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A new insight into meat toughness of callipyge lamb loins - The relevance of anti-apoptotic systems to decreased proteolysis

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ABSTRACT

The objective of this study was to determine associations of small heat shock proteins (sHSPs) in tenderness development of loins from callipyge and normal genotype lambs. Loins (*M. longissimus lumborum*) from sixteen lambs across four genotypes were collected throughout 9 days of postmortem aging. The loins from callipyge lambs had more intact desmin and troponin T throughout aging periods, as well as less μ-calpain autolysis and more calpastatin compared to loins from other genotypes ($P < 0.05$). Delayed onset of apoptosis was found in the callipyge loins indicated by less cytochrome *c* and more inactive procaspase-3 compared to normal lamb loins ($P < 0.05$). Less degraded HSP27 was also consistently found in the callipyge loins compared with loins from normal lambs ($P < 0.001$). The results found up-regulation of anti-apoptotic activities coincided with toughness in callipyge loins, which suggest apoptosis is likely involved in postmortem proteolysis and subsequent meat tenderization.

1. Introduction

Programmed cell death, or apoptosis, has been suggested as the first step in the process of converting muscle into meat. In particular, it has been suggested that apoptosis may be involved with meat tenderization (Sentandreu, Coulis, & Ouali, 2002; Ouali et al., 2006). Under certain conditions (i.e. change in redox state of the cell) apoptosis is initiated by the release of cytochrome *c* from the mitochondrial membrane to the cytoplasm, which, in turn, activates initiator and effector caspases including caspase-3 (Green and Reed, 1998; Hongmei, 2012). As caspase-3 is a proteolytic enzyme that also cleaves calpastatin (Pörn-Ares, Samali, & Orrenius, 1998), a potential involvement of apoptosis in meat tenderness development has been proposed (Sentandreu et al., 2002). Conversely, heat shock proteins (HSPs) are chaperone proteins that have a protective role in cell death, namely anti-apoptotic activity. HSPs inhibit the onset of apoptosis by binding to cytochrome *c* and/or preventing the activation of caspase-3 (Beere, 2005; Ba, Reddy, & Hwang, 2014). There is increasing evidence suggesting that anti-apoptosis, mainly through small HSPs (sHSPs), may be related in meat toughness (Balan, Kim, & Blijenburg, 2014; Ouali et al., 2006; Pulford et al., 2008).

Callipyge lambs have a paternally inherited genetic mutation that causes muscular hypertrophy of the loins and hindquarters (Freking et al., 1999). The callipyge phenotype is only inherited from sire (known as polar overdominance; *C* = mutant allele, + = wildtype allele). Thus, only +/*C* genotype lambs express the unique callipyge muscle phenotype, whereas other genotypes, such as *C*/*C*, *C*/+ and +/+, show in normal phenotypes (Bidwell et al., 2004). Affected lambs result in a more efficient feed-to-gain ratio and allow producers to grow more meat on less feed; however, the meat produced is considerably tougher (Sainz, 2001). The primary cause for this phenomenon was identified as an increase of calpastatin that inhibits μ-calpain activity thus preventing timely meat tenderization (Delgado, Geesink, Marchello, Goll, & Koohmarie, 2001). However, a recent study found lower caspase activities in callipyge muscles compared to normal counterparts, suggesting a possible involvement of apoptosis in callipyge lamb meat toughness (Kemp, King, Shackelford, Wheeler, & Koohmaraie, 2009). While several studies found the high levels of sHSPs in beef toughness (Lomiwes, Farouk, Frost, Dobbie & Young, 2013; Lomiwes, Farouk, Wiklund & Young, 2014; Pulford et al., 2008; Balan et al., 2014), the relevance of sHSPs (or anti-apoptosis) in callipyge lamb toughness has not been fully elucidated. Therefore, the working objective of this study was to determine the involvement of sHSPs in tenderness development of loins from callipyge and normal genotype lambs. The ultimate goal of this study was to elucidate the role of anti-apoptotic systems in the postmortem meat tenderization by using callipyge lambs as a model. This investigation is further elaboration of our recent study (Penick et al., 2017), where meat quality

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and oxidative stability results of callipyge loin muscles were previously reported.

2. Materials and methods

2.1. Muscle sample collection

The current study used the frozen stored meat samples from Penick et al. (2017) for the protein analyses that were associated with apoptosis cascades and myofibrillar proteolysis. The data were further used for correlation analyses with the previously reported meat quality attributes, such as tenderness and water-holding capacity. The detailed information regarding lamb selection and live animal performance was reported in our previous study (Penick et al., 2017). A total of 16 lambs (wethers and ewes; at 7 months of age and live weight of around 48.1 kg) from across four possible callipyge genotypes (*C/C*, $n = 4$; *+/C*, n = 4; *C/+*, n = 4; *+/+*, n = 4) were harvested. Lambs were slaughtered at the Purdue University Meat Lab. Muscle samples from the loin (*M. longissimus thoracis et lumborum*) were collected (approximately 5.0 g) about 15 min postmortem and snap-frozen. Lamb carcasses were chilled in a 1 °C cooler for 3 days, and then right-side loin muscles were separated from each carcass. Two chops collected from each right-side loin after 3, 6, and 9 days postmortem aging and were stored in a − 80 °C freezer for biochemical and qualitative analysis.

2.2. Whole muscle protein extraction and sample preparation

Whole muscle protein was extracted in accordance with Kim, Huff-Lonergan, Sebranek, and Lonergan (2010) with some modifications. One gram of frozen muscle sample was minced and homogenized in 10 mL of whole muscle protein extraction buffer (10 mM phosphate with 2% sodium dodecyl sulfate, pH 7.0 at 20 °C). Gel samples for SDS-PAGE analysis were prepared with protein extract (a concentration of 4 mg/mL protein) with extraction buffer to total 1 mL volume, 500 μL tracking dye (3 mM EDTA, 3% [wt/vol] SDS, 20% [vol/vol] glycerol, 0.003% [wt/vol] bromphenol blue, and 30 mM Tris-HCl; pH 8.0 at 20 °C) and 100 μL 2-mercaptoethanol. Gel samples were incubated in a 50 °C bath for 20 min and stored at −80 °C for Western blot analysis.

2.3. SDS-PAGE and western blot

Western blots were performed in accordance with Kim et al. (2010) with some modifications. SDS-PAGE separating gels were made with 100:1 bis-acrylamide (8% for μ-calpain; 10% for desmin, troponin T, calpastatin, caspase 3; 15% for small heat shock proteins and cytochrome *c*), 15 mL 2 M Tris-HCl buffer (pH 8.8 at 20 °C), 300 μL ammonium sulfate (10% wt/vol), and 30 μL tetramethylethylenediamine (TEMED). Stacking gel was made with 5% bis-acrylamide (100:1), 4 mL 1 M Tris-HCl buffer (pH 6.8 at 20 °C), 120 μL ammonium sulfate (10% wt/vol), and 20 μL TEMED. Gels were loaded with protein samples (40 μg desmin, troponin T, μ-calpain, calpastatin; 60 μg small heat shock proteins and caspase 3; 80 μg cytochrome *c*) and electrophoresis was run overnight in running buffer (3.021% Tris, 14.4% glycine, 2% SDS, 0.058% EDTA). Protein was then transferred to polyvinylidene fluoride membrane in 1 °C Tris-glycine buffer at 90 V for 90 min.

Membranes were blocked in PBS-Tween solution (1.632 M sodium phosphate dibasic, 4.08 M sodium phosphate monobasic monohydrate, 0.5 M sodium chloride) containing 5% nonfat dry milk at room temperature for 1 h. Membranes were incubated in primary antibody solution containing 3% nonfat dry milk and the following antibodies: 1:10,000 monoclonal mouse anti-desmin (Sigma-Aldrich; St Louis, MO); 1:10,000 monoclonal mouse anti-troponin T (Sigma Aldrich); 1:10,000 monoclonal mouse anti-μ-calpain (ThermoFisher Scientific; Waltham, MA); 1:4000 monoclonal mouse anti-calpastatin (ThermoFisher Scientific); 1:250 polyclonal rabbit anti-caspase 3 (Abcam; Cambridge, MA); 1:200 monoclonal mouse anti-cytochrome *c* (Abcam); 1:1000 monoclonal mouse anti-HSP27 (Abcam); 1:1000 polyclonal mouse anti-HSP20 (Abcam); 1:1000 polyclonal rabbit anti-αβ-crystallin (ThermoFisher Scientific). Membranes were incubated in primary antibody solution at 4 °C for 18 h overnight and were washed 3 times for 10 min each with PBS-T solution. Membranes were incubated with the following secondary antibody preparations for one hour at room temperature: goat anti-mouse IgG (H + L) horseradish peroxidase conjugate (dilution of 1:20,000 for desmin, troponin T, μ-calpain; 1:2000 for calpastatin, HSP27; Bio-Rad; Hercules, CA) or goat anti-rat IgG peroxidase conjugated (dilutions of 1:2000 for HSP20, αβ-crystallin; 1:200 for caspase 3, cytochrome *c*; ThermoFisher Scientific). After three ten-minute washes, membranes were developed with ECL Western blotting reagents (ThermoFisher Scientific) and visualized (UVP GelDoc-It). For each image intensity volume of each band was measured with UVP VisionworksLS Analysis Software (UVP, LLC; Upland, CA, USA) and were compared with an internal reference to normalize data and quantification.

2.4. Statistical analysis

The experimental design was split-plot, with animals from four genotypes (*C/C*, *+/C*, *C/+*, *+/+*) as whole-plot and aging periods (0, 3, 6, 9 days) as sub-plot. Data were analyzed by analysis of variance using PROC mixed procedures of SAS statistical software (Version 9.4, SAS Inst.; Cary, NC), where genotype and aging time were fixed effects and animal was a random effect. Least square means were separated (F test, $P < 0.05$) by least significant differences. Pearson correlation was performed between quantified Western blot data and meat quality data, which were collected by Penick et al. (2017).

3. Results

3.1. Calpain, calpastatin and myofibrillar protein degradation

μ-Calpain was quantified as three bands (80, 78, and 76 kDa) in order to indicate the extent of autolysis, commonly associated with calpain activity (Kim et al., 2013; Melody et al., 2004). Throughout postmortem aging, intact μ-calpain (80 kDa) decreased ($P < 0.0001$), while its 76 kDa subunit increased in abundance ($P < 0.0001$; Fig. 1 and Table 1). Within each timepoint (0, 3, 6, or 9 days postmortem), the loins from callipyge (*+/C* lamb) carcasses were found to have a higher abundance of intact μ-calpain and less abundance of the 76 kDa subunit ($P = 0.002$ and $P = 0.004$, respectively) than the loins from other normal genotypes (*C/C*, *C/+*, *+/+*) (Table 1). However, there was no difference in abundance of each μ-calpain subunit between normal genotypes ($P > 0.05$; Table 1). No difference in the 78 kDa subunit was

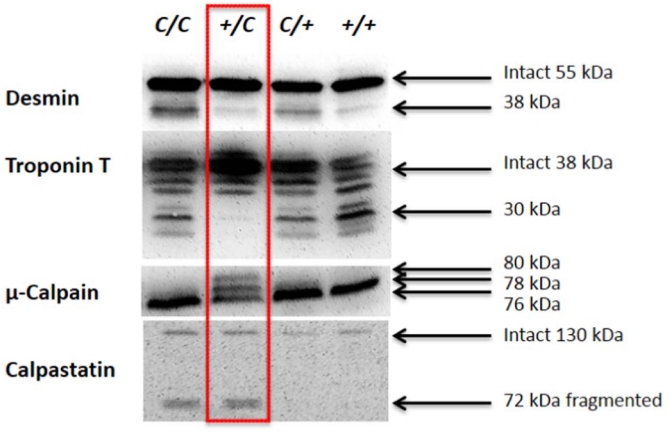

Fig. 1. Representative Western-blots for myofibrillar proteins, μ-calpain and calpastatin from loins from callipyge lamb (*+/C*) and other non-callipyge phenotype lamb (*C/C*, *C/+*, *+/+*) carcasses after 9 days postmortem aging.