

Postmortem proteolysis is reduced in transgenic mice overexpressing calpastatin^{1,2}

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ABSTRACT: Using both in vitro and in vivo approaches, numerous studies have provided evidence that μ -calpain is responsible for postmortem proteolysis. This paper reports the effect of overexpression of calpastatin on postmortem proteolysis in transgenic mice. Transgenic mice ($n = 8$) with a human calpastatin gene, whose expression was driven by the human skeletal muscle actin promoter, were killed along with control nontransgenic littermates ($n = 5$). Hind limbs were removed and stored at 4°C, and muscle samples were dissected at 0, 1, 3, and 7 d postmortem and analyzed individually. At time 0, active human calpastatin was expressed in transgenic murine skeletal muscle at a level 370-fold greater ($P < 0.001$) than calpastatin in control mice. Although the native isoform of this protein was degraded with storage, at 7 d postmortem, approximately 78% of at-death activity remained, indicating that degraded calpastatin retains activity. Calpain (μ - and m -) expression was unaffected ($P > 0.05$) by the

transgene as assessed by immunoreactivity at d 0. Over 7 d, 33% of at-death 80-kDa isoform immunoreactivity of μ -calpain was lost in transgenics compared to an 87% loss in controls, indicating that autolysis of μ -calpain was slowed in transgenic mice. Desmin degradation was also inhibited ($P < 0.05$) in transgenics when compared to controls. Control mice lost 6, 78, and 91% of at-death native desmin at 1, 3, and 7 d postmortem, respectively; conversely, transgenic mice lost only 1, 3, and 17% at the same times. A similar trend was observed when examining the degradation of troponin-T. Interestingly, m -calpain seemed to undergo autolysis in control mice, which in postmortem tissue is indicative of proteolysis. Further investigation revealed that both μ - and m -calpain are active postmortem in normal murine skeletal muscle. In conclusion, a high level of expression of active calpastatin was achieved, which, by virtue of its inhibitory specificity, was determined to be directly responsible for a decrease in postmortem proteolysis.

Key Words: Calpastatin, Calpain, Postmortem, Proteolysis, Transgenic Mice

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Introduction

Current evidence indicates that μ -calpain is responsible for degradation of myofibrillar proteins in postmortem skeletal muscle, and that this degradation is responsible for improvements in meat tenderization (Goll et al., 1991; Koohmaraie, 1992c; Koohmaraie et al., 2002). To date, evidence for this has been collected using

a variety of approaches, including in vitro experiments (myofibrillar incubations; Koohmaraie et al., 1986), in vivo experiments with animal models (Callipyge; Koohmaraie et al., 1995; Geesink and Koohmaraie, 1999b), castration (Morgan et al., 1993), and treatment with β -adrenergic agonist (BAA; Kretchmar et al., 1990; Wheeler and Koohmaraie 1992; Koohmaraie et al., 1996). Limitations of these approaches, however, have prevented the gathering of direct evidence under authentic postmortem conditions. For example, there are many variables affecting the conversion of muscle to meat that cannot be recreated in vitro, and data collected from controlled in vitro experiments do not perfectly represent postmortem muscle events. Moreover, the ability to interpret in vivo data gathered from animal models is restricted because it is not possible to conclude with certainty that a particular measured change results directly from a particular treatment or genotype. Therefore, at this time, research relating cal-

¹Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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pain activity to postmortem proteolysis will benefit from additional supporting evidence. To address this, we examined postmortem myofibril degradation in transgenic mice overexpressing calpastatin. The tissue-specific, ectopic expression of calpastatin in skeletal muscle, and its exclusive inhibitory action over calpain, will allow us to test the hypothesis that μ -calpain mediates postmortem degradation of myofibrillar proteins, and draw confident conclusions.

Materials and Methods

Generation of Calpastatin Transgenics

Transgenic (**Tg**) mice were produced containing the full-length human calpastatin complementary DNA driven by the human skeletal muscle actin (**HSA**) promoter. Detailed information regarding this construct may be found in Tidball and Spencer (2002). This promoter has been used previously to successfully generate Tg mice with muscle-specific expression (Crawford et al., 2000; Spencer, 2002; Spencer and Mellgren, 2002).

Breeding and Sample Collection

Normal female mice (C57BL/10ScN) were mated with heterozygous Tg males (that had been backcrossed to C57BL/10 for at least 10 generations) derived from the 74.1 line described by Spencer and Mellgren (2002). All comparisons were made between age-matched mice. At 4 wk of age, mice were screened for the transgene by PCR and tagged for identification. At 8 wk of age, control ($n = 5$) and transgenic ($n = 8$) mice were killed by decapitation, and both hind limbs were removed and skinned. From each mouse, approximately half the muscle from one hind limb was immediately dissected, diced, and snap frozen in liquid nitrogen prior to storage at -80°C . The remaining hind limb was dipped in 1 mM NaN_3 solution to prevent microbial growth, blotted to remove excess liquid, and wrapped in plastic wrap. These portions were stored at 4°C until 1, 3, and 7 d postmortem, when additional portions of hind limb were dissected, snap frozen, and stored at -80°C .

PCR Screening

At 4 wk of age, DNA was extracted from 5 mm of tail and PCR was used to identify the presence of the transgene construct as described by Spencer et al. (2002) and Tidball and Spencer (2002) using upstream primers in the HSA promoter (5' CCC GAG CCG AGA GTA GCA GT 3') and downstream primers in the vp1 intron (5' CCC TTC CCT GTT GGC TAC T 3').

Sample Preparation

A portion of the frozen muscle samples (approximately 200 mg) was weighed and extracted in five volumes of ice-cold extraction buffer (100 mM Tris-HCl [pH 8.3] and 5 mM EDTA). Tissue was homogenized

for 15 s using a polytron set on high. One-fifth of the total homogenate was removed and prepared for SDS-PAGE, whereas the remaining four-fifths was used to determine calpastatin activity. The homogenate for SDS-PAGE analysis was prepared by adding an equal volume of 2 \times treatment buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, and 20% glycerol) and heating the mixture at 50°C for 20 min. After centrifuging the solution at $8,800 \times g$ for 20 min, the supernatant was collected for protein determination with a Pierce BCA protein assay kit (Pierce Laboratories, Rockford, IL). The sample was diluted to 2 mg/mL total protein using 2 \times treatment buffer containing 0.5% 2-mercaptoethanol (**MCE**) and bromophenol blue (0.04%), and reheated at 50°C for 10 min.

Quantification of Calpastatin Activity

In preparation for determination of calpastatin activity, the remaining total homogenate was centrifuged at $10,000 \times g$ for 30 min at 4°C . The supernatant was collected, heated at 100°C for 15 min, and then cooled on ice for 10 min before being centrifuged for 30 min in a microfuge set at $8,800 \times g$. The supernatant (containing heat stable proteins) was then dialyzed overnight against elution buffer (40 mM Tris-HCl [pH 7.35], 0.5 mM EDTA, and 0.05% MCE) in a Slide-A-Lyzer cassette (Pierce Laboratories) with a 10-kDa cut-off. The supernatant was assayed for calpastatin activity using [^{14}C]casein as described by Koohmaraie (1992b). Briefly, [^{14}C]casein was incubated with heated calpastatin sample and semipurified m-calpain from ovine lung in a 100- μL reaction (10 mM Tris-HCl; pH 7.5, 10 mM CaCl_2). The reaction was stopped, after incubation for 1 h at 25°C , with the addition of 100 μL of cold 10 mg/mL BSA and 200 μL of cold 10% trichloroacetic acid. The reactions were centrifuged at $8,800 \times g$ for 15 min at 4°C , and 200 μL of the trichloroacetic acid-soluble proteins was mixed with 5 mL of scintillation fluid (ScintiVerse; Fisher Scientific, Pittsburgh, PA). The radioactivity of the mixture was measured using a Packard TriCarb 1600TR liquid scintillation analyzer (Packard Instruments, Meriden, CT), and activity of calpastatin was expressed as micrograms of [^{14}C]casein not degraded per gram of tissue.

SDS-PAGE and Western Blotting

The SDS-PAGE was performed as described by Laemmli (1979) using $8 \times 10 \times 0.075$ -cm minigels. The acrylamide percentage of the resolving gel varied depending on the protein to be analyzed: 15% gels were used for troponin T, 10% gels were used for desmin and calpastatin, and 7.5% gels were used for μ -calpain and m-calpain. All gels included 4% stacker gels and were made using 37.5:1 acrylamide to bisacrylamide solution.

After electrophoresing at 200 V for 1 h, proteins were transferred onto Hybond-P polyvinylidene difluoride

(PVDF) membrane (Amersham Biosciences, Uppsala, Sweden) at 200 mA for 1 h using a wet transfer apparatus (BioRad Laboratories, Hercules, CA). Membranes were blocked with 3% non-fat dry milk in Tris-buffered saline containing Tween (TTBS; 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.05% Tween-20). After blocking for 1 h, membranes were exposed to primary antibodies diluted in TTBS as follows: 1:20 monoclonal anti-desmin clone D3, Hybridoma Bank (Danto and Fischman, 1984), 1:5 monoclonal anti- μ -calpain clone B2F9, MARC-USDA (Geesink and Koohmaraie, 1999b), 1:500 polyclonal anti-human calpastatin SC-7560 (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1,000 monoclonal anti-m-calpain clone C-0728 (Sigma Chemical Co., St. Louis, MO), and 1:1,600 monoclonal anti-rabbit troponin-T clone T-6277 (Sigma Chemical Co., St. Louis, MO). Blots were incubated with primary antibody for 1 h at room temperature before being washed with TTBS. Secondary antibodies conjugated with peroxidase were diluted in TTBS and used as follows: anti-mouse IgG (31430; Pierce Laboratories) was used to detect desmin (1:2,000) and μ -calpain (1:5,000); anti-goat IgG (sc-2020; Santa Cruz Biotechnology) was used to detect human calpastatin (1:2,000); and anti-rabbit IgG (A-6154; Sigma Chemical Co.) was used to detect m-calpain antibodies (1:500). Anti-mouse IgG secondary antibody (A-2179; Sigma Chemical Co.) conjugated to alkaline phosphatase was diluted 1:1,000 in TTBS and used to detect troponin-T and m-calpain antibodies. Blots were exposed to secondary antibodies for 1 h at room temperature before being extensively washed with TTBS. Antibody binding was visualized by incubating Hybond-P PVDF membranes either with chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate, Pierce Laboratories) or alkaline phosphatase conjugate substrate kit (BioRad Laboratories, Hercules, CA). Images were captured and analyzed using a ChemImager 5500 digital imaging system (Alpha Innotech Corp., San Leandro, CA). The amount of immunoreactive protein remaining at 1, 3, and 7 d postmortem was expressed as a percentage of the amount measured at d 0.

Zymography and Nondenaturing PAGE

Portions (approximately 100 mg) of frozen skeletal muscle were homogenized in five volumes of extraction buffer containing 0.05% MCE for 15 s using a polytron set on high. After centrifuging the homogenate at 8,800 $\times g$ for 30 min, the supernatant was collected and its volume recorded. Casein zymography was performed according to the procedure of Veiseth et al. (2001). Polyacrylamide gels (12.5%; 8 \times 10 \times 0.075 cm) were loaded with supernatant equivalent to 1.5 mg of muscle and electrophoresed at 150 V for 4 h at 4°C before incubation and staining. Nondenaturing PAGE was performed following the zymography protocol with the exception that casein was excluded from the separating gel. After elec-

trophoresis, protein was transferred and probed with antibodies as described previously.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC) for a split-plot design. The model included the main effects of genotype, day postmortem, genotype \times day, and animal. The error term used to test genotype effects was animal. When the main effect or interaction was significant ($P < 0.05$), least squares means were separated using a pair-wise t -test (PDIFF option).

Results and Discussion

There was no obvious effect of the transgene on mouse size or weight (results not shown). This is consistent with previous reports that showed that the effects of the calpastatin transgene on mouse phenotype were evident only at the microscopic level in transgenic dystrophic *mdx* mice (Spencer and Mellgren, 2002) or following treatment to induce muscle atrophy (Tidball and Spencer 2002).

Calpastatin

The presence of the transgene resulted in high expression of human calpastatin protein in skeletal muscle of Tg mice (Figure 1a). The efficacy of the HSA promoter to drive gene expression was first characterized by Brennan and Hardeman (1993), who reported the advantages of this promoter to be its tissue specificity, developmentally correct expression, level of expression, and the fact that α -skeletal actin gene is expressed naturally in all types of skeletal muscle. To verify that the expressed protein was functional, calpastatin activity was quantified using [¹⁴C]casein as a substrate. Our results demonstrated that the presence of the transgene caused a 370-fold increase ($P < 0.001$) in at-death calpastatin activity in Tg mice relative to controls (Table 1), which is comparable to the 340-fold increase in calpastatin activity previously reported for this line of mice (line 74.1; Spencer and Mellgren, 2002). This increase is greater than the increase reported in studies using other animal models, which have reported calpastatin increases of 83 to 114% for Callipyge lambs (Koohmaraie et al., 1995; Delgado et al., 2001) and 60% for BAA-treated lambs (Wheeler and Koohmaraie, 1992). Relative to at-death values, calpastatin activity decreased by 22% in Tg mice and 31% in control mice by 7 d postmortem. Compared with other species and treatments, this is a relatively small percentage decline. Relative to at-death levels, calpastatin activity at 7 d decreased as much as 81.5% in beef (Wheeler and Koohmaraie 1992), 62.5% in lambs (Koohmaraie et al., 1995), 48.8% in BAA-treated steers (Wheeler and Koohmaraie 1992), 50% in BAA-treated lambs (Koohmaraie et al., 1996), and 62% in callipyge lambs (Kooh-

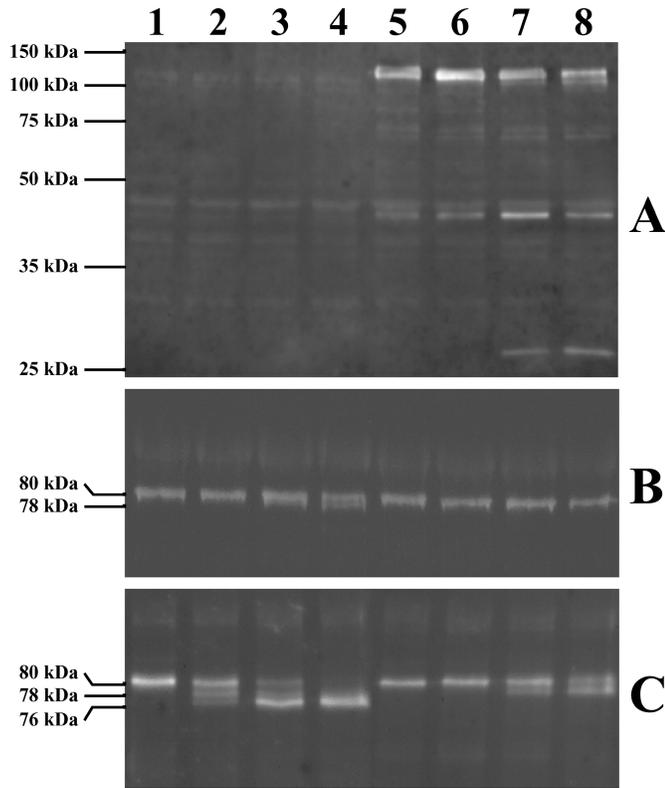


Figure 1. Western blots showing degradation of human calpastatin, m-calpain, and μ -calpain in skeletal muscle from transgenic and control mice during storage. Each panel represents a blot probed with anti-human calpastatin (A), anti-m-calpain (B), and anti- μ -calpain (C). Lanes 1 through 4 correspond to samples from control mice aged 0, 1, 3, and 7 d, respectively, whereas Lanes 5 through 8 correspond to samples from transgenic mice aged 0, 1, 3, and 7 d, respectively.

marai et al., 1995). Examination of Western blots showed that the amount of full-length immunoreactive human calpastatin protein dropped during storage, whereas several smaller immunoreactive bands became apparent (Figure 1a). Relative to starting levels, full-length immunoreactive human calpastatin protein was reduced by 5, 21, and 80% at 1, 3, and 7 d postmortem, respectively, in samples from Tg mice (data not shown). The discrepancy between the declines in immunoreactive human calpastatin (80%) and detectable activity (22%) over 7 d suggests that the epitope was lost with-

Table 1. Calpastatin activity (μ g casein not degraded/g of fresh muscle) in control and transgenic mice at 0 and 7 d postmortem

Item	d 0	d 7
Transgenic (n = 8)	279 \pm 24 ^a	218 \pm 34 ^b
Control (n = 5)	0.74 \pm 0.06 ^c	0.51 \pm 0.09 ^c

^{a,b,c}Within a row or column, means without a common superscript letter differ ($P < 0.05$).

Table 2. Mean percentage of decline in immunologically detectable m-calpain, μ -calpain, and desmin during post-mortem aging from five control and eight transgenic mice

Protein	Days postmortem	Percent remaining	
		Control	Transgenic
m-calpain (80 kDa)	1	94 ^a	96 ^a
	3	99 ^a	98 ^a
	7	93 ^a	91 ^a
	SEM	2.8	2.2
μ -calpain (80 kDa)	1	81 ^b	97 ^a
	3	29 ^d	94 ^a
	7	13 ^e	67 ^c
	SEM	4.4	3.5
Desmin (native)	1	94 ^a	99 ^a
	3	22 ^c	97 ^a
	7	9 ^d	83 ^b
	SEM	3.1	2.4

^{a,b,c,d,e}Within a protein, means without a common superscript letter differ ($P < 0.05$).

out an ensuing loss of activity. This agrees with the results of Geesink and Koohmaraie (1999b), who, using an antibody specific for full-length calpastatin, found that calpastatin activity was detectable in the absence of immunoreactive protein in postmortem ovine samples. Based on two lines of evidence, it is likely that calpain is responsible for this degradation. Firstly, post-mortem infusion of carcasses with calcium chloride activates calpain and causes a reduction in calpastatin (Koohmaraie et al., 1989), whereas zinc chloride eliminates calpain activity and prevents the loss of calpastatin (Koohmaraie 1990). Secondly, similarities between the calpastatin fragments produced postmortem in vivo and the degradation products produced by incubating calpastatin with μ -calpain in vitro indicates that calpain is responsible for postmortem degradation of calpastatin in lamb (Doumit and Koohmaraie 1999).

Calpain

A significant body of literature exists to indicate that μ -calpain is responsible for postmortem degradation of myofibrillar proteins during the early postmortem period (Koohmaraie, 1987; Veiseth et al., 2001). Therefore, revealing what impact the transgene has on μ -calpain was a vital consideration. Examination of μ - and m-calpain Western blots suggested that at-death expression of these proteases was unaffected ($P < 0.05$) by the presence of the transgene (Figures 1b and 1c), which agrees with a similar assessment made by Spencer and Mellgren (2002). With respect to m-calpain, there was no decline in detectable protease between d 1 and 7 ($P > 0.05$; Table 2). However, examination of Western blots revealed a second band that appeared at d 7 in some control samples. Although this second band is not clear on the reproduced chemiluminescent Western blot (Figure 1b), it is evident on alkaline phosphatase

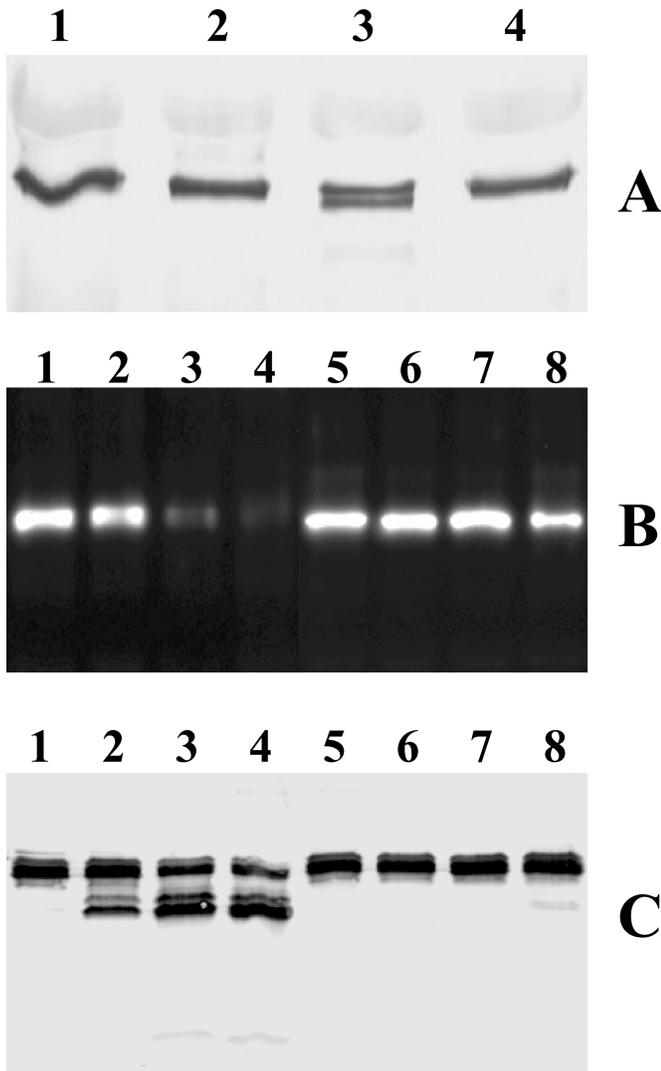


Figure 2. Western blots showing degradation of m-calpain, desmin, and troponin-T during storage. A) Alkaline phosphatase developed anti-m-calpain blots of skeletal muscle collected from control and transgenic (Tg) mice at 0 and 7 d postmortem (Lanes 1 and 2 are samples from control and Tg mice at 0 d, respectively, whereas Lanes 3 and 4 are samples from control and Tg mice at 7 d, respectively). B) Chemiluminescent developed anti-desmin blots of aged skeletal muscle from control and Tg mice. Lanes 1 through 4 correspond to samples from control mice aged 0, 1, 3, and 7 d, respectively, whereas Lanes 5 through 8 correspond to samples from Tg mice aged 0, 1, 3, and 7 d, respectively. C) Alkaline phosphatase developed anti-troponin-T blots of aged skeletal muscle from control and Tg mice. Lanes 1 through 4 correspond to samples from control mice aged 0, 1, 3, and 7 d, respectively, whereas Lanes 5 through 8 correspond to samples from transgenic mice aged 0, 1, 3, and 7 d, respectively.

tase developed blots (Figure 2a). The presence of this smaller, immunoreactive band in control samples may be evidence of autolysis and hence, postmortem m-calpain activity. There was no difference ($P < 0.05$) between

Tg and control mice in the quantity of m-calpain at any time postmortem (Table 2). The loss of immunoreactive μ -calpain was defined as the decrease in full-length large subunit (80 kDa). Abundance of the 80-kDa μ -calpain subunit declined in both Tg and control mice during the 7-d storage period; however, the rate and extent of this decline appeared to be reduced in Tg mice (Table 2). Although declines ($P < 0.05$) in the 80-kDa μ -calpain subunit were detected at all time intervals in control mice, Tg mice showed negligible changes ($P > 0.05$) over the initial 3 d of storage (6% loss in Tg mice vs. 71% loss in control mice), followed by a decrease ($P < 0.05$) between 3 and 7 d. Between 0 and 7 d, the overall loss of the 80-kDa subunit was 33% in Tg mice and 87% in controls. With increased postmortem storage, samples from control mice exhibited the distinctive postmortem profile of μ -calpain immunoreactive bands, specifically the generation of 78- and 76-kDa polypeptides (Figure 1c). Transgenic mice, however, did not display this typical pattern of degradation (Figure 1c); the 78-kDa polypeptide appeared only at 3 d postmortem, and the 76-kDa polypeptide was not detected at any time. Because autolysis of μ -calpain is an intermolecular proteolytic event (Cottin et al., 2001) and calpastatin is a reversible inhibitor, the surplus of active calpastatin in Tg mice would be expected to slow down autolysis rather than stop it completely. This agrees with results from studies showing that Callipyge sheep (which have elevated calpastatin activities) also show a delayed generation of μ -calpain autolytic fragments (Geesink and Koohmaraie 1999b). The fact that μ -calpain autolyses in the presence of excess calpastatin agrees with in vitro studies by Koohmaraie (1992a), who demonstrated that co-incubation of μ -calpain and calpastatin in the presence of calcium did not stop the initial autolysis of μ -calpain to 78 kDa, but did prevent generation of the 76-kDa polypeptide.

Conditions in normal postmortem muscle usually dictate that autolysis of calpain and substrate proteolysis parallel each other closely. Examples of this can be found in Callipyge lamb (Geesink and Koohmaraie, 1999b) and BAA-treated animals (Koohmaraie et al., 1991). However, autolysis is not obligatory for μ -calpain proteolysis (Cottin et al., 2001), and the 76-kDa isoform is not always proteolytically active (Geesink and Koohmaraie, 1999a); therefore, the degradation of desmin was used as an indicator of calpain-mediated postmortem proteolysis. The rationale for quantifying proteolysis using desmin are as follows: 1) desmin is a substrate for calpain and degradation of this cytoskeletal protein correlates well with tenderization (Taylor et al., 1995; Robson et al., 1997) and 2) other proteolytic systems, lysosomal and multicatalytic proteinase complexes, do not have access to, or the ability to degrade, desmin (Goll et al., 1992; Koohmaraie, 1992b). Therefore, analysis of desmin degradation is a good estimate of in situ calpain activity. Table 2 was generated from densitometric analysis of desmin Western blots (see Figure 2b), and shows that desmin degradation was

reduced in transgenic mice, and demonstrated that hind limb skeletal muscle suitably reflects the treatment effect. The amount of immunoreactive desmin decreased between 1 and 3 d ($P < 0.001$), and between 3 and 7 d ($P < 0.01$) in control mice. Degradation of desmin was slower in Tg mice and the only reduction in native desmin ($P < 0.01$) took place between 3 and 7 d. As a result, differences between control and Tg mice were detected at d 1 ($P < 0.10$), 3 ($P < 0.001$), and 7 ($P < 0.001$). At 7 d, Tg mice retained 83% of at-death desmin, whereas control mice retained only 9% intact desmin. In addition, degradation of troponin-T was examined qualitatively. This myofibrillar protein has been used previously to illustrate postmortem proteolysis in skeletal muscle (Taylor et al., 1995; Geesink and Koohmarai, 1999b). Consistent with the desmin data, degradation of troponin-T was reduced in Tg mice (Figure 2c).

Zymography and Nondenaturing Gels

Casein gel zymography is a sensitive method for detection of calpain protease and has the capacity to differentiate between autolysed and unautolysed isoforms (Veiseth et al., 2001). Previously, reports have noted that the ability of zymography to detect both μ - and m-calpain is compromised in Tg mice (Tidball and Spencer, 2002), which led Spencer and Mellgren (2002) to hypothesize that this could be due to comigration of calpain with calpastatin in the gel or complexing of the protease with its inhibitor during sample preparation. The formation of a calpain/calpastatin complex has an absolute dependence on calcium and, because chelating agents are included during sample preparation, the complexing of protease and inhibitor is unlikely. To test the alternative suggestion of comigration, zymograms were compared with Westerns blots generated from nondenaturing gels. In agreement with previous reports (Spencer and Mellgren, 2002; Tidball and Spencer, 2002), μ - and m-calpain from control mice were easily detected on zymograms. Interestingly, both proteases migrate more slowly than their ovine equivalent (Figure 3a). However, it was not possible to detect μ -calpain in samples from Tg mice, whereas m-calpain was only detected in d-0 samples from Tg mice. Western blots generated from nondenaturing gels and probed with anti-human calpastatin antibody produced a strong reaction in all Tg samples. Although the immunoreactivity was limited to the top half of the gel at d 0, at later sampling times, calpastatin became more smeared and ultimately extended throughout the length of the lane. When this appearance is considered together with the denaturing gel (Figure 1a), which showed evidence of degradation, it is likely that the smear represents human calpastatin in various stages of degradation. Using the ovine zymogram control lane as a reference, it is possible to align and compare the zymogram and nondenatured μ - and m-calpain Western blots (Figures 3c and 3d) with the nondenatured calpastatin Western blot (Figure 3b). It appears that

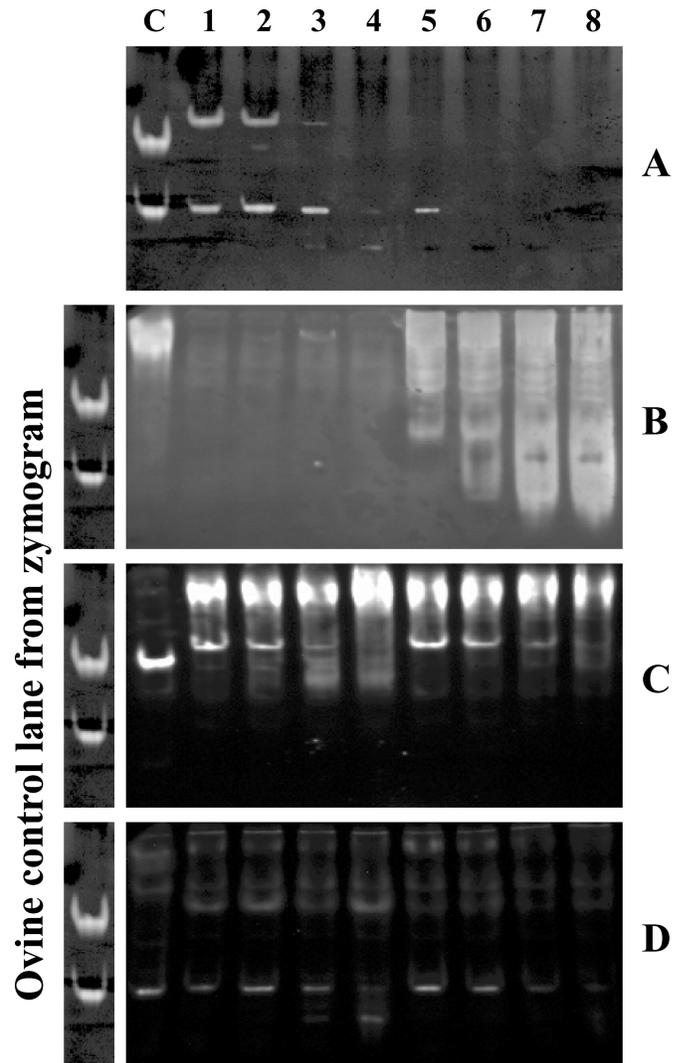


Figure 3. Zymogram showing calpain activity (A). Nondenaturing gels separated total proteins, which were then transferred and probed with anti-human-calpastatin (B), anti- μ -calpain (C) or anti-m-calpain (D) antibodies. Lanes 1 through 4 correspond to samples from control mice aged 0, 1, 3, and 7 d, respectively, whereas Lanes 5 through 8 correspond to samples from transgenic mice aged 0, 1, 3, and 7 d, respectively (Lane C represents a d 0 ovine control sample).

human calpastatin and/or its degradation products would comigrate with μ -calpain at times postmortem preventing its detection. With regard to m-calpain, its activity is only detectable in d-0 samples. The reason that calpastatin activity is not detectable in samples from other times is that the degradation of calpastatin has produced smaller fragments, which pass through the region containing m-calpain and prevent its detection. Evidently, these fragments maintain inhibitory activity, which supports our previous suggestion that, at later postmortem times, loss of full-length calpastatin does not represent a loss of activity.

These zymograms and nondenaturing Western blots provide several other insights into the behavior of cal-

pain in murine muscle. Significantly, m-calpain appears to be active in postmortem muscle from control mice. This was suggested in Figure 2a, which revealed the presence of a double band in 7-d samples, and is confirmed by Figures 3a and d, which, when considered together, show the appearance of an active, immunoreactive band that migrates faster than the native m-calpain band in 3- and 7-d control samples. Our conclusion that this band is caused by m-calpain autolysis and does not involve other enzymes is based on three considerations. First, because the degraded m-calpain bands are not seen in Tg mice, which over-express calpastatin, the protease responsible for the degradation of m-calpain must be either μ - or m-calpain. Second, by performing zymography on aged muscle samples, Veiseth et al. (2001) showed that μ -calpain is active postmortem, whereas m-calpain levels remained unchanged. This indicates that μ -calpain does not degrade m-calpain in vivo. Finally, our zymograms were similar to those of Veiseth et al. (2001), who showed, by deliberately activating m-calpain with calcium in vitro, that it is possible to generate a proteolytically active m-calpain fragment that migrates faster than the native enzyme on non-denaturing gels. With the exception of experiments where intracellular calcium is artificially elevated (e.g., by CaCl_2 infusion; Koohmaraie et al., 1989), this is the first example of postmortem m-calpain activity in normal skeletal muscle. In cell culture, however, the activation of m-calpain without calcium elevation has been reported by Temm-Grove et al. (1999), who observed that microinjection of bovine m-calpain into rat satellite cells accelerated cell fusion. These authors suggested that these cells contain some mechanism that enables m-calpain to be active in vivo at calcium concentrations less than those required for in vitro activity. Our preliminary in vitro investigations have shown that murine m-calpain is active at calcium concentrations under which ovine m-calpain remains inactive; however, it is not possible to speculate what factor(s) contribute to its activation postmortem in skeletal muscle. Another observation of interest is that the abundance of native μ - and m-calpain declines with storage in Tg samples (Figure 3c and d). Because we can be confident that these isoforms possess proteolytic potential (Figure 3a), it seems likely that their decline during storage reflects in situ activity. Although there is good evidence that calpain can degrade calpastatin in postmortem muscle, it is reasonable to suggest that calpain (possibly both μ - and m-) is responsible for the degradation of human calpastatin.

Implications

The results of the present study establish firmly that calpastatin regulates calpain activity postmortem and that this activity alone is responsible for postmortem proteolysis of key cytoskeletal proteins. A high level of expression of active calpastatin can be achieved apparently without indiscriminate collateral effects, and this

leads directly to a decrease in proteolytic activity as demonstrated by reduced autolysis and reduced degradation of substrates. This transgenic model has potential application for experiments investigating the role of calpain and calpastatin in postmortem proteolysis, as well as research seeking to define the role of calpains and calpastatin in muscle growth and protein turnover in the live animal.

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