Interaction between myofibril structure and proteolytic tenderization in beef

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Abstract

The tenderization process of beef is a multifaceted process. Tenderness development is dependent on the architecture and the integrity of the skeletal muscle cell and on the activity of endogenous proteases within the cell. Also, the intracellular environment is an additional controlling factor that must be considered. In fact, it is in actuality, the interplay between these systems that is the critical factor in determining the rate of tenderization. Given the intricacy of the structure of the muscle cell, coupled with the complexity of the regulation of protease activity, and the ever-changing intracellular environment it is not surprising that this area of research is a very dynamic field. Just as the overall integrity and function of muscle cells does not depend on a single protein, but rather on the coordinated interaction of several proteins, the structural weakening of muscle cells during postmortem aging also must not depend on the degradation of a single myofibrillar or other cytoskeletal protein. The proteins mentioned in this review are located in different regions of the muscle cell, and most have been implicated in some manner as being important in maintaining the structure and function of the muscle cell. In addition, these proteins are located at regions that are affected during postmortem aging, including areas at or near the Z-line and in the I-band. Degradation of proteins such as desmin and filamin, located at the periphery of the Z-line, may disrupt the lateral register and integrity of the myofibril themselves as well as the attachments of the peripheral layer of myofibril to the sarcolemma. Degradation of the proteins within the myofibril that are associated with the thick and thin filaments may allow lateral movement or breaks to occur within the sarcomeres of postmortem aged samples. Titin, nebulin, and troponin-T, by their ability to directly interact with, or modulate the interaction between, major proteins of the thick and thin filaments and(or) the Z-line, play key roles in muscle cell integrity. Disruption of these proteins, especially titin and nebulin, could initiate further physicochemical and structural changes that result in myofibril fragmentation and loss of muscle cell integrity, and ultimately in tenderization of the muscle. In order to make real progress in this area, the scientific community must have a global appreciation of how both the structural proteins and the key proteases are influenced by the vast changes that occur during the conversion of muscle to meat.

Introduction

The tenderization process that beef undergoes during postmortem aging is a multifaceted process. From a physical standpoint, the development of tenderness is dependent on both the architecture and the integrity of the skeletal muscle cell and on the activity of endogenous proteases within the cell. To make matters more complex, the intracellular environment, the availability and the timing of the availability of metabolites are additional controlling factors that must be considered. In fact, it is in actuality, the interplay between these systems that is the critical factor in determining the rate of tenderization. Given the intricacy of the structure of the muscle cell, coupled with the complexity of the regulation of protease activity, and the ever changing intracellular environment it is not surprising that this area of research is a very dynamic field, and has been for numerous years.

It is interesting to that in 1948, Bate-Smith noted “because of the bewildering rate of growth of this fundamental knowledge (of physiological and biochemical properties and behavior of muscle) and the constantly changing conception of muscle which has resulted, there has not been so striking an advance in knowledge of the particular processes involved in the prolonged storage of meat, nor any striking application of the principles of modern biochemistry to the technology of handling of animals and meat.” (Bate-Smith, E.C., 1948). Even though the scientific community has continued to make great progress in understanding the physiology and biochemistry of muscle, we are still discovering a bewildering array of factors that can influence meat quality, and still are struggling with the ideal predictors of beef tenderness. Lowe (Lowe, B., 1948) noted that in poultry, microscopic changes were noted that seemed to parallel changes in tenderness. This work has was landmark and has been shown numerous times to be the same in multiple species including beef and point out the importance of understanding muscle structure and its relationship to tenderness.

Muscle Structure and Metabolism

Muscle cells are among the most highly organized cells in the animal body. This is because they perform a diverse array of mechanical functions. They are required for movement of limbs for locomotion and other gross movements, but also, they must perform finer tasks such as maintaining balance and coordination. Muscle movement and metabolism are also associated with other diverse functions such as aiding in maintaining body heat and movement of blood and lymph. Few cells are required to generate as much force and undergo as dramatic shifts in rate of metabolism as muscle cells. Thus the organization, structure and metabolism of the muscle is key to its function and to the maintenance of its integrity both during contraction and during the early postmortem period.

Muscle cells are striated, meaning that when viewed under a microscope, distinct banding patterns or striations are observed. This appearance is due to specialized organelles, myofibrils, found in muscle cells. Myofibrils are the contractile "machinery" of the cell, and, like the cells they reside in are very highly organized. When examining a myofibril, one of the first observations that can be made is that the cylindrical organelle is made up of repeating units. These repeating units are known as sarcomeres. Contained in each sarcomere are all of the structural elements needed to perform the physical act of contraction at the molecular level. Current proteomic analysis estimates that over 65 proteins make up the structure of the sarcomere (Fraterman, S., Zeiger, U., Khurana, T.S., Wilm, M. & Rubinstein, N.A., 2007). Given that this the sarcomere is the most basic unit of the cell and that the number quoted in this analysis did not take into account the multiple isoforms of the proteins, this number is quite high. Many of the proteins interact with each other in a highly coordinated fashion, and some of the interactions are just now being discovered. Protolysis or other modification of a relatively more minor or less studied protein may impact the structure of the sarcomere/myofibril in a subtle manner that may not be realized until a later time postmortem. This paper will describe some of the major changes in the myofibril during postmortem storage and will explore some of the possible changes that may be related to tenderness.

The structure of the sarcomere is responsible for the striated appearance of the muscle cell. The striations arise from alternating, protein dense A-bands and less dense I-bands within the myofibril. Bisecting the I-bands are dark lines known as Z-lines. The structure between two Z-lines is a sarcomere. The less dense I-band is made up primarily of thin filaments while the A-band is made up of thick filaments and some overlapping thin filaments (Goll, D.E., Robson, R.M. & Stromer, M.H., 1984). The backbone of the thin filaments is made up primarily of the protein actin while the largest component of the thick filament is the protein myosin. Myosin consists of a tail or rod region that forms the backbone of the thick filament and a globular head region that extends from the thick filament and interacts with actin in the thin filament. In order for contraction to occur, the thick and thin filaments interact via the head region of myosin. The complex formed by the interaction of myosin and actin is often referred to as actomyosin. In electron micrograph images of contracted muscle or of post-rigor muscle the actomyosin looks very much like cross-bridges between the thick and thin filaments, indeed, it is often referred to as such. In post-mortem muscle these bonds become irreversible and are also known as known as rigor bonds as they are the genesis of the stiffness that develops in postmortem muscle. The globular head of myosin also has enzymatic activity; it can hydrolyze ATP and liberate energy. In living muscle during contraction, the ATPase activity of myosin provides energy for myosin bound to actin to swivel and ultimately pull the thin filaments toward the center of the sarcomere. This shortens the myofibril, the muscle cell and eventually the muscle to produce contraction. The myosin and actin can disassociate when a new molecule of ATP is bound to the myosin head (Goll, D.E. et al., 1984). In post-rigor muscle, the supply of ATP is depleted, resulting in the actomyosin bonds becoming essentially permanent.

As muscle is converted to meat, many changes occur, including: ***1***) a gradual depletion of available energy, ***2)*** a shift from aerobic to anaerobic metabolism favoring the production of lactic acid resulting in the pH of the tissue declining from near neutrality to 5.4-5.8, ***3)*** a rise in ionic strength, in part, because of the inability of ATP-dependent calcium, sodium, and potassium pumps to function, and ***4)*** an increasing inability of the cell to maintain reducing conditions. All of these changes can have a profound effect on numerous proteins in the muscle cell, especially on one of the proteolytic enzyme systems that is thought to play a significant role in the tenderization that occurs during postmortem aging. Among some of the microenvironmental factors that can have a major influence on proteolysis and specifically, the calpain system are pH and ionic strength and oxidative and nitrosylation status of the proteins in the cell.

The Calpain Proteinase System.

The calpain system is composed of several isoforms of the proteolytic enzyme calpain, and an endogenous inhibitor of the calpains, calpastatin. The two best-characterized isoforms are µ - calpain and m-calpain. These isoforms require the presence of calcium to be active and are named in reference to the amount of calcium each requires for activity. In general, µ-calpain requires between 5 and 65 µM Ca^{2+} for half-maximal activity, while m-calpain requires between 300-1000 µM Ca^{2+} for half-maximal activity (Goll, D.E., Thompson, V.F., Taylor, R.G. & Christiansen, J.A., 1992). These two enzymes cleave the same myofibrillar proteins that are degraded during postmortem aging (Huff-Lonergan, E., Mitsuhashi, T., Beekman, D.D., Parrish, F.C., Olson, D.G. & Robson, R.M., 1996a; Kendall, T.L., Koohmaraie, M., Arbona, J.R., Williams, S.E. & Young, L.L., 1993) *without* degrading actin and myosin (Dayton, W.R., Reville, W.J., Goll, D.E. & Stromer, M.H., 1976). Both µ- and m-calpain are heterodimers composed of an 80 kDa and a 28 kDa subunit (Suzuki, K., 1990). The 28 kDa subunit is identical in both µ-calpain and m-calpain. The C-terminus of this subunit contains amino acid sequences that predict calcium binding E-F hand structures, however, the exact function of this subunit is not known. The 80 kDa subunits of µ- and m-calpains are similar, but are encoded for by different genes(Suzuki, K., 1990). The 80 kDa subunits of both proteins are composed of four domains. Domain I, the N-terminal domain, has no sequence homology to any known polypeptide. Domain II is the catalytic domain. This domain contains a cysteine residue as well as a histidine residue that are in relative positions that are conserved in all cysteine proteinases (including papain) (Suzuki, K., 1990). Domain III is not homologous to any other known protein, but has sequences that predict E-F hand Ca^{2+} binding sites (Goll, D.E., Thompson, V.F., Li, H.Q., Wei, W. & Cong, J.Y., 2003). Domain IV is a calmodulin-like domain that has sequences that predict E-F hand Ca^{2+} binding sites. Studies using fragments of µ- and m-calpain that contain domain II (catalytic domain) have shown that these fragments may not have catalytic activity by themselves (Nishimura, T. & Goll, D.E., 1991). This suggests that activity of these proteolytic enzymes may depend on regions of the molecule more distant from the active site. Thus the conformational state of µ - calpain and m-calpain may directly affect activity of these enzymes (Strobl, S., Fernandez-Catalan, C., Braun, M., Huber, R., Masumoto, H., Nakagawa, K. et al., 2000).

Although calcium is necessary for their activity, both µ- and m-calpain will also autolyze when incubated with calcium. Autolysis reduces the mass of the 80 kDa subunit of µ-calpain to 76 kDa, and the mass of the 80 kDa subunit of m-calpain to 78 kDa. The 28 kDa subunit of both enzymes is reduced to 18 kDa. Brief autolysis also reduces the Ca^{2+} concentration required for half-maximal activity of both enzymes. Extended autolysis leads to inactivation of the enzymes (Edmunds, T., Nagainis, P.A., Sathe, S.K., Thompson, V.F. & Goll, D.E., 1991). Autolysis occurs under situations that allow activity, both in living cells and in postmortem muscle, but the physiological significance of autolysis is not clear (Goll, D.E., Thompson, V.F., Taylor, R.G. & Zalewska, T., 1992; Johnson, G.V.W. & Guttmann, R.P., 1997). Both autolyzed and unautolyzed forms of the enzymes have been shown to have activity. However, the autolyzed form of µ-calpain appears to be more hydrophobic and binds tightly to subcellular organelles, including myofibrils (Boehm, M.L., Kendall, T.L., Thompson, V.F. & Goll, D.E., 1998). Presence of the autolyzed form of µ-calpain in post-mortem tissue has been suggested to indicate µ-calpain has been active.

In addition to the ubiquitous forms of calpain (µ-calpain and m-calpain), there are several other isoforms of the enzyme. The enzyme p94 was the first tissue specific calpain isoform to be discovered (Sorimachi, H., Imajohohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y. et al., 1989). This isoform is also known as calpain 3 in reference to the fact that it was the third isoform identified. The enzyme p94 is predominantly located in skeletal muscle in association with the myofibril, specifically with the megaprotein titin. This enzyme is similar in structure to the large subunit of µ-calpain and m-calpain. It has additional amino acid sequences making its predicted MW 94,000 compared to 80,000 for the large subunit of µ- and m-calpain. Also, it does not have the smaller 30,000 Da subunit that µ-calpain and m-calpain have. This isoform has proven very difficult to study as it apparently has a very low Ca^{2+} requirement and it autolyzes very rapidly when it is not associated with titin. Disruption of the gene for p94 has been shown to be causative for limb girdle muscular dystrophy type 2A. Its role in muscle remains somewhat of an enigma (Goll, D.E., Neti, G., Mares, S.W. & Thompson, V.F., 2008) and it role in postmortem muscle seems to be even more elusive (Geesink, G.H., Taylor, R.G. & Koohmaraie, M., 2005; Ilian, M.A., Bekhit, A.E.-D. & Bickerstaffe, R., 2004; Ilian, M.A., Bekhit, A.E.-D.A., Stevenson, B., Morton, J.D., Isherwood, P. & Bickerstaffe, R., 2004; Ilian, M.A., Bickerstaffe, R. & Greaser, M.L., 2004; Koohmaraie, M. & Geesink, G.H., 2006).

Both µ-calpain and m-calpain have slower rates of activity against myofibrillar protein substrates at pH

values and ionic strengths similar to those found in postmortem muscle (Geesink, G.H. & Koohmaraie, M., 1999; Huff-Lonergan, E. & Lonergan, S.M., 1999; Kendall, T.L. et al., 1993). Alterations in pH and/or ionic strengths may cause conformational changes that allow an increase in the hydrophobicity and aggregation of the enzyme. Likewise, pH/ionic strength changes may alter the conformation of substrate proteins and render them less susceptible to cleavage by µ-calpain (Huff-Lonergan, E. & Lonergan, S.M., 1999).

Because of the limited specificity of µ- and m-calpain, they do not degrade proteins to their constituent amino acids, nor do they degrade major myofibrillar proteins such as myosin or actin. A hypothesized role for calpains in muscle is the specific proteolysis of cytoskeletal proteins (titin and nebulin) and intermediate filaments (desmin) to initiate myofibrillar protein degradation. In one proposed model (Goll, D.E. et al., 2003)) calpains catalyze the release of myofilaments from the myofibril and are made available to the proteosome and lysosomes for complete degradation to amino acids. Purintrapiban et al. (Purintrapiban, J., Wang, M.C. & Forsberg, N.E., 2003) have provided compelling evidence that the calpain system works in concert with proteosome and lysosomal proteinases to degrade cytoskeletal and sarcomeric proteins. The calpain system has been implicated in several models of muscle hypertrophy including models using exogenous ß-adrenergic agonists (Wheeler, T.L. & Koohmaraie, M., 1992) and in sheep exhibiting the callipyge muscle hypertrophy phenotype (Koohmaraie, M., Shackelford, S.D., Wheeler, T.L., Lonergan, S.M. & Doumit, M.E., 1995). In these models, elevated expression and activity of calpastatin are documented, and it is hypothesized that calpastatin can inhibit protein degradation and allow for more efficient protein accretion. Calpains may also have a role in muscle development as it is frequently observed that calpain activity is necessary for fusion of developing myoblasts to form myotubes (Balcerzak, D., Cottin, P., Poussard, S., Cucuron, A., Brustis, J.J. & Ducastaing, A., 1998; Schollmeyer, J.E., 1986a, b), possibly by allowing cell migration and proper alignment of myoblasts prior to fusion (Dedieu, S., Poussard, S., Mazeres, G., Grise, F., Dargelos, E., Cottin, P. et al., 2004). Furthermore, there is strong evidence that calpastatin regulates this activity (Barnoy, S., Glaser, T. & Kosower, N.S., 1998; Dedieu, S. et al., 2004).

Rapid proteolysis of intermediate filament proteins in meat has been associated with improved water-holding capacity (Morrison, E.H., Mielche, M.M. & Purslow, P.P., 1998) and tenderness (Melody, J.L., Lonergan, S.M., Rowe, L.J., Huiatt, T.W., Mayes, M.S. & Huff-Lonergan, E., 2004b) in pork. There is strong evidence that the calpain enzymes are responsible postmortem proteolysis observed in pre- and post-rigor muscle (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a; Koohmaraie, M., 1992). Calpain proteinases are considered primary candidates of muscle protein degradation (including intermediate filament proteins such as desmin) initiated during the first 24 hours postmortem (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a; Koohmaraie, M., 1992).

Calpastatin

Calpastatin, the endogenous inhibitor of the calpain enzymes, has been found in all the tissues that contain calpains. Interestingly, calpastatin requires calcium to bind calpain. The amount of calcium required to allow half-maximal binding of calpastatin to calpains is generally lower than that required for half-maximal activity of the unautolyzed and autolyzed forms of m-calpain and µ-calpain (Kapprell, H.P. & Goll, D.E., 1989). This calpastatin binding is reversible as calcium chelators can cause calpastatin to dissociate from calpain (Otsuka, Y. & Goll, D.E., 1987).

Calpastatin has most commonly been described as a structure of four repeats of mutually homologous sequences at an interval of about 140 amino acids (domains 1, 2, 3, and 4) and an alkaline N-terminal region is referred to as domain L (Maki, M., Hatanaka, M., Takano, E. & Murachi, T., 1990). The intact calpastatin molecule is capable of inhibiting multiple molecules of calpain (Otsuka, Y. & Goll, D.E., 1987) due to the multiple inhibitor domains 1-4. Three regions (A, B, C) within domains 1-4 are responsible for binding to calpain and inhibiting calpain activity. Region A binds calpain domain IV, region C binds calpain domain VI. Region C appears to bind near the active site in calpain domain II or possibly in domain III. *Region B does not bind the active site cysteine, as numerous reports have shown that calpain with a chemically modified cysteine retains the capacity to bind to calpastatin* (Anagli, J., Vilei, E.M., Molinari, M., Calderara, S. & Carafoli, E., 1996; Kapprell, H.P. & Goll, D.E., 1989; Takano, E. & Maki, M., 1999)). Takano and Maki (Takano, E. & Maki, M., 1999) have proposed a "Tripartite Interaction Model" that suggests that binding by calpastatin regions A and C positions region B in the catalytic cleft of calpain.

Calpastatin is degraded in postmortem muscle (Lonergan, S.M., Huff-Lonergan, E., Rowe, L.J., Kuhlers,

D.L. & Jungst, S.B., 2001a) and there is good evidence that calpains are responsible for this activity (Doumit, M.E. & Koohmaraie, M., 1999). The rate of calpastatin degradation and inactivation is related to the rate of proteolysis and tenderization observed in meat (Geesink, G.H. & Koohmaraie, M., 1999; Lonergan, S.M. et al., 2001a; Lonergan, S.M., Huff-Lonergan, E., Wiegand, B.R. & Kriese-Anderson, L.A., 2001b)). However, the exact factors or sets of conditions that regulate the degradation of calpastatin by calpain are not known.

Even though there has been much research done on the calpain system over the years, still relatively little is known about its regulation. Certainly, the endogenous inhibitor of the calpains, calpastatin is involved, but there is evidence to suggest that other mechanisms may also be important, particularly in meat.

Protein Oxidation

Another change that occurs in postmortem muscle during aging of whole muscle products is increased oxidation of myofibrillar proteins (Martinaud, A., Mercier, Y., Marinova, P., Tassy, C., Gatellier, P. & Renerre, M., 1997; Rowe, L.J., Maddock, K.R., Lonergan, S.M. & Huff-Lonergan, E., 2004a, b). This results in the conversion of some amino acid residues, including histidine, to carbonyl derivatives (Levine, R.L., Williams, J.A., Stadtman, E.R. & Shacter, E., 1994; Martinaud, A. et al., 1997) and can cause the formation of intra and/or inter protein disulfide cross-links (Martinaud, A. et al., 1997; Stadtman, E.R., 1990). In general, both of these changes reduce the functionality of proteins (Xiong, Y.L. & Decker, E.A., 1995). Because µ-calpain and m-calpain enzymes contain both histidine and SH-containing cysteine residues at their active sites, they are particularly susceptible to inactivation by oxidation (Lametsch, R., Lonergan, S. & Huff-Lonergan, E., 2008). Therefore, oxidizing conditions in postmortem muscle lead to inactivation or modification of calpain activity (Harris, S.E., Huff-Lonergan, E., Lonergan, S.M., Jones, W.R. & Rankins, D., 2001; Maddock, K.R., Huff-Lonergan, E., Rowe, L.J. & Lonergan, S.M., 2006; Rowe, L.J. et al., 2004a, b). In fact, evidence suggests oxidizing conditions inhibits proteolysis by µ-calpain, but might not completely inhibit autolysis(Guttmann, R.P., Elce, J.S., Bell, P.D., Isbell, J.C. & Johnson, G.V., 1997; Guttmann, R.P. & Johnson, G.V., 1998; Maddock, K.R. et al., 2006). In postmortem muscle, there are differences between muscles in the rate that postmortem oxidation processes occur (Martinaud, A. et al., 1997). It has been noted that differences in the rate of oxidation in muscle tissue are seen when comparing the same muscles between animals and/or carcasses that have been handled differently (Juncher, D., Ronn, B., Mortensen, E.T., Henckel, P., Karlsson, A., Skibsted, L.H. et al., 2001). These differences may arise because of differences in diet, breed, antemortem stress, postmortem handling of carcasses, etc. In fact, there have been reports of differences between animals and between muscles in the activity of some enzymes involved in the oxidative defense system of muscle (Daun, C., Johansson, M., Onning, G. & Akesson, B., 2001). Therefore, there may be genetic differences in susceptibility to oxidation that could be capitalized on to improve meat quality. It is therefore reasonable to hypothesize that differences in the antioxidant defense system between animals and/or muscles would influence calpain activity, proteolysis, and thus tenderization.

Exposure to oxidizing conditions (H_2O_2) under post-mortem-like conditions inhibits calpain activity (Carlin, K.R., Huff-Lonergan, E., Rowe, L.J. & Lonergan, S.M., 2006). In a series of in vitro assays using either a fluorescent peptide or purified myofibrils as the substrate it was shown that the presence of oxidizing species does significantly impede the ability of calpains to degrade their substrates**.** Oxidation with H_2O_2 significantly limits proteolytic activity of µ- and m-calpain against the fluorescent peptide Suc-Leu-Leu-Val-Tyr-AMC, regardless of the pH or ionic strength. Similar results were seen when using purified myofibrils as the substrate). This inhibition was reversible as addition of reducing agent (DTT) to the oxidized samples restored activity. Oxidation also has been shown to slow the rate of µ-calpain autolysis and could be part of the mechanism underlying some of the retardation of activity (Carlin, K.R. et al., 2006; Guttmann, R.P., Elce, J.S., Bell, P.D., Isbell, J.C. & Johnson, G.V.W., 1997).

Oxidation does occur early in postmortem meat and it does influence proteolysis (Harris, S.E. et al., 2001; Rowe, L.J. et al., 2004b). Rowe et al., 2004 showed that, there was a significant increase in proteolysis of troponin-T in steaks from alpha-tocopherol fed steers after 2 days of postmortem aging compared to steers fed a conventional feedlot diet. This indicates that very low levels of oxidation ***can*** influence proteolysis and that increasing the level of antioxidants in meat may have merit in improving tenderness in future studies. In fact, low levels of oxidation may be the cause of some heretofore-unexplained variations in proteolysis and tenderness that have been observed in meat.

S-Nitrosylation

Nitric oxide is produced in biological systems by a family of enzymes known as nitric oxide synthases (NOS). There are three major isoforms of NOS, neural, inducible and endothelial. Skeletal muscle expresses all three isoforms, however, the neural form, nNOS is thought to be the predominant isoform (Kaminski, H.J. & Andrade, F.H., 2001). Nitric oxide is important in biological systems, particularly because of its role as a second messenger. However, while nitric oxide rapidly diffuses through tissues, nitric oxide itself is a relatively short-lived species. It does have the ability to combine with other biomolecules that also have physiological importance. One example of this is its ability to combine with superoxide to form the highly oxidizing molecule peroxynitrite. Proteins are important biological targets of peroxynitrite – particularly proteins containing cysteine, motioning and/or tryptophan (Radi, R., Denicola, A., Alvarez, B., Ferrer-Sueta, G. & Rubbo, H., 2000). Several enzymes have been shown to be inactivated by peroxynitrite. Among these is the sarcoplasmic reticulum Ca^{2+}-ATPase (Klebl, B.M., Ayoub, A.T. & Pette, D., 1998). One indirect effect of nitric oxide is S-nitrosylation. In most cases S-nitrosylation events involve amines and thiols. Nitric oxide can interact with cysteines to form nitrosothiols that can alter the activity of the protein. Because of this it has been suggested that S-nitrosylation may function as a post-translational modification much like phosphorylation (Jaffrey, S.R., Erdjument-Bromage, H., Ferris, C.D., Tempst, P. & Snyder, S.H., 2001). Some proteins like the ryanodine receptor and the cysteine protease caspase-3 have been shown to be endogenously nitrosylated – further supporting the suggestion that formation of nitrosothiols may be an important regulatory step (Hess, D.T., Matsumoto, A., Kim, S.O., Marshall, H.E. & Stamler, J.S., 2005; Hess, D.T., Matsumoto, A., Nudelman, R. & Stamler, J.S., 2001). µ- Calpain is also a cysteine protease that could be influenced by S-nitrosylation. Small thiol peptides like glutathione can be impacted by nitorsative stress to form compounds like S-nitrosoglutathione (GSNO). These compounds can in turn, influence other proteins by transnitrosating other reduced thiols (Miranda, K., Epsey, M.G., Jourd'heul, D., Grisham, M.B., Fukuto, J., Freelisch, M. et al., 2000). Because muscle contains all of the compounds needed to form these intermediates, it stands to reason that they could be important in the conversion of muscle to meat.

Major Postmortem Changes in Muscle Architecture

Another important question that must be answered is: What are the major changes that occur in muscle that affect tenderness? Certainly, as muscle goes into rigor, there is a loss of extensibility and along with that, a change in the texture of the meat. During storage, however, that product becomes more tender because of proteolytic changes occurring in the architecture of the myofibril and its associated proteins. There are several key proteins that are degraded during postmortem aging.

Titin

Titin (aka connectin) is a mega protein that is approximately 3 mega Daltons in size. In addition to being the largest protein found in mammalian tissues, it is also the third most abundant. A single titin molecule is estimated to be between 2 and 2.5 µM in length. In striated muscle, titin thus spans fully half of a sarcomere with its C-terminal end localizing in the M-line and the N-terminal forming an integral part of the Z-line. Titin aids in maintaining sarcomeric alignment of the myofibril during contraction. Titin integrates the z-line and the thick filaments – thus maintaining the location of the thick filaments between the Z-lines. Titin is also hypothesized to play a role in generating at least a portion of the passive tension that is present in skeletal muscle cells. During development of the myofibril, titin is one of the earliest proteins expressed and it is thought to act as a "molecular ruler" by providing a scaffolding or template for the developing myofbril (Clark, K.A., McElhinny, A.S., Beckerle, M.C. & Gregorio, C.C., 2002).

Titin also plays a role as a framework for specific ligands that are associated with it. These ligands are the major Z-line protein sarcomeric alpha-actinin, telethonin, muscle specific RING-finger protein-1 (MURF-1) and p94 (also known as calpain 3). These ligands bind to titin as specific locations. For example, sarcomeric alpha-actinin binds at the Z-line and p94 at both the N2 lines in the I-band and also at the M-line region (Clark, K.A. et al., 2002). This interaction between p94 and titin is particularly intriguing as the N_2 line in the I-band has long been known to be particularly labile (Taylor, R.G., Geesink, G.H., Thompson, V.F., Koohmaraie, M. & Goll, D.E., 1995). In addition, in the absence of titin, p94 autolyzes very rapidly, therefore, it is possible that titin may regulate the activity of this protease by providing a stabilizing force. Another interesting feature of p94 is the fact that its binding at the M-line region of titin is associated with the state of contraction of the myofibril. Its localization to this region is favored in contracted and/or relaxed myofibrils, however in stretched myofibrils,

p94 is redistributed to the N2 region of titin/myofibrils (Ojima, K., Ono, Y., Doi, N., Yoshioka, K., Kawabata, Y., Labeit, S. et al., 2007). One hypothesis is that p94 has to be proteolytically active for its redistribution in response to stretch to occur. The relationship between p94 and titin is complex and merits more investigation from both muscle biologists and meat scientists (Ojima, K. et al., 2007; Ono, Y., Kakinuma, K., Torii, F., Irie, A., Nakagawa, K., Labeit, S. et al., 2003; Ono, Y., Shimada, H., Sorimachi, H., Richard, I. & Saido, T.C., 1998).

Due to the aforementioned roles of titin in living cells, it is quite conceivable that its degradation in postmortem muscle would lead to weakening of the longitudinal structure of the myofibrillar sarcomere and integrity of muscle. This weakening, in conjunction with other changes in postmortem muscle, could lead to enhanced tenderness. The degradation of titin has been observed in several studies (Astier, C., Labbe, J.P., Roustan, C. & Benyamin, Y., 1993; Huff-Lonergan, E., Parrish, F.C. & Robson, R.M., 1995; Lusby, M.L., Ridpath, J.F., Parrish, F.C. & Robson, R.M., 1983; Melody, J.L., Lonergan, S.M., Rowe, L.J., Huiatt, T.W., Mayes, M.S. & Huff-Lonergan, E., 2004a; Rowe, L.J. et al., 2004a, b; Zeece, M.G., Robson, R.M., Lusby, M.L. & Parrish, F.C., 1986). When titin is degraded, a major degradation product is observed that migrates only slightly faster under SDS-PAGE conditions than intact titin and it is termed T_2. This product migrates at approximately 2,400 kDa (Huff-Lonergan, E. et al., 1995; Kurzban, G.P. & Wang, K., 1987, 1988).. Another titin degradation product that has been observed migrates at approximately 1,200 kDa by SDS-PAGE analysis (Huff-Lonergan, E. et al., 1995; Matsuura, T., Kimura, S., Ohtsuka, S. & Maruyama, K., 1991). This latter polypeptide has been shown to contain the portion of titin that extends from the Z-line to near the N_2 line in the I-band (Kimura, S., Matsuura, T., Ohtsuka, S., Nakauchi, Y., Matsuno, A. & Maruyama, K., 1992), although the exact position that the 1200 kDa polypeptide reaches in the sarcomere is still not certain. The 1,200-kDa polypeptide has been documented to appear earlier postmortem in myofibrils from aged beef that had lower shear force (and more desirable tenderness scores) than in samples than from product that higher shear force and/or less favorable tenderness scores (Huff-Lonergan, E. et al., 1996a; Huff-Lonergan, E., Mitsuhashi, T., Parrish, F.C. & Robson, R.M., 1996b; Huff-Lonergan, E. et al., 1995). The T2 polypeptide can also be subsequently degraded or altered during normal postmortem aging. Studies that have used antibodies against titin have been shown to cease to recognize T2 after prolonged periods of postmortem storage or µ-calpain digestion (Ho, C.Y., Stromer, M.H. & Robson, R.M., 1994; Huff-Lonergan, E. et al., 1996a)

Nebulin

Nebulin is another mega-protein (Mr 600-900 kDa) that is part of the fourth filament system in muscle. This protein extends from the Z-line to the pointed ends of the thin filament. The C-terminal end of nebulin is embedded into the Z-line. Nebulin is highly non-extensible and has been referred to as a molecular ruler that, during development may serve to define the length of the thin filaments (Kruger, M., Wright, J. & Wang, K., 1991). Nebulin, via its intimate association with the thin filament (Lukoyanova, N., VanLoock, M.S., Orlova, A., Galkin, V.E., Wang, K. & Egelman, E.H., 2002) has been hypothesized to constitute part of a composite nebulin/thin filament (Pfuhl, M., Winder, S.J. & Pastore, A., 1994; Robson, R.M., Huff-Lonergan, E., Parrish, F.C., Ho, C.-T., Stromer, M.H., Huiatt, T.W. et al., 1995), may aid in anchoring the thin filament to the Z-line (Komiyama, M., Zhou, Z.H., Maruyama, K. & Shimada, Y., 1992; Wang, K. & Wright, J., 1988). Degradation of nebulin postmortem could weaken the thin filament linkages at the Z-line, and(or) of the thin filaments in the nearby I-band regions (Taylor, R.G. et al., 1995), and thereby weaken the structure of the muscle cell. Nebulin has also been shown to be capable of linking actin and myosin (Root, D.D. & Wang, K., 1994a, b). It has been hypothesized that nebulin may also have a regulatory function in skeletal muscle contraction (Bang, M.-L., Li, X., Littlefield, R., Bremner, S., Thor, A., Knowlton, K.U. et al., 2006; Root, D.D. & Wang, K., 1994a, b). Portions of nebulin that span the A-I junction have the ability to bind to actin, myosin and calmodulin (Root, D.D. & Wang, K., 2001). More interestingly, this portion of nebulin (spanning the A-I junction) has been shown to inhibit actomyosin ATPase activity (Lukoyanova, N. et al., 2002; Root, D.D. & Wang, K., 2001). This region of nebulin also has been suggested to inhibit the sliding velocities of actin filaments over myosin If the latter role is confirmed, then it is also possible that nebulin's postmortem degradation may alter actin-myosin interactions in such a way that the alignment and interactions of thick and thin filaments in postmortem muscle is disrupted. This, too, could lead to an increase in postmortem tenderization. Nebulin degradation, does seem to be correlated to postmortem tenderization, although the exact cause and effect relationship remains to be substantiated (Huff-Lonergan, E. et al., 1996a; Huff-Lonergan, E. et al., 1995; Melody, J.L. et al., 2004b; Taylor, R.G. et al., 1995).

Troponin-T

For many years it has been recognized that the degradation of troponin-T and the appearance of polypeptides migrating at approximately 30 kDa are strongly related to, or correlated with, the tenderness of beef (MacBride, M.A. & Parrish, F.C., 1977; Olson, D.G. & Parrish, F.C., 1977; Olson, D.G., Parrish, F.C., Dayton, W.R. & Goll, D.E., 1977; Penny, I.F., Voyle, C.A. & Dransfield, E., 1974)). It has been shown that purified bovine troponin-T can be degraded by µ-calpain in vitro to produce polypeptides in the 30-kDa region (Olson, D.G. et al., 1977). In addition, polypeptides in the 30-kDa region found in aged bovine muscle specifically have been shown to be products of troponin-T by using Western blotting techniques (Ho, C.Y. et al., 1994). Often more than one fragment of troponin-T can be identified in postmortem muscle. Increasing postmortem time has been shown to be associated with the appearance of two major bands (each is likely a closely spaced doublet of polypeptides) of approximately 30 and 28 kDa, which label with monoclonal antibodies to troponin-T (Huff-Lonergan, E. et al., 1996a). In addition, the increasing postmortem aging time was also associated with a loss of troponin-T, as has been reported in numerous studies (Ho, C.Y. et al., 1994; Koohmaraie, M., Kennick, W.H., Anglemier, A.F., Elgasim, E.A., Jones, T.K. & 1984a; Koohmaraie, M., Kennick, W.H., Elgasim, E.A. & Anglemier, A.F., 1984b; Olson, D.G. et al., 1977). It has recently been shown that Troponin-T is cleaved in its glutamic acid-rich amino-terminal region (Muroya, S., Ohnishi-Kameyama, M., Oe, M., Nakajima, I. & Chikuni, K., 2007). Some studies have shown to labeling of two very closely spaced bands corresponding to intact troponin-T. This is likely due to isoforms of troponin-T that are known to exist in skeletal muscle (Briggs, M.M., Mcginnis, H.D. & Schachat, F., 1990; Malhotra, A., 1994; Muroya, S. et al., 2007) including specifically bovine skeletal muscle (Muroya, S. et al., 2007). Both the appearance of the 30- and 28-kDa bands and the disappearance of the intact troponin-T in the myofibril are very strongly related to the shear force (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996b; Lonergan, S.M. et al., 2001b; Penny, I.F., 1976; Rowe, L.J. et al., 2004a; Rowe, L.J., Maddock, K.R., Trenkle, A., Lonergan, S.M. & Huff-Lonergan, E., 2003). Troponin-T is a substrate for µ-calpain and it is hypothesized that µ-calpain is at least partly responsible for the postmortem degradation of troponin-T and the concomitant production of the 28- and 30-kDa polypeptides. Degradation of troponin-T may simply be an indicator of overall postmortem proteolysis (i.e., it occurs as meat becomes more tender). However, because troponin-T is an integral part of skeletal muscle thin filaments (Greaser, M.L. & Gergely, J., 1971), its role in postmortem tenderization may warrant more careful examination as has been suggested (Ho, C.Y. et al., 1994; Huff-Lonergan, E. et al., 1996b; Taylor, R.G. et al., 1995; Uytterhaegen, L., Claeys, E. & Demeyer, D., 1994). Indeed, the troponin-T subunit makes up the elongated portion of the troponin molecule and through its interaction with tropomyosin aids in regulating the thin filament during skeletal muscle contraction (Greaser, M.L. & Gergely, J., 1971; Hitchcock, S.E., 1975; Lehman, W., Rosol, M., Tobacman, L.S. & Craig, R., 2001; McKay, R.T., Tripet, B.P., Hodges, R.S. & Sykes, B.D., 1997). It is conceivable that postmortem degradation of troponin-T and disruption of its interactions with other thin filament proteins aid in the disruption of the thin filaments in the I-band, possibly leading to fragmentation of the myofibril and overall muscle integrity. During postmortem aging, the myofibrils in postmortem bovine muscle are broken in the I-band region (Taylor, R.G. et al., 1995). Because troponin-T is part of the regulatory complex that mediates actin-myosin interactions (Greaser, M.L. & Gergely, J., 1971; Hitchcock, S.E., 1975; Lehman, W. et al., 2001; McKay, R.T. et al., 1997), it is also conceivable that its postmortem degradation may lead to changes involving thick and thin filament interactions. Regardless of whether or not troponin-T aids in disruption of the thin filament in the I-band, alters thick and thin filament interactions, or simply reflects overall protein degradation, its degradation and appearance of polypeptides in the 30-kDa region seem to be an indicator of beef tenderness (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a; Huff-Lonergan, E. et al., 1995; Koohmaraie, M., 1992; Koohmaraie, M. et al., 1984a; Koohmaraie, M. et al., 1984b; Olson, D.G. & Parrish, F.C., 1977; Olson, D.G. et al., 1977).

Desmin

It has been suggested that desmin, a member of the type III group of intermediate filament proteins (O'Shea, J.M., Robson, R.M., Huiatt, T.W., Hartzer, M.K. & Stromer, M.H., 1979; Robson, R.M., 1989), localized at the periphery of the myofibrillar Z-disk in skeletal muscle (Richardson et al., 1981), plays a role in the development of tenderness (Boehm, M.L. et al., 1998; Huff-Lonergan, E. et al., 1996a; Melody, J.L. et al., 2004b; Taylor, R.G. et al., 1995). The desmin intermediate filaments surround the Z-lines of myofibrils. They connect adjacent myofibrils at the level of their Z-lines, and the myofibrils to other cellular structures, including

the sarcolemma (Robson, R.M., 1989; Robson, R.M. et al., 1995) and thus may be important in maintaining the structural integrity of muscle cells (Robson et al., 1981, 1991). It is possible that degradation of structural elements that connect the major components (i.e., the myofibrils) of a muscle cell together, as well as the peripheral layer of myofibrils to the cell membrane, could affect the development of tenderness. Desmin is known to be degraded during postmortem storage (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a; Hwan, S.F. & Bandman, E., 1989; Melody, J.L. et al., 2004a; Rowe, L.J. et al., 2004b; Zhang, W.G., Lonergan, S.M., Gardner, M.A. & Huff-Lonergan, E., 2006). Furthermore, it has been documented that desmin is degraded more rapidly in myofibrils from samples with low shear force and higher water-holding capacity (Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a; Melody, J.L. et al., 2004a; Rowe, L.J. et al., 2004b; Zhang, W.G. et al., 2006). A major degradation product that is often seen in beef is a polypeptide of approximately 38 kDa. This degradation product also has been shown to be present µ-calpain digested myofibrils (Carlin, K.R. et al., 2006; Huff-Lonergan, E. & Lonergan, S.M., 1999; Huff-Lonergan, E. et al., 1996a). Thus, µ-calpain may be, at least in part, responsible for desmin degradation under normal postmortem aging conditions. Whether or not this degradation is truly directly linked to tenderization or is simply an indicator of overall postmortem proteolysis remains to be determined.

Filamin

Filamin is a large (*M*r = 245,000 in skeletal and cardiac muscle) actin binding protein that exists in numerous cell types(Loo, D.T., Kanner, S.B. & Aruffo, A., 1998; Thompson, T.G., Chan, Y.M., Hack, A.A., Brosius, M. & Rajala, M., 2000; van der Flier, A., Kuikman, I., Kramer, D., Geerts, D. & Kreft, M., 2002). There are several different isoforms of filamin (Hock, R.S., Davis, G. & Speicher, D.W., 1990). The amount of filamin in skeletal and cardiac muscle is very low (approximately < .1% of the total muscle protein). In skeletal and cardiac muscle, filamin is localized at the periphery of the myofibrillar Z-disk, and it may be associated with intermediate filaments in these regions (Loo, D.T. et al., 1998; Thompson, T.G. et al., 2000; van der Flier, A. et al., 2002). Thus, postmortem degradation of filamin conceivably could disrupt key linkages that serve to help hold myofibrils in lateral register. Degradation of filamin may also alter linkages connecting the peripheral layer of myofibrils in muscle cells to the sarcolemma by weakening interactions between peripheral myofibrillar Z-disks and the sarcolemma via intermediate filament associations or costameres (Robson, R.M. et al., 1995). A study using myofibrils from beef showed that some filamin was degraded to form an approximately 240-kDa degradation product that migrated as a doublet in both myofibrils from naturally aged muscle and in µ-calpain-digested myofibrils (Huff-Lonergan, E. et al., 1996a). This same doublet formation (composed of intact and degraded filamin) has been seen in cultured embryonic skeletal muscle cells and was attributed to calpain activity (Robson, R.M. et al., 1995). Uytterhaegen et al. (Uytterhaegen, L. et al., 1994)) have shown increased degradation of filamin in muscle samples injected with $CaCl_2$, a process that has been shown to stimulate proteolysis and postmortem tenderization (Harris, S.E. et al., 2001; Wheeler, T.L., Crouse, J.D. & Koohmaraie, M., 1992). Compared to other skeletal muscle proteins, relatively little has been done to fully characterize the role of this protein in postmortem tenderization o f beef. Further studies that employ a combination of sensitive detection methods (e.g., one and two-dimensional gels, Western blotting, immunomicroscopy) are needed to determine the role of filamin in skeletal muscle systems and postmortem tenderization.

Summary

Just as the overall integrity and function of muscle cells does not depend on a single protein, but rather on the coordinated interaction of several proteins, the structural weakening of muscle cells postmortem also must not depend on the degradation of a single myofibrillar or other cytoskeletal protein. The proteins mentioned in this review are located in different regions of the muscle cell, and most have been implicated in some manner as being important in maintaining the structure and function of the muscle cell. In addition, these proteins are located at regions that seem to be affected during postmortem aging, including areas at or near the Z-line and in the I-band. Degradation of proteins such as desmin and filamin, located at the periphery of the Z-line, may disrupt the lateral register and integrity of the myofibril themselves as well as the attachments of the peripheral layer of myofibril to the sarcolemma. Degradation of the proteins within the myofibril that are associated with the thick and thin filaments may allow lateral movement or breaks to occur within the sarcomeres of postmortem aged samples. Titin, nebulin, and troponin-T, by their ability to directly interact with, or modulate the interaction between, major proteins of the thick and thin filaments and(or) the Z-line, have the opportunity to

play key roles in muscle cell integrity . Disruption of these proteins, especially titin and nebulin, could initiate further physicochemical and structural changes that result in myofibril fragmentation and loss of muscle cell integrity, and ultimately in tenderization of the muscle.

The protease, µ-calpain has the ability under postmortem-like in vitro conditions of relatively low pH and temperature to catalyze the degradation of titin, nebulin, filamin, desmin, and troponin-T into many of the same degradation products produced in myofibril from naturally aged muscle samples. This further implicates µ-calpain as a catalyst for at least some of the changes occurring in postmortem muscle. Some reports have indicated that the activity of µ-calpain, as measured by caseinolytic activity of supernatants of muscle extracts, declines significantly within the first 24 to 72 hours postmortem (Koohmaraie, M., 1990). In contrast, it has been reported as postmortem aging time increases, µ-calpain becomes associated with the myofibrillar component of skeletal muscle tissue samples (Boehm, M.L. et al., 1998; Melody, J.L. et al., 2004b). This observation leads to the hypothesis that postmortem activity of µ-calpain may be sustained over a longer period of time than was originally indicated by µ-calpain activity in muscle extracts however, this has proven difficult to examine (Boehm, M.L. et al., 1998). Additional research is needed to determine the factors that govern the µ-calpain activity and interactions with substrates within the postmortem skeletal muscle cell in order to develop methods whereby tenderness can be effectively altered or predicted.

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