STRUCTURAL WEAKENING OF INTRAMUSCULAR CONNECTIVE TISSUE DURING CONDITIONING OF BEEF

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SUMMARY

Structural changes in intramuscular connective tissue during conditioning of beef were studied by electron microscopy.

Bovine *semitendinosus* muscle was dissected from carcass 48 h post-mortem and stored at 4°C. The three-dimensional organization of collagen fibrils in the endomysium and perimysium was investigated using the cell-maceration/scanning electron microscope method, by which cellular elements were eliminated and collagen fibrils and fibres were exposed. The arrangement of proteoglycans in intramuscular connective tissue was examined under a transmission electron microscope, using Cuprolinic Blue, a cationic dye for identification of proteoglycans. Perimysial fractions were isolated from conditioned beef and the amount of hexuronic acid was measured by carbazole method.

Scanning electron microscopic studies revealed that the endomysium resolved into individual collagen fibrils and thick sheets of the perimysium separated into collagen fibres in beef conditioned for 28 days. These results show directly the structural weakening of the endomysium and perimysium during conditioning of beef. Transmission electron microscopic studies displayed that most proteoglycans were dissociated from collagen fibrils in beef conditioned for 28 days. The amount of hexuronic acid in perimysial fractions decreased with time. These results suggest that the interaction between collagen fibrils and proteoglycans weaken during conditioning of beef.

The structural changes in intramuscular connective tissue described above were minimal until 7 days post-mortem, but clearly observable after 14 days post-mortem. Therefore, we conclude that intramuscular connective tissue shows the effect on tenderization in extended conditioning (2-4 weeks) of beef.

Introduction

Conditioning is the process in which meat toughened by rigor mortis is naturally tenderized (Lawrie, 1985). Though it has not been conclusively proven, the tenderness of meat is believed to be the result of the weakening of the myofibrils (Takahashi, 1992) and the intramuscular connective tissues (Etherington, 1987). Collagen is the major connective tissue component of meat (Light et al., 1985), which has been implicated in contributing to meat toughness (Bailey & Light, 1989). In early studies it has been shown that the solubility of collagen is affected by neither temperature nor time of conditioning (Sharp, 1963; Pierson & Fox, 1976; Chizolini et al., 1977). These results suggest that collagen remains unchanged at the molecular level during conditioning. However, Stanton & Light (1988, 1990) have presented data which proved that perimysial collagen is damaged and partially solubilized during conditioning. Using differential scanning calorimetry, Judge & Aberle (1982) have shown that the thermal shrinkage temperature of bovine intramuscular collagen decreases by 7-8°C within 7 days post-mortem. It has also been shown that the isometric tension of the intramuscular collagen decreases at 21 days post-mortem in beef (Etherington, 1987). These results suggest post-mortem alterations in the intramuscular connective tissue.

Collagen fibrils of intramuscular connective tissue are embedded in ground substances such as proteoglycans and glycoproteins. It has been reported that proteoglycans bind to collagen fibrils at a specific site in connective tissue, such as tendon (Scott, 1991). We found that proteoglycans are arranged along collagen fibrils of the perimysium with a regular interval of 65 nm, which is quite agreement with the periodicity of collagen fibrils (Nishimura et al., manuscript in preparation). Their precise role remains to be elucidated but they bind to collagen fibrils and may have an important function in tissue stability.

The objective of this study was to investigate the structural changes in intramuscular connective tissues during conditioning of beef by electron microscopy. We show here that the intramuscular connective tissues resolve into individual collagen fibrils and most proteoglycans are dissociated from collagen fibrils in beef conditioned for 28 days.

Materials and Methods

Preparation of muscle samples.

Japanese Black steers aged 32 months were stunned and slaughtered conventionally. *Semitendinosus* muscles were dissected from the carcasses 48 h post-mortem and were treated antiseptically by dipping them in a solution containing 1 mM NaN_3. Then they were wrapped with polyethylene films and stored for 28 days at 4°C.

Scanning electron microscopy.

According to the cell-maceration method of Ohtani et al. (1988), small pieces of muscle (10x10x15 mm) were cut out and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer solution, pH 7.3, for 1 day. The pieces were immersed in 10% NaOH for 7 days and then rinsed in distilled water for 5 days at room temperature. They were put in 1% tannic acid for 3 h, rinsed in distilled water for several hours, and post-fixed in 1% osmium tetroxide for 1 h. The specimens were dehydrated in a series of graded concentrations of ethanol, freeze-fractured with a razor blade in liquid nitrogen, and dried by the t-butyl alcohol freeze-drying method. The dried specimens were coated with gold and observed using a scanning electron microscope (S-800, Hitachi, Tokyo) with an accelerating voltage of 10 kV.

Transmission electron microscopy.

According to the method of Scott (1980), samples (1x1x2 mm) were cut out and were fixed and stained in a solution containing 2.5% gultaraldehyde, 0.3 M $MgCl_2$ and 0.025 M sodium acetate buffer, pH 5.6, with 0.1% Cuprolinic Blue (POLYSCIENCE INC., USA). After 24 hours, the tissue samples were washed three times in a same solution without Cuprolinic Blue, and immersed in a same solution with 0.5% sodium tungstate for 1 hour. Tissue samples were subsequently washed, dehydrated, embedded in epon, and sectioned. Ultrathin sections on formvar-coated grids (80 μm in thickness) were post-stained with 10% uranyl acetate for 10 min. Each sample was observed with a transmission electron microscope (H-800, HITACHI) with an accelerating voltage of 75 kV.

Preparation of perimysial fractions.

Perimysial fraction was extracted and purified, using the method described by Stanton & Light (1987), which, briefly, involve the following steps. Muscle samples (50 g wet weight) were minced and homogenized in 200 ml of ice-cold 0.05 M $CaCl_2$ for 10 s at 10,000 rpm in a Virtis homogenizer (The Virtis Co., New York). The homogenate was filtered through a graded copper grid (1 mm^2 perforations) and the material retained on the filter was re-homogenized in 0.05 M $CaCl_2$ and re-filtered. The process was repeated twice, and the retained material was denoted the perimysial fraction.

Results and Discussion

Figure 1A shows an electron micrograph of intramuscular connective tissue of bovine *semitendinosus* muscle immediately post-mortem. The honeycomb structure of the endomysium, the sheaths 60-100 mm in diameter for housing individual muscle fibres, was clearly observed (Fig. 1B). The sheaths of endomysium were membranous and consisted of tightly arranged collagen fibrils 30-70 nm in diameter (Fig. 1C). The perimysium was composed of several layers of 100-200 mm-thick sheets surrounding the endomysia (Fig. 1A). The wavy sheets of perimysium consisted of collagen fibres in which the fibrils were in close contact with each other (Fig. 1D). Some collagen fibrils formed loose networks at the surface of the perimysial sheets and between two sheets. The well-ordered anatomical arrangement of collagen fibrils and fibres in the endomysium and perimysium seems to have an important function in tissue stability of skeletal muscle.

These structures of the intramuscular connective tissue remained unchanged for up to 7 days post-mortem, but a progression of structural alterations was clearly visible after 14 days post-mortem. In intramuscular connective tissue of bovine *semitendinosus* muscle conditioned for 28 days at 4°C, gaps of various sizes opened everywhere (Fig. 2A); the honeycomb structure of endomysium markedly deformed, and

the perimysial sheets disintegrated into ribbon-like structures. Endomysial sheaths became lacy (Fig. 2B). At higher magnification, the endomysium resolved into individual collagen fibrils that were arranged loosely, though neither broken nor torn (Fig. 2C). A closer view of perimysium shows that the thick sheets of perimysium separated into collagen fibres of 4-8 mm in diameter, where collagen fibrils lay more or less in parallel (Fig. 2D). These results indicate that the structural changes in intramuscular connective tissue are induced by the dissociation of collagen fibrils and fibres from endomysial sheaths and perimysial sheets, respectively.

Fig. 3A shows the transverse section along the axis of muscle fibre of *semitendinosus* muscle immediately post-mortem. Proteoglycans of 20-40 nm in length, being heavily stained with Cupurolinic Blue, were arranged in the lamina lucida of basal laminae and seemed to be attached each other. Proteoglycans of 40-70 nm in length were randomly distributed in the reticular layer and the endomysium. In the perimysium, proteoglycans of 40-50 nm in length were arranged along collagen fibrils with a regular interval of 65 nm, which is quite agreement with the periodicity of collagen fibrils (Fig. 3B). They seemed to be linked to collagen fibrils at the specific binding site and appeared to be contributed to linking collagen fibrils in the perimysium.

In bovine *semitendinosus* muscle conditioned for 28 days at 4°C, no proteoglycan was observed in the basement membrane (Fig. 4A). A few of proteoglycans were randomly distributed in the endomysium. In the perimysium, most proteoglycans were dissociated from collagen fibrils (Fig. 4B). The amount of hexuronic acid in perimysial fractions decreased with time. These results suggest that the interaction between collagen fibrils and proteoglycans weakened during conditioning of beef. Proteoglycans may tightly bind collagen fibrils to each other in intramuscular connective tissue *in vivo*, and the binding properties may diminish during conditioning of beef, resulting in the separation of collagen fibrils and fibres.

Conclusion

Electron microscopic studies demonstrated that the intramuscular connective tissue resolves into individual collagen fibrils and most proteoglycans are dissociated from collagen fibrils in beef conditioned for 28 days. The structural changes in intramuscular connective tissue described above were minimal until 7 days post-mortem, but clearly observable after 14 days post-mortem. Therefore, we conclude that intramuscular connective tissue shows the effect on tenderization in extended conditioning (2-4 weeks) of beef.

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Figure Legends

Fig. 1. Scanning electron micrographs of intramuscular connective tissue of bovine *semitendinosus* muscle immediately post-mortem. (**A**) Low magnification view of intramuscular connective tissue. (**B**) Endomysial sheaths. (**C**) A closer view of a part of (B). (**D**) A closer view of a part of the perimysium. Scale bars indicate 100 (A), 25 (B), 1.0 (C) and 5.0 mm (D). E, Endomysium; P, perimysium.

Fig. 2. Scanning electron micrographs of intramuscular connective tissue of bovine *semitendinosus* muscle conditioned for 28 days at 4°C. (**A**) Low magnification view of intramuscular connective tissue. (**B**) Endomysial sheaths. (**C**) A closer view of a part of (B). (**D**) A closer view of a part of the perimysium. Scale bars indicate 100 (A), 25 (B), 1.0 (C), 5.0 (D) mm. E, Endomysium; P, perimysium.

Fig. 3. Transmission electron micrographs of intramuscular connective tissue of bovine *semitendinosus* muscle immediately post-mortem. (**A**) The cross section along the axis of muscle fibre. (**B**) Longitudinal section of the bundle of collagen fibrils in the perimysium. Arrows indicate proteoglycans stained with Cuplolinic Blue. Scale bars indicate 200 nm (A) and 200 nm (B).

Fig. 4. Transmission electron micrographs of intramuscular connective tissue of bovine *semitendinosus* muscle conditioned for 28 days at 4°C. (**A**) The cross section along the axis of muscle fibre. (**B**) Longitudinal section of the bundle of collagen fibrils in the perimysium. Arrows indicate proteoglycans stained with Cuplolinic Blue. Scale bars indicate 200 nm (A) and 200 nm (B).