

Is Z-Disk Degradation Responsible for Postmortem Tenderization?¹

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ABSTRACT: A number of studies have suggested that Z-disk degradation is a major factor contributing to postmortem tenderization. These conclusions seem to have been based largely on experimental findings showing that the calpain system has a major role in postmortem tenderization, and that when incubated with myofibrils or muscle strips, purified calpain removes Z-disks. Approximately 65 to 80% of all postmortem tenderization occurs during the first 3 or 4 d postmortem, however, and there is little or no ultrastructurally detectable Z-disk degradation during this period. Electron microscope studies described in this paper show that, during the first 3 or 4 d of postmortem storage at 4°C, both costameres and N₂ lines are degraded. Costameres link myofibrils to the sarcolemma, and N₂ lines have been reported to be areas where titin and nebulin filaments, which form a cytoskeletal network linking thick and thin filaments, respectively, to the Z-disk, coalesce. Filamentous

structures linking adjacent myofibrils laterally at the level of each Z-disk are also degraded during the first 3 or 4 d of postmortem storage at 4°C, resulting in gaps between myofibrils in postmortem muscle. Degradation of these structures would have important effects on tenderness. The proteins constituting these structures, nebulin and titin (N₂ lines); vinculin, desmin, and dystrophin (three of the six to eight proteins constituting costameres); and desmin (filaments linking adjacent myofibrils) are all excellent substrates for the calpains, and nebulin, titin, vinculin, and desmin are largely degraded within 3 d postmortem in semimembranosus muscle. Electron micrographs of myofibrils used in the myofibril fragmentation index assay show that these myofibrils, which have been assumed to be broken at their Z-disks, in fact have intact Z-disks and are broken in their I-bands.

Key Words: Calpains, Costameres, Myofibril Fragmentation Index, N₂ Lines, Postmortem Tenderization, Z-Disks

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Introduction

Light and electron microscope studies in the late 1960s first showed that Z-disks occasionally were disrupted and, in some instances in muscle stored at higher temperatures, were lost entirely during post-

mortem storage (Davey and Gilbert, 1969; Fukazawa and Yasui, 1967; Goll, 1968; Henderson et al., 1970). It seemed likely that loss of Z-disk integrity would contribute to increased tenderness, and indeed, myofibrils prepared from muscle after 3 to 20 d of postmortem storage at 0 to 2°C were fragile and were broken into short segments of four to seven sarcomeres each by homogenization (Davey and Gilbert, 1969; Henderson et al., 1970). Immediately after these structural observations, it was discovered that incubating muscle strips in a Ca²⁺-containing solution resulted in total degradation of Z-disks (Goll et al., 1970; Busch et al., 1972), and efforts were begun to identify the Ca²⁺-activated factor (called CAF at that time) responsible for this degradation. Even before CAF had been completely purified, it was shown that muscles having more CAF activity underwent a greater degree of postmortem tenderization than muscles having less CAF activity (Goll et al., 1974). Moreover, incubation of myofibrils with purified CAF (now called m-calpain) caused the same or similar

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structural changes (Olson et al., 1976) and polypeptide degradation (Olson et al., 1977) that postmortem storage did. Both CAF treatment and postmortem storage resulted in appearance of several polypeptide fragments migrating in the 30,000-Da range in SDS-PAGE. Earlier studies (Hay et al., 1973) had reported the appearance of a 30-kDa polypeptide in postmortem muscle, but the origin and cause of this fragment was unknown at that time. It is now known that the 30-kDa polypeptides result from degradation of troponin-T (Olson et al., 1977; Ho et al., 1994), and that density of the 30-kDa band is related to ultimate tenderness (McBride and Parrish, 1977).

Calpain Activity, Z-disks, and Postmortem Tenderization

Since these early observations, a great deal of evidence has accumulated indicating that the calpains have an important role in postmortem tenderization (see Figure 1 for a summary). This evidence has led to a prevailing view that the calpains are responsible for 90% or more of the tenderization that occurs during postmortem storage (Goll et al., 1992a), and furthermore, that this tenderization is the direct result of calpain's ability to degrade Z-disks in skeletal muscle. Several observations, however, are not readily accommodated by the "Z-disk dogma" of postmortem tenderization.

Troponin T is Not Located in the Z-Disk, and Its Degradation and Appearance of the 30-kDa Frag-

ments Are Not the Result of Z-Disk Degradation. It has been suggested that postmortem degradation of troponin T is simply an in situ estimate of calpain activity, but this argument would be more convincing if degradation of a Z-disk protein could be shown to be related to tenderization.

There is Little or No Degradation of the Known Z-Disk Proteins During the First 3 Days Postmortem When Most Tenderization Occurs. α -Actinin is the major Z-disk protein, and the filaments in Z-disks contain actin (Luther, 1991). Western blot analysis finds no detectable degradation of α -actinin until after 2 wk of postmortem storage at 2°C (Hwan and Bandman, 1989), and functional α -actinin can be isolated from muscle after 13 d postmortem (Arakawa et al., 1970). Purified α -actinin and actin are not degraded by purified calpain in in vitro assays (Goll et al., 1991). It seems likely, therefore, that any postmortem degradation of α -actinin or actin is due to the cathepsins, which do degrade these two proteins (Okitani et al., 1980; Matsukara et al., 1981), and that this degradation occurs late, probably after 7 to 10 d postmortem.

Two other proteins, titin and nebulin, are anchored at their N- and C-terminal ends, respectively, in the Z-disk. The titin and nebulin polypeptide chains, however, both extend through the I-band and into the A-band areas, and it seems unlikely that degradation of these two proteins would result in loss of Z-disks. Filamin and desmin are both located at the periphery

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- A. Very little degradation of myosin and actin occurs during postmortem storage at 2 to 4°C for 72 h (Bandman and Zdanis, 1988), even though most of the total postmortem tenderization occurs during this period.
 - 1. The calpains are unique among the known proteolytic enzymes in that they do not degrade undenatured myosin and actin.
 - a. The known cathepsins all degrade myosin and actin.
 - B. Very little degradation of α -actinin, the major protein in skeletal muscle Z-disks, occurs during postmortem storage at 2 to 4°C for 72 h (Hwan and Bandman, 1989).
 - 1. Several of the cathepsins and many other proteases (e.g., trypsin; Stromer et al., 1967) degrade the Z-disk structure, and this degradation is accompanied by degradation of α -actinin; the calpains, however, are unique in that they degrade the Z-disk structure, but they *release* α -actinin from this structure without degrading it to small fragments.
 - 2. Degradation of myosin, actin, and α -actinin occurs during postmortem storage at 37°C, and this degradation may be due to the cathepsins.
 - C. There is *very little* proteolysis of muscle proteins during postmortem storage at 2 to 4°C.
 - 1. The sarcomere structure remains largely intact, with the exception of loss of the N₂ line and some variable but small loss of Z-disk integrity (Stromer et al., 1967).
 - 2. Functional actomyosin (Robson et al., 1967) and α -actinin (Arakawa et al., 1970) can be isolated from skeletal muscle even after 13 d of postmortem storage at 2 to 4°C.
 - 3. Many studies have failed to find significant increases in free amino acids even after long periods of postmortem storage (Parrish et al., 1969).
 - a. The cathepsins cause extensive degradation of proteins (Whipple and Koohmaraie, 1991), but the calpains cause relatively few cleavages and have little activity after muscle pH falls below 6.0.
 - D. Several studies have shown that increasing Ca²⁺ concentration in muscle results in increased tenderness (Penny et al., 1974; Koohmaraie et al., 1988b, Koohmaraie and Shackelford, 1991).
 - 1. Neither the cathepsins nor the multicatalytic protease is activated by Ca²⁺ (Koohmaraie, 1992).
 - E. A number of studies have shown that tenderness increases to a greater extent during postmortem storage in those muscles that have higher calpain (especially μ -calpain) or lower calpastatin activities than in other muscles; low calpastatin activity especially seems highly associated with increased postmortem tenderization (Koohmaraie, 1988, 1992; Wheeler et al., 1990).

Figure 1. Summary of some of the evidence that the calpain system has a major role in postmortem tenderization. This figure is adapted from a similar summary that appeared in Goll et al. (1992a).

of the Z-disk and are not part of the Z-disk structure (Granger and Lazarides, 1978). Although these two proteins are readily degraded by the calpains (Davies et al., 1978; O'Shea et al., 1979), it seems unlikely that their degradation would by itself cause loss of Z-disks.

The Time During Which Most Postmortem Tenderization Occurs Does Not Coincide with the Time That Changes in Z-Disk Structure or Z-Disk Proteins Are Observed. A number of studies have reported that most postmortem tenderization occurs during the first 3 or 4 d after death (Goll et al., 1964; Davey and Gilbert, 1966; Parrish et al., 1973; Marsh et al., 1981; Dransfield, 1992; Wheeler and Koohmaraie, 1994). The SDS-PAGE of myofibrils from postmortem muscle, however, indicates that the complex of polypeptides migrating at 30 kDa does not begin to appear until after 3 d of postmortem storage at 2°C (Koohmaraie et al., 1984). Moreover, although Z-disks lose some structural integrity and density during prolonged periods of postmortem storage at temperatures of 25°C or higher (Henderson et al., 1970), very few structural changes can be detected in Z-disks during the first 3 d postmortem at 2°C, and most Z-disks show little ultrastructural signs of degradation even after 13 d or longer at 2 to 4°C (Stromer et al., 1967).

It has been argued that only a few Z-disks, possibly less than 5% of the total, would need to be degraded or partly degraded to have a large effect on tenderness, and that the sampling problems inherent to electron microscope analysis would make it difficult to detect this small number of disrupted Z-disks. Consequently, the myofibril fragmentation index (MFI) has been widely adopted as a method of estimating Z-disk degradation in postmortem muscle. Myofibrils prepared from postmortem muscle are shorter than those prepared from at-death muscle (Stromer et al., 1974; Olson et al., 1976), suggesting that myofibrils are weakened during postmortem storage and therefore are more easily broken into short fragments by homogenization. The MFI is a measure of the average length of myofibrils and is significantly related to tenderness (shorter myofibrils result in higher MFI, which is related to greater tenderness; Møller et al., 1973). Most studies on the MFI have related MFI at some chosen time postmortem (usually between 3 and 14 d) with tenderness at that same time, and only a few studies have monitored changes in the MFI during postmortem storage (see Olson et al., 1976; Koohmaraie et al., 1987, 1988a; Whipple et al. 1990a, b; Shackelford et al., 1991). Although meat tenderness decreases markedly during the first 24 h postmortem and then increases rapidly between 24 and 72 h postmortem (see Figure 2 and discussion in the following paragraph), the MFI increases uniformly during the first 72 h postmortem (Olson et al., 1976; Koohmaraie et al., 1987, 1988a; Whipple et al., 1990a, b; Shackelford et al., 1991) and does not reflect the

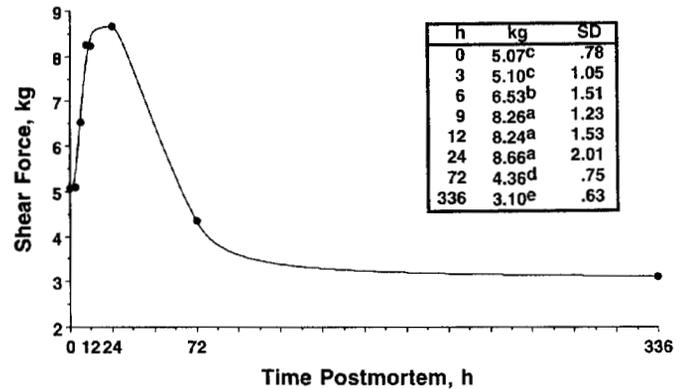


Figure 2. Changes in Warner-Bratzler shear force of lamb longissimus thoracic et lumborum muscle during postmortem storage. Insert shows the means and standard deviations of the numbers plotted in the figure. Means without a common superscript differ significantly at $P < .05$. This figure is taken from Wheeler and Koohmaraie (1994).

large changes in tenderness that occur during this period. A study comparing *bos taurus* and *bos indicus* found that the MFI for either of these breeds did not change between 1 and 3 d postmortem and then increased significantly after 3 d of postmortem storage (Whipple et al., 1990b). Conversely, two studies monitoring postmortem changes in the MFI beginning at death and continuing until 7 or 14 d postmortem found that 61 to 64% of the increase in MFI had occurred by d 3 postmortem compared with d 7 (Koohmaraie et al., 1988a) or d 14 (Koohmaraie et al., 1987). Other studies that have monitored changes in MFI during the first 6 or 7 d postmortem indicate that only 13 (Whipple et al., 1990b) to 30 to 38% (Olson et al., 1976; Whipple et al., 1990a; Shackelford et al., 1991) of the increase in MFI had occurred after 3 d postmortem compared with 6 or 7 d postmortem. Hence, studies on changes in the MFI during the first 3 to 4 d postmortem have produced inconsistent results. More importantly, perhaps, no studies have been done to determine whether changes in the MFI are indeed due to breakage at the Z-disk, as suggested by the Z-disk theory, or whether these breaks occur elsewhere in the myofibril.

Estimates of percentage of total tenderization that occurs during the first 3 d after death depend to a large extent on the value obtained for tenderness at death. At-death muscle contains ATP and undergoes severe contraction during cooking. It, therefore, has been difficult to obtain accurate estimates of at-death tenderness. Modeling studies based on activity of μ -calpain as a primary cause of postmortem tenderization predict toughness at death as approximately 7.2 to 11.0 kg, toughness at 3 d postmortem as approximately 5.5 to 7.2 kg, toughness at 5 d as approximately 5 to 7 kg, and ultimate toughness (25 d) as approximately 3.5 to 4.0 kg (Dransfield, 1992).

Hence, this model suggests that approximately 65% of total postmortem tenderization occurs by d 3 postmortem. Warner-Bratzler shear measurements of bovine semitendinosus muscle stored at 2 to 4°C have been reported to change from 7.3 kg at death to 4.1 kg after 3 d postmortem, and to 3.4 kg after 13 d of postmortem storage (Goll et al., 1964). Consequently, 82% of all postmortem tenderization had occurred within the first 3 d postmortem in this study.

Recently, a concerted effort was made to obtain an accurate estimate of changes in meat toughness during the first 3 d postmortem (Wheeler and Koohmaraie, 1994). At-death muscle was clamped before excision from the carcass to prevent shortening and was then frozen until all ATP in the muscle had been hydrolyzed. Shear force measurements indicated that muscle was only moderately tough at death, increased in toughness during the first 24 h postmortem, and then decreased rapidly in toughness during the next 48 h postmortem (Figure 2). Based on the maximum toughness at 24 h, 77% of all postmortem tenderization had occurred after 72 h postmortem. The

results of these three studies indicate that approximately 65 to 80% of all postmortem tenderization occurs during the first 72 h postmortem, before any large ultrastructural changes in Z-disk structure or any significant degradation of actin or α -actinin occurs.

Costamere Structures in Skeletal Muscle

In 1983, Craig and associates observed filamentous structures that linked myofibrils to the sarcolemma (Craig and Pardo, 1983; Pardo et al., 1983; Figure 3). These structures, which were named costameres, contained γ -actin, vinculin, β -spectrin, talin, and the intermediate filament proteins, desmin and vimentin, and occurred periodically along the myofibril at the level of each I-band in skeletal muscle or Z-disk in cardiac muscle (Craig and Pardo, 1983; Pardo et al., 1983). Subsequent studies have shown that costameres also contain β_1 integrin (the integrin that binds to collagen), clathrin, ankyrin, β -spectrin, and dystrophin, the protein missing in Duchenne and Becker

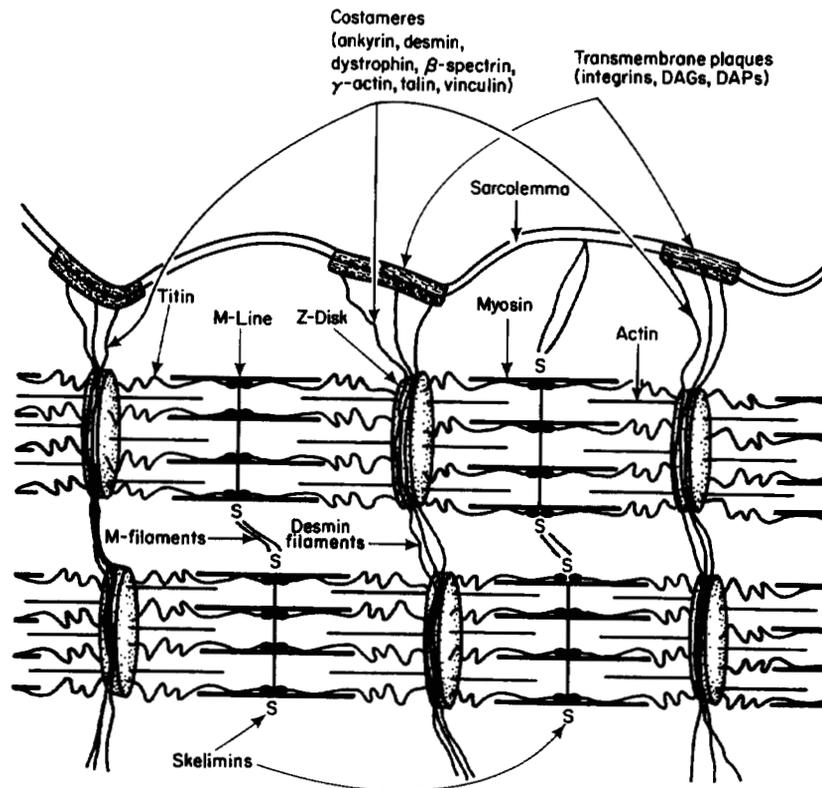


Figure 3. Schematic diagram showing the structure and protein composition of costameres in striated muscle relative to Z-disks and the myofibrillar lattice. The probable position of titin filaments is shown in this diagram, but nebulin filaments have been omitted to simplify the diagram. The N-terminal end of the large titin molecule is anchored in the Z-disk. Desmin, vinculin, and ankyrin, three of the protein constituents of costameres, extend into the muscle cell where they encircle myofibrils at the Z-disk and run from myofibril to myofibril to link adjacent Z-disks laterally (Granger and Lazarides, 1978; Nelson and Lazarides, 1984; Terracio et al., 1990). DAPs = dystrophin associated proteins; DAG = dystrophin-associated glycans; S = skelemins. This diagram has been adopted from a similar diagram shown in Price (1991).

muscular dystrophy (Nelson and Lazarides, 1983, 1984; Shear and Block, 1985; Byers et al., 1991; Danowski et al., 1992; Porter et al., 1992; Straub et al., 1992), and that a second costamere-like network extends from the M-line region of myofibrils to the sarcolemma (Figure 3). Recent studies indicate that costameres are more robust structures than their ethereal appearance would indicate and that they are sites of force transduction to the substratum (Street, 1983, Danowski et al., 1992). Consequently, degradation of costameres may significantly weaken muscle structure. Two earlier studies (Young et al., 1980; Robson et al., 1984) suggested that the cytoskeletal proteins, desmin, nebulin, and titin, may have an important role in tenderness, but these studies did not mention costameres.

With the possible exception of γ -actin, whose susceptibility to the calpains has not been tested, all protein components of costameres, including dystrophin (Cottin et al., 1992), are rapidly degraded by the calpains in *in vitro* assays. Also, the costameres are located at the surface of the muscle cell where they would be immediately exposed to any extracellular Ca^{2+} leaking into the muscle cell due to damage or weakening of the sarcolemma during postmortem storage (Jeacocke, 1993). Taylor et al. (1994) found that costamere structures are lost during the first 24 to 72 h postmortem and that this loss parallels loss of N_2 lines in postmortem muscle. Both these structural changes can be mimicked by calpain treatment of muscle strips (Yamaguchi et al., 1983; Goll et al., 1991, 1992b; Taylor et al., 1994). Hence, loss of costameres and the N_2 line occur during the same period that the major increase in tenderization occurs.

The available evidence, therefore, suggests that something besides or in addition to Z-disk degradation is involved in postmortem tenderization. Consequently, we have examined more closely the nature of the ultrastructural changes that occur during the first 7 d postmortem, when the major increase in tenderization occurs, and have monitored the rate of degradation of nebulin, titin, and several costamere proteins during postmortem storage to learn whether degradation of these proteins parallels major changes in tenderness. Finally, we have examined ultrastructurally the myofibrils produced during the MFI assay to determine whether these myofibrils are broken at the Z-disk, as is commonly assumed, or whether they have been broken elsewhere along the myofibril. The results indicate that Z-disks remain fairly robust at least during the first 7 d of postmortem storage, and that myofibrils seem to be weakest in the I-band area.

Experimental Procedures

Materials

Acrylamide (99.9%), bisacrylamide (99.99%), and sodium dodecyl sulfate (99%) were from Schwartz/Mann; Tris (ultrapure, 99.8%) was from Amresco

(Solon, OH); and the protease inhibitors used in the preparation of myofibrils (Edmunds et al., 1991) were from Sigma (St. Louis, MO). The reagents used for the enhanced chemiluminescence (ECL) reaction on Western blots were purchased from DuPont NEN Research Product (Boston, MA). The resins and reagents used for fixation in electron microscopy were obtained from Ted Pella (Redding, CA). All other chemicals were analytical reagent grade or better.

Myofibril Preparation and Myofibril Fragmentation Index

Bovine semimembranosus and biceps femoris muscles from each of four animals were sampled within 30 min after exsanguination, and myofibrils were prepared according to Goll et al. (1977). The muscle on the right side of the animal was used for the at-death sample, and samples at 1, 3, 6 or 7, and 14 d postmortem were taken from the left side to prevent any effects associated with shortening of the muscle that had been sampled within 30 min after exsanguination. Myofibril fragmentation index was done as described by Culler et al. (1978).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Samples for SDS-PAGE were prepared at 0, 1, 3, and 6 d postmortem by homogenizing a 5-g sample of whole muscle tissue in 15 mL of 2% SDS, pH 7.0, using three 30-s bursts of a Brinkmann Polytron homogenizer at 25,000 rpm. Five hundred-microliter aliquots of the homogenate were dissolved by adding an equal volume of sample buffer (8 M urea, 2 M thiourea, 3% SDS, .7% MCE, 50 mM Tris-HCl, pH 6.8,) and heating for 4 min at 70°C. This treatment resulted in complete dissolution of the muscle sample. Protein concentration in the dissolved sample was measured by using Coomassie Brilliant Blue G-250 (Coomassie Plus Protein Assay, Pierce, Rockford, IL), and the samples were diluted to a final concentration of 4.0 mg/mL with a 50:50 mixture of 2% SDS and the urea sample buffer. The diluted samples were clarified by centrifuging 2 min at 5,000 $\times g$ in a table-top microcentrifuge at 22 to 25°C, and bromophenol blue tracking dye was added to a final concentration of .1%. This sampling procedure ensured that all muscle proteins were retained in the sample being assayed and that no fragments that might be produced during postmortem storage were lost by washing steps. The SDS-PAGE was done according to Wolfe et al. (1989), or for titin, nebulin, and dystrophin assays, according to Fritz et al. (1989). Both procedures used tall minigels (.75 mm \times 8.0 cm \times 10.9 cm). After electrophoresis, the gels were transferred electrophoretically to a nitrocellulose membrane (Hoefler Scientific Instruments, San Francisco, CA) using a semi-dry blotting apparatus and the procedure recommended by Hoefler.

Transfer was for 90 min at 100 mA (or for 120 min at 150 mA for dystrophin, nebulin, and titin) in 20 mM Tris, 150 mM glycine, .1% SDS, 10 mM MCE, 15% methanol. Membranes containing the transferred proteins were then incubated overnight either with a monoclonal antibody (MAb) to vinculin (V284, Boehringer Mannheim); to nebulin (Nb₂, Sigma), to titin (T₁₂, Sigma), or to desmin (Allen et al., 1991). A polyclonal antibody elicited against a peptide whose sequence was identical to residues 3495–3544 from the dystrophin molecule (Ishiura et al., 1990) was used in the dystrophin Western blots, and alkaline phosphatase detection involving the reaction with 5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium was used instead of ECL detection. These residues are from the C-terminal part of the dystrophin polypeptide and elicit antibodies that can cross-react with dystrophin-related proteins (Ishiura et al., 1990; Khurana et al., 1991). After washing, the nitrocellulose membranes were incubated for 1 h with a second antibody conjugated with horseradish peroxidase, and the signal from the second antibody was detected by using the ECL procedure (exposure times up to 50 min, depending on the experiment). Films from the ECL assay were scanned densitometrically using a Molecular Dynamics Computing Densitometer with a helium/neon laser light source.

Electron Microscopy

Biceps femoris muscle strips approximately 4 mm × 30 mm were isolated parallel with the long axis of the muscle, were immobilized in muscle clamps (Fine Science Tools, Foster City, CA), and were then removed from the muscle and fixed immediately with 2.5% glutaraldehyde in .1 M cacodylate buffer, pH 7.3, for 4 h at 4°C. Samples were rinsed with cacodylate buffer, *en bloc* stained with 1% tannic acid for 30 min, postfixed with 1% osmium for 1 h, rinsed with cacodylate buffer followed by three rinses with 25% ethanol, and then *en bloc* stained with 1% uranyl acetate in 25% ethanol for 30 min. The samples were dehydrated using a graded series of ethanol and were embedded in Spurr's resin. Thin sections were stained with lead citrate and uranyl acetate. Myofibrils prepared for the myofibril fragmentation index were

also examined ultrastructurally using this same protocol.

Results

Ultrastructural Changes During Postmortem Storage

Figure 4 is a panel of electron micrographs showing bovine skeletal muscle sampled at death and after 1, 3, and 16 d of postmortem storage. The sarcolemma in at-death muscle has periodic areas of invaginations at the level of the Z-disk and the M-line; the densely staining diffuse regions underlying these invaginations are costameres. The sarcolemmal invaginations disappear during the first 24 h of postmortem storage, and in approximately 50% of the fibers, the sarcolemma is pulled away from the underlying myofibril (Figure 4C). Because costameres evidently are responsible for attachment of the sarcolemma to the myofibril, these structural changes indicate that the costameres have been extensively degraded already within the first 24 h postmortem. After 72 h postmortem, the sarcolemma has moved away from the myofibril in all the fibers (Figure 4D), and only remnants of the densely staining transmembrane plaques that connected the sarcolemma to the myofibril in at-death muscle remain (Figure 4D). The nature of the proteins constituting these densely staining patches of material is unclear. Most costamere proteins (ankyrin, desmin, dystrophin, spectrin, talin, vinculin) are excellent substrates for the calpains, as are many of the transmembrane proteins (β -integrin, the DAPs and DAGs; see Matsumura and Campbell, 1994). Consequently, it would be expected that most of the proteins constituting the costamere/transmembrane complex would be destroyed by the calpains during the first 72 h postmortem. The densely staining patches may be complexes of membrane phospholipids, calpain-resistant transmembrane proteins, and large polypeptides that remain after calpain cleavage of proteins such as filamin, talin, and vinculin. The degree of separation between the sarcolemma and the myofibrils in postmortem muscle was variable and seemed to depend in part on

Table 1. Densitometric scans of Western blots of desmin, nebulin, and vinculin at different times postmortem^a

Time postmortem, d	Desmin		Vinculin		Nebulin	
	Biceps femoris	Semimembranosus	Biceps femoris	Semimembranosus	Biceps femoris	Semimembranosus
0	100	100	100	100	100	100
1	107	94.8	92.2	61.5	75.4	71.0
3	88.0	44.2	75.4	39.1	26.0	16.8
6	90.5	25.2	53.7	14.6	22.4	3.5

^aFigures are expressed as percentage of densitometric units obtained from the blot of at-death muscle.

whether adjacent muscle cells were close together and thereby hindered sarcolemmal separation or whether an open space allowed considerable separation (note that the sarcolemma cannot be seen in Figure 4E). The important point, however, is not the extent of the sarcolemma/myofibril separation, but rather that it was a consistent structural change observed in all postmortem muscle examined and that it indicates loss of the structure that attaches myofibrils to the sarcolemma in living muscle.

Another characteristic ultrastructural change during postmortem aging of muscle is that adjacent myofibrils within the muscle fiber become separated (Figure 4A and 4E). It is unclear whether this separation is due to loss of water as sarcolemmal integrity is lost; to the increased osmolarity that occurs in postmortem muscle (Ouali, 1990), to degradation of the desmin and M-filaments that connect adjacent myofibrils at the level of the Z-disk and M-line, respectively (see Figure 3), or to a combination of these and perhaps other factors. Although the lattice spacing between actin and myosin filaments decreases as the pH decreases below 6.0 (Matsuda and Podolsky 1986), the extent of this shrinkage is very low in "rigor" fibers (i.e., fibers having no ATP, which would be the case in postmortem muscle). Matsuda and Podolsky (1986) found that lattice spacing between filaments in rigor muscle decreases by approximately 8 to 9%, not enough to account for the large gaps seen between adjacent myofibrils in postmortem muscle. Moreover, measurement of myofibril diameter indicates that the myofibrils in postmortem muscle have approximately the same diameter as those in at-death muscle (allowing for variations due to the plane at which they were sectioned; see Figure 4). Consequently, it is unlikely that the separation between adjacent myofibrils in postmortem muscle is caused by a change in lattice spacing of the filaments.

Most Z-disks in postmortem muscle stored at 4°C remain almost unchanged ultrastructurally, even after 16 d of postmortem storage (Figure 4F). Except for degradation of the costameres and loss of sarcolemmal integrity, the ultrastructural changes most frequently observed in postmortem muscle are "tears" or gaps in the I-band area (Figure 4F). These tears or breaks often occur near intact Z-disks in the area previously occupied by the N₂ line. Although not shown here, it is possible to find areas in postmortem muscle where the Z-disks seem to have lost some structural integrity and appear diffuse with occasional breaks along their length (as has been reported previously; Fukazawa and Yasui, 1967; Davey and Gilbert, 1969; Goll, 1968; Henderson et al., 1970). In muscle stored at 4°C, however, such areas of Z-disk disruption are rare during the first 4 to 7 d postmortem, and total loss of Z-disks is not observed. Conversely, loss of costamere structures within 72 h postmortem is a consistent

ultrastructural change. "Tears" or breaks in the I-band begin to appear after 3 d postmortem and become increasing prevalent with long periods of postmortem storage (Ouali, 1990).

Ultrastructure of Myofibrils Used in the Myofibril Fragmentation Index and Changes in Myofibril Fragmentation Index During Postmortem Storage. The MFI is related to tenderness and has been used widely as a method of estimating meat tenderness. It evidently has generally been assumed that postmortem storage weakens myofibrils at the Z-disk and that this weakening results in rupture of the Z-disk when postmortem muscle is exposed to the shearing forces of blender blades. In agreement with earlier studies, the MFI in the present study increased continuously during 14 d of postmortem storage (Figure 5). Absolute values of the MFI can vary significantly from animal to animal, but the relative increases in MFI shown in Figure 5 are similar to those that have been reported earlier (Koochmaraie et al., 1987, 1988a; Whipple et al., 1990a,b; Shackelford et al., 1991). Consequently, our MFI assays are measuring the same changes as those described in previous studies, which showed that the MFI is highly related to tenderness. If the change in MFI between 0 and 14 d postmortem is taken as 100%, then 75% (semimembranosus; a "fast-aging" muscle) to 14% (biceps, a "slow-aging" muscle) of the total change in MFI occurred between 0 and 3 d postmortem. These changes compare with those reported in earlier studies in which 61 to 64% of the total change in MFI of longissimus dorsi muscle after 7 (Koochmaraie et al., 1988a) or 14 d (Koochmaraie et al., 1987) of postmortem storage had occurred during the first 3 d. Electron micrographs of the myofibrils used in the MFI assays showed that Z-disks in these myofibrils were intact and that rupture of myofibrils in the MFI assay occurs in the I-band and not at the Z-disk (Figure 6). Detailed ultrastructural analysis of myofibrils used in a MFI assay is difficult because the myofibrils are randomly oriented. However, contrary to the assumption that the increase in MFI during postmortem storage is caused by weakening of Z-disks, no instances of Z-disk breakage were observed in the myofibrils examined in this study.

Western Analysis of Postmortem Changes in Costamere Proteins and Nebulin and Titin

Vinculin and desmin are two of the important proteins found in costameres. Moreover, these two proteins extend from the costamere structure to encircle Z-disks within the muscle cells (Figure 3, Granger and Lazarides, 1978; Richardson et al., 1981; Robson et al., 1984; Terracio et al., 1990). Consequently, Western blots were done to learn whether these proteins are degraded during postmortem storage and whether this degradation temporally parallels the loss of costameres observed ultrastructurally.



Western blots of whole skeletal muscle tissue showed that little or no degradation of desmin occurs in either the biceps femoris or the semimembranosus muscle during the first 24 h postmortem (Figure 7, Table 1). Over half the total desmin in the semitendinosus muscle, however, was degraded between 24 and 72 h after death (Figure 7, Table 1), a period during which tenderness increases dramatically (Figure 2). Earlier studies using Western analysis of bovine semitendinosus muscle (Hwan and Bandman, 1989) also found that desmin was degraded in postmortem muscle, although desmin degradation was not detectable until after 72 h postmortem in this muscle. The difference between the biceps femoris and the semimembranosus muscles in rate of postmortem desmin degradation (Figure 7, Table 1) is remarkable and underscores the need to specify the muscle used in postmortem tenderization studies. Vinculin is very susceptible to degradation in postmortem muscle, and vinculin degradation begins during the first 24 h postmortem (Table 1, Figure 7). Almost half the vinculin in semimembranosus was degraded after 24 h postmor-

tem, and substantial vinculin degradation occurs by 3 d postmortem even in the biceps femoris, a slow-aging muscle (Table 1, Figure 7). This loss of vinculin may be associated with the changes in costamere structure seen during the first 24 h postmortem (Figure 4).

Because vinculin seems very susceptible to postmortem degradation, we examined six lamb longissimus muscles that had been classified as either tough or tender on the basis of Warner-Bratzler shear force and MFI measurements (Table 2). Western analysis of these six samples showed that the ratio of degraded/undegraded vinculin was 4- to 50-fold higher in the tender muscles than in the tough muscles (Figure 7, Table 2). A major polypeptide fragment that reacts with the anti-vinculin antibody is prominent in samples from the tender muscles (Figure 7). This polypeptide fragment migrates in SDS-PAGE at a relative molecular weight of 90 kDa, which is the same size as the major proteolytic fragment produced by calpain digestion of purified vinculin (Goll et al., 1983b). Consequently, rate and extent of postmortem degradation of vinculin seem related to rate and extent of postmortem tenderization.

Dystrophin is the protein encoded by the gene that is missing or disrupted in patients afflicted with Duchenne or Becker's muscular dystrophy (Matsumura and Campbell, 1994). Extensive immunolocalization studies have shown that dystrophin

Figure 4. Electron micrographs of sections of bovine biceps femoris muscle sampled after different times of postmortem storage at 4°C. (A) Electron micrograph of bovine skeletal muscle sampled within 45 min after exsanguination. Arrow and arrowhead indicate areas of density (costameres) where the sarcolemma is attached to the Z-disk and M-line, respectively. (B and C) Bovine biceps femoris muscle sampled 24 h after death. Both these micrographs show structural disintegration and broadening of the sarcolemma compared with the structure of at-death sarcolemma. The sarcolemma is clearly detached and has been displaced from the myofibril in C. This detachment is representative of approximately 50% of the structures observed after 24 h postmortem. (D and E) Bovine biceps femoris muscle after 3 d of postmortem storage. Arrowheads (D) point to patches of densely staining material at the level of the Z-disk. These densely staining areas may be remnants of the transmembrane patches containing integrins, DAGs, and DAPs (see Figure 3). The arrow in D shows the detached membrane that has now been pulled away a considerable distance from the myofibril. E shows examples of widening of the distance between adjacent myofibrils in postmortem muscle and loss of material that seems to connect Z-disks from adjacent myofibrils (arrow). This material probably contains desmin and filamin (Richardson et al., 1981). (F) Bovine biceps femoris muscle after 16 d postmortem. The Z-disk structure is well-preserved even after 16 d postmortem. Arrowheads point to gaps in the I-band adjacent to the intact Z-disk. These gaps are observed in muscle that has been stored for 4 d or longer at 4°C. Bar = 1 μ m.

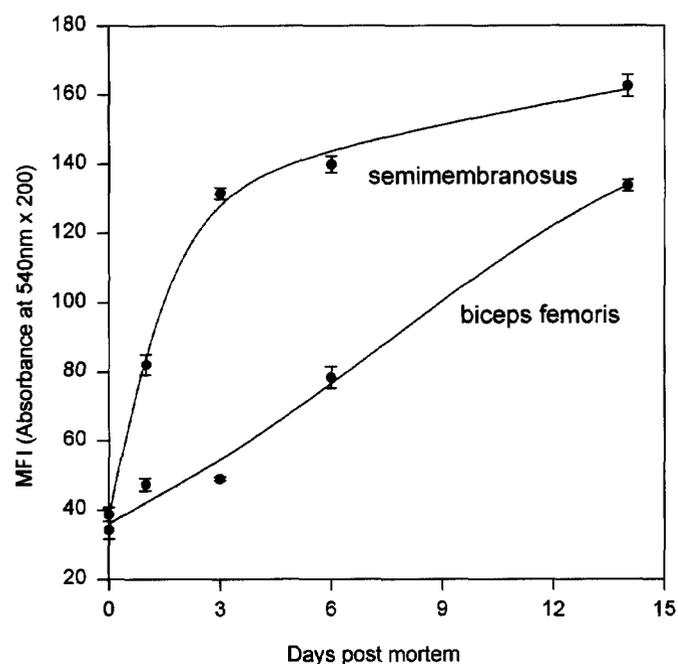


Figure 5. Changes in MFI of two different muscles sampled after different times of postmortem storage. Points are means plus or minus standard errors (vertical lines) of measurements in six separate muscle samples each done in duplicate.

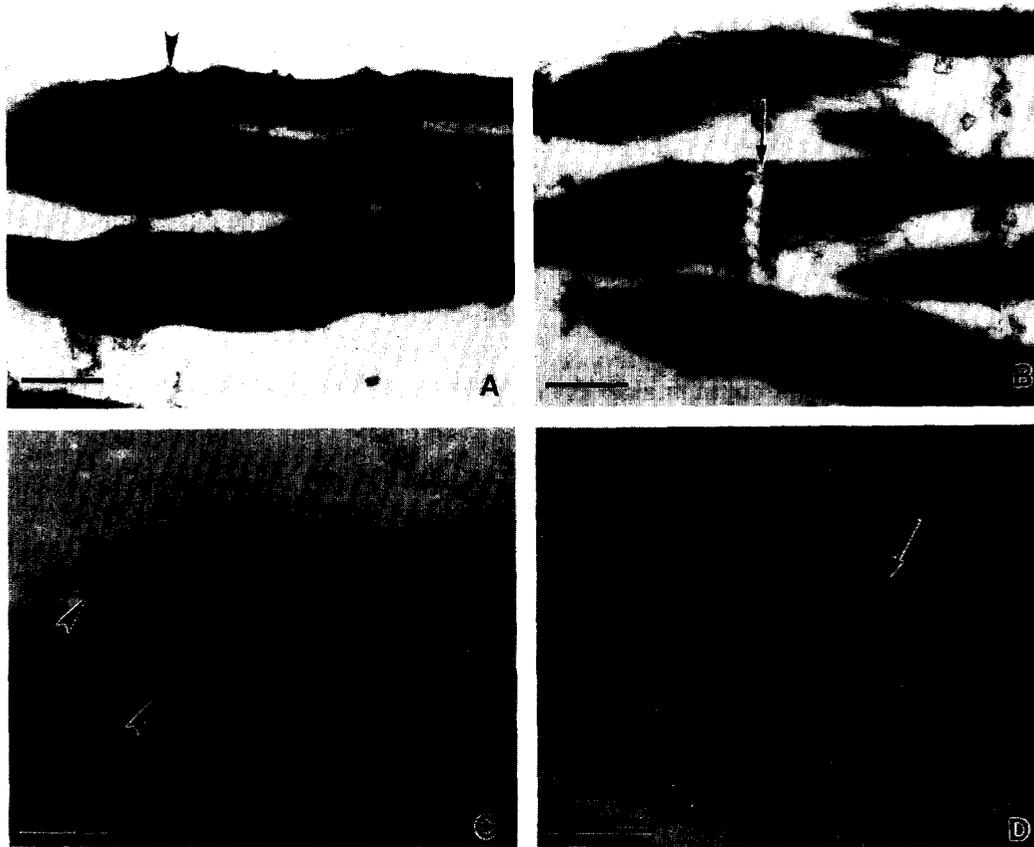


Figure 6. Electron micrographs showing structure of biceps femoris myofibrils used in the MFI assay. (A) Myofibrils used in a MFI assay done after 24 h of postmortem storage. (B, C, and D) Myofibrils used in a MFI assay done after 7 d of postmortem storage. Myofibrils after both 1 and 7 d of postmortem storage at 4°C are sheared in the I-band or A-band areas of the myofibril and not at the Z-disk. These breaks seem to occur most frequently at an area closer to the Z-disk (Figure 6C and 6D) than the A-I junction. The break next to the Z-disk is especially evident at higher magnification (Figure 6D). As the MFI assay shows, the breaks are more frequent and the myofibril segments are shorter after 7 d postmortem than they are after 1 d of postmortem storage. All Z-disks are intact in the myofibrils used in the MFI assay both after 1 and after 7 d postmortem. Bar = .5 μm for A, B, and C and .25 μm for D.

is located on the intracellular surface of the sarcolemma in normal skeletal muscle cells and is a component of the costamere (Byers et al., 1991; Porter et al., 1992; Straub et al., 1992). Dystrophin is present in at-death bovine skeletal muscle and is

degraded during postmortem storage (Figure 8). Very little dystrophin degradation occurs during the first 24 h after death, but in the semitendinosus, dystrophin is degraded rapidly between 24 and 72 h postmortem and is barely detectable after 6 d postmortem. Less

Table 2. Densitometric scans of Western blots of vinculin from tough and tender muscles^a

Sample	Lane in Figure 7	Warner-Bratzler shear force	Myofibril fragmentation index	Vinculin (area of degradation fragment) area of intact vinculin
Tough	1	10.67	44.0	.16
Tough	2	9.90	35.0	.11
Tender	3	3.08	89.0	5.55
Tender	4	4.03	93.5	2.66
Tender	5	3.96	84.0	1.33
Tough	6	13.18	43.5	.37

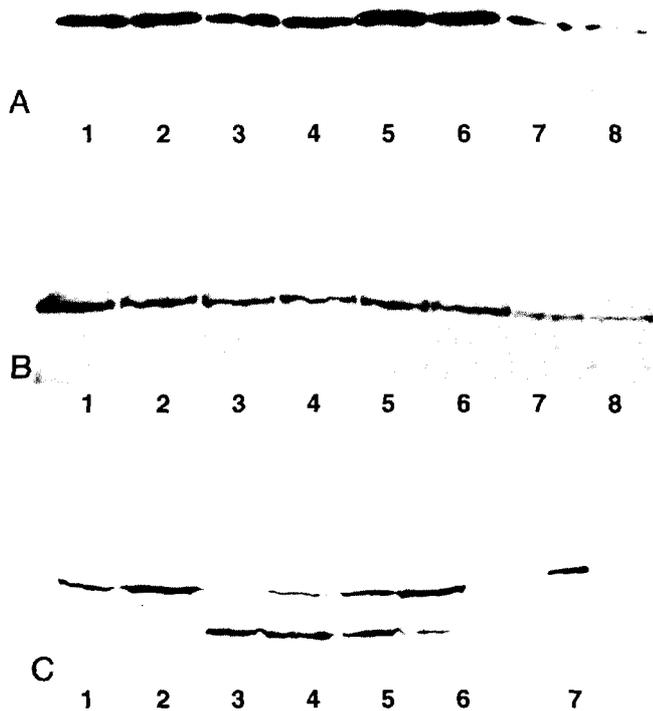


Figure 7. Western blot analysis showing rates of degradation of desmin and vinculin during postmortem storage and the relative extent of vinculin degradation in tough and tender muscle. (A) Anti-desmin blots of bovine biceps femoris muscle (lanes 1–4) and bovine semimembranosus muscle (lanes 5–8). These muscle samples were taken from the same animal and, therefore, the ratio of postmortem desmin degradation can be compared directly in these two sets of blots. Lane 1, at-death biceps; lane 2, 1-d biceps; lane 3, 3-d biceps; lane 4, 6-d biceps; lane 5, at-death semimembranosus; lane 6, 1-d semimembranosus; lane 7, 3-d semimembranosus; lane 8, 6-d semimembranosus. Postmortem degradation of desmin obviously occurs more rapidly in the semimembranosus, a "fast-aging" muscle, than in the biceps femoris. (B) Anti-vinculin blots of bovine biceps femoris (lanes 1–4) and bovine semimembranosus muscle (lanes 5–8). These muscle samples were also taken from the same animal, and the rate of postmortem vinculin degradation in these two sets of blots can be compared directly. Lane 1, at-death biceps; lane 2, 1-d biceps; lane 3, 3-d biceps; lane 4, 6-d biceps; lane 5, at-death semimembranosus; lane 6, 1-d semimembranosus; lane 7, 3-d semimembranosus; lane 8, 6-d semimembranosus. The rate of postmortem vinculin degradation is much faster in the semimembranosus than in the biceps femoris. (C) Anti-vinculin blots of tough (lanes 1, 2, and 6) and tender (lanes 3, 4, and 5) lamb longissimus muscle (see Table 2 for details). Lane 7 contained purified bovine cardiac muscle vinculin. The major fragments in lanes 3, 4, and 5 migrate at an approximate molecular weight of 90 kDa, corresponding to the major polypeptide fragment produced by calpain digestion of purified vinculin.

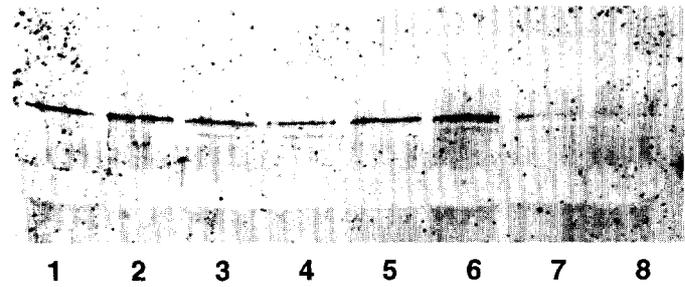


Figure 8. Western blots showing rate of degradation of dystrophin during postmortem storage. SDS-polyacrylamide gels of samples from the biceps femoris and semitendinosus muscles were run, were transferred to nitrocellulose membranes as described in Experimental Procedures, and the nitrocellulose membranes were probed with an anti-dystrophin antibody. Lanes 1–4 are biceps femoris muscle sampled at death (lane 1) and after 1 d (lane 2), 3 d (lane 3), and 6 d (lane 4) postmortem. Lanes 5–8 are semimembranosus muscle sampled at death (lane 5) and after 1 d (lane 6), 3 d (lane 7), and 6 d (lane 8) postmortem. The Western analysis shown in this experiment used alkaline phosphatase detection rather than ECL detection, and the "grainy" appearance is from the reaction with 5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium used in alkaline phosphatase detection. No prominent reaction with dystrophin-related proteins was detected in our studies.

than 50% of the dystrophin in at-death muscle remains after 6 d postmortem, even in the slow-aging biceps femoris (Figure 8). Loss of dystrophin, therefore, may not be related to destruction of costameres during the first 24 h postmortem but may contribute to the postmortem tenderization that occurs between 24 and 72 h postmortem.

Titin and nebulin have been reported to be constituents of the N₂ line (Funatsu et al., 1990), and ultrastructural studies have shown that the N₂ line is the one of the first structural entities degraded by calpain (Goll et al., 1991, 1992b; Taylor et al., 1994). The titin and nebulin molecules both extend through the entire I-band, where myofibrils seem to rupture when being prepared for MFI assays (Figure 5), and anchor in the Z-disk (Fürst et al., 1988; Wang and Wright, 1988). Therefore, Western analysis was used to determine the rates at which nebulin and titin are degraded during postmortem storage and whether their degradation occurs during the first 3 d postmortem at the same time as the large changes in tenderness occur. Approximately 25% of skeletal muscle nebulin is degraded during the first 24 h after death in both the biceps femoris and semitendinosus muscles (this degradation is not obvious from the Western blots shown in Figure 9 but is revealed when the blots are scanned (Table 1). Degradation of

nebulin may be related to loss of the N₂ line in postmortem muscle. The Nb₂ anti-nebulin MAb recognizes an epitope near the N₂ line in the large nebulin molecule (Fürst et al., 1988). That this anti-nebulin MAb also recognizes large fragments of the nebulin molecule, only slightly smaller than the 600- to 900-kDa intact nebulin molecule, indicates that postmortem degradation of nebulin occurs at one end of the nebulin polypeptide rather than in its center. If this degradation is at the Z-disk end (C-terminal end) of the nebulin polypeptide, it would contribute to weakening of the Z-disk attachment at this point and may account for breaks in this area observed in the MFI myofibrils (Figure 6).

Titin was already partly degraded in the 0-time (at-death) samples, because they contained approximately a 50-50 mixture of the T₁ and T₂ forms of titin (Figure 9B). Earlier studies have reported

that it is difficult to avoid partial degradation of titin to the T₂ form during sampling. Little additional degradation of titin occurs during the first 24 h postmortem, but the remaining T₁ form of titin is entirely degraded to the T₂ form between 24 and 72 h of postmortem storage (Figure 9B). The T₁₂ anti-titin MAb recognizes an epitope approximately .1 μm from the Z-disk, and degradation of titin from the T₁ to the T₂ form results from cleavage at the N-terminal end (the Z-disk end) of the titin polypeptide (Fürst et al.,

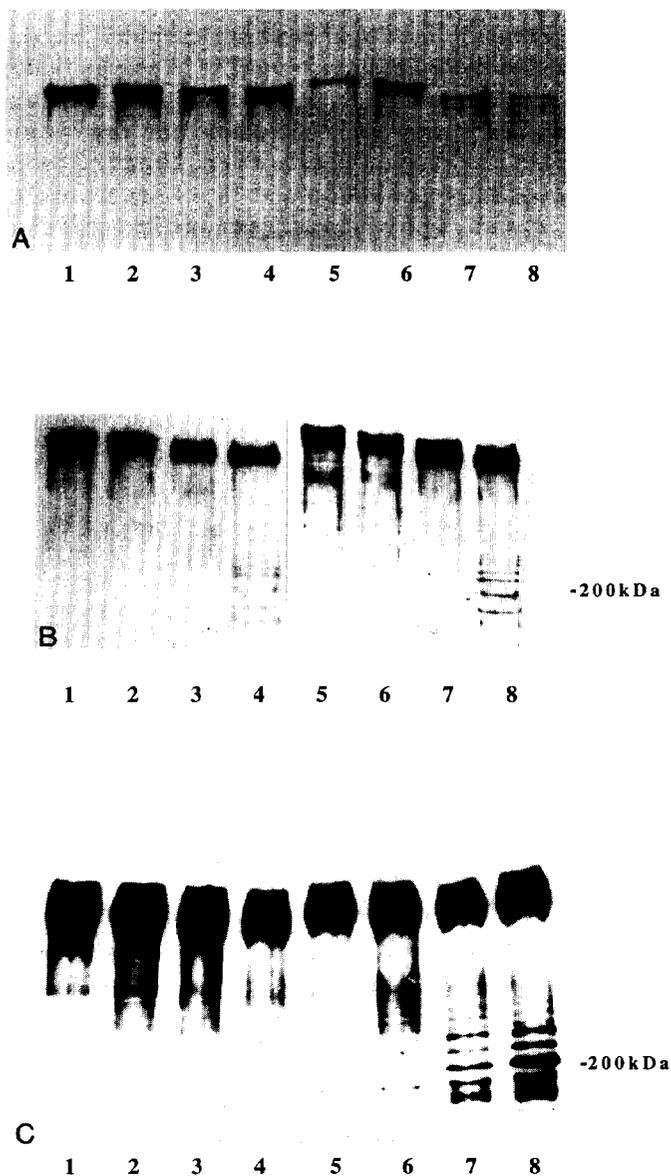


Figure 9. Western blots showing rates of degradation of nebulin and titin during postmortem storage. (A) Anti-nebulin blots of bovine biceps femoris and semimembranosus muscles at death and after 1, 3, and 6 d of postmortem storage at 4°C. Lanes 1-4 are biceps femoris muscle at death (lane 1) and after 1 d (lane 2), 3 d (lane 3), and 6 d (lane 4) postmortem. Lanes 5-8 are semimembranosus muscle at death (lane 5) and after 1 d (lane 6), 3 d (lane 7), and 6 d (lane 8) postmortem. Lane 5, the at-death semimembranosus sample, inadvertently had less protein loaded on it than the other lanes shown in this figure. (B) Anti-titin blots of bovine rectus abdominis muscle (lanes 1-4) and of myofibrils prepared from the same rectus abdominis muscle (lanes 5-6). Lanes 1-4 are rectus abdominis muscle at death (lane 1), and after 1 d (lane 2), 3 d (lane 3), and 6 d (lane 4) of postmortem storage at 4°C. Lanes 5-6 are myofibrils from rectus abdominis muscle at death (lane 5), and after 1 d (lane 6), 3 d (lane 7), and 6 d (lane 8) of postmortem storage at 4°C. The polypeptides migrating in the range of 150 to 180 kDa (6 d) resemble polypeptides that are produced by digestion of myofibrils with purified calpain and that react with the T₁₂ anti-titin MAb (Thompson et al., 1993). The polypeptides above 200 kDa are not normally observed in calpain-treated muscle. The pattern of titin degradation observed in myofibrils is identical to that observed in whole muscle strips, so preparing myofibrils did not wash titin fragments produced by postmortem storage from the myofibril. Even the smaller polypeptides migrating in the range of 150 to 350 kDa are identical to those seen in Western blots of intact muscle fibers. (C) Anti-titin blots of bovine biceps femoris and semitendinosus muscles at death and after 1, 3, and 6 d of postmortem storage at 4°C. Large amounts of protein were loaded onto the gels in this experiment so the fragments produced by degradation of titin postmortem can be more easily detected. Lanes 1-4 are biceps femoris muscle at death (lane 1) and after 1 d (lane 2), 3 d (lane 3), and 6 d (lane 4) postmortem. Lanes 5-8 are semimembranosus muscle at death (lane 5) and after 1 d (lane 6), 3 d (lane 7), and 6 d (lane 8) postmortem. The T₁ and T₂ forms of titin are not distinguishable at these heavy loads and appear only as a broad band.

1988). Consequently, postmortem storage results in cleavage of the titin polypeptide at a site closer than .1 μm from the Z-disk; this cleavage occurs sometime between 24 and 72 h postmortem at 4°C. This degradation of titin together with nebulin degradation (Figure 9A) likely contributes to the increased I-band fragility in myofibrils during postmortem storage. The N_2 line is approximately .22 μm from the Z-disk, so postmortem cleavage of both titin and nebulin occurs at a site that is closer to the Z-disk than the N_2 line. Some additional degradation of titin to polypeptides recognized by the T_{12} MAb occurs between 72 and 144 h postmortem, but the major T_2 polypeptide remains (Figure 9B and 9C). Hence, the major fraction of postmortem titin degradation occurs between 24 and 72 h postmortem and temporally coincides with the major increase in tenderness (Figure 3).

We prepared myofibrils from muscle at different times postmortem and then subjected these myofibrils to Western blot analysis to learn whether the washing steps used in preparing myofibrils removed any titin fragments produced during postmortem storage. The results of this experiment showed that the major titin fragments that accumulate during postmortem storage remain associated with the myofibril and that similar results concerning the postmortem degradation of titin are obtained whether using whole muscle or myofibrils (Figure 9B).

These results on rate of postmortem titin degradation differ from those reported earlier by Fritz and Greaser (Fritz and Greaser, 1991; Fritz et al., 1993) but agree with Bandman and Zdanis (1988), who also used Western blotting to show that significant titin degradation occurs in bovine semitendinosus muscle between 24 and 72 h postmortem. Most investigators (see Paxhia and Parrish, 1988; Anderson and Parrish, 1989) find substantial degradation of titin to the T_2 form during the first 3 d postmortem, especially in "light" (Paxhia and Parrish, 1988) or "tender" (Longergan, Parrish, and Robson, Iowa State Univ., unpublished results) muscles. It is not clear whether the T_1 and T_2 forms of titin were resolved in Fritz and Greaser (1991); the titin band in this study is broad and is barely separated from nebulin in the gels shown. The Fritz et al. (1993) study used only two time periods, 2 d and 16 d postmortem, and the titin again may have already been in the T_2 form in the first sample. Hence, it is possible the Fritz and Greaser studies measured degradation of the T_2 form of titin, which is stable during postmortem storage, rather than the conversion of T_1 to T_2 .

Discussion

The results of this study indicate that postmortem tenderization involves a complex interplay among at least three interacting events.

1. *An increased rigidity or strengthening followed by a weakening of the actin/myosin interaction.* The nature of this strengthening and weakening is not understood. Strengthening of the actin/myosin interaction in postmortem muscle markedly increases toughness and occurs during the first 24 h postmortem (in bovine skeletal muscle stored at 4°C or earlier in other species such as poultry). This strengthening is usually accompanied by sarcomere shortening such as occurs to an extreme extent during cold shortening. Shortening increases the degree of toughening that occurs during strengthening of the actin/myosin interaction, but it seems unlikely that shortening alone is responsible for the large increase in toughness that occurs during the first 24 h postmortem. The large decrease in toughness (increase in tenderness) that occurs between 24 and 72 h postmortem (Figure 2) is due partly to a weakening of the actin/myosin interaction. Again, the nature of this weakening is unknown, but it results in gradual lengthening of rigor-shortened sarcomeres (Stromer et al., 1967; Goll et al., 1970), in a 20 to 80% increase in the Mg^{2+} -modified ATPase activity of myofibrils (Goll and Robson, 1967), and in an increase in the rate of actomyosin superprecipitation (Goll et al., 1970). It seems likely that the postmortem changes in ATPase and superprecipitation involve the actin/myosin interaction rather than changes in myosin itself because the EDTA-modified ATPase activity of myofibrils, which measures myosin ATPase activity alone at an ionic strength of .5M, remains unchanged during postmortem storage (Goll and Robson, 1967).

2. *Disruption and weakening of the connections between thin filaments in the I-band and the Z-disk.* This disruption may involve partial degradation of some as yet unidentified Z-disk proteins, but it probably is principally due to degradation of titin and nebulin, which extend into the Z-disk and are anchored there by interaction with a Z-disk protein (possibly α -actinin). The two major Z-disk proteins, α -actin and α -actinin, are not degraded during postmortem storage, at least not to any appreciable extent, during the first 14 to 18 d postmortem at 4°C.

3. *Degradation of the costameres and intermyofibril linkages.* As shown in the present study, costameres are partly degraded within 24 h postmortem and are almost completely destroyed after 72 h postmortem. Even partial degradation of costameres may cause weakening of the sarcolemma and lead to increased leakage of extracellular Ca^{2+} into muscle cells and to the increased osmolarity observed in postmortem muscle (Ouali, 1990). Muscle osmolality increases by 60 to 70% during the first 24 h postmortem and then remains almost constant for 9 d postmortem (Ouali 1990). Consequently, osmolality per se does not seem to be causally related to the large changes in postmortem tenderness that occur during this period.

Depending on the physiological state of the animal at slaughter and on the conditions and time of

postmortem storage, any one or any combination of the three events described in the preceding paragraphs could have a predominant effect on postmortem tenderness. The very large increase in toughness that occurs during the first 24 h postmortem (Figure 2) is almost certainly due to event 1, strengthening of the actin/myosin interaction. It is unclear how much of the large decrease in toughness that occurs between 24 and 72 h postmortem is due to weakening of the actin/myosin interaction and how much is due to events 2 and 3. It seems likely, however, that weakening of the actin/myosin interaction accounts for only part of this large decrease, and that events 2 and 3 are responsible for part of this change (perhaps up to 50%). Degradation of desmin, nebulin, titin, and vinculin would contribute continuously to increased tenderness beginning shortly after death and continuing for 4 to 6 d postmortem, at which time these proteins have been largely degraded (or for nebulin and titin, clipped to smaller polypeptides). The contribution of this degradation to tenderness during the first 24 to 48 h postmortem is overshadowed by the large effect that strengthening of the actin/myosin interaction has on tenderness. As the actin/myosin interaction weakens between 24 and 72 h postmortem, the effects of desmin, nebulin, titin, and vinculin degradation become evident.

It also is difficult to estimate the extent to which disruption of the thin filament/Z-disk interaction contributes to the major decrease in toughness between 24 and 72 h postmortem. After 72 h of postmortem storage at 4°C, nebulin is almost completely degraded, and titin has been almost completely converted to the T₂ form. Because the T₂ form of titin lacks the N-terminal end (the Z-disk end) of the intact titin molecule (the T₁ form of titin is approximately 2,600 to 2,800 kDa; the T₂ form is approximately 1,600 to 2,100 kDa, so the T₂ form is still a very large polypeptide), attachment of titin to the Z-disk has been destroyed after 72 h postmortem at 4°C. This degradation of titin and nebulin probably results in a significant weakening of the thin filament/Z-disk interaction.

Costameres, also, are completely destroyed during the first 72 h of postmortem storage at 4°C, and Western analysis shows that two of the important proteins constituting costameres, desmin and vinculin, are almost completely degraded during this period. Dystrophin, also a component of costameres, is substantially degraded after 72 h postmortem. Consequently, to the extent that costameres contribute to muscle fiber stability, degradation of costameres would contribute significantly to postmortem tenderization. It also seems likely that degradation of the filamentous linkages between adjacent myofibrils would contribute to postmortem tenderization. The model for postmortem tenderization proposed in the present paper, therefore, differs from previous models

in that important roles are ascribed to degradation of costameres, intermyofibril linkages, and disruption of the actin filament/Z-disk interaction, and less importance is given to degradation of the Z-disk itself. The model proposed also indicates that postmortem tenderization is a complex process and involves a number of changes including degradation of certain cytoskeletal proteins and changes in the actin/myosin interaction.

Although the model for postmortem tenderization proposed in this paper ascribes less importance to Z-disk degradation than earlier models, it nevertheless retains a central role for the calpain system in this process. Costamere proteins are all excellent substrates and are readily degraded by the calpains. Intermyofibril linkages contain desmin and filamin, both of which are rapidly cleaved by the calpains. The calpains also rapidly degrade nebulin and convert titin from the T₁ to T₂ form, both in vitro and in situ in myofibrils (Thompson et al., 1993). Consequently, it seems likely that most proteolytic degradation associated with the postmortem tenderization that occurs during the first 72 to 96 h postmortem (see Figure 2) is due to the calpain system. The evidence that the calpain system has an important role in postmortem tenderization has been summarized earlier (Goll et al., 1983a, 1992a; Koohmaraie, 1988, 1992; Figure 1). Perhaps the most compelling part of this evidence is that neither myosin nor α -actinin is degraded to any significant extent during postmortem storage at 4°C (Bandman and Zdanis, 1988; Hwan and Bandman, 1989). The calpains are unique in that they do not degrade α -actinin and myosin (Goll et al., 1991, 1992b), whereas both these proteins are rapidly degraded by cathepsins B, D, and L (Okitani et al., 1980; Matsukara et al., 1981).

The ability of calpain to remove N₂ lines and to degrade nebulin and titin suggests that calpain is responsible for postmortem weakening of the thin filament/Z-disk interaction. It has been proposed that the N₂ line results from a coalescence of titin and nebulin filament in this region of the sarcomere (Funatsu et al., 1990). Degradation of N₂ may be responsible for the "tears" or breaks in the I-band region of postmortem muscle. These tears are the most consistently observed ultrastructural changes found in postmortem muscle (Henderson et al., 1970; Penny, 1980; Ouali, 1990) and are seen more frequently than frank Z-disk degradation (Taylor et al., 1994). Our electron microscope observations of the myofibrils used in MFI assays suggest that weakening of the thin filament/Z-disk interaction is a major contributor to the postmortem increase in MFI. Ouali (1990) originally suggested that degradation of the N₂ line has an important role in postmortem tenderization. Recently, Tatsumi and Takahashi (1992) found that Ca²⁺ induced fragmentation of nebulin filaments and suggested that such fragmentation would destabilize

thin filaments and would be a key factor in postmortem tenderization. It could also be suggested that degradation of troponin T to 30-kDa fragments weakens thin filaments and that degradation of troponin T, therefore, may be a direct contributor to postmortem tenderization rather than being simply an *in situ* indicator of calpain activity as previously suggested. Because the cathepsins are localized in the sarcoplasmic reticulum in the I-band of skeletal muscle (Taylor et al., 1994), and because incubation of muscle with crude cathepsin mixtures results in I-band degradation (Ouali, 1990), the cathepsins may contribute to proteolysis in postmortem muscle after 6 or 7 d postmortem when muscle pH is low and small amounts of myosin and α -actinin degradation are observed (Bandman and Zdanis, 1988; Hwan and Bandman, 1989).

It seems likely, therefore, that the calpains are a major cause of proteolysis in skeletal muscle during the first 72 to 96 h postmortem, but that this proteolysis probably results in destruction of costameres and intermyofibrillar linkages and in weakening of the thin filament/Z-disk interaction rather than in direct Z-disk degradation, as previously proposed. Hence, the answer to the question posed by the title of this paper is, probably not a major role. However, the questions as to how the calpains, with their high Ca^{2+} requirement for proteolytic activity and in the presence of excess calpastatin, which can completely inhibit their activity, can ever be active in postmortem muscle remain (Goll et al., 1983a, 1994).

Implications

The findings reported in this paper indicate that degradation of intermyofibrillar linkages and of costameres, structures that link myofibrils to the sarcolemmal cell membrane of muscle cells, and weakening of the thin filament/Z-disk interaction have major roles in postmortem tenderization of muscle. Z-disk degradation, however, which was previously thought to have a major role in postmortem tenderization, does not occur to any significant extent during the first 3 or 4 d when most postmortem tenderization occurs. If this conclusion is correct, future studies attempting to enhance the rate and uniformity of postmortem tenderization should focus on determining how to increase the rate of degradation of costamere proteins such as desmin and vinculin, and of thin filament/Z-disk proteins such as nebulin and titin.

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