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Role of β-Adrenoceptor Signaling and AMP-Activated Protein Kinase in Glycolysis of Postmortem Skeletal Muscle

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Postmortem glycolysis is directly linked to the incidences of PSE (pale, soft, and exudative) and DFD (dark, firm, and dry) meats, which cause significant economic loss to the meat industry. However, mechanisms controlling postmortem glycolysis are unclear. The objective of this study was to determine the role of β-adrenoceptor signaling and AMP-activated protein kinase (AMPK) in postmortem glycolysis. Eighteen 2 month old C57BL/6J female mice were randomly separated into three groups. Group I received an intraperitoneal injection of saline solution only and served as the control; group II received a saline injection and then were forced to swim for 1 min; and group III received an injection of propranolol (1 mg/kg) in saline. In addition, six C57BL/6J female AMPK knockout mice were assigned to group IV, which received a saline injection and were forced to swim for 1 min. The *longissimus dorsi* muscle was sampled at 0, 1, and 24 h postmortem for pH and enzyme activity measurements. The objective is to elucidate the roles of β-adrenoceptor signaling and AMPK in the glycolysis of postmortem muscle. Results showed that AMPK activity had a major role in determining the ultimate muscle pH, with an ultimate pH for control mice of 6.16 and AMPK knockout mice of 6.48. The β-adrenoceptor signaling is essential for initial rapid glycolysis. Blocking β-adrenoceptor signaling prevented the initial pH decline induced by stress. Activation of β-adrenoceptor signaling due to preslaughter stress activates glycogen phosphorylase, resulting in a rapid glycolysis shortly after slaughter. On the other hand, the activation of AMPK is important for maintaining the activity of glycogen phosphorylase and pyruvate kinase, leading to a sustained glycolysis and a low ultimate pH.

KEYWORDS: AMP-activated protein kinase; β-adrenoceptor; skeletal muscle; glycogen phosphorylase; pyruvate kinase; mice; PSE; meat

INTRODUCTION

The high incidences of PSE (pale, soft, and exudative) meat in pork, turkey, and chicken cause significant loss to the meat industry due to their high drip loss, low cooking yield, and tough texture after cooking (*1*−*3*). Fast and/or excessive glycolysis in postmortem muscle causes PSE syndrome and is also related to "acid meat" in Hampshire pigs (*4*−*6*). On the other hand, insufficient postmortem glycolysis, usually due to a lack of glycogen, leads to DFD (dark, firm, and dry) meat. Beef and lamb have relatively high incidences of DFD meat, which has an unattractive dark color and has a stale off-flavor after cooking. Because of its inferior quality, the dark cutter in beef also causes significant loss to the meat industry (*7*). To solve these problems, however, it is necessary to understand the underlying mechanisms, which remain largely unclear.

Recent biomedical studies indicate that AMP-activated protein kinase (AMPK) plays a crucial role in the glycolysis of skeletal and cardiac muscle in vivo (*8*). AMPK, a heterotrimeric enzyme with α-, β-, and γ-subunits, is mainly recognized as a critical regulator of energy metabolism (*9*). AMPK is switched on by an increase in the AMP/ATP ratio in muscle cells, which leads to the phosphorylation of AMPK at Thr^{172} by an unidentified kinase (*9*). Once activated, AMPK switches on glycolysis (*8*, *10*). In the current study, we hypothesize that AMPK plays an important role in the glycolysis of postmortem muscle and the incidence of PSE meat.

β2-Adrenoceptor is the main adrenoceptor in skeletal muscle (*11*). In vivo studies show that β-adrenoceptor activation is essential for the rapid initialization of glycolysis in skeletal muscle following stress. Stresses instantly increase the catecholamine levels in serum (*12*−*14*). Epinephrine, one of the main catecholamines secreted during stress, is a potent stimulator of lactate production in skeletal muscle (*15, 16*). The observation that propranololol, a competitive inhibitor of the β-adrenergic agonist binding to these receptors, completely blocks epinephrine-stimulated muscle lactate production (*15*) and directly supports a role for β-adrenoceptor activation in glycolysis. There are no reports on the role of β-adrenoceptor in the glycolysis of postmortem muscle. We hypothesize that epinephrine secreted during preslaughter stress leads to the activation of β-adrenoceptor and initiates rapid lactate production in skeletal muscle

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shortly after slaughter; the rapid pH decline results in a high incidence of PSE meat. Altogether, the objective of the current study is to elucidate the importance of β-adrenoceptor signaling and AMPK in the glycolysis of postmortem muscle.

EXPERIMENTAL PROCEDURES

Treatments. Eighteen 3 month old C57BL/6J female mice were randomly separated into three groups. Group I received an intraperitoneal injection of saline solution only and served as the control (control); group II received a saline injection and then were forced to swim for 1 min (swim); and group III received an injection of propranolol (1 mg/kg) in saline solution (swim + blocker). In addition, six C57BL/6J female AMPK knockout mice (3 months old) were assigned to group IV, which received a saline injection and were forced to swim for 1 min (swim + AMPK knockout). Forced swim was used to mimic the stress physically and emotionally experienced by pigs before slaughter. The AMPK knockout mice were originally obtained from Dr. M. J. Birnbaum (Department of Medicine and Howard Hughes Medical Institute, University of Pennsylvania) and bred in the PD's laboratory. These AMPK knockout mice express a dominant negative AMPKα2 subunit under the control of the muscle specific creatine kinase promoter (*17*). This dominant negative AMPKα2 replaces functional α1, α2, and α3 in AMPK and results in a very low AMPK activity in skeletal muscle (*17*, *18*). Forced swimming was conducted 10 min after propranolol or saline injection. Mice were then anesthetized by carbon dioxide inhalation and killed by cervical dislocation immediately. Within 30 s, the pelt was removed and ~0.2 g each of upper-right (last cervical vertebrae to 8th thoracic vertebrae) and lower-left (14th lumbar to first sacral vertebrae) *longissimus* muscle was removed and combined. Part of this muscle (~0.1 g) was used to measure pH, and the rest was snap-frozen in liquid nitrogen for the analysis of enzyme activity. Carcasses were eviscerated and suspended in a glass chamber at 4 °C. Subsequent samples were taken in a similar fashion from the middle part (8th thoracic to 14th lumbar veterbrae) of both sides of the muscle after 1 h and from the remaining lower-right and upper-left regions after 24 h. Muscle samples were snap-frozen in liquid nitrogen and used to assess glycolytic potential and the activities of key glycolytic enzymes.

Muscle Homogenate. Frozen *longissimus* muscle samples were cut into small pieces and mixed. A 0.1 g amount of *longissimus* muscles was weighed and then homogenized in a polytron homogenizer (7 mm diameter generator) with 5 vol of ice-cold lysis buffer [137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% nonylphenyl-poly(ethylene glycol), 10% glycerol, 2 mM phenylmethanesulfonyl fluoride, 10 mM sodium pyrophosphate, 2.5 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 100 mM NaF]. Following centrifugation at 12000*g* for 5 min at 4 °C, the supernatant was used for enzyme activity measurements.

AMPK Activity Measurement. The activity was measured using a method previously described (*19*, *20*). Briefly, a SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg; Invitrogen) was used as a substrate for AMPK phosphorylation. The muscle homogenate obtained above was centrifuged at 12000*g* for 5 min at 4 °C. The supernatant (10 μL) was incubated for 10 min at 37 °C in 40 mM HEPES, 0.2 mM SAMS peptide, 0.2 mM AMP, 80 mM NaCl, 8% (w/v) glycerol, 0.8 mM EDTA, 0.8 mM 1,4-dithio-DL-threitol, 5 mM $MgCl_2$, and 0.2 mM ATP + 2 μCi [^{32}P]ATP, pH 7.0, in a final volume of 50 μL. An aliquot (20 μL) was removed and spotted on a 2 cm × 2 cm piece of Whatman P81 filter paper. The [^{32}P]ATP was removed with six washes in 1% phosphoric acid, and radioactivity was quantified after immersing the filter paper in 3 mL of Scintiverse (Fisher Scientific, Hanover Park, IL). The activity was expressed as nmol of ATP-P incorporated into the SAMS peptide × min^{-1} × g muscle^{-1}.

Glycogen Phosphorylase Activity Measurement. For glycogen phosphorylase activity measurement, 200 μL of supernatant of muscle homogenate obtained above was diluted 1:1 with ice-cold solution B (50 mM MES, 50 mM KF, and 60 mM β-mercaptoethanol, pH 6.1). The diluted supernatant (60 μL) was mixed with 100 μL of solution C (400 mM KF, 2 mg/mL glycogen, 27.88 mg/mL glucose-1-phosphate, and 0.5 μCi/ml [U-^{14}C]glucose-1-phosphate; Amersham, Piscataway, NJ) and incubated for 1 h at 30 °C. A portion of the mixture (80 μL) was removed and spotted on a 20 mm × 20 mm piece of 3M chromatography paper. The paper was immediately dropped into ice-cold 66% (v/v) ethanol. Three separate washes in ice-cold 66% ethanol were preformed. The paper was incubated in acetone for 2−3 min before being dried for scintillation counting. The activity was expressed as the incorporation of glucose (mg) into glycogen × min^{-1} × g muscle^{-1} (*21*).

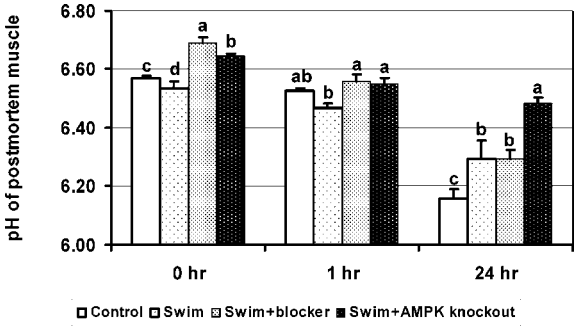

Figure 1. pH values of postmortem muscle from mice received various preslaughter treatments. Control, received an injection of saline solution only; swim, received a saline injection and then were forced to swim for 1 min; swim + blocker, received an injection of propranolol (1 mg/kg) in saline solution; and swim + AMPK knockout, AMPK knockout mice received a saline injection and were forced to swim for 1 min. *Longissimus* muscle was sampled at 0, 1, and 24 h postmortem for pH measurements. Mean values and standard errors of the mean were reported. a and b mean that there was a significant difference among treatments within a single time point; $n = 6$, $P < 0.05$.

Pyruvate Kinase Activity Measurement. The supernatant of muscle homogenate obtained above (200 μL) was diluted 1:1 with solution C (0.3 mM NADH, 1.5 mM phosphoenolpyruvic acid, 16 mM $MgSO_4$, 150 mM KCl, 60 U/mL lactic dehydrogenase, and 0.1 M Tris-HCl (pH 7.4), mixed, and incubated at 37 °C for 5 min. Then, 0.64 mM ADP was added and the change in absorbance at 340 nm was recorded. The activity was expressed as μmol of NAD^+ formed × min^{-1} × g muscle^{-1}.

pH Measurement. *Longissimus* muscle (0.1 g) was homogenized in 0.9 mL of 5 mM iodoacetate solution, and the pH of the homogenate was measured directly with a pH meter (*22*).

Statistical Analysis. Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). The activities of AMPK, glycogen phosphorylase, and pyruvate kinase and the pH of *longissimus dorsi* were analyzed. The differences in the mean values were compared by the Fisher's protected least significant difference test ($P < 0.05$). Mean values and standard errors of the mean were reported.

RESULTS AND DISCUSSION

The pH values in postmortem muscle were significantly affected by preslaughter stress treatment, propranolol injection, and AMPK knockout (**Figure 1**). For the initial pH, it was significantly lower in mice stressed by swimming and higher in mice with β-adrenoceptor blockade (**Figure 1**), showing that β-adrenoceptor is important for the initial glycolysis. Not only β-adrenoreceptor blocker but also AMPK had a higher pH than the control, indicating that AMPK is involved in initial glycolysis, which results in a pH decline in muscle (**Figure 1**). At 1 h, group II mice (swim) had a significantly lower pH as compared with mice administered the β-adrenoceptor blocker (swim + blocker) (**Figure 1**), confirming a role of β-adrenoceptor in initial glycolysis. This result is in agreement with other reports in live animals, where β-adrenoceptor was shown to be a potent stimulator of lactate production in skeletal muscle (*15*, *16*). After 24 h, the pH of the muscle from AMPK knockout

(group IV) mice remained very high, indicating that glycolysis was very slow after 1 h postmortem and suggesting that AMPK is required for sustained glycolysis in postmortem muscle. The difference in pH between mice without (swim) and with (swim + blocker) β-adrenoceptor blockade disappeared at 24 h (**Figure 1**), suggesting that the β-adrenoceptor pathway has little effect on the ultimate pH of postmortem muscle (**Figure 1**). One interesting observation is that the control mice (control) had a lower pH than mice stressed by swimming (swim and swim + blocker) (**Figure 1**), which may be related to glycogen availability. Stress may cause the consumption of part of the glycogen, thereby leaving less glycogen available for postmortem glycolysis. In pigs, it has been shown that short-term preslaughter stress slightly but significantly increases the pH at 24 h (*23*), consistent with our results. These results strongly support the hypothesis that β-adrenoceptor mainly contributes to the initial glycolysis. The stress induced by forced swimming hastened glycolysis and the pH decline in postmortem muscle. Treatment of mice with propanolol inhibited this effect, strongly suggesting that the β-adrenoceptor pathway controls the rate of initial glycolysis in postmortem muscle. The activation of glycolysis through β-adrenoceptor involves the activation of adenylyl cyclase associated with the receptor. Adenylyl cyclase catalyzes the production of cAMP, which further activates protein kinase A (PKA) (*24*). Activated PKA then activates glycogen phosphorylase, a key enzyme in glycolysis, through the activation of phosphorylase kinase (*25*).

The large difference in ultimate pH between control and AMPK knockout mice at 24 h postmortem shows the importance of AMPK in sustaining glycolysis in postmortem muscle. These data supported the view that the prevalence of "acid" meat in RN− pigs, in which AMPK is continuously active via mutation, resulted in a low ultimate pH (*26*). The important role of AMPK in the control of glycolysis in vivo has been demonstrated by several studies. Administration of AICAR, a specific activator of AMPK, dramatically increases the lactic acid content in muscle (*27*). Also, knockout AMPK significantly reduced the pH decline in ischemic cardiac muscle (*28*).

Blockade of the β-adrenoceptor pathway prevented the activation of glycogen phosphorylase. At 0 and 1 h postmortem, the activity of glycogen phosphatase, a key enzyme involved in glycogen utilization and glycolysis, was significantly lower in stressed mice with β-adrenoceptor blockade (swim + blocker) as compared with mice without blockade (swim) and control mice (control) (**Figure 2**). These results clearly show that β-adrenoceptor signaling contributes to the phosphorylation and activation of glycogen phosphorylase. At 24 h postmortem, the difference in the activity of glycogen phosphorylase disappeared among control mice and mice with and without β-adrenoceptor blockade. However, at both 1 and 24 h postmortem, the activity of glycogen phosphorylase in the muscle of AMPK knockout mice was significantly lower than other mice, showing that AMPK is required for the sustained activation of glycogen phosphorylase (**Figure 2**), consistent with the higher ultimate pH in muscle from AMPK knockout mice (**Figure 1**).

The activity of pyruvate kinase did not differ at 0 and 1 h postmortem (**Figure 3**), suggesting that pyruvate kinase was not affected by the β-adrenoceptor pathway. At 24 h postmortem, however, the activity of pyruvate kinase was significantly lower in AMPK knockout mice (swim + AMPK knockout), indicating that AMPK participates in the sustained activation of pyruvate kinase (**Figure 3**). A previous study showed that pyruvate kinase in PSE meat is phosphorylated, resulting in higher enzyme activity as compared with normal meat. This

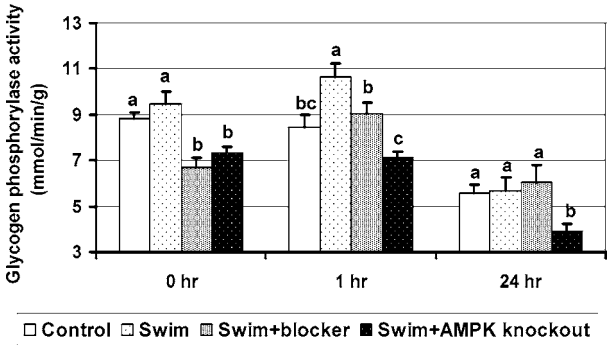

Figure 2. Glycogen phosphorylase activity of postmortem muscle from mice received various preslaughter treatments. Control, received an injection of saline solution only; swim, received a saline injection and then were forced to swim for 1 min; swim + blocker, received an injection of propranolol (1 mg/kg) in saline solution; and swim + AMPK knockout, AMPK knockout mice received a saline injection and were forced to swim for 1 min. *Longissimus* muscle was sampled at 0, 1, and 24 h postmortem for glycogen phosphorylase measurements. Mean values and standard errors of the mean were reported. a and b mean that there was a significant difference among treatments within a single time point; $n = 6$, $P < 0.05$.

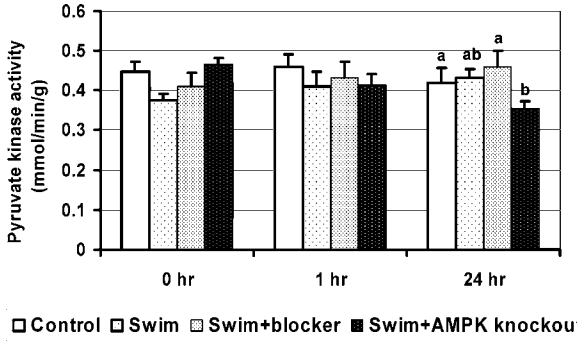

Figure 3. Pyruvate kinase activity of postmortem muscle from mice received various preslaughter treatments. Control, received an injection of saline solution only; swim, received a saline injection and then were forced to swim for 1 min; swim + blocker, received an injection of propranolol (1 mg/kg) in saline solution; and swim + AMPK knockout, AMPK knockout mice received a saline injection and were forced to swim for 1 min. *Longissimus* muscle was sampled at 0, 1, and 24 h postmortem for pyruvate kinase activity measurements. Mean values and standard errors of the mean were reported. a and b mean that there was a significant difference among treatments within a single time point; $n = 6$, $P < 0.05$.

result suggests that overactivation of pyruvate kinase contributes to the incidence of PSE meat (*29*). Furthermore, the phosphorylation of pyruvate kinase is responsible for the increased activity of this enzyme in PSE meat (*29*). Although the direct phosphorylation/activation of pyruvate kinase by AMPK has not been reported, it is quite possible that this reaction contributes to the rapid or excessive glycolysis in PSE meat.

Figure 4 shows the activity of AMPK. At 0 h postmortem, the AMPK activity was lower in stressed mice with β-adrenoceptor blockade (swim + blocker) than control and stressed mice without blockade (control and swim), suggesting that β-adrenoceptor signaling can activate AMPK (**Figure 4**). However, the exact mechanisms associated with this activation are unclear. The AMPK activity in AMPK knockout mice was significantly lower than that in other mice, while the difference in AMPK activity among the remaining three groups of mice disappeared at 1 h postmortem. At 24 h postmortem, the activity of AMPK

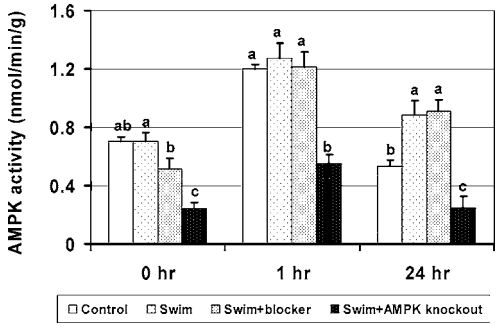

Figure 4. AMPK activity of postmortem muscle from mice received various preslaughter treatments. Control, received an injection of saline solution only; swim, received a saline injection and then were forced to swim for 1 min; swim + blocker, received an injection of propranolol (1 mg/kg) in saline solution; and swim + AMPK knockout, AMPK knockout mice received a saline injection and were forced to swim for 1 min. *Longissimus* muscle was sampled at 0, 1, and 24 h postmortem for AMPK activity measurements. Mean values and standard errors of the mean were reported. a and b mean that there was a significant difference among treatments within a single time point; $n = 6$, $P < 0.05$.

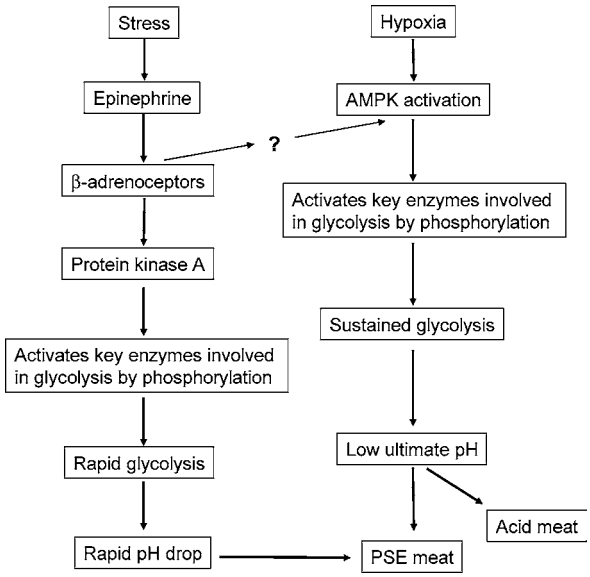

Figure 5. Proposed roles of β-adrenoceptor and AMPK in the incidence of PSE and acid meats.

was higher in stressed mice (swim and swim + blocker) than control mice (control) (**Figure 4**). Comparing mice of (swim + blocker) and (swim + AMPK knockout), a significant difference in the activities of glycogen phosphorylase and pyruvate kinase at 24 h was detected (**Figures 2** and **3**). Because both groups of mice were stressed and the only difference between them was the activity of AMPK (**Figure 4**), this result suggested an important role for AMPK in the sustained activation of both glycogen phosphorylase and pyruvate kinase, thus supporting our hypothesis that AMPK is mainly responsible for sustained glycolysis in postmortem muscle.

On the basis of these results, we propose mechanisms of β-adrenoceptor signaling and AMPK in the postmortem glycolysis (**Figure 5**). Preharvest stresses quickly activate the β-adrenoceptors and PKA signaling in skeletal muscle, which leads to the rapid activation of key glycolytic enzymes and results in a fast pH decline in postmortem muscle; glycolysis is maintained by the activation of AMPK, which results in a low ultimate pH (**Figure 5**). We detected sustained activation of AMPK in postmortem muscle, which should be due to the high level of AMP present in the muscle. During postmortem change, ATP is rapidly consumed and a high level of AMP accumulates, which constantly stimulates AMPK activity.

PSE and DFD syndromes have been recognized for decades. The incidence of PSE and DFD meats causes significant economic loss to the animal industry. Both PSE and DFD meats result from either rapid and excessive or insufficient postmortem glycolysis. However, the detailed mechanisms associated with this abnormal glycolysis are still unclear (*22*, *30*, *31*). In this study, for the first time, we proposed the roles of β-adrenoceptor and AMPK in the glycolysis of postmortem muscle. Elucidating mechanisms controlling postmortem glycolysis are very conducive to solve PSE and DFD problems in meats. However, this study was conducted in mice. Although mice are an excellent animal model for mechanism studies due to the availability of transgenic mice and its small body size facilitating the application of drugs to inhibit selected pathways, we cannot directly assess meat quality in mice. Therefore, further studies in livestock are needed.

In summary, both β-adrenoceptor signaling and AMPK play important roles in postmortem glycolysis. Activation of β-adrenoceptor signaling due to preslaughter stress activates glycogen phosphorylase, resulting in a rapid glycolysis shortly after slaughter. On the other hand, the activation of AMPK is important for maintaining the activity of glycogen phosphorylase and pyruvate kinase, leading to a sustained glycolysis and a low ultimate pH.

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