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REVIEW

The role of calpain in skeletal muscle

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Calpains are a class of proteins that belong to the calcium-dependent, non-lysosomal cysteine proteases. There are three major types of calpains expressed in the skeletal muscle, namely, μ-calpain, m-calpain, and calpain 3, which show proteolytic activities. Skeletal muscle fibers possess all three calpains, and they are Ca^{2+}-dependent proteases. The functional role of calpains was found to be associated with apoptosis and myogenesis. However, calpain 3 is likely to be involved in sarcomeric remodeling. A defect in the expression of calpain 3 leads to limb-girdle muscular dystrophy type 2A. Calpain 3 is found in skeletal muscle fibers at the N2A line of the large elastic protein, titin. A substantial proportion of calpain 3 is activated 24 h following a single bout of eccentric exercise. *In vitro* studies indicated that calpain 3 can be activated 2–4 fold higher than normal resting cytoplasmic [Ca^{2+}]. Characterization of the calpain system in the developing muscle is essential to explain which calpain isoforms are present and whether both μ-calpain and m-calpain exist in differentiating myoblasts. Information from such studies is needed to clarify the role of the calpain system in skeletal muscle growth. It has been demonstrated that the activation of ubiquitous calpains and calpain 3 in skeletal muscle is very well regulated in the presence of huge and rapid changes in intracellular [Ca^{2+}].

Keywords: calpains; calpastatin; skeletal; protein; proteases

Introduction

Calpains are a class of proteins that belong to the Ca^{2+}-dependent, non-lysosomal cysteine proteases (Koohmaraie and Geesink 2006). There are three major types of calpains expressed in skeletal muscle, namely, μ-calpain, m-calpain, and calpain 3 (p94), which show proteolytic activities. μ-Calpain and m-calpain are calcium-activated proteases and require micro- and millimolar concentrations of Ca^{2+}, respectively, for their activation (Goll et al. 2003). They are expressed ubiquitously in the skeletal muscle. Calpain 3 requires very little or no Ca^{2+} for its activation and is characterized by a rapid and complete autolysis (Sorimachi et al. 1993). In addition to calpains, their specific inhibitor, calpastatin, is also found in the skeletal muscle, which is a highly polymorphic protein and calpain-specific endogenous inhibitor. It is found to be associated with calpain proteolytic enzyme family (Wendt et al. 2004). It was originally proposed that calpains are responsible for initiating metabolic turnover of myofibrillar proteins and therefore they affect the rate of muscle protein degradation (Dayton et al. 1975). Recent investigations have shown that the calpain activity is mandatory for myoblast fusion and for cell proliferation (Barnoy et al. 1996). Calpains also affect the number of skeletal muscle cells in domestic animals by changing the rate of myoblast proliferation and myoblast fusion modulation. In this review, we focus on the role of the calpain system in the skeletal muscle.

Background information on calpain system

Calpain history dates back to 1964 when the calpains were detected in the brain and eye lens. Calpains activity was caused by intracellular cysteine proteases and having an optimal activity at neutral pH (Ohno et al. 1984). Calpains activity was found to be attributable to two main isoforms, μ-calpain and m-calpain. These two isoforms differ in their calcium requirements *in vitro*, and their names suggest that they are activated by micro- and millimolar concentrations of Ca^{2+} within the cells (Glass et al. 2002). The terms μ-calpain and m-calpain were first used in 1989 to address the requirement of micromolar and millimolar amounts of Ca^{2+} for their activation, and they are referred to as ubiquitous calpains (Cong et al. 1989).

Calpain 3 is the third member of the calpain family, and it was discovered 13 years ago. Mutations in calpain 3 result in an autosomal recessive and progressive form of limb-girdle muscular dystrophy called limb-girdle muscular dystrophy type 2A (Beckmann and Spencer 2008). Calpastatin that shows both calpain and calpain inhibitor activities was discovered during purification of m-calpain from the muscle extracts (Goll et al. 1990). The name calpastatin was given by Takashi Murachi (Murachi 1989). Calpastatin system is involved in the membrane fusion events such as neural vesicle exocytosis and platelet and red-cell aggregation. Calpastatin inhibits both μ- and m-calpain, but calpain 3 is not inhibited and may regulate

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the activity of μ- and m-calpain by degrading calpastatin (Ono et al. 2004).

Role of calpain system in skeletal growth

There is ample evidence that the calpains have main roles, both in normal and postnatal skeletal muscle growth, muscle wasting, and loss of muscle mass. A number of experiments have reported that the calpain system also has a role in normal skeletal muscle growth. Administration of β-adrenergic agonists to an animal results in a 10–30% elevation in the rate of accumulation of muscle mass (Yang and McElligot 1989). Even though various experiments were carried out using various species with variable β-adrenergic agonists, different conditions produced slightly different results. Several studies agree that the administration of β-adrenergic agonists elevates both the rate and efficiency. It has been reported that the administration of β-adrenergic agonist affects the activity of calpain system (Forsberg et al. 1989) and elevates the muscle calpastatin activity (Higgins et al. 1988). This elevation ranges from 52% to 430% (Kretchmar et al. 1989). Muscle μ-calpain activity is either reduced or unchanged, while m-calpain activity seems to be elevated (Koohmaraie and Shackelford 1991). It has been reported that the activity of both μ- and m-calpain is reduced. The skeletal muscle mass in the callipyge phenotype sheeps was 30–40% greater than that in half-siblings not expressing the callipyge trait (Cockett et al. 1994). Calpastatin activity in the affected muscles from callipyge lambs are 68–126% greater than in the same muscles from normal lambs, while calpastatin activity in the unaffected callipyge muscles. This finding suggests that elevated rates of skeletal muscle growth can result from a reduction in the rate of muscle protein degradation. This reduced rate of muscle protein degradation is associated with the reduction in the activity of calpain system (Koohmaraie et al. 1995).

Recent investigation reports that the calpain activity is necessary for cells to progress through the G1 to S phase of the mitotic cycle and is also required for myoblast fusion (Barnoy et al. 1997). This suggests that the enhanced calpain activity during muscle development may be associated with an elevated number of myoblasts. Growing C2C12 myoblasts contain m-calpain only (Cottin et al. 1994). This is due to the administration of β-adrenergic agonist to growing animals, which seems to have less effect on m-calpain than on calpastatin.

Role of calpains in skeletal muscle

Calpains are believed to play a role in protein modification bylimiting protein degradation and consequent targeting of the ubiquitin proteasome degradation pathway (Murphy et al., 2006). The μ-calpain undergoes autolysis from the full-length 80 kDa form to the 78 kDa form by increasing its Ca^{2+} sensitivity greater than 10-fold. The pre-activated μ-calpain is localized under the surface membrane, and it has been found that in the presence of micromolar concentration of Ca^{2+}, the freely diffusible, full-length μ-calpain binds quickly at least in part to cell membranes and stays bound for some time. The Ca^{2+} sensitivity of μ-calpain autolysis is elevated in the presence of phospholipids. The pre-activated calpain is ideally placed for facilitating the rapid membrane resealing, a process known to be dependent on dysferlin and local remodeling of the cytoskeleton by calpain (Gailly et al. 2007). *In vivo*, the μ-calpain has been shown to play a role in membrane repair, and its protective role in skeletal muscle has also been demonstrated.

Many reports have indicated that the calpains have very limited and specific effects on skeletal muscle proteins. Myosin degrades very slowly and even this slow degradation is limited to a few cleavages of the light chains. Non-denatured actin is not cleaved by either μ-calpain or m-calpain. Both μ- and m-calpain rapidly cleave troponin T, desmin, vinculin, talin, spectrin, nebulin, and titin; more slowly cleave troponin I, filamin, C-protein, dystrophin, and tropomyosin; and very slowly cleave alpha-actinin and M protein (Yang and McElligot 1989).

Incubation of skeletal muscle proteins with the calpains results in the loss of N2 line and then in complete loss of Z-disks, leaving a gap in the middle of sarcomere. Simultaneously, loss of periodicity in the I-band area occurs due to troponin and tropomyosin degradation. The loss of Z-disk structure is caused by release of alpha-actinin from Z-disks in a nearly intact form (Clark 1993). Even though many experiments have indicated that the myosin, actin, and alpha-actinin are degraded by the calpains, it is unclear whether these investigations have utilized non-denatured proteins. Both μ- and m-calpain rapidly degrade denatured actin, myosin, and alpha-actinin. The calpains specifically cleave several cytoplasmic proteins, which include mostly protein kinases and phosphatases. They do not cause bulk degradation of sarcoplasmic proteins into small fragments (Tan et al. 1988). Most of the experiments attempting to determine muscle protein turnover use release of free amino acids or 3-methyl histidine to estimate the rate of muscle protein degradation (Clark 1993).

The myofibrillar structure must remain intact in skeletal muscle cells in order to be functional. The turnover of myofibrillar proteins, which represent the major fraction of total protein in muscle cells, must proceed through a different mechanism. Even though

the mechanism by which myofibrillar proteins turn over is still not clear and remains an area of active research; presently, it seems that this turnover proceeds via two steps: (1) removal of the proteins from the myofibrillar structure, which must occur without disruption of the myofibril that extends continuously from one end of the muscle cell to the other; (2) degradation of the individual myofibrillar proteins to small peptides and free amino acids.

Kinetic investigations have reported that the proteins in adult cardiac myocytes are degraded from two different pools: (1) 10% of total muscle protein turns over rapidly with a mean half-life of 11.9 h and (2) remaining 90% of the total muscle protein turns over more slowly with a half-life of 15.6 d. Similar experiments have not been done on skeletal muscle cells, but the structure of cardiac myofibrils, the proteins of which constitute a smaller percentage of total muscle protein (45–55%) than in skeletal muscle, is very similar to the structure of skeletal muscle myofibrils and the myofibrillar proteins probably turn over via the same mechanism in the two types of cells. The presence of proteins turning over at different rates in muscle cells does not prove that the myofibrillar proteins must be removed from the myofibril before they can be degraded to amino acids (Clark 1993).

Many experiments reported that 5–15% of the total myofibrillar protein in the skeletal and cardiac muscle cells can be dissociated from intact myofibrils in the form of myofilaments by using gentle agitation in an adenosine triphosphate (ATP)-containing solution (Van der Westhuyzen et al. 1981). These easily releasable filaments lack alpha-actinin, desmin, titin, and other cytoskeletal proteins such as filamin having molecular masses above 200 kDa but contain the major myofibrillar proteins, actin and myosin. Proteins in easily releasable filament fraction turn over rapidly, indicating that they are in a pool of degrade proteins. The easily releasable filament levels in the muscle are elevated in response to treatments that increase calpain activity and decrease in response to treatments that inhibit calpain activity (Dahlmannn et al. 1986; Reville et al. 1994).

The calpains make specific cleavages in cytoskeletal proteins that are involved in maintaining the myofibrillar structure: (1) degradation of desmin, vinculin, talin, dystrophin, and spectrin; (2) loss of Z-disks and release of alpha-actinin evidently due to cleavage of the N-terminal end of the large titin polypeptide and to the rapid cleavage of nebulin (Ohtsuka et al. 1997); (3) degradation of C-protein, which encompasses thick filaments like staves around a barrel; (4) degradation of troponin T and tropomyosin, which would contribute to 'weakening' of the thin filament; (5) degradation of M proteins along with the degradation of C protein. The calpains are unique among proteolytic enzymes in that they do not rapidly degrade the major muscle proteins, actin and myosin, which also are the major components of the easily releasable filaments. Large size and ordered structure of intact myofibrils would prevent them from being taken up into lysosomes (Lowell et al. 1986). Neither myofibrils nor structurally recognizable fragments of myofibrils have been observed in lysosomal structures. The proteasome has no effect on myofibrillar proteins when they are in the myofibrillar structure (Goll et al. 1989; Solomon and Goldberg 1996).

The available evidence suggests that the myofibrillar proteins in skeletal muscle cells are first removed from the myofibril either in the form of filaments or as individual proteins. These protein molecules in the filaments are then degraded to amino acids by proteolytic systems in the cell cytoplasm. Some experimental results have suggested that individual proteins are removed from myofibrils. The mechanism by which this exchange is accomplished without disrupting the myofibrillar structure is not known. The presence of easily releasable filaments suggests that myofibrillar proteins are released from the surface of myofibrils. This mechanism would leave functionally intact, although smaller, myofibrils as turnover progressed. The removed myofilaments could either reassemble back onto the surface of the myofibril or be degraded to amino acids/small peptides by cytoplasmic proteinases (Yang and McElligot 1989).

Calpains initiate disassembly of the myofibril by specific cleavages of Z-disk proteins at the surface of the myofibril, releasing the thin filaments from their attachments to the myofibril. The myosin thick filaments that are attached to the released thin filaments dissociate in the presence of ATP in the cell. Calpain-induced cleavage of C-protein and M-protein leads to further dissociation of thick filaments to individual myosin molecules, and these are degraded by proteasome or taken up into lysosomes and degraded by lysosomal cathepsins. Calpain-induced cleavage of tropomyosin and troponin T and I together with the degradation of nebulin favors dissociation of thin filaments to actin monomers, and these are degraded by the proteasome.

The disassembly and degradation process is teleologically attractive and proposes definite roles for calpains and proteasome, which are consistent with the known properties of these systems. An experiment done over 35 years ago suggested that a newly synthesized myofibrillar protein was added to the surface of growing myofibrils. This mechanism also indicates that the interior of myofibrils would be immortal unless the entire myofibril was turned over. Even though there is remarkable circumstantial

evidence indicating that the calpains play a main role in initiating turnover of the myofibrillar proteins, it is clear that the calpains cannot degrade the myofibrillar or any other class of proteins to amino acids. Consequently, at least two or three proteolytic systems are involved in the turnover of the myofibrillar proteins. Calpain cleavage frequently ablates the regulation that normally governs the activity of these kinases/phosphatases, and leaves constitutively active enzymes. The effects of active enzymes on signal transduction are not clear, and it is ironic that the clearly defined property of the calpains does not involve a function in living cells, but rather its role in the postmortem tenderization (Boehm et al. 1998).

Calpains have already been demonstrated to participate in the turnover of several other muscle cytoskeletal proteins under different conditions. For example, desmin and dystrophin were shown to be calpain targets in cultured myotubes during periods of serum withdrawal (Purintrapiban et al. 2003). Similarly, desmin, talin, and fibronectin were shown to be cleaved by calpain during membrane cytoskeleton rearrangement concomitant with myoblast fusion (Otani et al. 2004). Recent evidence corroborating the idea that calpain regulates physiological muscle protein turnover was obtained in transgenic mice. In this study, the muscle-specific overexpression of a calpastatin transgene resulted in significant muscle hypertrophy and accumulation of several proteins, presumably as a consequence of reduced degradation. Preactivated calpain seems ideally placed for facilitating rapid membrane resealing if there is localized damage, a process known to be dependent on dysferlin and local remodeling of the cytoskeleton by calpain (Murphy et al. 2006).

A huge amount of evidence acquired during the past 35 years has indicated that the calpains are responsible for up to 95% of all proteolytically induced postmortem tenderization that occurs during the first 7–14 d of postmortem storage at 2–4°C. Storage for longer durations causes postmortem to involve some catheptic proteolysis, and it is still unknown whether postmortem proteolysis involves primarily μ- or m-calpain or both (Boehm et al. 1998). Experiments on animals that have received β-agonists or animals having a *Bos indicus* have indicated less tender meat. Muscle calpain activity seems to vary little among these different groups of animals. High calpastatin activity decreases the ability of calpains to degrade myofibrillar proteins during postmortem storage. This is because the muscle calpastatin levels vary more widely in response to different treatments than muscle calpain activities.

Even though the physiological function of μ-calpain, along with the other ubiquitously expressed calpain, m-calpain, is not clear, it has been implicated in a number of cellular functions linked to Ca^{2+} signaling, including apoptosis, myogenesis, cell signaling, and cell differentiation (Saido et al. 1994). Calpains are believed to play a role in protein modification with one course of action involving limited degradation of proteins and consequently targeting to the ubiquitin proteasome degradation pathway (Verburg et al. 2009). It has been suggested that a specific role of m-calpain in skeletal muscle is to prevent major degradation by reducing Ca^{2+} release from the sarcoplasmic reticulum, following periods of excessive Ca^{2+} release (Mellgren et al. 2007). This intervention seems to occur through interference with excitation–contraction matched, in which the communication at the triad junction between the voltage sensors and the calcium release channels is hindered. The m-calpain plays a role in membrane repair *in vivo* (Kapprell and Goll 2006). μ-Calpain is inhibited by endogenously expressed calpastatin (Murphy et al. 2006).

Following exposure to micromolar [Ca^{2+}], full-length 80 kDa μ-calpain autolyzes to its 78 and 76 kDa isoforms. The presence of autolyzed μ-calpain is an indicator of an activated protease. Biochemical assays have demonstrated that the 80 kDa isoform of μ-calpain can itself be active if the [Ca^{2+}] is sufficiently enough (Goll et al. 2003). Autolyzed μ-calpain isoforms have a huge Ca^{2+} sensitivity than the full-length 80 kDa isoform. The skeletal muscle μ-calpain was active at a similar rate in the presence of either 20 μM Ca^{2+} or 2 μM Ca^{2+}. Above a baseline level of autolysis in the human skeletal muscle, there was an additional 23%, 28%, and 46% autolysis following 1 min exposure to 2.5, 10, and 25 μM Ca^{2+}, respectively (Ruegg et al. 2002). Calpains participate in the physiological turnover of utrophin in muscle cells, particularly in dystrophin-deficient muscle cells, where impaired Ca^{2+} homeostasis has been reported. A major advance created in this study compared to previous biochemical-based studies is that the μ-calpain was expressed endogenously in the skeletal muscle and was examined in the presence of all other muscle constituents, including factors such as DUK114 (Isabelle and Alexandre 2006).

The role of calpain-3 in muscle repair and maintenance in myogenesis and apoptosis has been proposed. The absence of calpain-3 in the mouse skeletal muscle produces an overt phenotype (Fougerousse et al. 2003). Muscular dystrophy with myositis is a mouse model of the human diseases human tibial muscular dystrophy and limb-girdle muscular dystrophy type 2A (Haravuori et al. 2001). Calpain-3 has been shown to regulate nuclear IkBak (Baghdiguian et al. 2001). Calpain-3 is inactive in full form and active

on removal of the IS1 region, the process being Ca^{2+} dependent. The cleaved domains must remain associated with each other for the protease to be active (Rey and Davies 2002). Calpain-3 was found to be spontaneously autolyzed *in vivo*. Calpain-3 is quite stable in the skeletal muscle and not inhibited by calpastatin. The specific endogenous substrates for calpain-3 have not been identified yet. The identification of the substrates for calpain-3 is important in order to define the role of calpain-3 in the skeletal muscle. Many proteins, such as filamin, vinexin, ezrin, talin, and myosin light chain, have been identified as substrates for calpain *in vitro* (Hayashi et al. 2008).

The intracellular [Ca^{2+}] often changed in the skeletal muscle, and there was a large transient elevation in intracellular [Ca^{2+}], with cytoplasmic [Ca^{2+}] reaching 2–20 mM during normal contraction (Chin and Allen 1996). Mild and prolonged elevation in intracellular [Ca^{2+}] duration causes fatigue such as that which follows eccentric exercise, and there was a little persistent increase in resting intracellular [Ca^{2+}] in certain disease states and aging. The cytoplasmic [Ca^{2+}] is enhanced two- to threefold above resting levels for 48 h following eccentric exercise in the mouse skeletal muscle. There are two mechanisms involved in calpain-3 activation following an eccentric bout of exercise. Firstly, the muscle fibers are lengthened during muscle contraction. Secondly, the intracellular [Ca^{2+}] would be expected to remain elevated slightly above normal resting cytoplasmic levels for 24–48 h following the exercise (Lynch et al. 1997). Calpain-3 would be activated if its IS2 region was not bound to titin and its activation might be sensitive to a muscle fiber stretching (Lowell et al. 1986).

Calpains in muscle pathology

In the muscular dystrophies and other muscle pathologies the intracellular free Ca^{2+} concentration is enhanced. This increased intracellular Ca^{2+} stimulates calpain activity. The structural alteration observed in the atrophying muscles (Kretchmar et al. 1989) is mimicked by treatment of myofibrils from normal muscle with μ-calpain or m-calpain (Koohmaraie et al. 1995). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has reported that the calpains cause similar degradative changes in myofibrils as those seen in Duchenne muscular dystrophy.

Summary and conclusions

Skeletal muscle fibers contain m-calpain, μ-calpain, muscle-specific calpain-3, and Ca^{2+}-dependent proteases. Their physiological roles are unclear. Even though ubiquitous calpains have been associated with apoptosis and myogenesis, calpain-3 is likely to be involved in sarcomeric remodeling. A defect in the expression of calpain-3 results in limb-girdle muscular dystrophy type 2A. At the rest intracellular [Ca^{2+}], calpains are present in full length in unautolyzed forms. Calpain-3 is bound in skeletal muscle fibers at the N2A line of the large elastic protein, titin. Ubiquitous calpains or calpain-3 is activated immediately following a sprint, endurance despite the frequent episodes of high cytoplasmic [Ca^{2+}]. A substantial proportion of calpain-3, but not ubiquitous calpains, is activated 24 h after a single bout of eccentric exercise. *In vitro* studies indicated that calpain-3 becomes activated two- to fourfold higher than normal resting cytoplasmic [Ca^{2+}] if exposed for a prolonged period of time, and this suggests the sustained increase in intracellular [Ca^{2+}].

Measurement of calpastatin isoforms present in different muscles shows whether change in these isoforms in response to treatments can alter muscle growth. Moreover, this indicates whether the relationship between calpastatin activity and rate of skeletal muscle growth in mature animals is the result of a change in a calpain isoform with different calpain inhibitory properties. Characterization of the calpain system in the developing muscle is needed to indicate as to which calpain isoforms are present and whether both μ-calpain and m-calpain exist in differentiating myoblasts. Information from studies such as this is needed to provide a basis for more detailed studies that would clarify the role of the calpain system in skeletal muscle growth. It has been demonstrated that the activation of ubiquitous calpains and calpain-3 in the skeletal muscle is very well regulated in the presence of huge and rapid change in intracellular [Ca^{2+}].

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