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The protection of bovine skeletal myofibrils from proteolytic damage *post mortem* by small heat shock proteins

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ABSTRACT

This study aimed to determine how small heat shock proteins (sHSPs) protect myofibrillar proteins from μ-calpain degradation during ageing. Immunoprecipitation experiments with *M. longissimus dorsi* (LD) from Angus heifers (n = 14) examined the interaction between αβ-crystallin, desmin, titin, HSP20, HSP27 and μ-calpain. Results showed that αβ-crystallin associated with desmin, titin, HSP20, HSP27 and μ-calpain. Exogenous αβ-crystallin reduced desmin and titin degradations in myofibrillar extracts and attenuated μ-calpain activity. In a second experiment, bull LD (n = 94) were aged at −1.5 °C for up to 28 days *post mortem*. μ-Calpain autolysed faster in high ultimate pH (pH_u) meat ($pH_u \geq 6.2$) and this was concomitant with the more rapid degradation of titin and filamin in this pH_u group. Desmin stability in intermediate pH_u meat (pH_u 5.8 to 6.19) may be due to the protection of myofibril-bound sHSPs combined with the competitive inhibition of μ-calpain by sHSPs.

1. Introduction

The integrity of the myofibrillar framework within a sarcomere is essential to the function of skeletal muscle. The intracellular environment surrounding myofibrils within a working muscle is in a constant state of flux, which may expose myofibrillar structural proteins to possible damage. During muscle trauma, muscle homeostasis is disrupted which can lead to the fragmentation of myofibrillar structural proteins and loss of sarcomere integrity and muscle function (Gissel & Clausen, 2001; Zhang, Yeung, Allen, Qin, & Yeung, 2008).

Under normal conditions, intracellular calcium concentrations are maintained at low levels in the sarcoplasmic reticulum by calcium ATPase pumps. However, muscle stress, such as strenuous exercise may lead to the accumulation of intracellular calcium and result in irreversible damage (Gissel & Clausen, 2001). A consequence of elevated calcium concentration is the potential activation of the endogenous calcium-dependent protease μ-calpain (Belcastro, Shewchuk, & Raj, 1998). Because the myofibrillar structural proteins including desmin and titin are known μ-calpain substrates and are integral to Z-disc myofibrillar structure (Baron, Jacobsen, & Purslow, 2004; Raynaud et al., 2005), the degradation of these proteins may result in the destabilisation of the sarcomere.

In response to the detrimental changes taking place within a muscle cell during stress, a series of compensatory cellular mechanisms are activated to limit damage. These include inflammatory/repair responses (Tidball, 2005) and an increased expression of selective stress proteins (McArdle, Vasilaki, & Jackson, 2002). Small heat shock proteins (sHSPs), also known as chaperone proteins, have been shown to contribute to the maintenance and/or remodelling of structural as well as functional proteins during stress (Sun & MacRae, 2005; Tytell & Hooper, 2001). The role of sHSPs at maintaining muscle structural integrity due to intracellular stresses such as changing pH has significant implications for meat quality. For example, studies have shown that variation in *post mortem* muscle ultimate pH affects the redistribution of sHSPs in the muscle and the nature of this intracellular distribution together with the intracellular pH is correlated with meat tenderness (Pulford et al., 2008).

The sHSP αβ-crystallin is an important chaperone in preserving the infrastructure of various cells. In skeletal and cardiac muscles, αβ-crystallin associates with myofibrillar proteins in both the Z-disc and I band by interacting with exposed hydrophobic residues of myofibrillar structural proteins like desmin and titin (Bennardini, Wrzosek, & Chiesi, 1992; Raman & Rao, 1994).

In *post mortem* muscle, calcium accumulates in the sarcoplasm, activating μ-calpain and initiating the proteolytic degradation of myofibrillar proteins leading to meat tenderisation (Koohmaraie, 1992b). To prevent the inevitable apoptotic death of muscle cells *post mortem*, it has been proposed that sHSPs are induced and recruited to maintain

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cell homeostasis (Ouali et al., 2006). The contribution of sHSPs to meat quality is not yet understood. However, studies have demonstrated the expression of αβ-crystallin and HSP27 in muscle are correlated with shear force and colour (Bernard et al., 2007; Kim et al., 2008).

This study first explores the ability of the sHSP αβ-crystallin to maintain myofibrillar protein structure following the activation of exogenous μ-calpain. Second, the relationship between sHSPs expression, shear force, ultimate pH (pH_u) and myofibrillar protein degradation was investigated to determine whether the interactions of sHSPs with myofibrillar proteins *in vitro* are mimicked in muscle during *post mortem* ageing.

2. Materials and methods

2.1. Experiment 1

2.1.1. Animals and sample collection

M. longissimus dorsi (LD) from Angus heifers (n = 14) all stunned by captive bolt then slaughtered on the same day. Carcasses were not electrically stimulated. After the LD muscles were hot-boned from the carcass at approximately 30 min *post mortem*, they were packed in vacuum bags and held in a 37 °C water bath. Muscle pH was monitored using a combination puncture electrode pH metre (Mettler-Toledo GmbH, Switzerland). Approximately 10 g of muscle samples was collected from each LD at pH 7.4, 7.2, 7.0, 6.8 and 6.5 and used for subsequent biochemical analyses.

2.1.2. Extraction of muscle fractions and protein measurement

For the extraction of the soluble muscle fraction, 2 g of muscle was homogenised in soluble fraction extraction buffer (50 mM Tris–HCl, 10 mM EDTA, 1 mM DTT, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) with COMPLETE™ protease inhibitors (Roche, 11 836 170 001); pH 7.4). The homogenate was then centrifuged at 3000 *g* for 10 min at 4 °C and the soluble muscle fraction stored at −80 °C until analysed.

Bovine myofibrillar fractions with associated proteins were prepared as described by Wang et al. (1988). Muscle fibres were teased apart in myofibrillar extraction buffer (50 mM Tris–HCl, 10 mM EGTA, 2 mM $MgCl_2$, 0.1 mM PMSF, 0.1% Triton X-100; pH 7.0) and stored in the buffer at 4 °C overnight. The muscle fibres were then gently homogenised in the same buffer and centrifuged at 3000 *g* for 5 min. The supernatant was discarded and the myofibrillar pellet was washed three times in myofibril extraction buffer without Triton X-100. The myofibrillar fraction was stored in 50% glycerol in a standard salt solution (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM NaN_3; pH 6.8) at −20 °C until analysed.

For the preparation of stripped myofibrillar fractions, 0.5 g of muscle sample was teased apart in stripped myofibrillar extraction buffer (20 mM NaH_2PO_4:Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.1 PMSF. 0.1 mg mL^{-1} soybean trypsin inhibitor, 0.5% Triton X-100; pH 7.0), finely chopped with scissors then agitated at 200 rpm in an oscillating shaker in extraction at room temperature for 1 h. The resulting suspension was centrifuged at 2000 *g* at 4 °C for 5 min. The supernatant was discarded and the pellet was washed four times with BASS buffer (0.1 M NaH_2PO_4:$NaHPO_4$, 25 mM KCl, 39 mM boric acid, 0.1 mM PMSF, 0.1 mg mL^{-1} soybean trypsin inhibitor; pH 7.0). The pellet was then resuspended in BASS buffer containing 0.5 mg mL^{-1} collagenase type 1 (Roche, 04 834 606) and 0.1 mM $CaCl_2$ and digested at 25 °C for 30 min before redispersing the digested pellet in the buffer followed centrifugation at 2000 *g* for 5 min. The resulting pellet was washed four times with BASS buffer and resuspended in ice cold phosphate buffer (0.1 M NaH_2PO_4:Na_2HPO_4; pH 7.0) followed by centrifugation at 300 *g* for 5 min. Myofibrils remaining in the suspension were pelleted from the supernatant by centrifugation at 3000 *g* for 5 min. Some myofibrils were then 1: stripped further using phosphate buffer containing 0.5 M KCl for 10 min on ice, washed three times with phosphate buffer, suspended in phosphate buffer containing 0.1 mM PMSF and 50% glycerol then stored at −20 °C (this treatment removed all myofibril associated proteins, but maintained the myofibrillar structural protein integrity) or 2: resuspended in phosphate buffer (these myofibrils had most associated proteins removed, including μ-calpain, but still had αβ-crystallin attached).

Sample protein concentrations for all soluble and myofibrillar fractions were determined using a Bradford Protein Assay kit (BioRad, 500-0202).

2.1.3. Purification of bovine αβ-crystallin

Bovine LD muscle was homogenised in extraction buffer (10 mM Tris–HCl, 10 mM EDTA, 2 mM DDT; pH 7.5) then filtered through a fine wire mesh to remove large particles. The suspension was centrifuged at 15,000 *g* and the supernatant was stored at −80 °C until purified.

Protein purification was conducted at 4 °C using GE Pharmacia FPLC system, as described by Atomi et al. (1991) and Schoenmakers et al. (1969). Briefly, the soluble muscle suspension was initially passed through a Sephacryl S-200 size exclusion column (Amersham Biosciences, 17-0584-10), and the fractions containing αβ-crystallin, as determined by immunoblot were passed through a DEAE Sepharose fast flow FPLC column (Amersham Biosciences, 17-0907-01). Finally, αβ-crystallin was passed through a SP Sepharose fast flow column (Amersham Biosciences, 17-0729-01) and it was eluted using a linear salt gradient (0 to 0.5 M KCl), with αβ-crystallin eluting at approximately 0.3 M KCl. αβ-Crystallin fractions were pooled and dialysed against a buffer (10 mM NaH_2PO_4:$NaHPO_4$; pH 7.0), then concentrated to approximately 10 mg mL^{-1} using Centricon-10 filters (Millipore, 4206) and stored at −80 °C. Purity and renaturation of the bovine αβ-crystallin were verified by sodium dodecyl gel electrophoresis (SDS-PAGE) (data not shown).

2.1.4. Gel electrophoresis and Western blotting

Protein samples (10 μg) were mixed with Laemmli sample loading buffer with reductant (mercaptoethanol) (Laemmli, 1970), heated in a boiling water bath for 5 min before resolution on a 10% SDS-PAGE gel. For the separation of titin fragments, the myofibril fractions were suspended in sample loading buffer devoid of reductant (50 mM Tris–HCl, 0.2% SDS, 0.2% bromophenol blue), heated at 50 °C for 15 min then resolved on 5% SDS-PAGE gels.

Proteins were subsequently blotted onto Immobilon-P PVDF membranes (Millipore, IPVH00010) and then blocked overnight at 4 °C in 5% non-fat dry milk powder in PBS-Tween (0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.1 M NaCl, 0.1% Tween). The blots were probed with anti-αβ-crystallin (Abcam, ab13496), anti-HSP20 (Hytest, HSP20-11), anti-HSP27 (Hytest, #4HSP27), anti-μ-calpain (Thermo Scientific, MA3-940), anti-desmin (Sigma, D1033) or anti-titin (Sigma, 9D10) monoclonal antibodies for 1 h at ambient temperature. All monoclonal antibodies were derived from mouse cells. αβ-Crystallin, HSP20 and HSP27 primary antibodies were diluted 1:10,000 in PBS-Tween. Desmin, titin and μ-calpain antibodies were diluted 1:5000, 1:200 and 1:5000, respectively, in PBS-Tween. Membranes were washed four times for 10 min with PBS-Tween, then incubated with goat anti-mouse IgG (H + L) horseradish peroxidase (BioRad, #172-1011) diluted to 1:5000 in PBS-Tween. Following four washes with PBS-Tween, as previously described, membrane bound antibody was detected with ECL Western blot substrate kit (Pierce, 32106). Western blot images were captured with a G:Box Chemi HR16 image capture instrument (Syngene, Cambridge, UK).

2.1.5. Immunoprecipitation of myofibrillar and soluble proteins

αβ-Crystallin antibody (10 μg mL^{-1}) was covalently bonded to 100 μL Dynabeads® Protein G magnetic beads (Invitrogen, 100-03) according to the manufacturer's protocol. Immunoprecipitation of soluble and myofibrillar proteins was performed in 1 mL immunoprecipitation buffer (50 mM Tris–HCl, COMPLETE™ protease inhibitor (Roche, Cat. No. 11 836 170 001), 10 mM EDTA, 10 mM EGTA; pH 7.4) containing