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Short communication

Antemortem stress regulates protein acetylation and glycolysis in postmortem muscle

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ABSTRACT

Although exhaustive research has established that preslaughter stress is a major factor contributing to pale, soft, exudative (PSE) meat, questions remain regarding the biochemistry of postmortem glycolysis. In this study, the influence of preslaughter stress on protein acetylation in relationship to glycolysis was studied. The data show that antemortem swimming significantly enhanced glycolysis and the total acetylated proteins in postmortem *longissimus dorsi* (LD) muscle of mice. Inhibition of protein acetylation by histone acetyltransferase (HAT) inhibitors eliminated stress induced increase in glycolysis. Inversely, antemortem injection of histone deacetylase (HDAC) inhibitors, trichostatin A (TSA) and nicotinamide (NAM), further increased protein acetylation early postmortem and the glycolysis. These data provide new insight into the biochemistry of postmortem glycolysis by showing that protein acetylation regulates glycolysis, which may participate in the regulation of preslaughter stress on glycolysis in postmortem muscle.

1. Introduction

PSE (pale, soft, exudative) meat is characterized by low pH, a pale and exudative appearance, and a soft texture. Currently, it is accepted that fast glycolysis and a rapid buildup of lactic acid in early-stage postmortem muscle, when the muscle temperature is high, which results in protein denaturation, is the direct cause of the PSE syndrome (Owens, Hirschler, McKee, Martinez-Dawson, & Sams, 2000; Solomon, Van Laack, & Eastridge, 1998; Woelfel, Owens, Hirschler, Martinez-Dawson, & Sams, 2002), thus insight into the biochemistry of glycolysis in postmortem muscle will help to prevent the occurrence of PSE meat.

Protein acetylation at lysine residues is a post-translational modification that was first discovered in histone in 1963 (Phillips, 1963). Recently, it has been reported that protein acetylation and deacetylation plays an important role in cellular metabolism from bacteria to human (Wang et al., 2010; Zhao et al., 2010). Most enzymes involved in intermediate metabolism have been shown to be acetylated (Guan & Xiong, 2011; Menzies & Auwerx, 2013). In addition, exercise and energetic stress have been shown to regulate the activity of protein deacetylases and energy metabolism in skeletal muscle (Canto et al., 2009; McGee et al., 2014; Menzies & Auwerx, 2013). Based on these reports, we hypothesize that pre-slaughter stress regulates the acetylation/deacetylation of glycolytic enzyme in skeletal muscle and subsequently the postmortem glycolysis.

In this study, the influence of pre-slaughter stress on protein acetylation and the relationship between protein acetylation and glycolysis in postmortem muscle were investigated. The data obtained suggest that protein acetylation regulates glycolysis in postmortem muscle. Pre-slaughter stress might act on postmortem glycolysis through its regulation of protein acetylation though the exact mechanism is to be defined. This study reveals a new mechanism that regulates postmortem energy metabolism. The conversion of muscle into meat is accomplished by a series of physicochemical changes which are not fully understood, our study provides new information about this process and may ignite a new topic in food biochemistry.

2. Materials and methods

2.1. Animal treatments

A total of 32 three-month-old C57BL/6J mice (~25 g, male) were randomly assigned to four treatments: (1) control, mice were injected with vehicle before killed; (2) swim, mice were injected with vehicle and force to swim for 2 min before killed; (3) S+HATi,

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mice were intraperitoneally injected with 185 μg/g histone acetyltransferase inhibitor II (Sigma), waited for 15 min to let it go into muscle, and then forced to swim; (4) S+HDACi, mice were injected with 8.0 μg/g trichostatin A (TSA, an inhibitor of HDAC I and II) and 250 μg/g nicotinamide (NAM, an inhibitor of the Sirt family deacetylases) (Sigma), waited for 15 min, and then forced to swim. Muscle samples were obtained as previously described (Shen, Gerrard, & Du, 2008).

2.2. Glycolytic assay

For muscle pH measurement, 0.1 g of muscle was homogenized in 0.9 mL of 5 mM iodoacetate solution, and pH of the homogenate was measured with a pH meter (Sartorius Scientific Instruments Co., Ltd., Beijing, China).

Glycogen, glucose, glucose-6-phosphate and lactate concentrations were determined as in the literature (Shen, Jones, Kalchayanand, Zhu, & Du, 2005). Briefly, frozen LD muscle (0.1 g) was homogenized in 0.5 mL of distilled water plus 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/mL, and 10 μL of pepstatin/mL. An aliquot of 100 μL homogenate was incubated with 0.5 mL of 1 mg/mL amyloglucosidase in 0.2 M acetate buffer (pH 4.8) for 2 h at 37 °C. Samples were cooled on ice and centrifuged at 12,000×*g* at 4 °C for 2 min. The extract (50 μL) was incubated with 1 mL of assay buffer (1 mM ATP, 1 mM NADP, 3 U of glucose-6-phosphate dehydrogenase/mL, 3 U of hexokinase/mL, and 0.1 M Tris–HCl; pH 7.4) for 1 h at 25 °C. Total concentrations of glycogen, glucose, and glucose-6-phosphate were determined simultaneously by the change in absorbance at 340 nm.

For lactate measurement, 0.05 g muscle was homogenized in 450 μL of 0.9 M $HClO_4$. The homogenates was centrifuged at 13,000×*g*, 4 °C for 5 min. The supernatants was removed and neutralized with 2 M KOH and centrifuged again to precipitate potassium perchlorate. The extracts were used for lactate measurement using a commercial kit (Sigma, St. Louis, MO, USA).

Glycolytic potential was calculated using the formula of Monin and Sellier (Monin & Sellier, 1985), where glycolytic potential = 2 × ([glycogen] + [glucose] + [glucose-6-phosphate]) + [lactate].

Both pyruvate kinase (PK) and hexokinase (HK) activities in muscle were measured using commercially available analysis kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. PK activity was measured based on the coupled reaction of PK with lactic dehydrogenase which results in the formation of NAD^+. One unit of PK is defined as the amount of enzyme to transfer a phosphate group from PEP to ADP to generate 1.0 μmol of pyruvate per minute at 37 °C and pH 7.6. HK activity was measured based on the coupled reaction of hexokinase with glucose-6-phosphate dehydrogenase which results in the formation of NADH. One unit of HK is defined as the amount of enzyme to generate 1.0 mmol of NADH per minute at 37 °C and pH 7.6. Both PK and HK activities in muscle are expressed as enzyme units per gram of muscle protein (U/g protein).

2.3. Western blotting analysis

Frozen mouse *longissimus dorsi* (LD) muscle (0.05 g) was homogenized in 300 μL T-PER® Tissue Protein Extraction Reagent (Thermo, Rockford, IL, USA) containing 10 mmol/L nicotinamide, 1 μmol/L TSA and protease inhibitors (Roche, Basel, Switzerland). A total of 40 μg proteins were separated by 12% SDS–PAGE gel and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked at room temperature with 1% BSA for 1 h and incubated overnight at 4 °C with rabbit Acetylated-Lysine Antibody (Cell Signaling Technology, Beverly, MA, USA). Images were obtained using the ChemiDoc XRS$^+$ imaging system (Bio-Rad, Hercules, CA, USA) and Quantity One software.

2.4. Statistical analysis

All data were subjected to ANOVA as a completely randomized design using the GLM procedure of SAS (SAS 9.1, 2002). The differences in the mean values were compared by the Fisher's Protected Least Significant Difference (LSD) test ($P < 0.05$). Mean values and standard errors of the mean were reported.

3. Results and discussion

3.1. HAT and HDAC were involved in the regulation of postmortem glycolysis

To test if reversible protein acetylation plays a role in postmortem glycolysis, mice were forced to swim, intraperitoneally injected with HAT and HDAC inhibitors before slaughter. As expected, preslaughter swim decreased pH and increased lactic acid concentration ($P < 0.05$) in LD muscle both 45 min and 24 h postmortem when compared to control (Fig. 1A and B). No difference in glycolytic potential was determined (Fig. 1C), suggesting that the altered glycolysis among treatments was not related to muscle glycogen storage. These data support previous studies that pre-slaughter stress induced by swimming accelerates glycolysis in postmortem muscle (Shen & Du, 2005).

Antemortem injection of HDAC and HAT inhibitors altered glycolysis in postmortem muscle (Fig. 1A and B). Injection of HDAC inhibitors, TSA and NAM, further reduced ($P < 0.05$) muscle pH in combination to swim. Consistent with pH values, the S+HDACi mice had the highest lactic acid concentration in muscle postmortem (Fig. 1B). Opposite to HDAC inhibitors, preslaughter injection of HAT inhibitors diminished the effect of antemortem swimming on glycolysis in postmortem muscle. Injection of HAT inhibitor II significantly increased muscle pH and decreased ($P < 0.05$) lactic acid in muscle postmortem when compared to those of muscle from mice forced to swim alone (Fig. 1A and B). The altered glycolysis in postmortem muscle injected with HAT and HDAC inhibitors suggest that protein acetylation is involved in the regulation of postmortem glycolysis.

3.2. Pre-slaughter stress altered protein acetylation in postmortem muscle

A time course change in total acetylated proteins was shown in Fig. 2. In the control muscle, no change in total acetylated proteins was detected between 0 and 45 min postmortem. However, the total acetylated proteins were significantly decreased ($P < 0.05$) 24 h after animals were killed (Fig. 2A and C), indicating increased protein deacetylation late postmortem. Pre-slaughter stress induced by swimming increased ($P < 0.05$) the total amount of acetylated proteins in muscle 45 min postmortem (Fig. 2B and D), showing that pre-slaughter stress up-regulated protein acetylation in early-stage postmortem muscle.

Variation in total acetylated proteins was shown in Fig. 3. At 0 min postmortem, no difference in total acetylated proteins among the four treatments was detected. However, varied protein acetylation was detected in muscle 45 min postmortem. When compared to the control, antemortem swim significantly increased ($P < 0.05$) the total amount of acetylated proteins in muscle 45 min postmortem. Injection of HDAC inhibitors in combination with stress further increased the amount of total acetylated proteins, which had the highest content of acetylated protein in muscle 45 min postmortem. Inversely, the preslaughter stress induced