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The effects of gender and muscle type on the mRNA levels of the calpain proteolytic system and beef tenderness during *post-mortem* aging

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ABSTRACT

The objective of this study was to determine the effects of gender and muscle type on the mRNA levels of the calpain system and the tenderization of beef meat. The *Longissimus thoracis* (LT) and the *Semimembranosus* (SM) were sampled from each bull, steer and heifer after routine slaughter (Six animals per group). The mRNA levels of μ-calpain, m-calpain, calpain-3 and calpastatin were quantified using real-time PCR. Concurrently, tenderness was determined following the Warner–Bratzler Shearforce (WBSF) procedure and rate of tenderization during *post-mortem* storage was calculated from the WBSF values of 7d and 35d aged steaks. The results show that bulls had significantly lower ($P < 0.01$) WBSF values than heifers which were accompanied by higher ($P < 0.01$) levels of μ-calpain and calpain-3 mRNA but similar levels of calpastatin as compared to heifers. There was a significantly higher ($P < 0.05$) calpastatin expression in steers, as compared to heifers. However, μ-calpain expression was lower ($P < 0.05$) in heifers whose meat was significantly tougher ($P < 0.05$) than that of steers. Steer meat was slightly tougher than that of bulls, while steers had had a tendency to express higher levels of calpastatin but similar μ-calpain and calpain-3 mRNA. The LT had lower ($P < 0.05$) WBSF values than the SM but these muscles tenderised at the same rate, and had similar mRNA levels for all investigated genes. M-calpain mRNA levels were not significantly affected by muscle and gender ($P > 0.05$). Moreover, calpain 3 was negatively correlated to 7d WBSF values ($P < 0.05$). Despite the small sample size, these results suggest that variations in beef tenderness could be modulated through the differential expression of the members of the calpain system, specifically, μ-calpain, calpain 3 and calpastatin.

1. Introduction

The calpain system consists of a large family of proteolytic enzymes whose *post-mortem* activities have been associated with enhanced meat quality characteristics. Specifically, the roles of the micromolar Ca^{2+} requiring μ-calpain, the millimolar Ca^{2+} requiring m-calpain (Dayton, 1982), the skeletal muscle-specific calpain-3 or p94 (Sorimachi et al., 1989) and calpastatin (Goll et al., 2003) have been extensively investigated in the degradation of several myofibrillar proteins.

Research has shown that Calpastatin regulates the activities of μ-calpain and m-calpain (Goll et al., 2003), where higher calpastatin activity leads to less tender meat (Morgan et al., 1993a). Therefore, as calpastatin losses its inhibitory effects during *post-mortem* aging, the ubiquitous calpains becomes increasingly active and cleaves myofibrillar proteins such as titin, desmin and vinculin which leads to increased tenderness (Taylor et al., 1995; Kemp et al., 2010).

Despite this, evidence about the activities of the individual members of the calpain system and their specific roles in the degradation of skeletal muscle proteins remain contentious. Huff-Lonergan et al. (1996) demonstrated that the *in vitro* degradation of desmin, nebulin, titin, vinculin, troponin-T, and other cytoskeletal proteins by μ-and m-calpain were similar to those observed in *post-mortem* muscle. However, Boehm et al. (1998) argued that m-calpain would not autolyse under the Ca^{2+} levels observed in *post-mortem* muscle. In addition, the degradation of myofibrillar proteins were significantly reduced in μ-calpain knock-out mouse,

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suggesting that m-calpain may be inactive in *post-mortem* muscle (Geesink et al., 2006). However, others argue that the levels of calcium *in vivo* may not be accurately represented through *in vitro* assays and there are possibly other unknown cofactors that may be involved and may allow m-calpain to be active (Goll et al., 2003).

Similarly, no differences in *post-mortem* protein degradation were found between calpain-3 knockout and wild type mice (Geesink et al., 2005) despite the strategic location of the protease within the sarcomere (Sorimachi et al., 1996). Besides, calpastatin does not regulate calpain-3 activities (Ono et al., 2004), suggesting no significant role for this protease in meat tenderization. In contrast, significant correlations between the appearance of the autolysed calpain-3 and the degradation of nebulin in the *Longissimus* muscle of goats were reported (Ilian et al., 2004). Results from the *Longissimus* muscles of pigs showed a positive relation between calpain-3 mRNA and tenderness (Yang et al., 2012), conflicting those of Gandolfi et al. (2011) who found high calpain 3 expression in relation to higher shear force values.

This suggests that all three calpains may have proteolytic properties but the extent of their involvement during *post-mortem* aging may sometimes be difficult to ascertain. In other words, direct evidence at the protein levels are challenging in terms of quantification and the emulation of *in vivo* activity levels. In that case, quantification of the mRNA levels of candidate genes under varying experimental and physiological conditions may offer insights into the variable cellular demand of a specific protease.

It was thus presumed that these genes would be differentially regulated in bulls, steers and heifers and the variation would be indicative of differences in meat quality characteristics. Taking into consideration post-transcription modifications, the variations observed in mRNA concentrations of a particular gene may correlate to a great extent with the quantity and activity of the translated protein, as demonstrated by others for m-calpain and calpastatin (Parr et al., 1992; Ilian et al., 1999) and other genes (Tang et al., 2010).

Such information may be useful particularly to the meat industry as it would enhance the understanding of the molecular basis of meat tenderization, and may allow amendment of pre-slaughter handling practices and *post-mortem* treatments that improves meat quality.

Therefore, the objective of this study was to quantify the mRNA levels of the large subunits of μ-calpain, m-calpain, calpain-3 and calpastatin in bovine skeletal muscles and to test the hypothesis that gender and muscle type have an effect on the expression of these genes in association with meat tenderness.

2. Materials and methods

2.1. Sample acquisition and storage

Eighteen Hereford-cross cattle were obtained from a commercial farm in Yorkshire, North-East England and slaughtered at equivalent liveweight of approximately 550 kg following the regulations in an EU approved abattoir and in accordance with EU Directive 2010/63/EU. The carcases were weighed and then graded by expert meat graders following the EUROP carcass classification.

Six carcasses from each gender group, namely young bulls, steers and heifers, were selected for use in this study. Muscle tissue sub-samples for RNA extraction were collected from each carcass by making a deep incision approximately 10 cm into the forerib and topside section to access the *Longissimus thoracis* (LT) and the *Semimembranosus* (SM), respectively. These sub-samples were then immediately submerged in tubes containing RNA*later*™ stabilisation reagent (Sigma-Aldrich, St. Louis, USA). This was carried out within 10 minutes after exsanguination. After 24 h, the sub-samples were subsequently frozen at −20 °C in accordance with manufacturer recommendations (Sigma-Aldrich, St. Louis, USA). The rest of the carcass was stored in a 4 °C chiller to age for 7 days, for subsequent use in meat tenderness analysis.

2.2. Measurement of pH

Specifically, Carcass pH was taken at the centre of Longissimus muscle, at approximately 15 min and 24 h after slaughter for pH0 and pH2d, respectively. This was then repeated after 1 week to obtain the pH7d. The pH0 and pH2d readings for bull carcasses were not available and thus only the pH7d could be reported for this group.

2.3. RNA extraction

Total RNA were extracted from 100 mg bovine skeletal muscle subsamples using the RNeasy fibrous tissue extraction kit (Qiagen, Germany), following manufacturer's instructions. To ensure that any carry-over genomic DNA was eliminated, the eluates were treated with DNase-I (Sigma-Aldrich, St. Louis, USA). Total isolated RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA), were the A260/A280 ratios between 1.40 and 2.16 indicated samples with optimal purity. Quality was assessed by performing electrophoresis on a 2% agarose gel and by exposure to UV light using the SYNGENE system (SynGene Ltd., Cambridge, UK). The RNA was then aliquoted into microtubes and stored at −80 °C until required in downstream processes.

2.4. Quantitative PCR

The mRNA levels of μ-Calpain, m-Calpain, Calpain-3 and Calpastatin in the two skeletal muscles were determined by real-time quantitative PCR (qPCR). Firstly, the total RNA was reverse-transcribed to cDNA using the Verso™ reverse transcriptase and oligo-dT primers from the Verso® cDNA synthesis kit (Abgene Ltd., Epsom, UK). Then an aliquot was taken from each tube to create a pooled cDNA mixture which was subsequently serially diluted 5-fold to generate a standard curve. Real-time PCR was performed in a 25 μl amplification reaction mixture containing 5 μl of cDNA, 12.5 μl ABsolute™ Blue QPCR SYBR® Green master mix (ABgene Ltd., Epsom, UK), 300 nM of gene specific primers (Primerdesign Ltd., Southampton, UK) and PCR-grade water. Duplicate reactions for each study sample and triplicate reactions for standard curve subsamples were performed as a means of controlling between wells variation. The sequences of the primers used in the amplification of target genes are shown in Table 1. On the other hand, commercially available primers sets for the *Bos taurus* housekeeping genes (HKGs), namely Glyceraldehyde-3-phosphate dehydrogenase (GAPD), Peptidylprolyl isomerase-A (PPIA) and Eukaryotic initiation factor-2B Subunit 2 (EIF2B2), were used to amplify the reference genes (Primer Design, Southampton, UK). The GeneBank Accession Numbers for these HKGs genes are NM_001034034, NM_178320 and NM_001015593, respectively.

The following PCR cycling conditions were used in the Mastercycler® ep realplex thermal cycler (Eppendorf AG, Hamburg, Germany): One 15 min cycle of enzyme activation at 95 °C, 40 cycles at 95 °C for 15 s, 30 s annealing at 60 °C and 30 s elongation at 72 °C. The threshold cycle value (Ct value) of each reaction tube was automatically determined using the realplex 2.2 software (Eppendorf AG, Hamburg, Germany).