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Correlation between the Presence of Ubiquitin Conjugated Protein Phosphatase Inhibitor 1 and Postmortem Muscle Glycogen Metabolism

Purevdorj NYAM-OSOR[1], Kenichiro SHIMADA[2*], Kyu-Ho HAN[2], Michihiro FUKUSHIMA[2] and Mistuo SEKIKAWA[3]

[1] *Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan*

[2] *Department of Food Science, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan*

[3] *Department of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, 2-11 Inada, Obihiro, Hokkaido 080-8555, Japan*

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Recently, we have identified the ubiquitin conjugate, protein phosphatase inhibitor 1 (I1), from postmortem muscles. Since I1 is one of the important regulators of glycogen metabolism, the effect of this conjugate (Ub-I1) present in postmortem muscle on glycolysis was investigated. Two sample groups were distinguishable: those not containing Ub-I1 (I, *n=5*) and those containing Ub-I1 (II, *n=27*). Due to the enormous variation in ultimate pH at 48 h postmortem, the latter group was subcategorized into a IIa group showing lower or normal pH and a IIb group showing higher or dark, firm, dry meat pH. Postmortem glycolysis occurred rapidly and vigorously among group I samples. The rate and extent of postmortem glycolysis was typical in group IIa samples while it was slower and weaker in group IIb samples although they had similar glycolytic potential. This discrepancy between sample groups IIa and IIb corresponded to the Ub-I1 abundance. Thus, the relative Ub-I1 abundance appears to correlate with postmortem glycolysis.

Keywords: ubiquitin conjugate, protein phosphatase inhibitor 1, glycogen, glycolysis

Introduction

Postmortem conversion of muscle to meat involves several biochemical modifications and processes. The first process is the metabolic shift from an aerobic to anaerobic state. This is followed by transformation of glycogen into lactic acid and consequent pH reduction, activation of different proteinase systems with degradation of muscle proteins and activity of other enzymatic mechanisms with effects and/or production of specific metabolites (Lawrie, 1985). New approaches and technologies have been changing the way in which these mechanisms and their final results and products are investigated. The primary emphasis has been towards the use of proteomics to analyze the muscle postmortem processes and to identify biomarkers associated with meat quality traits and technological processing (Bendixen, 2005; Hollung *et al.*, 2007; Lametsch and Bendixen, 2001; Wiel and Zhang, 2007). Recent studies have suggested that the ubiquitin system, consisting of ATP, proteasomes and ubiquitin, plays an important role in the degradation of muscle proteins under various catabolic conditions (Galban *et al.*, 2001). Although this ubiquitin system of cellular protein degradation has been investigated in various fields, such as clinical medicine and cellular biology, it has received little attention in the field of meat science (Sekikawa *et al.*, 1998). Recently, an ubiquitin conjugate around 27 kDa has been identified as protein phosphatase inhibitor 1 (I1) based on amino acid sequence and western blot analyses of proteins in the sarcoplasmic fraction obtained from bovine postmortem skeletal muscle (Nyam-Osor *et al.*, 2009). I1 was first indentified from rabbit skeletal muscle in 1976 as an inhibitor of protein phosphatase 1 (PP1) and a regulator of glycogen metabolism (Huang and Glinsmann, 1976). The primary role of I1 in glycogen metabolism is activation of glycogen phosphorylase function by inhibit-

*To whom correspondence should be addressed.
E-mail: kshimada@obihiro.ac.jp

ing PP1 action. Once activated, glycogen phosphorylase switches on glycogenolysis/glycolysis (Foulkes and Cohen, 1979; Aleem *et al.*, 2004). Upon phosphorylation by cAMP dependent protein kinase, I1 becomes a potent inhibitor of PP1 (Sahin *et al.*, 2006) and acts as a remarkable switch that amplifies cAMP signals to glycogen metabolism.

However, the relationship between ubiquitination and I1 in muscle cells has not been clarified. Considering the role of I1 in glycogen metabolism, this study investigated whether Ub-I1 plays a role in muscle carbohydrate metabolism and effects glycolysis of postmortem muscle.

Materials and Methods

Animals and sample preparation of muscle sarcoplasmic proteins Samples (7 ± 1 h after slaughter) were obtained from neck area muscle (*Semispinalis capitis*) of intact carcasses of Holstein Frisian steers (*n=32*) that were slaughtered at a local commercial line after checking for bovine spongiform encephalopathy according to Japanese regulations. Sample preparation was performed as described previously (Nyam-Osor *et al.*, 2009). Briefly, muscle tissues were ground and boiled for 5 min with thrice their weight of distilled water and allowed to cool to room temperature. Homogenization was performed with an ultra high speed homogenizer (Physcotron NS-50, Microtec Co., Ltd., Funabashi, Japan). Homogenates were centrifuged under refrigeration (0°C, 6000 × *g*, 30 min) to obtain a pellet and clear supernatant. The supernatant was dialyzed against distilled water and freeze dried, and the final lyophilized samples were considered to be the sarcoplasmic fraction. The protein concentration was determined by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) and suspended in 0.5 M Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue to give a final concentration of 2 mg/ml.

pH measurement and biochemical assays The pH was measured at 8, 24, and 48 h postmortem. Muscle samples (1 g) were homogenized in 10 ml of 5 mM sodium iodoacetate solution in 150 mM KCl (Bendall, 1973), and the pH of the homogenate was measured directly with a pH meter. Residual glycogen and lactic acid were measured at 48 h postmortem. Glycogen-glucose and lactate concentrations were determined as described by Immonen *et al.* (2000b) using commercial kits from Sigma (St. Louis, MO, USA; GAHK-20) and R-Biopharm AG (Darmstadt, Germany; No. 10 139 084 035), respectively. Briefly, muscle samples were homogenized in ice-cold phosphate buffer (pH 7.0) with a Polytron homogenizer. The homogenate (10 ml) was hydrolyzed in 200 ml of 0.1 M HCl at 100°C for 2 h and then adjusted to pH 6.5-7.5. Glucose concentration was determined pectrophotometrically at 340 nm based on a NAD-linked assay catalyzed by hexokinase and glucose-6-phosphate dehydrogenase. The assay determines the concentration of total glucose in the hydrolyzed muscle specimen, including mainly glycogen, glucose-1-phosphate, glucose-6-phosphate and free glucose, which were considered as total carbohydrates when applicable. Lactate concentration in the homogenate was determined spectrophotometrically at 365 nm based on the reactions catalyzed by lactate dehydrogenase and glutamate pyruvate transaminase.

Glycolytic potential (GP)' was calculated according to the formula by Monin and Sellier (1985): GP = 2 × (glycogen + glucose + glucose-6-phosphate) + lactate

SDS-polyacrylamide gel electrophoresis and western blotting SDS-polyacrylamide gel electrophoresis was performed using the method of Laemmli (1970), with 6% gels. After electrophoresis, proteins were transferred to a polyvinylidenedifluoride membrane with a 0.2-μm pore size (Bio-Rad Laboratories, Hercules, USA) and then the membrane was blocked with 10% skim milk in PBS overnight at room temperature. After blocking, the membrane was incubated for 40 min with rabbit anti-ubiquitin antibody (Sigma, St. Louis, MO, USA; U5379). After three washes in Tween-PBS for 5 min each, the membrane was incubated with biotin-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) for 30 min. After three more washes, the membrane was incubated with avidin-DH and biotin-conjugated HRP (vectorstain, ABC kit, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. After the final three more washes, the membrane was stained with a 3,3'-diaminobenzidine substrate.

Image analysis Results of western blotting were quantified using ImageJ 1.43j (Windows version of NIH Image, http://rsbweb.nih.gov/ij/) as described by Gassmann *et al.* (2009) and Vierck *et al.* (2001).

Statistical analysis Data were analyzed with ANOVA, using the General Linear Model procedures of SPSS 18. Duncan's test was performed to identify means that differed significantly from each other. The relationship between conjugate existence and glycolytic potential was examined by regression analysis. Mean values and standard errors of the meat were reported.

Results and Discussion

The proteins in the sarcoplasmic fraction from postmortem muscle were separated by SDS-PAGE and analyzed by western blotting with an antibody for ubiquitin (Fig. 1A and B). The target ubiquitin conjugate around 27 kDa which was previously identified as Ub-I1 (Nyam-Osor *et al.*, 2009) was the major detected band. Western blotting results showed two

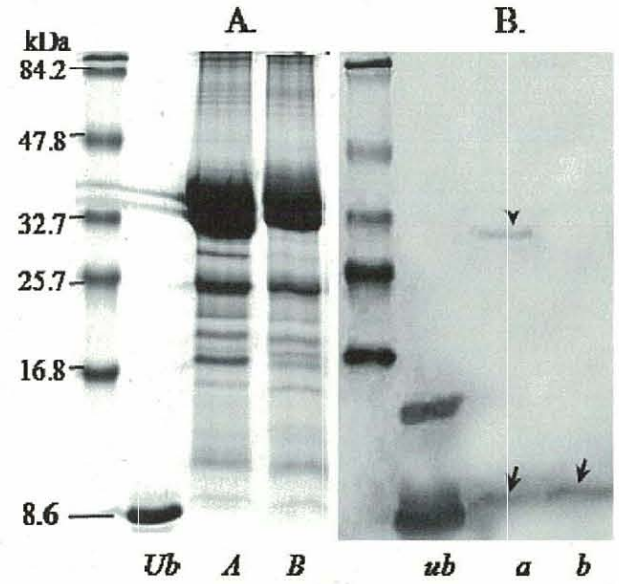

Fig. 1. Representative SDS-PAGE (A) and western blot results (B) of sarcoplasmic proteins. Protein (30 μg) was loaded into each lane. Lanes Ub and ub, purified commercially available ubiquitin used as the positive controls; *A* and *a*, Ub-I1 is present; and *B* and *b*, Ub-I1 is absent. Arrow head, Ub-I1; arrows, free ubiquitin present in the samples. Molecular weight markers are indicated on the left.

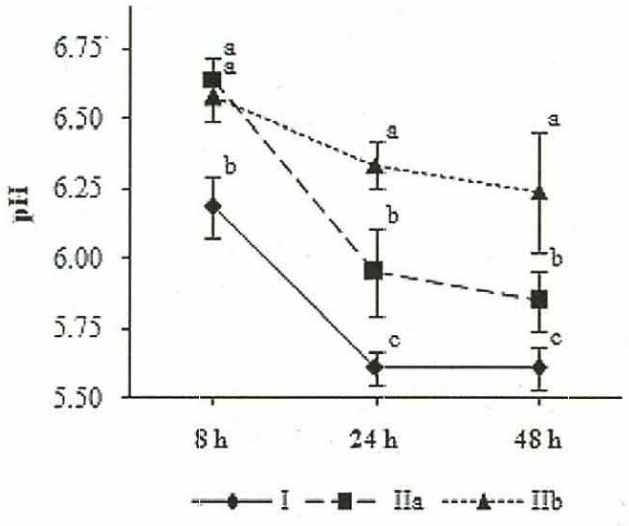

Fig. 2. Patterns of pH decline for I, IIa and IIb meat sample groups. Data are presented as mean ± S.E. and the different superscript letters at each pH measurement indicate significant differences ($P<0.01$) in pH between meat sample groups.

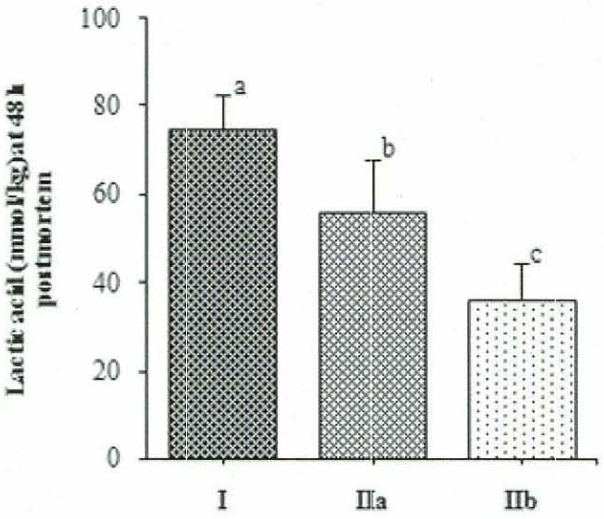

Fig. 3. Lactic acid concentration in each meat sample group. Data are presented as mean ± S.E. Different superscript letters indicate significant differences ($P< 0.01$).

distinct sample groups: those lose lacking Ub-I1 (I, *n=5*) and those containing Ub-I1 (II, *n=27*). The pattern of pH decline was notably different between these two groups, with a wide range of variation observed particularly in group II at 24 and 48 h postmortem. Therefore, this group was further divided (Fig. 2) into group IIa which showed lower or normal pH <6 (*n=20*) and group IIb which showed higher or dark, firm, dry (DFD) meat pH >6 (*n=7*) (Dransfield, 1981).

The concentration of lactic acid (LA) was 74.82 mmol/kg in group I, 55.95 mmol/kg in group IIa and 36.12 mmol/kg in group IIb at 48 h postmortem (Fig. 3). Comparison of pH values at 48 h and the corresponding LA content indicated a linear pH dependence on LA concentration, as demonstrated previously (Immonen and Puolanne, 2000a). Accelerated postmortem glycolysis results in rapid accumulation of lactate and hydrogen ions in muscle that has become pale, soft and exudative (PSE) (Allison *et al.*, 2003). The mechanisms associated with such abnormal glycolysis in postmortem muscle and the incidence of PSE meat are largely unclear (Shen and Du, 2005). Group I meat samples lacking Ub-I1 showed relatively rapid postmortem glycolysis with pH 6.2 at 8 h postmortem compared to pH 6.54 and 6.61 of groups IIa and IIb meat samples, respectively. Furthermore, pH reached a minimum at 24 h postmortem for group I samples while further decline was observed until 48 h postmortem for groups IIa and IIb samples. These results suggest that in the absence of Ub-I1, muscle tissue tends to develop PSE characteristics. Although, Ub-I1 was present in both IIa and IIb meat samples the rate and extent of postmortem glycolysis were different between groups. Variation in pH depends mainly on muscle type and level of pre-slaughter stress, which can affect the content of available glucose (Puolanne *et al.*, 2002). The amount of LA needed to produce a certain pH is relatively constant, and therefore the pre-slaughter concentration range of glycogen reflects its residual concentration (Immonen *et al.*, 2000d). The amount of residual glycogen at

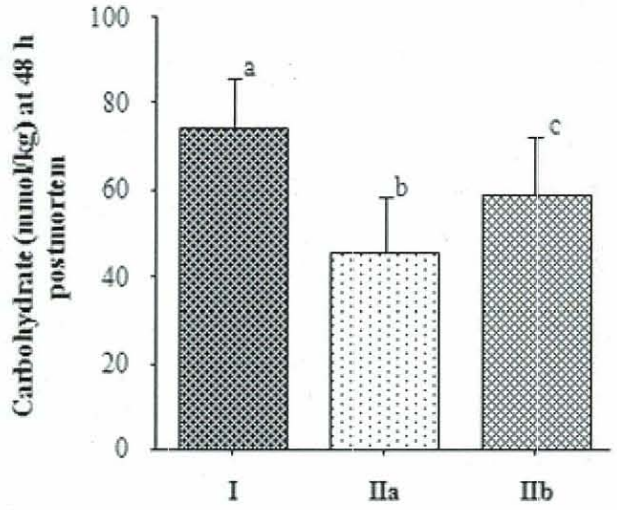

Fig. 4. Carbohydrate concentration in each meat sample group at 48 h postmortem. Data are presented as mean ± S.E. Different superscript letters indicate significant differences (P <0.01).

48 h postmortem differed significantly among the groups (Fig. 4), with the largest amount found in group I samples (74.89 mmol/kg). Moreover, the glycogen amount was higher in IIb samples (59.37 mmol/kg) than in IIa samples (45.7 mmol/kg), suggesting that glycolysis in the former samples did not occur successfully, despite sufficient glycogen. In the study of Immonen and Puolanne (2000b), bovine muscle residual glycogen concentration varied from 10 to >80 mmol/kg at lower pH around 5.75. Moreover, several samples has had residual glycogen concentrations at higher pH >6, which is sufficient for decreasing pH, similar to our results.

Since concentrations of LA and residual carbohydrate at 48 h postmortem varied among the sample groups, we calculated the corresponding glycolytic potential (GP) of each group based on LA and residual glucose concentrations to predict the total carbohydrate amount. Group I meat samples tended the have high GP (224.47 mmol/kg), which was significantly different from meat samples of groups IIa and IIb (P<0.001); however, no significant difference in GP was found between the IIa and IIb meat samples. Table 1 compares the absence/presence of U-I1 relative to variations in corresponding GP with pH values; the corresponding GP and pH values are summarized by group.

However, it is unknown whether the presence of Ub-I1 in muscle is linked to its physiological function and targeted ubiquitination. The group 1 meat samples lacking Ub-I1 had high GP and resulted in relatively rapid glycolysis. To a certain extent, the absence of Ub-I1 in conjunction with higher GP may indicate the alteration of muscle carbohydrate metabolism at the time of slaughter. In the case of pigs, it is generally accepted that if GP is high (>180-200 mmol/kg), the animals considered a Rendement Napole gene carrier and develops PSE pork (Monin and Seller, 1985; Fernandez *et al.*, 1992). Aalhus *et al.* (1998) reported that PSE in beef is less common than in pork, probably due to the relatively low proportion of fast, glycolytic fibers in beef compared to that in pork. In contrast, Ub-I1 was detected in the majority of beef samples (*n=27*) in the present study, suggesting that the presence or formation of this conjugate in muscle is common in meat-producing animals at the time of slaughter. Furthermore, IIa meat samples show typical characteristics of postmortem glycolysis compared to IIb meat samples, showing DFD characteristics; however, both groups have similar GP. The abundance of Ub-I1, as determined densitometrically from the western blot, was about 1.5- to 3-folds greater in IIb meat than in IIa meat. Thus, the extent of postmortem glycolysis may relate to the abundance of Ub-I1 in the meat.

Few studies have reported on the presence or formation of Ub-I1 in muscle despite concerns related to meat quality.

Table 1. Meat categorization and relationship between Ub-I1 and muscle glycolytic potential.

Group	Ub-I1	pH range	Glycolytic potential (mmol lactate eq. per kg)	
			Mean	Range
I	Absent	5.56-5.71	224.61*	204.23-248.49
IIa	Present	5.62-5.98	147.61	98.58-182.02
IIb	Present	6.00-6.62	154.14	109.62-181.60

*Significantly different, compared to other meat groups.

In rat muscle, ubiquitin conjugates were reportedly increased 50-250% after food deprivation (Wing *et al.*, 1995), but the precise substrates were poorly characterized (Taillandier *et al.*, 2004). Immonen *et al.* (2000c) found that beef bulls lost 11-33 mmol/kg glycogen during processing from the farm to slaughter depending on the diet and season. As I1, an inducer of glyconeolysis/glycolsis, is an important regulator of glycogen metabolism, ubiquitination in muscles of animals exposed to stress during the farm-to-slaughter process may restrict the function of I1 (Ub-I1 formation), preventing intensive glycogen degradation. To reveal the underlying mechanisms associated with Ub-I1 formation and its effect on postmortem glycolysis further studies such as determination of the ratio of I1 and Ub-I1 are needed.

Conclusions

The absence or presence of Ub-I1 may relate to the status of muscle carbohydrate metabolism and subsequently glycolysis of postmortem muscle. Thus, understanding the significance of Ub-I1 in muscle may provide additional knowledge in the field of meat science.

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