Calpain 3/p94 is not involved in postmortem proteolysis[1,2]

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ABSTRACT: Studies on the correlation between expression and/or autolysis of calpain and postmortem proteolysis in muscle have provided conflicting evidence regarding the possible role of calpain 3 in postmortem tenderization of meat. Thus, the objective of this research was to test the effect of postmortem storage on proteolysis and structural changes in muscle from normal and calpain 3 knockout mice. Knockout mice (n = 6) were sacrificed along with control mice (n = 6). Hind limbs were removed and stored at 4°C; muscles were dissected at 0, 1, and 3 d postmortem and subsequently analyzed individually for degradation of desmin. Pooled samples for each storage time and mouse type were analyzed for degradation of nebulin, dystrophin, vinculin, and troponin-T. In a separate experiment, hindlimb muscles from knockout (n = 4) and control mice (n = 4) were analyzed for structural changes at 0 and 7 d postmortem using light microscopy. As an index of structural changes, fiber detachment, cracked or broken fibers, and the appearance of space between sarcomeres were quantified. Cumulatively, the results of the first experiment indicated that postmortem proteolysis of muscle occurred similarly in control and in calpain 3 knockout mice. Desmin degradation did not differ ($P > 0.99$), and there were no indications that degradation of nebulin, dystrophin, vinculin, and troponin-T were affected by the absence of calpain 3 in postmortem muscle. Structural changes were affected by time postmortem ($P < 0.05$), but not by the absence of calpain 3 from the muscles. In conclusion, these results indicate that calpain 3 plays a minor role, if any, in postmortem proteolysis in muscle.

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

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Introduction

Skeletal muscle contains two well-characterized, calcium-dependent neutral proteinases, μ- and m-calpain, their specific inhibitor, calpastatin, and a number of calpains whose characteristics are largely unknown (Goll et al., 2003). Considerable evidence indicates that μ-calpain, but not m-calpain, plays an important role in postmortem degradation of myofibrillar proteins in postmortem muscle, resulting in meat tenderization (Geesink et al., 2000; Hopkins and Thompson, 2002; Koohmaraie et al., 2002).

Of the other calpains, p94, skeletal muscle specific (**SKM**), or calpain 3 is of interest to meat scientists because it binds to titin at the N_2 line (Sorimachi et al., 1995; Kinbara et al., 1998; Spencer et al., 2002), a site where proteolysis has been linked to tenderization (Taylor et al., 1995). Characterization of calpain 3 has been hampered by the fact that it autolyzes rapidly, even in the absence of Ca, when it is not bound to titin (Sorimachi et al., 1995; Kinbara et al., 1998; Spencer et al., 2002); however, using Western blotting, it has been established that calpain 3 autolyzes in postmortem muscle (Anderson et al., 1998; Parr et al., 1999; Ilian et al., 2004a). Moreover, μ- and m-calpain autolysis is indicative of activation (Goll et al., 2003). Because it is difficult to purify active calpain 3, its role in tenderization has been assessed by correlating expression or autolysis of calpain 3 and postmortem proteolysis and tenderization (Parr et al., 1999; Ilian et al., 2001a, 2004a). Parr et al. (1999) found no evidence that calpain 3 abundance or rate of autolysis was associated with variability in meat tenderness. Conversely, Ilian et al.

[1]Names 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 products to the exclusion of others that may also be suitable.

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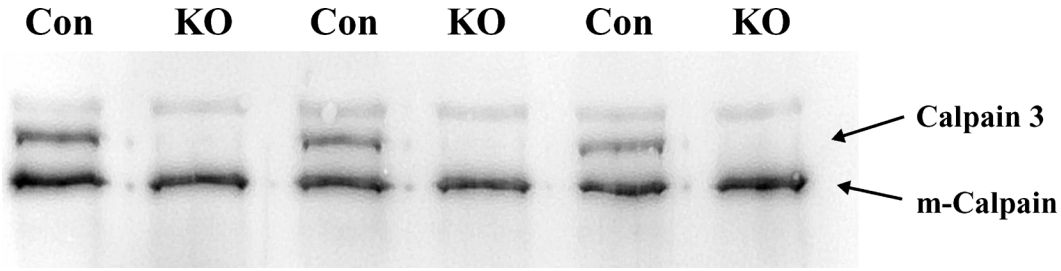

Figure 1. Western blot analysis of calpain 3 and m-calpain in whole muscle extracts of control (Con) and calpain 3 knockout (KO) mice.

(2001a,b) reported significant correlations between calpain 3 expression and tenderization between and within muscles and the rate of autolysis of calpain 3 and postmortem proteolysis and tenderization (Ilian et al., 2004a,b).

In contrast with μ- and m-calpain, calpain 3 is not inhibited by calpastatin (Sorimachi et al., 1993). Moreover, it was recently suggested that calpain 3 may regulate the activity of μ- and m-calpain by degrading calpastatin (Ono et al., 2004). The fact that calpain 3 is not inhibited by calpastatin suggests it has a minimal role in postmortem proteolysis and tenderization because 1) it is well-established that an increase in calpastatin levels inhibits these events; 2) calpastatin activity is the trait most highly correlated with the rate and extent of postmortem proteolysis; and 3) most importantly, overexpression of calpastatin in mouse muscle significantly decreases postmortem proteolysis (Koohmaraie et al., 1991, 1995; Kent et al., 2004).

Our objective was to investigate whether calpain 3 affects postmortem proteolysis either directly by degrading structural proteins or indirectly by degrading calpastatin and thereby promoting the action of μ-calpain. To this end, we compared proteolysis and ultrastructural changes in muscles of normal and calpain 3 knockout mice during postmortem storage.

Materials and Methods

Generation of Calpain 3 Knockouts

Calpain 3 knockout (**KO**) mice were kindly supplied by M. J. Spencer (Dept. of Pediatrics and UCLA Duchenne Muscular Dystrophy Research Center, Univ. Calif., Los Angeles) and produced as described by Kramerova et al. (2004).

Sample Collection

At 5 wk of age, control (n = 6) and KO (n = 6) 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 and snap-frozen in liquid N_2 before storage at –70°C. The remaining hind limb was dipped in 1 m*M* 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 and 3 d postmortem, when additional portions of the hind limb were dissected, snap frozen, and stored at –70°C. In a separate experiment, hind limbs of control (n = 4) and KO (n = 4) 5-wk-old mice were sampled as described above at 0 and 7 d postmortem and prepared for structural examination.

In addition to the samples from the calpain 3 KO mice, we also used muscle extracts from mice overexpressing calpastatin. These samples were used for Western blots against calpain 3, and were prepared as described in Kent et al. (2004).

Sample Preparation

A portion of the frozen muscle (approximately 150 mg) was weighed and extracted in five volumes of ice-cold extraction buffer (100 m*M* Tris·HCl; pH 8.3; 5 m*M* EDTA). Tissue was homogenized for 15 s using a polytron set on high. Half the total homogenate was removed and centrifuged at 16,000 × *g* (maximum force value) for 10 min at 4°C. After centrifugation, the supernatant fraction was collected. The total homogenate and the soluble muscle fraction were mixed with an equal volume of 2× SDS-PAGE sample buffer (0.125 *M* Tris·HCl; pH 6.8; 4% SDS and 20% glycerol) (vol/vol) and heated at 50°C for 20 min. After centrifuging the solution at 16,000 × *g* (maximum force value) for 5 min at room temperature, the supernatant fraction was collected, and protein concentration was determined using a Pierce BCA protein assay kit (Pierce Laboratories, Rockford, IL). Samples were diluted to 2 mg/mL of total protein using SDS-PAGE sample buffer containing 0.5% (vol/vol) 2-mercaptoethanol (**MCE**) and bromophenol blue (0.04%) (vol/vol).

SDS-PAGE and Western Blotting

Western blotting and SDS-PAGE were performed on pooled muscle samples from the hindlimbs of the respective groups. In addition, each individual muscle was analyzed for desmin content for quantitative analysis of postmortem proteolysis. The SDS-PAGE was performed as described by Laemmli (1979) using 8 × 10 × 0.075 cm minigels. The acrylamide percentage of resolv-

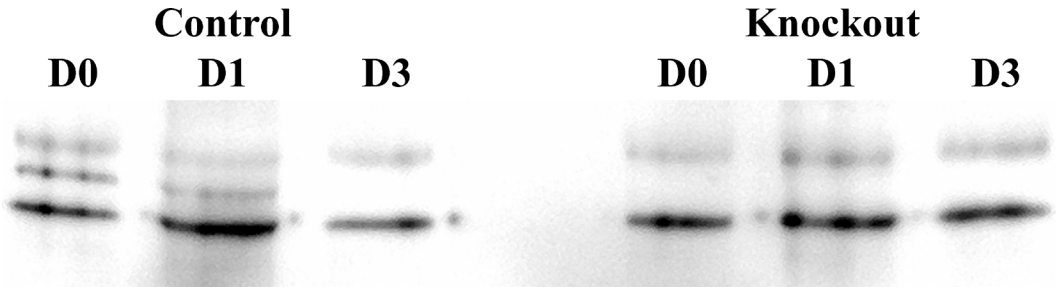

Figure 2. Western blot analysis of calpain 3 and m-calpain in whole muscle extracts of control and calpain 3 knockout mice at death (D0) and after 1 (D1) and 3 d (D3) storage at 4°C.

ing gel varied depending on the protein of interest: for μ-calpain, 7.5% gels were used; for calpain 3, desmin, and vinculin, 10% gels were used; for troponin T, 12.5% gels were used; and for nebulin and dystrophin, 5% gels were used. All gels, except the 5% gels, included 4% stacking gels, and were made using a 37.5:1 acrylamide to bisacrylamide solution. The 5% gels were made using a 100:1 acrylamide to bisacrylamide solution.

After electrophoresis at 200 V for 1 h, proteins were transferred onto Hybond-P polyvinylidine fluoride (**PVDF**) membranes (Amersham Biosciences, Uppsala, Sweden) at 200 mA for 1 h using a wet transfer apparatus (BioRad Laboratories, Hercules, CA). All of the following steps were performed at room temperature. Membranes were blocked with 3% nonfat dry milk in Tris-buffered saline containing Tween (**TTBS**; 20 m*M* Tris·HCl, 137 m*M* NaCl, 5 m*M* KCl, and 0.05% Tween 20). After blocking for 1 h, membranes were exposed to the following primary antibodies diluted in 3% (wt/vol) nonfat dry milk in TTBS: mouse monoclonal anti-calpain 3 clone NCL-CALP-12A2 (dilution 1:50; Novocastra Laboratories, Newcastle upon Tyne, U.K.; Anderson et al., 1998); mouse monoclonal anti-μ-calpain (dilution 1:5; MARC-USDA; Geesink and Koohmaraie, 1999); mouse monoclonal anti-desmin clone D3 (dilution 1:10; Hybridoma Bank, Iowa City, IA; Danto and Fischman, 1984); mouse monoclonal anti-vinculin clone V284 (dilution 1:500; Accurate, Westbury, NY); mouse monoclonal anti-troponin-T clone JLT-12 (dilution 1:5000; Sigma Chemical Co., St. Louis, MO); mouse monoclonal anti-nebulin clone NB2 (Sigma Chemical Co.); and mouse monoclonal anti-dystrophin clone NCL-DYS1 (dilution 1:100; Novocastra Laboratories). Blots were incubated with primary antibody for 1 h at room temperature before being washed with TTBS. The secondary antibody used was anti-mouse IgG conjugated with peroxidase (dilution 1:5000; 31430; Pierce Laboratories). Blots were exposed to the secondary antibody for 1 h at room temperature before being extensively washed with TTBS. Antibody binding was visualized by incubating PVDF membranes with chemiluminescent substrate (SuperSignal West Dura extended duration substrate; Pierce Laboratories). Images were captured and the intensity of the bands was analyzed using a ChemiImager 5500 digital imaging system (Alpha Innotech Corp., San Leandro, CA). The amount of immunoreactive desmin remaining at 1 and 3 d postmortem was expressed as a percentage of the amount on d 0.

Light Microscopy

Samples were prepared as described previously by Taylor and Koohmaraie (1998). Briefly, at death and after 7 d postmortem storage at 4°C, muscle samples were cut to approximately $2 \times 2 \times 5$ mm cubes and fixed overnight by immersion in cold 2.5% glutaraldehyde (vol/vol) in 0.1 *M* sodium cacodylate buffer (pH 7.3). Samples were stained en bloc with 1% tannic acid (vol/vol), postfixed in 1% osmium (vol/vol), stained en bloc with uranyl acetate in 25% ethanol, dehydrated in etha-

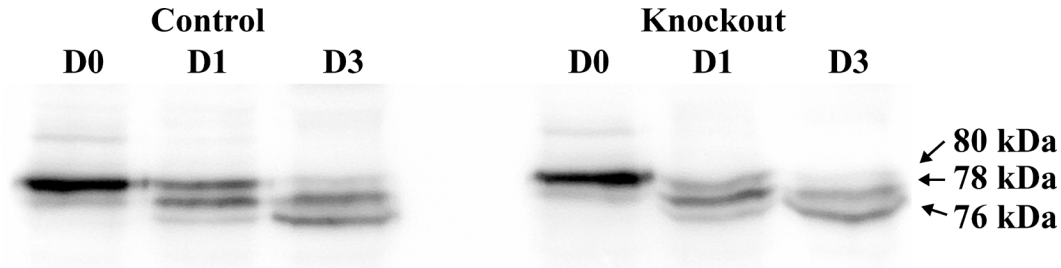

Figure 3. Western blot analysis of μ-calpain in the soluble muscle fraction of control and calpain 3 knockout mice at death (D0) and after 1 (D1) and 3 d (D3) storage at 4°C. 80 kDa denotes the intact large subunit of μ-calpain, whereas 78 and 76 kDa denote autolysis products of the large subunit of μ-calpain.

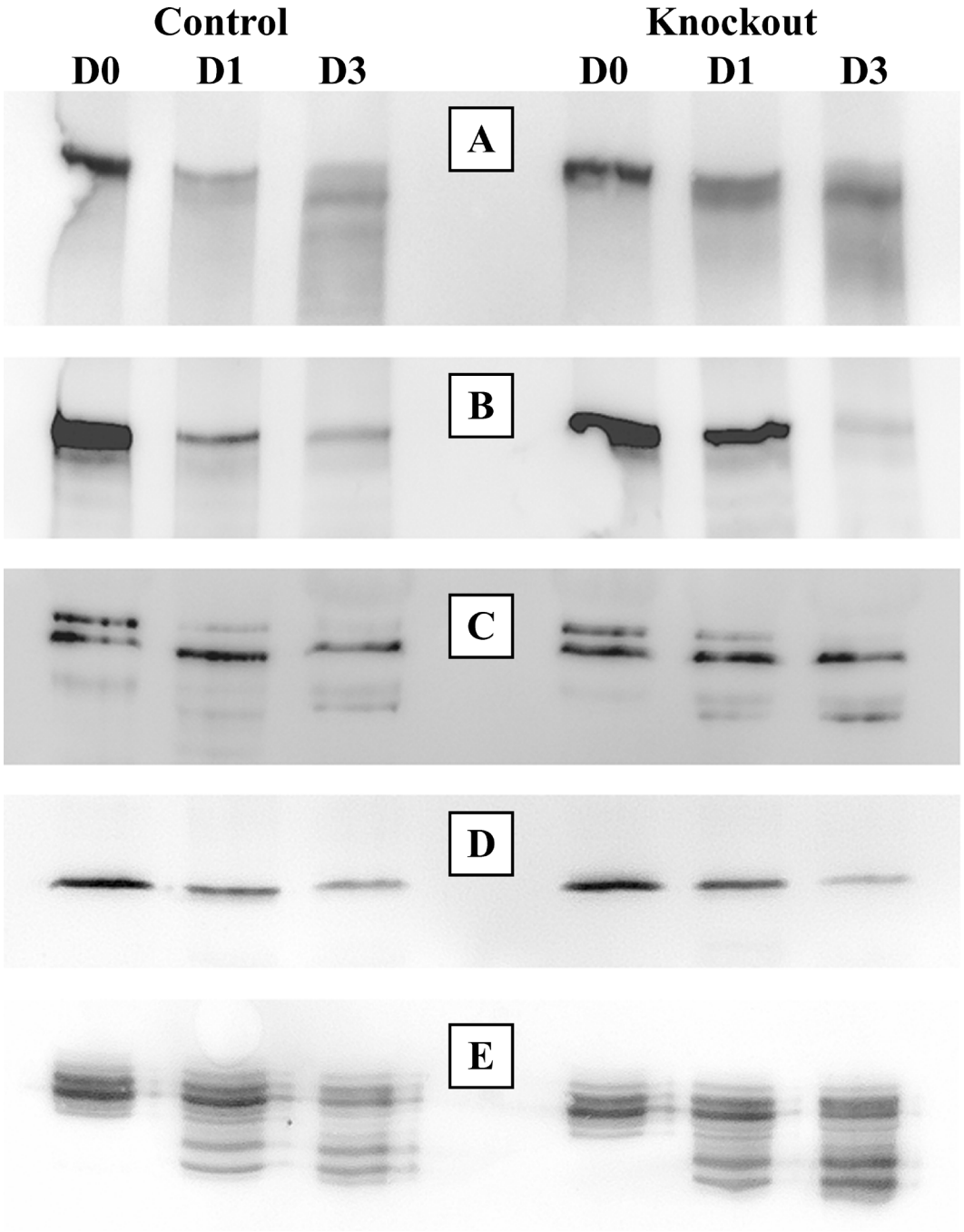

Figure 4. Western blot analysis of nebulin (A), dystrophin (B), metavinculin and vinculin (C), desmin (D), and troponin-T (E) in whole muscle extracts of control and calpain 3 knockout mice at death (D0) and after 1 (D1) and 3 d (D3) storage at 4°C.

nol, and then embedded in Spurr's resin. Sections of 1-μm thickness were stained with toluidine blue and examined by light microscopy. At the light microscopic level, the evident changes included fiber detachment from adjacent fibers, fibers that were partially cracked or broken entirely, and the development of space between the sarcomeres. These measurements were quantified for 40 fibers per sample.

Statistical Analyses

Data were analyzed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The model included the main effects of genotype, day postmortem, and their interaction. When the main effect or interaction was significant ($P < 0.05$), least squares means separation was accomplished by the PDIFF option (a pair-wise *t*-test).

Table 1. Mean percent decrease in immunologically detectable desmin during postmortem aging of control (n = 6) and calpain 3 knockout (n = 6) murine hind limb muscle[a]

Days postmortem	Percent remaining	
	Control	Knockout
1	69	74
3	38	34

[a]Main effect of genotype was not significant, $P > 0.99$. Main effect of day postmortem was significant, $P < 0.001$. Genotype × day postmortem was not significant, $P > 0.54$.

Results and Discussion

Calpain 3 Autolysis

To verify calpain 3 KO, Western blots of whole muscle extracts of at-death samples (Figure 1) were probed with an antibody developed by Anderson et al. (1998). Besides calpain 3, this antibody also recognizes m-calpain (Anderson et al., 1998). The results confirmed the PCR screening results of the KO parents (Kramerova et al., 2004). During postmortem storage, detectable calpain 3 in control decreased to zero at 3 d postmortem, whereas the amount of m-calpain in both groups did not seem to decrease noticeably (Figure 2). The observed rate of autolysis of calpain 3 in postmortem murine muscle seemed comparable to that in ovine muscle (Ilian et al., 2004a), but slower than that in porcine muscle (Parr et al., 1999).

Autolysis of μ-calpain occurred at a slightly faster rate than that observed for lamb muscle by Geesink and Koohmaraie (1999; Figure 3). The amount and rate of autolysis of μ-calpain seemed similar and not affected by elimination of the calpain 3 gene. Possible differences in the degradation of muscle proteins, therefore, cannot be explained by differences in the activity of μ-calpain.

Degradation of Myofibrillar Proteins

Postmortem proteolysis of a number of muscle proteins was visualized using Western blotting (Figure 4). In agreement with earlier studies on bovine (Fritz and Greaser, 1991; Taylor et al., 1995) and ovine muscle (Koohmaraie et al., 1995; Geesink and Koohmaraie, 1999), murine nebulin and dystrophin were very susceptible to postmortem proteolysis (Figure 4A, B). Proteolysis of metavinculin (Figure 4C, upper band) and vinculin (Figure 4C, lower band) occurred in a fashion similar to that observed for lamb muscle (Geesink and Koohmaraie, 1999). Metavinculin was quite susceptible to proteolysis, whereas vinculin seemed relatively resistant to proteolysis. Proteolysis of desmin at 1 d postmortem was more extensive than was observed by Kent et al. (2004) for normal mice. At 3 d postmortem, however, the opposite was observed. Nevertheless, in the present study, proteolysis of desmin did not differ between the control and KO mice (Table 1). Proteolysis of troponin-T, with the simultaneous appearance of degradation products with a molecular weight of 27 to 30 kDa, is the most frequently reported change in myofibrillar proteins during postmortem storage of muscle of various species (for review see Robson et al., 1997). In murine muscle, a similar pattern was observed, but again no difference in proteolysis was observed between control and KO mice (Figure 4E). Dystrophin was the only protein for which an indication of a possible difference between control and KO mice was observed. At 1 d postmortem, the dystrophin band seemed more intense in the KO than in control mice; however, at 3 d postmortem, the opposite was observed. As mentioned in the Materials and Methods section, with the exception of the results for desmin, the conclusions regarding the results of the Western blots were drawn from visual appraisal on pooled muscle samples, and thus were not analyzed statistically.

Table 2. Structural changes during postmortem aging of control (n = 4) and calpain 3 knockout (n = 4) murine hind limb muscle

Item	Detached[a]	Broken[b]	Gaps[c]
Genotype			
Control	36.9	6.6	19.4
Knockout	35.3	7.2	22.8
SEM	5.2	1.9	5.5
P-value	0.83	0.82	0.66
Day			
0	14.1	0.0	0.0
7	58.1	13.8	42.2
SEM	5.2	1.9	5.5
P-value	0.001	0.003	0.001

[a]Frequency (%) of fibers that were detached from adjacent fibers.
[b]Frequency (%) of fibers were partially cracked or broken entirely.
[c]Frequency (%) of observed space between sarcomeres.

Structural Changes

A previous study described in detail the muscle structural changes due to calpain 3 KO mice (Kramerova et al., 2004). Our interest was to examine the structural changes associated with normal postmortem aging of meat, the major observation being that sarcomeres break in the I-band (first reported by Gothard et al., 1966, using optical microscopy, and reported by Davey and Dickson, 1970, using electron microscopy). Quantitative differences during aging included I-band breaks (Taylor et al., 1995; Ho et al., 1997; Taylor and Koohmaraie, 1998) and sarcomere detachment from endomysium (Taylor et al., 1995; Taylor and Koohmaraie, 1998). In the current study, we used light microscopy to quantify structural changes in control vs. KO mice. Fiber breaks across the entire width of the fiber (Table 2) were only observed at long postmortem storage times and did not differ, nor were there gaps or spaces between the fibers. Fiber detachment was present in d-1 samples, indicating very rapid evolution postmortem and/or that this assay was sensitive to sample manipu-

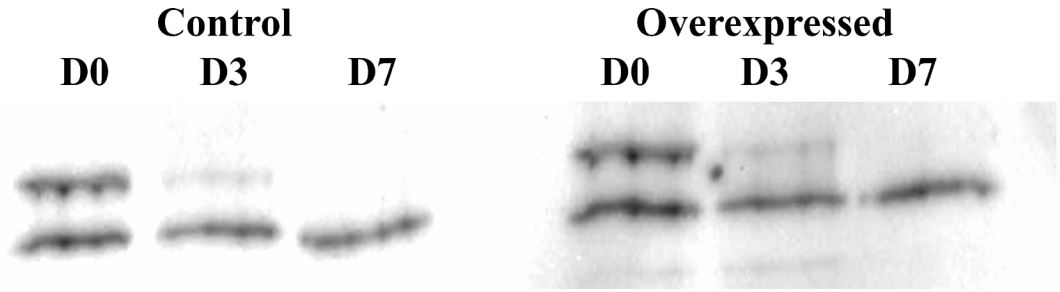

Figure 5. Western blot analysis of calpain 3 and m-calpain in whole muscle extracts of control and calpastatin overexpressed mice at death (D0) and after 3 (D3) and 7 d (D7) storage at 4°C.

lation. Fiber detachment was higher at d 7, but not different between controls and KO mice (Table 2).

The fact that calpain 3 is not inhibited by calpastatin (Sorimachi et al., 1993), combined with the well-established significance of calpastatin in postmortem proteolysis (Geesink et al., 2000; Hopkins and Thompson, 2002; Koohmaraie et al., 2002), should be sufficient to exclude a significant role for calpain 3 in postmortem proteolysis. Kent et al. (2004) examined postmortem proteolysis in transgenic mice overexpressing calpastatin (at time 0, active human calpastatin was expressed in transgenic murine skeletal muscle at a level 370-fold greater than calpastatin in control mice). Overexpression of calpastatin resulted in a large decrease in both proteolysis of muscle proteins and autolysis of μ-calpain (Kent et al., 2004). In the present study, we probed the muscle extracts from the study by Kent et al. (2004) with the antibody against calpain 3 and m-calpain (Figure 5). It is clear that overexpression of calpastatin did not result in detectable inhibition of autolysis of calpain 3. Thus, when autolysis is taken as an indication that calpain 3 is activated and is degrading structural muscle proteins (Ilian et al., 2004a,b), its role in postmortem proteolysis is negligible because proteolysis was minimal in muscle of transgenic mice overexpressing calpastatin.

The hypothesis of Ilian et al. (2001a, 2003, 2004a) with respect to the role of calpain 3 in postmortem proteolysis and meat tenderization can essentially be summarized as follows: 1) unlike ubiquitous forms of the calpain, there are no methods for purifying and quantifying calpain 3; 2) native calpain 3 has never been isolated from skeletal muscle; hence, neither the proteolytic capacity nor substrates is known; 3) the only methods available that can be used to study calpain 3 are mRNA quantification and autolysis and their correlation to postmortem proteolysis and meat tenderization; and 4) because mRNA level and pattern of calpain 3 autolysis are correlated with postmortem proteolysis, calpain 3 must be involved in this process. Correlations and cause and effect are very different phenomena. Results of this study indicate that although calpain 3 autolysis occurred as reported by Ilian et al. (2001a, 2003, 2004a), such autolysis is independent of degradation of proteins that are involved in postmortem meat tenderization. Therefore, because the absence of calpain 3 does not affect postmortem proteolysis, overexpression of calpastatin, which shuts down postmortem proteolysis, has no effect on the pattern of autolysis of calpain 3, and autolysis of calpain 3 in postmortem muscle is the sole basis for suggesting a role for calpain 3 in postmortem muscle proteolysis (Ilian et al., 2001a,b, 2004a), it seems that calpain 3 plays a minor role, if any, in postmortem proteolysis of the proteins analyzed.

Implications

The results of the present study indicate that calpain 3 plays a minor role, if any, in postmortem proteolysis of muscle proteins. Therefore, based on the large body of existing evidence, μ-calpain is the major proteolytic enzyme that causes postmortem proteolysis of key myofibrillar and associated proteins that result in meat tenderization. Therefore, understanding the regulation of μ-calpain activity in postmortem muscle should be the focus of further research.

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