# The Molecular Mechanisms of Calpains Action on Skeletal Muscle Atrophy

## Jiaru HUANG<sup>1</sup>, Xiaoping ZHU<sup>2</sup>

<sup>1</sup>Ningxia Medical University, Yinchuan, Ningxia, China, <sup>2</sup>Department of Respiratory Diseases, YangPu Hospital of Tongji University, Shanghai, China

Received June 2, 2015 Accepted November 12, 2015 On-line March 15, 2016

#### Summary

Skeletal muscle atrophy is associated with a loss of muscle protein which may result from both increased proteolysis and decreased protein synthesis. Investigations on cell signaling pathways that regulate muscle atrophy have promoted our understanding of this complicated process. Emerging evidence implicates that calpains play key roles in dysregulation of proteolysis seen in muscle atrophy. Moreover, studies have also shown that abnormally activated calpain results muscle atrophy *via* its downstream effects on ubiquitin-proteasome pathway (UPP) and Akt phosphorylation. This review will discuss the role of calpains in regulation of skeletal muscle atrophy mainly focusing on its collaboration with either UPP or Akt in atrophy conditions in hope to stimulate the interest in development of novel therapeutic interventions for skeletal muscle atrophy.

#### **Key words**

Cell signaling • Calpains • UPP • Akt • Muscle wasting

#### **Corresponding author**

X. Zhu, Department of Respiratory Diseases, YangPu Hospital of Tongji University, Yangpu Tengyue Road No. 450, Shanghai, China. E-mail: z\_xping@hotmail.com

## Introduction

Skeletal muscle atrophy, defined as the unintentional loss of 5-10 % of muscle mass (Kotler 2000), is present in numerous pathologies such as cancer (Sarah *et al.* 2011), sepsis (Smith *et al.* 2008), neuromuscular disorders (Park *et al.* 2012, Cho *et al.* 

2015) and diabetes (Yoshikawa *et al.* 2000). Muscle atrophy can also occur in the absence of diseases due to prolonged periods of muscle inactivity (Sieck and Mantilla 2008, Salazar *et al.* 2010), which can contribute greatly to reduced life quality and to increased mortality. Thus understanding the molecular contributors to muscle atrophy is a prerequisite for development of therapeutic strategies to improve clinical outcomes on muscle atrophy and reduce the burden on health care systems.

Many molecular events contribute to muscle atrophy, including events like protein synthesis, protease activation, ubiquitin conjugation, and autophagy (Mammucari et al. 2007, Stitt et al. 2004, Wing 2005, Zhao et al. 2007). In the past few years, there have been significant advances in elucidation of signal transduction pathways that regulate the balance between skeletal muscle protein synthesis and degradation. An increased rate of proteolysis has been identified as a major step in muscle atrophy (Krawiec et al. 2005). More significantly, the most studied proteases in skeletal muscle are lysosomal proteases, caspase-3, Ca<sup>2+</sup>-activated proteases (calpain) and the ubiquitin-proteasome pathway (UPP). This review will mainly focus on modulation of protein degradation by calpain and its crosstalk with UPP and Akt along with their contribution towards muscle atrophy.

#### Calpains

Calpains are  $Ca^{2+}$ -dependent cysteine proteases that are located in all vertebrate cells (Goll *et al.* 2003). The calpain family is comprised of 14 members, and

© 2016 Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

Fax +420 241 062 164, e-mail: physres@biomed.cas.cz, www.biomed.cas.cz/physiolres

muscle tissue expresses mainly three distinct calpains: the ubiquitous calpain1 and calpain2 (also called u- and m-) and calpain3 (also called p94). The typical structure of a calpain is composed of four distinct domains (Fig. 1). Calpain1 and calpain2 are heterodimers composed of two subunits of 80 and 30 kDa, respectively. The 80 kDa subunit is bond non-covalently to 30 kDa subunit which contains hydrophobic residues (domain V) and calcium binding sites (domain VI) (Fig. 1, Lin *et al.* 1997). The larger subunit contains the catalytic domain, whereas the

smaller unit has regulatory functions. In addition to the structure elements found on calpain1 and calpain2, calpain3 possesses three unique sequences not found in other calpains including NS at N-terminus, IS1 within the catalytic domain and IS2 upstream of Ca<sup>2+</sup>-binding domain. The latter two sequences confer specific properties to calpain3: IS1 possesses autolytic sites and IS2 comprises a nuclear localization signal and a binding site to titin (also called connectin), a giant elastic protein present in the sarcomere (Beckmann and Spencer 2008).



**Fig. 1.** (**A**) Four domains have been identified in both calpain1 and calpain2: (I) the N-terminal domain, (II) a domain containing a sequence characteristic of cysteine proteases, (III) a connecting domain, and (IV) a  $Ca^{2+}$ -binding domain. (**B**) Calpain3 carries three unique sequences: NS, IS1 and IS2.

Based on the many substrate proteins identified in vivo and in vitro, calpains are implicated in cytoskeletal remodeling via regulation of attachment of cytoskeletal proteins to plasma membrane (Mazères et al. 2006). This process has been known to be important for the physiological functions including cell fusion and cell motility. Disruption of murine calpain4 resulted inactivation of both calpain1 and calpain2, which together led to lethal phenotype in which embryos died at midgestation with defects in cardiovascular system, hemorrhaging and accumulation of erythroid progenitors (Arthur et al. 2000). These are characteristics for loss of physiological cytoskeletal remodeling essential for normal embryo development. Disruption of murine calpain3 was not lethal but the myotubes from calpain3deficient mice lacked well-organized sarcomeres (Kramerova et al. 2004). Thus, calpain3 plays an important role in sarcomere remodeling. Indeed, loss-offunction mutations in calpain3 gene have been associated with limb-girdle muscular dystrophy type 2A and tibial muscular dystrophy (Charton et al. 2015). In addition, calpains are also important for signal transduction (Jungwirth et al. 2014), cell cycle (Liang et al. 2015),

apoptosis (Nozaki *et al.* 2011), regulation of gene expression (Qin *et al.* 2010), and even for long-term potentiation which is believed to be molecular basis of memory response in neurons (Lynch 1998). Because of the wide spectrum of calpain activities, deregulation of calpain activation have been implicated in various pathological conditions including traumatic spinal cord and brain injuries, cataract formation, cerebral and heart ischemia, hypertension, arthritis, etc. (Branca 2004).

#### Calpains in muscle wasting

Several studies have established that abnormally enhanced calpain activation is commonly observed in atrophic conditions like disuse, denervation, glucocorticoid treatment and sepsis (Nelson et al. 2012, Matsumoto et al. 2014, Fareed et al. 2006). Forsberg's team applied several strategies to downregulate intracellular calpain activities like inhibition of calpain expression by use of calpain inhibitor, overexpression of dominant-negative form of calpain2 or endogenous inhibitor calpastatin. They found that under conditions of accelerated degradation, inhibition of calpain2 reduced protein degradation by 30 %, whereas calpastatin reduced

degradation by 63 %. Western blot analysis showed that cytoskeletal proteins, like fodrin and nebulin, were stabilized by inhibition of calpain2. These observations indicate that calpains play key roles in the disassembly of sarcomeric proteins (Huang and Forsberg 1998, Purintrapiban et al. 2003). In addition, the availability of transgenic mice or calpastatin-overexpressing mdx mice confirmed that calpains are involved in muscle wasting (Tidball and Spencer 2002, Spencer and Mellgren 2002). Researchers found that expression of the transgene resulted in increase of calpastatin concentration and elimination of calpain activity. Moreover, calpastatin overexpression completely prevented the shift in myofibrillar myosin content from slow to fast isoforms. Other researchers (Williams et al. 1999, Fischer et al. 2001) observed that sepsis increased expression of calpains and calcium-dependent release of myofilaments in skeletal muscle in septic rats. Either in vitro or in vivo, it has shown that null mutation of calpain3 in mice causes abnormal sarcomere formation (Kramerova et al. 2004).

#### Mechanisms of calpains in muscle wasting

Since proteolytic activities are potentially deleterious to the cell, calpains should be in inactivated state on most of the time (Goll et al. 2003, Taveau et al. 2003). Ca<sup>2+</sup> spikes, removal of N-terminus region via autolysis, phosphorylation, membrane association and calpastatin all seem to be involved in the regulation of calpain activities (Goll et al. 2003). Among them, calcium and endogenous inhibitor calpastatin are the two major regulators on calpains activation. Calpain1 and calpain2 have different in vitro calcium sensibility with range of 5-50 µM for calpain1 and 250-1000 µM for calpain2 (Elce et al. 1997). It is clear that skeletal muscle atrophy induced by calpains is associated with an increase in cytosolic calcium levels (Kourie 1998). For example, intracellular concentrations of calcium are increased in skeletal muscle during sepsis (Fischer et al. 2001).

Calpains exists in the cytosol as an inactive form and translocates to membranes in response to increases in intracellular  $Ca^{2+}$  level. At the membrane, calpain is activated in the presence of  $Ca^{2+}$  and phospholipids. Process of calpains activation by  $Ca^{2+}$  concludes two steps. First, domain interaction imposes release of structural constraints which then leads to dissociation of 30 kDa from 80 kDa. Second, rearrangement of the active cleft is caused by binding of two  $Ca^{2+}$  atoms to the protease domain. In the cases of calpains without 30 kDa, the first step of activation might be bypassed and they are activated directly by the second stage. Ca<sup>2+</sup>-induced structural changes that release the constraints are prerequisite for activation to form a functional catalytic site. Activated calpain or 80 kDa hydrolyzes substrate proteins at membranes or in cytosol after release from membranes. Phosphorylation of calpain might be another important mechanism for activity regulation. Phosphorylation of calpain at Ser369 in domain III by protein kinase A restricts domain movement and freezes calpain2 in an inactive state (Shiraha et al. 2002). Of note, anchoring to titin keeps calpain3 from autolytically degrading itself and maintains it in a proteolytically inactive state. Robyn et al. (2006) stated that calpain3 is bound tightly within a fiber, whereas most calpain1 is initially freely diffusible in the cytoplasm at resting [Ca<sup>2+</sup>] under physiological conditions. These findings demonstrated that the process is precisely attuned to avoid uncontrolled proteolytic activity under normal circumstances. Moreover, these findings indicated that substantial proteolytic damage may be resulted if resting or localized calcium is elevated, which is likely to occur during eccentric contraction and in dystrophic muscle.

As we know, skeletal muscle contains 3 groups of proteins: myofibrillar proteins, sarcoplasmic proteins, stroma proteins. The myofibrillar proteins are not only the largest class of skeletal muscle proteins but also are responsible for the contractile properties of muscle. The contractile function of myofibrils requires the myofibrillar structure extend continuously from one end of the muscle cell to the other. Thus, turnover of myofibrillar proteins must be accomplished without disrupting this continuous structure. This mechanism is consistent with the observations that atrophying muscle in different metabolic conditions has smaller diameter myofibrils than unaffected muscle (Badalamente and Stracher 2000).

Myofibrils are composed of functional mixtures of proteins which include sarcomeric (i.e. contractile) and cytoskeletal proteins, and the latter actually account for nearly 50 % of adult protein mass. Calpains residing within sarcomere are also associated with formation of sarcolemma mainly through controlling of early events of sarcomeric protein disassembly. Cytoskeletal proteins like desmin, vimentin, dystrophin, filamin and sarcomeric proteins have all been reported to be substrates of calpains *in vivo* or *in vitro* (Table 1). Due to the strong cleavage activities on these critical cytoskeletal proteins demonstrated by calpains, Dayton *et al.* (1976a, b) proposed that the calpains might be responsible for release of myofilaments from the surface of myofibrils.

Table 1. Some cytoskeletal proteins known as substrates of calpains (Goll et al. 2003).

Polypeptide name	Effects of calpain cleavage
Adducin	Both the 103-kDa-subunit and the 97-kDa-subunit are degraded to produce stable 57- and 49-kDa fragments, respectively; the 49-kDa-subunit fragment does not bind calmodulin as its parent97-kDa does (Scaramuzzino and Morrow 1993).
Ankyrin	Both the brain (212 kDa) and erythrocyte (239 kDa) isoforms are degraded to a 160-kDa polypeptide (Harada et al. 1997).
Caldesmon	Degraded to polypeptide fragments of 125-, 115-, 100-, 105-, and 88-90 kDa; the latter fragment is stable (Croall <i>et al.</i> 1996).
Cadherin	E-cadherin is not degraded by calpain (Bush <i>et al.</i> 2000), whereas the intracellular domain of N-cadherin is degraded by calpain1 (Covault <i>et al.</i> 1991) or calpain2 (Sato <i>et al.</i> 1995).
Calponin	Degraded to 30-, 27-, and 19.5-kDa fragments; the latter fragment is stable (Croall <i>et al.</i> 1996, Tsunekawa <i>et al.</i> 1989).
Catenin	$\alpha$ -catenin is not degraded by calpain but calpain removes the NH <sub>2</sub> terminus of $\beta$ -catenin to produce 90- and 75-kDa fragments from the 97-kDa $\beta$ -catenin.
C-protein	Small fragment removed from the native 140-kDa polypeptide leaving a 120-kDa fragment (SDS-PAGE); this degradation can occur while the C-protein is bound to the myofibril (Dayton <i>et al.</i> 1975).
Desmin	Degraded to a 32- to 37-kDa stable fragment; an 18-kDa fragment appears after longer digestion (O'Shea <i>et al.</i> 1979); a 9-kDa fragment is removed from the NH <sub>2</sub> terminus leaving the central rod domain; calpain degradation destroys the ability of desmin to self-assemble and to bind nucleic acids (Nelson and Traub 1983).
Dystrophin	Degraded to a 30-kDa NH <sub>2</sub> -terminal fragment and several 50- to 140-kDa fragments (Yoshida <i>et al.</i> 1992).
Gelsolin	Degraded to a 40-kDa fragment that contains the actin-binding segment of gelsolin and a 45-kDa fragment that contains the $Ca^{2+}$ -binding properties of gelsolin.
Filamin/actin-binding protein	Degraded to 240- and 10-kDa fragments that no longer have the cross-linking abilities of the undegraded filamin (Davies <i>et al.</i> 1978).
Myosin	Degradation of undenatured myosin; the 210-kDa large subunit is degraded to fragments of 150-, 165-, and 180-kDa; degradation of LC2 light chain (Pemrick and Grebenau 1984).
Nebulin	Degraded to a series of smaller polypeptides ranging from 30 to several hundred kDa; fragments produced by calpain may remain bound to actin (Taylor <i>et al.</i> 1995); calpain degradation severs nebulin connection to Z-disc.
all-spectrin	Degraded to 145- and 150-kDa fragments; the 150-kDa fragment may be detected by a specific antibody; the 145- and 150-kDa fragments are then degraded to smaller fragments (Yoshida <i>et al.</i> 1995); the initial calpain sensitive site is at $Tyr_{1176}$ -Gly <sub>1177</sub> (Stabach <i>et al.</i> 1997).
Talin	Cleaved between $Q_{433}$ and $Q_{434}$ (chicken talin) to produce a 190-kDa COOH-terminal actin binding fragment and a 47-kDa NH <sub>2</sub> -terminal fragment; after cleavage, can no longer cross-link integrin to cytoskeletal elements (Hemmings <i>et al.</i> 1996, Muguruma <i>et al.</i> 1995).
Titin	The ~3000-kDa titin polypeptide is cleaved to a large ~2000-kDa fragment by removal of a 1200-kDa NH <sub>2</sub> -terminal fragment; this cleavage severs the connection of titin to the Z-disc; the 1200-kDa fragment is degraded to smaller fragments of 100-500 kDa, with the 500-kDa fragment being stable (Taylor <i>et al.</i> 1995); chicken titin is quickly degraded to a T2 (2000 kDa) fragment which is then degraded to a 1700- and a 400-kDa fragment; the 1700-kDa fragment is further degraded to a 1400-kDa fragment (Suzuki <i>et al.</i> 1996).
Tropomyosin	Cleaved to a 14-kDa fragment and several smaller polypeptides (Dayton et al. 1975).
Troponin I	Degraded to smaller polypeptides (the 32-kDa cardiac troponin I is degraded to a 26-kDa fragment as measured by SDS-PAGE) at a moderate rate; not protected while in the myofibrillar structure (Dayton <i>et al.</i> 1975).
Troponin T	Degraded to smaller fragments of 35-, 30-, and 28-kDa as measured by SDS-PAGE; the 30-kDa fragment is stable; a 15-kDa fragment is produced by longer digestion (Ho <i>et al.</i> 1994); not protected when in the myofibrillar structure (Dayton <i>et al.</i> 1975).
Tubulin	Degraded from a 55- to a 50- to 52-kDa fragment (Billger <i>et al.</i> 1988); or not degraded at all (Sandoval and Weber 1978).
Vinculin	Cleaved to a ~90-kDa fragment (Taylor <i>et al.</i> 1995) that can no longer cross-link talin, paxillin, and $\alpha$ -actinin filaments.



**Fig. 2.** (**A**) Calpains mainly reside in Z line of intact myofilaments; (**B**) calpains are able to rapidly cleave cytoskeletal proteins like titin and nebulin at sites near the Z-disc sequentially dissociation of myofilaments; (**C**) myofilaments release from myofibril; (**D**) polypeptides and polypeptide fragments degrade to amino acids by proteosome.

In muscle wasting, preferential site of calpain proteolysis seems to be at Z-disc, a dense multiproteinaceous network located on both sides of the sarcomeres. Calpains are able to rapidly cleave cytoskeletal proteins like titin and nebulin at sites near the Z-disc once they are activated, thereby severing their attachment to Z-disc. Moreover, the calpains cleave Z-disc-associated desmin, an intermediate filament protein, in the sarcolemma. Consequently, the major Z-disc proteins  $\alpha$ -actinin are released which results dissolvement of Z-disc leaving a hollow space in myofibril (Dayton et al. 1975, 1976b). On other hand, calpains are also able to rapidly cleave sarcomeric proteins like troponins T and I, tropomyosin and C-protein, although they cleave myosin and actin very slowly. Additionally, calpains can also cleave M proteins that are components of M line, the anchoring points for

myofilaments (Goll et al. 1992, 1999). Furthermore, cleavage of the M proteins and titin severs the attachments of the thick filament to the myofibril. The thick filaments would be released from the myofibril in the presence of ATP to dissociate myosin crossbridge binding to thin filaments. Calpain cleaved products like molecules of actins and myosins or polypeptide fragments of titin, nebulin, desmin, troponin, tropomyosin, and C-proteins can all subsequently be ubiquitinated and degraded to amino acids by proteosome, intracellular peptidases and lysosomal cathepsins. These activities together lead to final dissociation of myofibril and muscle wasting is destined (Fig. 2).

## UPP pathways

The essential feature of the UPP is that proteins are tagged with a polyubiquitin chain, which marks them for degradation by the 26S proteasome, a tubular multisubunit complex containing proteolytic enzymes on the luminal side of the proteasome chamber. The whole proteasome complex (26S) is comprised of a core proteasome subunit (20S) that is coupled with a regulatory complex (19S) at both ends (Hasselgren 1999, Tidball and Spencer 2002, Grune and Davies 2003, Grune et al. 2003). Interestingly, proteins can be degraded by either the 26S proteasome or the 20S protease core. 26S proteasome degradation pathway is only active machinery when protein substrates are ubiquitinated and marked for degradation. Addition of ubiquitin to a protein substrate is believed to be an exquisitely modulated process. This process requires three distinct components: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligating enzyme (Hershko and Ciechanover 1998, Cohen et al. 2009). UPP-mediated protein degradation is a selective process with two distinct and successive steps which include covalent attachment of multiple ubiquitin molecules to the target protein substrates and subsequent degradation of the tagged substrates by 26S-proteasome (Schwartz and Ciechanover 2009). Conjugation of ubiquitin to the substrate proceeds via a three-step enzymatic mechanism with E3 ubiquitin ligases being the rate-limiting step (Ciechanover et al. 2000). Ubiquitin is first activated by ubiquitin activating enzyme (E1). This activated ubiquitin is then transferred to E2. E3 transfers an activated form of ubiquitin from E2 to the lysine residue on the substrate. The E3 enzymes are the primary determinant of substrate specificity because they are able to recognize several structural motifs like the amino terminal residue of the substrate, specific phosphorylated domains or the "destruction box" (Lecker *et al.* 1999a). Individual E3 ubiquitinate specific classes of proteins; hence the E3s play an important role in determining which proteins are targeted for degradation by the proteasome.

#### Muscle atrophy induced by UPP

It has been shown that degradation through the UPP may account for up to 80 % of proteolysis during skeletal muscle wasting (Tawa et al. 1997). Studies of experimental animal models and patients have consistently demonstrated that the UPP is responsible for degradation of muscle proteins in bulk volume. More importantly, UPP becomes activated in catabolic states associated with atrophy (Sandri 2013, Smuder et al. 2014). In most atrophy models studied, levels of UPP components transcripts are increased, and higher rates of ubiquitin conjugation were found in vitro (Jagoe et al. 2002, Lecker et al. 2004, 1999b, Stevenson et al. 2003). Studies of differentiated myotube cultures demonstrated that treatment of myotubes with cachectic glucocorticoid dexamethasone increased expression of genes broadly involved in UPP proteolytic pathway correlating well with enhanced protein breakdown (Du et al. 2000, Hong and Forsberg 1995, Wang et al. 1998). The unique ubiquitin E3 ligases, muscle atrophy F-box (MAFbx) and muscle ring finger-1 (MuRF-1) exist in skeletal muscle, and these ligases play essential roles in skeletal muscle atrophy (Foletta et al. 2011). In vitro treatment of myotubes with DEX induces atrophy was accompanied with specifically increased expression of MAFbx and MuRF1 (Sandri et al. 2004, Stitt et al. 2004), indicating MAFbx and MuRF1 are negatively associated with presence of muscle atrophy. Indeed, mice lacking MAFbx or MuRF1 genes were found to be resistant to atrophy (Bodine et al. 2001a, Furlow et al. 2013). For example, both MAFbx and MuRF1 knockout mice showed a significant attenuation in loss of muscle mass after denervation comparing to wild-type mice.

#### Relationship between calpains and UPP

Proteasome is able to degrade only sarcomeric proteins in monomeric form (including  $\alpha$ -actin) but not when they are in higher-ordered structures. Higherordered proteins are much more stable when being associated with each other in the actomyosin complex (accounted for 50-70 % of muscle proteins) or intact myofibrils (Tidball and Spencer 2002). On one hand, the size of proteasome is so large that it is not possible for them to enter the sarcomere and to digest their proteins. On the other hand, the entrance to the central cavity of the proteasome containing the active sites is only 10-13 Å in diameter which is much narrower for entry of myofibrils ranging from 10 to 100  $\mu$ m in diameter. Thus, the first step in degradation of myofibrillar proteins (actin and myosin) during atrophy requires release of myofilaments from the sarcomere (Solomon and Goldberg 1996, Solomon *et al.* 1998). These initial steps in myofibrillar proteolysis appear to rely on the calpain activation and caspase systems.

Overall, loss of myofibrillar proteins in muscle wasting requires the concerted action of at least two proteolytic systems. Calpains can be regarded as the initiators of myofibrillar degradation. As previously stated, several proteins important for the structural integrity of the sarcomere such as nebulin, titin and filamin are readily cleaved by calpain (Huang and Forsberg 1998, Smith et al. 2008). The consequences are deattachment of these structural proteins in the Z-disc and release of principal Z-disc proteins like α-actinin. Thus, calpains rapidly cleave sarcomeric proteins and result in filament release from the myofibril. Once filament released, UPP plays an essential role in their proteolysis. E3 ubiquitin ligases, MuRF1 and MAFbx are essential for UPS-mediated proteolysis of filament proteins (Clarke et al. 2007, Cohen et al. 2009). MuRF1 and MAFbx mediate atrophy by ubiquitinating particular protein substrates that further degraded by proteasome. MuRF1's substrates include several components of the sarcomeric thick filament, e.g. myosin heavy chain (Clarke et al. 2007). It also has been showed that MuRF1 binds to myofibrillar protein titin at the M line (Centner et al. 2001, McElhinny et al. 2002, Pizon et al. 2002). Subsequently, it was shown that several other proteins in the thick filament of muscle were also degraded by MuRF1, including myosin light chain and myosin binding protein C (Cohen et al. 2009). Smith and Dodd (2007) designed ex vivo experiments using isolated rat diaphragm muscle treated with Ca<sup>2+</sup>, calpain inhibitor calpeptin or proteasome inhibitor epoxomicin. They found that calpain activation increased total protein degradation and proteasome-dependent proteolysis by 65 % and 144 %, respectively. In addition, when proteasomes were inhibited, the increase in proteolysis following calpain activation was ameliorated. Hence, calpain and UPP could have synergistic reaction on

muscle proteins proteolysis and calpain could act as the upstream of UPP during muscle atrophy.

## Akt signaling pathway

The serine/threonine kinase Akt, also known as protein kinase B, is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. As a transducer it stimulates signaling to its downstream cascades through phosphorylation of a myriad of substrates, resulting in the integration of anabolic, catabolic and mechanical responses (Clemmons 2009, Wu *et al.* 2010). Two major downstream pathways of Akt relevant to muscle hypertrophy are the mammalian target of rapamycin (mTOR) and glycogen synthase kinase  $3\beta$  (GSK $3\beta$ ). Both contribute to control of protein synthesis. A third downstream pathway of Akt is the Forkhead Box O (FoxO) transcription factor which controls protein degradation.

## Akt and muscle hypertrophy

Evidences both in rats and humans have shown that Akt activity is increased in response to muscle contraction and hormonal or growth factor stimulation (Nader and Esser 2001, Sakamoto et al. 2002, 2003). For example, insulin or IGF1 can phosphorylate and activate Akt both in vitro (Takahashi et al. 2002) and in vivo (Bodine et al. 2001b, Pallafacchina et al. 2002). Furthermore, presence of a constitutively active form of Akt in skeletal muscle cells is able to result muscle hypertrophy probably via abnormal activation of its downstream pathways. Phosphorylated Akt subsequently phosphorylates and activates mTOR which then activates p70<sup>S6K</sup> and increases inhibitory effect of PHAS-1/4E-BP1 (Terada et al. 1994). All these can promote protein synthesis (Stitt et al. 2004, Glass 2005, Rena et al. 1999). Meanwhile, Akt activation reduces GSK-3<sup>β</sup> kinase activity through phosphorylation of Ser9 on GSK-3β (Harwood 2001). Phosphorylated GSK-3B is able to enhance activities of eIF2B (eukaryotic initiation factor 2B) resulting increased mRNA translation (Welsh et al. 1998). Therefore, the IGF-I/Akt/mTOR and IGF-I/Akt/GSK-3ß pathways are playing mediating effects for muscle hypertrophy caused by dysregulated Akt.

#### Akt and muscle atrophy

In vast majority of atrophy conditions, especially in rodents, the reduction of Akt activation is associated with activation in the FoxO/MAFbx pathway. The reduced activity of Akt found in various models of muscle atrophy lead to decreased phosphorylation of FoxO which hence results its accumulation in the nucleus since phosphorylated FoxO by Akt will export out of nucleus (Calnan and Brunet 2008). The translocation and transcriptional activation of FoxO members is sufficient to promote both a MAFbx and MuRF1 expression (Bodine et al. 2001a, Gomes et al. 2001). Moreover, studies revealed that TNF-a activation of FoxO transcription of MAFbx was paralleled by an increase in Akt activity (Stitt et al. 2004). Léger et al. (2006) found that Akt was reduced while MAFbx mRNA and protein levels were increased in amyotrophic lateral sclerosis (ALS) patients and ALS G93A mice. This occurred without a change in nuclear FoxO levels, suggesting that MAFbx transcription may be regulated by another Akt stimulated pathway.

Research by Sugita et al. (2005) found that burn injury impairs phosphorylation of Akt and activation of GSK-3 $\beta$  in skeletal muscle. These findings suggested that attenuated Akt activation was involved in disturbed metabolism and muscle wasting. Other research by Fang et al. (2007) reported that burn injury increased GSK-3β kinase activity in atrophying muscle and significantly lowered Akt kinase activity, which was further confirmed by reduced levels of phosphorylated Akt. In addition, hindlimb unloading induced by suspending rats for 14 days, results in muscle atrophy with a decrease in total and phosphorylated Akt, reduction of phosphorylation of p70<sup>S6K</sup> and increase in binding of PHAS-1 to eIF4E (Bodine et al. 2001b). These indicate that interfering with other pathways of Akt, mTOR or GSK-3β, may also regulate muscle atrophy.

## Relationship between calpains and Akt

As mentioned previously, calpain mainly destructs skeleton structural proteins whereas Akt mainly perturbs translation or transcription of mRNA of skeleton proteins. Studies by Sato et al. (2000) revealed that molecular chaperone heat shock protein90 (HSP90) can bind and protect phosphorylated Akt from dephosphorylation by phosphatase2A to maintain Akt activation. In addition, they also demonstrated that HSP90 is a calpain substrate in which calpain activation reduces HSP90-client proteins binding (Stalker et al. 2003). These suggest that calpain activation may diminish HSP90-Akt binding in skeletal muscle thus reducing Akt activation. A study by Smith and Dodd (2007) demonstrated that presence of  $Ca^{2+}$  significantly decreased HSP90 content by 33 % which can be prevented by inhibition of calpain activation. Therefore, increased calpain-dependent protein degradation seen in calcium-treated muscles could be associated with reduced HSP90 and hence reduced Akt activation. Moreover, reduction of Akt activities will allow nuclear translocation and activation of FoxO transcription factors which are vital in regulating the expression and activity of MAFbx and MuRF1, thereby initiation of UPP-mediated muscle proteolysis (Sandri *et al.* 2004, Stitt *et al.* 2004, Glass 2005).

#### Association of calpains with other signaling pathway

Reports indicated that oxidative stress is capable of promoting calpain expression in muscle cells in culture. Exposure of C2C12 myotubes and human myoblasts to H<sub>2</sub>O<sub>2</sub> resulted an increase in expression of calpains (McClung et al. 2009, Dargelos et al. 2010). Studies have reported that intracellular production of reactive oxygen species (ROS) could play a role in disturbances of calcium homeostasis (Kandarian and Stevenson 2002). A potential mechanism to link oxidative stress with calcium overload is that ROSmediated formation of reactive aldehydes which is known to be able to inhibit plasma membrane Ca<sup>2+</sup>-ATPase activity (Siems et al. 2003). Therefore, an oxidative stress-induced decrease in membrane Ca<sup>2+</sup>-ATPase activity would impede Ca2+ removal from the cell and promote intracellular Ca<sup>2+</sup> accumulation and calpains activation.

Calpain activation may also regulate degradation of various transcription factors involved in muscle wasting which include signal transducer, transcription family (STAT) and nuclear factor- $\kappa$ B (Oda *et al.* 2002, Wei *et al.* 2006). In addition, intracellular signaling molecules, like protein kinase C, calcineurin and Cdc42/RhoA, were also cleaved by calpain to regulate muscle atrophies (Chockalingam *et al.* 2002, Sneddon *et al.* 2000, Wang *et al.* 1989). Therefore, we predict that new concepts concerning calpain regulation of muscle wasting will soon emerge.

## Clinical implications

Skeletal muscle atrophy is a major clinical problem, as it occurs in a large group of patients, such as neuromuscular disorders and sepsis, prolonged mechanical ventilation (MV). It increases the risk of complications and puts a huge financial burden on the healthcare system. No therapies are currently available that improve skeletal muscle weakness. A recent data on the effects of MV on the human diaphragm indicates that diaphragm muscle fibers display atrophy, contractile weakness, and activation of UPP (Pleuni et al. 2015). However, studies showed that absence effect of inhibition of UPP on MV-induced muscle atrophy (Smuder et al. 2014, Agten et al. 2012). Meanwhile, inhibition of the calpain activity preserves sarcomeric structure, prevents the development of muscle weakness and muscle atrophy both in peripheral muscle and diaphragm (Nelson et al. 2012, Salazar et al. 2010). Therefore, to investigate the molecular mechanisms of calpains on muscle atrophy is of clinic importance in hope to stimulate the interest in development of novel therapeutic interventions.

### **Conflict of Interest**

There is no conflict of interest.

## Acknowledgements

The authors acknowledged the contribution of Prof. Xiaoping Chen for her critical review for this manuscript. This research was supported by grants from the National Science Funds 81170074, China.

## References

- AGTEN A, MAES K, THOMAS D, CIELEN N, VAN HEES HW, DEKHUIJZEN RP, DECRAMER M, GAYAN-RAMIREZ G: Bortezomib partially protects the rat diaphragm from ventilator-induced diaphragm dysfunction. *Crit Care Med* **40**: 2449-2455, 2012.
- ARTHUR JS, ELCE JS, HEGADORN C, WILLIAMS K, GREER PA: Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol* **20**: 4474-4481, 2000.
- BADALAMENTE MA, STRACHER A: Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. *Muscle Nerve* 23: 106-111, 2000.
- BECKMANN JS, SPENCER M: Calpain 3, the "gatekeeper" of proper sarcomere assembly, turnover and maintenance. *Neuromuscul Disord* **18**: 913-921, 2008.

- BILLGER M, WALLIN M, KARLSSON J-O: Proteolysis of tubulin and microtubule-associated proteins 1 and 2 by calpain I and II. Difference in sensitivity of assembled and disassembled microtubules. *Cell Calcium* **9**: 33-44, 1988.
- BODINE SC, LATRES E, BAUMHUETER S, LAI VK, NUNEZ L, CLARKE BA, POUEYMIROU WT, PANARO FJ, NA E, DHARMARAJAN K, PAN ZQ, VALENZUELA DM, DECHIARA TM, STITT TN, YANCOPOULOS GD, GLASS DJ: Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294: 1704-1708, 2001a.
- BODINE SC, STITT TN, GONZALEZ M, KLINE WO, STOVER GL, BAUERLEIN R, ZLOTCHENKO E, SCRIMGEOUR A, LAWRENCE JC, GLASS DJ, YANCOPOULOS GD: Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014-1019, 2001b.
- BRANCA D: Calpain-related diseases. Biochem Biophys Res Commun 322: 1098-1104, 2004.
- CALNAN DR, BRUNET A: The FoxO code. Oncogene 27: 2276-2288, 2008.
- CENTNER T, YANO J, KIMURA E, MCELHINNY AS, PELIN K, WITT CC, BANG ML, TROMBITAS K, GRANZIER H, GREGORIO CC, SORIMACHI H, LABEIT S: Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J Mol Biol* **306**: 717-726, 2001.
- CHARTON K, SARPARANTA J, VIHOLA A, MILIC A, JONSON PH, SUEL L, LUQUE H, BOUMELA I, RICHARD I, UDD B: CAPN3-mediated processing of C-terminal titin replaced by pathological cleavage in titinopathy. *Hum Mol Genet* 24: 3718-3731, 2015.
- CHO K, CHO MH, SEO JH, PEAK J, KONG KH, YOON SY, KIM DH: Calpain-mediated cleavage of DARPP-32 in Alzheimer's disease. *Aging Cell* 14: 878-886, 2015.
- CHOCKALINGAM PS, CHOLERA R, OAK SA, ZHENG Y, JARRETT HW, THOMASON DB: Dystrophinglycoprotein complex and Ras and Rho GTPase signaling are altered in muscle atrophy. *Am J Physiol Cell Physiol* 283: C500-C511, 2002.
- CIECHANOVER A, ORIAN A, SCHWARTZ AL: Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22: 442-451, 2000.
- CLARKE BA, DRUJAN D, WILLIS MS, MURPHY LO, CORPINA RA, BUROVA E, RAKHILIN SV, STITT TN, PATTERSON C, LATRES E, GLASS DJ: The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell Metab* **6**: 376-385, 2007.
- CLEMMONS DR: Role of IGF-I in skeletal muscle mass maintenance. Trends Endocrinol Metab 20: 349-356, 2009.
- COHEN S, BRAULT JJ, GYGI SP, GLASS DJ, VALENZUELA DM, GARTNER C, LATRES E, GOLDBERG AL: During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. J Cell Biol 185: 1083-1095, 2009.
- COVAULT J, LIU QY, EL-DEEB S: Calcium-activated proteolysis of intracellular domains in the cell adhesion molecules NCAM and N-cadherin. *Brain Res* **11**: 11-16, 1991.
- CROALL DE, CHACKO S, WANG Z: Cleavage of caldesmon and calponin by calpain: substrate recognition is not dependent on calmodulin binding domains. *Biochim Biophys Acta* **1298**: 276-284, 1996.
- DARGELOS E, BRULÉ C, STUELSATZ P, MOULY V, VESCHAMBRE P, COTTIN P, POUSSARD S: Up-regulation of calcium-dependent proteolysis in human myoblasts under acute oxidative stress. *Exp Cell Res* **316**: 115-125, 2010.
- DAVIES PJA, WALLACH D, WILLINGHAM MC, PASTAN I: Filamin-actin interaction. Dissociation of binding from gelation by Ca<sup>2+</sup>-activated proteolysis. *J Biol Chem* **253**: 4036-4042, 1978.
- DAYTON WR, GOLL DE, STROMER MH, RVILLE WJ, ZEECE MG, ROBSON RM: Some properties of a Ca<sup>2+</sup>activated protease that may be involved in myofibrillar protein turnover. In: *Proteases and Biological Control. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 2.* REICH E, RIFKIN DB, SHAW E (eds), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1975, pp 551-577.
- DAYTON WR, GOLL DE, ZEECE MG, ROBSON RM, REVILLE WJ: A Ca<sup>2+</sup>-activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry* **15**: 2150-2158, 1976a.

- DAYTON WR, REVILLE WJ, GOLL DE, STROMER MH: A Ca<sup>2+</sup>-activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry* **15**: 2159-2167, 1976b.
- DU J, MITCH WE, WANG X, PRICE SR: Glucocorticoids induce proteasome C3 subunit expression in L6 muscle cells by opposing the suppression of its transcription by NF-kappa B. *J Biol Chem* **275**: 19661-19666, 2000.
- ELCE JS, HEGADORN C, ARTHUR JS: Autolysis, Ca<sup>2+</sup> requirement, and heterodimer stability in m-calpain. *J Biol Chem* **272**: 11268-11275, 1997.
- FANG CH, LI B, JAMES JH, YAHYA A, KADEER N, GUO X, XIAO C, SUPP DM, KAGAN RJ, HASSELGREN PO, SHERIFF S: GSK-3beta activity is increased in skeletal muscle after burn injury in rats. Am J Physiol Regul Integr Comp Physiol 293: R1545-R1551, 2007.
- FAREED MU, EVENSON AR, WEI W, MENCONI M, POYLIN V, PETKOVA V, PIGNOL B, HASSELGREN PO: Treatment of rats with calpain inhibitors prevents sepsis-induced muscle proteolysis independent of atrogin-1/MAFbx and MuRF1 expression. *Am J Physiol Regul Integr Comp Physiol* **290**: R1589-R1597, 2006.
- FISCHER DR, SUN X, WILLIAMS AB, GANG G, PRITTS TA, JAMES JH, MOLLOY M, FISCHER JE, PAUL RJ, HASSELGREN PO: Dantrolene reduces serum TNFalpha and corticosterone levels and muscle calcium, calpain gene expression and protein breakdown in septic rats. *Shock* **15**: 200-207, 2001.
- FOLETTA VC, WHITE LJ, LARSEN AE, LÉGER B, RUSSELL AP: The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflugers Arch* **461**: 325-335, 2011.
- FURLOW JD, WATSON ML, WADDELL DS, NEFF ES, BAEHR LM, ROSS AP, BODINE SC: Altered gene expression patterns in muscle ring finger 1 null mice during denervation- and dexamethasone-induced muscle atrophy. *Physiol Genomics* 45: 1168-1185, 2013.
- GLASS DJ: Skeletal muscle hypertrophy and atrophy signaling pathways. Int J Biochem Cell Biol 37: 1974-1984, 2005.
- GOLL DE, THOMPSON VF, TAYLOR RG, CHRISTIANSEN JA: Role of the calpain system in muscle growth. *Biochimie* 74: 225-237, 1992.
- GOLL DE, THOMPSON VF, TAYLOR RG, OUALI A, CHOU RGR: The calpain system in muscle tissue. In: *Calpain: Pharmacology and Toxicology of Calcium-Dependent Protease*. WANG KKW, YUEN P-W (eds), Taylor & Francis, Philadelphia, 1999, pp 127-160.
- GOLL DE, THOMPSON VF, LI H, WEI W, CONG J: The calpain system. Physiol Rev 83: 731-801, 2003.
- GOMES MD, LECKER SH, JAGOE RT, NAVON A, GOLDBERG AL: Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci U S A* **98**: 14440-14445, 2001.
- GRUNE T, DAVIES KJ: The proteasomal system and HNE-modified proteins. Mol Aspects Med 24: 195-204, 2003.
- GRUNE T, MERKER K, SANDIG G, DAVIES KJ: Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* **305**: 709-718, 2003.
- HARADA F, KUNIMOTO M, YOSHIDA K-I: Distribution of ankyrin isoforms and their proteolysis after ischemia and reperfusion in rat brain. *J Neurochem* **69**: 371-376, 1997.
- HARWOOD AJ: Regulation of GSK-3: a cellular multiprocessor. Cell 105: 821-824, 2001.
- HASSELGREN PO: Role of the ubiquitin-proteasome pathway in sepsis-induced muscle catabolism. *Mol Biol Rep* 26: 71-76, 1999.
- HEMMINGS L, REES DJG, OHANIAN V, BOLTON SJ, GILMORE AP, PATEL B, PRIDDLE H, TREVITHICK JE, HYNES RO, CRITCHLEY DR: Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. *J Cell Sci* 109: 2715-2726, 1996.
- HERSHKO A, CIECHANOVER A: The ubiquitin system. Annu Rev Biochem 67: 425-479, 1998.
- HO CY, STROMER MH, ROBSON RM: Identification of the 30 kDa polypeptide in post mortem muscle as a degradation product of troponin-T. *Biochimie* **76**: 369-375, 1994.
- HONG DH, FORSBERG NE: Effects of dexamethasone on protein degradation and protease gene expression in rat L8 myotube cultures. *Mol Cell Endocrinol* **108**: 199-209, 1995.
- HUANG J, FORSBERG NE: Role of calpain in skeletal-muscle protein degradation. *Proc Natl Acad Sci U S A* **95**: 12100-12105, 1998.
- JAGOE RT, LECKER SH, GOMES M, GOLDBERG AL: Patterns of gene expression in atrophying skeletal muscles: response to food deprivation. *FASEB J* 16: 1697-1712, 2002.

- JUNGWIRTH U, GOJO J, TUDER T, WALKO G, HOLCMANN M, SCHÖFL T, NOWIKOVSKY K, WILFINGER N, SCHOONHOVEN S, KOWOL CR, LEMMENS-GRUBER R, HEFFETER P, KEPPLER BK, BERGER W: Calpain-mediated integrin deregulation as a novel mode of action for the anticancer gallium compound KP46. *Mol Cancer Ther* 13: 2436-2449, 2014.
- KANDARIAN SC, STEVENSON EJ: Molecular events in skeletal muscle during disuse atrophy. *Exerc Sport Sci Rev* 30: 111-116, 2002.

KOTLER DP: Cachexia. Ann Intern Med 133: 622-634, 2000.

- KOURIE JI: Interaction of reactive oxygen species with ion transport mechanisms. *Am J Physiol Cell Physiol* 275: C1-C24, 1998.
- KRAMEROVA I, KUDRYASHOVA E, TIDBALL JG, SPENCER MJ: Null mutation of calpain 3 (p94) in mice causes abnormal sarcomere formation in vivo and in vitro. *Hum Mol Genet* **13**: 1373-1388, 2004.
- KRAWIEC BJ, FROST RA, VARY TC, JEFFERSON LS, LANG CH: Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. *Am J Physiol Endocrinol Metab* 289: E969-E980, 2005.
- LECKER SH, SOLOMON V, MITCH WE, GOLDBERG AL: Muscle protein breakdown and the critical role of theubiquitin-proteasome pathway in normal and disease states. *J Nutr* **129**: 227-237, 1999a.
- LECKER SH, SOLOMON V, PRICE SR, KWON YT, MITCH WE, GOLDBERG AL: Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. *J Clin Invest* **104**: 1411-1420, 1999b.
- LECKER SH, JAGOE RT, GILBERT A, GOMES M, BARACOS V, BAILEY J, PRICE SR, MITCH WE, GOLDBERG AL: Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J* **18**: 39-51, 2004.
- LÉGER B, VERGANI L, SORARÙ G, HESPEL P, DERAVE W, GOBELET C, D'ASCENZIO C, ANGELINI C, RUSSELL AP: Human skeletal muscle atrophy in amyotrophic lateral sclerosis reveals a reduction in Akt and an increase in atrogin-1. *FASEB J* **20**: 583-585, 2006.
- LIANG Z, BROWN RC, FLETCHER JC, OPSAHL-SORTEBERG HG: Calpain-mediated positional information directs cell wall orientation to sustain plant stem cell activity, growth and development. *Plant Cell Physiol* 56: 1855-1866, 2015.
- LIN GD, CHATTOPADHYAY D, MAKI M, WANG KK, CARSON M, JIN L, YUEN PW, TAKANO E, HATANAKA M, DELUCAS LJ, NARAYANA SV: Crystal structure of calcium bound domain VI of calpain at 1.9 A resolution and its role in enzyme assembly, regulation, and inhibitor binding. *Nat Struct Biol* **4**: 539-547, 1997.
- LYNCH G: Memory and the brain: unexpected chemistries and a new pharmacology. *Neurobiol Learn Mem* 70: 82-100, 1998.
- MAMMUCARI C, MILAN G, ROMANELLO V, MASIERO E, RUDOLF R, DEL PICCOLO P, BURDEN SJ, DI LISI R, SANDRI C, ZHAO J, GOLDBERG AL, SCHIAFFINO S, SANDRI M: FoxO3 controls autophagy in skeletal muscle in vivo. *Cell Metab* **6**: 458-471, 2007.
- MATSUMOTO A, FUJITA N, ARAKAWA T, FUJINO H, MIKI A: Influence of electrical stimulation on calpain and ubiquitin-proteasome systems in the denervated and unloaded rat tibialis anterior muscles. *Acta Histochem* **116**: 936-942, 2014.
- MAZÈRES G, LELOUP L, DAURY L, COTTIN P, BRUSTIS JJ: Myoblast attachment and spreading are regulated by different patterns by ubiquitous calpains. *Cell Motil Cytoskeleton* **63**: 193-207, 2006.
- MCCLUNG JM, JUDGE AR, TALBERT EE, POWERS SK: Calpain-1 is required for hydrogen peroxide-induced myotube atrophy. *Am J Physiol Cell Physiol* **296**: C363-C371, 2009.
- MCELHINNY AS, KAKINUMA K, SORIMACHI H, LABEIT S, GREGORIO CC: Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J Cell Biol* **157**: 125-136, 2002.
- MUGURUMA M, NISHIMUTA S, TOMISAKA Y, ITO T, MATSUMURA S: Organization of the functional domains in membrane cytoskeletal protein talin. *J Biochem* **117**: 1036-1042, 1995.

- NADER GA, ESSER KA: Intracellular signaling specificity in skeletal muscle in response to different modes of exercise. *J Appl Physiol* **90**: 1936-1942, 2001.
- NELSON WB, SMUDER AJ, HUDSON MB, TALBERT EE, POWERS SK: Cross-talk between the calpain and caspase-3 proteolytic systems in the diaphragm during prolonged mechanical ventilation. *Crit Care Med* **40**: 1857-1863, 2012.
- NELSON WJ, TRAUB P: Proteolysis of vimentin and desmin by the Ca<sup>2+</sup>-activated proteinase specific for these intermediate filament proteins. *Mol Cell Biol* **3**: 1146-1156, 1983.
- NOZAKI K, DAS A, RAY SK, BANIK NL: Calpeptin attenuated apoptosis and intracellular inflammatory changes in muscle cell. *J Neurosci Res* **89**: 536-543, 2011.
- ODA A, WAKAO H, FUJITA H: Calpain is a signal transducer and activator of transcription (STAT) 3 and STAT5 protease. *Blood* **99**: 1850-1852, 2002.
- O'SHEA JM, ROBSON RM, HUIATT TM, HARTZER MK, STROMER MH: Purified desmin from adult mammalian muscle: a peptide mapping comparison with desmins from adult mammalian and avian smooth muscle. *Biochem Biophys Res Commun* **89**: 972-980, 1979.
- PALLAFACCHINA G, CALABRIA E, SERRANO AL, KALHOVDE JM, SCHIAFFINO S: A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fibre type specification. *Proc Natl Acad. Sci U S A* **99**: 9213-9218, 2002.
- PARK S, NOZAKI K, GUYTON MK, SMITH JA, RAY SK, BANIK NL: Calpain inhibition attenuated morphological and molecular changes in skeletal muscle of experimental allergic encephalomyelitis rats. *J Neurosci Res* **90**: 2134-2145, 2012.
- PEMRICK SM, GREBENAU RC: Qualitative analysis of skeletal myosin as a substrate of Ca<sup>2+</sup>-activated neutral protease: comparison of filamentous and soluble, native, and L2-deficient myosin. *J Cell Biol* **99**: 2297-2308, 1984.
- PIZON V, IAKOVENKO A, VAN DER VEN PFM, KELLY R, FATU C, FÜRST DO, KARSENTI E, GAUTEL M: Transient association of titin and myosin with microtubules in nascent myofibrils directed by the MURF2 RING-finger protein. *J Cell Sci* **115**: 4469-4482, 2002.
- PLEUNI EH, ALBERTUS B, CHRISTIAN C: Diaphragm muscle fiber weakness and ubiquitin-proteasome activation in critically ill patients. *Am J Respir Crit Care Med* **191**: 1126-1138, 2015.
- PURINTRAPIBAN J, WANG MC, FORSBERG NE: Degradation of sarcomeric and cytoskeletal proteins in cultured skeletal muscle cells. *Comp Biochem Physiol B Biochem Mol Biol* **136**: 393-401, 2003.
- QIN QY, LIAO GH, MICHEL B, BI XN: Role of calpain-mediated p53 truncation in semaphorin 3A-induced axonal growth regulation. *Proc Natl Acad Sci US A* **107**: 13883-13887, 2010.
- RENA G, GUO S, CICHY SC, UNTERMAN TG, COHEN P: Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J Biol Chem* **274**: 17179-17183, 1999.
- ROBYN MM, ESTHER V, GRAHAM DL: Ca<sup>2+</sup> activation of diffusible and bound pools of μ-calpain in rat skeletal muscle. *J Physiol* **576**: 595-612, 2006.
- SAKAMOTO K, HIRSHMAN MF, ASCHENBACH WG, GOODYEAR LJ: Contraction regulation of Akt in rat skeletal muscle. *J Biol Chem* 277: 11910-11917, 2002.
- SAKAMOTO K, ASCHENBACH WG, HIRSHMAN MF, GOODYEAR LJ: Akt signaling in skeletal muscle: regulation by exercise and passive stretch. *Am J Physiol Endocrinol Metab* **285**: 1081-1088, 2003.
- SALAZAR JJ, MICHELE DE, BROOKS SV: Inhibition of calpain prevents muscle weakness and disruption of sarcomere structure during hindlimb suspension. *J Appl Physiol* **108**: 120-127, 2010.
- SANDOVAL IV, WEBER K: Calcium-induced inactivation of microtubule formation in brain extracts. Presence of a calcium-dependent protease acting on polymerization-stimulating microtubule associated proteins. *Eur J Biochem* 92: 463-470, 1978.
- SANDRI M: Protein breakdown in muscle wasting: role of autophagy-lysosome and ubiquitin-proteasome. Int J Biochem Cell Biol 45: 2121-2129, 2013.
- SANDRI M, SANDRI C, GILBERT A, SKURK C, CALABRIA E, PICARD A, WALSH K, SCHIAFFINO S, LECKER SH, GOLDBERG AL: Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**: 399-412, 2004.

- SARAH SJ, NEIL CO, MARGARET FC, TIM P, STEWART MG: The calpain system and cancer. *Nat Rev Cancer* 11: 364-374, 2011.
- SATO N, FUJIO Y, YAMADA-HONDA F, FUNAI H, WADA A, KAWASHIMA S, AWATA N, SHIBATA N: Elevated calcium level induces calcium-dependent proteolysis of A-CAM (N-cadherin) in heart analysis by detergent-treated model. *Biochem Biophys Res Commun* **217**: 649-653, 1995.
- SATO S, FUJITA N, TSURUO T: Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci U S A* **97**: 10832-10837, 2000.
- SCARAMUZZINO DA, MORROW JS: Calmodulin-binding domain of recombinant erythrocyte β-adducin. *Proc Natl Acad Sci U S A* **90**: 3398-3402, 1993.
- SCHWARTZ AL, CIECHANOVER A: Targeting proteins for destruction by the ubiquitin system: implications for human pathobiology. *Annu Rev Pharmacol Toxicol* **49**: 73-96, 2009.
- SHIRAHA H, GLADING A, CHOU J, JIA Z, WELLS A: Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol Cell Biol* **22**: 2716-2727, 2002.
- SIECK GC, MANTILLA CB: Effect of mechanical ventilation on the diaphragm. N Engl J Med 358: 1392-1394, 2008.
- SIEMS W, CAPUOZZO E, LUCANO A, SALERNO C, CRIFO C: High sensitivity of plasma membrane ion transport ATPases from human neutrophils towards 4-hydroxy-2, 3-trans-nonenal. *Life Sci* **73**: 2583-2590, 2003.
- SMITH IJ, DODD SL: Calpain activation causes a proteasome-dependent increase in protein degradation and inhibits the Akt signalling pathway in rat diaphragm muscle. *Exp Physiol* **92**: 561-573, 2007.
- SMITH IJ, LECKER SH, HASSELGREN PO: Calpain activity and muscle wasting in sepsis. Am J Physiol Endocrinol Metab 295: E762-E771, 2008.
- SMUDER AJ, NELSON WB, HUDSON MB, KAVAZIS AN, POWERS SK: Inhibition of the ubiquitin-proteasome pathway does not protect against ventilator-induced accelerated proteolysis or atrophy in the diaphragm. *Anesthesiology* **121**: 115-126, 2014.
- SNEDDON AA, DELDAY MI, MALTIN CA: Amelioration of denervation-induced atrophy by clenbuterol is associated with increased PKC-alpha activity. *Am J Physiol Endocrinol Metab* **279**: E188-E195, 2000.
- SOLOMON V, GOLDBERG AL: Importance of the ATP-ubiquitin-proteasome pathway in the degradation of soluble and myofibrillar proteins in rabbit muscle extracts. *J Biol Chem* **271**: 26690-26697, 1996.
- SOLOMON V, LECKER SH, GOLDBERG AL: The N-end rule pathway catalyzes a major fraction of the protein degradation in skeletal muscle. *J Biol Chem* 273: 25216-25222, 1998.
- SPENCER MJ, MELLGREN RL: Overexpression of a calpastatin transgene in mdx muscle reduces dystrophic pathology. *Hum Mol Genet* 11: 2645-2655, 2002.
- STABACH PR, CIANCI CD, GLANTZ SB, ZHANG Z, MORROW JS: Site-directed mutagenesis of αII spectrin at codon 1175 modulates its μ-calpain susceptibility. *Biochemistry* **36**: 57-65, 1997.
- STALKER TJ, SKVARKA CB, SCALIA R: A novel role for calpains in the endothelial dysfunction of hyperglycemia. *FASEB J* 17: 1511-1513, 2003.
- STEVENSON EJ, GIRESI PG, KONCAREVIC A, KANDARIAN SC: Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. *J Physiol* **551**: 33-48, 2003.
- STITT TN, DRUJAN D, CLARKE BA, PANARO F, TIMOFEYVA Y, KLINE WO, GONZALEZ M, YANCOPOULOS GD: The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* **14**: 395-403, 2004.
- SUGITA H, KANEKI M, SUGITA M, YASUKAWA T, YASUHARA S, MARTYN JA: Burn injury impairs insulinstimulated Akt/PKB activation in skeletal muscle. *Am J Physiol Endocrinol Metab* **288**: E585-E591, 2005.
- SUZUKI A, KIM K, IKEUCHI Y: Proteolytic cleavage of connectin/titin. Adv Biophys 33: 53-64, 1996.
- TAKAHASHI A, KUREISHI Y, YANG J, LUO Z, GUO K, MUKHOPADHYAY D, IVASHCHENKO Y, BRANELLEC D, WALSH K: Myogenic Akt signaling regulates blood vessel recruitment during myofiber growth. *Mol Cell Biol* 22: 4803-4814, 2002.
- TAVEAU M, BOURG N, SILLON G, ROUDAUT C, BARTOLI M, RICHARD I: Calpain3 is activated through autolysis within the active site and lyses sarcomeric and sarcolemmal components. *Mol Cell Biol* 23: 9127-9135, 2003.

- TAWA NE, ODESSEY R, GOLDBERG AL: Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. *J Clin Invest* **100**: 197-203, 1997.
- TAYLOR RG, GEESINK GH, THOMPSON VF, KOOHMARAIE M, GOLL DE: Is Z-disk degradation responsible for postmortem tenderization? *J Anim Sci* **73**: 1351-1367, 1995.
- TERADA N, PATEL HR, TAKASE K, KOHNO K, NAIRN AC, GELFAND EW: Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc Natl Acad Sci U S A* **91**: 11477-11481, 1994.
- TIDBALL JG, SPENCER MJ: Expression of a calpastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse. *J Physiol* **545**: 819-828, 2002.
- TSUNEKAWA S, TAKAHASHI K, ABE M, HIWADA K, OZAWA K, MURACHI T: Calpain proteolysis of free and bound forms of calponin, a troponin T-like protein in smooth muscle. *FEBS Lett* **250**: 493-496, 1989.
- WANG KK, ROUFOGALIS BD, VILLALOBO A: Characterization of the fragmented forms of calcineurin produced by calpain I. *Biochem Cell Biol* 67: 703-711, 1989.
- WANG L, LUO GJ, WANG JJ, HASSELGREN PO: Dexamethasone stimulates proteasome- and calcium-dependent proteolysis in cultured L6 myotubes. *Shock* 10: 298-306, 1998.
- WEI W, YANG H, CAO P, MENCONI M, CHAMBERLAIN C, PETKOVA V, HASSELGREN PO: Degradation of C/EBPβ in cultured myotubes is calpain-dependent. *J Cell Physiol* **208**: 386-398, 2006.
- WELSH GI, MILLER CM, LOUGHLIN AJ, PRICE NT, PROUD CG: Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. FEBS Lett 421: 125-130, 1998.
- WILLIAMS AB, DECOURTEN-MYERS GM, FISCHER JE, LUO G, SUN X, HASSELGREN PO: Sepsis stimulates release of myofilaments in skeletal muscle by a calcium-dependent mechanism. *FASEB J* 13: 1435-1443, 1999.
- WING SS: Control of ubiquitination in skeletal muscle wasting. Int J Biochem Cell Biol 37: 2075-2087, 2005.
- WU M, FALASCA M, BLOUGH ER: Akt/protein kinase B in skeletal muscle physiology and pathology. J Cell Physiol 226: 29-36, 2010.
- YOSHIDA K, INUI M, HARADA K, SAIDO TC, SORIMACHI H, ISHUIRA S, ISHIHARA T, KAWASHIMA S, SOBUE K: Reperfusion of rat heart after brief ischemia induces proteolysis of calspectrin (nonerythroid spectrin or fodrin) by calpain. *Circ Res* 77: 603-610, 1995.
- YOSHIDA M, SUZUKI A, SHIMIZU T, OZAWA E: Proteinase-sensitive sites on isolated rabbit dystrophin. *J Biochem* **112**: 433-439, 1992.
- YOSHIKAWA Y, MUKAI H, HINO F, ASADA K, KATO I: Isolation of two novel genes, down-regulated in gastric cancer. *Jpn J Cancer Res* **91**: 459-463, 2000.
- ZHAO J, BRAULT JJ, SCHILD A, CAO P, SANDRI M, SCHIAFFINO S, LECKER SH, GOLDBERG AL: FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab* **6**: 472-483, 2007.