μ-Calpain is essential for postmortem proteolysis of muscle proteins\textsuperscript{1,2}

G. H. Geesink,* S. Kuchay,† A. H. Chishti,† and M. Koohmaraie‡\textsuperscript{3}

*CCL Research, Veghel, The Netherlands NL-5462; †Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL 60612-3725; and ‡USDA, ARS, Roman L. Hruska US Meat Animal Research Center, Clay Center, NE 68933-0166

**ABSTRACT:** The objective of this investigation was to test the hypothesis that μ-calpain is largely responsible for postmortem proteolysis of muscle proteins. To accomplish this objective, we compared proteolysis of known muscle proteins in muscles of wild type and μ-calpain knockout mice during postmortem storage. Knockout mice (n = 6) were killed 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 for degradation of nebulin, dystrophin, metavinculin, vinculin, desmin, and troponin T. In a separate experiment, hind limb muscles from knockout (n = 4) and control mice (n = 4) were analyzed at 0, 1, and 3 d postmortem using casein zymography to confirm that μ-calpain activity was knocked out in muscle and to determine whether or not m-calpain is activated in murine postmortem muscle.

Cumulatively, the results of the first experiment indicated that postmortem proteolysis was largely inhibited in μ-calpain knockout mice. The results of the second experiment established the absence of μ-calpain in the muscle tissue of knockout mice and confirmed the results of an earlier study that m-calpain is active in postmortem murine muscle. The results of the current study show that even in a species in which m-calpain is activated to some extent postmortem, μ-calpain is largely responsible for postmortem proteolysis. This observation excludes a major role for any of the other members of the calpain family or any other proteolytic system in postmortem proteolysis of muscle proteins. Therefore, understanding the regulation of μ-calpain in postmortem muscle should be the focus of further research on postmortem proteolysis and tenderization of meat.

**Key words:** μ-calpain, meat tenderization, postmortem, proteolysis

\textsuperscript{1}Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, 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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\textsuperscript{3}Corresponding author: koohmaraie@email.marc.usda.gov

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**INTRODUCTION**

Skeletal muscle contains 2 well-characterized calcium-dependent neutral proteinases, μ- and m-calpain, their specific inhibitor, calpastatin, and a number of other calpains, including the muscle-specific calpain 3, whose characteristics are less well known (Goll et al., 2003). Considerable evidence now indicates that μ-calpain, but not m-calpain, plays an important role in postmortem degradation of myofibrillar proteins and tenderization of muscle during refrigerated storage (Geesink et al., 2000; Hopkins and Thompson, 2002; Koohmaraie et al., 2002). However, the available evidence indicating that μ-calpain is largely responsible for postmortem proteolysis is indirect. Proteolysis patterns in postmortem muscles can be mimicked by incubation of myofibrillar proteins with μ- and m-calpain, a high level of calpastatin inhibits postmortem proteolysis and tenderization, and μ-calpain, but not m-calpain, is activated in postmortem muscle as evidenced by the autolysis of the activated enzyme (Koohmaraie and Geesink, 2006).

Using calpain 3 knockout mice, we have recently shown that calpain 3 is not involved in postmortem proteolysis of muscle proteins (Geesink et al., 2005). Nonetheless, the results of that study illustrate that knockout models are powerful tools to test a hypothesis related to postmortem muscle. The experimental testing of the hypothesis that μ-calpain is largely responsible for postmortem proteolysis in muscle became feasible with the development of μ-calpain knockout mouse...
model. This mouse model has been previously used to explore the role of \( \mu \)-calpain in platelet function (Azam et al., 2001), invasion and growth of the malaria parasite in erythrocytes (Hanspal et al., 2002), activation of transglutaminase in erythrocytes (O’Neill et al., 2003), and long-term potentiation and fear conditioning learning (Grammer et al., 2005).

Our objective of this study was to test the hypothesis that \( \mu \)-calpain is the dominant proteinase responsible for postmortem proteolysis of muscle proteins. To this end, we compared proteolysis of muscle proteins in muscles of wild type and \( \mu \)-calpain knockout mice during postmortem storage.

**MATERIALS AND METHODS**

**Generation of \( \mu \)-Calpain Knockout Mice**

The Roman L. Hruska US Meat Animal Research Center Animal Care and Use Committee approved the use of the animals in this study. \( \mu \)-Calpain knockout mice were produced as described by Azam et al. (2001). Mice with a mixed genetic background of C57BL/6J and 129svJ/B6 were used in this study.

**Sample Collection**

Aged matched control \((n = 10)\) and \( \mu \)-calpain knock-out \((n = 10)\) mice were killed at 5 mo of age by decapitation, and both hind limbs were removed and skinned. From each mouse, the muscle of 1 hind limb was immediately dissected and snap-frozen in liquid N\(_2\) before storage at −20°C. The remaining hind limb was dipped in a 1.0 mM NaN\(_3\) solution to prevent microbial growth, blotted to remove excess liquid, and stored in plastic wrap. The hind limbs were stored at 4°C until 1 and 3 d postmortem, when approximately half the muscle was dissected, snap frozen, and stored at −20°C. Muscles from 6 mice of each treatment group were used for SDS-PAGE and Western blotting. Muscles from 4 mice of each treatment group were used for zymography.

**SDS-PAGE and Western Blotting**

A portion of the frozen muscle (approximately 150 mg) was weighed and extracted in 5 volumes of ice-cold extraction buffer (100 mM Tris/HCl; pH 8.3; 5 mM EDTA). Tissue was homogenized for 15 s using a polytron on high speed. Half of the total homogenate was removed and centrifuged at 16,000 \( \times \) g (maximum force value) for 10 min at 4°C. After centrifugation, the supernatant fraction was collected. The collected fractions (total homogenate and 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; percentages indicate vol/vol) and heated at 50°C for 20 min. After centrifuging the solution at 16,000 \( \times \) g (maximum force value) for 5.0 min at room temperature, the supernatant fraction was collected, and the protein concentration was determined using a Pierce BCA protein assay kit (Pierce Laboratories, Rockford, IL). Samples were diluted to 2.0 mg/mL of total protein using SDS-PAGE sample buffer containing 0.5% (vol/vol) 2-mercaptoethanol and bromophenol blue (0.04%; vol/vol).

The SDS-PAGE was performed as described by Laemmli (1979) using 8 \( \times \) 10 \( \times \) 0.075-cm minigels. The acrylamide percentage varied depending on the protein of interest: for troponin T, 12.5% gels were used; for desmin, 10% gels were used; for metavinculin and vinculin, 7.5% 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, proteins were transferred onto Hybond-P polyvinylidene fluoride membranes (Amersham Biosciences, Uppsala, Sweden) at 200 mA for 1.0 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% (wt/vol) nonfat dry milk (Instant Fat Free Dry Milk, Shurfine Intl. Inc., Northlake, IL) in Tris-buffered saline containing Tween (TTBS; 20 mM Tris/HCl, 137 mM NaCl, 5 mM KCl, and 0.05% Tween 20). After blocking for 1 h, the membranes were exposed to the following primary antibodies diluted in 3% nonfat dry milk in TTBS: mouse monoclonal antidesmin clone D3 (dilution 1:10; Hybridoma Bank, Iowa City, IA; Danto and Fischman, 1984); mouse monoclonal antitroponin T clone JLT-12 (dilution 1:1,000; Sigma-Aldrich, St. Louis, MO); mouse monoclonal antivinculin clone V284 (dilution 1:100; Accurate, Westbury, NY); mouse monoclonal antinebulin clone NB2 (dilution 1:1,000; Sigma-Aldrich); and mouse monoclonal antidystrophin clone NCL-DYS1 (dilution 1:100; Vector Laboratories, Burlingame, CA).

Blots were incubated with primary antibody for 1.0 h before being washed with TTBS. The secondary antibody used was goat anti-mouse IgG conjugated with peroxidase (dilution 1:1,000; Pierce Laboratories, Rockford, IL). Blots were exposed to the secondary antibody for 1.0 h before being extensively washed with TTBS. Antibody binding was visualized by incubating the polyvinylidene fluoride 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 protein remaining at 1 and 3 d postmortem was expressed as a percentage of the amount on d 0.

**Zymography**

Portions (approximately 300 mg) of frozen skeletal muscle were homogenized in 5 volumes of extraction
buffer containing 0.05% 2-mercaptoethanol for 15 s using a polytron on high. After centrifuging the homogenate at 8,800 × g for 30 min, the supernatant was collected. Casein zymography was performed in duplicate for each sample according to the procedure of Veiseth et al. (2001). Polyacrylamide gels (12.5%, 8 × 10 × 0.075 cm) were loaded with supernatant equivalent to 4.0 mg of muscle and electrophoresed at 100 V for 6 h before incubation and staining.

Images were captured and the intensity of the bands was analyzed using a ChemiImager 5500 digital imaging system (Alpha Innotech Corp.). The amount of proteolytic activity remaining at 1 and 3 d postmortem was expressed as a percentage of the amount on d 0.

**Statistical Analysis**

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 \( P < 0.05 \), separation of least squares means was accomplished by the PDIFF option (a pairwise t-test).

**RESULTS AND DISCUSSION**

**Zymography**

Casein zymography is a sensitive method to determine calpain activity and can be used to differentiate between autolyzed and native calpains (Veiseth et al., 2001). The zymograms confirmed the absence of μ-calpain (upper band), and all its associated autolyzed forms, in the knockout muscle tissue (Figure 1). In agreement with the results of Kent et al. (2004), m-calpain (lower band) appears to be active in postmortem murine muscle of wild type and μ-calpain knockout mice (Figure 1). This can be inferred from the appearance of a proteolytically active m-calpain fragment (see arrow) with a higher mobility than native m-calpain (Veiseth et al., 2001). Using Western blotting, Kent et al. (2004) have shown that this band is autolyzed m-calpain. Also, the loss of m-calpain activity during postmortem storage (Table 1) suggests that m-calpain is activated and loses its activity as a result of autolysis. This phenomenon is not observed in bovine or ovine muscles. In these muscles, the m-calpain activity is remarkably stable unless the intracellular calcium concentration is artificially elevated by calcium infusion (Koohmarai et al., 1987, 1989; Geesink and Koohmarai, 1999b; Veiseth et al., 2001).

**Protein Degradation**

Postmortem proteolysis of a number of muscle proteins was visualized using Western blotting (Figure 2). With the exception of nebulin, the extent of proteolysis of the different proteins was quantified as a percentage of the amount of intact protein at death (Tables 2–6). For nebulin this approach was omitted because intact nebulin and its degradation products are not well separated (Figure 2, panel A). Nevertheless, it is obvious that virtually no intact nebulin remains at 3 d postmortem in control muscles. In knockout muscles, nebulin appears mostly intact after 3 d of aging.

Degradation of dystrophin was largely reduced in μ-calpain knockout mice, although some degradation was

| Table 1. m-Calpain activity during postmortem aging of control and μ-calpain knockout mouse hind limb muscle

<table>
<thead>
<tr>
<th>Day postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110.8</td>
<td>95.4</td>
</tr>
<tr>
<td>3</td>
<td>84.8</td>
<td>73.5</td>
</tr>
</tbody>
</table>

*Main effect of genotype was significant, \( P < 0.002 \). Main effect of day postmortem was significant, \( P < 0.0001 \). Genotype × day postmortem was not significant, \( P > 0.47 \).

*Mean percentage of at-death values.*
Figure 2. Western blot analysis of nebulin (A), dystrophin (B), metavinculin and vinculin (C), desmin (D), and troponin T (E) in whole muscle extracts of 2 control and 2 μ-calpain knockout mice at death (D0) and after 1 (D1) and 3 d (D3) storage at 4°C.

The degradation of dystrophin in the knockout mice can possibly be attributed to the action of μ-calpain. The most dramatic effect of the absence of μ-calpain was observed for metavinculin (Table 3; Figure 2, panel C, upper band). At 1 d postmortem, about 90% of the protein was degraded in control muscles, whereas in knockout muscles no degradation was observed throughout the aging period. In agreement with earlier observations (Geesink and Koohmaraie, 1999a; Geesink et al., 2005), vinculin was relatively resistant to postmortem proteolysis (Table 4; Figure 2, panel C, lower band).

The degradation pattern of desmin (Table 5; Figure 2, panel D) and troponin T (Table 6; Figure 2, panel E) appeared similar to that detected earlier in wild-type mice and calpain 3 knockout mice (Geesink et al., 2005). The degradation pattern, or lack thereof, of these proteins in μ-calpain knockout mice appeared to be similar to that detected for mice overexpressing calpastatin (Kent et al., 2004). This observation suggests that the large reduction in postmortem proteolysis in mice over-
Table 2. Immunologically detectable dystrophin during postmortem aging of control and μ-calpain knockout mouse hind limb muscle

<table>
<thead>
<tr>
<th>Days postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45.2</td>
<td>107.5</td>
</tr>
<tr>
<td>3</td>
<td>15.1</td>
<td>52.6</td>
</tr>
</tbody>
</table>

1Main effect of genotype was significant, $P < 0.0001$. Main effect of day postmortem was significant, $P < 0.0001$. Genotype × day postmortem was not significant, $P > 0.07$.

2Mean percentage of at-death values.

Table 3. Immunologically detectable metavinculin during postmortem aging of control and μ-calpain knockout mouse hind limb muscles

<table>
<thead>
<tr>
<th>Days postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.5</td>
<td>105.5</td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>109.2</td>
</tr>
</tbody>
</table>

1Main effect of genotype was significant, $P < 0.0005$. Main effect of day postmortem was not significant, $P > 0.89$. Genotype × day postmortem was not significant, $P > 0.74$.

2Mean percentage of at-death values.

Table 4. Immunologically detectable vinculin during postmortem aging of control and μ-calpain knockout murine hind limb muscle

<table>
<thead>
<tr>
<th>Days postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>109.7</td>
<td>102.6</td>
</tr>
<tr>
<td>3</td>
<td>64.4</td>
<td>79.2</td>
</tr>
</tbody>
</table>

1Main effect of genotype was not significant, $P > 0.85$. Main effect of day postmortem was not significant, $P > 0.14$. Genotype × day postmortem was not significant, $P > 0.60$.

2Mean percentage of at-death values.

Table 5. Immunologically detectable desmin during postmortem aging of control and μ-calpain knockout murine hind limb muscles

<table>
<thead>
<tr>
<th>Days postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69.7</td>
<td>112.3</td>
</tr>
<tr>
<td>3</td>
<td>40.1</td>
<td>87.4</td>
</tr>
</tbody>
</table>

1Main effect of genotype was significant, $P < 0.0001$. Main effect of day postmortem was significant, $P < 0.0001$. Genotype × day postmortem was not significant, $P > 0.07$.

2Mean percentage of at-death values.

Table 6. Immunologically detectable troponin T during postmortem aging of control and μ-calpain knockout murine hind limb muscle

<table>
<thead>
<tr>
<th>Days postmortem</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96.7</td>
<td>102.5</td>
</tr>
<tr>
<td>3</td>
<td>83.8</td>
<td>99.9</td>
</tr>
</tbody>
</table>

1Main effect of genotype significant, $P < 0.005$. Main effect of day postmortem was significant, $P < 0.03$. Genotype × day postmortem was not significant, $P > 0.12$.

2Mean percentage of at-death values.

Expressing calpastatin is largely due to inhibition of μ-calpain.

**General Discussion**

The strong association between postmortem proteolysis and tenderization of meat has been demonstrated in numerous studies. Differences in meat tenderness between *Bos taurus* (on average more tender) and *Bos indicus* (on average less tender) cattle are related to a reduced rate of postmortem proteolysis in *Bos indicus* cattle (Whipple et al., 1990; Shackelford et al., 1991). Treatment of sheep and cattle with β-adrenergic agonists results in a decrease in postmortem proteolysis and tenderization (Fiems et al., 1990; Kretchmar et al., 1990; Koohmaraie et al., 1991a). Finally, differences in postmortem tenderization rate among pigs, sheep, and cattle can be explained by the different rates of postmortem proteolysis in these species (Ouali and Talmant, 1990; Koomaraie et al., 1991).

In skeletal muscle, 3 different proteinase systems have been studied for their possible role in postmortem proteolysis: the calpain system, the lysosomal cathepsins, and the multicatalytic proteinase complex (MCP). Incubation of myofibrillar proteins with cathepsins results in different degradation patterns than occur during postmortem storage of muscle, and it is doubtful that cathepsins are released from the lysosomes in postmortem muscle (Koohmaraie, 1988). A significant role for MCP can be excluded because myofibrils are very poor substrates for this proteinase system (Koohmaraie, 1992). Moreover, the degradation pattern of myofibrillar proteins by MCP does not mimic the degradation pattern observed in postmortem muscle (Taylor et al., 1995). This leaves the calpain system, or potentially another not yet investigated proteolytic system, responsible for postmortem proteolysis of key myofibrillar proteins and the resultant meat tenderization. The evidence for the involvement of the calpain system in postmortem proteolysis and tenderization comes from a variety of studies: (a) Incubation of myofibrils with calpains produces the same proteolytic pattern as observed in postmortem muscle (Koohmaraie et al., 1986; Huff-Lonergan et al., 1996; Geesink and Koohmaraie, 1999b); (b) Infusion or injection of muscles with calcium
accelerates postmortem proteolysis and tenderization (Koohmaraie et al., 1988; Koohmaraie et al., 1990; Wheeler et al., 1992), whereas infusion or injection of muscles with calpain inhibitors inhibits postmortem proteolysis and tenderization (Koohmaraie, 1990; Uytterhaegen et al., 1994); (c) Differences in the rate of postmortem proteolysis and tenderization between species can be explained by the variation in calpastatin activity (Ouali and Talmant, 1990; Koohmaraie et al., 1991); (d) Differences in the rate of postmortem proteolysis and tenderization between Bos taurus and Bos indicus cattle can be explained by the variation in calpastatin activity (Whipple et al., 1990; Shackelford et al., 1991); (e) The toughening effect of treatment with β-agonists can be explained by an increase in calpastatin activity (Koohmaraie et al., 1991; Garssen et al., 1995); (f) The greatly reduced rate and extent of postmortem proteolysis and tenderization in callipyge lamb can be attributed to elevated levels of calpastatin in these animals (Koohmaraie et al., 1995; Geesink and Koohmaraie, 1999b); (g) Overexpression of calpastatin in transgenic mice results in a large reduction in postmortem proteolysis of muscle proteins (Kent et al., 2004).

From the above-cited studies and others, it is clear that the calpain system plays an important role in postmortem proteolysis and tenderization. The remaining important question is which of the calpains is responsible for postmortem proteolysis and tenderization. An important characteristic of the calpains is that they autolyze once activated, ultimately leading to loss of activity (Suzuki et al., 1981; Dayton, 1982; Nagainis et al., 1983). In bovine and ovine postmortem muscle, the extractable activity of μ-calpain declines but the activity of m-calpain is remarkably stable (Ducastaing et al., 1985; Geesink and Koohmaraie, 1999b; Kretchmar et al., 1990, Veiseth et al., 2001). This observation led Koohmaraie et al. (1987) to conclude that μ-calpain, but not m-calpain, is responsible for postmortem tenderization. Western blotting has established that calpain 3 does autolyze in postmortem muscle (Anderson et al., 1998; Parr et al., 1999; Ilian et al., 2004). In contrast with μ-calpain and m-calpain, calpain 3 is not inhibited by calpastatin (Sorimachi et al., 1993). This observation excludes a major role of calpain 3 in postmortem proteolysis and tenderization given the large influence of calpastatin activity on these events. This conclusion was further corroborated by results of a recent study showing that postmortem proteolysis is not affected in calpain 3 knockout mice (Geesink et al., 2005).

The results of the current study show that even in a species where m-calpain is activated postmortem to some extent, μ-calpain is largely responsible for postmortem proteolysis. This observation excludes a major role for any of the other members of the calpain family or any other proteolytic system in postmortem proteolysis of muscle proteins.

**IMPLICATIONS**

The results of the current study provide strong evidence in support of the large body of previously published indirect evidence that μ-calpain is the major proteolytic enzyme that causes postmortem proteolysis of myofibrillar and associated proteins. Therefore, a precise understanding of the regulation of μ-calpain in postmortem muscle should be the focus of further research on postmortem proteolysis and tenderization of meat.

**LITERATURE CITED**


