Time course of changes in the activities of μ- and m-calpain during muscle denervation

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Abstract

It has been hypothesized that calpains, Ca^{2+}-dependent proteases, may be involved in the initiation of myofibrillar and cytoskeletal protein breakdown. Therefore, calpains may play key roles in inducing muscle atrophy. However, the pattern of changes in their activities during muscle denervation remains unclear. We characterized the time course of changes in the activities of μ- and m-calpains during muscle denervation. Female mice underwent surgery to transect the sciatic nerve. Gastrocnemius muscles were isolated 1, 3, 5, 10, 20, or 30 days after surgery. The activities of μ- and m-calpains were analyzed using casein zymography. Casein zymography was performed using 0.02% casein copolymerized in a 10% polyacrylamide gel. The muscle-to-body weight ratio began to decrease 5 days after muscle denervation and progressively decreased until 20 days. Total calpain activity (μ- and m-calpains) slightly increased at 5 days and then gradually decreased during the 10- to 30-day period after muscle denervation. The change in the activity of m-calpain was similar to that of total calpain activity. The activity of μ-calpain was slightly increased at 5 days and thereafter progressively decreased through 30 days. Until 10 days after muscle denervation, the relative abundance of μ- and m-calpain was approximately 70% and 30% of total calpain, respectively. At 30 days, the relative abundance of m- and m-calpain was each 50% of total calpain. Taken together, μ- and m-calpains respond differently to muscle denervation, suggesting that the possible roles of μ- and m-calpains may be distinct from each other in the denervated muscle.

KEY WORDS : calpain, denervation, zymography

Introduction

Skeletal muscles adapt to decreased neuromuscular activity and reduced load bearing. Muscle atrophy occurs as a consequence of spaceflight, hind-limb unweighing, prolonged bed rest, joint immobilization, tenotomy, spinal cord isolation, or muscle denervation. These models of muscle atrophy induce a significant loss of myofibrillar proteins. It has been proposed that lysosomal, Ca^{2+}-dependent or ubiquitin-proteasome pathways may be associated with muscle atrophy. However, the molecular mechanism(s) underlying how these pathways induce muscle atrophy is not fully understood. Recent evidence has suggested that activation of the ubiquitin-proteasome pathway is primarily responsible for the rapid loss of muscle proteins in various types of atrophy[13]. Indeed, the ubiquitin-proteasome pathway plays a major role in muscle wasting during various catabolic states[6]. However, intact myofilaments cannot be degraded directly by the ubiquitin-dependent process[14]. This observation leads us to hypothesize that the disassembly of intact myofilaments occurs prior to degradation by the ubiquitin-proteasome pathway. Calpain releases α-actinin from the Z-disk without degradation[8]. *In vitro*, calpain cleaves a variety of substrates, cytoskeletal proteins (i.e., desmin, α-actinin, vimentin, spectrin, integrin, cadherin), and

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myofibrillar proteins (i.e., troponin, tropomyosin, myosin light chain kinase)[1]. Therefore, it has been proposed that calpains may play key roles in the disassembly of sarcomeric proteins.

Calcium-activated neutral protease (CANP, calpain, EC 3.4.22.17) is an intracellular non-lysosomal cystein protease. This protease is a heterodimer with a molecular weight of 108,000 Da, consisting of one 80,000-Da subunit and one 28,000-Da subunit[7]. Two isoforms of calpain, differing in Ca^{2+} requirement, exist ubiquitously in mammalian tissue[1]. The low (μM) and high (μM) Ca^{2+}-requiring isoforms are designated μ-calpain and m-calpain, respectively[1]. It is known that the Ca^{2+} concentration ($[Ca^{2+}]$) requires 3-50 μM for half-maximal proteolytic activity of m-calpain and 200-1000 μM for half-maximal proteolytic activity of m-calpain[7]. Calpastatin is an endogenous inhibitor of calpain and is equally effective in inhibiting all isoforms[7]. The Ca^{2+}-dependent proteolytic pathway is activated at the early stage of muscle denervation[6]. Subcellular localization of μ-calpains, m-calpains, and calpastatin was reviewed by Kumamoto and colleagues[10]. They have demonstrated that μ-calpains, m-calpains, and calpastatin are approximately two times greater at the Z-disk of myofibrils than at the I- or A-band regions. Additionally, Kumasmoto and colleagues reported that the concentration of μ-calpains, m-calpains, and calpastatin increased during 15 days of muscle denervation. However, the pattern of changes in the activities of μ-calpains and m-calpains during prolonged muscle denervation has yet to be elucidated. Therefore, in the present study, we characterized the time course of changes in the activities of μ-calpains and m-calpains in the gastrocnemius muscles from hindlimb denervated animals.

Materials and Methods

Animal care

Female 7-week-old CD1 mice (CLEA, Tokyo, Japan) were used in the present studies. The mice were housed in the animal facility under a 12-h light/12-h dark cycle at room temperature (23 ± 2 ℃) and 55 ± 5% humidity. The mice were maintained on a diet of CE-2 rodent chow (CLEA) and given water ad libitum. All procedures in the animal experiments were performed in accordance with the guidelines presented in the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, published by the Physiological Society of Japan. This study was also approved by the Animal Committee of the National Institute of Fitness and Sports, Japan.

Surgical procedure

Mice underwent mid-thigh sciatic nerve transection. A surgical level of anesthesia was induced by intraperitoneal injection of pentobarbital sodium (30 mg/kg). All surgical procedures were performed under aseptic conditions. The right sciatic nerve was isolated mid-thigh and transected 5-8 mm proximal to the trifurcation. Gastrocnemius muscles (superficial portion) were isolated at 1, 3, 5, 10, 20, or 30 days after surgery and immediately stored for biochemical analysis.

Preparation of muscle extract

The muscles, frozen with liquid nitrogen, were pulverized in a steel pestle and mortar. The following procedures were performed at 4℃. Muscle powder was homogenized at intense cooling by an ice-salt mixture in a five-fold volume of 20 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 10 μg/ml Pefabloc, and 10 μg/ml pepstatin A. Muscle extracts were separated from

debris by 10-min centrifugation at 1,000*g*. The supernatant was subjected to 60-min centrifugation at 16,000*g* to separate soluble and myofibril fractions. Protein concentration was determined by the Bradford method[3] with bovine serum albumin as a standard.

Casein zymography

The soluble fraction was assessed by casein zymography. Casein zymography was performed using the original assay[12] with minor modifications. Briefly, 0.02% (wt/vol) casein was copolymerized in a 10% (wt/vol) polyacrylamide gel (pH 8.8). The casein gel was subjected to 15 min of preelectrophoresis (100 V, 30-40 mA) with Tris-glycine buffer (pH 8.3) containing 1 mM EGTA and 1 mM DTT. After protein loading (50 μg), electrophoresis was started (2-3 h, 100 V, 30-40 mA, 4℃). The gel was washed twice for 30 min in a buffer containing 20 mM Tris-HCl (pH 7.4) and then incubated at room temperature overnight in activation buffer (pH 7.4) containing 20 mM Tris-HCl, 10 mM DTT, and 4 mM $CaCl_2$. Finally, the casein gel was fixed with solution containing 10% acetic acid (vol/vol) and 25% methanol (vol/vol) and then stained for 2 h with acid-based Coomassie Brilliant Blue R-250, followed by vigorous washing in distilled water. We verified whether the amount of soluble protein was equally loaded using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Image analysis

The gels were photographed using a digital camera (CAMEDIA E-10, OLYMPUS, Tokyo, Japan). The intensity of the bands was densitometrically quantified using Image-Pro Analyzer Ver. 4.0 for Windows (Media Cybernetics, MD, USA) and was expressed as integrated optical density (IOD). Therefore, calpain activity was expressed as intensity of IOD. Total calpain corresponds to μ-calpain plus m-calpain. All data are expressed as mean ± SD (n=3 per each time point).

Results

As shown in Fig. 1, the muscle-to-body weight ratio began to decrease 5 days after muscle denervation and continued to decrease until 20 days.

A zymographycal assay was used to assess calpain activity in muscle homogenates. This assay is based on electrophoretic separation before activity is measured. Therefore, calpastatin-bounded calpain was liberated to quantify calpain activity and to distinguish calpain isoforms. As shown in Fig. 2, crude muscle homogenates yielded two major electrophoretically separated caseinolytic bands. The faster migrating band was identified as m-calpain and the slower migrating band was identified as μ-calpain[16]. Minor band migrating ahead of μ-calpain was assumed to be the autoproteolyzed form of μ-calpain. As shown in Fig. 3, total calpain activity (μ- and m-calpain) slightly increased 5 days and then gradually decreased during the 10- to 30-day period after muscle denervation. As shown in Fig. 4, the change in activity of μ-calpain was similar to that of total calpain activity. The activity of μ-calpain

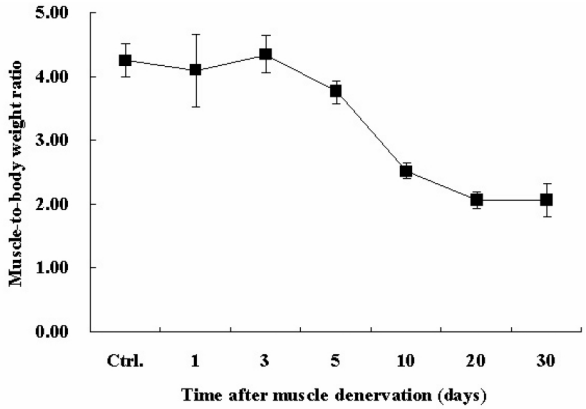

Fig. 1. Time course of changes in the muscle-to-body weight ratio during muscle denervation. Muscle wet weight (mg) was divided by body weight (g). Data are expressed as mean ± SD (n=3 per each time point).

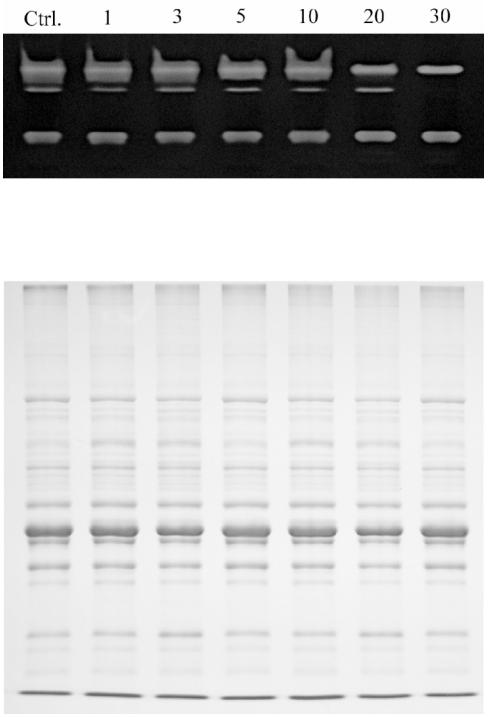

Fig. 2. Representive gel for characterization of skeletal muscle calpains by zymographical analysis. Caseinolytic bands remain unstained in the acid-based Coomassie Brilliant Blue R-250-stained gels. The activity of calpain is expressed as integrated optical density (IOD) by image analysis of casein zymography. We verified whether the amount of soluble protein was equally loaded using SDS-PAGE.

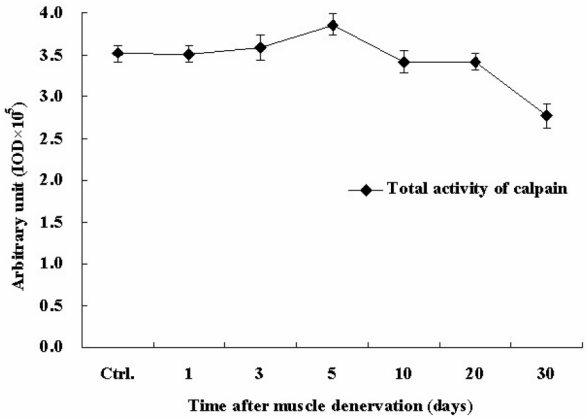

Fig. 3. Time course of changes in the total activity of calpain during muscle denervation. Data are expressed as mean ± SD (n=3 per each time point).

was slightly increased at 5 days and thereafter progressively decreased through 30 days. As shown in Fig. 5, until 10 days after muscle denervation, the relative abundance of μ- and m-calpain was 70% and 30%, respectively. By 30 days, the relative

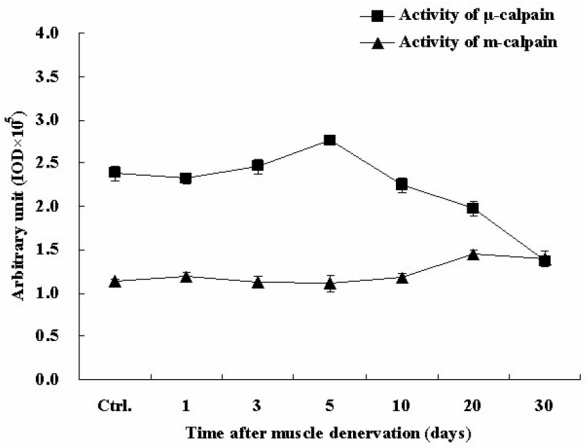

Fig. 4. Time course of changes in the activities of m- and m-calpain during muscle denervation. Data are expressed as mean ± SD (n=3 per each time point).

abundance of μ-calpain was decreased by 50% of total calpain while that of m-calpain was increased by 50% of total calpain.

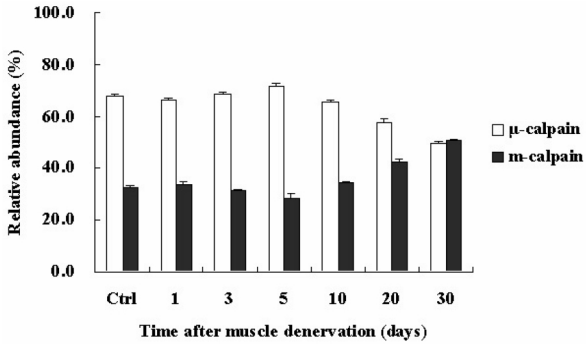

Fig. 5. Relative abundance of calpain isoforms (m- and m-calpain) during muscle denervation. Data for each isoform are given as the percentage of total calpain and are expressed as mean ± SD (n=3 per each time point).

Discussion

At the early stage of muscle denervation, the total concentration of μ- and m-calpains increases and the Ca^{2+}-dependent proteolytic pathway is activated[6, 10]. However, the activities of μ- and m-calpains have not been separately analyzed. The purpose of this study was to characterize changes in the activities of μ- and m-calpains during muscle denervation. We were able to analyze μ- and m-calpains using casein

zymography, and found that each responds differently to muscle denervation. This indicates that the possible roles of μ- and m-calpains may be distinct from each other in the denervated muscle.

In this experiment, a muscle denervation model was used to study the disassembly and degradation mechanisms of myofibrils and the role of calpain in this process. The muscle fibers of the denervated gastrocnemius muscles pathologically atrophied (data not shown). This is in line with changes in the muscle-to-body weight ratio, which began to decrease at 5 days and continued to drastically decrease until 20 days after muscle denervation. This trend is generally in agreement with a previous study[2].

In agreement with an earlier study[10], we found that the activity of μ-calpain was slightly increased at the early stage of muscle denervation. This suggests that m-calpain may play a role in disassembling sarcomeric proteins and in making the myofibril susceptible to proteolytic attack by the ubiquitin-proteasome complex. If this is so, μ-calpain-dependent degradation may be an important step in the control of myofibrillar protein turnover during muscle denervation. While the activity of μ-calpain increases, the activity of m-calpain remains unchanged. This finding is inconsistent with a previous study, which demonstrated that the concentration of m-calpain is increased in the denervated muscle[10]. Although the reason for this discrepancy is unclear, we believe that m-calpain contributes to the disassembly of sarcomeric proteins because it can completely remove Z-disks from skeletal muscle myofibrils[8].

We found that the activity of μ-calpain gradually decreased below control levels when skeletal muscle is denervated over a prolonged period. This finding suggests that the decreased activity of μ-calpain may result in a reduction of myofibrillar protein turnover. This decrease may be, at least partly, explained by the decreases in transcriptional and post-transcriptional activities of the μ-calpain gene in response to muscle denervation.

Calpains are highly expressed in myotubes and regenerating muscle fibers in dystrophin-deficient mice[15]. Additionally, when myoblasts enter a quiescent/G0 stage, m-calpain is detected only in the cytoplasm. If myoblasts are proliferating, m-calpain is present in the nucleus throughout the cell cycle[13]. Therefore, it is assumed that m-calpain controls the late events of cell-cell fusion in skeletal muscle tissue through its involvement in cell membrane and cytoskeleton component reorganization. In this study, the activity of m-calpain slightly increased at the late stage of muscle denervation when activated satellite cells and myotube (small-caliber fibers with centrally located nuclei) were frequently observed (unpublished data). Therefore, our findings may support the assumption that m-calpain is involved in the process of fusion of myogenic cells.

The mechanism regulating the activity of the calpain system in vivo remains unknown[7]. It has been proposed that the activity of the calpain system may be regulated by an association of the calpains with a phospholipid in cell membranes, and subsequently autolysis (the conversion of an inactive proenzyme into an active protease). For example, phosphatidylinositol lowers the [Ca^{2+}] required for autolysis of μ-calpain from 10-50 μM to 1-5 μM[4, 11] and for autolysis of m-calpain from 550-750 μM to 90-350 μM[4, 5], suggesting that autolysis may be an important step in activating calpains. However, this hypothesis does not accommodate immunohistochemical data. Kumamoto and colleagues have demonstrated that μ- and m-calpains were localized mainly in the Z-disk, the I-band, and the A-band, and that muscle denervation does not change their distribution[10], which suggests that other mechanisms, independent of the cell membrane, that activate the calpain system

may exist in skeletal muscle.

The membrane-activation hypothesis does not include any specific role for calpastatin. In this study, we assessed the activity of calpains in the absence of endogenous calpastatin because calpastatin-bounded calpains were separated by electrophoresis. Therefore, it is unclear to what extent calpains are inhibited by endogenous calpastatin. Calpastatin completely inhibits both the autolyzed and unautolyzed form of μ- and m-calpain at [Ca^{2+}] lower than those required to initiate either proteolytic activity or autolysis[9]. Immunohistochemical evidence indicates that the calpains and calpastatin are co-localized in muscle fibers and that the concentrations of μ- and m-calpains increase concomitantly with that of calpastatin[10]. This suggests that calpastatin may play a physiological role in regulating the activity of calpains in skeletal muscle. Further studies are needed to elucidate the mechanism underlying the regulation of the calpain-calpastatin system during muscle denervation.

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除神経筋における μ- and m-カルパイン活性の経時的変化

我妻　玲*

要　約

カルパインは筋原線維及び細胞骨格タンパク質の分解を誘発することから，筋萎縮の誘発に重要な役割を果たすと考えられる。しかしながら，除神経筋における m-カルパインと m-カルパイン活性の経時的変化についてはほとんど報じられていない。そこで本研究では，除神経がm-カルパイン と m-カルパインの活性に及ぼす影響について，マウス（CD1系雌性，７週齢）骨格筋を用いて検討した。体重を測定後，麻酔下でマウスの坐骨神経を切断した。手術後1，3，5，10，20，30日目に腓腹筋を摘出し，湿重量を測定した。カルパインの活性は，カゼインザイモグラフィーを用いて測定した。体重当たりの筋湿重量は除神経後５日目から減少し始め，20日目まで減少し続けた。全カルパイン活性（m-カルパインと m-カルパイン）は除神経後５日目までに増加傾向を示し，10－30日間では減少傾向を示した。m-カルパインも同様の傾向を示した。m-カルパインは10-30日間で増加傾向を示した。除神経10日目までは，m-カルパインとm-カルパインの占める割合はそれぞれ70%，30%であったが，30日目にはそれぞれ50%とその割合が変化した。これらの結果は，除神経の初期においては m-カルパインと m-カルパインは筋タンパク質の分解に関与すると考えられる。また，除神経の後期においては m-カルパインの活性上昇は，除神経に伴う筋管細胞の形成に関与している可能性が考えられる。

KEY WORDS : calpain，denervation，zymography

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