The Caspase System: a Potential Role in Muscle Proteolysis and Meat Quality?

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Introduction

Meat quality is the generic term used to describe the properties and perceptions of meat which are generally considered to be colour, flavour, texture, tenderness and juiciness (Maltin et al., 2003), with tenderness considered as one of the most important traits (Miller et al., 2001). Meat tenderisation occurs primarily from the weakening of the myofibrillar structure in muscle fibres via the actions of proteolytic enzymes (Sentandreu et al., 2001). There are several proteolytic systems that could have a potential role in post-mortem proteolysis and meat tenderisation, including the cathepsins, the multicatalytic protease (or proteasome) and calpains. The contribution of cathepsins to meat tenderisation is no longer considered to be a strong candidate as they are contained within the lysosomes and appear not to have access to the myofibrillar structure at critical stages of the post-mortem proteolysis period (Hopkins and Taylor, 2004). There is also little association between cathepsin activity and the variation in tenderness in meat (Whipple et al., 1990). Although the proteasome is found at relatively high levels in skeletal muscle and is well established as having a role in muscle function, it is thought that it does not contribute to meat tenderisation as it is unable to cleave myofibrillar proteins to generate a similar pattern to that seen in situ during the conditioning period (Taylor et al., 1995). Calpains are probably the most extensively studied protease family with regard to meat science and it is widely accepted that calpain-mediated proteolysis does play a major role in the process of meat tenderisation (Sentandreu et al., 2001). Although there is considerable evidence to support the role of the calpain system in the postmortem conditioning period and the development of meat tenderness, it has been suggested that the calpain system cannot be solely responsible for all the biochemical and structural changes that take place, and therefore other proteolytic systems must be involved (Jiang, 1998). Recently, several reviewers have examined the role of proteolytic systems in postmortem proteolysis and meat tenderisation and have proposed that the caspase family of proteases could be activated postmortem and influence the rate of tenderisation (Sentandreu et al., 2001; Herrera-Mendez et al., 2006; Ouali et al., 2006).

The Caspase Protease System

Caspase Structure and Function

Caspases are a family of intracellular cysteine aspartate-specific proteases (Alnermi et al., 1996). To date 14 members of the caspase family have been identified and phylogenetic analysis indicates that there are two subfamilies, namely those involved in the inflammatory response and those involved in apoptosis (Earnshaw et al., 1999). Apoptosis or programmed cell death is the organised dismantling of the cell, characterised by cell shrinking, DNA fragmentation, membrane blebbing and the formation of apoptotic bodies without inducing an inflammatory response (Wyllie et al., 1980). This contrasts with necrosis which usually involves an inflammatory response. The caspases involved in apoptosis can be further subdivided into initiator caspases such as caspases 8, 9, 10 and 12 and effector caspases such as 3, 6 and 7, depending on their position in the cell death pathway (Earnshaw et al., 1999). Initiator caspases are activated in response to pro-apoptotic signals and in turn activate the downstream effector caspases that are involved in the direct targeting and cleavage of specific proteins resulting in cell disassembly (Fuentes-Prior and Salvesen, 2003).

Caspase Activation Pathways

There are three main caspase activation pathways; the extrinsic pathway, the intrinsic pathway and the endoplasmic reticulum (ER)-mediated pathway (Figure 1). The extrinsic pathway, also referred to as the death receptor pathway, is triggered by cell surface receptors. The initiator caspases, caspases 8 and 10, are activated via this type of receptor-mediated signalling (Boatright and Salvesen, 2003). The intrinsic pathway involves caspase 9 and is activated in response to environmental stress such as hypoxia and...
ischemia, acting via mitochondrial damage and release of cytochrome C (Earnshaw et al., 1999). The ER-mediated pathway is activated via stress directly upon the ER, such as disruption in Ca\(^{2+}\) homeostasis, which in turn activates initiator caspase 12.

**Caspase Regulation**

Inadvertent activation of caspases can have devastating effects and is therefore strictly regulated to protect from inappropriate caspase-directed proteolysis. There are a number of mechanisms that are involved in the regulation of caspase activation, including those acting via the Bcl-2 family, IAPs (Inhibitors of Apoptosis Proteins), ARC (Apoptosis Repressor with CARD (caspase recruitment domain)) or FLIP (FLICE (Fas associated death domain-like IL-\(\beta\)-converting enzyme)-inhibitory protein) (Earnshaw et al., 1999). Interactions between these inhibitory proteins and caspases are outlined in Figure 1.

**Caspase Substrates**

Caspases function in a mainly executive role, switching off protective pathways and turning on downstream activities, which in turn lead to cellular destruction (Earnshaw et al., 1999). To date more than 280 targets of caspase-mediated proteolysis have been identified (Fischer et al., 2003). Proteins targeted and cleaved by caspases include cytoskeletal proteins, nuclear structural proteins, cytokines, membrane receptors, proteins involved in DNA synthesis and repair, RNA synthesis and also transcription factors (Schuh and Schulze-Osthoff, 1998).

**Interactions between the Calpain and Caspase Systems**

There is increasing evidence for interactions between the calpain and caspase protease systems, predominantly focusing on the protein substrates that are targeted. Indeed, some authors have claimed that certain calpain isoforms and caspase family members exhibit virtually identical substrate specificity (Wang, 2000). Furthermore, Nakagawa and Yuan (2000) demonstrated that a disruption in Ca\(^{2+}\) homeostasis as a result of ischemic injury, induced calpain-mediated activation of caspase 12 and that the anti-apoptotic protein Bcl-XL was cleaved by m-calpain transforming it into a pro-apoptotic protein. The endogenous calpain inhibitor calpastatin is also cleaved by caspases 1, 3 and 7, generating distinct degradation patterns (Wang et al., 1998). Therefore if caspases are indeed active in the muscle postmortem they may influence meat quality either directly or by proteolysis of calpastatin. If the latter, this in turn could result in the activation of calpain which are proven to be involved in meat tenderisation and thus reduced toughness. The potential for interaction between the calpain and caspase systems was highlighted further in a study by Neumar et al. (2003), who demonstrated that calpain inhibition though over-expression of calpastatin promoted caspase 3 activity and apoptosis.

**Caspases and Skeletal Muscle**

Caspase expression has shown to be up-regulated in a number of skeletal muscle diseases and conditions including sarcopenia (Leeuwenburgh, 2003), Duchenne muscular dystrophy (Martini, 2001) and hypoxia/ischemia (Adams et al., 2001). Quite apart from their probable involvement in skeletal muscle atrophy, caspases have also been shown to play key roles in skeletal muscle development, with caspase expression being essential for normal muscle differentiation during myogenesis (Fernando et al., 2000).
Table 1. Changes in caspase 3/7 and 9 activities and protein levels of poly (ADP-ribose) polymerase (PARP 89kDa) and alpha II spectrin breakdown degradation product 120 kDa (SBDP120), the caspase-mediated proteolysis breakdown products, over time in porcine longissimus dorsi.

<table>
<thead>
<tr>
<th>Time h</th>
<th>n</th>
<th>Caspase 3/7 activity</th>
<th>Caspase 9 activity</th>
<th>PARP 89 kDa</th>
<th>SBDP120</th>
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<tr>
<td></td>
<td></td>
<td>fluorescence/µg protein</td>
<td>luminescence/µg protein</td>
<td>units/mg protein</td>
<td>units/mg protein</td>
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<tr>
<td>0</td>
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<tr>
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<tr>
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<tr>
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<td>5.22</td>
<td>5.14</td>
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<tr>
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<td>P&lt;0.001</td>
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</tbody>
</table>

ND = not detectable
1 arbitrary densitometry units

Caspases and Postmortem Proteolysis

In meat animals the process of exsanguination occurs during slaughter, depriving all cells and tissues of nutrients and oxygen (Herrera-Mendez et al., 2006). After death essentially normal metabolism continues in muscle for a short time, but inevitably muscle cells will soon start to die. It has been suggested that apoptosis rather than necrosis is the most likely process by which cell death occurs (Sentandreu et al., 2003). In our recent study, several components of the caspase system were analysed across longissimus dorsi (LD), trapezius (TZ), psoas (PS) and semitendinosus (ST) muscle samples in Large White pigs. This work has shown that caspases and the skeletal muscle-specific caspase inhibitor ARC can be detected in all these muscle types and that levels of expression and activity vary significantly; for example, there is 13 fold more caspase 12 in ST in comparison to LD muscle (Kemp et al., 2006a). This suggests that different muscles will vary in their capacity to carry out caspase-mediated proteolysis. However our research found no association between the pattern of caspase activity and the fibre type profile of particular muscle types based on myosin heavy chain analysis (Kemp et al., 2006a).

Caspase Activity across the Postmortem Conditioning Period

The study described above indicated that caspases were present in a range of muscle types and that caspase activity could be found at and around the point of slaughter, indicating that apoptotic processes are in place and potentially primed to make a contribution to postmortem proteolysis (Earnshaw et al., 1999). In a subsequent preliminary study with ten animals, we examined the levels of caspase activity in porcine LD muscle during the meat conditioning period up to eight days post-mortem (Kemp et al., 2006b). The activities of effector caspases 3 and 7 (combined activity assay) and the initiator caspase 9 were found to decrease over the postmortem conditioning period (Table 1). Caspases 3/7 and 9 activities were highest in the early stages of the postmortem conditioning period, with less than 6% of at death activity remaining after 192 h. Amongst the individual animals, the post-mortem changes in caspase activity were quite variable and the ratio of activities at 0 and 32 h was taken as an indication of the residual caspase activity in an individual animal during the early postmortem period. Thus a high value of the ratio could indicate that most of the caspase had been activated and used prior to autolytic removal during the previous 32h conditioning. There was a negative relationship between shear force and the 0:32 h ratio of caspase 9 (r = -0.68, P = 0.044) (Figure 2A) and caspase 3/7 activities (r = -0.62, P = 0.053) (Figure 2B). Caspase substrates alpha II spectrin and poly (ADP-ribose) polymerase (PARP) were analysed in muscle samples taken across the conditioning period and their specific caspase-mediated degradation products were detected by Western blot analysis (Table 1). The caspase-specific cleavage products of PARP and alpha II spectrin are both known indicators of apoptosis and the changes observed in their protein levels corresponded with those detected in caspase activities. 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) (Figure 2C). These preliminary findings indicate that changes in caspase activity and caspase-mediated cleavage do in fact take place in muscle during the conditioning pe-
riod and that this appeared to be associated with the develop-
ment of more tender meat.

Recombinant Caspase 3 Proteolysis of Myofibrillar Proteins

To gain an indication whether caspases were capable of degrading myofibrillar proteins we carried out in vitro expe-
riments examining the effect of co-incubation of recombi-
nant caspase 3 on purified myofibrils. A full length human recombinant caspase 3 (kind gift from Henning R. Sten-
nicke, Nova Nordisk, Bargværd, Denmark) was expressed in E.coli and purified to homogeneity. Myofibrils were ex-
tracted from porcine LD muscle according to the procedure of Goll et al. (1974) and incubated with recombinant caspase 3 (rC3) in a buffer designed to simulate conditions found in muscle postmortem, according to Winger and Pope (1981). The purified myofibrils were incubated with either increasing concentrations of rC3 or for different dura-
tion. The resulting digests were examined by SDS-
polyacrylamide gel electrophoresis. Although there was a visible change in some bands, indicating degradation of myofibrillar proteins, the major proteins myosin heavy chain and actin appeared to be mainly intact. However there was a visible decrease in the band intensity of proteins identified as desmin and troponin I (based on apparent mo-
lecular weight) which correlated with increasing concentra-
tions of rC3 (Figure 3A). In addition the appearance of bands at approximately 32, 28 and 18 kDa was observed, which were presumed to be the result of caspase 3-
mediated protein degradation. Using MALDI-TOF mass spectrometry these bands were identified as being derived from actin, troponin T and myosin light chain respectively. These degradation patterns were not observed in myofibrils incubated in the presence also of the caspase 3 specific inhibitor Ac-DEVD-CHO (Figure 3B). However co-
incubation with either the endogenous calpain inhibitor calpastatin or the Ca²⁺ chelator EDTA in the presence of rC3 still showed an increase in band intensities at 32, 28 and 18 kDa (Figure 3B), suggesting that conditions where calpain is inhibited still permitted recombinant caspase 3 activity.

Myofibrils were subsequently incubated with rC3 at 4°C for up to 8 d to simulate the postmortem conditions in vitro. Incubation of isolated myofibrils with rC3 over this time period resulted in the visible degradation of a number of protein bands identified as desmin and troponin I, with tro-
ponin 1 disappearing completely after 8 d of incubation (Figure 3C). The 28 kDa troponin T degradation product, which has been shown to positively associate with meat tenderness (Huff-Lonergan et al., 1996), increased, as did the 18 kDa myosin light chain degradation product. In comparison these degradation patterns were not observed in myofibrillar preparations incubated for 8 d at 4°C in the absence of rC3 (Figure 3C), indicating that rC3 was responsi-
bile for the degradation observed. These incubation studies have shown that rC3 is capable of causing proteolysis of key myofibrillar proteins under conditions that are similar to those found in the muscle in the postmortem conditioning period.
Conclusion

The ultimate tenderness of meat is to a large extent dependent on the degree of alteration and weakening of myofibrillar structures and has been largely attributed to endogenous proteolytic enzymes (Sentandreu et al., 2002). Our research into the potential role of caspases in the postmortem conditioning period and meat tenderness has shown that caspases are expressed in a number of different muscle types and their activity can be detected across the postmortem conditioning period, on a similar time scale as µ-calpain. Additionally caspase-mediated myofibrillar degradation patterns detected in vitro are similar to those observed in situ. These preliminary investigations have indicated that the caspase proteases appear to fulfill some of the criteria that were stipulated by Koohmaraie (1988) as a requirement for a protease system to influence meat quality. Firstly caspases are endogenous to skeletal muscle fibres and secondly it appears that they have the ability to degrade myofibrillar proteins. Our investigations are seeking to build on our observations to investigate whether caspases do contribute to the process of meat tenderisation in situ and whether variability within this system or in its response to stimuli at slaughter could be contributory to the factors that are known to influence meat quality.

References


Huff-Lonergan E; Mitsushashi T; Beekman DD; Parrish FC, Jr.; Olson DG & Robson RM. (1996). Proteolysis of specific muscle structural proteins by μ-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. J Anim Sci 74, 993-1008.


Figure 3. The effects of incubating porcine myofibrils with recombinant caspase 3 (rC3). A) The effect of increasing concentrations of rC3 at 37°C for 24 h. B) The effect of co-incubation: lane 1 10 units rC3 + 5 mM EDTA, lane 2 no rC3 + 5 mM EDTA, lane 3 10 units rC3 + 50 µl semi-purified calpastatin, lane 4 10 units rC3 + Ac-DEVD-CHO (0.1 µg/µl). C) The effect of incubating myofibrils for 8 d at 4°C with or without 10 units rC3. In both figures the major degradation products generated by caspase-mediated proteolysis are indicated by their molecular weights. Abbreviations: M, myosin heavy chain; α, α-actinin; D, desmin; A, actin; T, troponin I.


Ouali A; Herrera-Mendez CH; Coulis G; Becila S; Boudjellal A; Aubry L & Sentandreu MA. (2006). Revisiting the conversion of muscle into meat and the underlying mechanisms. Meat Science 74, 44-58.


