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Activity and expression of nitric oxide synthase in pork skeletal muscles

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ABSTRACT

The objective of this study was to investigate the biochemical changes of nitric oxide synthase (NOS) in pork skeletal muscles during postmortem storage. Longissimus thoracis (LT), psoas major (PM) and semimembranosus (SM) muscles of pork were removed immediately after slaughter and stored under vacuum condition at 4 °C for 0, 1 and 3 d. Results showed that all three muscles exhibited NOS activity until 1 d while SM muscle retained NOS activity after 3 d of storage. The content of nNOS in SM muscle was stable across 3 d of storage while decreased intensity of nNOS was detected at 1 and 3 d of aging in PM and LT muscles due to the degradation of calpain. Immunostaining showed that nNOS was located at not only sarcolemma but also cytoplasm at 0 and 1 d of storage. Our data suggest that postmortem muscles possess NOS activity and nNOS expression depends on muscle type.

1. Introduction

Nitric oxide (NO) is an endogenous molecule involved in diverse developmental and physiological processes (Pacher, Beckman, & Liaudet, 2007). In skeletal muscles, NO could regulate muscle contractility, energy metabolism, calcium homeostasis, and protein proteolysis (Stamler & Meissner, 2001). NO is generated during the conversion of L-arginine to L-citrulline in skeletal muscle catalyzed by nitric oxide synthase (NOS). There are three isoforms of NOSs in skeletal muscle including the calcium dependent neuronal NOS (nNOS) and endothelial NOS (eNOS), and the calcium independent inducible NOS (iNOS). Among three isoforms, the nNOS is thought to be the predominant isoform in skeletal muscle (Kaminski & Andrade, 2001). Activity of skeletal muscle is closely related to nNOS expression, and mislocalization of nNOS has been reported in muscular dystrophies, cachexia/atrophic syndromes and heart failure (Stamler, Sun, & Hess, 2008). nNOS is generally located at cell membrane of skeletal muscles and its expression depends on muscle types and species (Kobzik, Reid, Bredt, & Stamler, 1994).

During last decades, few studies have been conducted to investigate the potential role of NO in the regulation of fresh meat quality during postmortem aging. Cook, Scott, and Devine (1998) reported that the injection of NO enhancers and NO inhibitor in beef longissimus lumborum at 2 h postmortem could influence the tenderization of beef during aging over 2–6 d. Cottrell, McDonagh, Dunshea, and Warner (2008) reported that preslaughter infusion of NOS inhibitor in ovine muscle could result in improved tenderness in longissimus thoracis et lumborum (LTL) muscle. In the study of Zhang, Marwan, Samaraweera, Lee, and Ahn (2013), the degree of lipid oxidation and protein oxidation could be affected by managing the levels of NO in chicken breast muscle at d 1 and 4 of refrigerated storage.

Since NO is produced by catalyzing NOS in skeletal muscle, studies on the biochemical changes of NOS in postmortem muscle are necessary to further understand how the NO and NO induced nitrosylation could be involved in meat quality during postmortem aging. Therefore, the objective of this study was to investigate the NOS activity, the expression and the localization of nNOS in porcine skeletal muscles during postmortem storage.

2. Materials and methods

2.1. Sample preparation and storage

Six longissimus thoracis (LT), psoas major (PM) and semimembranosus (SM) muscles were removed from six-month-old crossbred pigs (100 ± 10 kg) within 45 min postmortem at a commercial meat processing company (Sushi Meat Co. Ltd., Huai'an, China). Samples were excised immediately and frozen in liquid nitrogen, and then stored at −80 °C for 0 d analysis. Six samples of LT, PM and SM were vacuum-packaged and aged for 1, 3 or 7 d. At each time point, NOS activity, protein expression and localization were detected. To determine whether nNOS was degraded by calpain, six LT muscles post-slaughter within 45 min were rapidly removed and dissected into small pieces (0.2 g/piece). These samples were randomly assigned to one of two treatments: calpain inhibitor solution containing 100 μM calpain inhibitor (MDL 28170,

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Sigma-Aldrich Corporation, St. Louis, MO, USA), 100 mM NaCl and 2 mM NaN_3 or control solution (same with calpain inhibitor solution without MDL 28170 reagent) in the ratio 1:1 (w/v) (meat:solution). The samples were stored at 4 °C for 1, 3 and 7 d to investigate the degradation of nNOS at different aging times.

2.2. NOS activity

Total NOS activity was assessed on extracts from the skeletal muscle using a NOS detection assay kit (Nanjing Jiancheng Bioengineering Inc., Nanjing, China). The protein extraction was prepared according to Brannan and Decker (2002) with a slight modification. One gram of well mined meat samples was homogenized twice at a speed of 1900 g for 15 s in 5 volumes (w/v) of homogenization buffer (25 mM Tris–HCl, 1 mM EDTA and 1 mM EGTA, pH 7.4). The homogenate was centrifuged at 20,000 *g* for 15 min at 4 °C. The supernatant was collected and used for the determination of the NOS activity. The protein concentration was determined using the method described by Lowry, Rosebrough, Farr, and Randall (1951) and the total NOS activity was expressed as nmoles per min per milligram protein.

2.3. Muscle fiber type

Differences in muscle fiber type were evaluated in 0 d samples from three skeletal muscles by the separation of myosin heavy chain (MHC) isoforms on high porosity SDS-PAGE gels according to Lonergan, Huff-Lonergan, Rowe, Kuhlers, and Jungst (2001). Half gram finely minced skeletal muscle was homogenized in 9 volumes of ice-cold homogenization buffer (250 mM sucrose, 100 mM KCl, 5 mM EDTA, and 20 mM Tris, pH 6.8) followed by centrifugation at 1500 *g* for 15 min at 4 °C. The pellets were then homogenized in 25 volumes of whole muscle extraction buffer (2% sodium dodecyl sulfate (SDS) (wt./vol.), 10 mM sodium phosphate, pH 7.0) and then centrifuged at 1500 *g* for 15 min at 20 °C. Protein concentration was determined with BCA Protein Assay Kit (Pierce, IL, USA). Protein concentration was adjusted to 0.256 mg/ml diluted with deionized water. One volume of the diluted samples was added to one volume of tracking dye solution (50% [vol/vol] glycerol, 2% [wt./vol.] SDS, 0.1% [wt./vol.] bromophenol blue, and 60 mM Tris–HCl, pH 6.8) and 0.1 volume β-mercaptoethanol. Spontaneously, the final concentration of those samples was 0.125 mg/ml. Samples were heated in 95 °C water bath for 5 min and then stored at −80 °C refrigerator for subsequent analysis.

Two micrograms of total protein was loaded on each lane of 15 cm × 18 cm × 1.5 cm SDS-PAGE gels. The stacking gels were composed of 30% glycerol, 4% acrylamide-N,N′-methylene-bis-acrylamide (acrylamide-bis) (50:1), 200 mM Tris–HCl (pH 6.7), 4 mM EDTA, and 0.4% SDS. The separating gels were composed of 30% glycerol, 6% acrylamide-bis (50:1), 200 mM Tris–HCL (pH 8.8), 100 mM glycine, and 0.4% SDS. Polymerization of stacking and separating gels was initiated with 0.05% N,N,N′,N′-tetramethylethylenediamine (TEMED) and 0.1% ammonium persulfate (APS). Separate upper and lower running buffers were used. The upper running buffer consisted of 200 mM Tris, 300 mM glycine, 0.1% β-mercaptoethanol and 0.2% SDS. The lower running buffer consisted of 100 mM Tris, 150 mM glycine, 0.1% β-mercaptoethanol and 0.1% SDS. The gels were run at 125 V on Hoefer SE 600 Ruby (Hoefer Scientific Instruments, SF, USA) for approximately 66 h and then the gels were stained with Coomassie brilliant blue R-250. The gels were scanned with a scanner (GT-800 F, Epson, Nagano, Japan) at a resolution of 600 dpi, and then the densities of bands were quantified by Quality One software (Version 4.6, Bio-Rad, CA, USA). The relative value for each MHC isoform (type I or type IIA/X or type IIB) was calculated as the ratio that the density of each isoform over the total density of MHC isoforms in each lane.

2.4. Western blots

Samples from skeletal muscles were homogenized in ice cold buffer (50 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA and 5 mM dithiothreitol (DTT), pH 7.4) and centrifuged at 20,000 *g* for 15 min at 4 °C. Protein concentration of supernatant was assessed by BCA protein assay kit and adjusted to 4 mg/ml. One volume of diluted solutions was combined with one volume loading buffer (10 mM Tris–HCl, 2.5% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue, pH 6.8). Before they were stored at −80 °C, the combined solutions were heated at 95 °C for 5 min. Forty microgram proteins were loaded per lane and separated by 7.5% gel of SDS-PAGE. Gels were electrophoresed using a Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, CA, USA) setting at 150 V for 75 min at 4 °C. For Western blot analysis, the proteins were blotted by electrodiffusion for 1.5 h at 380 mA on nitrocellulose membranes (Bio-Rad Laboratories, CA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS: 20 mM Tris, 137 mM NaCl and 5 mM KCl) with 0.05% Tween 20 (TBS-T) for 1.5 h and incubated with primary antibody to nNOS (a rabbit polyclonal antibody, sc-8309, Santa Cruz Biotechnology, CA, USA) overnight at

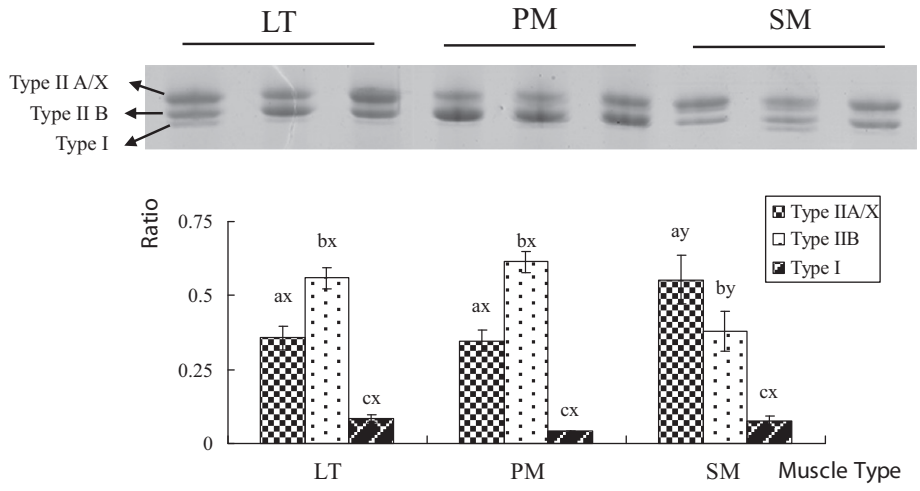

Fig. 1. Electrophoretic separation of MHC isoforms in three porcine skeletal muscles. The top bands in the each lane were the MHC type IIA/X isoform, the intermediate bands were the MHC type IIB and the bottom bands were MHC type I isoform. Percentage of each isoform was calculated as the intensity of band over whole intensity of three bands together with the densitometric analysis, mean ± SE, n = 6. "a–c": means of proportion of each fiber type in the same muscle with different letters are significantly different at $P < 0.05$; "x–y": means of proportion in each muscle of same fiber type with different letters are significantly different at $P < 0.05$.