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Inhibition of protease activity 2. Degradation of myofibrillar proteins, myofibril examination and determination of free calcium levels

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Abstract

The structure of muscle injected with specific cysteine protease inhibitors was examined to determine whether inhibitors cause denaturation and the degradation post-mortem of myofibrillar proteins was followed using SDS electrophoresis. Given the central role of calcium in theories of tenderisation the level of free calcium was measured during the early post-mortem period. The protease enzyme inhibitor E-64 was injected into the *m. longissimus et thoracis lumborum* (LTL) on the right side of 12 lamb carcasses within 15 min of death and in another 12 carcasses with the protease inhibitor Z-Phe-Ala-CHN_2. The left LTL (control) was injected with saline (0.25 M NaCl). Muscle samples were obtained at death, pH 6.2 and 6.0 and then at 1 and 2 days post-mortem ($n=215$). Muscle samples were selected from eight portions of the LTL (1-day post-mortem, from six different carcasses) for examination by transmission electron microscopy. Matching light images of myofibrils were obtained after determination of myofibrillar fragmentation. Free calcium concentration was determined for all samples ($n=191$) using an ion selective electrode excluding those 'at death'. Light images of myofibrils from treated samples showed normal striations and no evidence of denaturation or aggregation compared to control samples. This also applied to the samples processed for examination by electron microscopy. Appearance of the 30-kDa subunit increased with time ($P<0.001$) post-mortem. The interaction between ageing and stimulation had an effect ($P<0.001$) on the amount of a protein designated M1. The amount of M1 measured pre-rigor was greater for stimulated muscle, but the rate of decline was also greater through to day 2 post-mortem. Proteolysis was very rapid in the first 24 h post-mortem in ovine muscle. Ageing had an effect ($P<0.001$) on the free calcium concentration, which increased as muscle aged. As a covariate pH also had an effect ($P<0.05$). Based on a non-linear model when the concentration of free calcium reached a plateau ($\sim$110 μM) the predicted pH was 5.5 (ultimate). From the qualitative observation of images and the levels of free calcium in injected muscle there is no support for the view that the inhibitors bind to sarcomere proteins, occupying sites to which calcium might bind. The levels of free calcium do not provide support for the view that m-calpain has a role in post-mortem tenderisation, but do suggest along with results of protein degradation that activation of μ-calpain is likely to occur before the pH drops to 6.2–6.1.

Keywords: Inhibitors; Proteases; Proteolysis; Myofibrils; Free calcium; Lamb

1. Introduction

A small number of studies have used specific inhibitors as a method to elucidate which proteases are active in post-mortem muscle and a brief summary of the outcomes was provided by Dransfield (1999). In the accompanying paper (Hopkins, & Thompson, 2001), we showed that the cysteine protease inhibitor, E-64 can prevent tenderisation when injected into pre-rigor muscle and that a peptidyl-diazomethane compound, that selectively inhibited the activity of cathepsins B and L, did not prevent tenderisation. This has added to the evidence that the cysteine proteases, in particular the calpains, are largely responsible for myofibrillar protein degradation (Uytterhaegen, Claeys, & Demeyer, 1994), whilst cathepsins B and L are unlikely to play a significant role in tenderisation.

Use of specific inhibitors offers the opportunity to determine the contribution that myofibrillar protein degradation makes to improvements in tenderness.

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Although the largest improvement in tenderness subsequent to rigor was observed within 1 or 2 days (Wheeler & Koohmaraie, 1994), myofibrillar protein degradation was not considered by some to be extensive during this period (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). It may be however, that degradation of strategic proteins has a significant effect on meat structure and tenderness (Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson, & Robson, 1996). Although some studies have provided estimates of the relationship between the disappearance of specific proteins and changes in tenderness (Penny & Dransfield, 1979; Taylor et al., 1995) there has been little attempt to model these relationships.

Tatsumi, Hattori, and Takahashi (1998) questioned the action of protease inhibitors, suggesting that a number of these inhibitors bound to proteins such as titin and nebulin causing deterioration and as a result, suppressing their ability to bind calcium. This result was advanced as evidence in support of the calcium theory of tenderisation. In this theory a role for enzymes in tenderisation was dismissed (Takahashi, 1996) and it was claimed that tenderisation was due to the binding of calcium to specific sites on proteins. However the claim of the binding of protease inhibitors to sarcomere proteins appears in conflict with the reported action of inhibitors such as E-64 (Fujishima, Imai, Nomura, Fujisawa, Yamamoto, & Sugawara, 1997).

To support the notion that inhibitors cause deterioration of sarcomere proteins, Tatsumi et al. (1998) presented light microscope images of homogenised myofibrils, which had been incubated in specific protease inhibitors. If, this was a real effect, then electron microscope images should provide an opportunity for closer examination of the localities within the sarcomere, which are denatured by inhibitors. To our knowledge such images have not been previously reported. If, as suggested by the 'calcium theory of tenderisation' calcium ions are bound to myofibrillar proteins, resulting in cleavage of these proteins (Takahashi, 1996), then it would be expected that the concentration of 'free' calcium would be higher in muscle treated with inhibitors such as E-64, as the inhibitor would occupy sites otherwise taken by calcium.

This, the second paper in this series, describes aspects of a study, which was designed to investigate the mechanisms that control proteolysis and the interactions with tenderness. A qualitative examination of muscle treated with inhibitors, using images from light and electron microscopy is also outlined. 'Free' calcium concentrations were determined to aid interpretation of the mechanisms by which inhibitors work and also to help clarify how these concentrations change post-rigor, as this has ramifications for the activation of calpains. A preliminary report of some aspects of this study has been presented (Hopkins, Littlefield, & Thompson, 2000c).

2. Materials and Methods

2.1. Experimental design

A full description of the design of the experiment is given by Hopkins and Thompson (2001). Briefly, 24 lambs were slaughtered in groups of eight over a 3-week period. The protease enzyme inhibitor E-64 (trans-Epoxsuccinyl-L-Leucylamindo- (4-Guandino) Butane (50 ml) was injected into the *m. longissimus thoracis et lumborum* on the right side of 12 carcasses (four per slaughter) within 15 min of death, firstly between the 10th and 12th ribs and then between the first lumbar vertebrae and the chump. In the remaining 12 carcasses (four per slaughter) the protease inhibitor Z-Phe-Ala-CHN_2 (N-carbobenzoxy-phenylalanyl-alanyl-diazomethylketone) was injected using the same procedure as for the E-64 inhibitor (50 ml). Muscle sections for the left side of each carcass ($n = 24$) were injected with 50 ml of saline (0.25 M NaCl) at the same time as the right side was injected. Muscle samples were obtained 'at death', pH 6.2 and 6.0 and then at 1 and 2 day's post-mortem for measurement of pH (1 g), free calcium (2 g) and SDS electrophoresis (2 g). For the three latter sampling times, samples were also taken for determination of the myofibrillar fragmentation index (MFI).

2.2. Light and electron microscopy

From the last slaughter group, samples of muscle were collected from portions of the *m. longissimus lumborum* (LL) 1-day post-mortem for examination by transmission electron microscopy. For preparation of electron microscopic images, samples were selected from eight portions of the LL (eight samples from six carcasses). The number of samples used was constrained by limitations on processing the samples fresh. The rationale in selecting the eight samples was to match injected samples with a control sample from some of the carcasses, and also to sample more carcasses injected with E-64. Sample 1 represented muscle injected with E-64 and not stimulated, whereas samples 2a and 2b came from muscle injected with saline and E-64, respectively that was also not stimulated. Samples 3 and 4 came from muscle injected with Z-Phe-Ala-CHN_2 and E-64, respectively, which was stimulated, whereas samples 5a and 5b came from muscle not stimulated and injected with saline and Z-Phe-Ala-CHN_2, respectively. Sample 6 was taken from muscle injected with E-64 and stimulated.

Samples 2a and 2b came from the same carcass, as did samples 5a and 5b, with the remaining samples coming from different carcasses. Ten to 12 sections (1–2 g) of fresh LL (approximately 4–3 mm long by 2 mm wide) were dissected parallel to the fibre axis and then processed as described by Hwang (1999). Duplicate blocks

from each sample were sectioned using a microtome with a diamond knife. Sections were examined with a transmission electron microscope (JEOL JEM-1200 EX TEM) at an accelerating voltage of 60 kV. Photographic images were captured (25,000×), with several additional images captured at 60,000× to allow closer examination of sarcomere structure. Care was taken to ensure that the captured images were representative of the sample.

Samples of frozen (−20°C) muscle were thawed, homogenised and the MFI determined as described by Hopkins, Littlefield and Thompson (2000a). After homogenization, a drop of suspension containing myofibrils was placed on a microscope slide and examined under a light microscope (Lecia DMLB, Leica Cambridge Ltd, UK). Representative images were captured using an image processing and analysis system linked to a CCD camera at ×250 magnification. Light microscope images are shown with their matching TEM image.

2.3. Electrophoresis

Electrophoresis was performed on muscle samples obtained 'at death', pH 6.2 and 6.0 and then at 1 and 2 days post-mortem ($n=215$). Muscle samples (2 g) held at −70°C were used for electrophoresis (SDS-PAGE). Muscle tissue was homogenised with an Ultra Turrax in 15 ml of buffer (100 mM KCl, 1 mM EGTA, 8 mM KH_2PO_4, 12 mM K_2HPO_4, 1 mM NaN_3, and 2 mM $MgCl_2$, pH 7.0 at 4–5°C) for three bursts of 15 s at full speed with 30-s rests on ice. This buffer composition was based on the report of Goll, Young and Stromer (1974). The shaft of the homogeniser was washed down with 5 ml of buffer and the muscle homogenate centrifuged at 2000 ***g*** (Model Allegra™ 6R, Beckman Instruments, CA, USA) at 5°C for 10 min and the supernatant discarded. The pellet was re-suspended in 15 ml of buffer, mixed and filtered through a mesh grid (0.5 mm) into a 50-ml centrifuge tube. Washing the homogenate with 5 ml of cold buffer facilitated filtration through the mesh.

The filtrates were centrifuged (2000 *g*) at 5°C for 10 min and the supernatant discarded. The myofibrils were re-suspended in volumes of 5 mM Tris–HCl, pH 8.0, ranging from 15 to 25 ml, with smaller volumes used for pre-rigor samples. Protein concentration was determined using the BCA microplate method. Aliquots (20 μl) of each protein suspension were diluted with 80 μl of 5 mM Tris–HCl, pH 8.0 in quadruplicate for the assay and the protein concentration adjusted to 3.0 mg/ml with 5 mM Tris–HCl, pH 8.0.

A 0.45 ml aliquot of each sample was mixed with an equal volume of sample buffer (125 mm Tris, 4% sodium dodecyl sulphate, SDS (w/v), 20% glycerol (v/v) and 0.0075% bromophenol blue adjusted to pH 8.0 with HCl). Fifty μl of BSA at 2 mg/ml and 50 μl of 2-mercaptoethanol were also added to each sample and the solutions heated for 5 min at 90°C in a Dri-bath (Type 17600, Thermolyne Corporation, IA, USA) after which they were centrifuged for 2 min at 8500 *g*.

An 8 μl aliquot of each sample was loaded onto 10 well Tris-HCl mini gels containing 12% polyacrylamide (Bio-Rad Laboratories, CA, USA) with 4% polyacrylamide in the stacking zone. These gels have 2.6% cross-linking and were run at 180 V for an average of 50 min in a Mini-Protean ll Cell. The running buffer was 25 mM Tris, 192 mM glycine with 0.1% SDS.

Each sample was run on duplicate gels with known molecular weight markers run on every gel to aid protein identification. Samples for the same animal were run at the same time in the same lane order (i.e. at death sample lane 1, pH 6.2 samples lanes 2 and 3, pH 6.0 samples lanes 4 and 5 etc.). Brilliant Blue G-Colloidal was used to stain proteins, which contained 0.1% (w/v) Brilliant Blue G, 0.29 M phosphoric acid, and 16% saturated ammonium sulfate (Sigma-Aldrich Corporation, MO, USA). Gels ($n=2$ for each animal) were stained in 100 ml of the stain according to the suppliers instructions for 1.5 h with gentle mixing (Bio-line Platform Rocker, Edwards Instrument Company, NSW, Australia). The gels were destained for 16 h in a solution of 25% methanol made up in deionised water and then air dried between cellophane sheets.

Images of the gels were captured using an image processing and analysis system (Phoretix 1D, Version 3.01, Phoretix International, Newcastle upon Tyne, UK). A set aperture was used for the lens and the gel illuminated with white light. The peak area volume of all proteins in each lane was determined after the system automatically determined the background for the gel. Proteins of interest (α-actinin, actin, 30 kDa subunit and one designated M1) were identified using the molecular markers as a reference and the peak area volume then adjusted using the internal BSA standard and the amount of each protein determined relative to the standard at 100 μg of BSA as described by Claeys, Uytterhaegen, Buts, and Demeyer (1995). To minimise operator bias, gels were processed without reference to treatments. Repeat gels were run for some samples and the need for this was exacerbated by several batches of defective gels.

2.4. Free calcium concentration

Muscle samples (2 g) held at −70°C were used for determination of free calcium concentration. These samples were taken at pH 6.2 and 6.0 sampling times and at 1 and 2 days post-mortem. The samples were removed from storage at −70°C, 2 weeks prior to measurement and stored frozen at −20°C. On the day of measurement, the samples were removed from storage and held chilled at 2–3°C for 10 min after which they were finely diced, held on ice and then centrifuged at 120,000 *g* for 20 min (Model L8-M, Beckman Instruments,

CA, USA) at 5°C. The supernatant was removed and 300 μl aliquots were mixed with 6 μl of 4 M KCl and these samples incubated in a waterbath at 20°C. The calcium concentration was determined using a calcium selective electrode (Cole-Parmer Instrument Company, Illinios, USA) coupled to an Orion meter (Model 250 A, Orion Research International, MA, USA) with a measurement range of 1 M to 5×10^{-6} M. The electrode had a high selectivity for Ca^{2+} ions over Mg^{2+} and Zn^{2+} ions, such that these ions would not interfere unless they were present at 653 and 293 times the level of calcium ions, respectively.

A calibration curve was determined prior to each measurement run to establish the relationship between calcium concentration and millivolts. The electrode was soaked in a 1×10^{-3} M calcium solution for 1 h prior to calibration. To calibrate the electrode it was placed in 100 ml of deionised water at 20°C, to which 2 ml of 4 M KCl was added. Using a titration approach increasing volumes of a 1-mM calcium solution were added to the base solution and the millivolts recorded. The effective calcium concentrations were 3.9, 9.7, 46.7, 89.3 and 227 μM, which were converted to log values, making the curve linear when plotted against millivolts. For each sample the average of 2 mV readings was used for analysis and repeat measurements were taken for any samples that provided unusual results and for six samples (out of 191), spare 2-g samples were tested to verify absolute measures. One sample returned an unusually high reading, but the level could not be verified and thus the data for this sample was treated as missing. For each corresponding muscle sample tested for free calcium there was a matching 1-g sample used for measurement of pH using iodoacetate as outlined by Hopkins and Thompson (2001).

2.5. Statistical analysis

2.5.1. Linear model (amounts of proteins and calcium concentration)

The amounts of specific proteins determined by electrophoresis and calcium concentration were analysed using a mixed model procedure (SAS, 1997) which contained fixed effects for stimulation (stimulated or control), injection (inhibitor A, B or control) and post-mortem age of sample (pH 6.2, pH 6.0, 1 or 2 days post-mortem), all first order interactions and a random term (animal nested within stimulation). Non significant interactions ($P>0.05$) were removed from the model until the final model was obtained. Predicted means were compared using the PDIFF statement, which enables multiple comparisons. For amounts of the 30 kDa subunit, α-actinin and the protein designated M1, the effect of the rate of pH fall (pHk, where this was estimated previously; Hopkins & Thompson, 2001) as a covariate was tested (with the stimulation term dropped from these models).

2.5.2. Non-linear models (degradation and accretion of proteins)

The disappearance of the protein designated M1 was modeled individually for the two injection treatments within each animal against time using the exponential function;

$$Y = A + B\exp^{-kt} \quad (1)$$

where Y = the concentration of M1 in BSA equivalents μg/ml, A = the concentration of M1 in BSA equivalents μg/ml at $t=0$, B = the difference in the concentration of M1 in BSA equivalents μg/ml at $t=0$ and at 2 days post-mortem. k = rate of M1 decline and t = the time in hours.

Estimates of A, B and k were made and these used to derive the coefficients using non-linear regression (SAS, 1997) using data for the two injection treatments within each lamb (i.e. a total of 48 samples). Of the 48 data groups (each made up of five data points), the function converged for 42 data sets. Examination of the data revealed that for two data sets the value of A was lower than subsequent values. Since this was against the general trend these data points were removed and the models refitted for both sides of the lamb. Of the remaining data sets that did not converge, two were for E-64 injected samples, and two for control samples. The coefficients A, B and k [from Eq. (1)] were analysed using the mixed model, which contained the fixed effects stimulation and injection and their interaction along with the random term animal nested within stimulation.

Using non-linear regression (SAS, 1997) an attempt was made to model the appearance of the 30-kDa subunit against time. However, of the 48 data sets, the model converged for only 31 and of the data sets, which did not converge, six were for E-64 injected samples, three for Z-Phe-Ala-CHN_2 injected samples and the remaining eight were control samples. Given the poor convergence subsequent analysis of A, B and k was not undertaken.

2.5.3. Non-linear model (calcium concentration)

Given that ageing was the only significant fixed effect on calcium concentration and that this was likely a reflection of the drop in pH post-mortem, pH was included as a covariate and found to be significant ($P<0.05$). So as to predict the concentration of calcium at different pHs, an overall relationship between pH and calcium concentration was established using all the data points.

Based on the plot of calcium concentration against pH (Fig. 6), a non-linear procedure (SAS, 1997) was used to derive an equation that would describe the decrease in pH as calcium concentration increased. An exponential function as shown below was tested;

$$\mathrm{pH_c} = pH_\mathrm{f} + (pH_\mathrm{i} - pH_\mathrm{f})\exp^{-kc} \quad (2)$$

Table 1
Predicted means (S.E.) of the 30-kDa subunit (BSA equivalents μg/ml) from models containing the terms for fixed effects and interactions as indicated, including a random term (animal nested within stimulation)[a]

Terms	d.f.[b]	*F*-ratio	Levels	30 kDa	Average S.E.
Injection	2/162	0.7	Inhibitor — E-64 Inhibitor — Z-Phe-Ala-CHN_2 Control		
Ageing	3/162	39.7***	pH 6.2 pH 6.0 1 day 2 days	13.1x 14.9x 23.6y 26.8z	1.8
Stimulation	1/22	0.0			

[a] Values followed by the same letter in a column within a main term (x, y, z) are not significantly different ($P > 0.05$).
[b] d.f., Degrees of freedom (numerator/denominator).
***$P < 0.001$.

where pH_c = the pH at calcium concentration c in μM, pH_f = the ultimate pH, pH_i = the pH when $c = 0$, k = rate constant of pH decline relative to calcium concentration and c = the calcium concentration in μM. This function described the data with a high level of confidence with a very small residual sum of squares for the final model. The fitted line is shown in Fig. 6. Using the derived equation the predicted pH at any calcium concentration could be determined.

3. Results

3.1. Examination of muscle structure

Light microscope images were obtained for all samples ($n = 144$) subjected to homogenisation for determination of MFI values at pH 6.0, 1 and 2 days post-mortem samplings. There was no evidence that the inhibitors caused denaturation of myofibrils and normal striations were apparent. Further to this, of the samples prepared for electron microscope analysis ($n = 8$) there was no evidence that the inhibitors caused denaturation and so for brevity only images for samples 1, 2a and 2b are presented here to illustrate these observations.

For sample 1, which was injected with E-64 and stimulated (Fig. 1), there did appear to be some gaps or breakdown in the Z-disk (shown with →; Fig. 1b). This, however, was not a consistent observation as seen by comparing these images with image 1c and it was also observed in sample 2 (not shown) which was injected with the cathepsin inhibitor Z-Phe-Ala-CHN_2 and stimulated. Inter myofibril linkages between the Z-disk can be seen in Fig. 1d (shown with →) and in both Fig. 1a and d clear A-bands can be seen. Images obtained for sample 2b from muscle injected with E-64 and not simulated showed normal striations in the myofibrils (Fig. 2) and no evidence of denaturation of sarcomere proteins (Fig. 2b) when compared to control muscle (Fig. 2a). As for other images inter myofibril linkages between the Z-disk could be seen.

3.2. Protein degradation measured by electrophoresis

The location of the proteins measured on the gels is shown in Fig. 3. This gel shows clearly the increase in the amount of what is considered the 30-kDa subunit and the disappearance of the protein M1 during the post-mortem period.

There was little effect of injection, stimulation or ageing on the amounts of α-actinin and actin and no further results are reported. Appearance of the 30 kDa subunit increased with time post-mortem ($P < 0.001$) after a pH of 6.0 was reached (Table 1), although there was no effect of injection or stimulation, or the covariate pHk ($P > 0.05$). The pattern of increase in the 30-kDa subunit with time is shown in Fig. 4. This shows a slower increase in the 30 kDa subunit in muscle treated with E-64, but in this muscle and the control muscle there was large variation around each mean and this partly explains the lack of significance between the two groups of samples. This large variation was also observed in the Z-Phe-Ala-CHN_2 treated muscle and its matching control (not shown) which showed similar patterns of accretion to each other. The plots also show that the 30-kDa subunit appeared very early in the post-mortem period.

The amount of the protein M1, was affected by stimulation, ageing and the interaction between ageing and stimulation (Table 2, $P < 0.05$, 0.001 and 0.001, respectively). The amount of M1 measured pre-rigor was greater for stimulated muscle, but the rate of decline with ageing was also greater, yet the covariate pHk had no effect and neither did injection ($P > 0.05$). The pattern of decrease in the protein M1 with time is shown in Fig. 5.

There was no effect ($P > 0.05$) of stimulation, injection or their interaction on parameters A or B for the exponential function which described the amount of the protein M1, as a function of time. There was however an effect ($P < 0.05$) of injection on k with E-64 injected samples having a higher k compared with control samples (0.23 vs 0.13), but there was no difference ($P > 0.05$)

Table 2
Predicted means (S.E.) for the protein M1 (BSA equivalents μg/ml) from models containing the terms for fixed effects and interactions as indicated, including a random term (animal nested within stimulation)[a]

Terms	d.f.[b]	*F*-ratio	Levels	M1		Average S.E.
Injection	2/159	1.5	Inhibitor — E-64 Inhibitor — Z-Phe-Ala-CHN_2 Control			
Ageing	3/159	163.3***	pH 6.2 pH 6.0 1 day 2 days	30.1x 28.3x 13.6y 10.3z		1.2
Stimulation	1/22	4.8*	Stimulated Control	22.9x 18.3y		1.5
				Stimulation		
			Ageing	*Stimulated*	*Control*	
Ageing×stimulation	3/159	13.7***	pH 6.2	34.8ax	25.5bx	
			pH 6.0	33.1ax	23.4bx	
			1 day	14.1ay	13.0ay	
			2 days	9.4az	11.2ay	1.8

[a] Values followed by the same letter in a row (a, b) are not significantly different ($P > 0.05$). Values followed by the same letter in a column within a main or interaction term (x, y, z) are not significantly different ($P > 0.05$).
[b] d.f., degrees of freedom (numerator/denominator).
*$P < 0.05$; ***$P < 0.001$.

between E-64 injected samples and those injected with Z-Phe-Ala-CHN_2 (0.23 vs 0.15). The exponential decline constant (k) did not explain any additional variance in shear force over the fixed effects and interactions previously found significant.

3.3. Free calcium levels

Ageing had an effect ($P < 0.001$) on calcium concentration but, there was no effect ($P > 0.05$) of stimulation or injection. The predicted means for the significant effects are shown in Table 3. With pH included, only pH and ageing had an effect ($P < 0.05$ and $P < 0.001$, respectively; Table 3). The overall model describing the relationship between pH and calcium concentration was as follows;

$$\text{pH} = 5.04 + 2.00\exp^{(-0.013\times \text{ calcium concentration in micromoles})}$$

A plot of all the data points is shown in Fig. 6 along with the fitted line derived from the equation. This shows that when the calcium concentration reached a plateau of ~110 μM the predicted pH was 5.5.

Table 3
Predicted means (S.E.) of calcium concentration (μM) from a model containing terms for fixed effects and the covariate pH as indicated, including a random term (animal nested within stimulation)[a]

Terms	d.f.[b]	*F*-ratio	Levels	Calcium concentration (μM)	Average S.E.
Injection	2/157	0.1	Inhibitor — E-64 Inhibitor — Z-Phe-Ala-CHN_2 Control		
Ageing	3/157	14.2***	pH 6.1 pH 5.8 1 day 2 days	59w 67x 91y 104z	4.3
Stimulation	1/22	0.1			
Covariate					
pH	1/157	9.5*			

[a] Values followed by the same letter in a column within a main or interaction term (w, x, y, z) are not significantly different ($P > 0.05$).
[b] d.f., Degrees of freedom (numerator/denominator).
*$P < 0.05$; ***$P < 0.001$.

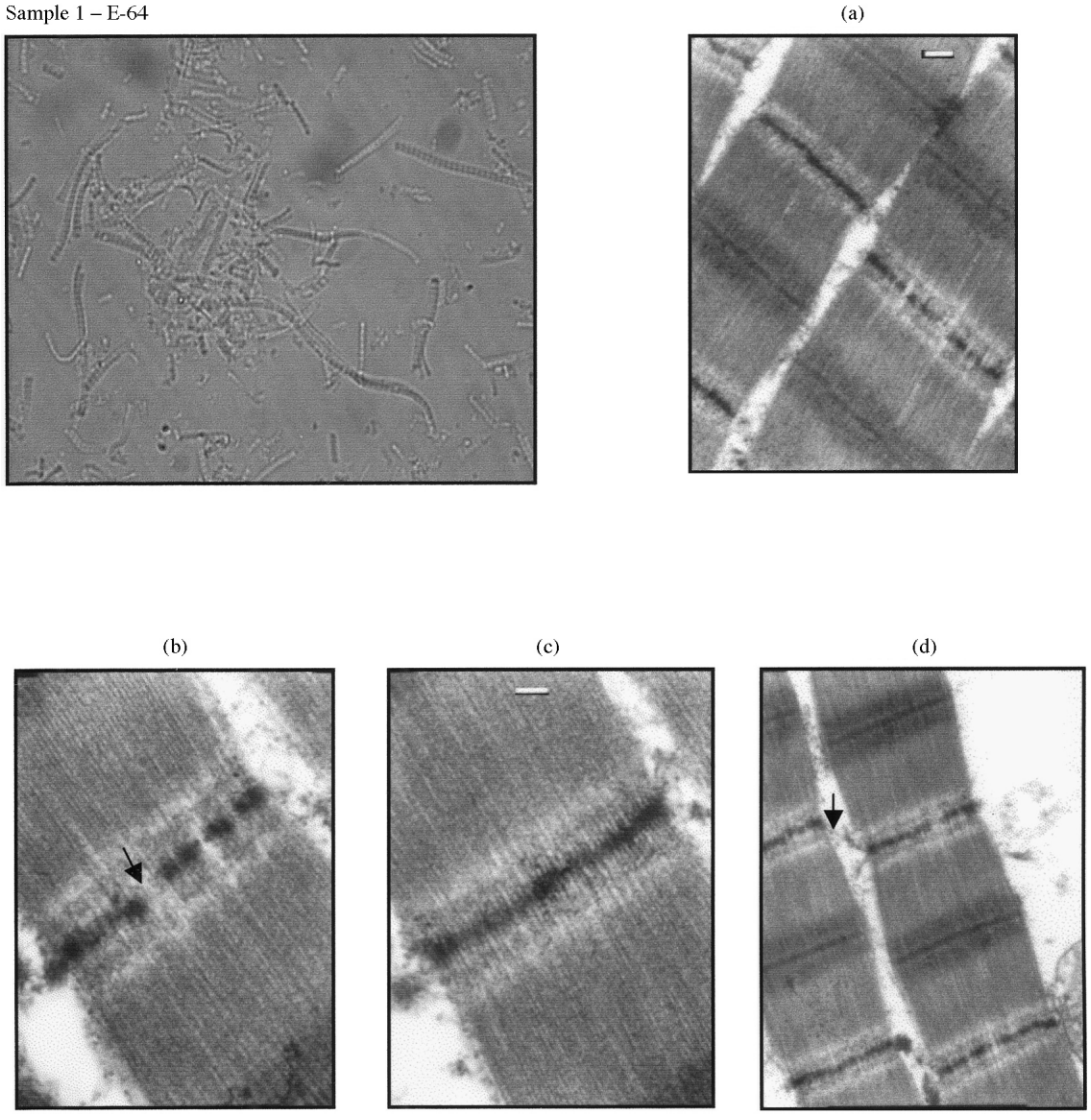

Fig. 1. Light (250×) and electron microscope (25,000 and 60,000×) images for sample 1 (injected with E-64, no stimulation, cranial portion). Images (a) and (d) are from two different blocks (25,000×) and images (b) and (c) (60,000×) are taken from image (d). The white bar in image (a) = 200 nm and can be used as a reference for other images and in image (c) the white bar = 100 nm.

4. Discussion

4.1. Muscle structure and free calcium levels

It is apparent from examination of the light microscope images (Figs. 1 and 2) that unlike the images of Tatsumi et al. (1998) the injection of an inhibitor (in this case either E-64 or the peptidyl diazomethane) did not cause denaturation and aggregation of the myofibril fragments. Normal striations could readily be seen, suggesting that the integrity of the myofibrils was retained and even post-mortem samples held for 48 h did not show denaturation (Hopkins, Littlefield, & Thompson, 2000b).

Electron microscope images did not reveal any modification that could be ascribed to either of the inhibitors. In all samples examined there was no visual evidence of denaturation and treated samples showed no structural differences compared with control samples. For example, the Z-disk and A-band of the sarcomeres were clear and intact as indicated for Fig. 2a. In some of the sections for different samples, gaps were apparent in the Z-disk, but this was not isolated to samples from a particular treatment. Similar gaps were evident in images presented by Ho, Stromer, and Robson (1996) and Hwang (1999) for beef muscle 24 h post-mortem, which had not been treated with inhibitors.

Tatsumi et al. (1998) claimed that the inhibitors used in their study bound to titin and nebulin preventing the binding of calcium ions, although this was not the case when 1-mM leupeptin was used. Tatsumi et al. (1998) provided no explanation of how E-64 bound to sarco-

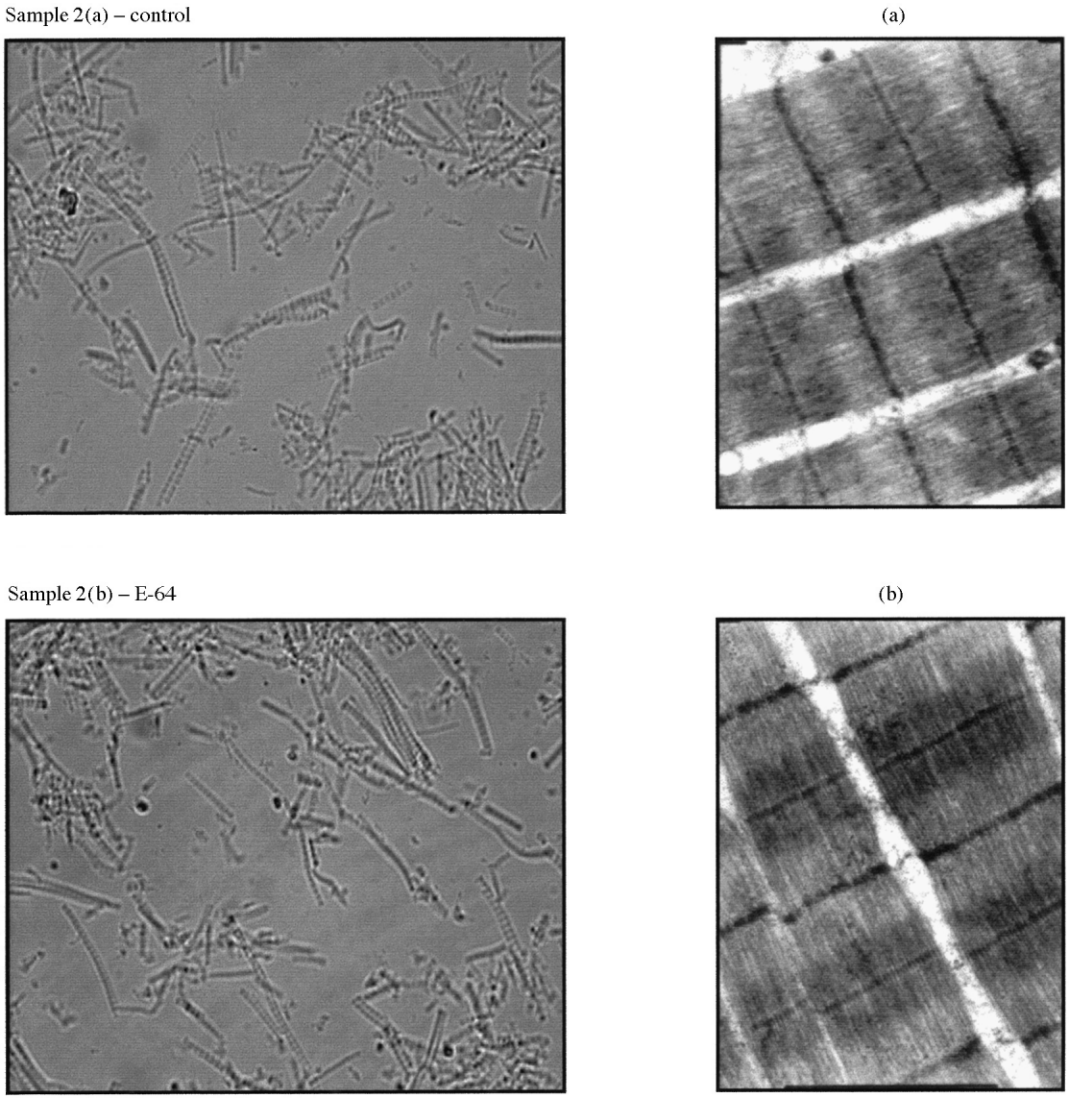

Fig. 2. Light (250×) and electron microscope (25,000×) images for samples 2a and 2b (injected with saline, no stimulation, cranial portion and injected with E-64, no stimulation, caudal portion, respectively).

mere proteins, but a mode of binding between leupeptin and proteins such as titin was proposed. This was not, however, consistent with the reported interaction of the aldehyde group of leupeptin and the thiol group of cysteine 25 in proteases, such as the calpains (Barrett, 1986).

If inhibitors such as E-64 bind to sarcomere proteins and, as a result, prevent calcium binding to these proteins as proposed by Hattori and Takahashi (1982) and Tatsumi et al. (1998), then the level of free calcium in treated muscle should be higher. The results in Table 3 show that this is not the case, and there was no difference between muscle injected with either of the inhibitors and control muscle. This provides further evidence that inhibitors such as E-64 do not alter the structure of sarcomere proteins as proposed by Tatsumi et al. (1998).

The claim by Tatsumi et al. (1998) about the binding of inhibitors to myofibrillar proteins was linked to the report by Takahashi, Hattori, Tatsumi, and Takai (1992) which suggested that calcium ions bind to titin. More recently, Tatsumi, Maeda, & Takahashi (1999) reported that the binding was to the major sub-fragment of degraded titin, where the 1200-kDa fragment is cleaved. Given that calpains bind calcium ions for activation and titin has been shown a good substrate for these enzymes (Robson et al., 1997), these results are not inconsistent, and it is reasonable to suggest that wherever calpains bind to proteins there will be evidence of calcium ions. This explanation may reconcile two differing viewpoints.

4.2. Degradation of myofibrillar proteins

Degradation of troponin-T and an unidentified protein designated M1 was evident very soon after death in this study as evidenced by examining Figs. 4 and 5. In

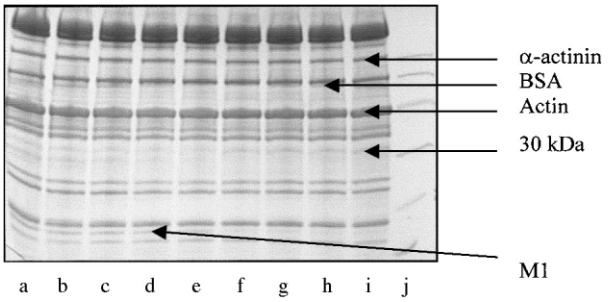

Fig. 3. Electrophoresis pattern for proteins extracted from muscle samples taken from a carcass injected with either the protease inhibitor (Z-Phe-Ala-CHN_2) or isotonic saline and aged for different periods. Lane a, at death muscle; Lane b, muscle injected with saline, pH 6.2; Lane c, muscle injected with inhibitor, pH 6.2; Lane d, muscle injected with saline, pH 6.0; Lane e, muscle injected with inhibitor, pH 6.0; Lane f, muscle injected with inhibitor, aged 1 day; Lane g, muscle injected with saline, aged 1 day; Lane h, muscle injected with inhibitor, aged 2 days; Lane i, muscle injected with saline, aged 2 days; Lane j, low molecular weight markers, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa).

the case of troponin-T there was evidence of the 30-kDa subunit at death in muscle taken from the majority of carcasses (20 of the 24).

Study of the timing of the commencement of post-mortem proteolysis is important because it will help to clarify the mechanisms controlling this process. Dransfield (1993) suggested that μ-calpain is not fully activated until a pH of ~6.1 is reached and that this coincided with the start of tenderisation. However, this does not preclude proteolysis occurring prior to this pH as the prediction by Dransfield (1993) sees an increase in μ-calpain activity up to a maximum at 10 h. Thus, proteolysis could proceed before tenderisation commences. Given that calpains have been implicated in the deposition and degradation of protein in living muscle (Goll, Thompson, Taylor, & Ouali, 1998; McDonagh, 1998) it seems logical that degradation products could be detected either in muscle at death or soon after death. As we found, Geesink and Koohmaraie (1999b) reported the appearance of the 30-kDa subunit very early post-mortem in beef muscle.

Data on MFI values and protein solubility as presented in the first paper in the series for samples taken at pH 6.0 and 1 and 2 days post-mortem provide a picture of the rate with which myofibrillar protein structure changes as pH falls. The average time post-mortem at which samples with a pH of 6.0 were taken was 9 h. Between this time and 1-day post-mortem protein solubility increased by 78%, whereas for the subsequent 24 h it only increased by 26%. In a similar way, MFI values changed by 38% between 9- and 24-h post-mortem and to a much lesser extent (12.5%) between 24- and 48-h post-mortem (Hopkins & Thompson, 2001). This indicates that considerable proteolysis had occurred before shear force was measured at 1-day post-mortem. This was supported by the rate of degradation of the protein M1 and the appearance of the 30 kDa subunit which was faster in the first 24-h post-mortem compared to the subsequent 24 h (Figs. 4 and 5, Tables 1 and 2), even though it was not always possible to mathematically describe these changes. The results presented here on proteolysis are of particular relevance to the argument that only a 'small amount of proteolysis occurs in the first 72 h post-mortem and that this does not result in any appreciable increase in tenderness' (Goll, Boehm, Geesink, & Thompson, 1997). Equally the results are at odds with predictions from the Dransfield model (Dransfield, 1993), because the rate of proteolysis was fastest during the early post-mortem period.

4.3. Free calcium levels and enzyme action

Data presented here shows that as pH drops and rigor develops the level of free calcium rises. In the present study, when the level of free calcium reached a plateau (~110 μM) the predicted pH was 5.5 (ultimate). This calcium level is more than sufficient to fully activate μ-calpain, but not m-calpain (Etherington, 1984; Boehm et al., 1998). Recently, Geesink and Koohmaraie (1999a) reported that the activity of m-calpain was largely unaltered in lamb muscle 56 days post-mortem, suggesting this was because free calcium levels were insufficient to activate this enzyme.

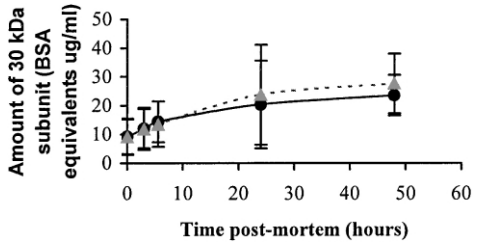

Fig. 4. A plot of the mean (S.D.) increase of the 30 kDa subunit in muscle treated with E-64 (•–•) and control muscle (Δ–Δ) over the post-mortem period.

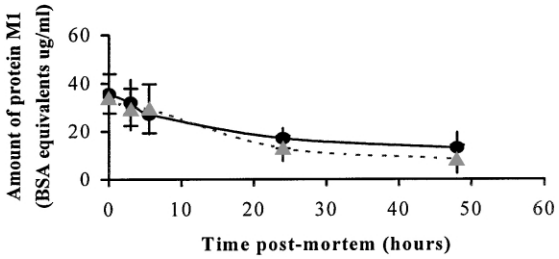

Fig. 5. A plot of the mean (S.D.) decrease of the protein M1 in muscle treated with E-64 (•–•) and control muscle (Δ–Δ) over the post-mortem period.

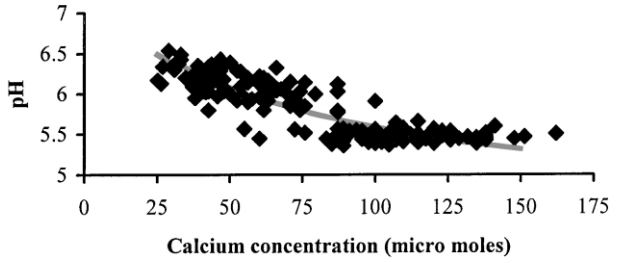

Fig. 6. A plot of the increase in calcium concentration as rigor progresses and pH falls including the fitted line based on predicted values.

The high levels of free calcium (up to 1000 μM) at 10–14 days post-mortem reported by Parrish, Selvig, Culler, and Zeece (1981) for beef muscle were similar to those published by Kudryashov, Kudryashova, and Lisitsyn (1999) when the pH was 5.67 at 24 h post-mortem. These levels would be high enough to activate m-calpain, but interestingly when the meat exhibited PSE characteristics with a lower pH (not given) the level of free calcium only reached ~100 μM. This level is in closer agreement to that reported by Takahashi (1999) and Jeacocke (1993) and the level found in the current study. The much lower level reported by Jaime, Beltran, Cena, Lopez-Lorenzo, and Roncales (1992) for the same muscle as used in this study and also from lamb could be due to the use of EDTA in homogenisation buffer. Since significant tenderisation and proteolysis had occurred in muscle samples in this study by 2 days post-mortem (Hopkins & Thompson, 2001). It is difficult to see how m-calpain could be active and make a contribution to these processes, based on the levels of free calcium measured and our current understanding of activation.

Using the relationship derived between pH and free calcium concentration the predicted level of free calcium at pH 7.0 was 1.55 μM having a strong similarity to the level reported by Kurebayashi, Harkins, and Baylor (1993) in living frog skeletal muscle. Such levels would seem to support the prediction by Dransfield (1993) that μ-calpain will reach maximum activity several hours after death when calcium levels reach critical levels for activation of the enzyme. However, this pattern of activity is different to that reported by Ducastaing, Valin, Schollmeyer, and Cross (1985) and Boehm et al. (1998) who showed the activity of extractable μ-calpain, m-calpain and calpastatin maximal at death with a subsequent decline post-mortem. If the prediction of Dransfield (1993) was correct, then the role of calpains in the deposition and degradation of protein in living muscle would seem unlikely, but there is ample evidence that this is not the case (Goll et al., 1998; McDonagh, 1998). The data of Marban, Rink, Tsien, and Tsien (1980) for heart muscle may help to reconcile these differences as they reported that during heart muscle contraction the concentration of free calcium rose to 10 μM. Thus, it is apparent that stimulation of living cells will cause an elevation in calcium ion levels intracelluarly and this is a plausible explanation for the activation of μ-calpain. This rise and fall of free calcium concentration has been verified recently in 'real time' by Kerr, Lev-Ram, Baird, Vincent, Tsien, and Schafer (2000) using optical imaging techniques and cameleon proteins which fluoresce in the presence of calcium.

As the pH and temperature fall in post-mortem muscle, the activity of the calpains will be significantly reduced. Hence, even if post-mortem levels of free calcium rose to the levels required for activation of m-calpain, by the time this occurred the conditions for its activity would be sub-optimal. Given the results of SDS electrophoresis it is apparent that degradation of some proteins occurred before the muscle pH dropped to the 6.1–6.2 level cited by Dransfield (1993) as the pH when μ-calpain would be fully activated. In this study, the free calcium concentration had not risen to the level predicted by Dransfield (1993) for a pH of 6.1–6.2, yet some protein degradation was apparent. Related to this, the use of specific inhibitors provided strong evidence that the calpains were the enzyme group responsible for post-mortem tenderisation so they were clearly active before these pH levels were reached.

5. Conclusions

There was no evidence that E-64 changed sarcomere structure causing denaturation or aggregation. From this qualitative observation and the levels of free calcium in injected muscle there is no support for the view that this inhibitor binds to sarcomere proteins, occupying sites to which calcium might bind. This evidence and that provided in the previous paper in this series raise serious doubts that the calcium theory of tenderisation can stand alone as the sole explanation for improvements in tenderness post-rigor. The levels of free calcium do not support the view that m-calpain has a role in post-mortem tenderisation. The results suggest that activation of μ-calpain is likely to occur before the pH drops to 6.2–6.1, as proteolysis was very rapid in the first 24 h post-mortem in ovine muscle.

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