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Identification of S-nitrosylated proteins in postmortem pork muscle using modified biotin switch method coupled with isobaric tags

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ABSTRACT

The objective of this study was to identify the S-nitrosylated proteins in aging samples of pork longissimus thoracis muscle (aged 0 and 3 d) and to study the effects of exogenous S-nitrosoglutathione (GSNO, concentration at 10 and 100 μM) treatments of aged 0 d sample. After validating modified biotin switch method, the samples were labeled with tandem mass tags (TMT126-129) for the LC-MS/MS analysis. A total of 366 peptides were identified to be S-nitrosylated corresponding to 339 proteins. Comparison of total intensity and individual S-nitrosylated sites between aging samples revealed that S-nitrosylation did occur in pork muscle during postmortem aging through possible pathways of denitrosylation and transnitrosylation. GSNO treatment groups showed a considerable number of potential cysteines could be modified with high thiol-reactivity. It was deduced that S-nitrosylation could be involved in the postmortem metabolic process possibly through the regulation of activity or function of glycolytic enzymes, calcium release, heat shock proteins, antioxidant enzymes and myofibrillar proteins.

1. Introduction

Nitric oxide (NO) is an essential signaling molecule and acts as a regulator of the muscle functions including force production, respiration and glucose uptake (Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). During the last decades, several studies have been conducted to investigate the potential role of NO in fresh meat quality by managing the NO content in postmortem muscle cell (Cook, Scott, & Devine, 1998; Cottrell, McDonagh, Dunshea, & Warner, 2008; Cottrell, Ponnampalam, Dunshea, & Warner, 2015). However, the results were inconsistent across the reports. When the NO production in skeletal muscle was derived from the catalyzation of nitric oxide synthase (NOS) from L-arginine to L-citrulline, we discovered that expression and activity of NOS were dependent on the muscle types (Liu, Zhang, Fu, Liu, & Zhou, 2015). It has also been shown that NOS activity is different between species (Brannan & Decker, 2002). Different amounts of NO in postmortem muscle cells may participate in distinct biochemical reactions during the postmortem aging process resulting in the multiple mechanisms regulating meat quality (Liu, Warner, Zhou, & Zhang, 2018). Thus, it is important to explore how NO exerts its effect in postmortem meat.

NO is able to affect the end-target proteins through cyclic guanosine monophosphate (cGMP) which could activate the cGMP-dependent protein kinase and cGMP-independent signaling pathways termed as protein S-nitrosylation (Ziolo, 2008). Protein S-nitrosylation, covalent attachment of nitric oxide (NO) to the sulfur of a cysteine residue to form S-nitrosothiol (SNO), has been recognized as a ubiquitous post-translational modification in cellular signaling pathways (Hess et al., 2005). S-nitrosylation can either positively or negatively regulate diverse physiological or pathophysiological process by altering protein conformation, stability and function (Hess et al., 2005). The extent of protein S-nitrosylation was highly correlated with NOS expression and localization (Iwakiri et al., 2006). We revealed that neuronal NOS (the predominant isoform of NOSs in skeletal muscle) was not only localized at sarcolemma but also cytoplasm. We also showed that long duration of NOS activity was maintained within 24 h postmortem aging in pork (Liu et al., 2015). Thus, identification of S-nitrosylated proteins in postmortem muscle would help us achieve a better understanding of the

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role NO plays in the biochemical changes during postmortem aging.

During the last decades, numerous efforts have been made toward proteomics identification of protein S-nitrosylation in various biological systems. However, due to the low mass, the lability, and the low abundance of S-nitrosylation modification, it is difficult to have unbiased identification of SNO-modified proteins and specific cysteines. However, those issues were initially surmounted by Jaffrey, Erdjument-Bromage, Ferris, Tempst, and Snyder (2001) who created the biotin switch method (BSM) that utilized a sulphydryl-specific biotinylated reagent to react with free cysteine which were reduced from S-NO bond by ascorbate. Afterwards, modified technologies are developed to improve the efficacy and precision of the biotin switch method. These included SNO site identification (SNOSID) applying trypsinolysis before enrichment (Hao, Derakhshan, Shi, Campagne, & Gross, 2006), resin-assisted capture (SNO-RAC) utilizing thiol reactive resin instead of thiol reactive biotin (Forrester et al., 2009), and site-specific high-throughput identification of protein S-nitrosylation (SHIPS) employing two alkylation agents (Liu et al., 2010). Recently, cysteine tandem mass tags (Cys TMT) with the reporter ions ranging from 126 to 131 Da were employed to specifically identify and quantify SNO-modified sites in multiple samples (Murray, Uhrigshardt, O'Meally, Cole, & Van Eyk, 2012). However, the enrichment efficiency of the TMT affinity resin is poor resulting in the loss of S-nitrosylated peptides. Therefore, we modified the biotin switch method based on the previous results and then combined with tandem mass tag (TMT 126–129) to characterize the S-nitrosylated proteins in postmortem pork muscle. In addition, the potential S-nitrosylated proteins and cysteine sites were also explored using endogenous S-nitrosothiol, S-nitrosoglutathione (GSNO) as NO donor to induce protein S-nitrosylation.

The objective of this study was to identify the S-nitrosylated proteins in postmortem muscle to give a better understanding of how NO and protein S-nitrosylation exert the effect on the meat quality and in which pathways they were involved during postmortem aging.

2. Materials and methods

2.1. Pork sample collection and muscle extracts preparation

Six Duroc × Landrace × Yorkshire crossbred pigs (live weight 100 ± 10 kg, aged for 6 months) were electrically stunned and then slaughtered at a commercial meat processing company (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. Six longissimus thoracis (LT) muscles were removed from the right side of the carcasses within 45 min post-slaughter. Samples collected at 45 min were designated as A0. Subsequently, LT muscles were vacuum-packaged and stored at 4 °C for 3 d postmortem aging and designated as A3. Parameters of pH at 45 min and 1 d, color at 1 d, and purge loss at 1 and 3 d of aging were measured as shown in Table S1. The protein was extracted by homogenizing one gram of well-minced muscle with 5 volumes (w/v) of homogenization buffer (50 mM NaCl, 50 mM NH_4HCO_3) according to Su et al. (2013). The homogenate was centrifuged at 5000 *g* for 15 min at 4 °C. The supernatant was collected and protein concentration was measured by the BCA Protein Assay Kit (Pierce, IL, USA). The supernatant was stored at −80 °C.

2.2. GSNO-induced protein S-nitrosylation

To induce protein S-nitrosylation *in vitro*, protein concentration of A0 was adjusted to 4 mg/mL by buffer 1 (250 mM HEPES, pH 7.7, 1 mM EDTA) and then incubated with either 10 or 100 μM GSNO (Sigma-Aldrich, Chemie GmbH, Germany) dissolved in 1% (v/v) dimethyl sulfoxide (DMSO). Those samples were named as G10 and G100 groups. For control, the samples of A0 and A3 were treated with 1% DMSO. Solutions were incubated at 37 °C for 30 min on a rocker in the dark. Excess GSNO was removed by precipitating with cold methanol/acetone (1:12, v/v) solution in 1:3 ratio overnight at −20 °C. Then, the solution was centrifuged at 10,000 *g* for 15 min at 4 °C and the pellet was washed two times with methanol/acetone (1:12). Finally, the pellet was stored at −80 °C and used for protein S-nitrosylation detection.

2.3. Saville assay

The Saville assay was employed to detect the S-nitrosothiol (SNO) content as described by Su et al. (2013). Briefly, 50 μL of aging and exogenous GSNO treatment samples or standard (GSNO) was reacted with 50 μL either buffer A (1% (w/v) sulfanilamide in 0.5 M HCl) or buffer B (buffer A containing 0.2% (w/v) $HgCl_2$) at room temperature for 5 min in a 96-well microtiter plate. Then, 100 μL buffer C (0.02% (w/v) N-(1-naphthyl)ethylenediaminedihydrochloride in 0.5 M HCl) was added to each well for another 5 min incubation at room temperature. Absorbance was measured at 540 nm. A standard curve for GSNO was generated that ranged from 1.17 to 75 μM. The SNO content of the samples were determined by the difference in absorbance between the samples treated with buffer B and those with buffer A. Concentration of SNO content was determined according to the SNO standard curves.

2.4. Modified biotin switch method

The biotin switch method to detect protein S-nitrosylation refers to Murray et al. (2012) with some modification. Samples of A0, A3, G10, and G100 were precipitated by acetone and fully suspended in HENS buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 1 mM neocuproine and 1% SDS) containing 8 M urea. The protein concentration of each sample was adjusted with HENS buffer to 2 mg/mL. Free thiols were blocked in darkness with 20 mM N-ethylmaleimide (NEM) at 60 °C for 30 min. Excess NEM was removed by acetone precipitation. Pellets were re-suspended in 1.5 mL of HENS buffer. S-nitrosylated proteins were reduced with 20 mM sodium ascorbate and simultaneously labeled with 2 mM N^{α}-(3-malemidylpropionyl)biocytin (MPB, Life Technology, Eggenstein-Leopoldshafen, Germany). The reduction was performed at room temperature protected from light for 2 h. Reduced proteins were precipitated and suspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Proteins were digested with 10 μg of trypsin at 37 °C for 24 h. After pre-washing and equilibration with PBS, 100 μL of high capacity streptavidin agarose (No. 20361, Pierce, Germany) beads were mixed with samples and incubated at room temperature for 1 h on a rocker. Biotinylated peptides were eluted by 8 M guanidine hydrochloride (pH 1.5). Eluted peptides were then desalted with C18 column (WAT094225, Waters, USA) and dried in a spin vacuum for 30 min. Dried peptides were re-suspended in 100 μL of tetraethylammonium bromide (TEAB, pH 8.0) for tandem mass tags (TMT) labeling (No.90066, Pierce, Germany). Finally, samples were combined and dried in spin vacuum and then dissolved in 0.1% formic acid (FA) for LC-MS/MS analysis.

2.5. Validation of modified biotin switch method

Bovine serum albumin (BSA, Sigma-Aldrich) was reduced by 1 mM tris(2-carboxyethyl)phosphine and utilized as the substrate to verify the efficacy of modified biotin switch method (Fig. S1). BSA of 1 mg/mL was incubated with NEM at 60 °C for 30 min and subsequently trypsinization as described above to detect the NEM-modified cysteines. The reduced BSA was labeled by 2 mM MPB and followed by trypsinization. The cleaved BSA peptides were directly converted to high capacity streptavidin agarose enrichment procedure. The eluant and the washing buffer flowing through the beads were collected and further analyzed by LC-MS/MS for labeled MPB modification. In addition, 100 μM GSNO was used to induce BSA S-nitrosylation to identify the modified cysteines using this modified biotin switch method. The S-nitrosylated