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A mitochondrial protein increases glycolytic flux

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ABSTRACT

The purpose of this study was to determine the role of mitochondria in postmortem muscle metabolism. Isolated mitochondria were incorporated into a reaction buffer that mimics postmortem glycolysis with or without mitochondrial electron transport chain inhibitors. Addition of mitochondria lowered pH values at 240 and 1440 min regardless of inhibitors. Reduction in pH was accompanied by enhanced glycogen degradation and lactate accumulation. To explore the mechanism responsible for this exaggerated metabolism, mitochondrial preparations were mechanically disrupted and centrifuged. Resulting supernatants and pellets each were added to the *in vitro* model. Mitochondrial supernatants produced similar effects as those including intact mitochondria. To narrow further our target of investigation, mitochondrial supernatants were deproteinized with perchloric acid. The effect of mitochondrial supernatant was lost after perchloric acid treatment. These data indicate that a mitochondrial-based protein is capable of increasing glycolytic flux in an *in vitro* model and may partially explain acid meat development in highly oxidative AMPK$\gamma3^{R200Q}$ mutated pigs.

1. Introduction

During the process of converting muscle to meat, hydrogen ions (H^+) accumulate in the muscle and gradually lower the pH from 7.2 to an ultimate pH around 5.5. The rate and extent of postmortem pH decline can profoundly affect the quality characteristics of fresh meat. While the rate of pH decline is a function of muscle ATPase activity (Scopes, 1974), the exact biochemical mechanisms responsible for determining the ultimate pH of meat remain imprecise. Although great efforts have been made to determine factors influencing the extent of pH decline (England, Matarneh, Scheffler, Wachet, & Gerrard, 2014; Matarneh, England, Scheffler, Oliver, & Gerrard, 2015; Scopes, 1974; Van Laack & Yang, 2001), gaps in our knowledge still exist. This is partly a result of the ambiguity and over-simplification of events controlling postmortem metabolism.

Postmortem metabolism is usually viewed as exclusively anaerobic process, with glycogen being converted to lactate and H^+ that accumulate in the muscle and cause a drop in pH. However, attempts to predict accurately the extent of pH decline using only anaerobic metabolism have proven unreliable (Monin & Sellier, 1985; Scheffler, Scheffler, Kasten, Sosnicki, & Gerrard, 2013), suggesting other mechanisms may be involved in determining the extent of postmortem metabolism. Because mitochondrial respiration is impeded by the lack of oxygen, mitochondria are often considered irrelevant to postmortem metabolism. Yet, mitochondria preserve functionality and structural integrity for several hours postmortem (Tang et al., 2005), and therefore represent a potential mechanism for altering postmortem metabolism. Indeed, we have recently shown that inclusion of functioning mitochondria to an *in vitro* model designed to mimics postmortem metabolism reduced the rate of ATP hydrolysis (Scheffler, Matarneh, England, & Gerrard, 2015), which strongly argues mitochondria impact postmortem metabolism. Further, mitochondria may alter cytosolic redox state (NAD^+/NADH ratio), which could impact glycolysis (Jong & Davis, 1983).

Our interest in the possible role of mitochondria in postmortem metabolism has mostly risen from studying pork quality in pigs harboring the AMPK$\gamma3^{R200Q}$ (RN^-) mutation. The postmortem pH decline in muscle from pigs carrying the AMPK$\gamma3^{R200Q}$ mutation is usually characterized by a normal rate initially, but continues to drop abnormally to an ultimate pH around 5.3 (Milan et al., 2000). The lower ultimate pH of AMPKγ_3^{R200Q} pigs is often attributed to a greater than normal glycolytic potential in resting muscle of these pigs compared to wild-types (Monin & Sellier, 1985). Yet, we have recently proposed that greater flux through glycolysis in muscle from AMPK$\gamma3^{R200Q}$ pigs causes a lower ultimate pH rather than greater tissue glycogen deposition (Matarneh et al., 2015). On the other hand, however, these pigs contain more mitochondria and retain greater oxidative capacity (Estrade, Ayoub, Talmant, & Monin, 1994; Scheffler et al., 2014),

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suggesting mitochondria may contribute in some way to the lower ultimate pH of muscle in these mutant pigs. Therefore, the purpose of this research was to examine the role of mitochondria in postmortem metabolism, specifically as related to acid meat development. However, testing the role of mitochondria *in vivo* is difficult. This is particularly onerous because modifying mitochondrial content of a muscle can alter confounding factors (*i.e.*, glycolytic capacity) that can also impact postmortem pH decline (Petersen, Henckel, Maribo, Oksbjerg, & Sørensen, 1997). To eliminate the potentially confounding effect, we used an *in vitro* system designed to recapitulate postmortem glycolysis (England et al., 2014; Scopes, 1973) and added 0.5 mg/ml isolated mitochondria with or without mitochondrial electron transport chain (ETC) inhibitors.

2. Materials and methods

2.1. Muscle sampling

All pigs used for this study were raised under the same feeding and management conditions at the Virginia Tech Swine Center to approximately 115 kg live weight. Six crossbred (Yorkshire × Duroc; 3 males and 3 females) pigs were transported to the Virginia Tech Meat Science Center and harvested using standard commercial procedures. Muscle samples were excised from the *longissimus lumborum* muscle within 5 min of exsanguination. Samples were used for mitochondrial extraction or immediately snap frozen in liquid nitrogen, and stored at − 80 °C.

2.2. Mitochondria isolation and characterization

Mitochondria were isolated using the differential centrifugation method as described by Scheffler et al. (2014) with minor modification. Briefly, muscle samples were finely minced at 1:5 (wt/vol) in ice-cold isolation buffer (100 mM sucrose, 180 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 10 mM EDTA, 1 mM K-ATP, pH 7.4). Protease (subtilisin A) was added to the tissue suspension at 0.4 mg/ml before homogenization with a Potter-Elvehjem type homogenizer system (Glas-Col, Terre Haute, IN. USA). Homogenates were diluted with isolation buffer to achieve ~20 ml/g of tissue followed by filtering through cheese-cloth. Homogenates were then centrifuged at 1000 × *g* for 10 min at 4 °C. Resulting supernatants were filtered again through cheese-cloth followed by centrifugation at 8000 × *g* for 10 min at 4 °C. Mitochondrial pellets were re-suspended by repeated pipetting in mitochondrial suspension buffer (220 mM mannitol, 70 mM sucrose, 10 mM Tris–HCl, and 1 mM EGTA, pH 7.4). Mitochondrial protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL. USA).

Intactness of mitochondria was evaluated by measuring citrate synthase activity in mitochondria preparations and the same preparations subjected to sonication for 2 min. Briefly, mitochondria were added to a buffer containing 85 mM Tris (pH 8.3), 0.5 mM oxaloacetate, 0.45 mM acetyl-CoA, and 0.1 mM dithionitrobenzoic acid. The assay was carried out at 37 °C and the increase in absorbance due to the formation of thionitrobenzoic acid was measured spectrophotometrically at 412 nm. Because inner mitochondrial membrane is impermeable to the substrates, the activity in the sonicated preparations (ruptured mitochondria) represents total citrate synthase activity as substrates are readily available to the enzyme. Percentage of intact mitochondria was calculated by comparing citrate synthase activity of the non-sonicated mitochondrial samples to that of the sonicated samples (Scheffler et al., 2015).

2.3. In vitro glycolysis model and experimental design

Frozen muscle samples were pulverized under liquid nitrogen and homogenized at 1:10 (wt/vol) in reaction buffer containing 40 mM glycogen, 60 mM KCl, 5 mM $MgCl_2$, 10 mM Na_2HPO_4, 30 mM creatine, 25 mM carnosine, 10 mM sodium acetate, 5 mM ATP, 0.5 mM ADP, and 0.5 mM NAD^+ (pH 7.4) (England et al., 2014). Either 0 or 0.5 mg/ml isolated mitochondria were incorporated into the *in vitro* model with or without a mitochondrial inhibitor cocktail (20 μM rotenone, 10 mM potassium cyanide, and 10 μM oligomycin, to inhibit mitochondrial complexes I, IV, and V, respectively). Aliquots were removed at 0, 30, 120, 240, and 1440 min for pH and metabolite analysis. Reaction vessels were maintained at 25 °C for the duration of the trial. *Longissimus lumborum* muscles from six different pigs were used for mitochondrial isolation, while frozen portions were powdered and split between treatments (n = 6 per treatment).

In the second experiment, mitochondrial samples were homogenized with a Polytron homogenizer (Polytron PT-MR 2100, Kinematica AG, Switzerland) then centrifuged at 13,000 rpm for 5 min at 4 °C. The resulting supernatants were transferred to new tubes while pellets were re-suspended again in the mitochondrial suspension buffer. Mitochondrial supernatant and pellet were then tested in the *in vitro* model.

In the third experiment, mitochondrial supernatants were mixed with equal volume of 1 M perchloric acid (PCA) and incubated on ice for 15 min. After centrifugation at 13,000 rpm for 5 min at 4 °C, resulting supernatants were transferred to new tubes and the pH was adjusted to 7.4. Mitochondrial and PCA-treated supernatants were tested in the *in vitro* model. Number of experimental units per treatment, time of aliquots removal and conditions in the second and third experiments were consistent with the first experiment.

2.4. pH measurement

pH samples were prepared using the iodoacetate method as described by Bendall (1973). Briefly, four volumes of homogenate were removed from the reaction vessel and placed in a new tube containing one volume of 25 mM sodium iodoacetate and 750 mM KCl (pH 7.0). Samples were centrifuged at 13,000 rpm for 5 min at room temperature, equilibrated to 25 °C and measured directly using an Orion Ross Ultra pH glass electrode (Thermo Scientific, Pittsburgh, PA, USA).

2.5. Metabolite analysis

Aliquots for glycogen analysis were mixed at 1:1 ratio with 2.5 M HCl, heated at 90 °C for 2 h, centrifuged at 13,000 rpm for 5 min, and the resulting supernatant was neutralized with 1.25 M KOH (Bergmeyer, 1984). Aliquots for glucose, glucose 6-phosphate (G6P), lactate, adenine nucleotides (ATP, ADP, AMP), and inosine monophosphate (IMP) were incubated for 20 min on ice with an equal volume of 1 M PCA. Following, samples were centrifuged at 13,000 rpm for 5 min, and the resulting supernatants were transferred to new tubes and neutralized with 2 M KOH (Bergmeyer, 1984). Glycogen, glucose, G6P, and lactate concentrations were determined with enzymatic methods (Bergmeyer, 1984) modified for a 96-well plate (Hammelman et al., 2003). All metabolites were analyzed at room temperature and measured spectrophotometrically at 340 nm. ATP, ADP, AMP, and IMP contents were quantified by high-performance liquid chromatography (HPLC). Breifly, samples were filtered (PTFE 0.22 μm pore size) then injected into an HP Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using continuous gradient elution with two mobile phases using a C18 2.6 μm 50 × 4.6 mm column (Thermo Scientific, Pittsburgh, PA, USA). Flow rate was maintained at 1.25 ml/min. Detections were performed at room temperature at a wavelength of 260 nm (Bernocchi et al., 1994; Williams, Vidt, & Rinehart, 2008).

2.6. Statistical analysis

Metabolite and pH data collected at different time points were