Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle

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ABSTRACT: The objective of this study was to investigate the protease family caspases in skeletal muscle and their potential contribution to postmortem proteolysis and meat tenderization. Ten Large White gilts were slaughtered, and samples of LM were taken at 0, 2, 4, 8, 16, 32, and 192 h after slaughter and immediately snap frozen in liquid nitrogen. Samples were subsequently analyzed for caspase 3/7 and caspase 9 activity, protein levels of known caspase substrates, alpha II spectrin and poly (ADP-ribose) polymerase (PARP), as well as, at 192 h, shear force. Specific degradation products of alpha II spectrin and PARP, which are known indicators of caspase activity, and apoptosis were detected on immunoblots of muscle samples taken over the postmortem period. The relationships between the changes observed in caspase activities and protein levels of PARP and spectrin across the entire postmortem conditioning period were investigated (n = 70). Protein levels of alpha II spectrin cleavage products across the conditioning period were found to correlate positively to caspase 3/7 activity ($r = 0.38$, $P = 0.003$) and caspase 9 activity ($r = 0.32$, $P = 0.012$), indicating that caspase-mediated cleavage was occurring in situ. There was a negative relationship between shear force and the 0 to 32 h ratio of caspase 3/7 ($r = -0.62$, $P = 0.053$) and caspase 9 activities ($r = -0.68$, $P = 0.044$). In addition, there was also a negative relationship between shear force and the level of the caspase-generated alpha II spectrin 120 kDa degradation product ($r = -0.75$, $P = 0.012$). The findings of this study indicate that changes in caspase activity and caspase-mediated cleavage take place in muscle during the conditioning period, and this could be associated with the development of tender meat.

Key words: caspase, meat, pig, skeletal muscle, tenderization

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INTRODUCTION

The extent and rate of tenderization does not occur equally in all animals, resulting in variable meat quality. The final toughness of meat depends in part on the degree of alteration of the structural components of muscle and associated proteins postmortem. Although calpain proteolytic activity is a significant contributor to meat tenderization, other proteolytic enzymes are likely to contribute to the biochemical and structural changes that take place (Sentandreu et al., 2002). To date, research on the cysteine protease family caspases has focused on their involvement in programmed cell death where they function as vital executioners of apoptosis, mediating the disassembly of cellular structures and formation of apoptotic bodies (Tews, 2005). The caspase system has a hierarchy of initiating isoforms, with caspases 8, 9, 10, and 12 activating downstream executioner caspases such as caspases 3, 6, and 7 that are involved in cleaving specific target proteins (Earnshaw et al., 1999).

Recent studies have shown that caspases are involved in skeletal muscle development and remodeling, with expression being essential for normal muscle differentiation during myogenesis (Fernando et al., 2002). Caspases are upregulated in conditions such as in sarcopenia (Dupont-Versteegden, 2005), muscular dystrophies (Sandri et al., 2001), and are activated early in pathological events associated with hypoxia/ischemia (Gustafsson and Gottlieb, 2003), which is similar to the hypoxic conditions that occur postmortem. With this unique relationship between skeletal muscle and caspases in mind, it is conceivable that caspases could be involved in postmortem proteolysis and meat tenderization.
The aims of this study were to measure caspase activities and changes in the levels of their specific substrates in porcine LM during the postmortem conditioning period and investigate any potential relationship with shear force.

MATERIALS AND METHODS

Animals and Sample Collection

Animal studies were conducted according to the provisions of the UK Home Office Animals (Scientific Procedures) act of 1986. Ten female Large White pigs (81.2 ± 1.98 kg of BW) were slaughtered by electrical stunning and severance of the carotid arteries. After slaughter and subsequent dressing, a core sample of LM (approximately 2 g) was taken from the area anterior to the last rib within 15 min of slaughter, which was designated 0 h. Carcasses were then hung in the chiller at 4°C, and core samples of LM were taken from the area anterior to 0 h sampling point at 2, 4, 8, 16, and 32 h after slaughter and immediately snap frozen in liquid nitrogen. At 48 h, triplicate chops were taken (one for time point 192 h and duplicates for shear force analysis) from the region proximal to the sampling area, vacuum-packed, and conditioned at 4°C for a further 6 d before being frozen in liquid nitrogen. All samples were stored at −80°C until analysis.

Determination of Caspase Activity

Caspase-3/7 activity was measured in LM samples using Apo-One Homogeneous Caspase-3/7 Assay (Promega, Southampton, UK), adapted for tissue samples (Wagner et al., 2003). Frozen tissue samples were pulverized in liquid nitrogen, and 1 g was homogenized in 3 mL of extraction buffer [25 mM HEPES (pH 7.5), 0.1% (vol/vol) Triton X-100, 5 mM MgCl₂, 2 mM 1,4-Dithiothreitol (DTT), 74 mM antipain, 0.15 mM aprotinin, 1.3 mM EDTA, 20 μM leupeptin, and 15 μM pepstatin] for 2 × 20 s at full speed on ice. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant was removed for the assay. A 1:1 ratio of caspase reagent to sample supernatant was used for the assay, and this mixture was incubated at room temperature for 4 h. The end-point fluorescence was measured at an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm on a Fluostar Galaxy spectrometer (BMG Labtechnologies, Aylesbury, UK).

Caspase 9 activity was measured in the muscle samples using the Caspase-Glo 9 Assay (Promega, Southampton, UK). Samples were prepared as described for the caspase 3/7 assay, and a 1:1 mixture of caspase reagent to supernatant was again used for the assay. The reaction was incubated at room temperature for 3 h and end-point luminescence measured on a Fluostar Galaxy spectrometer (BMG Labtechnologies).

The caspase 3/7 and 9 assays involve an activation step cleaving any procaspase into its active isoforms; therefore the amount of fluorescence or luminescence product generated was directly proportional to the amount of caspase cleavage activity in the sample. Protein concentrations of sample supernatants used in the activity assays were determined using a Bradford assay (BioRad, Hemel Hempstead, UK).

Immunochemoical Analysis

Whole LM homogenates were prepared from each muscle sample for each time point for assessment of PARP and alpha II spectrin protein levels. Crushed tissue samples were homogenized in extraction buffer as described above, and an equal volume of double-concentrated SDS sample buffer [125 mM Tris-HCl pH 6.8, 4% (wt/vol) 10% SDS, 0.1 M DTT, and 10% glycerol 0.01% (wt/vol) bromophenol blue] was added. Samples were prepared by boiling for 5 min before centrifuging at 15,000 × g for 3 min. Volumes of whole muscle homogenates equivalent to equal wet weight of tissue were subjected to 6% SDS-PAGE. The gels were electrophoresed on a vertical, dual plate unit (Fisher, Loughborough, UK), and the separated proteins were transferred onto a polyvinylidene difluoride membrane (GE Healthcare Life Sciences, Little Chalfont, UK) using a Trans-Blot cell with plate electrodes (BioRad) according to the procedure by Towbin et al. (1979). Blots were immunoprobed with anti-human alpha II spectrin antibody diluted 1:1000 (Chemicon International, Temecula, CA) or with anti-human poly (ADP-ribose) polymerase (PARP) antibody diluted 1:200 (Abcam, Cambridge, UK). Protein bands were detected using the Enhanced Chemiluminescence (ECL) Plus detection system (GE Healthcare Life Sciences, Little Chalfont, UK) and subsequent exposure to ECL-Hyperfilm (GE Healthcare Life Sciences), and their intensity was quantified using a Quantity-One Multi Analyst (BioRad). Protein concentrations of extracted samples were determined using a Bradford assay (BioRad), and band intensities were normalized for protein concentration.

Shear Force

The shear force was measured by cooking the 192-h conditioned LM chops in a water bath at 80°C to an internal temperature of 78°C. The samples were cooled overnight at 4°C before shearing cored samples using a Stevens CR Analyzer fitted with Volodkevich-type jaws (Stable Micro Systems Ltd., Godalming, UK). The mean values of 8 shearings on subsamples of 1 cm² cross-section were recorded (Brown et al., 1998).

Data Analysis

Statistical analysis was performed using General ANOVA (Genstat for Windows, version 7.2, Hemel Hempstead, UK) to determine if caspase activities and protein levels of PARP and spectrin changed significantly during the postmortem conditioning period. Cor-
**Table 1.** Changes in caspase 3/7 and 9 activities, and protein levels of poly (ADP-ribose) polymerase (PARP) and alpha II spectrin breakdown products (SBDP), over time in porcine LM

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Caspase 3/7 activity, fluorescence/µg of protein</th>
<th>Caspase 9 activity, luminescence/µg of protein</th>
<th>PARP 89 kDa, units/µg of protein</th>
<th>SBDP120, units/µg of protein</th>
<th>SBDP150, units/µg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>110.49</td>
<td>55.3</td>
<td>21.52</td>
<td>2.59</td>
<td>1.44</td>
</tr>
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<td>129.88</td>
<td>58.58</td>
<td>11.14</td>
<td>2.98</td>
<td>1.19</td>
</tr>
<tr>
<td>4</td>
<td>115.87</td>
<td>56.81</td>
<td>19.65</td>
<td>2.82</td>
<td>1.73</td>
</tr>
<tr>
<td>8</td>
<td>110.66</td>
<td>39.9</td>
<td>18.84</td>
<td>1.23</td>
<td>2.25</td>
</tr>
<tr>
<td>16</td>
<td>120.52</td>
<td>48.07</td>
<td>ND¹</td>
<td>1.01</td>
<td>2.47</td>
</tr>
<tr>
<td>32</td>
<td>73.16</td>
<td>25.71</td>
<td>ND²</td>
<td>1.03</td>
<td>2.43</td>
</tr>
<tr>
<td>192</td>
<td>5.49</td>
<td>3.01</td>
<td>ND²</td>
<td>1.08</td>
<td>4.78</td>
</tr>
<tr>
<td>SED</td>
<td>10.32</td>
<td>5.22</td>
<td>5.14</td>
<td>0.57</td>
<td>0.59</td>
</tr>
</tbody>
</table>

P-value: P < 0.001  P < 0.001  P < 0.001  P < 0.001  P < 0.001

¹Arbitrary densitometry units.
²ND = Not detectable.

relations were performed using SPSS for Windows (version 14, Chicago, IL) between caspase activities, PARP, and alpha II spectrin across the time-course, with data being blocked for animal variation (58 df). Correlations between single-point values such as shear force and caspase activities, PARP, and alpha II spectrin protein levels at specific time points were performed.

**RESULTS**

**Caspase Activity**

Caspase 3/7 activity was found to decrease over the 192-h conditioning period. The highest activity was found at 2 h after slaughter, which was significantly higher than activity at 0 and 8 h (P = 0.042). By 16 h postmortem the majority of caspase 3/7 activity had occurred with the activity at 32 and 192 h being significantly lower than all the other time points (P < 0.001) (Table 1).

Like caspase 3/7 activity, over the 192-h postmortem period there was a general decrease in caspase 9 activity with the highest activity being at 2 h postmortem. Activity at time points 0, 2, and 4 h were all found to be significantly higher than that at 8, 32, and 192 h (P < 0.001; Table 1). Caspase 9 activity was found to positively correlate to caspase 3/7 activity across the whole time course (r = 0.92, P < 0.001).

**Immunochromical Analysis of Alpha II Spectrin and PARP**

Caspase-mediated cleavage of full length 214 kDa alpha II spectrin generates 150 and 120 kDa degradation products (SBDP150 and SBDP120, respectively). Alpha II spectrin can also be cleaved by calpains producing another 150 kDa peptide and a 145 kDa peptide (SBDP145; Warren et al., 2005). Western blots probed with anti-human alpha II spectrin detected immunopositive bands at 150 and 120 kDa that could correspond to the cleavage products of alpha II spectrin (Figure 1); SBDP145 is not recognized by this antibody. The level of SBDP150 was found to steadily increase with time postmortem, with the band intensity at 192 h being significantly higher than all the other time points (P < 0.001; Table 1). The intensity of SBDP120 immunopositive band peaked at 2 h, with levels at 8, 16, 32, and 192 h all significantly lower than those at time points 0, 2, and 4 h (P < 0.001; Table 1). Across the postmortem time course SBDP120 band intensity positively correlated to caspase 3/7 (r = 0.38, P = 0.003) and caspase 9 activities (r = 0.32, P = 0.012).

Protein levels of PARP during the postmortem conditioning period were investigated in this study as an indicator of caspase-mediated proteolysis and that apoptosis was occurring in the skeletal muscle sampled. Degradation of the 113 kDa full length PARP by caspases generates a distinct 89 kDa fragment containing the catalytic domain and a 24-kDa fragment, which contains the DNA binding domain (Soldani and Scovassi, 2002). The anti-human PARP antibody used in this study detected an 89-kDa band that could correspond to the caspase-generated cleaved isoform of PARP (Figure 2), the 24-kDa peptide is not recognized by this antibody. There was no significant difference in the 89kDa band intensity over the first 8 h postmortem, and after this period it was no longer detectable. Protein levels of PARP at 0, 2, 4, and 8 h were therefore all

![Figure 1](image-url)
significantly higher than at 16, 32, and 192 h \((P < 0.001; \text{Table 1})\). There was no association found between caspase 3/7 activity or caspase 9 activity and the 89 kDa PARP fragment; however, the appearance of the 89 kDa PARP peptide early in the postmortem period and its subsequent disappearance with time does indicate caspase activity and proteolysis of full length PARP during the postmortem conditioning period.

**Shear Force**

Shear force was determined in duplicate LM chops for all 10 pigs. The shear force values obtained would classify the LM from the carcasses examined as being within a tender cohort, with a mean shear force value of 4.54 kg ± 0.29 SEM. The relationships between shear force and the change in caspase 3/7 and 9 activities were investigated with the ratio of activities at 0 and 32 h being taken as an approximation of the change in caspase activity during the early postmortem period. It is in the early conditioning period that caspases appear to be most active, with activity levels of both caspas 3/7 and 9 being less than 6% of at death activity by 192 h. There was a significant correlation between 0:32 h caspase 9 activity ratio and shear force \((r = -0.68, P = 0.044; \text{Figure 3A})\), whereas there was a trend observed between the 0:32 h ratio of caspase 3/7 activity and shear force \((r = -0.62, P = 0.053; \text{Figure 3B})\). The caspase activity assays used in this study cleave all the procaspase present in the sample into the active caspase isoform. Therefore a high 0:32 h caspase ratio suggests a more rapid decline in caspase activity over this early time period, which according to this relationship was associated with a low shear force value. The relationships between shear force and the 89 kDa PARP isoform and SBDP120 were also investigated. Peak protein levels of SBDP120 at 2 h were found to negatively correlate to shear force \((r = -0.75, P = 0.012; \text{Figure 3C})\). No correlation between the 89 kDa PARP isoform protein levels and shear force was observed.

**DISCUSSION**

Herrera-Mendez et al. (2006) suggest that meat tenderization is a multienzymatic process involving not only the well studied systems of calpains and cathepsins but also proteasomes and caspases whose functions in postmortem muscle are less clear. In meat animals the last phase of slaughter is exsanguination, all cells and tissues will therefore be deprived of oxygen and nutrients, and under these extreme environmental condi-

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**Figure 3.** Relationships observed between shear force and a) caspase 9, 0:32 h activity ratio; b) caspase 3/7, 0:32 h activity ratio, and c) protein levels of the specific caspase 3, spectrin 120 kDa degradation product at 2 h postmortem, \(n = 10\). \(^1\)Arbitrary densitometry units.
After 8 h postmortem the 89 kDa PARP fragment may not be detected, although it is very probable that the 89kDa PARP peptide fragment is generally considered as a marker for apoptosis. Poly (ADP-ribose) polymerase is a zinc finger nuclear protein that is activated by the electron transfer protein cytochrome c is released, which in turn binds to apoptotic peptidase activating factor (Apaf-1), a cytoplasmic protein containing a caspase recruitment domain (CARD). Together Apaf-1 and cytochrome c undergo a conformational change allowing the binding of procaspase 9 and its activation. Downstream of caspase 9 are the executioner caspases 3 and 7, which are in turn activated and cleave specific target substrates including spectrin, actin, PARP, calpastatin, and lamins (Earnshaw et al., 1999). The entire process of apoptosis from the initial stimuli to the complete destruction of the cell can take hours or even days. However, from the first mitochondrial changes to the activation of caspases only takes approximately 10 min, and once committed cell death is inevitable (Green, 2005).

The changes in caspase activities and protein levels of PARP and alpha II spectrin fragments observed in this study signifies that caspases are active during the conditioning period in pigs. The positive correlation shown between the initiator and executioner isoforms indicate that these changes correspond to the cascade of events that occurs in situ when tissue is stimulated by hypoxic stress, suggesting that this may also be occurring in the LM sampled.

The activated caspases function in an executive role switching off protective pathways and switching on downstream activities that in turn lead to cellular destruction. During apoptosis many proteins undergo degradation by caspases; PARP was identified as the first protein to be proteolyzed and the appearance of the 89-kDa PARP peptide fragment is generally considered as a marker for apoptosis. Poly (ADP-ribose) polymerase is a zinc finger nuclear protein that is activated by breaks in DNA and is involved in regulating poly (ADP-riboseylation), a posttranslational modification that plays a role in DNA repair, replication, differentiation, and the activation of cellular defenses in response to DNA damage (Soldani et al., 2001). The PARP can be cleaved by all caspases in vitro, although in vivo only caspases 3 and 7 are capable of causing degradation (Soldani and Scovassi, 2002). Whether this specificity exists in situ in postmortem muscle has not been determined, although it is very probable that the 89kDa PARP peptide product is a marker of caspase-mediated proteolysis. The detection of 89 kDa PARP isoform by Western blot analysis and its degradation over the postmortem period indicates that caspase-mediated proteolysis does occur in the skeletal muscle postmortem. After 8 h postmortem the 89 kDa PARP fragment may have been degraded further to the 24 kDa peptide; however, this can only be speculated because the antibody used was not specific for this isoform of PARP. Alvarez-Gonzalez et al. (1999) studied the location of PARP in HeLa cells and found that the 89 kDa isoform translocated from the nucleoli during apoptosis. It is therefore plausible to suggest that this translocation may also be happening in situ and could also be an explanation for the disappearance of the 89 kDa fragment. Importantly, this PARP degradation was over a time period, which was coincident with the greatest change in caspase activity.

The other caspase specific substrate investigated in this study was alpha II spectrin, a tetrameric actin cross-linking protein that is involved in the maintenance of membrane structural integrity. The alpha II spectrin antibody used in this study is not able to distinguish between the calpain and caspase-generated SBDP150; therefore, it is not possible to conclude which protease is involved in the generation of the SBDP150 detected by Western blotting. The level of SBDP150 in the LM increased during the conditioning period, with the highest levels occurring at 192 h, but there was no association found between SBDP150 levels and caspase 3/7 activity or caspase 9 activity. This could suggest that SBDP150 is not created by caspase-mediated proteolysis of alpha II spectrin and that calpains may be responsible for its generation, or that caspase cleavage is masked by that of calpains.

Western blots probed with the anti-human alpha II spectrin antibody also detected SBDP120, which has been used as a marker for caspase-mediated proteolysis (Nath et al., 2000). Western blot analysis showed that peak SBDP120 levels occurred within the first few hours after death, corresponding with the patterns observed for both caspase 3/7 and caspase 9 activity. The degradation of alpha II spectrin during apoptosis could significantly compromise the membrane permeability and is also likely to compromise cytoskeletal integrity (Wang, 2000), a process that is associated with meat tenderization (Taylor et al., 1995). These observations, combined with the positive correlations found between SBDP120 protein levels and caspase 3/7 and 9 activities and those made regarding the detection and degradation of the 89 kDa PARP peptide product, strengthen the suggestion that caspase-mediated proteolysis does occur in skeletal muscle during the postmortem conditioning period.

The hypothesis that caspases contribute to the development of tender meat and that this is likely to be an initiating event early in the meat conditioning period is supported by the negative relationships between shear force and SBDP120 band intensity and also the negative relationships between shear force and the 0.32 h caspase 3/7 and 9 activity ratios. Unfortunately, very little is known about the molecular mechanisms behind the caspase activation pathways with respect to postmortem proteolysis, and this study must therefore be regarded as a preliminary investigation into their po-
tential connection with meat tenderization. However, in this study we have shown that caspase 3/7 and 9 activities are detectable in skeletal muscle and that activity declines significantly after the initial phase of postmortem storage. Additionally, protein levels of caspase-generated degradation peptides 89 kDa PARP and SBDP120 peaked within the first 4 h after death. This preliminary study suggests that it is in the early stages of the conditioning period that caspases are at their most active, and because of their substrate specificity, they could contribute to early postmortem proteolysis and meat quality. Our ongoing investigations are seeking to determine the extent of degradation of myofibrillar proteins by caspases in situ in order to try and ascertain their potential role in tenderization.

**LITERATURE CITED**


