

## The role of ubiquitin–proteasome-dependent proteolysis in the remodelling of skeletal muscle

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In skeletal muscle, as in any mammalian tissue, protein levels are dictated by relative rates of protein synthesis and breakdown. Recent studies have shown that the ubiquitin–proteasome-dependent proteolytic pathway is mainly responsible for the breakdown of myofibrillar proteins. In this pathway proteins that are to be degraded are first tagged with a polyubiquitin degradation signal. Ubiquitination is performed by the ubiquitin-activating enzyme, ubiquitin-conjugating enzymes and ubiquitin–protein ligases, which are responsible for the recognition of specific substrates. Polyubiquitinated protein substrates are then specifically recognised and degraded by the 26S proteasome. The present review focuses on: (1) the mechanisms of ubiquitination–deubiquitination that make the system highly selective; (2) the mechanisms of proteolysis in skeletal muscle. In particular, the role of the system in the remodelling of skeletal muscle during exercise and disuse and in recovery or regeneration that prevails during post-atrophic conditions is reviewed.

### Muscle activity and disuse: Exercise: Muscle wasting: Ubiquitin–proteasome-dependent proteolysis

In skeletal muscle, as in any mammalian tissue, protein levels are dictated by relative rates of protein synthesis and breakdown. Detailed information is available on the mechanisms that regulate protein synthesis in muscle. By contrast, much less is known about proteolysis, mainly because of the lack of reliable techniques for estimating rates of proteolysis *in vivo* and the complexity of protein breakdown. In brief, skeletal muscle is believed to contain at least five proteolytic pathways (i.e. the lysosomal, the Ca<sup>2+</sup>-activated and the ubiquitin (Ub)–proteasome-dependent systems, caspases and matrix metalloproteinases). Unfortunately, the precise substrates of these systems are still poorly characterised. However, both lysosomal and Ca<sup>2+</sup>-activated proteases (i.e. cathepsins and calpains respectively) are not directly responsible for the breakdown of myofibrillar proteins (Attaix & Taillandier, 1998; Jagoe & Goldberg, 2001; Hasselgren *et al.* 2002). Furthermore, a systematic activation of either cathepsins (except cathepsin L; see Deval *et al.* 2001; Jagoe *et al.* 2002) or calpains is not observed in all muscle-wasting conditions (for detailed information, see Attaix & Taillandier, 1998; Jagoe *et al.* 2002). By contrast, the activation of the Ub–proteasome

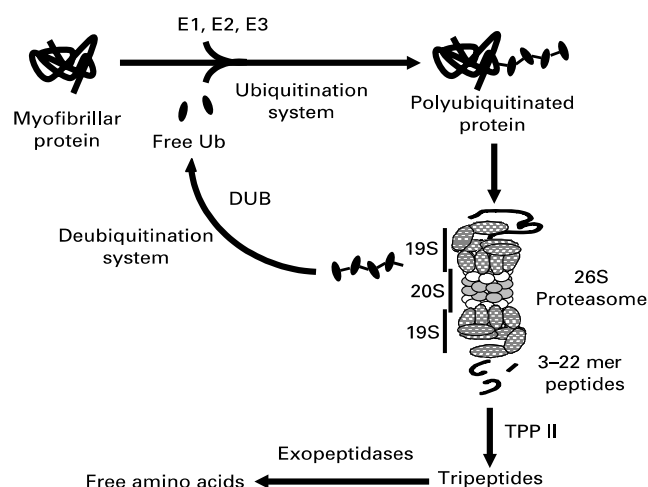
pathway is mainly responsible for the muscle loss that occurs in various animal models of wasting, except muscular dystrophies (Attaix & Taillandier, 1998; Jagoe & Goldberg, 2001; Hasselgren *et al.* 2002; Attaix *et al.* 2003), and in patients when muscle wasting is rapid and pronounced (Mansoor *et al.* 1996; Bossola *et al.* 2003). In addition, this system is critical for the breakdown of myofibrillar proteins (Attaix & Taillandier, 1998; Jagoe & Goldberg, 2001; Hasselgren *et al.* 2002). In the present paper the mechanisms of ubiquitination and proteasome-dependent proteolysis will be critically reviewed. The regulation of this proteolytic machinery in the remodelling of skeletal muscle will then be discussed, with a particular emphasis on its control during exercise and disuse and during repair and regeneration.

### Ubiquitin–proteasome-dependent proteolysis

The Ub–proteasome pathway is the major non-lysosomal process responsible for the breakdown of most short- and long-lived proteins in eukaryotic cells. Several hundred genes, including genes of the ubiquitination–deubiquitination system (approximately 200–300) and

**Abbreviations:** E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin–protein ligase; RING finger E3, really interesting new gene finger E3; Ub, ubiquitin.

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**Fig. 1.** Schematic representation of the breakdown of myofibrillar proteins. The ubiquitination enzymes, ubiquitin (Ub)-activating enzyme (E1), Ub-conjugating enzymes (E2) and Ub-protein ligases (E3), tag disassembled myofibrillar proteins with a polyUb degradation signal. The polyUb chain binds to the 19S complex (19S) of the 26S proteasome. ATPases within 19S provide energy for the recognition of the polyUb degradation signal, unfolding of the substrate, the gating of the 20S proteasome (20S) channel, the injection and progression of the substrate into the catalytic chamber of the 20S proteasome, protein hydrolysis and peptide release. (●), The  $\beta$  catalytic subunits of the 20S proteasome; (○), the  $\alpha$  non-catalytic subunits of the 20S proteasome. The polyUb chain is recycled into free Ub by the deubiquitinating activity intrinsic to the 19S complex and/or deubiquitinating enzymes (DUB). The final hydrolysis of the peptides generated by the 26S proteasome into free amino acids is then sequentially performed by the tripeptidyl peptidase II (TPP II) and exopeptidases.

genes (approximately fifty) encoding different proteasome subunits or endogenous proteasome activators and inhibitors, control this complex proteolytic machinery. The pathway carries out housekeeping functions in basal protein turnover and the elimination of abnormal proteins (e.g. miscoded, misfolded or mislocalised proteins). In addition, the system also controls several major biological functions that include class I antigen presentation, signal transduction, protein sorting and the control of transcription and of cell division (Glickman & Ciechanover, 2002). Basically, there are two main steps in the pathway: (1) covalent attachment of a polyUb chain to the substrate; (2) specific recognition of the polyUb degradation signal and subsequent breakdown of the targeted protein by the 26S proteasome (Fig. 1).

#### Ubiquitination–deubiquitination

Ubiquitination is the covalent attachment of Ub (a seventy-six amino acid polypeptide) to a protein substrate. This widespread post-translational modification has diverse proteolytic and non-proteolytic functions. Importantly, only substrates tagged with a polyUb degradation signal that consists of at least four Ub moieties are degraded by the 26S proteasome (Attaix *et al.* 2003). Polyubiquitination is a complex multiple-step process. Ub is initially activated

by the Ub-activating enzyme (E1). E1 then transfers the activated Ub to one of the Ub-conjugating enzymes (E2). E2 bind the first Ub molecule to protein substrates through the formation of an isopeptide bond that results in the formation of a monoubiquitinated protein. Some E2 catalyse the continued addition of Ub molecules to form polyubiquitinated conjugates. However, the formation of polyUb chains generally requires the presence of a Ub–protein ligase (E3) that specifically recognises one or a few protein substrates.

There is a single E1, which is an extremely-active enzyme capable of charging excess amounts of E2 with Ub, providing activated Ub for the entire downstream Ub conjugation pathways. By contrast, there are at least twenty to thirty E2 in mammals, but only a limited number of E2 play a role in the formation of the polyUb degradation signal. The formation of polyUb chains is generally achieved in the presence of an E3 enzyme. Most E2 interact with several E3 (and conversely), which in turn recognise their specific protein substrates, and a given protein substrate can be ubiquitinated by different combinations of E2 and E3. This enzyme system results in a wide range of alternative ubiquitination pathways (Attaix *et al.* 2003).

E3 play a critical role in polyubiquitination, as they are responsible for the selective recognition of protein substrates. All known E3 are either homologous to E6-AP C-terminus domain E3, really interesting new genes (RING) finger E3, or U-box-containing E3. Very few U-box E3 have been characterised so far. By contrast, mammalian genome-sequencing projects have identified numerous potential uncharacterised homologous to E6-AP C-terminus E3. In addition, there are several hundred cDNA encoding RING finger proteins in the GenBank database and many unrelated RING finger proteins with unknown functions behave *in vitro* as E3 (Attaix *et al.* 2003). The simplest RING finger E3 are monomeric ‘N-end rule’ enzymes such as E3 $\alpha$ , which binds to proteins bearing basic or bulky hydrophobic N-terminal amino acid residues. However, most RING-finger E3 are high-molecular-weight multi-subunit complexes. These complexes contain adapter subunits, e.g. F-box proteins in the Skp1-Cdc53-F-box E3 family that recognise different substrates through specific protein–protein interaction domains such as leucine-rich repeats or WD-40 domains.

Finally, eukaryotic cells also contain deubiquitinating enzymes that form a huge family with at least ninety member proteins. The putative roles of deubiquitinating enzymes in proteasome-dependent proteolysis are: (1) to maintain free Ub levels by processing polyUb degradation signals and Ub precursors into free monomers; (2) to proof-read and deubiquitinate substrates erroneously tagged for degradation; (3) to keep 26S proteasomes free of bound-polyUb chains (Fig. 1; Attaix *et al.* 2003).

#### The 26S proteasome

The second major step in the Ub–proteasome pathway is the degradation of polyubiquitinated proteins by the 26S proteasome complex. The 26S proteasome is formed by the association of the 20S proteasome with two 19S

regulatory complexes (Fig. 1; for review, see Glickman & Ciechanover, 2002; Attaix *et al.* 2003). The 20S proteasome is the core of the proteolytic machinery. This barrel-shaped particle is organised as a stack of four rings, each ring containing seven subunits (Fig. 1). The non-catalytic  $\alpha$ -subunits form the two outer rings and the catalytic  $\beta$ -subunits form the two inner rings. The  $\beta$ -subunit active sites are located inside the cylinder and thus the proteasome is a self-compartmentalising protease, as substrates must enter the catalytic chamber delimited by the  $\beta$ -rings to be degraded into peptides. In eukaryotes the 20S proteasome contains at least two chymotrypsin-, two trypsin- and two caspase-like active sites. 19S complexes bind to both outer  $\alpha$ -rings of the 20S proteasome. They contain at least six ATPase and twelve non-ATPase subunits. The ATPase subunits provide energy for assembly of the 26S proteasome, gating of the 20S proteasome channel, unfolding and injection of protein substrates into the catalytic chamber of the proteasome, proteolysis and energy for peptide release (Fig. 1). Both the non-ATPase subunit S5a and the ATPase S6' subunit bind to polyUb chains and the latter interaction also depends on ATP hydrolysis (for review, see Attaix *et al.* 2003).

### Regulation of ubiquitin–proteasome-dependent proteolysis in muscle wasting

Goldberg and colleagues (for review, see Jagoe & Goldberg, 2001) have provided the first evidence for a role of the Ub–proteasome pathway in the muscle wasting seen during denervation atrophy and starvation. Most of the increased proteolysis seen in such conditions is ATP-dependent. ATP hydrolysis is required for the activation of Ub by E1 and proteolysis by the 26S proteasome (see above). Similar observations have been reported in other rodent catabolic models and correlated with many alterations in both the ubiquitination and proteasome machineries (Table 1). Importantly, only proteasome inhibitors such as lactacystin or MG132 suppress the enhanced rates

**Table 1.** Adaptations in the ubiquitin–proteasome pathway in various muscle-wasting conditions

Adaptations in the ubiquitination–deubiquitination system	
Increased transcription of ubiquitin	
Increased expression of 14 kDa E2 and E3 $\alpha$ (N-end rule system)	
Increased rates of ubiquitination in soluble muscle fractions blocked by inhibitors of E3 $\alpha$	
Increased expression of muscle-specific E3 (atrogin-1/MAFbx and MuRF-1)	
Increased expression of some deubiquitinating enzymes	
Accumulation of ubiquitin conjugates in the myofibrillar fraction	
Adaptations in the proteasome system	
Increased proteasome-dependent proteolysis	
Increased total or myofibrillar proteolysis blocked by proteasome inhibitors	
Increased proteasome activities	
Increased expression of many subunits of the 20S proteasome	
Increased transcription of subunit C3 of the 20S proteasome	
Increased expression of some subunits of the 19S complex	
Alterations in the protein content of some proteasome subunits	

E2, E3, ubiquitination enzymes.

of overall proteolysis in wasting conditions, including elevated 3-methylhistidine release by incubated atrophying muscles (Jagoe & Goldberg, 2001; Hasselgren *et al.* 2002; Attaix *et al.* 2003). 3-Methylhistidine is formed by post-translational modifications of actin and pale myosins, and its rate of appearance in incubation media only reflects the breakdown of these myofibrillar proteins (Attaix & Taillandier, 1998). Further strong support for a major role of the Ub–proteasome system is the demonstration that knock-out mice for the muscle-specific E3 atrogin-1/MAFbx and MuRF-1 are partially resistant to muscle atrophy (Bodine *et al.* 2001). Thus, it is now clear that Ub–proteasome-dependent proteolysis plays a critical role in the control of muscle mass.

### Regulation of ubiquitin–proteasome-dependent proteolysis with exercise and disuse and in muscle recovery or regeneration

#### *Eccentric exercise*

Eccentric exercise results in skeletal muscle damage and stimulates muscle proteolysis. In accord with observations made in muscle-wasting conditions (i.e. trauma, cancer and sepsis) eccentric exercise in human subjects (e.g. damaging eccentric–isokinetic actions of the biceps muscle with the non-dominant arm, or leg press then knee extension) results in increased levels of Ub conjugates in muscle biopsies (Thompson & Scordilis, 1994; Stupka *et al.* 2001). Accordingly, proteasome enzyme activities increase after 14 d of eccentric exercise in vastus lateralis muscle biopsies from healthy volunteers (Feasson *et al.* 2002).

#### *Endurance exercise*

Endurance exercise is known to have hypertrophic effects in skeletal muscle. Kee *et al.* (2002) have reported that in incubated muscles harvested from rats 24 h after five consecutive days of progressive treadmill exercise proteolysis is markedly reduced compared with that of unexercised rats. This reduction is associated with a reduction in the chymotrypsin-like activity of the proteasome and the rate of Ub–proteasome-dependent casein hydrolysis in muscle extracts from exercised rats compared with unexercised rats. In contrast, exercise has no effect on the rates of total mixed muscle protein synthesis in incubated muscles 24 h post exercise. These results suggest that suppression of Ub–proteasome proteolysis after endurance exercise may contribute to the acute post-exercise net protein gain. Furthermore, passive leg cycling for 12 weeks in patients with spinal cord injury, which has anabolic effects, increases expression of type IIa and IIx myosin heavy chains and decreases the expression of Ub, an E2 and a 20S proteasome subunit (Willoughby *et al.* 2000).

#### *Disuse*

Immobilisation, denervation and hindlimb suspension in rodents are highly catabolic treatments. For example, exposure to microgravity and hindlimb suspension result in

severe atrophy of anti-gravity muscles (e.g. soleus) accompanied by various metabolic modifications of the contractile apparatus that comprises the synthesis of new myofibrillar protein isoforms (Taillandier *et al.* 2003). Taillandier *et al.* (1996) have shown that 9 d of hindlimb suspension results in atrophy (–55%) and loss of protein (–53%) in rat soleus muscle as a result of a marked elevation in non-lysosomal Ca<sup>2+</sup>-independent proteolysis. Accordingly, increased mRNA levels for Ub, the 14 kDa E2 and the C2 and C9 subunits of the 20S proteasome are observed in the atrophying unweighted muscles and the mRNA for the 14 kDa E2 and C9 subunit are actively translated, as shown by the analysis of polyribosomal profiles. Similar observations have been observed in muscles of rats exposed to 16 d spaceflight (Ikemoto *et al.* 2001). More recently, Bodine *et al.* (2001) and Gomes *et al.* (2001) have identified two muscle-specific E3 (atrogin-1/MAFbx and MuRF-1) that are overexpressed in immobilisation, denervation, hindlimb suspension and other catabolic treatments, and are essential for muscle wasting.

#### *Ubiquitin–proteasome proteolysis in muscle recovery and remodelling*

Surprisingly, there are very few data on the proteolytic adaptations responsible for muscle recovery following a catabolic state (Samuels & Baracos, 1995). In chemotherapy-treated healthy and tumour-bearing mice Tilignac *et al.* (2002a, b) have shown that muscle proteolysis progressively decreases below basal levels observed in healthy control mice and contributes to the cessation of muscle wasting. Proteasome-dependent proteolysis is inhibited by mechanisms that include reduced mRNA levels for 20S and 26S proteasome subunits, decreased protein levels of 20S proteasome subunits and the S14 non-ATPase subunit of the 26S proteasome and reduced chymotrypsin- and trypsin-like activities of the proteasome.

Taillandier *et al.* (2003) have recently investigated protein turnover and the role of proteolysis in the reorganisation of the soleus muscle in unweighted rats that are reloaded. During early recovery (18 h of reloading) both muscle protein synthesis and breakdown are elevated. However, only the activation of non-lysosomal and Ca<sup>2+</sup>-independent proteolysis is responsible for increased protein breakdown. Accordingly, mRNA levels for Ub and 20S proteasome subunits C8 and C9 are markedly elevated (from +89 to +325%) and actively translated, as shown by the analysis of polyribosomal profiles. Following 7 d of reloading, protein synthesis is still elevated and there is no marked change in protein breakdown, although an accumulation of Ub conjugates is still detectable in the myofibrillar fraction. This finding suggests that soleus muscle remodelling is still going on. The up-regulation of the Ub–proteasome pathway is presumably required to target specific classes of substrates (atrophy-specific protein isoforms and damaged proteins induced by reloading as in eccentric exercise) for replacement and/or elimination.

Remodelling of skeletal muscle in response to altered patterns of contractile activity is also achieved, in part, by protein breakdown. Ordway *et al.* (2000) have investigated

the role of the proteasome system in rabbit tibialis anterior muscle electrically stimulated for  $\leq 28$  d. There are marked and coordinated increases in protein levels of the 20S proteasome and of the 19S complex and in mRNA levels for a 20S proteasome subunit in contracting muscles. Chronic contractile activity also increases proteasome activity and ATP–Ub-dependent hydrolysis of an exogenous protein substrate. These results clearly support a role for the Ub–proteasome pathway in the contraction-induced remodelling of muscle.

Finally, skeletal muscle has a remarkable capacity to regenerate after injury. Duguez *et al.* (2003) have recently determined whether changes in the expression of proteinases contribute to the remodelling response of muscle tissue. In tibialis anterior muscles of rats injected with bupivacaine (0.75 ml/l) and removed at 3, 5, 7, 10, 14, 21 or 35 d post injection, immunohistochemical analysis indicates the presence of myoblasts (days 3–7), inflammatory cells (days 3–7), degenerating myofibres (days 3–7), regenerating myofibres (days 5–10) and growing mature myofibres (days 10–21) in regenerating muscles. There are marked increases in the enzyme activities of matrix metalloproteinases 2 and 9 and plasminogen activators (days 3–14), calpains (days 3–7), cathepsins B and L (days 3–10) and proteasome (days 3–14). Proteasome activity is strongly correlated with proliferating cell nuclear antigen protein level, suggesting that proteasome plays a key role in myoblast proliferation.

#### **Conclusions and future directions**

There is now strong evidence that the Ub–proteasome system is the critical pathway for the breakdown of muscle proteins in exercise, disuse, recovery and remodelling. However, it should be pointed out that other proteolytic enzymes also play a role in these processes. For example, the 26S proteasome degrades proteins only into peptides, which must undergo further hydrolysis into free amino acids. The extralysosomal tripeptidyl-peptidase II and then exopeptidases degrade peptides generated by the proteasome into free amino acids (Fig. 1; Hasselgren *et al.* 2002). Conversely, other proteinases may act upstream of the proteasome. The rate-limiting step in the breakdown of myofibrillar proteins is probably their dissociation from the myofibril, since specific interactions protect them from Ub-dependent degradation (Solomon & Goldberg, 1996). Calpains play key roles in the disassembly of sarcomeric proteins and in Z-band disintegration, suggesting that they are acting upstream of the proteasome (Hasselgren *et al.* 2002). There is also recent evidence that caspase-3 is crucial for muscle proteolysis (Du *et al.* 2004). In addition, Deval *et al.* (2001) and Jagoe *et al.* (2002) have shown that cathepsin L is differentially up regulated in various muscle-wasting conditions. Whether there are functional connections between calpains, cathepsin L, caspase-3 and the Ub pathway is still unknown. Interestingly, the expression of cathepsin L and several components of the Ub–proteasome system is down regulated in knock-out mice for the muscle-specific calpain p94 (Combaret *et al.* 2003).

Future studies should aim to elucidate: (1) whether the Ub–proteasome system is functionally associated with

other proteolytic pathways; (2) the mechanisms that target protein substrates for ubiquitination and the various E2 and E3 that are effective in muscle; (3) the precise substrates of the pathway; (4) its 'rate-limiting step' (e.g. substrate ubiquitination–deubiquitination and/or proteasome activities); (5) the signalling pathways of catabolic and anabolic treatments and/or molecules that are still ill-defined, including in exercise and disuse.

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