have long been debated. Systematic reviews have concluded that insulin does not have a stimulatory or inhibitory effect on muscle protein synthesis [23], but instead plays an important role in attenuating muscle protein breakdown independent of amino acid availability [42]. The absence of increased muscle BCAA concentrations at 30 and 60 min (where serum concentrations are elevating and peaking, respectively) reveals that the intramuscular disappearance rate of amino acids equals the influx. We did not follow the amino acid tracers further in the intramuscular metabolic pathways, but we suggest that muscle protein synthesis and energy metabolism are responsible for the utilization of excess amino acids. This is due to the concomitant intramuscular availability of glucose, which would meet the major requirement for energy production, dampening the anaplerotic processes. The drop at 180 min in the muscle BCAA concentrations (Fig. 3f) agrees with serum EAA (Fig. 3c), phenylalanine (Fig. 3b), BCAA (Fig. 3e), and insulin concentrations (Fig. 3d) as these substrates had all returned to basal levels. Hence, it is likely that participants were in the postabsorptive period around 180 min post-exercise drink, and that the muscle BCAA pool at that time point was drained by either a net outflux into the circulation or by a request from translation processes or both [43].

It is generally agreed that 20 g of high-quality protein intake is required following resistance exercise (e.g., leg press and knee extension) to maximally stimulate muscle protein synthesis in younger individuals [2, 4]. However, Macnaughton et al. [28] reported findings that may suggest


a
Akt^{Ser473} phosphorylation/total (Fold change from baseline)
GLC
CDX
****
****
*
Baseline
30 min
60 min
180 min
b
p70S6K^{Thr389} phosphorylation/total (Fold change from baseline)
*
*#
c
4E-BP1$^{Thr37/46}$ phosphorylation/total (Fold change from baseline)
*
****
d
rpS6$^{Ser240/244}$ phosphorylation/total (Fold change from baseline)
*
*#
e
eEF2^{Thr56} phosphorylation/total (Fold change from baseline)
**
f
ERK$^{Thr202/Tyr204}$ phosphorylation/total (Fold change from baseline)
*#
g
AMPKα^{Thr172} phosphorylation/total (Fold change from baseline)
#

◂**Fig. 5** The phosphorylation of Akt^{Ser473} (**a**), $p70S6K^{Thr389}$ (**b**), $4E\text{-}BP1^{Thr37/46}$ (**c**), $rpS6^{Ser240/244}$ (**d**), $eEF2^{Thr56}$ (**e**), $ERK1/2^{Thr202/Tyr204}$ (**f**), $AMPK\alpha^{Thr172}$ (**g**) at 30, 60, 180 min after the ingestion of meat protein hydrolysate (0.6 g protein * FFM^{-1}) with either GLC ($n=10$) or CDX ($n=10$). Data were analyzed with the use of a 2-factor [time × group (GLC compared with CDX)] ANOVA with Turkey's multiple comparisons test to locate individual differences. The data were expressed relative to baseline. Values are means ± SEM. There was a main effect of time for Akt^{Ser473}, $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, $4E\text{-}BP1^{Thr37/46}$, $eEF2^{Thr56}$, $ERK1/2^{Thr202/Tyr204}$, and $AMPK\alpha^{Thr172}$ ($P<0.05$). There was a main effect of group (GLC compared with CDX) for Akt^{Ser473} $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, $ERK1/2^{Thr202/Tyr204}$ ($P<0.05$). There was a time × group interaction effect for $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, $ERK1/2^{Thr202/Tyr204}$, $AMPK\alpha^{Thr172}$ ($P<0.05$). Significance was set at $P<0.05$. *, **, **** denotes significant difference from baseline in respective group ($P<0.05$, $P<0.01$, $P<0.0001$). # indicates significant difference between CDX and GLC at the same time point ($P<0.05$). *GLC* glucose, *CDX* cluster dextrin

the amount of protein required to stimulate muscle protein synthesis may depend on the amount of muscle recruited during resistance exercise. This is because the demand for exogenous amino acids might be increased when more muscles are used during resistance exercise (whole-body vs unilateral leg) although the amount of lean body mass (LBM) itself does not affect muscle protein synthesis [28]. Macnaughton et al. [28] provided either 0.34 g or 0.68 g protein * LBM^{-1} whey protein in the lower LBM group (59 kg LBM on average), and either 0.26 g protein or 0.52 g protein * LBM^{-1} in the higher LBM group (77 kg LBM on average). In the present study, participants performed a whole-body resistance exercise followed by meat protein hydrolysate (0.6 g * FFM^{-1}) intake, which ended up as a mean of 36.3 g of meat protein hydrolysate (range 32.0 to 41.6 g) to our participants, which should provide a stimulus to maximally stimulate myofibrillar FSR following a whole-body resistance exercise. However, as we did not measure the baseline myofibrillar FSR we cannot say whether the myofibrillar FSR was enhanced by an acute bout of whole-body resistance exercise.

Akt/mTORC1 signaling pathway is crucial for muscle protein synthesis and skeletal muscle hypertrophy [7, 44, 45]. Previous studies showed that protein/amino acid feeding [7, 46–48], resistance exercise [35, 49–52], or a combination of both [12, 14, 35, 36, 53] activate Akt/mTORC1 signaling in younger individuals. In line, our whole-body resistance exercise protocol with post-exercise meat protein hydrolysate ingestion increased the phosphorylation of Akt^{Ser473}, $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, $4E\text{-}BP1^{Thr37/46}$, and $ERK1/2^{Thr202/Tyr204}$ and decreased the phosphorylation of $eEF2^{Thr56}$ over the course of 180 min post-exercise period (a main effect for time, $P<0.05$). Interestingly, the phosphorylation of $p70S6K^{Thr389}$, $rpS6^{Ser240/244}$, and $ERK1/2^{Thr202/Tyr204}$ was greater in CDX compared to GLC during the recovery from a whole-body resistance exercise (a time × group interaction effect, $P<0.05$), indicating an enhanced translation initiation and elongation in CDX. However, the enhanced mTORC1 signaling did not result in an increased postprandial myofibrillar FSR in CDX (Fig. 4), which is in line with previous studies that demonstrated that co-ingestion of carbohydrate does not further increase FSR compared to protein intake alone [15–17]. The absence of an enhanced postprandial myofibrillar FSR in CDX despite the increased mTORC1 signaling could be explained by no changes of amino acids availability in the circulation as well as muscle BCAA concentrations (Fig. 3). Previous studies have shown that metabolic flux in vivo cannot be predicted by intracellular signaling [54] or mRNA expression level [55]. In support, dissociation between Akt/mTORC1 signaling and muscle protein synthesis in response to amino acids and insulin was previously reported by Greenhaff et al. [56].

The present randomized controlled crossover trial is a robust study design with high statistical power. However, the absence of a meat protein hydrolysate group alone makes it impossible to reveal any effects of CDX or GLC per se, which could have been interesting now that the hypothesized beneficial effects of CDX could not be verified.

Conclusions

In moderately trained younger males, co-ingestion of CDX with meat protein hydrolysate does not enhance the availability of protein-derived amino acids and myofibrillar FSR as compared to GLC with meat protein hydrolysate during the recovery from a whole-body resistance exercise despite an increased intramuscular signaling.

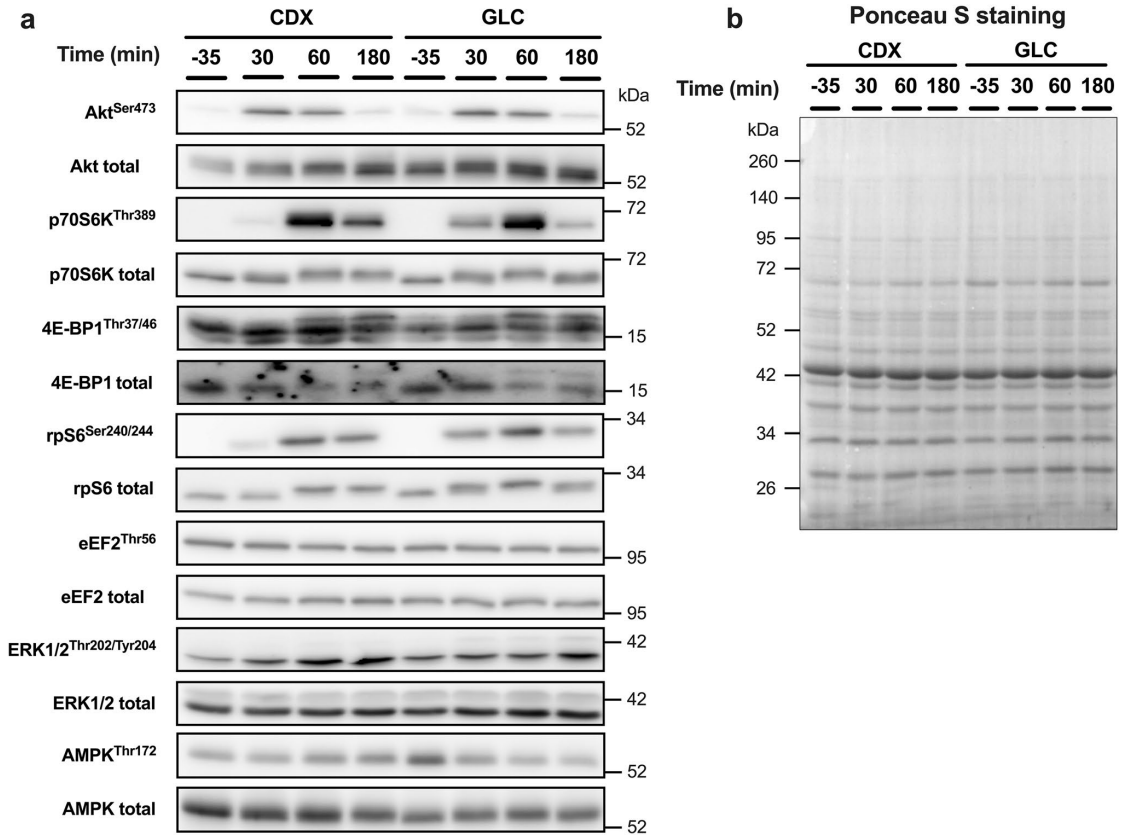


Fig. 6 Representative western blot images for intracellular signaling (**a**) and Ponceau-S (**b**). *CDX* cluster dextrin, *GLC* glucose

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Availability of data and material The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This study was approved by the Ethics Committee of the Capital Region (H-17017363) and adhered with the Helsinki II declaration.

Consent to participate Before inclusion, each participant was informed of the purpose of the study, experimental procedures, and potential risks prior to obtaining written informed consent.

Consent for publication All authors read the final version of the manuscript and approved for publication.

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