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The calpain system in three muscles of normal and callipyge sheep

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ABSTRACT: Activities of μ- and m-calpain and of calpastatin were measured at four different times during postmortem storage (0, 1, 3, and 10 d) in three muscles from either callipyge or noncallipyge (normal) sheep. The weights of two muscles, the biceps femoris and the longissimus, are greater in the callipyge phenotype, whereas the weight of the infraspinatus is not affected. The activity of m-calpain was greater (P < 0.05) in the biceps femoris and longissimus from callipyge than in those from normal sheep, but it was the same in the infraspinatus in the two phenotypes. The extractable activity of m-calpain did not change (biceps femoris and infraspinatus) or decreased slightly (longissimus) during postmortem storage. Extractable activity of μ-calpain decreased to zero or nearly zero after 10 d postmortem in all muscles from both groups of sheep. The rate of decrease in μ-calpain activity was the same in muscles from the callipyge and normal sheep. At all time points during postmortem storage, calpastatin activity was greater (P < 0.05) in the biceps femoris and longissimus from the callipyge than from the normal sheep, but it was the same in the infraspinatus from callipyge and normal sheep. Calpastatin activity decreased (P < 0.05) in all three muscles from both phenotypes during postmortem storage; the rate of this decrease in the callipyge biceps femoris and longissimus and in the infraspinatus from both the callipyge and normal sheep was slow, especially after the first 24 h postmortem, whereas calpastatin activity in the biceps femoris and longissimus from the normal sheep decreased rapidly. During postmortem storage, the 125-kDa calpastatin polypeptide was degraded, but the 80-kDa subunit of μ-calpain was cleaved only to 76- and 78-kDa polypeptides even though extractable μ-calpain activity declined nearly to zero. Approximately 50 to 60% of total μ-calpain became associated with the non-extractable pellet after 1 d postmortem. The myofibril fragmentation index for the biceps femoris and longissimus from normal sheep increased significantly during postmortem storage. The fragmentation index for the infraspinatus from the callipyge and normal sheep increased to an intermediate extent, whereas the index for the biceps femoris and longissimus from the callipyge did not change during 10-d postmortem storage. The results suggest that postmortem tenderization is related to the rate of calpastatin degradation in postmortem muscle and that calpastatin inhibition of the calpains in postmortem muscle is modulated in some as yet unknown manner.

Key Words: Callipyge, Calpains, Calpastatin, Postmortem Tenderization


Introduction

At 169 d of age, the biceps femoris (42.1%), semimembranosus (38.3%), longissimus (32.1%), and semitendinosus (26.4%) muscle weights were larger in the callipyge than in the noncallipyge sheep, whereas the infraspinatus and supraspinatus, two muscles from the front quarter, had approximately the same weights in the two phenotypes (Koohmaraie et al., 1995). A striking parallelism was observed between the increased muscle weight and calpastatin activities in these muscles; calpastatin was cleaved only to 76- and 78-kDa polypeptides even though extractable μ-calpain activity declined nearly to zero. Approximately 50 to 60% of total μ-calpain became associated with the non-extractable pellet after 1 d postmortem. The myofibril fragmentation index for the biceps femoris and longissimus from normal sheep increased significantly during postmortem storage. The fragmentation index for the infraspinatus from the callipyge and normal sheep increased to an intermediate extent, whereas the index for the biceps femoris and longissimus from the callipyge did not change during 10-d postmortem storage. The results suggest that postmortem tenderization is related to the rate of calpastatin degradation in postmortem muscle and that calpastatin inhibition of the calpains in postmortem muscle is modulated in some as yet unknown manner.

1This work was supported by grants from the USDA National Research Initiative Competitive Grants Program, 9801191 and 980361; the Muscular Dystrophy Association; and the Arizona Agriculture Experiment Station, Project 28, a contributing project to USDA Regional Research Project NC-131. Eduardo Delgado gratefully acknowledges scholarship support from the Ministry of Education, CAPES (Brazil). We thank Steven Shackelford and Dan Nonneman for helpful discussions, Sue Havur and Patty Beska for expert technical assistance, Valery Thompson for her assistance in preparing the figures, and Janet M. Christner for her assistance in preparing the manuscript. Mention of a trade name, proprietary product, or specific equipment is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Accepted March 3, 2000.

Accepted September 7, 2000.
pastatin activity was higher in the biceps femoris (124%), semimembranosus (126%), and longissimus (106%) muscles from callipyge sheep than from noncallipyge sheep, but was approximately the same in the infraspinatus (3.7% higher) and the supraspinatus (3.6% lower) from callipyge and noncallipyge sheep (Koohmaraie et al., 1995). The correlation between muscle weight and calpastatin activity in callipyge sheep is 0.96 (Shackelford et al., 1997).

Muscle calpastatin activity, especially when measured 24 h postmortem, also is highly related to meat toughness (Whipple et al., 1990; Shackelford et al., 1994). Warner-Bratzler shear force measurements of the longissimus from callipyge and noncallipyge sheep showed that the callipyge longissimus was considerably less tender than the noncallipyge longissimus (Koohmaraie et al., 1995). These studies used only the longissimus, and it was unclear whether this increased toughness was due to callipytsis or was a direct effect of the callipyge gene on tenderness. Consequently, we have measured the activities of both μ- and m-calpain and of calpastatin at four different times of postmortem storage in three muscles; two, the biceps femoris and longissimus, whose weight is increased in the callipyge phenotype, and one, the infraspinatus, whose weight is not affected by the callipyge phenotype. The results suggest that the relationship between the callpain system and postmortem tenderization may be more complex than has sometimes been supposed.

**Experimental Procedures**

**Animals.** The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study. Twelve Dorset sheep (all ewes), six of them carriers and six of them noncarriers of the callipyge gene as assessed by leg score, averaging 3 yr of age, were slaughtered in pairs (one callipyge, one normal) at 2-wk intervals. These animals were older than the animals used in previous studies of the callipyge, but age of animal does not fundamentally change the muscle differences between callipyge and normal sheep; if anything, it may accentuate the differences slightly. After slaughter, the carcasses were placed in a cooler at 4°C.

**Muscle Sampling.** The biceps femoris, longissimus dorsi, and infraspinatus muscles were removed from the left side of the carcass within 30 min of exsanguination. The same three muscles were removed from the right side of the carcass after 24 h at 4°C. The muscles removed from the right side were weighed, and the weights were multiplied by 2 to obtain weights of these muscles for the entire animal. Samples taken within 30 min of exsanguination from the left side were used for separation and assay of μ-calpain, m-calpain, and calpastatin activities; for Western analysis of μ-calpain, m-calpain, and calpastatin; and for measurements of the myofibril fragmentation index. Longissimus muscles taken from the right side after 24 h postmortem were divided into three regions: costal, medial, and caudal, and samples from each region were randomly assigned to one of three times postmortem: 1 d; 3 d; or 10 d. The samples assigned to 3 d or 10 d postmortem were vacuum-packaged and were stored at 4°C until removed for analysis. The samples taken 24 h postmortem from the right side were used for measurements of sarcomere length and for Warner-Bratzler shear force measurements of tenderness in addition to assays of μ-calpain, m-calpain, and calpastatin activities, Western analysis of these three proteins, and myofibril fragmentation index; Warner-Bratzler shear measurements were done only on the longissimus. In addition, 10- to 15-g samples from all three muscles were taken at each of the four postmortem times (at-death and 1, 3, and 10 d); these samples were minced and then were frozen at −70°C until removed for analysis.

**Sarcomere Length.** Sarcomere lengths were determined on samples from biceps femoris, longissimus, and infraspinatus after 1 or 10 d of postmortem storage at 4°C by using the laser diffraction method (Koolmees et al., 1986). Thin transverse sections, 0.5 to 1 cm thick, were cut from the muscle tissue and were subdivided by cutting three pieces, each 3 cm long, parallel to the fiber length at the lateral, central, and caudal areas of the transverse section. These three samples were placed in separate vials that contained 5% glutaraldehyde, 0.1 M Na2HPO4, pH 7.2, for approximately 4 h at 10°C. After fixation, the samples were placed in 0.2 M sucrose, 0.1 M Na2HPO4, pH 7.2, and stored at 4°C for no more than 4 d. Three fiber bundles were teased from each of the three samples, and several fibers from each bundle were placed between a glass slide and cover slip in a drop of sucrose buffer. The diffraction pattern was determined by using a 2.0-mW monochromatic light from a helium-neon laser (632.8 nm). Six diffraction measurements were obtained for each of the three locations, and the sarcomere length was obtained by averaging the means of the six measurements taken at each of the three locations.

**Myofibril Fragmentation Index.** The myofibril fragmentation index (MFI) was determined on samples of biceps femoris, longissimus, and infraspinatus taken immediately after death or after 1, 3, or 10 d of postmortem storage at 4°C by using the method described by Olson et al. (1976). Two-gram muscle samples were homogenized for 30 s in 20 volumes (vol/wt) of 100 mM KCl; 20 mM K-phosphate, pH 7.1; 1 mM EGTA; 1 mM MgCl2; 1 mM Na3EDTA (MFI buffer) at 4°C. The homogenates were centrifuged for 15 min at 1,000 × gmax at 2°C, and the pellet was resuspended in the same volume of MFI buffer as before by using a stirring rod. The centrifugation step was repeated, the pellet was resuspended in 5 volumes (vol/wt) of MFI buffer by vortexing, and the suspension was filtered through a nylon strainer. The strainer was rinsed with 5 volumes of MFI buffer, and the protein concentration of the final suspension was determined by using the biuret method (Gornall et al., 1949). The myofibril suspension was
Figure 1. Elution profile of a DEAE-Sephacel cellulose column of the supernate from an ovine muscle homogenate. The 37,500 × g max supernate of muscle extract was loaded onto a 1.6-× 20-cm DEAE-Sephacel column and was washed with 50 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 10 mM MCE (≈ 3 column volume) until the A 278 reached the baseline. The bound proteins were eluted with a linear 25→400 mM NaCl gradient (500 mL of each) in 50 mM Tris-HCl, pH 7.5; 0.5 mM EDTA; 10 mM MCE. Flow rate was 30 mL/h; 3.0 mL fractions were collected.

diluted to 0.5 mg myofibrillar protein/mL in a final volume of 8 mL, the samples were vortexed, and absorbance of the vortexed suspension was measured at 540 nm with a B&L spectrophotometer (VWR Scientific, St. Louis, MO) and a large slit width. The absorbance was multiplied by 200 to give a MFI value.

Calpain and Calpastatin Activities. Twenty-five-gram samples from each of the three muscles were removed immediately after death or after 1, 3, or 10 d of postmortem storage. The samples were trimmed free from visible fat and connective tissue and were homogenized at 4°C in 3 volumes (vol/wt) of 100 mM Tris-HCl, pH 8.3; 10 mM EDTA; 10 mM 2-mercaptoethanol (MCE), and a cocktail of protease inhibitors (6 mg leupeptin/L, 100 mg ovomucoid/L, and 2 mM phenylmethylsulfonyl fluoride) by using a Waring blender at high speed three times for 30 s each time with 30-s cooling periods between each time. The homogenate was centrifuged at 37,500 × g max for 120 min, the volume of the supernate was recorded, and the supernate was placed in a dialysis bag and dialyzed overnight against 25 volumes of 40 mM Tris-HCl, pH 7.35; 5 mM EDTA; 10 mM MCE. After dialysis, the supernate was filtered through Miracloth (Calbiochem) and was loaded onto a 1.6- × 20-cm DEAE-Sephacel column. The DEAE columns were eluted as described in the caption to Figure 1. Calpastatin eluted between 20 and 105 mM NaCl, μ-calpain between 105 and 165 mM NaCl, and m-calpain between 220 and 310 mM NaCl from this column (Figure 1).

Calpain activity was measured using casein as a substrate and was done at 25°C in 100 mM Tris-acetate, pH 7.5; 10 mM MCE; 5 mM CaCl2; 7 mg Hammersten casein/mL for 60 min. Trichloroacetic acid (TCA) was added to a final concentration of 2.5% to stop the reaction and to precipitate undegraded protein. After centrifugation at 2000 × g max for 30 min, absorbance of the supernate was read at 280 nm. Each assay contained two controls: a tube containing 5 mM CaCl2 but no calpain and a second tube containing the calpain fraction but no CaCl2. The absorbance of these two controls was averaged and was subtracted from each measurement. All fractions eluting from the column and containing calpain activity were pooled, and activity of the pooled fractions was determined. Total μ-calpain and m-calpain activity was calculated by multiplying activity of the pooled fraction by the volume of the fraction and a dilution factor (calculated by multiplying the activity by [total mL of original fraction/mL in assay]). One unit of calpain activity was defined as an increase of 1.0 absorbance unit at 280 nm in 60 min at 25°C.

Calpastatin activity was determined by incubating calpastatin-containing fractions under the same conditions as those used to assay calpain activity, but the assays contained partly purified m-calpain. Calpastatin activity was determined by subtracting activity in the tubes containing calpastatin from activity in the tubes containing only m-calpain. One unit of calpastatin activity was defined as the amount of calpastatin that inhibits one unit of m-calpain activity. Total calpastatin activity was determined by measuring calpastatin activity of the pooled calpastatin fractions and multiplying that activity by the volume of the pooled fraction and a dilution factor (mL of total fraction/mL in assay) as described for the total calpain activity.

SDS-PAGE and Western Blotting. The pellets and the supernates obtained after centrifugation of the muscle
extract that was used for separation of calpastatin, \( \mu \)-calpain, and m-calpain (Figure 2) were subjected to SDS-PAGE and Western blotting analysis to determine the status of the \( \mu \)-calpain and calpastatin in these fractions (m-calpain was not assayed). Protein concentration in the supernatant fraction was diluted to 2 mg/mL by adding 62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.02% bromophenol blue; 0.5% MCE (protein denaturing buffer, PDB). The pellet was washed three times with 100 mM NaCl, 1 mM NaN\(_3\) before being resuspended at a final protein concentration of 2 mg/mL in PDB. The SDS-PAGE was done according to the method of Laemmli (1970) using either 8- x 10-cm minigels (10% acrylamide; 0.266% bis-acrylamide; calpastatin) or 14- x 17.7-cm gels (8% acrylamide; 0.0266% bis-acrylamide; \( \mu \)-calpain). The separated proteins were transferred in 25 mM Tris-HCl, pH 8.2; 193 mM glycine; 15% methanol to either Hybond-C nitrocellulose or Hybond-P polyvinylidine fluoride membranes for 2.5 h at 4°C and 200 to 300 mA. After blocking for 1 h with filtered sheep serum in 25 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20, the membranes were incubated with a primary antibody for 60 min in 1% bovine serum albumin, 4% nonfat dry milk at room temperature. The membranes were washed three times with TTBS between each incubation. The primary antibodies used were polyclonal anti-calpastatin (MARC-USDA; Doumit and Koohmaraie, 1999) at 1:500 dilution and monoclonal anti-\( \mu \)-calpain (clone B2F9; MARC-USDA; Geesink and Koohmaraie, 1999b) at a 1:5 dilution. The polypeptides labeled with the primary antibodies were detected by using an anti-mouse IgG (Fab specific/A; A-2179, Sigma Chemical Co., St. Louis, MO) for calpastatin or an anti-mouse IgG (A-5153. Sigma Chemical Co.) for \( \mu \)-calpain, both conjugated with alkaline phosphatase and both at a 1:1000 dilution. The secondary antibody was detected by using BCIP/NBT as a substrate.

Western analysis was done on all three muscles for all twelve animals; blots of those samples whose calpain or calpastatin activities were closest to the mean activity for that group of animals are shown.

**Warner-Bratzler Shear Force Measurements.** After 24 h at 4°C, three chops, 2.54 cm thick, were removed from the longissimus and either frozen immediately at −30°C or vacuum-packaged and stored at 4°C for either 2 or 9 d (3 or 10 d postmortem) before being frozen at −30°C. The frozen samples were equilibrated overnight at 5°C and then were broiled from the frozen state on a Faberware Open Hearth electric broiler (Faberware, Bronx, NY) to an internal temperature of 40°C before being turned and broiled to an internal temperature of 75°C. The chops were cooled (4°C) for 24 h and six cores, 1.27 cm in diameter (two from each chop), were removed parallel to the longitudinal orientation of the muscle fibers. Each core was sheared once with a Warner-Bratzler shear device attached to an Instron Universal Testing Machine (Instron, Canton, MA) having a 100-kg

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**Figure 2.** Schematic diagram of the procedures used to obtain samples for the MFI; for separation of calpastatin, \( \mu \)-calpain, and m-calpain from a muscle homogenate; and for Warner-Bratzler shear force measurements.
load cell and using a cross-head speed of 20 cm/min (18 shear force measurements per animal, muscle, and time postmortem).

Materials. Acrylamide, ammonium persulfate, N,N′-methylene-bis-acrylamide, N,N,N′,N′-tetra-methyleneethylethylene, and the BCIP/NBT substrate for alkaline phosphatase detection were purchased from Bio-Rad Laboratories, Hercules, CA; bovine serum albumin, EDTA, MCE, EGTA, PMSF, sodium azide, sodium dodecyl sulfate (SDS), the filtered shear serum for membrane blocking, and Tris were all from Sigma Chemical Co.; CaCl$_2$ was purchased from J. T. Baker, Phillipsburg, NJ; Hammersten casein was from United States Biochemical Corporation, Cleveland, OH; leu- peptin was from Peptides International, Louisville, KY; and the Hybond-C and Hybond-P membranes were purchased from Amersham, Buckinghamshire, United Kingdom. All other chemicals were reagent grade or purer. Deionized water was used in all the experiments.

Results

Callipyge Traits. The callipyge sheep used in this study had larger biceps femoris (44%) and longissimus (27%) weights than the noncallipyge sheep (Table 1), whereas the weights of the infraspinatus in the callipyge and noncallipyge sheep were nearly the same (0.04% smaller in the callipyge than in the noncallipyge). As reported previously (Koohmaraie et al., 1995), the callipyge gene has no effect on carcass weight of the sheep (Table 1).

Calpain and Calpastatin Activities. Activity of m-calpain in both the biceps femoris and longissimus, two muscles that are affected in the callipyge phenotype, was greater in the callipyge than in the noncallipyge sheep (Table 2). However, m-calpain activity in the infraspinatus, which is not affected by the callipyge phenotype, was the same in the callipyge and noncallipyge sheep (Table 2). Activity of m-calpain in the biceps femoris and infraspinatus did not change up to 10 d of postmortem storage in either the callipyge or the noncallipyge sheep and decreased only slightly in the longissimus during this period. A number of earlier studies have shown that m-calpain activity in bovine or rabbit skeletal muscle either does not change (Vidalenc et al., 1983; Ducastaing et al., 1985; Koohmaraie et al., 1987) or decreases slightly (to 63% of at-death activity; Boehm et al., 1998) during postmortem storage at 4°C.

Activity of extractable $\mu$-calpain was slightly but significantly lower in the biceps femoris and infraspinatus of the callipyge than in the same muscles from noncallipyge sheep (Table 2). In agreement with Koohmaraie et al. (1995), $\mu$-calpain activity was the same in the longissimus of callipyge and noncallipyge sheep. It is not clear why the longissimus should differ from the biceps femoris or infraspinatus muscles; differences in at-death $\mu$-calpain activity between the callipyge and noncallipyge sheep were small and may not have any physiological significance. The most striking change in $\mu$-calpain activity is the remarkable decrease in extractable activity in all three muscles with increasing time of postmortem storage (Table 2). Although it seems that $\mu$-calpain activity decreases at a slightly faster rate during postmortem storage in muscles from the noncallipyge sheep than in muscles from the callipyge sheep, there was no significant difference in $\mu$-calpain activity between the callipyge and the noncallipyge muscles at any of the three postmortem times sampled (Table 2). After 10 d of postmortem storage at 4°C, no extractable $\mu$-calpain activity was detected in the noncallipyge muscles, and extractable $\mu$-calpain activity was nearly nil in the callipyge muscles. It has been shown that a significant amount of total muscle $\mu$-calpain becomes tightly associated with the myofibrillar fraction during postmortem storage of bovine muscle (Boehm et al., 1998), and we found that over 50% of total muscle $\mu$-calpain activity in ovine muscle also becomes tightly associated with the myofibrillar fraction during postmortem storage. The properties of this myofibril-bound $\mu$-calpain will be discussed in detail in a successive article (Delgado, Geesink, Marchello, Goll, and Koohmaraie, unpublished data).

At-death calpastatin activity in the biceps femoris and longissimus from the callipyge sheep was 60.9% and 113.9% higher, respectively, than in the same muscles from the noncallipyge sheep (Table 3), in agreement with the earlier study (Koohmaraie et al., 1995). Calpastatin activity in the infraspinatus, however, was not significantly affected by the callipyge phenotype (13% higher in the callipyge than in the noncallipyge). As described previously for bovine muscle (Ducastaing et al., 1985; Koohmaraie et al., 1987; Boehm et al., 1998), muscle calpastatin activity decreases substantially during postmortem storage. This decrease occurred in all three muscles studied and in muscles from both the callipyge and from the noncallipyge sheep (Table 3). The rate of decrease in calpastatin activity during postmortem storage followed a classical first-order reaction.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Normal</th>
<th>Callipyge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot carcass wt, kg</td>
<td>33.5 (9.2)</td>
<td>30.8 (8.7)</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biceps femoris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>0.82 (0.23)</td>
<td>1.18 (0.33)</td>
</tr>
<tr>
<td>% of the carcass</td>
<td>2.5 (0.4)</td>
<td>3.8 (0.4)</td>
</tr>
<tr>
<td>Infraspinatus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>0.50 (0.12)</td>
<td>0.48 (0.14)</td>
</tr>
<tr>
<td>% of the carcass</td>
<td>1.5 (0.2)</td>
<td>1.6 (0.2)</td>
</tr>
<tr>
<td>Longissimus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight, kg</td>
<td>1.64 (0.65)</td>
<td>2.08 (0.60)</td>
</tr>
<tr>
<td>% of the carcass</td>
<td>4.8 (1.2)</td>
<td>6.7 (0.1)</td>
</tr>
</tbody>
</table>

*Weights were obtained by weighing muscle from one side of the carcass and multiplying the results by 2.

*Values within parentheses indicate standard deviation.
### Table 2. Least square means for the activities of μ- and m-calpain in biceps femoris (BF), longissimus dorsi (LD), and infraspinatus (IS) muscles of callipyge and normal lambs at four time points during postmortem storage (days postmortem, DPM)\(^a\)

<table>
<thead>
<tr>
<th>Phenotype and days postmortem</th>
<th>m-Calpain</th>
<th>μ-Calpain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BF</td>
<td>ID</td>
</tr>
<tr>
<td>Callipyge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>1.80(^*)</td>
<td>2.47(^*\w)</td>
</tr>
<tr>
<td>1 d</td>
<td>1.74(^*)</td>
<td>1.89(^*\w)</td>
</tr>
<tr>
<td>3 d</td>
<td>1.63(^*)</td>
<td>1.96(^*\w)</td>
</tr>
<tr>
<td>10 d</td>
<td>1.62(^*\w)</td>
<td>1.88(^*\w)</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>1.12</td>
<td>1.32(^w)</td>
</tr>
<tr>
<td>1 d</td>
<td>1.05</td>
<td>1.13(^w)</td>
</tr>
<tr>
<td>3 d</td>
<td>1.02</td>
<td>1.22(^w)</td>
</tr>
<tr>
<td>10 d</td>
<td>1.19</td>
<td>1.01(^w)</td>
</tr>
<tr>
<td>SE</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Probability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P(^b) × M(^c)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>DPM</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>M(^d) × DPM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Units are total caseinolytic activity/g of muscle, fresh weight.  
\(^b\)P: phenotype.  
\(^c\)M: muscle.  
\(^w\),\(^x\),\(^y\),\(^z\)Means having different superscripts within phenotype, muscle and enzyme were significantly different \((P < 0.05)\).  
SE: Standard error.  
ND: Not detectable; a null value was used for statistical analysis.  
*Differences between phenotypes within postmortem time were significant \((P < 0.05)\).

### Table 3. Least square means for the activity of calpastatin and the combined activities of μ- and m-calpain in the biceps femoris (BF), longissimus dorsi (LD), and infraspinatus muscles (IS) of callipyge and normal lambs at four different time points during postmortem storage (days postmortem, DPM)

<table>
<thead>
<tr>
<th>Phenotype and days postmortem</th>
<th>Calpastatin(^a)</th>
<th>μ- and m-Calpain(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BF</td>
<td>LD</td>
</tr>
<tr>
<td>Callipyge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>5.55(^*\w)</td>
<td>6.01(^*\w)</td>
</tr>
<tr>
<td>1 d</td>
<td>4.40(^*\w)</td>
<td>5.24(^*\w)</td>
</tr>
<tr>
<td>3 d</td>
<td>3.72(^*\w)</td>
<td>4.56(^*\w)</td>
</tr>
<tr>
<td>10 d</td>
<td>2.81(^*\w)</td>
<td>2.56(^*\w)</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 d</td>
<td>3.45(^*)</td>
<td>2.81(^*)</td>
</tr>
<tr>
<td>1 d</td>
<td>2.72(^*)</td>
<td>2.44(^*)</td>
</tr>
<tr>
<td>3 d</td>
<td>1.66(^*)</td>
<td>1.77(^*)</td>
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<tr>
<td>10 d</td>
<td>0.80(^*)</td>
<td>0.53(^*)</td>
</tr>
<tr>
<td>SE</td>
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<td>0.40</td>
</tr>
<tr>
<td>Probability</td>
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<tr>
<td>P(^e) × M(^f)</td>
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<tr>
<td>DPM</td>
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<tr>
<td>M(^g) × DPM</td>
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</table>

\(^a\)Inhibition of m-calpain hydrolysis of casein; units are total activity/g muscle, fresh weight.  
\(^b\)Units are total of μ-calpain plus m-calpain caseinolytic activity/g muscle, fresh weight.  
\(^P\): phenotype.  
\(^M\): muscle.  
\(^w\),\(^x\),\(^y\),\(^z\)Means having superscripts within phenotype and muscle were significantly different \((P < 0.05)\).  
*Differences between phenotypes within postmortem times were significant \((P < 0.05)\).
very closely. Although there are only three time points, the fit was so close that the first-order rate constants for decrease in calpastatin activity can be compared: normal biceps femoris, \( k = 0.13/d \) \( (r^2 = 0.96) \); callipyge biceps femoris, \( k = 0.05/d \) \( (r^2 = 0.97) \); normal longissimus, \( k = 0.17/d \) \( (r^2 = 0.99) \); callipyge longissimus, \( k = 0.08/d \) \( (r^2 = 0.99) \); normal infraspinatus, \( k = 0.05/d \) \( (r^2 = 0.98) \); callipyge infraspinatus, \( k = 0.07/d \) \( (r^2 = 0.86) \). Based on these calculations, calpastatin activity in the biceps femoris and longissimus muscles from the normal sheep decreased approximately two times faster during the first 10 d of postmortem storage than calpastatin activity from these two muscles in the callipyge sheep did. Calpastatin activity in the infraspinatus muscle, however, decreased at approximately the same rate in the normal and the callipyge sheep and this rate of decrease was similar to the rate of decrease in calpastatin activity in the callipyge biceps femoris and longissimus muscles (i.e., a slower rate). Also, at-death calpastatin activity in the noncallipyge infraspinatus was already significantly higher than calpastatin activity in the noncallipyge biceps femoris and longissimus (Table 3). Hence, calpastatin activity in the noncallipyge infraspinatus was significantly higher at all times of postmortem storage than calpastatin activity in the noncallipyge biceps femoris and longissimus muscles. The significance of this high calpastatin activity in the noncallipyge infraspinatus is unclear; as will be shown below, the high calpastatin activity in the infraspinatus did not significantly decrease the extent of postmortem tenderization as assessed by the MFI measurement. It may be related to relative rate of growth in this muscle.

Because the \( \mu \)- and m-calpain and calpastatin activities in this study were all done on the same samples and used the same assay procedures, it is possible to compare these activities directly. This comparison is confounded slightly because specific activity of m-calpain, when measured by the release of TCA-soluble peptides from casein, is approximately two times greater than the specific activity of \( \mu \)-calpain (Nishimura and Goll, 1991; Koohmaraie, 1992), and the calpastatin assays in this study used m-calpain. Hence, the calpastatin activities in Table 3 may be artificially high if considered in terms of ability to inhibit only \( \mu \)-calpain. Even allowing that the m-calpain activities in Table 2 may be only one-half as high if measured using myofibrils as a substrate, m-calpain constituted a significant proportion of total calpain activity in all three ovine muscles at death, and was an increasingly larger percentage of total calpain activity with increasing time of postmortem storage (Table 2). Considering these caveats, it can be estimated that during the first 3 d of postmortem storage, total calpastatin activity was greater than total \( \mu \)- plus m-calpain activity in both phenotypes and all three muscles used in this study, with the sole exception of the noncallipyge longissimus (Table 3). This is consistent with the finding that calpain activity cannot be detected in homogenates of whole muscle that contain both calpain and calpastatin (Okitani, et al., 1976; Waxman and Krebs, 1979). After 10 d of postmortem storage at 4°C, however, total \( \mu \)- plus m-calpain activity was greater than total calpastatin activity in all three muscles of the noncallipyge sheep but only in the infraspinatus of the callipyge sheep (Table 3).

**Status of \( \mu \)-Calpain and Calpastatin Polypeptides in Postmortem Muscle.** Western blot analysis showed that the 125-kDa calpastatin polypeptide that predominates in ovine skeletal muscle (Geesink et al., 1998) was degraded during postmortem storage at 4°C (Figure 3). Postmortem degradation of calpastatin was most rapid in the biceps femoris and longissimus from the noncallipyge sheep and was slowest in the biceps femoris and longissimus from the callipyge sheep (Figure 3). This rate of degradation of the calpastatin polypeptide paralleled the rate of decline in calpastatin activity in these two muscles from the callipyge and noncallipyge sheep (compare Figure 3 and Table 3). The rate of calpastatin degradation in the infraspinatus during postmortem storage also paralleled the rate of decline in calpastatin activity in this muscle during postmortem storage; rate of calpastatin degradation in the infraspinatus was approximately the same in the noncallipyge and callipyge sheep and was slower than the rate of calpastatin degradation in the biceps femoris and longissimus from the noncallipyge sheep. As indicated in the preceding section, it is unclear how this rate of postmortem calpastatin degradation in the infraspinatus muscle is related to the rate of postmortem tenderization of this muscle. The major product of calpastatin degradation in postmortem muscle as detected by the antibody used in this study was a ~65-kDa polypeptide (Figure 3). This degradative product evidently had calpain inhibitory activity because a significant level of calpastatin activity remained in the noncallipyge biceps femoris and longissimus after 3 d of postmortem storage (Table 3), even though no undegraded 125-kDa calpastatin polypeptide was present in these muscles at that time (Figure 3). Earlier studies (Boehm et al., 1998) have shown that the calpastatin polypeptide is also degraded during postmortem storage of bovine longissimus, although the polypeptides produced by postmortem degradation of bovine calpastatin differed from those observed in the present study.

Western analysis of \( \mu \)-calpain during postmortem storage was limited to the longissimus and infraspinatus (Figure 4). As reported previously for bovine longissimus (Boehm et al., 1998), the 80-kDa polypeptide of \( \mu \)-calpain was not degraded to small peptides during postmortem storage (cannot be observed in Figure 4) even though proteolytic activity of \( \mu \)-calpain declined rapidly (compare Table 2 and Figure 4). The 80-kDa \( \mu \)-calpain subunit was gradually converted to the 78-kDa and then to the 76-kDa autolysed forms during postmortem storage; the rate of this conversion is slightly more rapid in the noncallipyge than in the callipyge longissimus but occurs at approximately the same rate in the noncallipyge and callipyge infraspinatus (Figure...
**Figure 3.** Western blots for calpastatin in the supernate of a muscle extract (Figure 2) from A) biceps femoris, B) longissimus, and C) infraspinatus muscles from callipyge or “normal” sheep after different times of postmortem storage. Lane 1: calpastatin purified from bovine skeletal muscle; Lanes 2 and 6: samples from at-death muscle; Lanes 3 and 7: samples from d-1 postmortem muscle; Lanes 4 and 8: samples from d-3 postmortem samples; Lanes 5 and 9: samples from d-10 postmortem samples. All lanes were loaded with 60 μg of protein. Approximate molecular weights are indicated.
4). Boehm et al. (1998) showed that μ-calpain becomes increasingly associated with the myofibrillar fraction of bovine longissimus during postmortem storage, and μ-calpain also becomes associated with the pellet fraction (Figure 2) in the ovine longissimus and infraspinatus during postmortem storage (Figure 4). In the ovine muscles, the greatest increase in myofibrillar association occurs during the first day of postmortem storage, whereas in bovine longissimus the degree of myofibrillar association increased with increasing time of

**Figure 4.** Western blots for μ-calpain in the supernate and in the pellet fractions obtained after centrifugation of extracts (Figure 2) from longissimus (upper two panels) or infraspinatus (lower two panels) muscle of callipyge or “normal” sheep after different times of postmortem storage. Lane 1 in all blots is purified μ-calpain from bovine skeletal muscle; Lanes 2 and 6: samples from at-death muscle; Lanes 3 and 7: samples from d-1 postmortem muscle; Lanes 4 and 8: samples from d-3 postmortem muscle; Lanes 5 and 9: samples from d-10 postmortem muscle. All lanes were loaded with 60 μg of protein. Approximate molecular weights are indicated.
Figure 5. Least square means for the MFI in biceps femoris, longissimus, and infraspinatus from callipyge and normal phenotypes after different times (d) of postmortem storage.

postmortem storage up to 7 d postmortem (Boehm et al., 1998).

The MFI, Postmortem Tenderization, and Sarcomere Length. The MFI was used to estimate the tenderness of all three muscles in this study because the small size of the infraspinatus precluded careful Warner-Bratler shear force measurements on this muscle. A number of studies have shown that the MFI is highly related to tenderness (MacBride and Parrish, 1977; Culler et al., 1978; Koohmaraie et al., 1991). Measurements of MFI showed that the biceps femoris and longissimus from the callipyge phenotype did not tenderize during postmortem storage up to 10 d, whereas, during this same period, these two muscles from the noncallipyge phenotype underwent the greatest amount of tenderization among the muscles studied (Figure 5). Koohmaraie et al. (1995) has also found that the MFI of callipyge longissimus changes very little during the first 21 d of postmortem storage. The infraspinatus from both the noncallipyge and the callipyge phenotypes underwent the same amount of postmortem tenderization (change in MFI); the degree of postmortem tenderization of the infraspinatus muscle, however, was slightly less than that of the biceps femoris or longissimus from the noncallipyge sheep (Figure 5). Calpastatin activity in the infraspinatus of the noncallipyge and callipyge sheep also was intermediate between the calpastatin activities of the callipyge and the noncallipyge biceps femoris and longissimus at all times of postmortem storage (compare Table 3 and Figure 5).

As discussed previously (Taylor et al., 1995a; Koohmaraie et al., 1987; Boehm et al., 1998), the MFI increases most rapidly during the first 3 d of postmortem storage and then increases at a much slower rate during subsequent postmortem storage. In the present study, the MFI for the biceps femoris and longissimus from the noncallipyge sheep and for the infraspinatus from both the callipyge and noncallipyge sheep increased 35 to 66% or at the rate of 12 to 22%/d, during the first 3 d of postmortem storage, whereas, between 3 and 10 d postmortem, the MFI of these muscles increased 17 to 46% or at the rate of 2.4 to 6.6%/d. To the extent that the MFI indicates tenderness, these results confirm the earlier studies (Goll et al., 1964; Davey and Gilbert, 1966; Dransfield, 1992) indicating that most postmortem tenderization occurs during the first 72 to 96 h after death in most animals. Phase microscopy of myofibrils prepared from the longissimus of noncallipyge and callipyge sheep after 10 d of postmortem storage showed that myofibrils from the callipyge longissimus were longer and were much less susceptible to breakage than myofibrils from the noncallipyge longissimus (Figure 6).

Warner-Bratzler shear force measurements of the longissimus at 1, 3, and 10 d of postmortem storage confirmed the MFI results; the longissimus from the callipyge sheep did not undergo any tenderization between 1 and 10 d of postmortem storage, whereas the Warner-Bratzler shear force values of the longissimus from the noncallipyge sheep decreased significantly from approximately 5.5 to 3.9 kg between 1 and 10
These changes in Warner-Bratzler shear force values are similar to the changes reported earlier for the longissimus of callipyge and normal sheep (Koohmaraie et al., 1995, 1998; Shackelford et al., 1997). It was noted during this study that Warner-Bratzler shear force values differed (P < 0.05) among the different locations of the longissimus that were sampled in this study; samples from the center or medial location were less tender than samples from either the costal or caudal locations. Future studies using the longissimus should standardize the location to be used for that study and should specify this location.

Sarcomere length did not change between 1 and 10 d of postmortem storage in any of the three muscles included in this study, but sarcomere lengths of the infraspinatus from both the callipyge and the normal sheep were significantly longer than sarcomere lengths in the biceps femoris and longissimus from either the callipyge or the normal sheep (Table 4). The average sarcomere length of 1.71 µm for the longissimus muscle after 1 d postmortem is similar to the sarcomere length of 1.69 µm reported by Wheeler and Koohmaraie (1994) for sheep longissimus. Koohmaraie et al. (1995, 1998) previously found that sarcomere lengths for the longissimus from normal and callipyge sheep did not differ: 1.62 µm and 1.61 µm for normal and callipyge, respectively, in one study (Koohmaraie et al., 1995) and 1.63 µm for both the normal and callipyge sheep in another study (Koohmaraie et al., 1998).

Discussion

The callipyge phenotype was first identified in 1983 in a Dorset ram that transmitted an extremely heavily muscled phenotype to some of his offspring. The heavy muscling characterizing this phenotype was especially prominent in the hindquarters of the offspring. The gene, callipyge, has been mapped to the telomeric region of the ovine chromosome 18 and exhibits an unusual mode of inheritance, termed polar overdominance, in which only individuals that have received the allele from their sire express the callipyge phenotype (Freking et al., 1998).

This study shows that two muscles, the longissimus and biceps femoris, that have a significantly greater mass in market-weight callipyge animals than in normal animals also 1) have significantly higher calpastatin activity immediately after death and after 1, 3, or 10 d of postmortem storage at 4°C than the same muscles from “normal” animals and 2) are significantly tougher than the same muscles from “normal” animals and do not undergo an increase in MFI during at least 10 d of postmortem storage at 4°C. The at-death calpastatin activity of the infraspinatus, a muscle whose mass is not affected by the callipyge phenotype, however, is the same in the normal and callipyge phenotypes, and calpastatin activity in the infraspinatus from callipyge and normal sheep decreases at the same rate during postmortem storage. Moreover, tenderness of the infraspinatus muscle increases at the same rate in callipyge and normal sheep during postmortem storage. These findings extend the results of earlier studies (Koohmaraie et al., 1995, 1996b; Shackelford et al., 1997) showing that calpastatin activity in the longissimus from callipyge sheep was higher than in the longissimus from normal sheep and that the longissimus, gluteus medius, semimembranosus, semitendinosus, biceps femoris, adductor, quadriceps femoris, and triceps brachii from the callipyge all were less tender than the corresponding muscles from normal sheep. Hence, the results of this study add to the growing body of evidence that muscle...
calpastatin activity is related to postmortem tenderization and to ultimate tenderness.

Although details of the mechanism underlying this relationship remain unclear, the relationship is conceptually logical. A large amount of evidence indicates that the calpains are responsible for postmortem tenderization (Koohmaraie; 1992, 1996; Goll et al., 1998) and calpastatin specifically inhibits the calpains. Thus, the currently accepted concept is that elevated calpastatin activity inhibits the calpains and postmortem tenderization. The protein(s) encoded by the callipyge gene is not yet known (it is not calpastatin), so it is unclear whether this protein(s) is expressed in muscles such as the infraspinatus that do not have the increased mass and calpastatin activity of muscles such as the longissimus or the biceps femoris. If the “callipyge protein(s)” is expressed in the infraspinatus at the same level as it is in the longissimus or biceps femoris muscles, then the greater toughness of the longissimus and biceps femoris in the callipyge cannot be a direct effect of the callipyge gene on tenderness but would seem to result from the increased level of calpastatin in those muscles. If, on the other hand, the callipyge protein(s) is not expressed in the infraspinatus, the relationship between the callipyge gene and tenderness is less clear. One explanation in the latter situation would be that the callipyge protein(s) acts to up-regulate the expression of the calpastatin gene, thereby resulting in increased calpastatin activity in those tissues that express this protein(s). The promoter region of the bovine calpastatin gene has been sequenced (Cong et al., 1998a,b) and has been shown to contain a number of cis-acting regulatory elements. Very little is known about the ovine calpastatin gene, however.

This present study also indicated that the mechanism by which the calpain system causes postmortem tender-
ization is not a simple one. The differences in μ-calpain and m-calpain activities between the callipyge and normal sheep are not consistent with the large differences in tenderness between these two phenotypes. Activity of μ-calpain is nearly the same among all three muscles from the callipyge phenotype, although the MFI of the callipyge longissimus and biceps femoris muscles does not increase with postmortem storage, whereas the MFI callipyge infraspinatus undergoes significant increase during postmortem storage. Indeed, μ-calpain activity in the infraspinatus, a muscle that tenderizes, is actually significantly less after 3 and 10 d of postmortem storage than it is in the callipyge longissimus and biceps femoris, two muscles that do not tenderize during postmortem storage. On the other hand, at-death m-calpain activity is greater in the callipyge longissimus and biceps femoris than it is in these two muscles from normal sheep, and m-calpain activity remains greater in these two muscles that do not tenderize throughout the 10 d of postmortem storage. Calculations based on the assumptions described earlier indicate that calpastatin activity is higher than total μ- plus m-calpain activity in all muscles studied for the first 3 d of postmortem storage, even though this is the period during which most postmortem tenderization normally occurs (Koohmaraie et al., 1996a; Ho et al., 1996, 1997). Immunolocalization studies have shown that, in bovine skeletal muscle, the calpains become widely distributed throughout the muscle cell during postmortem storage (Goll et al., 1992; Taylor et al., 1995b). Similar studies have shown that calpastatin also is widely distributed throughout the interior of skeletal muscle cells (Goll et al., 1989; Kumamoto et al., 1992), so it is likely that calpastatin and the calpains are located in similar if not identical areas of the cell in postmortem muscle. The calpains can autolyze (Zimmerman and Schlaepfer 1991) or degrade calpastatin itself (Mellgren et al., 1989; Kumamoto et al., 1992), so it is likely that calpastatin activity may not be in excess of activity of autolyzed but not unautolyzed μ-calpain, with approximately 30 to 40% of the original proteolytic activity of autolyzed μ-calpain remaining after incubation for 45 min in 300 mM NaCl. The 130 to 140 mM lactic acid that accumulates during the first 48 h postmortem has a pK_a of 3.88 at 0°C and therefore is nearly 99% in the ionized form at the pH of postmortem muscle. Consequently, it is possible that either the high ionic strength in postmortem muscle or the 120 to 200 mM salt in the chromatographic steps used to isolate μ-calpain from postmortem muscle may have inactivated the autolyzed μ-calpain. Other recent studies have shown that autolyzed μ- and m-calpain, but not their unautolyzed forms, associate in a salt-containing solution to produce a discrete aggregate, likely a trimer or tetramer (Li, Thompson, and Goll, unpublished). The associated form of calpain is inactive proteolytically, explaining the observed inactivation of the autolyzed calpains in the presence of salt. It is uncertain at this point whether the high ionic strengths, which occur within the first 24 to 48 h postmortem, could induce aggregation and hence inactivation of the autolyzed μ-calpain in situ in postmortem muscle, whether the aggregation/inactivation occurs only during extraction and isolation of μ-calpain, or some combination of both. Additional studies will be required to distinguish between these two possibilities.

Consequently, it seems likely that activity of the calpains in postmortem muscle is modulated in some manner, although the nature of this modulation presently remains unclear. In addition to the possibilities already discussed, postmortem muscle cells may contain localized areas where the calpains are separated from calpastatin, thereby permitting calpain activity. Or, although the calpastatin fragments produced by degradation of the calpastatin polypeptide still possess
Inhibitory activity, they have much higher inhibition constants ($K_i$) than the native calpastatin molecule (on the order of $10^{-3}$ to $10^{-6} M$ compared with less than $10^{-9} M$ for the intact calpastatin; Maki et al., 1988; Kawasaki et al., 1989). The lower $K_i$ of the calpastatin inhibitory activity, they have much higher inhibition than the intact calpastatin. In this situation, rate of post-mortem tenderization would depend primarily on the rate of calpastatin degradation in postmortem muscle, which is consistent with the results found in this study.

Sarcomere lengths in the longissimus and biceps femoris from the callipyge and normal muscles did not differ even though the tenderness (as estimated with the MFI) of these two muscles from the callipyge and normal sheep differed greatly. An earlier study (Smulders et al., 1990) found no correlation between sarcomere length and tenderness in bovine longissimus muscle when the pH was 6.3 or less after 3 h postmortem.

Implications

In addition to increasing the mass of certain muscles, the presence of the calpastatin gene also results in an increased calpastatin activity and a marked increase in toughness of the affected muscles. Tenderness and calpastatin activity of muscles such as the infraspinatus, whose mass is not increased in the callipyge phenotype, are not affected by the callipyge gene. Although the callipyge phenotype seems to have a number of advantages for meat-producing animals, it will be necessary to devise commercially economical ways to tenderize those muscles affected by the callipyge phenotype. The callipyge sheep provides a useful system for studying the role of the calpain system in postmortem tenderization because there are large differences in calpastatin and tenderness between the callipyge and normal sheep.

Literature Cited


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