Adenosine monophosphate-activated protein kinase status modulates kinetics of post-mortem pH decline and meat quality in pig *Longissimus* muscle

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Abstract— **AMP-activated protein kinase (AMPK) is a sensor of cellular energy status involved in regulation of glycogen metabolism and it influences meat quality by modulating post-mortem (p.m.) energy metabolism. This study aimed at evaluating the relationships between AMPK activity in the pig *Longissimus* muscle (LM) and the kinetics of p.m. pH decline. A total of 117 pigs exhibiting a high variability in muscle glycolytic potential (GP) (84 up to 212 µmol lactate/g fresh muscle) were used. Samples of LM were taken just after exsanguination (T0) to represent the *in vivo* situation, and then at 30 min (T30) and 24h p.m.. GP, pH at T30 (pH1) and at 24h (pHu) were determined. Total AMPK and its level of activation (pAMPK/total AMPK) were quantified at both T0 and T30 by immunoblotting. AMPK activation at T30 was positively correlated with pHu ($r=0.40$, $p<0.001$) and negatively with GP ($r=-0.42$, $p<0.001$) and pH1 ($r=-0.23$, $p=0.01$). A stepwise regression method ($R^2=0.52$, $p<0.001$) identified GP, pH1 and pAMPK/total AMPK at T30 as the best predictors of pHu. Phosphorylation status of AMPK was also involved in the determination of pH1, but the model explained less variability of this trait ($R^2=0.22$, $p<0.001$). Moreover, AMPK status at T0 and T30 were highly correlated ($r=0.69$, $p<0.001$), suggesting that a high *in vivo* level of phosphorylation of this enzyme could increase the rate and reduce the extent of pH decline. Altogether, this indicates that the control of AMPK activity during the rearing period seems to be an interesting way for modulating pork quality.**

Keywords— **AMPK, post-mortem pH, pork quality**

I. INTRODUCTION

Kinetics of post-mortem pH decline influences meat quality development, defaults and variability. Understanding the mechanisms contributing to rapid or extended post-mortem (p.m.) pH fall is still a challenge for the control and prediction of sensory and technological quality attributes of pork. Characteristics of p.m. pH decline are determined by genetic factors, physiological animal conditions before slaughter, muscle metabolism and metabolic network capacities. After slaughter, when the blood circulation stops, the muscle tissue attempts to maintain homeostasis by preserving cellular ATP concentration only through the glycolysis anaerobic pathway. Thus, glycogen breakdowns and lactic acid accumulates, contributing to a decrease in muscle pH. Several studies agree to demonstrate a curvilinear relationship between initial glycogen content and ultimate pH [1]. However, even though the glycolytic potential (GP) combines in a single criteria the muscle capacity for p.m. glycolysis, the glycogen and lactate contents do not fully explain the extent of postmortem pH decline.

The AMP:ATP ratio is a sensitive indicator of cellular energy status and can activate allosteric enzymes involved in the glycolytic pathway. As part of the regulating network, the AMP-activated protein kinase (AMPK) enzyme has been shown to play a key role in regulating the level of muscle glycogen and p.m. metabolism [2]. In response to cellular stress and to an increase in the AMP:ATP ratio, AMPK is activated by phosphorylation on Thr172 of the AMPKα subunit [3]. Overall, AMPK activation inhibits anabolic pathways and stimulates catabolic pathways to restore cellular energy level [2]. Level of AMPK phosphorylation is thus involved in the variation of energy status of muscle during the p.m. period and thus influences meat quality traits. Nevertheless, the importance of AMPK activity in modulating the kinetics of p.m. muscle pH decline is still unclear. This study aimed at evaluating the relationships between AMPK status determined by the pAMPK/total AMPK ratio with the rate and extent of p.m. pH decline in the *Longissimus* muscle (LM).

II. MATERIALS AND METHODS

A. Animals and slaughter conditions

The present study includes a total of 117 Large White females and castrated pigs, issued from two divergent lines selected for residual feed intake [4]. All pigs are free of the Ryr1 as well as the PRKAG3 R225G gene mutation coding for an altered isoform of AMPK. These pigs exhibit a high variability in muscle glycolytic potential (GP) as well as ultimate pH (pHu). Thus, this animal data base (Table 1) is well suited to analyse relationships between LM metabolism and kinetics of p.m. pH decline.

All pigs, raised under the same rearing conditions in an INRA experimental farm (Le Magneraud, France), were fed *ad libitum* a standard diet and controlled individually during the growing-finishing period (31±5 up to 108±8 kg) for body weight and daily feed consumption. Pre-slaughter transport stress was reduced by delivering pigs 20 h prior to slaughter. Pigs were electrically stunned and exsanguinated under highly standardized conditions at INRA slaughter house (St Gilles, France). Carcass lean meat content was estimated from the percentage of primal cuts.

B. Muscle samples and analysis

Samples of LM were taken just after exanguination (T0) and thirty minutes after slaughter (T30), immediately frozen in liquid nitrogen and stored at -80°C. At T30, pH1, GP and activities of metabolic enzymes lactate dehydrogenase (LDH), citrate synthase (CS) and β-hydroxy-acyl-CoA dehydrogenase (HAD) were determined as previously described [5]. The day after slaughter, pHu of LM was determined directly on the carcass. The same day, a slice of LM was minced and freeze-dried before the determination of intramuscular fat content (IMF) [5].

C. Determination of AMPK phosphorylation by immunoblotting

Samples of LM taken at T0 and T30 were analysed by immunublotting to quantify the level of AMPKα phosphorylation (pAMPK/total AMPK).

Since AMPK activity increases after slaughter and its relationships with meat quality traits may depend on the time of muscle sampling [6], these samples represent the *in vivo* status of AMPK (T0, n=77) and the level of phosphorylation during the p.m. conversion of muscle to meat (T30, n=117).

The western blotting method was adapted for pig species from Sibut et al. [7]. After quantification, solubilisation and migration onto a 10% SDS-PAGE under reducing conditions, separated proteins were electrotransferred overnight at 4°C to PVDF membrane. Then, those membranes were incubated overnight with primary antibodies specific for AMPKα (α1+α2) and Thr172 phospho-AMPKα (α1+α2). Protein bands were visualized using enhanced chemiluminescence (ECL plus kit), scanned (ImageQuant LAS 4000) and quantified (ImageQuant TL program). Membrane normalization was realised based on a common sample (previously selected as exhibiting average values for lactate 30 min p.m. and pHu) repeated on each gel.

D. Statistical analysis

Relationships between variables were analysed using pearson's correlations (r).

Considering a large number of potential explanatory variables, we used a stepwise regression method to identify the best predictive variables of rate and extent of pH decline in LM. Variables included in the model were growth performance and carcass traits (birth weight, slaughter weight, age at slaughter, average feed intake, average daily gain, feed conversion ratio, lean meat content), the LM metabolic traits (glucose, lactate, IMF, GP, LDH, HAD, CS) and hypothetic factors influencing the kinetics of pH decline (AMPK T30, pAMPK/AMPK T30). Thus, two models have been established, one to predict pH1 and the other one for pHu. The stepwise method is a forward-selection method which adds variables one by one into the model. After one variable is added an automatic procedure looks at all the variables already included in the model and deletes any variable that does not produce a significant F-value at the 0.05 level. At the end of the procedure, the model accuracy is estimated by the model R-square (R^2) and normality of residual values. The partial R-square of each variable and the associated p-value characterize the statistical weight of each predictive variable included in the model.

Table 1 Statistical description of animal data base ([a]: µmol/g fresh muscle, [b]: µmol of substrate/min/g fresh muscle)

	Mean ± SE	Min	Max
pH1	6.40±0.15	5.92	6.71
pHu	5.63±0.14	5.39	6.19
Lactate[a]	51.8± 9.4	26.1	70.9
Glucose[a]	5.4±1.9	2.9	11.3
Glycogen[a]	31.9±11.0	6.6	79.0
GP[a]	139.5±21.7	84.4	212.1
LDH [b]	2.26±0.24	1.70	3.30
HAD [b]	3.8±0.5	2.7	9.0
CS [b]	5.65±0.7	4.2	8.1
IMF, %	1.3±0.4	0.6	2.4
Slaughter weight, kg	108.1±8.2	84	125
Age at slaughter, d	171±4	161	187
Average feed intake, g/d	2071±226	1566	2604
Average daily gain, g/d	763±69	520	930
Feed conversion ratio	2.69±0.24	2.11	3.32
Lean meat content, %	58.3±2.9	51.2	64.7

Table 2 Pearson's correlations coefficients between AMPK status at T30, meat quality traits and muscle metabolism in *Longissimus* muscle. (Significance level: ***p<0.001; **p<0.01; *p<0.05, NS p>0.10)

	AMPK T30	pAMPK/AMPK T30
pH1	NS	-0.23**
pHu	NS	0.40***
Lactate[a]	NS	0.43***
Glucose[a]	-0.28***	NS
Glycogen[a]	-0.20*	-0.58***
GP[a]	-0.25***	-0.42***
LDH [b]	NS	NS
HAD [b]	NS	NS
CS [b]	NS	NS
IMF, %	NS	NS

III. RESULTS AND DISCUSSION

AMPK level of phosphorylation at T30 is negatively associated with pH1 (r= -0.23, p=0.01), but positively with pHu (r=0.40, p<0.001), suggesting an important role of AMPK in both rate and extent of muscle pH decline (Table 2).

A. AMPK status as predictor of ultimate pH

The stepwise regression model identified GP, pAMPK/total AMPK and pH1 variables as the best predictors of ultimate pH. 44% of pHu variability can be explained by GP (Table 3). More than glycogen content alone, muscle GP represents the major predictor of pHu. However, variability in ultimate pH can be also explained at 3.7% by the level of AMPK phosphorylation at T30. This result identifies the AMPK activation as a biochemical mechanism influencing pHu and confirms that AMPK activity plays a key role in determining pHu [8]. Nevertheless, in contrast to p.m. mice muscle in which a high AMPK activity lead to a decreased pHu [8], a high AMPK level of phosphorylation at T30 in pig muscle increased pHu in the present experiment. By explaining only 52% of the variability of pHu despite complementary explanatory variables, the present study underlies the complexity of pHu prediction and suggests that other mechanisms are involved and should be studied using a multifactorial approach.

Table 3 Summary of best predictors of pHu after stepwise regression (***p<0.001; **p<0.01; *p<0.05)

	Partial R-squares	Cumulated Model R-squares	F value
GP	0.44	0.44	73.83***
pAMPK/AMPK T30	0.04	0.48	6.49 *
pH1	0.04	0.52	8.99**

B. AMPK status and rate of pH decline

The stepwise regression method selected lactate content, pHu and pAMPK/AMPK at T30 as the three best predictors of the rate of pH decline in LM (Table 4). Nevertheless, this model explained only 22% of pH1 variability, compared to 52% for the pHu model. About 5% of pH1 variability was explained by the level of AMPK phosphorylation at T30, with an increased level of phosphorylation leading to a decreased pH1. AMPK has been hypothesized to be a potential molecular target for the control of pH1 and thus the regulation of PSE incidence in pork [6]. However, no difference in pH1 has been observed between normal and AMPK$\gamma 3^{R200Q}$ mutated pigs, despite a higher AMPK activity in the latter [3-9].

Table 4 Summary of best predictors of pH1 after stepwise regression (***p<0.001; **p<0.01; *p<0.05)

	Partial R-squares	Cumulated Model R-squares	F value
lactate	0.13	0.13	13.50***
pHu	0.04	0.17	5.13*
pAMPK/AMPK T30	0.05	0.22	6.03*

Therefore, depending on the animal data base, AMPK can explain some variability in pH1 (5% in the present study). In this case, AMPK is rather a potential target for the control of pHu.

C. Relationships between estimated in vivo and post-mortem AMPK activation

Total AMPK at T0 and T30 were positively correlated (r=0.86, p<0.001). Interestingly, the level of AMPK phosphorylation at T30 was 7-fold higher than at T0, thus confirming the increase in AMPK activity (through phosphorylation) during the early p.m. period [6]. Moreover, the T0 and T30 pAMPK/total AMPK ratios were highly correlated (r=0.69, p<0.001), suggesting that p.m. muscle metabolism depends on *in vivo* AMPK activity. The role of AMPK in *in vivo* glycolysis has been well established in mice and pig species [8]. Our study hypothesizes the possible regulation of p.m. muscle metabolism, and thereby p.m. pH decline, through modulation of phosphorylation and activation of *in vivo* AMPK.

IV. CONCLUSIONS

Our study demonstrates that AMPK phosphorylation status influences kinetics of p.m. pH decline, even though it only explains less than 5% of pH1 and pHu variability in our models. This suggests that AMPK status can be considered as a new molecular target which could be taken into account to develop a systemic approach of meat quality evaluation.

Correlations between AMPK status at T0 and T30 make AMPK a good candidate for the control of pork quality during the rearing period. Relationships between *in vivo* and p.m. AMPK status and meat quality remain complementary experimental studies in order to better understand the relationships between variability of AMPK status and pigs rearing conditions (housing environment, ambient temperature, physical activity…) and genotype.

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