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A bioinformatics study on characteristics, metabolic pathways, and cellular functions of the identified S-nitrosylated proteins in postmortem pork muscle

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ABSTRACT

This study aimed to determine the characteristics, metabolic pathways and cellular functions of S-nitrosylated proteins from pork postmortem muscle using bioinformatics analysis. The results showed that S-nitrosylated proteins had a broad range of molecular weight and pI value and were mainly located in the functional region of secondary structure. The motif revealed the lysine (K) positioned at −5, −7, +1 and +5 through the S-nitrosocysteine while "C-X-X-C" was identified as the motif for non-S-nitrosylation-modified cysteine. The proteins were widely localized in cell compartments and mostly belonged to enzymes participating in the metabolic process. Glycolysis was the most significant pathways of S-nitrosylated proteins in postmortem muscle. The cell death of muscle cells was predicted to be inhibited by S-nitrosylation with the potential influence on the apoptosis. Those identified pathways and cellular functions of S-nitrosylation are proposed to have a profound influence on meat quality and should be highly regarded.

1. Introduction

Nitric oxide (NO) is a signaling molecule and plays multiple roles in physiological and pathophysiological activities. In skeletal muscle, it is regarded as a modulator to regulate muscle contraction and relaxation, myocyte differentiation, respiration and glucose uptake (Stamler & Meissner, 2001). In the last two decades, studies have investigated the effects of NO on fresh meat quality by the manipulation of NO level in the postmortem muscles using NO synthase (NOS) inhibitors and NO donors. NO could affect meat tenderness, water holding capacity, color and protein proteolysis in the postmortem muscles of beef, pork, lamb and chicken (Cottrell, McDonagh, Dunshea & Warner, 2008; Cook, Scott, & Devine, 1998; Li et al., 2014; Zhang et al., 2018; Zhang, Marwan, Samaraweera, Lee & Ahn, 2013). However, inconsistent results existed among the published studies with respect to the effects of NO on meat quality. It is possibly due to the variation in NOS expression and activity, animal species and muscle types, and different NOS inhibitors and NO donors (Liu et al., 2015; Liu, Warner, Zhou, & Zhang, 2018). However, the fundamental mechanism that how NO affects meat quality remains largely unknown.

Protein S-nitrosylation is a major and significant route in which NO exerts its ubiquitous biological effect. The S-nitrosothiol (SNO) is formed by attaching the NO moiety to the protein thiol to regulate an amount of enzymes activity, respiratory proteins, receptor/ion channels and transporters, cytoskeletal and structural components (Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). In postmortem pork muscle, NOS activity could be detected within 1 d postmortem aging of pork muscles (Liu et al., 2015). It was also reported that the NO content in beef longissimus thoracis et lumborum (LTL) muscle was approximately 2 μM (Cook et al., 1998). S-nitrosylated protein bands were detected in beef longissimus thoracis (LT) and psoas major (PM) muscle at 1 d post-slaughter (Zhang, 2009). To figure out how NO participates in the postmortem muscle metabolism, we have identified the S-nitrosylated proteins using the biotin switch method coupled with the isobaric tags in endogenous aging samples and exogenous S-nitrosoglutathione (GSNO)-treated samples in pork LT muscle (Liu et al., 2018). There are many SNO-modified cysteines corresponding to 339 proteins, suggesting that protein S-nitrosylation did occur in postmortem muscles. It was deduced that protein S-nitrosylation could affect the extent of postmortem muscle metabolism via glycolytic

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enzymes, calcium pumps, heat shock proteins, antioxidant enzymes and myofibrillar proteins (Liu et al., 2018).

As a post-translational modification, S-nitrosylation shows the specificity and selectivity on a particular cysteine residue of proteins. However, there are multiple pathways to form S-nitrosothiol including the enzymatic catalysis by NOS, un-catalyzed chemistry of reactive nitrogen species and metalloprotein intermediates (Hess et al., 2005). The predominant pathway to induce S-nitrosylation mainly depends on the subcellular localization of NOS and the compartmented redox state. The gaps remain existed between the known chemical biology of the S-nitrosothiol and alteration in protein functions. Whether the identified S-nitrosylated proteins exert the biological relevance in pork muscles during postmortem aging is still largely unknown. In addition, S-nitrosothiol in the biological system is dynamical and could be degraded and redistributed by denitrosylases and trans-nitrosylases. It is derived that denitrosylation and trans-nitrosylation could occur in the postmortem muscle of pork due to the variation of individual cysteine modification of proteins (Liu et al., 2018). Functional elucidation of these altered targets will yield the insight into SNO signaling in the context of the postmortem muscles.

Thus, the objective of this study was to characterize the S-nitrosylated proteins that were already identified in pork LT muscle and explore the significant metabolic pathways in which the S-nitrosylated proteins participated in postmortem aging. This will provide us the further understanding of how NO and protein S-nitrosylation are involved in postmortem aging and the effects on fresh meat quality.

2. Materials and methods:

2.1. S-nitrosylated protein dataset

The dataset of the S-nitrosylated proteins was obtained from our previous study (Liu et al., 2018). The pork LT muscles were obtained from the six carcasses within 45 min post-slaughter from a commercial slaughter plant (Sushi Meat Co. Ltd, Huai'an, China). The slaughter process was under the standard of commercial conditions according to the Regulations for the Administration of Pig Slaughtering of China. After sampled for 0 d aging samples, the LT muscles were vacuum-packaged and stored at 4 °C for 3 d postmortem aging. S-nitrosoglutathione (GSNO) at the concentration of 10 and 100 μM was incubated with protein extract from 0 d of pork muscles to explore the potential S-nitrosylated proteins and the cysteine sites. Proteomic identification and quantification of S-nitrosylated proteins were conducted in the postmortem aging samples of 0 and 3 d (A0 and A3 samples) as well as 10 and 100 μM GSNO treatment samples using tandem mass tags (TMT 126-129). A total of 339 proteins containing 366 individual S-nitrosylated peptides are presented in Table S1 (Liu et al., 2018). Those S-nitrosylated cysteines sites and proteins were used for the protein properties detection including molecular weight, pI, secondary structure, primary sequence motif and gene ontology analysis. The changes of S-nitrosylation level of individual Cys-site between A0 and A3 samples were considered as significant if its fold change was greater than 1.3. The differential proteins for S-nitrosylation among postmortem aging samples were used for the integrity pathway analysis.

2.2. Molecular weight and pI

The primary sequences of S-nitrosylated proteins were obtained from the database of UniProt (http://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov/protein/) according to their accessions. Molecular weight and pI of S-nitrosylated proteins were calculated by Expasy (http://web.expasy.org/compute_pi/) as shown in Table S2. The distribution of molecular weight and pI were plotted using R software.

2.3. Location in protein secondary structure

The location of S-nitrosylated cysteines in protein secondary structure was predicted by using the Garnier program from EMBOSS (European Molecular Biology Open Source Suite, http://emboss.sourceforge.net/). The four structure types (helix (H), strand (E), turn (T) and coil (C)) for individual cysteine and their proportions in proteins are recorded in Table S3. Then, the proportion of four structures for S-nitrosylated cysteines and proteins were plotted using SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA).

2.4. Primary sequence motif

Seven amino acids flanking each of the S-nitrosylated cysteine and *N*-ethylmaleimide (NEM) modified cysteine (i.e. non-S-nitrosylated cysteine) were fetched from porcine proteome (UniProt-protemo_UP000008227). The motifs were analyzed by motif-X (http://motif-x.med.harvard.edu/) and created by Weblogo3 (http://weblogo.threeplusone.com/create.cgi).

2.5. Gene ontology analysis

The UniProt accessions of S-nitrosylated proteins (Table S1) were employed to analyze the gene ontology by PANTHER (http://www.pantherdb.org/) which chose Sus scrofa as the organism to view the functional classification. The percentage of the genes of the single item for cellular component, biological process, molecular function and protein class was exported to plot by using SigmaPlot 10.0.

2.6. Integrity pathway analysis

The S-nitrosylated proteins of porcine were blasted to mouse, rat or human proteins (http://blast.ncbi.nlm.nih.gov/Blast.cgi) setting threshold of E-value $< 10^{-5}$ and similarity $> 60\%$. A total of 139 proteins were derived as shown in Table S4 which represents the NCBI protein accessions and their corresponding gene name with the S-nitrosylation fold change. Then, the differential proteins of A0 and A3 samples were subjected to Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) to generate the functional annotation. The most significant pathways were plotted by using SigmaPlot 10.0. The cellular function of cell death of muscle cells was selected to show and edited by the adobe illustrator CS5 (Adobe Systems Incorporated, San Jose, CA, USA).

3. Result and discussion

3.1. Properties of S-nitrosylated proteins and sites

The primary source of NO in biological system derives from the NOS catalyzation which enables the neighboring proteins to have the priority to be targets of S-nitrosylation for selective NO-based cell signaling. Skeletal muscle expresses all three isoforms of NOS including neuronal NOS, endothelial NOS and inducible NOS. In our previous study, a considerable number of S-nitrosylated proteins were identified in pork LT muscle due to the abundant nNOS expression and localization in muscle membrane and cytoplasm (Liu et al., 2018; Liu et al., 2015). Analogously, Su et al. (2013) also reported a total of 488 cysteines sites corresponding to 197 proteins those were identified to be S-nitrosylated in mouse muscle. We found that there were more proteins with less SNO-modified cysteines sites in pork LT muscle. In terms of the count of modified cysteine in a protein, the majority of proteins (82.2%) presented 1 cysteine to be S-nitrosylated and 13.7% of proteins had 2 SNO-modified cysteines. Three and four S-nitrosylated cysteines sites accounted for a small proportion of proteins (2.6% and 1.1%, respectively) and only serum albumin owned five modified cysteines. Only one or a few thiols of many available cysteines were modified by