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Thiamine accumulation and thiamine triphosphate decline occur in parallel with ATP exhaustion during postmortem aging of pork muscles

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ABSTRACT

We aimed to clarify the mechanisms affecting postmortem thiamine and its phosphoester contents in major edible pork muscles, namely the *longissimus lumborum* (LL) in addition to *vastus intermedius* (VI). Metabolomic analysis by capillary electrophoresis-time of flight mass spectrometry revealed that the level of thiamine triphosphate (ThTP), approximately 1.8-fold higher in LL than in VI muscle at 0 h postmortem, declined in the first 24 hrs, resulting in an undetectable level at 168 h postmortem in both muscles. In contrast, the thiamine content in both muscles increased after 24 h postmortem during the aging process. The thiamine accumulation and ThTP decline progressed in parallel with a drastic reduction of the ATP level. The intermuscular differences in pH at 24 h and in expression of thiamine transporter and thiamine pyrophosphokinase might result in delayed thiamine generation in LL. These results suggest that postmortem ATP exhaustion forced ThTP hydrolysis and further depyrophosphorylation of thiamine diphosphate in the porcine muscles, which resulted in thiamine accumulation.

1. Introduction

Thiamine is an essential vitamin in human nutrition, and it is converted to several phosphoester forms in various tissues and organs after being absorbed in animals. Of the phosphoesters, thiamine diphosphate (ThDP, or thiamine pyrophosphate) is the most important derivative due to its role as the active cofactor of a number of enzymes involved in carbohydrate and energy metabolism, such as the pyruvate dehydrogenase complex (Manzetti, Zhang, & van der Spoel, 2014). Despite the substantial coenzyme activity of ThDP, thiamine and its phosphoesters are primarily absorbed in the form of inactive thiamine cations in the intestine (Manzetti et al., 2014; Tallaksen, Sande, Bohmer, Bell, & Karlsen, 1993). After thiamine is taken up by intestinal epithelial cells, it is transferred to the blood in a manner dependent on intestinal thiamine concentration (Hayashi, Yoshida, & Kawasaki, 1981; Said et al., 1999). The dietary thiamine level has been shown to affect the ThDP level and ThDP-dependent enzyme activity in the rat liver (Blair et al., 1999).

Thiamine and its derivatives have been reported to be differently distributed between rat tissues and organs, and skeletal muscle is especially enriched with total thiamine and ThDP (Matsuda, Tonomura, Baba, & Iwata, 1989). It has been noted that porcine skeletal muscle is the tissue most enriched with thiamine derivatives, especially thiamine triphosphate (ThTP), among the animals investigated (Egi, Koyama, Shikata, Yamada, & Kawasaki, 1986; Makarchikov et al., 2003; Poel, Backermann, & Ternes, 2009), and it contains 10 times more total thiamine than beef and chicken (Souci, Fachmann, & Kraut, 2000). The contents of ThTP, ThDP, thiamine monophosphate (ThMP), and thiamine are 28.1–82.8, 22.8–35.3, 2.1–3.6, and 0.9–2.2 nmol/g of skeletal muscle tissue in pigs, respectively (Egi et al., 1986). As the ThTP and total thiamine contents in porcine skeletal muscles are the highest among animal resources, pork is one of the most important sources of vitamin B1 for human nutrition in addition to unrefined cereal grains. Thus, changes in thiamine and its phosphate derivatives during the postmortem aging in porcine skeletal muscles could determine the bioavailability of vitamin B1 in the pork and thus the subsequent pork nutritional value. The abundance of thiamine and its derivatives, however, is not only altered during postmortem aging in the porcine diaphragm (Poel et al., 2009), but is also differently distributed among muscle types in the rat, mouse, rabbit, and pig (Egi et al., 1986; Matsuda et al., 1989). Although thiamine content in pork muscles including *longissimus* has reported (Leonhardt & Wenk, 1997), the

Abbreviations: AK1, adenylate kinase isoform 1; CE-TOFMS, capillary electrophoresis-time of flight mass spectrometry; LL, *longissimus lumborum*; MyHC, myosin heavy chain; qPCR, quantitative PCR; RPL7, Ribosomal protein L7 (official gene symbol); ThDP, thiamine diphosphate; ThDPase, thiamine diphosphatase (enzyme); ThMP, thiamine monophosphate; THTPA, thiamine triphosphatase (official gene symbol); ThTPase, thiamine triphosphatase (enzyme); THTR1, thiamine transporter 1 (official gene symbol); THTR2, thiamine transporter 2 (official gene symbol); TPK1, thiamine pyrophosphokinase 1 (official gene symbol); ThTP, thiamine triphosphate; VI, *vastus intermedius*

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mechanism of absorbable thiamine accumulation in such major edible pork has not been elucidated so far.

The conversions between thiamine and its phosphate derivatives, namely ThDP, ThMP, and ThTP, are poorly unveiled in animal tissues including skeletal muscles. Nevertheless, thiamine pyrophosphokinase 1 (TPK1; EC: 2.7.6.2) and thiamine triphosphatase (THTPA; EC: 3.6.1.28) are known to catalyze thiamine generation (ThDP + AMP ↔ thiamine + ATP) and ThTP hydrolysis (ThTP → ThDP + Pi) in mammals, respectively (Bettendorff & Wins, 2009; Gangolf et al., 2010). In addition, the phosphorylation of ThDP to generate ThTP is catalyzed mainly by adenylate kinase isoform 1 (AK1, also called myokinase; EC: 2.7.4.3) in skeletal muscle with the reaction: ThDP + ADP ↔ ThTP + AMP (Miyoshi, Egi, Shioda, & Kawasaki, 1990; Shikata, Koyama, Egi, Yamada, & Kawasaki, 1989). ThTP synthesis by AK is coupled with the reaction by AK: ATP + AMP ↔ 2ADP, which will be combined as ThDP + ATP ↔ ThTP + ADP (Bettendorff, Lakaye, Kohn, & Wins, 2014). In early stages of postmortem period, generally global ATP exhaustion is progressing in muscle. Consequently, ATP exhaustion drives the coupled reaction ThTP + ADP ↔ ThDP + ATP to generate ATP in skeletal muscle. In addition, ThDP can be hydrolyzed into thiamine by TPK1 (ThDP + AMP ↔ thiamine + ATP). Given that ATP is exhausted in around half a day or less postmortem in the skeletal muscle of farm animals under a low temperature storage condition (Busch, P., & Goll, 1967; Muroya, Oe, Nakajima, Ojima, & Chikuni, 2014), we hypothesized that ThTP hydrolysis and thiamine generation progress toward ATP generation in response to the ATP exhaustion in postmortem porcine muscles.

To this end, we aimed to investigate the accumulation of thiamine and its phosphate derivatives in porcine *longissimus*, a major edible pork loin muscle. It is also possible that the postmortem changes in thiamine-related compounds are affected by muscle type that is determined by composition of fast and slow myofiber types, as the other metabolites differently change postmortem between different type muscles (Muroya et al., 2014). We determined the differences in the changes in the thiamine-related compounds between the *longissimus lumborum* (LL) and *vastus intermedius* (VI) muscles during postmortem aging to clarify the effect of muscle type on the accumulation of those thiamine-related compounds. The involvement of TPK1 and thiamine transporters in the accumulation of pork thiamine and its derivatives is also examined and discussed in the present study.

2. Material and methods

2.1. Animals and muscle samples

The animals were cared for as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee of the NARO Institute of Livestock and Grassland Science), and this committee approved the study. All efforts were made to minimize suffering. Three female pigs of Landrace × Large White × Duroc breed aged 4.5 months (90–100 kg) were bled and slaughtered. The muscle samples for capillary electrophoresis-time of flight mass spectrometry (CE-TOFMS) and quantitative PCR (qPCR) analyses were excised immediately after slaughter for the time 0. During storage of the carcasses at 2 °C, the CE-TOFMS samples of time 4, 24, and 168 h postmortem were further taken from each hanging carcass. For all samples, approximately 1 cm^3 cubes of LL and VI muscles were excised from the carcasses, frozen in liquid nitrogen, and then stored at − 80 °C until use. Profiles regarding postmortem changes in ATP concentration of these pork samples are shown in Table 1 (Muroya et al., 2014). Muscle pH was measured according to the method described previously (Muroya et al., 2014).

2.2. Total RNA preparation and cDNA synthesis

The total muscle RNAs for qPCR analysis were prepared using ISOGEN (Nippon Gene, Tokyo, Japan). The cDNAs from muscle samples were each synthesized from 1 μg of total RNA using the ReverTra Ace qPCR RT kit (Toyobo, Tokyo, Japan). These processes were conducted according to the manufacturers' protocols.

Table 1
Profiles of postmortem changes in ATP content in the porcine LL and VI muscles.

Postmortem time (h)	LL		VI		*P*-value
	Mean	SEM	Mean	SEM	
0	6.892^{a}	0.082	4.417^{a}	0.352	< 0.05
4	5.278^{a}	1.220	0.449^{b}	0.259	< 0.10
24	0.107^{b}	0.063	0.015^{b}	0.002	ns
168	0.008^{b}	0.001	0.022^{b}	0.006	ns

ATP content: μmol/g of muscle tissue. The values were determined previously (Muroya et al., 2014). Means with different superscript (^{a,b}) within a column differ at $P < 0.05$. ns: not significant.

2.3. qPCR analyses of myosin heavy chain (MyHC) isoform and genes related to thiamine phosphorylation and dephosphorylation

qPCR was conducted to analyze the expression of MyHC isoforms and genes related to thiamine and its phosphoester conversion, namely AK1, TPK1, and THTPA. The gene expression of thiamine transporter 1 (THTR1) was also analyzed by qPCR. The reaction was performed using a CFX96 thermal cycler (Bio-Rad, Hercules, CA, USA) under the following program: 15 min of enzyme activation at 95 °C, followed by 45 cycles of 15 s at 94 °C, 30 s at 55 °C (THTPA), 60 °C (THTR1), or 57 °C (the other genes), and 30 s at 72 °C, using the QuantiTect SYBR Green PCR kit (Qiagen, Tokyo, Japan). For the PCR analysis of MyHC isoforms, we designed four sense primers named pMYO305, pMYO207, pMYO107, and pMYO403, which are specific to MyHC-2b, -2x, -2a, and -slow type isoforms, respectively (Table 2), based on a previous study (Tanabe, Muroya, & Chikuni, 1999). These primers were used with a single common antisense primer pMYOn2, whose sequence was based on that of porcine MyHC-2a. THTPA primers were designed by the other researchers (Szyniarowski et al., 2005). The sequence, product size, and accession information of all the primers are shown in Table 2. Ribosomal protein L7 (RPL7) was used as an internal control for the expression of the genes tested in this study (Muroya et al., 2013).

2.4. Sample preparation for CE-TOFMS

The frozen muscle tissue was immediately plunged into methanol containing 50 μM Internal Standard Solution 1 (Solution ID: H33041002, Human Metabolome Technologies, Tsuruoka, Japan) at 0 °C and was homogenized 3 times at 1500 rpm for 120 s to inactivate enzymes. Then, Milli-Q water (Millipore, Billerica, MA) and chloroform were added at a ratio of 2:5 to the samples, thoroughly mixed, and then centrifuged for 4 min at 2300 × *g* and 4 °C. The upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove proteins. The filtrate was lyophilized and suspended in Milli-Q water and analyzed by CE-TOFMS.

2.5. Instrumentation and conditions of CE-TOFMS

CE-TOFMS was carried out using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time of Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). The system was controlled by Agilent G2201AA ChemStation software version B.03.01 for CE (Agilent Technologies, Waldbronn, Germany). The analytic conditions were the same as those used in a previous study (Muroya et al., 2014). Cationic and anionic metabolites were analyzed with a fused silica capillary