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WHAT INFORMATION DOES THE CALLIPYGE LAMB GIVE US ABOUT THE CALPAINS AND POSTMORTEM TENDERIZATION?

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Background:

Although a substantial amount of evidence has accumulated during the past 20 years to suggest that the calpain system has an important role in postmortem tenderization (Goll *et al.,* 1995; 1998; Koohmaraie, 1992; 1994; 1996), it is still unclear how this system functions in postmortem muscle. The three well-characterized components of the calpain system, μ-calpain, m-calpain, and calpastatin, are all present in varying ratios in the skeletal muscles of domestic animals. It has been established in *in vitro* assays that μ-calpain requires 3-50 μM Ca^{2+} for half-maximal proteolytic activity; that m-calpain requires 400-800 μM Ca^{2+} for half-maximal activity; and that calpastatin specifically inhibits the activity of both μ- and m-calpain but of no other proteolytic enzymes that have been tested thus far (Goll *et al.,* 1992). Ca^{2+} concentrations approximately equal to those required for proteolytic activity also cause autolysis of the two calpains, and the presence of an autolyzed calpain molecule has been used to indicate that the calpains have been "activated". Studies using a m-calpain that had been mutated so it could no longer undergo autolysis, however, have shown that unautolyzed calpain is fully active proteolytically, so autolysis is not an accurate indicator as to whether the calpain has been "activated".

The free Ca^{2+} concentration in skeletal muscle is 0.05-0.4 μM and increases to approximately 100 μM by the time that muscle enters rigor mortis (loss of extensibility). This increase in free Ca^{2+} occurs at the same time that the pH and temperature in postmortem muscle are decreasing. Because both μ- and m-calpain are optimally active at pH 7.5 and 25-37^0 C, and retain approximately only 15% of their optimal activity at pH 5.5, even at optimal temperature, the conditions in postmortem muscle are very unfavorable for calpain activity. Indeed, it has been suggested that Ca^{2+} acts directly on the myofibrillar structure and that its effects on postmortem tenderness are not mediated by the calpains (Takahashi, 1992). Only a few of the myofibrillar proteins (actin, myosin, and troponin C) have been demonstrated to bind Ca^{2+} with micromolar dissociation constants, however, and it seems unlikely that Ca^{2+} could affect the myofibrillar proteins directly without binding to them. The ultrastructurally observed changes in postmortem muscle occur in the thin filament near the Z-disk (Taylor *et al.*, 1995), and it is unlikely that Ca^{2+} binding to troponin C or myosin could cause these changes. Moreover, Uytterhaegen *et al.* (1995) have shown that injection of a variety of protease inhibitors capable of inhibiting the calpains in *in vitro* assays prevented postmortem tenderization; injection of inhibitors that inhibit serine or aspartic proteases, however, had no effect on postmortem tenderization. Hence, the evidence indicates that the calpains can act under the unfavorable conditions in postmortem muscle to increase tenderness, and research can therefore be focused on the mechanism that the calpains use to cause tenderization.

A number or studies have shown that calpastatin activities, especially the activity measured at 24 h postmortem, is highly related to extent of postmortem tenderization (see Koohmaraie, 1992; 1994, 1996 for reviews). The callipyge gene in sheep results in significantly increased muscle mass in certain muscles and in meat that undergoes very little tenderization during postmortem storage (Koohmaraie *et al.*, 1995). The calpastatin activities in those muscles affected by the callipyge gene (for example, the *biceps femoris, longissimus)* are 83% (*longissimus*) to 124% (*biceps femoris*) higher than in normal animals (Koohmaraie *et al.*, 1995). The activities of the two calpains and changes in calpastatin activity during postmortem storage of callipyge muscle have not been studied.

Objectives:

Earlier studies have shown that extractable μ-calpain activity decreases rapidly during the first 24-48 h of postmortem storage in bovine muscle; that calpastatin activities also decrease during postmortem storage but at a slower rate than μ-calpain activity; and that m-calpain activity remains nearly constant out to seven days of postmortem storage (Boehm *et al.*, 1998*)*. These results have raised questions about the role of μ-calpain in postmortem tenderization, even though the Ca^{2+} concentration required for μ-calpain activity is closer to the 100 μM Ca^{2+} that exists in muscle entering rigor mortis than the 400-800 μM Ca^{2+} required for activity of m-calpain. Because muscle from lambs having the callipyge gene undergo very tenderization during postmortem storage and also have high calpastatin activities, the changes in μ-calpain activity, m-calpain activity, and calpastatin activity during postmortem storage of muscles from callipyge and from normal lambs were monitored to learn whether the μ-calpain activities in callipyge muscle decreased more rapidly than in muscle from normal lambs. Such a finding would support the concept that μ-calpain is the primary calpain in postmortem tenderization.

Methods:

Twelve Dorset sheep (11 ewes and 1 ram), six of them carriers and six of them noncarriers of the callipyge gene, averaging 3 years of age were slaughtered in pairs. The *biceps femoris, longissimus, and infraspinitus* (the *infraspinitus* muscle is not affected by the callipyge gene) were removed from the left side of the carcass at-death and used for at-death assays. These same muscles were removed from the right side of the carcass after 24 h storage at 4^0 C. Activities of μ-calpain, m-calpain, and calpastatin were determined for all three muscles in at-death (0-time) muscle and in muscle after 1, 3 and 10 days of postmortem storage at 4^0 C. Western blot analysis was done for all three of these proteins at the same four sampling times. Tenderness (Warner-Bratzler shear) of the *longissimus* muscle was measured after 1, 3, and 10 days of postmortem storage, and the myofibril fragmentation index was determined for all three muscles at all four sampling times. Sarcomere lengths were determined for all three muscles after 1 and 10 days of postmortem storage. Earlier studies had shown that a significant amount of μ-calpain becomes associated with the myofibrillar fraction during postmortem storage (Boehm *et al.*,1998), so the amount of μ-calpain associated with the myofibrillar fraction of the

longissimus muscle was determined by using western blotting analysis of at-death muscle and of muscle after postmortem storage for 1, 3, and 10 days. The calpain activity associated with the *longissimus* myofibrillar fraction was also measured at these same four time-periods, and the effects of several different protease inhibitors on this activity was determined.

Results and Discussion:

Both μ-calpain and calpastatin activities decreased during postmortem storage of all three muscles and for muscles from both the callipyge and normal lambs. Calpastatin activity was higher in at-death muscle from the callipyge than in at-death muscle from normal lambs and stayed higher during the 10 days of postmortem storage; calpastatin activity in 10-day callipyge muscle was the same as calpastatin activity in 1-day muscle from normal lambs. Calpastatin activities remained high in the *infraspinitus* muscle even though the growth of this muscle is not affected by the callipyge gene. μ-Calpain activities decreased more rapidly during postmortem storage in muscle from normal lambs than in muscle from the callipyge lambs, even though the *longissimus* muscle from normal lambs was much more tender than the muscle from the callipyge lambs. Neither the tenderness (Warner-Bratzler shear) nor the myofibril fragmentation index of the *longissimus* muscle from the callipyge lambs changed significantly during the 10 days of postmortem storage although both the tenderness and myofibril fragmentation index of this muscle from the normal lambs increased significantly during this period. These results indicate that the relationship between μ-calpain activity and rate of postmortem tenderization is not a simple one.

m-Calpain activity changed very little in any of the three muscles studied during postmortem storage for up to 10 days, and there was no consistent difference between callipyge and normal muscles for any of the three muscles studied. Hence, differences in m-calpain activity also do not explain the differences in rate of postmortem tenderization between the callipyge and the normal *longissimus*.

The amount of μ-calpain associated with the myofibrillar fraction isolated from the *longissimus* muscle increased significantly during the first day of postmortem storage and then remained nearly constant during the next 9 days postmortem. Approximately 50-60% of total μ-calpain protein was associated with the myofibrillar fraction at day-1 postmortem, and almost all the 80 kDa subunit of this myofibril-bound μ-calpain was in the 78-and 76-kDa autolyzed form. Activity of the myofibril-bound calpain paralleled the activity of the extracted μ-calpain; this activity was higher for myofibrils from the callipyge (tougher) phenotype than for myofibrils from the normal phenotype. The activity measured in the assays was inhibited by E-64, leupeptin, and iodoacetate, indicating that it originated from a cysteine protease, almost certainly calpain. The myofibrillar bound calpain activity was only partly inhibited by calpastatin, even though the calpastatin completely inhibited μ-calpain activity in *in vitro* assays. It is possible that binding to a myofibrillar fraction partly protects μ-calpain from calpastatin inhibition, as has been suggested (Boehm *et al.*, 1998).

Western analysis showed that the calpastatin polypeptide was degraded during postmortem storage; the rate of this degradation was more rapid in muscles from the normal lambs than in muscles from the callipyge lambs. Also, the "autolytic" conversion of the 80 kDa subunit of μ-calpain to the 78- and 76-kDa forms occurred more rapidly in muscles from the normal lambs than in muscles from the callipyge lambs.

Conclusions.

The relationship between μ-calpain activity and tenderness or between m-calpain activity and tenderness is complex. The only activity that seemed to be consistently related to tenderness was calpastatin activity. Calpastatin activity is greater than the combined activity of μ-calpain and m-calpain in muscle at death, and this difference is much larger in callipyge muscles than in muscles from normal lambs. The excess of calpastatin activity over calpain activity decreases with increasing time of postmortem storage because calpastatin activity decreases while m-calpain activity remains nearly constant. The point at which total calpain activity begins to exceed calpastatin activity occurs about 1 to 3 days postmortem in muscles from normal lambs but does not occur even after 10 days of postmortem storage for muscles from callipyge lambs. A simple explanation would be that as long as calpastatin activity exceeds **total** calpain activity, no or very little postmortem tenderization occurs. This suggests that postmortem tenderization is the result of both μ- and m-calpain activity and not just one or the other.

References:

Boehm, M.L., Kendall, T.L., Thompson, V.F., and Goll, D.E. (1998). Changes in the calpains and calpastatin in postmortem muscle. J. Anim. Sci. **76**, 2415-2434.

Goll, D.E., Boehm, M.L., Geesink, G.H. and Thompson, V.F. (1998). What causes postmortem tenderization? Proc. 50th Annual Reciprocal Meat Conf., National Live Stock and Meat Board, Chicago, Il. pp. 60-67.

Goll, D.E., Geesink, G.H., Taylor, R.G., and Thompson, V.F. (1995). Does proteolysis cause all postmortem tenderization or are changes in the actin/myosin interaction involved? Proc. 41st Annual International Congress of Meat Science and Technology, National Live Stock and Meat Board, Chicago, Il. pp.537-544.

Goll, D.E., Thompson, V.F., Taylor, R.G., and Zalewska, T. (1992). Is calpain activity regulated by membranes and autolysis of by calcium and calpastatin? BioEssays **14**, 549-556.

Koohmaraie, M. (1992). The role of Ca^{2+}-dependent proteases (calpains) in post mortem proteolysis and meat tenderness. Biochimie **74**, 239-245.

Koohmaraie, M. (1994). Muscle proteinases and meat aging. Meat Sci. **36**, 93-104.

Koohmaraie, M. (1996). Biochemical factors regulating the toughening and tenderization processes of meat. Meat Sci. **43**, S193-S201.

Koohmaraie, M., Shackelford, S.D.,Wheeler, T.L., Lonergan, SM., and Doumit, M.E. (1995). A muscle hypertrophy condition in lamb (callipyge): characterization of effects on muscle growth and meat quality traits. J. Anim. Sci.**73**, 3569-3607.

Takahashi, K. (1992). Non-enzymatic weakening of myofibrillar structures during the conditioning of meat: calcium ions at 0.1 mM and their effect on meat tenderization. Biochimie **74**, 247-250.

Taylor, R.G., Geesink, G.H., Thompson, V.F., Koohmaraie, M., and Goll, D.E. (1995). Is Z-disk degradation responsible for postmortem tenderization? J. Anim. Sci. **73**,1351-1367.

Uytterhaegen, L., Claeys, E., and Demeyer, D. (1994). Effects of exogenous protease inhibitors on beef tenderness development and myofibrillar degradation and solubility. J. Anim. Sci. **72**, 1209-1223.