Effect of hindlimb immobilization on μ-calpain and m-calpain activities

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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 hindlimb immobilization has not been fully defined. We characterized the changes in the activities of μ- and m-calpains during hindlimb immobilization. The right foot of mice was immobilized in maximal plantar flexion to fix the gastrocnemius muscle in a shortened position. The left hindlimb served as the control muscle. The activities of μ- and m-calpains were analyzed using casein zymography. The muscle weight was decreased by 35.1% (at 7 days) and 44.6% (at 14 days) after hindlimb immobilization. The amount of easily releasable myofilaments increased in the immobilized muscles relative to control muscles. The amount of myosin heavy chain (MyHC) and actin decreased after hindlimb immobilization. The amount of easily releasable myofilaments paralleled the decrease of MyHC and actin proteins. Both μ-calpain and m-calpain activities increased in the immobilized muscles relative to the control muscle. These observations suggest that μ-calpain and m-calpain might play a role in protein degradation induced by hindlimb immobilization.

KEY WORDS : calpain, muscle atrophy, hindlimb immobilization

Introduction

Hindlimb immobilization leads to muscle atrophy. The process is highly regulated by several pathways of protein degradation. 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[16]. Indeed, the ubiquitin-proteasome pathway plays a major role in muscle wasting during various catabolic states[7]. However, intact myofilaments cannot be degraded directly by the ubiquitin-dependent process[17]. 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 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[9]. Two isoforms of calpain, differing in Ca^{2+} requirement, exist ubiquitously in mammalian tissue[1]. The low (μM) and high (mM) Ca^{2+}-requiring isoforms are designated μ-calpain and m-calpain, respectively[1]. It is known that the Ca^{2+}

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concentration ([Ca^{2+}]) requires 3-50 μM for half-maximal proteolytic activity of m-calpain and 200-1000μM for half-maximal proteolytic activity of m-calpain[9].

However, the pattern of changes in the activities of μ- and m-calpains when the muscle is immobilized in a shortened position has not been fully defined. Therefore, we characterized the time course of changes in the activities of μ- and m-calpains in the gastrocnemius muscles from the hindlimbs of immobilized mice.

Materials & methods

Animal care

The experiments were conducted on 6-week-old male CD1 mice (CLEA, Tokyo, Japan) (n＝18). 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.

Hindlimb immobilization

The right foot was immobilized in maximal plantar flexion, approximately 180 degrees between foot and leg, to fix the gastrocnemius muscle in a shortened position under ether anesthesia[19]. The left hindlimb served as the control. Great care was taken to make sure that the cast adequately immobilized the hindlimb without causing ischemia. The animals were sacrificed under ether anesthesia 7 and 14 days after hindlimb immobilization. Gastrocnemius muscles were carefully dissected and weighed.

Isolation of myofibrils and easily releasable myofilaments

Isolation of myofibrils and release of myofilaments were carried out according to previous procedures[6]. All steps were performed at 4℃. Low salt buffer (LSB) contained 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol, 10 mM Tris/maleate buffer (pH 7.0). After soaking with LSB for 90 min, the muscles were finely minced with scissors and homogenized in the soaking solution using a Dounce homogenizer (15 ml) with 10 strokes of the loose and 8 strokes of the tight pestle. The homogenate was centrifuged at 1500 g for 5 min and the pellet was suspended with a Pasteur pipette in 10 ml of LSB, filtered through 2 layers gauze cloth, and centrifuged. The myofibrillar pellet was washed once in LSB and then three times in LSB.

Filaments were released from the myofibrillar pellet by repeated pipetting (10 passages through a Pasteur pipette) in 1.5 ml of LSB containing 2 mM ATP. The suspension was immediately layered over 0.75 ml of LSB containing 20% glycerol in a conical tube (2 ml) and centrifuged at 1500 g for 5 min. The supernatant, including the glycerol-containing layer, was carefully collected with a Pateur pipette and was centrifuged through 0.5 ml of LSB containing 20% glycerol. The final supernatant contained the released myofilament fraction. The pellet constituted the residual myofibril fraction. The concentration of easily releasable myofilaments was expressed as the percentage of the fraction of easily releasable myofilaments divided by the amount of residual myofibril fraction plus easily releasable myofilament fraction.

Semi-quantitative sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Myosin heavy chain (MyHC) and actin proteins

were separated on 10% (wt/vol) polyacrylamide gel by using a standard SDS-PAGE technique[13]. Protein samples were denatured by placing 10 μg of sample in 30 μl of sample buffer (5% ß-mercaptoethanol, 100 mM Tris base, 5% glycerol, 4% SDS, and 0.05% bromophenol blue [pH 6.8]) and then heating the solution for 2 min at 100℃. Ten microliters of the denatured protein solution (equivalent to 2.5 μg of total muscle protein) were loaded per lane, and the gels were run at a constant current (20 mA) for 2.5 h at 22℃. The gels were fixed with solution containing 10% acetic acid and 25% methanol and then stained with Coomassie brilliant blue R-250, followed by vigorous washing in distilled water. The intensity of band was densitometrically quantified using Image J (http://rsb.info.nih.gov/ij/) and was expressed as integrated optical density (IOD).

Preparation of muscle extract

The muscle extract was prepared according to previous procedures[18]. The muscles, frozen in 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[2] 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[15] 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 and 25% methanol 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 SDS-PAGE (data not shown).

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 J and was expressed as IOD. Total calpain corresponds to μ-calpain plus m-calpain.

Results

As shown in Fig. 1, the muscle weight progressively

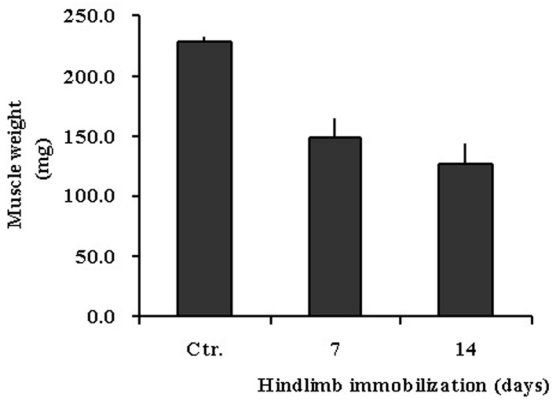

Figure 1. Effect of hindlimb immobilization on muscle weight. Data are expressed as mean ± SD (n=3 per each time point).

decreased in response to hindlimb immobilization. The amount of easily releasable myofilaments increased 7 and 14 days after hindlimb immobilization (Fig. 2). Proteins of myofibrils were analyzed by a semi-quantitative SDS-PAGE. The amount of MyHC and actin decreased in the immobilized muscles relative to control muscles (Fig. 3). 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. 4, 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[14]. The minor band migrating ahead of μ-calpain was assumed to be the autoproteolyzed form of μ-calpain. Both the activities

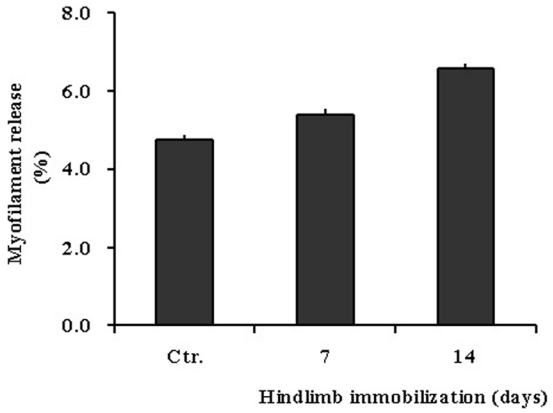

Figure 2. Effect of hindlimb immobilization on myofilament release. The concentration of easily releasable myofilaments is expressed as the percentage of the fraction of easily releasable myofilaments divided by the summation of the residual myofibril fraction plus the easily releasable myofilament fraction. Data are expressed as mean ± SD (n=3 per each time point).

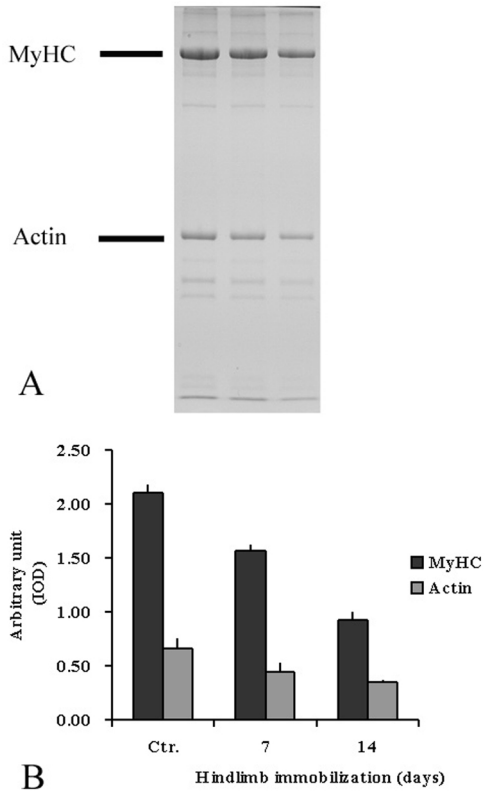

Figure 3. Semi-quantitative SDS-PAGE analysis (A). Effect of hindlimb immobilization on the amount of MyHC and actin proteins (B). The intensity of bands was densitometrically quantified and is expressed as integrated optical density (IOD). Data are expressed as mean ± SD (n=3 per each time point).

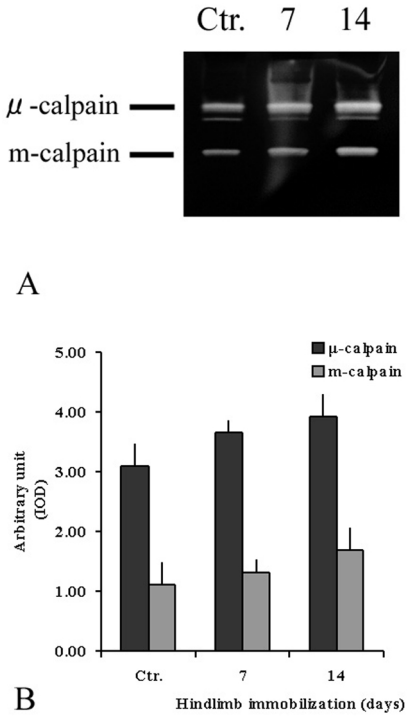

Figure 4. Representative gel for characterization of skeletal muscle calpains by zymographical analysis (A). Effect of hindlimb immobilization on μ- and m-calpain activity (B). Caseinolytic bands remain unstained in the acid-based Coomassie Brilliant Blue R-250-stained gels. The activity of calpain is expressed as integrated optical density (IOD) by image analysis of casein zymography. Data are expressed as mean ± SD (n=3 per each time point).

of μ-calpain and m-calpain increased in the immobilized muscles relative to the control muscle.

Discussion

In agreement with an earlier study[12], we found that the activities of μ-calpain and m-calpain increased in an immobilized muscle, suggesting that calpains 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- and m-calpain-dependent degradation may be an important step in the control of myofibrillar protein turnover during hindlimb immobilization.

In the present study, hindlimb immobilization (fixed in a shortened position) was used as disuse model. This model is known to affect muscle architecture[10]. In particular, the number of sarcomeres in series (sarcomere number) decreases in skeletal muscle when the muscle is fixed in a shortened position[11]. The remodeling of muscle architecture occurs because the muscle adapts to meet the change in muscle length induced by immobilization in a shortened position. Therefore, we can hypothesize that when the activities of μ- and m-calpain increase, the fixed position may modulate sarcomere number in skeletal muscle concurrently.

The fraction of easily releasable myofilaments contains intermediates of the degenerative pathway of myofibrils[20]. Under conditions such as starvation and chronic glucocorticoid administration, the fraction of easily releasable myofilaments is increased in rat skeletal muscle[5]. Similar to this observation, we found that the fraction of easily releasable myofilaments increased in the immobilized muscle, suggesting that a proteolytic step may be involved in the conversion of myofilaments into easily releasable myofilaments. Interestingly, the starvation-induced increase of the fraction of easily releasable myofilaments could be reduced by treatment with the protease inhibitor E-64, which inhibited calpain activity. Therefore, calpain could play an important role in the conversion of myofilaments into easily releasable myofilaments and contribute to muscle atrophy.

The mechanism regulating the activity of the calpain system *in vivo* remains unknown[9]. It has been proposed that the activity of the calpain system may be regulated by an association of the calpains with a phospholipids in cell membranes, and subsequent 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[3, 14] and for autolysis of m-calpain from 550-750 μM to 90-350 μM[3, 4], 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[12], suggesting that other mechanisms, independent of the cell membrane, that activate the calpain system may exist in skeletal muscle.

In conclusion, we found that both μ-calpain and m-calpain activities increased in the immobilized muscles concomitant with increases in easily releasable myofilaments. This suggests that μ-calpain and m-calpain might play a role in protein degradation induced by hindlimb immobilization.

Acknowledgements

This study was supported, in part, by the Grant-in-Aid for Scientific Research (B-17700499 to A. Wagatsuma) from Japan Society for the Promotion of Science, and the Grant-in-Aid for Scientific Research from the National Institute of Fitness and

Sports of Japan (President's Discretionary Budget 2005, to A. Wagatsuma).

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ギプス固定がカルパイン活性に及ぼす影響

我妻　玲*

要　　約

カルパインは Ca^{2+} に制御されるシステインプロテアーゼであり，筋タンパク質の分解に関与している。本研究の目的は，カルシウム感受性の異なる２つのアイソザイムであるμ-カルパインとm-カルパインに着目し，実験的に筋萎縮を誘発した時のそれらの活性変化について検討することである。

CD1 系の雄性マウスを用い，右足関節を最大限に底屈させた状態でギプス固定を行った。７，14日後に腓腹筋を採取し分析に供した。ミオシン重鎖及びアクチンタンパク質量は半定量的 SDS-PAGE により評価した。μ－カルパインと m-カルパインの活性はカゼインザイモグラフィーにより評価した。

腓腹筋の筋湿重量は７日後には35.1%，14日後は44.6%減少した。‘ Easily releasable myofilament’量は筋重量の減少に伴い増加傾向を示した。またミオシン重鎖およびアクチンタンパク質量も減少した。μ-カルパインおよび m-カルパインの活性は７日目および14日目に増加傾向を示した。これらの結果はμ-カルパインおよび m-カルパインがギプス固定によるタンパク質分解に関与している可能性を示唆すると考えられる。

KEY WORDS : calpain; muscle atrophy; hindlimb immobilization

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