The molecular mechanisms of calpains on muscle atrophy

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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

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 *et al.* 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^{2+}-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 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^{2+}-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).

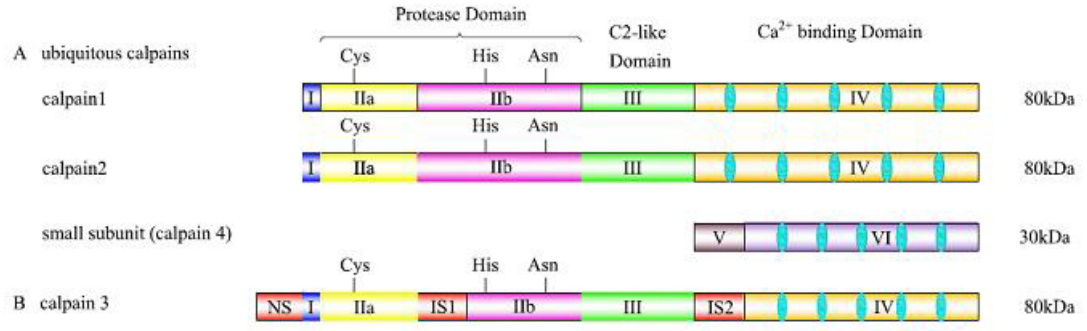

Fig.1 A, four domains have been identified in both calpain1 and calpain2: (I) the

N-terminal domain, (II) a domain containing a sequence characteristic of cysteine proteases, (III) a connecting domain, and (IV) a Ca2+-binding domain. B, calpain3 carries three unique sequences: NS, IS2 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 calpain3-defecient mice lacked well-organized sarcomeres (Kramerova *et al.* 2004). Thus, calpain3 plays an important role in sarcomere remodeling. Indeed, loss-of-function 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 and 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, Ayaka *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 calpain 2. 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 *et al.* 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 calpain 3 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^{2+} 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 5–50μM for calpain1 and 250–1000μM 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^{2+}-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.* stated that calpain3 is bound tightly within a fibre, whereas most calpain1 is initially freely diffusible in the cytoplasm at resting [Ca^{2+}] under

physiological conditions (Robyn *et al.* 2006). 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 known, skeletal muscle contains 3 groups of protein: 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 (table1). Due to the strong cleavage activities on these critical cytoskeletal proteins demonstrated by calpains, Dayton *et al.* proposed that the calpains might be responsible for release of myofilaments from the surface of myofibrils (Dayton, *et al.* 1976a, 1976b).

In muscle wasting, preferential site of calpain proteolysis seems to be at Z-disc, a dense multi-proteinaceous 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-disk once they are activated, thereby severing their attachment to Z-disk. Moreover, the calpains cleave Z-disk-associated desmin, an intermediate filament protein, in the sarcolemma. Consequently, the major Z-disk proteins α-actinin are released which results dissolvement of Z-disk 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, tropomosin 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).

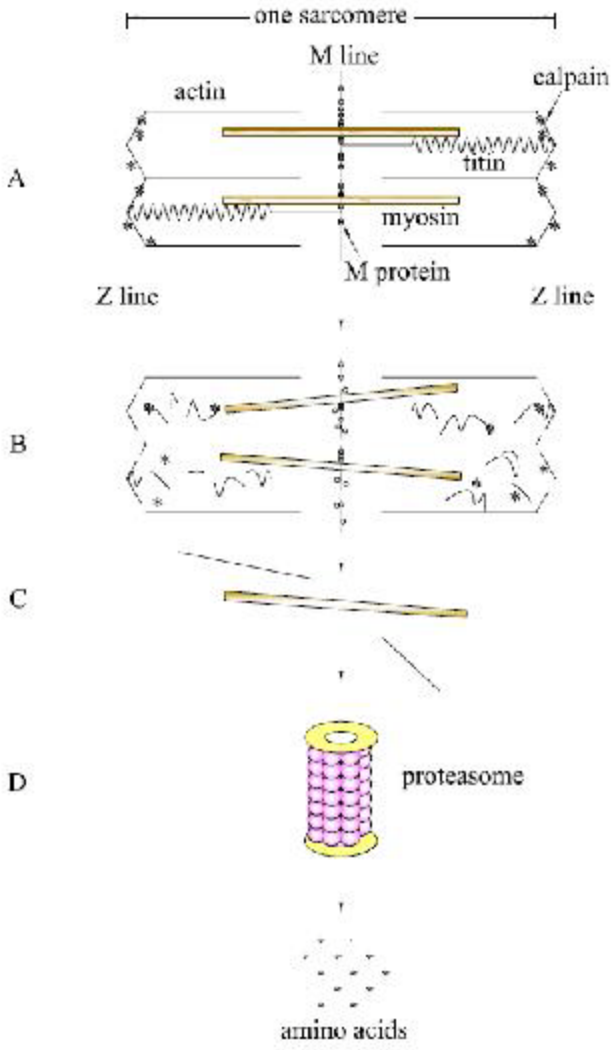

Fig.2 A，calpains mainly reside in Z line of intact myofilaments; B, calpains are able to rapidly cleave cytoskeletal proteins like titin and nebulin at sites near the Z-disk sequentially dissociation of myofilaments; C, myofilaments realse from myofibril; D, polypeptides and polypeptide fragments degrade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

Table1: 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 parent 97-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 *et al.* 1996).
Cadherin	E-cadherin is not degraded by calpain (Bush *et al.* 2000), whereas the intracellular domain of N-cadherin is degraded by calpain1 (Covault *et al.* 1991) or calpain2 (Sato *et al.* 1995).
Calponin	Degraded to 30-, 27-, and 19.5-kDa fragments; the latter

	fragment is stable (Croall *et al.* 1996, Tsunekawa *et al.* 1989).
Catenin	α-Catenin is not degraded by calpain but calpain removes the NH_2 terminus of β-catenin to produce 90- and 75-kDa fragments from the 97-kDa β-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 *et al.* 1975).
Desmin	Degraded to a 32- to 37-kDa stable fragment; an 18-kDa fragment appears after longer digestion (O'Shea *et al.* 1979); a 9-kDa fragment is removed from the NH_2 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_2-terminal fragment and several 50- to 140-kDa fragments (Yoshida *et al.* 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 *et al.* 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 *et al.* 1995); calpain degradation severs nebulin connection to Z-disk.
αII-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 *et al.* 1995); the initial calpain sensitive site is at Tyr_{1176}-Gly_{1177} (Stabach *et al.* 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_2-terminal fragment; after cleavage, can no longer cross-link integrin to cytoskeletal elements (Hemmings *et al.* 1996, Muguruma *et al.* 1995).
Titin	The ~3000-kDa titin polypeptide is cleaved to a large ~2,000-kDa fragment by removal of a 1,200-kDa NH_2-terminal fragment; this cleavage severs the

	connection of titin to the Z-disk; the 1,200-kDa fragment is degraded to smaller fragments of 100–500 kDa, with the 500-kDa fragment being stable (Taylor *et al.* 1995); chicken titin is quickly degraded to a T2 (2,000 kDa) fragment which is then degraded to a 1,700- and a 400-kDa fragment; the 1,700-kDa fragment is further degraded to a 1,400-kDa fragment (Suzuki *et al.* 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 *et al.* 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 *et al.* 1994); not protected when in the myofibrillar structure (Dayton *et al.* 1975).
Tubulin	Degraded from a 55- to a 50- to 52-kDa fragment (Billger *et al.* 1988); or not degraded at all (Sandoval and Weber 1978).
Vinculin	Cleaved to a ~90-kDa fragment (Taylor *et al.* 1995) that

can no longer cross-link talin, paxillin, and α–actinin filaments.

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 *et al.* 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, Alfred 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 sunstrates 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,

Stevenson *et al.* 2003, Lecker *et al.* 1999b). 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 *et al.* 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 appears to only able to degrade sarcomeric proteins of monomeric form (including a-actin) but not when they are in higher-ordered structures. Higher-ordered 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

et al. 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 theit 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 μm in diameter. Thus, the first step in degradation of myofibrillar proteins (actin and myosin) during atrophy requires release of myofilaments from the sarcomere (Solomon *et al.* 1996, 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-disk and release of principal Z disk 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 degradated 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 & Dodd designed an Ex vivo experiments using isolated rat diaphragm muscle treated with Ca^{2+}, calpain inhibitor calpeptin or proteasome inhibitor epoxomicin (Smith *et al.* 2007). They found that calpain activation increased total protein degradation and proteasome-dependent proteolys 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 *et al.* 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 3β (GSK3β). 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 $p70^{S6K}$ 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-3β kinase activity through phosphorylation of Ser9 on GSK-3β (Harwood 2001). Phosphorylated GSK-3β 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-3β pathways are playing mediating effects for muscle hypertroph 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 activitation 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.* found that Akt was reduced while MAFbx mRNA and protein levels were increased in amyotrophic lateral sclerosis (ALS) patients and ALS G93A mice (Léger *et al.* 2006). 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.* found that burn injury impairs

phosphorylation of Akt and activation of GSK-3β in skeletal muscle (Sugita *et al.* 2005). These findings suggested that attenuated Akt activation was involved in disturbed metabolism and muscle wasting. Other research by Fang *et al.* reported that burn injury increased GSK-3β kinase activity in atrophying muscle (Fang *et al.* 2007) 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 p70S6K 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.* revealed that molecular chaperone heat shock protein90 (HSP90) can bind and protect phosphorylated Akt from dephosphorylation by phosphatase2A to maintain Akt activation (Sato *et al.* 2000). In addition, they also demosntrated 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 (Smith *et al.* 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_2O_2 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 ROS-mediated formation of reactive aldehydes which is known to be able to inhibit plasma membrane Ca^{2+} -ATPase activity (Siems *et al.* 2003). Therefore, an oxidative stress-induced decrease in membrane Ca^{2+} -ATPase activity would impede Ca^{2+} removal from the

cell and promote intracellular Ca^{2+} accumulation and calpanis 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-κB (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 sepsis, neuromuscular disorders and 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

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