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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 activity (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 quality control, causing the accumulation of damaged and misfolded 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 degradation is crucial for regulating protein turnover and protein quality control in skeletal muscle.

The lack of studies on protein degradation from a molecular 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 spectrometry (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 mechanisms in protein degradation.

Recent studies began to unravel such a complex regulating 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 indicated that protein turnover rate decreased under aging and/or physical inactivity conditions (34–36), resulting in accumulated misfolded/damaged proteins and impaired protein quality control (37, 38). Exercise promotes protein turnover (6, 8, 9, 34), which facilitates the removal of misfolded/damaged 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 ubiquitin 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 discuss recent findings on how exercise modifies ubiquitin signaling 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 pathways, 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)

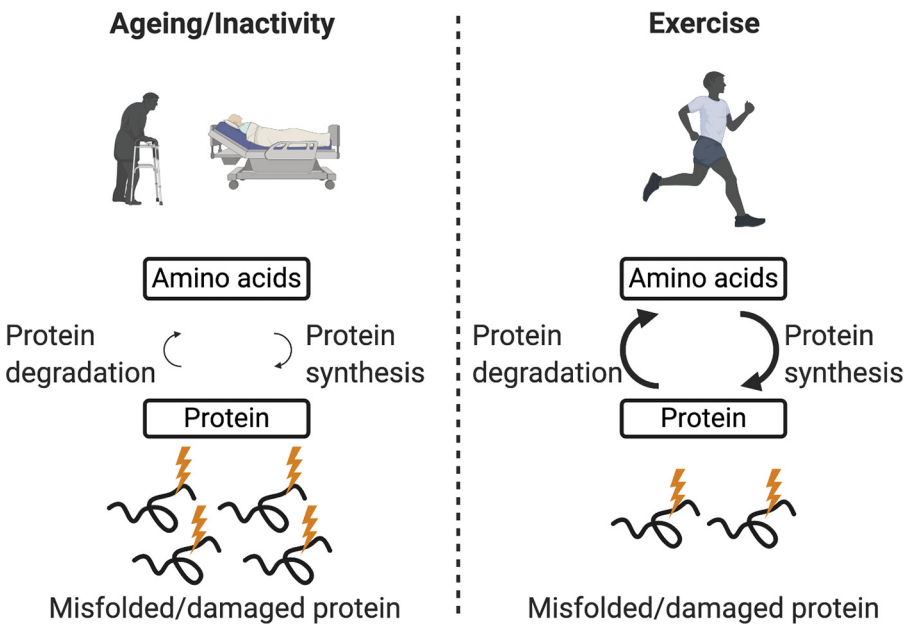

Figure 1. Proposed concept of the maintenance of protein turnover and protein quality control by exercise. Aging and inactivity result in an inevitable decline in protein turnover rate. This leads to the accumulation of damaged and misfolded proteins due to the inability to remove damaged and misfolded 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.

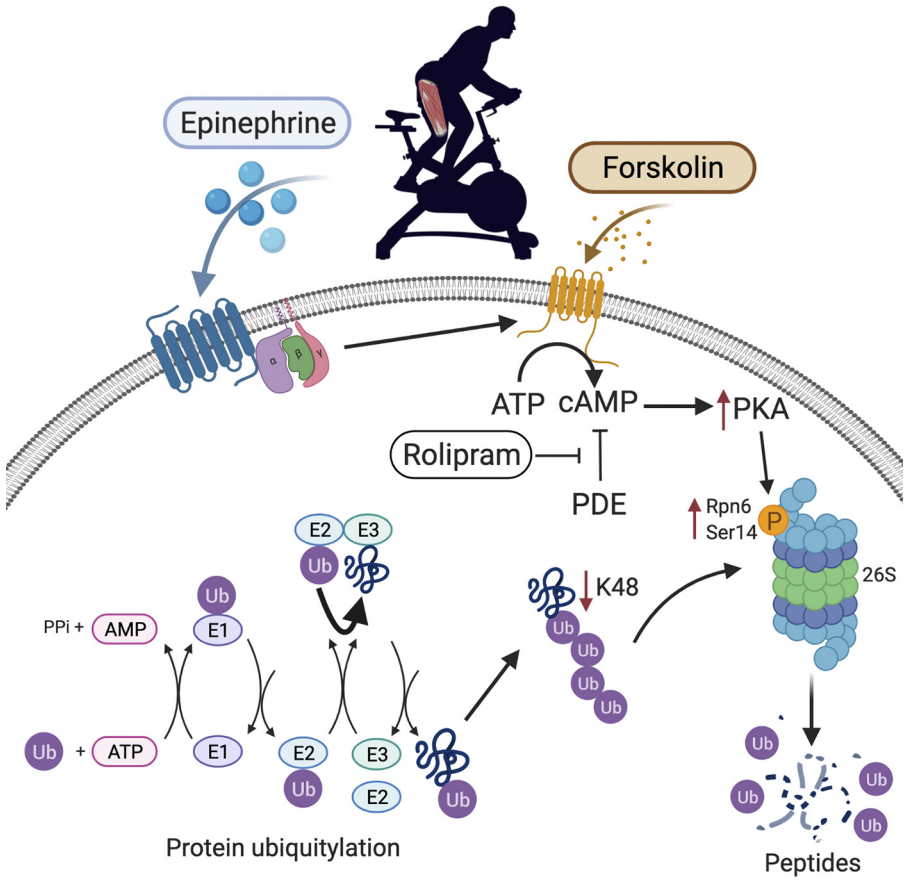

Figure 2. Schematic diagram of the crosstalk between phosphorylation and ubiquitylation in the 26S proteasome-mediated protein degradation in exercising skeletal muscle. Phosphorylation is involved in enhancing the 26S proteasome activity after exercise. Exercise increases epinephrine in circulation, 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

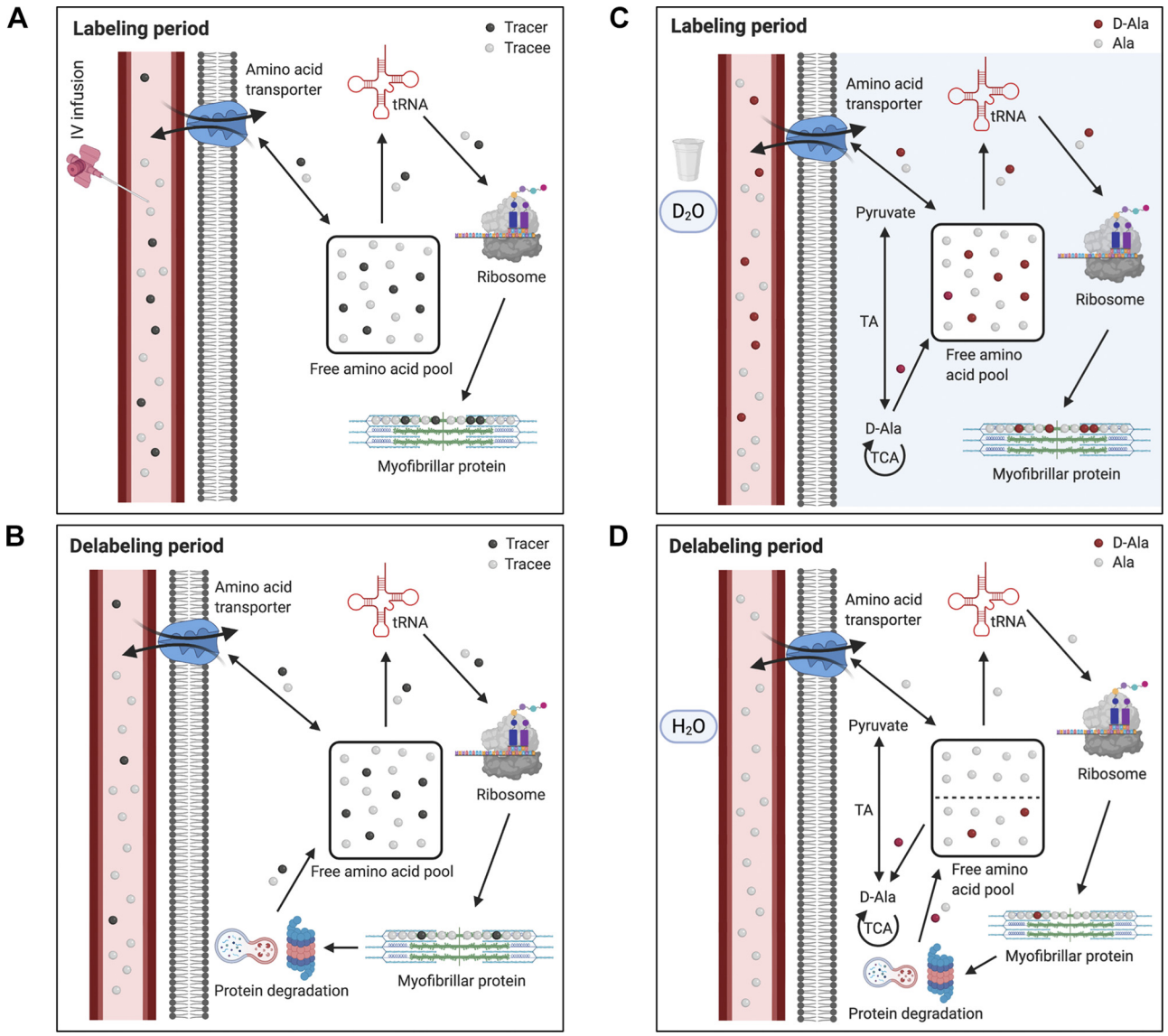

Figure 3. Principle of determining myofibrillar protein degradation using classical stable isotopically labeled amino acid tracers and D_2O. In principle, myofibrillar protein degradation rate can be measured in a two-step manner regardless of the use of classic stable isotopically labeled amino acid 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.

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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

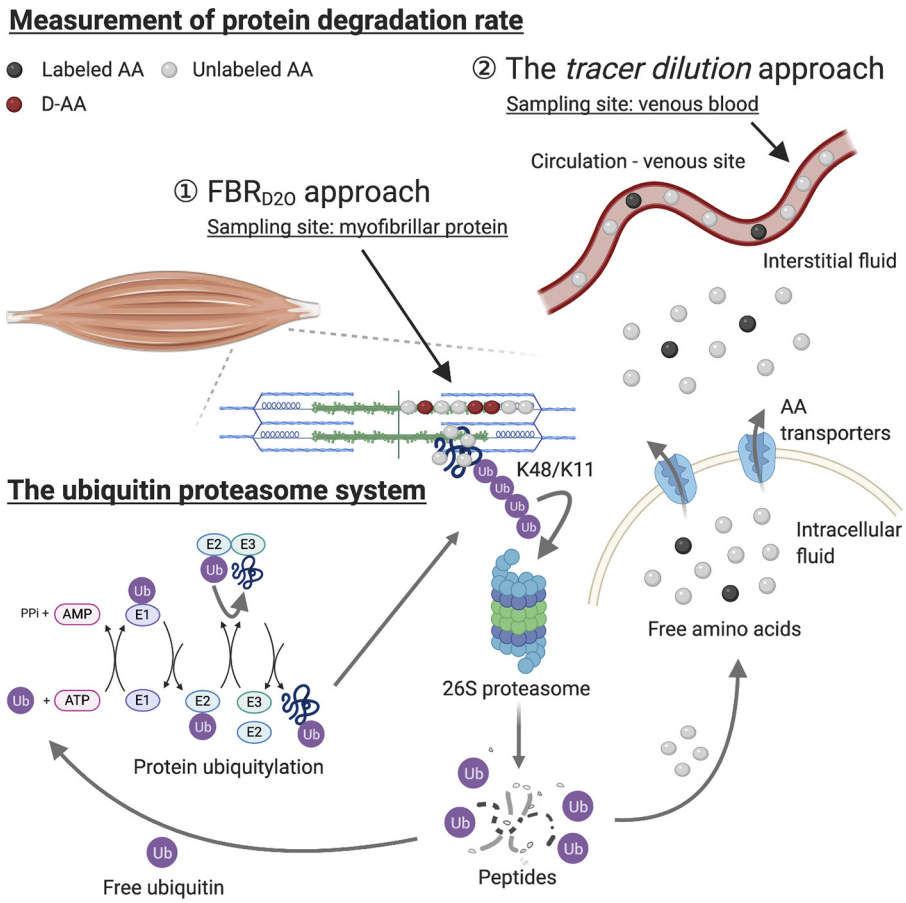

Figure. 4. The tracer-based approaches to measure protein degradation rate and the link to the ubiquitin proteasome system. Different tracer-based approaches inhere a temporal distinction of the measurement of protein degradation. Myofibrillar protein degradation rate can be measured by oral consumption 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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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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