ORIGINAL ARTICLE

Calpain/calpastatin activities and substrate depletion patterns during hindlimb unweighting and reweighting in skeletal muscle

Deborah L. Enns · Truls Raastad · Ingrid Ugelstad · Angelo N. Belcastro

Abstract Unloading of skeletal muscle by hindlimb unweighting (HU) is characterized by atrophy, protein loss, and an elevation in intracellular Ca^{2+} levels that may be sufficient to activate Ca^{2+}-dependent proteases (calpains). In this study, we investigated the time course of calpain activation and the depletion pattern of a specific structural protein (desmin) with unloading and subsequent reweighting. Rats underwent 12 h, 24 h, 72 h or 9 days of HU, followed by reweighting for either 0, 12 or 24 h. Total calpain-like activity was elevated with HU in skeletal muscle ($P < 0.05$) and was further enhanced with reweighting ($P < 0.05$). The increases in calpain-like activity were associated with a proportional increase in activity of the particulate fraction ($P < 0.05$). Activity of the µ-calpain isoform was elevated with 12 and 24 h of HU ($P < 0.05$) and returned to control levels thereafter. With reweighting, activities of µ-calpain were elevated above control levels for all HU groups except 9 days ($P < 0.05$). In contrast, minimal changes in m-calpain and calpastatin activity were observed with HU and reweighting. Although desmin depletion levels did not reach statistical significance, a significant inverse relationship was found between the µ-calpain/calpastatin ratio and the amount of desmin in isolated myofibrils ($R = -0.83$, $P < 0.001$). The results suggest that calpain activation is an early event during unloading in skeletal muscle, and that the majority of the increase in calpain activity can be attributed to the µ-isoform.

Keywords Protease · Atrophy · Hindlimb suspension · Skeletal muscle

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Introduction

The absence of weight-bearing activity produces a profound atrophy of skeletal muscles that are normally involved in maintaining posture (Booth and Criswell 1997; Ku and Thomason 1994; Thomason and Booth 1990). At the molecular level, the atrophy has been attributed to a rapid decrease in protein synthesis followed by an accelerated rate of protein degradation (Thomason et al. 1989). In an attempt to identify some of the degradative mechanisms responsible for muscle atrophy, a number of studies have focused on the role of calcium (Ca^{2+})-activated proteases (calpains) during conditions of muscle unloading (Ellis and Nagainis 1984; Spencer et al. 1997; Taillandier et al. 1996; Tischler et al. 1990). Calpains are a family of intracellular, non-lysosomal, Ca^{2+}-activated proteases that participate in a number of biological processes. Two isoforms—a micro-(or µ-)isoform and a milli-(or m-)isoform—are ubiquitously expressed in mammalian tissues (Goll et al. 2003). Since the isoforms require different intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) for activation (3–50 µM for µ-calpain and 400–800 µM for m-calpain in vitro), it has been suggested that the two isoforms may be differentially activated in vivo (Thompson et al. 2000). It has even been postulated that the µ-isoform, due to its lower Ca^{2+} requirement, may

proteolyze and subsequently activate the m-isoform (Tompa et al. 1996). Although this hypothesis has been tested with mixed results in vitro (Thompson et al. 2000; Tompa et al. 1996), the question as to whether the two isoforms are differentially activated in vivo remains to be answered.

Under normal physiological conditions, calpains display minimal activity, mainly because of a tight association with an endogenous inhibitor, calpastatin (Kapprell and Goll 1989). However, in the presence of increased intracellular Ca^{2+} levels calpastatin inhibition is released, and the protease undergoes a limited autoproteolysis through removal of a portion of its N-terminus (Cong et al. 1989). Once activated, the protease becomes localized to phospholipid-rich structures such as membranes (Melloni et al. 1996) where it is able to degrade a variety of cellular structures, including Z-disk proteins, myofibrillar proteins and structural cytoskeletal proteins (Goll et al. 2003; Whipple and Koohmaraie 1991).

A number of studies have attempted to address the role of the calpain system with unloading-induced atrophy. In vitro assessments have been invaluable for identifying the involvement of calpains during conditions where protein degradation is elevated (Furuno and Goldberg 1986; Zeman et al. 1985); however, the extent to which these reflect unloading in vivo is questionable. In vivo assessments of calpain involvement during muscle unloading have also been performed (Spencer et al. 1997; Taillandier et al. 1996); however, because different experimental and technical approaches have been used few generalizations can be made. For example, some investigators have reported increases in calpain mRNA levels with unloading (Taillandier et al. 1996), while others have found no changes (Ikemoto et al. 2001; Spencer et al. 1997). Although measuring changes in the amount of calpain mRNA and protein provides valuable information regarding transcription rates and mRNA stability during unloaded states, it does not necessarily reflect calpain activity in vivo, as $[Ca^{2+}]_i$ (Hosfield et al. 1999), intracellular localization (Melloni et al. 1996), and association with calpastatin (Barnoy et al. 1999) may all influence activity in vivo.

Other in vivo studies have monitored changes in calpain activities during unloading-induced atrophy. Direct assessments of calpain activity through caseinolysis of partially purified substrates have consistently demonstrated elevations in calpain activity in rat hindlimb muscles exposed to moderate periods of unloading (e.g. 5–9 days) (Ellis and Nagainis 1984; Taillandier et al. 1996). Recently, we demonstrated increases in calpain activity as early as 12 h after unloading in the soleus using a similar procedure (Enns and Belcastro 2006). Indirect evidence of calpain involvement during unloading has also been observed through studies employing various inhibitors and ionophores (Tischler

et al. 1990, 1991), as well as by measuring the appearance of calpain autolytic fragments (Spencer et al. 1997). Finally, a study using transgenic animals has revealed that overexpression of calpastatin not only reduces muscle atrophy during unloaded conditions, but also prevents the shifts in myofibrillar isoforms that normally accompany unloading (Tidball and Spencer 2002). Although the data from these studies strongly implicate involvement of the calpain–calpastatin system during muscle unloading, one major limitation is that calpain levels were assessed at only a single time point during unloading. Clearly the time course and/or mechanisms of calpain activation, as well as the interactions between calpain, calpastatin and target substrates need to be addressed in order to understand how the system functions during unloading in vivo.

Muscles that experience atrophy during unloading are more susceptible to injury when they are reloaded or reweighted. Riley et al. demonstrated that hindlimb muscles of rats removed ∼48 h following spaceflight exhibited sarcomeric disruptions, Z-line streaming, and an infiltration of inflammatory cells (Riley et al. 1990). Since similar events have also been observed during muscle injury following unaccustomed or eccentric exercise (Armstrong et al. 1991), it is possible that many of the same mechanisms are involved. During muscle injury, a loss of Ca^{2+} homeostasis in the muscle fibre leads to elevated $[Ca^{2+}]_i$. The Ca^{2+} then activates calpains, which cleave a number of myofibrillar and cytoskeletal proteins (Raj et al. 1998).

In this study we investigated the response of the calpain–calpastatin system and proteolysis of specific target proteins in the vastus during muscle unloading followed by reweighting. While our previous study (Enns and Belcastro 2006) was primarily concerned with identifying the time course of the total Ca^{2+}-activated protease response following HU and reweighting in different postural and locomotor muscles of the rat, our objectives in this study were as follows: (a) to characterize the time course of activity of specific calpain isoforms (μ- and m-) and calpastatin following unloading in skeletal muscle; (b) to visualize the time course and depletion patterns of a known calpain substrate, desmin, following unloading and reweighting; and (c) to determine whether a larger calpain response occurs in the vastus when skeletal muscles are reweighted following unloading. We hypothesized that activity of this protease system would be elevated following unloading in the rat vastus muscle and would be further enhanced with reweighting. A secondary hypothesis was that the amount of desmin (a cytoskeletal protein and known calpain target) present in myofibrillar extracts would be related to the increases, if any, in protease activities. A rat model and a number of hindlimb unweighting (HU) time points were used to assess the calpain–calpastatin response. We also

measured calpain activities during 12 and 24 h of reweighting, as elevations in $[Ca^{2+}]_i$ (Duan et al. 1990) and activation of calpains (Belcastro 1993) have been shown to be early events during muscle injury.

Methods

Experimental protocol

A total of 147 male Wistar rats were used for this study. The protocol was reviewed and approved by the The University of Western Ontario Animal Care and Ethics committee and all procedures were conducted in accordance with the Canada Council on Animal Care. The "Principles of laboratory animal care" (NIH publication No. 86–23) were followed at all times. Animals were housed either in standard cages (controls) or in cages specifically adapted for hindlimb unweighting (HU). The environment was temperature-controlled with a 12-h light/dark cycle. Rodent chow and water were available *ad libitum*. Control animals were weighed daily to monitor health and growth patterns. HU animals were weighed at the start of unloading and again at time of sacrifice.

Animals were divided into control and experimental groups as outlined in Fig. 1. Experimental animals were subdivided into one of four hindlimb unweighted (HU) groups: 12 h, 24 h, 72 h or 9 days ($n = 29$–31 per group). These experimental (HU) time points were chosen to (a) determine a time course of calpain and calpastatin activities during the early stages of HU (i.e. ≤ 72 h); and (b) compare the differences in protease activities between earlier and later HU time points. Animals in each of the four HU groups were further partitioned into 1–3 reweighting groups: 0 h or no reweighting, 12 or 24 h of reweighting ($n = 9$–11 per group). The reweighted groups were added to compare the response of the calpain–calpastatin system between unweighted and reweighted conditions. Animals exposed to reweighting were removed from the HU apparatus, placed in standard cages and allowed unrestricted movement. Control animals ($n = 16$) were terminated along with the experimental animals at each of the various HU time points. In addition, a pair-fed control group ($n = 12$) was included to determine whether decreased food consumption (typically exhibited by animals during the early stages of HU) leads to changes in calpain activity. Pair-fed animals were housed singly in standard cages and were given a quantity of food equivalent to the mean amount consumed by the 24 h HU animals for a 24-h period prior to sacrifice.

Hindlimb unweighting (HU) was performed by tail-casting and suspending the animals in a head-down position with the hindlimbs elevated above the floor of the cage at a
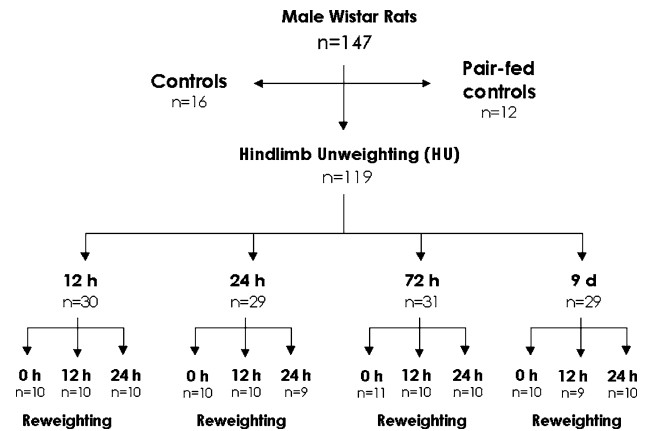

Fig. 1 Schematic outline of experimental protocol

30° angle as described previously (Munoz et al. 1993). Following each experimental protocol, animals were injected with sodium pentobarbitol (65 mg kg^{-1} i.p.), and the mixed vastus muscles were surgically removed. Previous work from our laboratory has revealed that use of this anaesthetic does not affect calpain activity (Belcastro, AN, unpublished observations). The mixed vastus muscle was chosen because significant amounts of tissue were required to perform the calpain isolations and other biochemical analyses. Tissues were rinsed to remove blood and all visible tendon was removed. All muscles were weighed prior to storage and analysis. In addition, a small sample of each muscle was removed and air-dried in order to assess any changes in muscle water content that may have occurred with the experimental protocols. The remaining muscle was immediately frozen in liquid nitrogen and stored at −80°C until analysis.

Determination of total and myofibrillar protein content

Individual muscle samples (approx. 100 mg) were homogenized for 3×10 s using an Ultra-Turrax homogenizer (IKA Laboratories, USA) in 20 volumes of a buffer containing 39 mM sodium borate (pH 7.1), 25 mM potassium chloride (KCl), 5 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM dithiothreitol (DTT). Tissues were visually inspected during cutting to ensure that each sample contained relatively equal proportions of red and white fibres. Following homogenization, a small aliquot of homogenate was removed and reserved for total protein analysis. Myofibrils were isolated from the remaining homogenate as described previously (Reid et al. 1994). Following isolation, an aliquot of myofibrils was reserved for quantification of myofibrillar protein. Total and myofibrillar protein yields were determined according to the method of Lowry (Lowry et al. 1951).

Calpain-like activities

Total calpain-like activity was measured using a microplate assay as described previously (Arthur et al. 1999) with minor modifications. Briefly, 200 μl of soluble or particulate extracts were added to reaction mixtures (final volume 500 μl) containing 1 mg ml^{-1} casein, 50 mM Tris (pH 7.5), and 20 mM DTT (in duplicate). Calcium chloride (5 mM) was added to one of the duplicates, while 5 mM EGTA was added to the other. After 30 min of incubation at 30°C, an aliquot (100 μl) of each sample was assayed for proteolysis in a total volume of 250 μl using diluted Bradford protein dye reagent (Bio-Rad Laboratories, Mississauga, ON) and an incubation time of 10 min. A change in A_{595} of 0.1 represented 1 unit of calpain-like activity. The activities are generally referred to as calpain-like activities because the assay measured Ca^{2+}-activated caseinolytic activity of cellular extracts. Total calpain-like activity was the sum of the activities of the soluble and particulate fractions.

Isolation and quantification of μ- and m-calpain and calpastatin

Tissue samples (1 g, containing relatively equal proportions of red and white fibres as determined by visual inspection) were homogenized at 4°C in 5 volumes of a homogenizing buffer containing 50 mM Tris (pH 7.5), 1 mM ethylene diamine tetraacetic acid (EDTA), 150 nM pepstatin A and 10 mM DTT using an Ultra-Turrax homogenizer for 3×30 s. Calpain isoforms (μ- and m-) and calpastatin were then isolated as described previously (Ilian and Forsberg 1992). Activities of each isoform were determined by measuring the release of trichloroacetic acid (TCA)-soluble peptides from casein at 280 nm. One unit of calpain activity was the amount of enzyme that produced a change of one absorbance unit at 280 nm after 30 min of incubation at 25°C. Calpastatin activity was assessed based on its ability to inhibit m-calpain activity. One unit of calpastatin activity is defined as inhibiting 1 unit of m-calpain completely. All activities were first assessed as Units g^{-1} protein and then expressed relative to control values.

Western blot analysis

Myofibrillar proteins were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10% Tris–HCl, 10-well Ready Gels, Bio-Rad Laboratories, Hercules, CA, USA, 20 μg protein per lane) and transferred to polyvinylidene difluoride (PVDF) membranes for 1 h at 100 V in a buffer containing 25 mM Tris (pH 8.3) and 192 mM glycine. Non-specific sites were blocked for 1 h at room temperature in a solution containing Tris-buffered saline (TBS, 20 mM Tris (pH 7.5) and 500 mM NaCl),

0.1% Tween-20, and 5% skim milk powder. After washing, membranes were incubated with a 1:5,000 dilution of primary antibody (mouse monoclonal anti-desmin, DE-R-11, DAKO, Glostrup, Denmark) overnight at 5°C. Following incubation, membranes were washed extensively with TBS-Tween, and goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL, USA), diluted 1:750 in TBS-Tween, were added for 60 min at 25°C. After washing, a chemiluminescent system was used to detect labelled proteins (SuperSignal West Dura, Pierce). Densitometric scans were performed using a Kodak IS2000R (Eastman Kodak Company, NY, USA). The density of the 53-kDa desmin protein band was quantified against known amounts of a desmin standard (rDesmin, RDI-PRO62016, Research Diagnostics, Flanders, NJ, USA) run concurrently on each gel.

Statistical analyses

All data are presented as means ± SE. Calpain-like activity distributions (soluble vs particulate fractions) were compared using Student's t-test. Correlative relationships were assessed using the Pearson product-moment correlation coefficient test. The remaining analyses were performed using a one-way ANOVA design. Where analysis of variance proved significant, differences were evaluated using Tukey's post hoc test. For all tests, the level of significance was set at $P < 0.05$.

Results

Body mass

Body mass was measured in control and experimental animals prior to and following the various HU protocols. Although some losses in body mass were evident during the shorter HU time points (12 and 24 h, $P < 0.05$), by 9 days of HU the animals were experiencing gains in body mass comparable to controls. Pair-fed animals also gained body mass during the 24-h period of food restriction. Although somewhat variable, reweighting did not change the loss of body mass that accompanied HU (data not shown).

Muscle mass

Muscle wet mass was measured to determine the degree of muscle atrophy that occurred with HU and reweighting (data not shown). Main effects were observed for both HU and reweighting at 9 days of HU compared to controls ($P < 0.05$). When muscle mass was normalized to body mass (mg per 100 g body weight) the differences in muscle wet mass with 9 days of HU and reweighting disappeared.

No differences in muscle mass were observed between control and pair-fed groups. In general, changes in muscle water content were minimal across all groups. Although some degree of edema was observed following 24 h of HU and 12 h of reweighting ($P < 0.05$), no other time points provided evidence of increased intramuscular water content (data not shown).

Total and myofibrillar protein

No changes in either total or myofibrillar protein contents (mg protein per g wet mass) were observed in the vastus for any of the unweighted or reweighted groups studied compared to controls. As well, no differences in either total or myofibrillar protein content were evident between control and pair-fed groups (data not shown).

Calpain-like activities

Total calpain-like activity was significantly elevated compared to controls at both 72 h and 9 days of HU ($P < 0.05$; Fig. 2a). Reweighting resulted in further increases in calpain-like activity during 12 and 24 h of HU compared to HU alone ($P < 0.05$; Fig. 2b). At 72 h and 9 days of HU, further increases compared to HU alone were not evident with reweighting; however, calpain-like activity remained elevated relative to the control condition at these time points ($P < 0.05$).

We also measured the distribution of calpain-like activities between soluble and particulate fractions to investigate the shifts in calpain activities between different intracellular pools (Fig. 3). Although no changes in activity of the soluble fraction were observed with HU, activity of the particulate fraction was elevated by approximately 200% compared to controls at 9 days of HU ($P < 0.05$; Fig. 3d). Calpain-like activity was also elevated in the particulate fraction for all reweighted groups compared to control values for 24 h and 9 days of HU ($P < 0.05$; Fig. 3b, d). There were minimal differences in calpain-like activities and distributions between control and pair-fed groups (not shown).

Activity of calpain isoforms and calpastatin

Activity of the μ-calpain isoform was elevated by 35% compared to controls ($P < 0.05$) as early as 12 h following the initiation of HU, remained elevated with 24 h of HU ($P < 0.05$), declined back to 30% above control values by 72 h of HU, and was only 10% higher than control levels by 9 days of HU (Fig. 4a). The elevations in μ-calpain activity were not enhanced further with reweighting compared to HU alone, although the 72 h HU group followed by 24 h of reweighting did exhibit higher μ-calpain activities compared to controls ($P < 0.05$). In contrast to μ-calpain
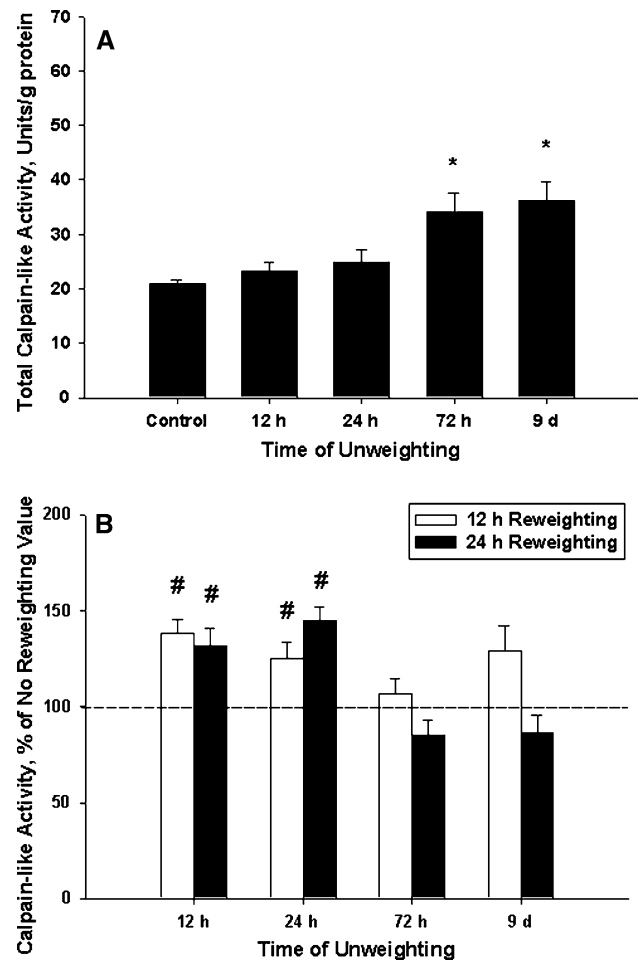

Fig. 2 Changes in total calpain-like activity following **a** various periods of HU and **b** reweighting for 12 and 24 h following HU. Data are presented as means ± SE. *Significantly different from control; #significantly different from "no reweighting" value for the same period of HU ($P < 0.05$)

activity, no changes in either m-calpain or calpastatin activities were observed with any of the experimental groups compared to controls (Fig. 4b, c).

The activities of the calpain isoforms were also expressed relative to calpastatin activities (i.e. as a calpain/calpastatin activity ratio; Fig. 5) because evidence suggests that the calpain/calpastatin ratio is a better indicator of net substrate proteolysis by calpains than calpain activities alone (Barnoy et al. 1998; Sorimachi et al. 1997). The elevations in the μ-calpain/calpastatin ratio with HU and reweighting were similar to those observed with μ-calpain activities; however, significant elevations in the μ-calpain/calpastatin ratio with 72 h of HU were also observed that were not evident as increases in μ-calpain activity only ($P < 0.05$; Fig. 5a). This was likely due to a slight (but non-significant) decline in calpastatin activity at this time point (compare with Fig. 4c). In contrast, no changes in the
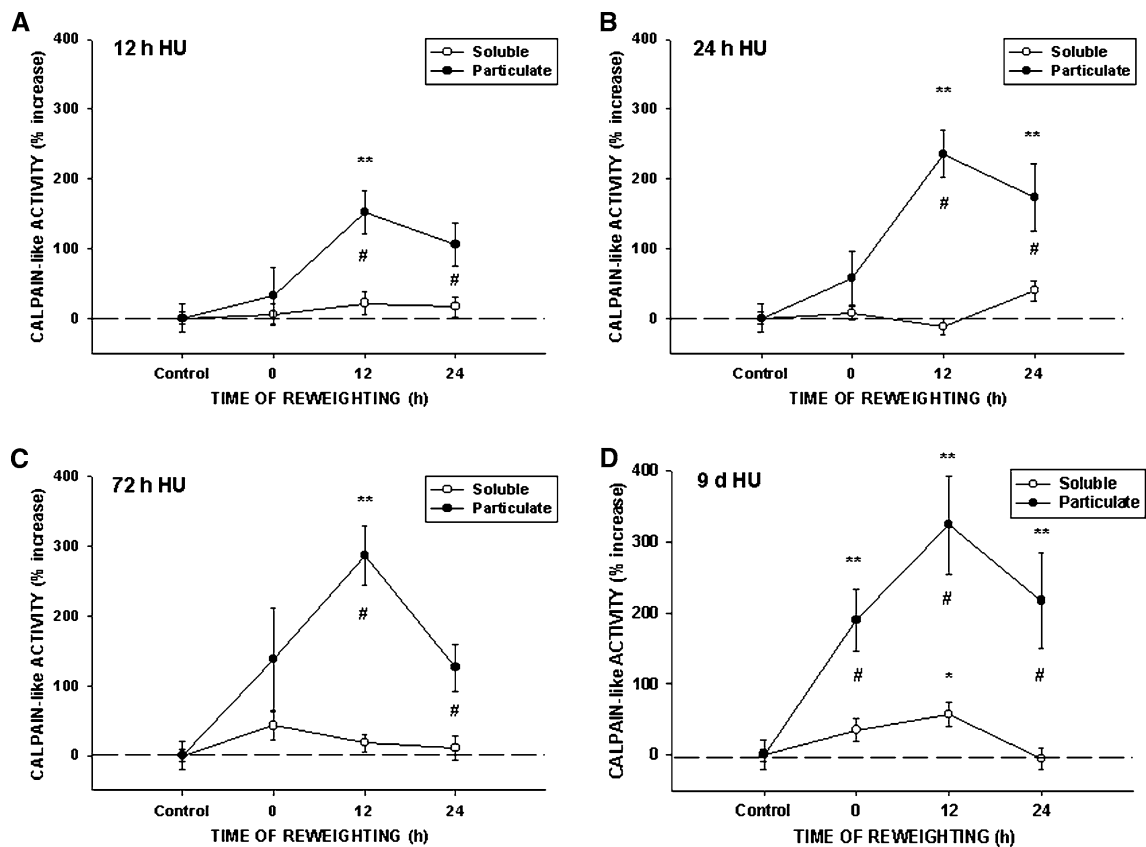
Fig. 3 Changes in relative distribution of calpain-like activities between soluble and particulate fractions during control conditions and following: **a** 12 h, **b** 24 h, **c** 72 h and **d** 9 days of HU. Following HU, hindlimb muscles were reweighted for 0, 12 or 24 h as indicated above.

Data are presented as means ± SE. *Significantly different from control soluble fraction; **significantly different from control particulate fraction; #significantly different from corresponding soluble fraction (P < 0.05)

m-calpain/calpastatin activity ratio were evident with any of the HU or reweighted groups (Fig. 5b).

Depletion patterns of desmin with HU and reweighting

To investigate the effects and time course of HU and reweighting upon proteolysis of a specific cytoskeletal calpain substrate, desmin, we employed a Western blotting technique. Two bands reacted with our monoclonal anti-desmin antibody: a 53-kDa protein that reacted almost exclusively with our rDesmin standard, and a 45-kDa fragment that was present in smaller quantities (not shown). We chose to evaluate the disappearance of the 53-kDa band because reaction of the antibody with the rDesmin standard and samples revealed that this band represented an undegraded form of desmin. Although desmin was depleted by 1, 47 and 36% with 12, 24 and 72 h of HU, respectively, these values did not reach statistical significance (Fig. 6). There were no differences in the amount of desmin between control and pair-fed groups (data not shown).

Although desmin depletion levels did not reach statistical significance with HU or reweighting, we did observe a

significant inverse relationship between the μ-calpain/calpastatin ratio and the amount of desmin protein present in myofibrils (R = −0.83, P < 0.001; Fig. 7). This finding suggests that as the proteolytic potential of the calpain–calpastatin system is enhanced, more desmin is depleted from myofibres.

Discussion

In this study, our objective was to establish a time course of activation of individual calpain isoforms with HU and correlate it with the depletion pattern of desmin, a cytoskeletal structural protein and known calpain target. The experiment was conducted to expand upon previous work from our laboratory (Enns and Belcastro 2006) in which we found rapid increases in calpain activity in the soleus following a similar HU protocol. The results of this study have demonstrated that (a) calpain activation is an early event during muscle unloading (i.e. ≤12 h) in the vastus, at least with respect to activation of the μ-calpain isoform; (b) vastus muscles that were unloaded for shorter time periods
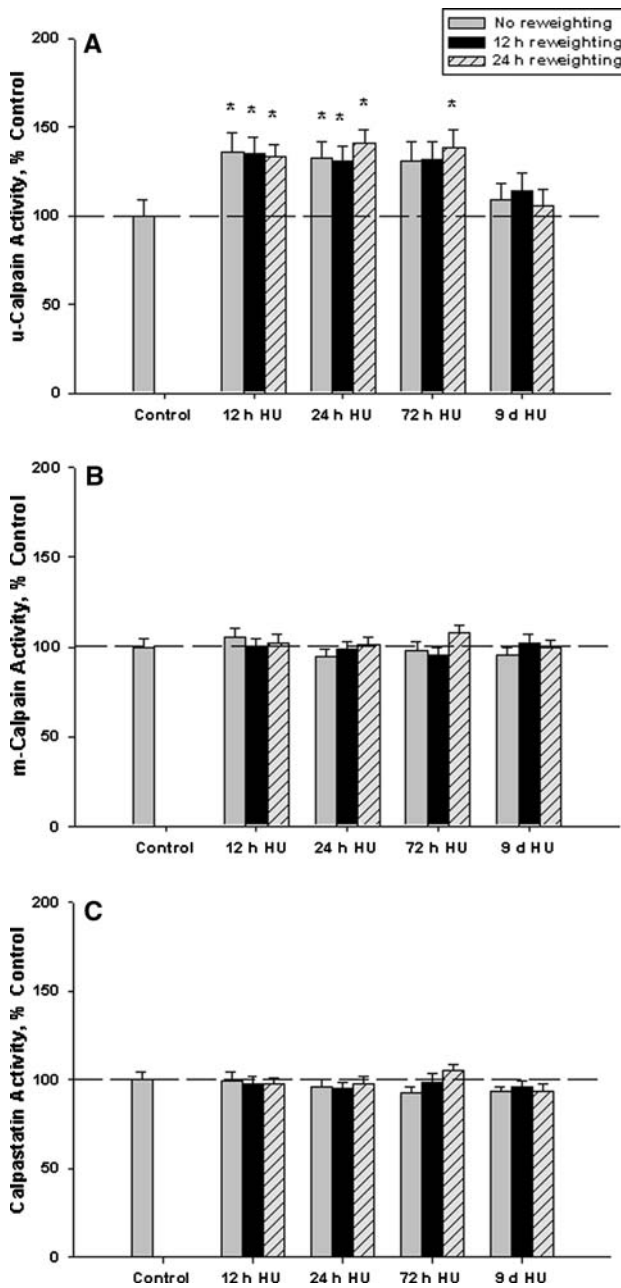
Fig. 4 Relative changes in **a** μ-calpain, **b** m-calpain and **c** calpastatin activities following various periods of HU and reweighting. Data are presented as means ± SE. *Significantly different from control ($P < 0.05$)

Fig. 5 Changes in **a** μ-calpain/calpastatin and **b** m-calpain/calpastatin activity ratios following various periods of HU and reweighting. Data are presented as means ± SE. *Significantly different from control ($P < 0.05$)

(i.e. ≤24 h) exhibited a larger Ca^{2+}-activated protease response during reweighting; (c) the increases in calpain activity were associated with a relative increase in the proportion of activity associated with the particulate fraction; and (d) the degree of elevation in the μ-calpain/calpastatin activity ratio was inversely related to the amount of desmin present in isolated myofibrils. Although evidence of calpain involvement in conditions of muscle unloading and reweighting is not a new finding (Spencer et al. 1997;
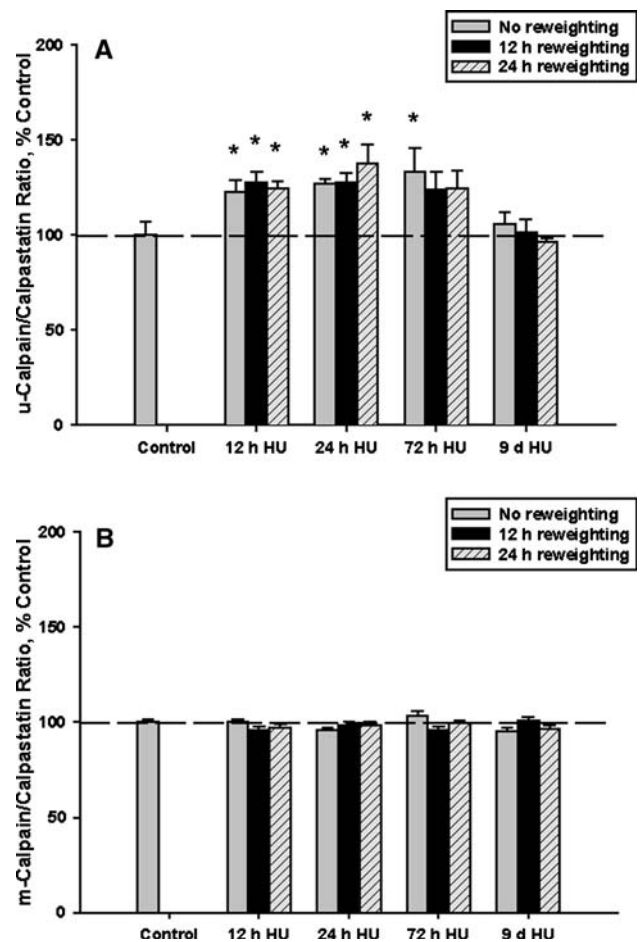
Taillandier et al. 1996, 2003), to our knowledge this is the first study to establish a time course of calpain activation and potential action upon a target substrate in vivo during HU and reweighting.

Muscle wet mass was measured to determine the degree of muscle atrophy that occurred with HU and reweighting. Absolute wet mass of the vastus muscle was reduced only with longer periods of HU (i.e. 9 days); although when wet masses were normalized to body mass the differences disappeared. The reductions in muscle mass could not be attributed to a loss of fluid to the interstitial space, as evidenced by the lack of changes in muscle water content. Losses in muscle mass, particularly in postural muscles, have been commonly observed in most studies involving muscle unloading (Fitts et al. 2000). Consistent with this finding, a recent study from our laboratory revealed that this same HU protocol reduced muscle wet mass in the soleus by up to 45% and produced elevations in calpain-like activity as early as 12 h following HU (Enns and Belcastro 2006). Although muscle mass did not decrease
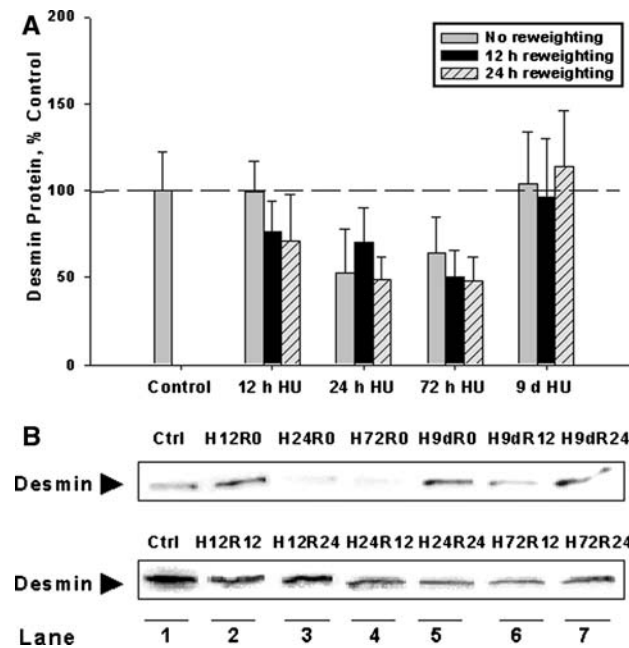
Fig. 7 Correlation between the μ-calpain/calpastatin activity ratio and the amount of desmin present in myofibrils following HU and reweighting. $R = -0.83$, $P < 0.001$

Fig. 6 Western blot illustrating desmin depletion patterns from isolated myofibrils following various periods of HU and reweighting. All densitometric values were standardized relative to rDesmin and then expressed relative to control values. **a** Mean densitometric values of desmin protein. Data are presented as means ± SE. *Significantly different from control ($P < 0.05$). **b** Representative blots showing depletion patterns of desmin following various HU and reweighting protocols. *Top panel* Lane 1, Control (Ctrl); Lane 2, 12 h HU (H12R0); Lane 3, 24 h HU (H24R0); Lane 4, 72 h HU (H72R0); Lane 5, 9 days HU (H9dR0), Lane 6, 9 days HU + 12 h reweighting (H9dR12); Lane 7, 9 days HU + 24 h reweighting (H9dR24). *Bottom panel* Lane 1, Control (Ctrl); Lane 2, 12 h HU + 12 h reweighting (H12R12); Lane 3, 12 h HU + 24 h reweighting (H12R24); Lane 4, 24 h HU + 12 h reweighting (H24R12); Lane 5, 24 h HU + 24 h reweighting (H24R24); Lane 6, 72 h HU + 12 h reweighting (H72R12); Lane 7, 72 h HU + 24 h reweighting (H72R24)

significantly until 9 days of HU in the vastus, we nevertheless observed similar early elevations (i.e. ≤12 h) in μ-calpain activity and the μ-calpain/calpastatin ratio in this muscle, thus demonstrating that significant losses in muscle mass are not necessarily an immediate outcome of enhanced calpain activity in skeletal muscle. Clearly other proteolytic systems are also involved in the atrophic process and likely play a more direct role in degrading larger proteins that would affect muscle mass, particularly during longer periods of unweighting (Ikemoto et al. 2001; Taillandier et al. 1996; Tischler et al. 1990).

Total calpain-like activity was measured to provide us with a general assessment of Ca^{2+}-activated protease activity in the vastus muscle prior to isolation of specific calpain isoforms. Although postural muscles such as the soleus tend to be most affected by exposure to unloaded conditions (Rapcsak et al. 1983), there is little or no evidence to support the contention that muscle atrophy and/or calpain activation occurs in the vastus during unloading. Although we
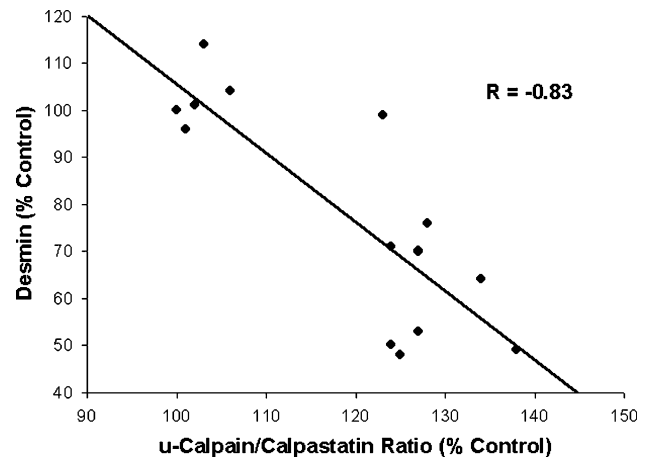
observed early elevations in total calpain activity with HU in the soleus in our previous study (Enns and Belcastro 2006), a significant amount of tissue (1 g) was required to isolate the calpain isoforms and calpastatin, making it impractical to use the soleus for these analyses.

Our results indicated that increases in total calpain-like activity occurred during 72 h and 9 days of unweighting in the vastus. In contrast, when we isolated the individual calpain isoforms we observed increases in μ-calpain activity as early as 12 h following the onset of HU. It is not known why the early increases in μ-calpain activities (i.e. <72 h HU) were not manifested as increases in total calpain-like activity at these time points. One possible explanation is that the relative amount and activity of μ-calpain in our extracts was too low to be detected by the calpain-like activity assay, which does not discriminate between the two isoforms and may also include contributions from other Ca^{2+}-activated proteases such as lysosomal proteases, which are reportedly active during longer periods of HU (Taillandier et al. 1996; Tischler et al. 1990).

A number of investigators have examined the contribution of individual calpain isoforms to muscle unloading. Although some studies have observed increases in the amount of m-calpain protein (Spencer et al. 1995) and mRNA (Taillandier et al. 1996) during unloaded conditions, these are not necessarily translated into increases in m-calpain activity in vivo (Spencer et al. 1995). It has also been established that calpain undergoes a limited autolysis of N-terminal peptides prior to, or during, its activation (Cong et al. 1989). In close agreement with our findings, Spencer and colleagues found an increased production of autolysis fragments from μ-calpain, but not m-calpain, in dystrophic muscles from *mdx* mice (Spencer et al. 1995). The appearance of the autolyzed peptides was independent

of any changes in μ-calpain mRNA or calpastatin levels, suggesting that activation of calpain depends more upon the intracellular environment and metabolic status of the cell than the amount of protease and inhibitor present during a given period of unloading.

The early increases in μ-calpain activity during HU most likely resulted from an early increase in $[Ca^{2+}]_i$. During muscle unloading, increases in passive Ca^{2+} leakage by the SR have been consistently shown to elevate resting $[Ca^{2+}]_i$ during the early stages of unloading (i.e. ≤3 days) (Ingalls et al. 1999, 2001; Stevens and Mounier 1992; Yoshioka et al. 1996). However, despite our early elevations in μ-calpain activity with HU, we did not observe similar elevations in m-calpain activity. Since the $[Ca^{2+}]_i$ requirement for half-maximal activity of m-calpain is much greater than that for μ-calpain (400–800 μM for m-calpain vs 3–50 μM Ca^{2+} for μ-calpain in vitro (Goll et al. 2003)), it is possible that the elevations in $[Ca^{2+}]_i$ that accompanied our unloading protocol were sufficient to activate the μ-isoform, but not the m-isoform.

The shift in distribution of calpain activities between soluble (cytosolic) and particulate (membrane-bound) fractions may also have contributed to the early increases in μ-calpain activity observed in this study. Activation and translocation of calpains between cytosolic and particulate pools has been reported in a number of cell systems with increases in $[Ca^{2+}]_i$ (Kuboki et al. 1990; Schollmeyer 1986). In addition, a recent study by Murphy and colleagues has provided new insight into the mechanisms involved in the translocation of μ-calpain between cytosolic and particulate pools in situ (Murphy et al. 2006). The authors found that most μ-calpain is freely diffusible in the cytoplasm at resting $[Ca^{2+}]_i$ levels; however, if $[Ca^{2+}]_i$ is raised, binding of the protease to structural proteins can occur within seconds. Once bound, the continued elevation of $[Ca^{2+}]_i$ levels promotes autolysis and enhances the proteolytic activity of μ-calpain. In the present study, both HU and reweighting were associated with greater elevations in activities of the particulate fraction compared to the soluble fraction. This redistribution of activities may preclude a greater potential for calpain-mediated degradation of protein substrates with HU and reweighting by localizing calpain to target substrates such as desmin. Unfortunately it was not possible to identify the exact mechanisms underlying the subcellular redistribution of calpain activities reported in this study.

Under the electron microscope, muscles exposed to either spaceflight or HU demonstrate atrophied and misaligned sarcomeres with disrupted Z-lines, suggesting that a limited degradation of cytoskeletal components occurs with microgravity (Widrick et al. 1999). We chose to analyze the depletion patterns of the calpain substrate desmin because desmin is a structural cytoskeletal protein responsible for maintaining sarcomeric alignment in intact muscle. As well, previous work in cultured cells supports desmin depletion during conditions of protein degradation (Purintrapiban et al. 2003). Although the amount of desmin depletion observed with HU did not reach statistical significance, there was nevertheless a tendency towards desmin depletion during the earlier stages of HU (i.e. 24 and 72 h) and a return to control levels during longer periods of HU (i.e. 9 days). While the lack of statistical significance could be attributed to the high degree of variability observed between animals, findings in the literature examining the desmin response to changes in muscle loading are equivocal. For example, Li and colleagues reported a decrease in the amount of desmin observed in histochemical sections of rat gastrocnemius muscles 3 and 7 days following strain injury (Li et al. 2005). In contrast, Féasson and colleagues reported no changes in the amount of desmin present in muscle extracts immediately after or one day following a session of eccentric exercise (Féasson et al. 2002).

Despite the lack of statistical significance with respect to desmin depletion, the amount of desmin present in myofibrillar extracts was significantly and inversely related to the increases in the μ-calpain/calpastatin ratio during HU and reweighting. This not only suggests a strong relationship between calpain activation and desmin depletion with unloading, but also supports the contention that the protease-to-inhibitor activity ratio is a better indicator of net substrate proteolysis than protease activity alone.

Although the results from this study and others have demonstrated that the calpain–calpastatin system plays a significant role in unloading-induced atrophy, current knowledge indicates that calpains are just one of a number of proteolytic systems involved in protein degradation during conditions of muscle unloading. While some investigators have suggested that calpains are minor contributors to muscle proteolysis during unloading (Ikemoto et al. 2001), others contend that calpains play an important role towards regulating protein degradation (Taillandier et al. 1996; Tidball and Spencer 2002). As calpain activation was found to be an early event during HU and was associated with degradation of the structural protein desmin, it is possible that one regulatory role of calpains during unloading might be to degrade structural Z-disk proteins such as desmin and expose and/or target the exposed myofibrillar proteins for future degradation by other proteolytic pathways, particularly the ubiquitin-proteasome system. Two lines of evidence support this theory: first, it is now generally accepted that the ubiquitin-proteasome pathway is the primary system responsible for the proteolysis of contractile proteins such as myosin and actin during unloaded conditions (Ikemoto et al. 2001; Taillandier et al. 1996); and second, in vitro studies have demonstrated that myofibrillar contractile proteins will only act as substrates for the

ubiquitin-proteasome pathway when they are added individually (Solomon and Goldberg 1996). Whether this putative regulatory role of calpain during unloading is limited to simply isolating myofibrillar proteins and exposing them for proteolysis, or whether calpain also acts by targeting myofibrillar proteins for degradation through modification of specific amino acids in the N-termini, as has been suggested by others (Solomon et al. 1998), is unknown.

The lack of change in calpastatin activity with unweighting and reweighting was surprising, as previous work from our laboratory (Enns et al. 2002) and others (Barnoy et al. 1998; Sorimachi et al. 1997) has shown that during both muscle injury and development–conditions where $[Ca^{2+}]_i$ is increased and calpains have been reported to be active—that calpastatin activity tends to be lowered, thus deregulating calpain activity and allowing proteolysis of substrates to occur. One possible explanation for the lack of change in calpastatin activity could be that our measurement of calpastatin activity was based on its ability to inhibit m-calpain, not µ-calpain, and no changes in m-calpain activity were observed. However, we are not aware of any studies that demonstrate a differential calpastatin response between the two isoforms at this time.

Atrophic muscles that are reweighted following unweighting experience weakness and exhibit signs of injury (Riley et al. 1990). Since many features of reweighted muscles resemble those of injured muscles following eccentric exercise (Armstrong et al. 1991), we speculated that similar mechanisms might be involved. A primary finding of this study was that calpain activities were greater during reweighting compared to both control conditions and HU only in the vastus, indicating that at least during shorter periods of HU, calpains are more active during reweighting-induced muscle damage than HU-induced muscle atrophy. The finding that calpain activities remained elevated during both 12 and 24 h of reweighting compared to controls suggests that the metabolic conditions (similar to those observed during muscle injury) favour increased protease activity during reweighting. Moreover, the finding that calpain activity during reweighting was greater than that observed with HU alone during the shorter HU time points suggests that muscle atrophy during unweighting is not necessary for calpain activation to occur during reweighting.

In conclusion, this study has demonstrated that calpain activation is an early event during unloading in skeletal muscle, and the majority of the increase can be attributed to the µ-calpain isoform. The finding that desmin depletion was inversely related to the increases in the calpain/calpastatin activity ratio lends further support for the theory that calpains perform a regulatory function during unloaded conditions by degrading cytoskeletal Z-disk structures such as desmin. In order to gain a clearer understanding of the mechanisms underlying muscle atrophy during unloading, future studies should be aimed at determining how the time course of calpain activity is related to the action of other proteolytic systems during similar unloaded conditions. With this knowledge, appropriate countermeasures and therapeutic strategies can then be developed to overcome the weakness and atrophy associated with conditions of muscle unloading.

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