Proteolysis may be controlled by postmortem energy metabolism

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ABSTRACT

Postmortem proteolysis of structural muscle proteins in the *psoas major* (PM) is dramatically faster than in the *longissiumus dorsi* (LD). Previous work has attributed this difference to an increased rate of pH decline and subsequent earlier activation of μ-calpain. However, the mechanisms responsible for the differences in pH decline between muscles are not well understood. Therefore, we hypothesized that early postmortem muscle energy metabolism controls the rate of pH decline. To test this, LD and PM muscle samples (10 g) were harvested at 3, 45, and 1440 min post-stunning from market-weight pigs (108.8 ± 3.8 kg). Muscle metabolites were measured with high-performance liquid chromatography (HPLC). Total muscle phosphate levels and muscle pH were also measured. Titin proteolysis was quantified with vertical agarose gel electrophoresis (VAGE). At 3 min post-stunning, the PM contained less (P < 0.05) ATP and creatine phosphate (CP), but more (P < 0.05) ADP and IMP. At 45 min postmortem, only IMP content was greater (P < 0.05) in the PM and no differences existed at 1440 min. Muscle pH was not different at 3 min postmortem, but was lower (P < 0.05) in the PM at 45 min, and higher (P < 0.05) in the PM at 1440 min. Greater titin proteolysis occurred (P < 0.05) in PM by 1440 min and no differences were detected in total phosphate levels. These data show that energy metabolism and proteolysis in the PM differs from that in LD and suggests that a more rapid pH decline may activate calpain-dependent proteolysis, resulting in increased proteolysis of the PM and a more tender product.

Keywords – titin, proteolysis, pH, HPLC

INTRODUCTION

Postmortem proteolysis by μ-calpain tenderizes meat. However, the postmortem mechanism that activates μ-calpain is unclear. Previous efforts have shown that a quicker pH decline activates μ-calpain earlier postmortem [1]. This is especially true in the *psoas major* (PM). However, alternate studies have shown that an extremely rapid pH decline is detrimental to the enzyme's activity and is often associated with pale, soft, and exudative (PSE) pork in the *longissiumus dorsi* (LD) [2]. Based on these studies, different muscles, such as PM and LD, which are more oxidative and glycolytic, respectively, differ in postmortem energy metabolism, pH decline, and consequently proteolytic activity. Therefore, we hypothesized that early postmortem metabolite levels of ATP, ADP, IMP, and creatine phosphate (CP) control pH decline and ultimately postmortem proteolysis of the LD and PM.

MATERIALS

Muscle Samples

Six market-weight pigs were harvested according to industry standards. Muscle samples were collected from the LD and PM at 3, 45, and 1440 min after stunning, frozen in liquid nitrogen, and stored at -80C until further analysis.

pH

Muscle pH was measured according to a previous method [3]. Approximately 1 g of muscle was powered with liquid nitrogen and homogenized using a Polytron (Brinkman Instruments, New York, NY) in 10 mL ice-cold buffer (5 mM sodium iodoacetate, 150 mM KCl, pH 7.0). Suspensions were centrifuged at 4,000 X *g* for 5 min at room temperature. Supernatants were collected, and the pH was measured at 25C.

	3		45		1440	
	LD	PM	LD	PM	LD	PM
IMP	0.78 ± 0.18^{a}	1.99 ± 0.87^{b}	1.22 ± 0.32^{a}	3.04 ± 0.43^{b}	3.98 ± 0.15^{a}	3.82 ± 0.15^{a}
ADP	0.38 ± 0.03^{a}	0.46 ± 0.02^{b}	0.49 ± 0.05^{a}	0.45 ± 0.08^{a}	0.20 ± 0.03^{a}	0.23 ± 0.04^{a}
ATP	5.10 ± 0.64^{a}	3.10 ± 0.45^{b}	4.04 ± 0.22^{a}	2.14 ± 0.88^{a}	BD	BD
PCR	3.23 ± 1.16^{a}	0.49 ± 0.24^{b}	0.02 ± 0.01^{a}	0.05 ± 0.05^{a}	BD	BD

Table 1. HPLC Data at 3, 45, and 1440 minutes postmortem. Data represent μmol/g muscle and is listed as mean ± standard error. Data were analyzed with a student's t-test. Values with different superscripts within time and metabolite are significantly different ($P < 0.05$) between muscles. BD = below detection limits. Detection limits are 0.01 μmol/g muscle for all metabolites.

Chromatography

Frozen muscle was powdered in liquid nitrogen, homogenized in 0.5M perchloric acid, and neutralized to pH 7.0 with 2M KOH. All metabolites were separated on an HP Agilent 1100 system using an UV/Vis detector and a Phenomenex Kinetex 2.6 μm C18 column (4.6 x 50 mm) with a flow rate of 2 mL/min. CP was isocratically separated using a previously published method [4]. The mobile phase (A) contained 100 mM KH_2PO_4, 5 mM tetrabutylammonium hydrogen sulfate (TBAHS), and 2.5% acetonitrile (pH 6.0). ATP, ADP, and IMP were separated using a modified procedure previously described [5] with a gradient mobile phase. A was the same and mobile phase B (B) contained 100 mM KH_2PO_4, 5 mM TBAHS, and 25% acetonitrile (pH 5.5). Following injection, the column was eluted for 2 min with A, for 2 min with A and B (increasing to 11% B), and for 4 min with A and B (increasing to 43% B). The column was reequilibrated with 100% A for 15 min.

Protein Analysis

Titin was electrophoretically separated according to a previous method [6]. Briefly, muscle samples were powdered with liquid nitrogen, solubilized, and analyzed for protein content with the RC DC protein assay (Bio-Rad, Hercules, CA). Titin was separated on SDS-VAGE gels, visualized with G-250 coomassie stain, and imaged. Intact titin was normalized to myosin heavy chain within the lane. Gels were imaged and analyzed with a LI-COR Odyssey (LI-COR, Lincoln, NE) imaging system.

Muscle phosphate

Total muscle phosphate was measured using a phosphate assay kit (BioChain, Hayward, CA). Briefly, the chromatography samples were incubated with the malachite green reagent according the manufacturer's instructions and measured with a spectrophotometer at 620 nm.

Statistical Analysis

Muscle metabolite, phosphate, and titin proteolysis data were analyzed with JMP using a student's t-test. Muscle pH was analyzed with JMP using the LSMeans analysis. Differences were determined significant at $P < 0.05$. Data are presented as mean ± standard error.

RESULTS AND DISCUSSION

The muscle metabolite concentrations (μmol/g muscle) between muscles at each time point are shown in Table 1. At 3 min postmortem, the PM contained less ($P < 0.05$) ATP and CP, but more ($P < 0.05$) ADP and IMP than the LD. The PM contained significantly more IMP ($P < 0.05$) at 45 min postmortem, but all other metabolites were not significantly different, although the PM tended to have less ATP than the LD ($P = 0.064$). No differences were detected at 1440 minutes. These data are important because it gives an insight into the initial energy charge of the muscle prior to and after harvesting. Differences in energy metabolite concentrations between the muscles are not surprising and the most intuitive explanation for the initial metabolite differences between these two muscles is differences in muscle fiber type. The

	3	45	1440
LD	6.25 ± 0.04^{a}	6.12 ± 0.03^{a}	5.52 ± 0.02^{d}
PM	6.16 ± 0.03^{a}	5.96 ± 0.08^{b}	5.68 ± 0.09^{c}

Table 2. Mean (±SE) muscle pH values at 3, 45, and 1440 min postmortem. Means bearing different superscripts differ ($P < 0.05$).

composition between fast and slow fibers dictates the energy stored in the muscle [7].

Previous efforts to associate changes in ATP and IMP with proteolysis concluded little correlation exists between energy metabolism and proteolysis [8]. However, the authors only measured ATP and IMP levels in the LD, *semitendinosus*, and *semimembranosus*. These muscles have similar fiber-type compositions when compared to the PM. In contrast, an alternate study using the LD and PM suggested that increased red (more oxidative) fibers in the PM contribute to the increased rates of proteolysis of titin [1]. Our data confirm these findings. While a cause and effect relationship between energy metabolism and proteolysis was not established, our data strongly support their association.

Muscle pH was not different at 3 min postmortem, but was lower ($P < 0.05$) in the PM at 45 min and greater in the PM ($P < 0.05$) at 1440 min (Table 2). Muscle phosphate was measured in an attempt to explain the ultimate pH differences between the muscles due to phosphate's buffering capacity. However, total phosphate was not different between the PM and LD (data not shown). Similar to previous results [1] and the aforementioned HPLC data, these pH differences show the PM reaches its ultimate pH quicker, which likely activates μ-calpain earlier due to the muscle's inability to sequester calcium to resting levels [9]. This raises the question of using pH alone to predict μ-calpain activity. The occurrence of PSE meat is highly probable with a rapid pH decline (typically with a 45 min pH <5.8) [10], yet this reduces μ-calpain activity [2]. While the PM in this study would not qualify as PSE, in a previous study [1], the PM had achieved a pH <5.8 at 45 min postmortem (classifying it as potentially PSE) despite the fact that proteolysis was more rapid. Muscle differences cannot be ignored (PSE is more often found in glycolytic muscles), however, it may be more prudent to clarify further the impact pH has on proteolysis in each situation by including the extent of postmortem metabolism by using metabolite levels like ATP, ADP, IMP, and CP.

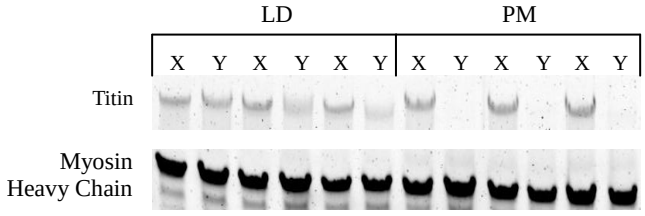

Figure 1. Representative agarose gel stained with coomassie G-250 and analyzed with a LI-COR Odyssey. The top band is intact native titin and bottom band is myosin heavy chain. Titin is analyzed relative to myosin heavy chain within lane. X and Y represent 3 and 1440 min postmortem, respectively.

Titin proteolysis was quantified to measure indirect μ-calpain activity. Figure 1 shows a representative agarose gel of intact and degraded titin. Titin was compared between muscles relative to 3 min. (Fig. 2). As expected, there was no difference between the amount of intact titin in either muscle at 3 min. At 1440 min, the PM has 11% remaining intact titin compared to 60% in the LD. This indirect evidence of μ-calpain activity further supports the notion that increased energy metabolism in the PM may activate proteolysis sooner.

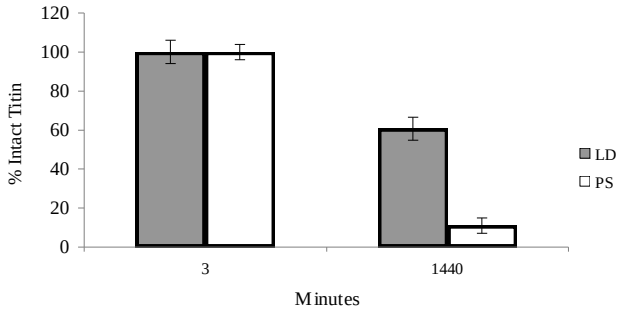

Figure 2. Percentage of intact titin (±SE) in the psoas major (PM) and the longissimus dorsi (LD) at 3 and 1440 min postmortem. At 1440 minutes, the relative percentages of intact titin in the LD and PM differ ($P<0.001$).

IMPLICATIONS

Previous work explaining the impact pH decline has on the activation of μ-calpain is important to understanding the tenderization processes. However, future work needs to include more detailed muscle metabolic data to understand better the differences between muscles, and the metabolic rates therein. We suggest that reporting ATP, ADP, IMP, and CP levels is useful in determining the rate of pH decline. All four metabolites are important components in postmortem metabolism and give greater insight into their significant impact upon subsequent proteolytic processes. With these additions, direct mechanisms may be discovered to understand the impact metabolism has upon proteolysis and ultimately tenderness.

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