NITRIC OXIDE SYNTHASE AND PROTEIN NITROSYLATION IN PORK DURING POSTMORTEM AGING

Rui Liu, Guanghong Zhou and Wangang Zhang*

College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

Abstract – This study was to investigate the changes of nitric oxide synthase (NOS) and nitrosylated proteins in pork skeletal muscles during postmortem aging. Longissimus thoracis (LT), psoas major (PM) and semimembranosus (SM) muscles of pork were removed immediately after slaughter and stored under vacuum condition at 4 ℃ for 0, 1 and 3 d. Results showed that NOS activity and content of nNOS (neuronal NOS) for LT and PM muscles gradually reduced from aging of 0 to 1 d and then almost disappeared at 3 d of postmortem. However, NOS activity and nNOS expression were not significantly different among 3 d of storage in SM muscle. Immunostaining showed that nNOS was located at not only sarcolemma but also cytoplasm at 0 and 1 d of storage in LT muscle. Proteomics revealed that 155 S-nitrosylation (SNO)-modified cysteine sites from 102 proteins were identified from pork LT muscle with different SNO levels between aging of 0 and 3 d. Our data suggest that NOS activity and nNOS expression depends on muscle type and protein nitrosylation could occur in postmortem pork.

Key Words – Activity and expression; Longissimus thoracis; Proteomics

I. INTRODUCTION

S-nitrosylation, the coupling of a nitric oxide (NO) moiety to a reactive cysteine thiol to form an S-nitrosothoil (SNO), has received considerable interest to modulate a broad spectrum of cellular signaling pathways by regulating enzyme activity and protein-protein interaction[1]. NO is produced by the reaction of L-arginine and oxygen to form L-citrulline catalyzed by nitric oxide synthase (NOS). Among NOSs, nNOS is the predominant isoform in skeletal muscle[2]. During last decades, few studies have been conducted to investigate the potential role of NO in regulation of fresh meat quality. Cook et al.[3] reported that the injection of NO enhancer and NOS inhibitor in beef longissimus lumbornm at 2 h postmortem could influence the tenderization of beef during 6 days of aging. Cottrell et al.[4] showed that preslaughter infusion of NOS inhibitor in ovine could improve tenderness in longissimus thoracis et lumborum (LTL) muscle. In the study of Zhang et al.[5], chicken breast meat from NO enhancer group showed the lowest water-holding capacity during refrigerated storage. In recent study, Cottrell et al.[6] found that the injection of NOS inhibitor preslaughter inhibited protein proteolysis and reduced tenderness in SM muscle while no significant difference was observed in LTL.

Reports about the effects of NOS inhibitor and NO enhancer on meat quality in different muscles are not consistent. Therefore, studies on the NOS in postmortem muscle are necessary to further understand the possible involvement of NO and NO induced nitrosylation in regulating meat quality during postmortem aging. Therefore, the objective of this study was to investigate the NOS activity, the expression and localization of nNOS and nitrosylated proteins in porcine skeletal muscles during postmortem storage.

II. MATERIALS AND METHODS

Six crossbred pigs (100±10 kg) were selected to remove longissimus thoracis (LT), psoas major (PM) and semimembranosus (SM) muscles within 45 min postmortem (Sushi, Huaian, China). Samples were randomly divided into three sections and vacuum-packaged for aging 0, 1 and 3 d. At each time point, NOS activity, protein expression and localization of nNOS were detected. LT samples at 0 and 3 d were selected to detect the nitrosylated proteins.

NOS activity of protein extraction was measured according to Brannan et al.[7] with minor modification. Meat samples were homogenized twice in homogenization buffer (25 mM Tris-HCl, 1 mM EDTA and 1 mM EGTA, pH 7.4). After centrifugation at 20,000 g for 15 min, supernatant was collected for protein concentration and NOS activity detection using BCA protein assay kit and

NOS detection assay kit (Nanjing Jiancheng Bioengineering, Nanjing, China), respectively. Protein concentration was 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 stored at -80 ℃, the combined solutions were heated at 95 ℃ for 5 min. Western-blot was employed to semi-quantify the content of nNOS. The localization of nNOS in LT muscle was assayed by SABC-Cy3 immumohistochemical staining kit (Boster Biological Engineering, Wuhan, China). Quantitative site-specific reactivity profiling of S-nitrosylation in pork skeletal muscle using cysteinyl peptide enrichment coupled with mass spectrometry was performed[8].

Statistical analysis used SAS statistical software (version 9.1.3). A Mixed Model was used to evaluate the differences ($P < 0.05$) between muscle type (MT), postmortem aging (PA) and muscle type × postmortem aging (MT × PA) which chose pigs as a random factor. Least-squares mean differences were assessed by the Bonferroni t-Test.

III. RESULTS AND DISCUSSION

NOS activity of three pork skeletal muscles during postmortem aging was presented in Table 1. LT and PM muscles had relatively less NOS activity than SM muscle at d 0 and 1 postmortem. NOS activity of SM muscle was not significantly different among 3 d of storage ($P > 0.05$). However, LT and PM muscle showed decreased NOS activity at d 1 compared to d 0. In consistent with a previous study[7], NOS activity could not be detected after 3 d of aging in LT and PM muscles.

In current study, nNOS appeared as a doublet of approximately 160 KDa and 140 KDa in three skeletal muscles (Fig. 1). Molecular weight of 160 kDa was chosen for semi-quantification by the software of Quality one (Table. 1). The relative value of nNOS at d 1 in LT and PM muscles were significantly decreased compared to d 0 ($P < 0.05$). However, the content of nNOS in SM muscle had no significant changes during 3 d of postmortem aging ($P > 0.05$). The nNOS intensity of SM muscle was significantly higher than that of LT and PM muscles after 1 and 3 d of aging ($P < 0.05$). The amount of 160 kDa nNOS seemed to have the parallel trend of NOS activity in three skeletal muscles during postmortem aging.

Table 1 Nitric Oxide synthase (NOS) activity (nmoles per milligram protein per min) and expression of nNOS of three skeletal muscles at d 0, 1 and 3 of postmortem aging

PA	MT	NOS Activity	Relative value of nNOS
0	SM	0.819^{d}	0.813^{d}
	PM	0.287^{b}	0.655^{c}
	LT	0.379^{c}	0.679^{c}
1	SM	0.612^{d}	0.772^{cd}
	PM	0.209^{a}	0.290^{b}
	LT	0.320^{b}	0.504^{b}
3	SM	0.602^{d}	0.645^{cd}
	PM	ND	0.062^{a}
	LT	ND	0.080^{a}
SED		0.035	0.060
Significance	PA	0.007	<0.001
	MT	<0.001	<0.001
	PA*MT	0.001	0.008

"ND": Not detected;
"a-d": mean with different letters were significantly different at $P < 0.05$ (n=6);
"PA": postmortem aging; "MT": muscle type

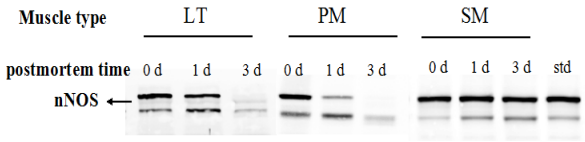

Figure 1. Representative graph of western-blot of nNOS in three skeletal muscles during porcine postmortem aging.

Immunostaining of nNOS in LT muscle was presented in Fig. 2. As shown in Fig. 2A, abundant nNOS was detected at surface membrane of muscle cell at 0 d postmortem, while lower fluorescence intensity was detected at d 1 of aging (Fig. 2B). At d 3, samples hardly showed fluorescence spot and muscle cells seem to break into smaller pieces to form cracks between cells (Fig. 2C). Those results were in consistent with the NOS activity and the content of nNOS in current study. The cytoplasm localization of pork skeletal muscle was also detected in 0 and 1 d samples indicating the potential interaction between NO and protein via s-nitrosylation.

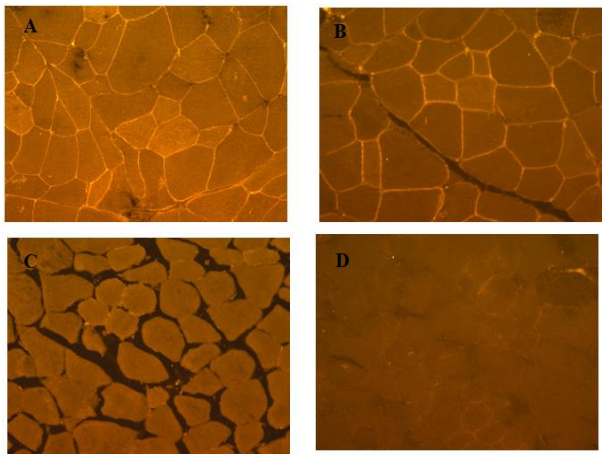

Figure 2. Immunofluorescent staining for nNOS in longissimus thoracis muscle during porcine postmortem aging of 0 d (A), 1 d (B), 3 d (C), and D was negative control. Magnification 200X.

LT samples at 0 and 3 d were chosen for quantitative site-specific reactivity profiling of S-nitrosylation. A total of 155 SNO-modified cysteine sites from 102 proteins were identified. Proteins with different SNO levels between the aging of 0 and 3 d implied that SNO could be generated or transferred between proteins. PANTHER was employed to analyze Gene ontology of cellular component (Fig. 3A) and molecular function (Fig. 3B). The identified nitrosylated proteins spread over membrane, macromolecular complex, extracellular matrix, cell part, organelle and extracellular region. These results indicate that the diversity of SNO-sensitive sites throughout muscle cell could play critical roles in message transferring between muscle cells.

Fig. 3B illustrates the molecular function of nitrosylated proteins. Among nitrosylated proteins, enzymes with catalytic activity possessed highest proportion of 44.60%. Those enzymes may be involved in energy metabolism and protein degradation which play important roles in regulating meat quality. Further study should focus on managing the SNO level of some particular proteins to evaluate the biochemistry changes of postmortem muscles.

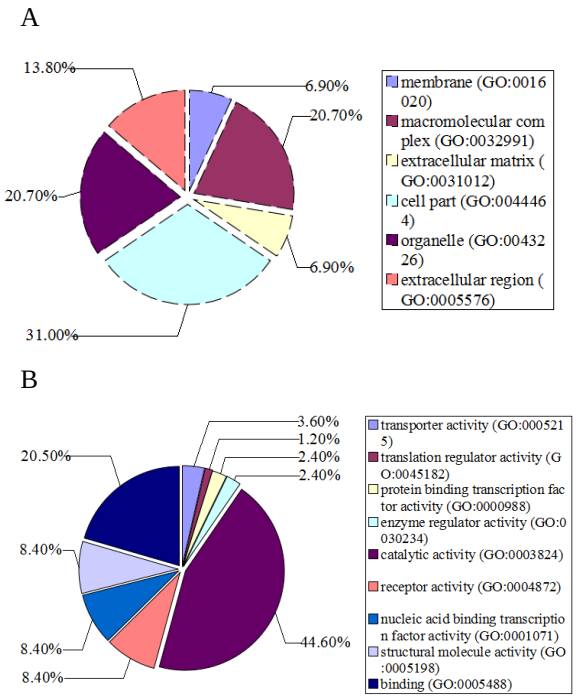

Figure 3. Cellular component (A) and molecular function (B) of identified nitrosylated proteins.

IV. CONCLUSION

NOS activity and nNOS content differed across different pork muscles during postmortem aging. S-nitrosylation modified cysteine sites of 155 from 102 proteins were identified and spread over LT muscle cells for major molecular function of catalytic activity. Whether and how protein nitrosylation could contribute to meat quality needs to be further studied.

ACKNOWLEDGEMENTS

This research was funded by National Natural Science Foundation of China (Grant NO: 31271899).

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