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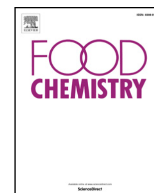
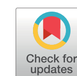


Effect of nitric oxide on myofibrillar proteins and the susceptibility to calpain-1 proteolysis



Rui Liu[a,b], Steven Lonergan[c], Edward Steadham[c], Guanghong Zhou[a], Wangang Zhang[a,*], Elisabeth Huff-Lonergan[c,*]

[a] Key Laboratory of Meat Processing and Quality Control, MOE; Key Laboratory of Meat Processing, MOA; Jiangsu Synergetic Innovation Center of Meat Processing and Quality Control, Nanjing Agricultural University, Nanjing 210095, PR China

[b] College of Food Science and Engineering, Yangzhou University, Yangzhou 225127, Jiangsu, China

[c] Department of Animal Science, Iowa State University, Ames 50011, USA

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ABSTRACT

This study was designed to investigate the nature of modification of myofibrillar proteins by nitric oxide (NO) and the extent to which S-nitrosylation alters their susceptibility to calpain-1 proteolysis. Isolated myofibrils from porcine semimembranosus muscle were incubated with the NO donor S-nitrosoglutathione (GSNO) at 0, 20, 50, 250, 1000 µM for 30 min at 37 °C and then incubated with purified calpain-1. GSNO treatment decreased the thiol content of myofibrillar proteins and increased their intensity and amount of S-nitrosylation. GSNO caused the formation of proteins cross-linkage through intermolecular disulfide. More desmin and titin (T2, the degraded fragment of original titin) were degraded by calpain-1 when myofibrils were incubated with 1000 µM GSNO. Incubation with 250 and 1000 µM GSNO suppressed calpain-1-catalyzed cleavage of troponin-T. The data suggest that NO could change the redox state of myofibrillar proteins and subsequently affect the extent of proteolysis by calpain-1 in a protein-dependent manner.

1. Introduction

Nitric oxide (NO) is a signaling molecule that is synthesized by nitric oxide synthase (NOS) in the mammalian cell. NO can be produced by NOS activation in response to hypoxic/ischemia conditions (Brannan & Decker, 2002; Man, Tsui, & Marsden, 2014). Hypoxic/ischemia conditions also occur in postmortem muscles. Modulations of NO level in pre-slaughter and post-slaughter muscle cells using NO donors and NOS inhibitors are reported to affect the meat quality attributes of tenderness, water holding capacity and color in a variety of animal species including beef, lamb, pork and chicken (Cook, Scott, & Devine, 1998; Cottrell, Ponnampalam, Dunshea, & Warner, 2015; Zhang, Marwan, Samaraweera, Lee, & Ahn, 2013; Zhang et al., 2018). However, variable NO effects on meat quality are observed across different animal species and muscles due to the pleiotropic functions of NO in vivo (Liu et al., 2015). Protein S-nitrosylation is defined as the formation of S-nitrosothiol (SNO) by covalent attachment of NO moiety with protein sulfhydryl (Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). Protein S-nitrosylation occurs in postmortem muscle as evidenced by the accumulation of S-nitrosylated proteins during postmortem aging of meat (Zhang, 2009). It is proposed that NO and protein S-nitrosylation play a regulatory role in postmortem aging and affect meat quality (Warner, Dunshea, Ponnampalam, & Cottrell, 2005; Liu, Warner, Zhou, & Zhang, 2018).

Myofibrillar proteins constitute a large portion of muscle proteins and contribute to the integrity of muscle cells. Many myofibrillar proteins including myosin, actin, desmin, titin and troponin have been identified to be endogenously S-nitrosylated in skeletal muscle and showed a high reactivity with the NO donor S-nitrosoglutathione (GSNO) (Su et al., 2013). Other studies have shown that incubation of intact mouse cardiomyocytes with GSNO and S-nitrosocysteine (CysNO) decreased the myofilament Ca^{2+} sensitivity and ATPase activity by modifying SNO-susceptible sarcomeric proteins including actin, myosin, myosin-binding protein C and troponin C (Figueiredo-Freitas et al., 2015). It has been hypothesized that the basal S-nitrosylated proteins in vivo may play a role in maintaining tissue homeostasis (Furuta, 2017).

Degradation of myofibrillar, cytoskeletal, and intermediate filament proteins during postmortem aging is known to weaken the longitudinal structure of myofibrillar sarcomere and affects fresh meat tenderness

* Corresponding authors at: National Center of Meat Quality and Safety Control, College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095 China (W. Zhang).

E-mail addresses: wangang.zhang@njau.edu.cn (W. Zhang), elonerga@iastate.edu (E. Huff-Lonergan).

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(Lana & Zolla, 2016). Calpain-1 is a major contributor to postmortem proteolysis of myofibrillar proteins including titin, nebulin, desmin and troponin-T (Huff-Lonergan, Zhang, & Lonergan, 2010). It has been reported that proteolysis was inhibited by the NOS inhibitor treatment pre-slaughter in lamb SM muscle resulting in tougher meat during postmortem aging (Cottrell et al., 2015). Calpain-1 can be modified by a NO donor and this modification inhibited calpain-1 autolysis and decreased degradation of myofibrillar proteins (Li et al., 2014; Liu, Li, Wang, Zhou, & Zhang, 2016). In addition, S-nitrosylated titin and α-actinin-1 were identified in sarcoplasmic protein extracts at 3 d of postmortem aging in pork longisiimus thoracis (Liu et al., 2018). Those detected myofibrillar proteins were presumed to be the degraded fragments as they were similar to fragmented desmin and myosin light chain 1 in the sarcoplasmic fraction (Anderson, Lonergan, & Huff-Lonergan, 2012; Carlson et al., 2017). It is possible that nitric oxide and protein S-nitrosylation are involved in calpain-1-induced proteolysis of myofibrillar protein. What is not known is how modification of calpain substrates affects their susceptibility to calpain-1-catalyzed proteolysis. Thus, the objectives of this study were to investigate the extent to which meat protein S-nitrosylation affects predisposition of calpain-1 degradation and to explore the basic mechanism for the effect of NO on fresh meat quality.

2. Materials and methods

2.1. Myofibril preparation

Porcine semimembranosus (SM) within 45 min post-exsanguination was obtained from Meat Laboratory of Iowa State University (Ames, Iowa, USA). The muscle was trimmed to remove visible connective tissue, cut into 1 cm cubes and frozen by liquid nitrogen. The frozen SM muscle was used to purify myofibrils according to Maddock Carlin, Huff-Lonergan, Rowe, and Lonergan (2006) with some modifications. Briefly, 150 g SM muscle was homogenized with 3 vol (w/t) of 4 °C extraction buffer containing 50 mM Tris-HCl (pH 8.5) and 1 mM EDTA. The homogenate was centrifuged at 20,000*g* for 30 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in 10 vol (w/v) standard salt solution (100 mM KCl, 20 mM potassium phosphate, pH 7.0, 2 mM EGTA, 2 mM $MgCl_2$, and 1 mM NaN_3) using the minimum homogenizing speed of the polytron (PT 3100 D, Kinematica, Bohemia, NY, USA) for 10 s. The homogenate was centrifuged at 1000*g* for 10 min at 4 °C to separate the supernatant. The suspension and centrifugation of the pellet were repeated 2 times with 10 vol standard salt solution and then 3 times with 10 vol 5 mM Tris-HCl, pH 8.0. The last pellet was suspended in 20 mM phosphate buffer (pH 7.0) and protein concentration was determined by the Biuret assay (Robson, Goll, & Temple, 1968). The myofibrillar protein concentration was adjusted to 10 mg/ml with 20 mM phosphate buffer, pH 7.0.

2.2. Incubation of myofibril with GSNO and the sample preparation

S-nitrosoglutathione (GSNO, N4148, Sigma, St. Louis, MO, USA) at 0, 20, 50, 250 or 1,000 μM was incubated with myofibrils (10 mg/ml) in 20 mM phosphate buffer, pH 7.0 on a rocker at 37 °C for 30 min. After reactions were completed, the samples were placed on ice and an aliquot (approximately 2.5 mg) was immediately removed and combined with an equal volume of 2.5% SDS and 10 mM phosphate buffer (pH 7.0). Reduced SDS-PAGE gel samples and non-reduced SDS-PAGE gel samples were made by adding 250 μl Wang's buffer (3 mM EDTA, 3% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.003% (wt/vol) pyronin Y, and 30 mM Tris-HCl, pH 8.0) with or without 0.1% (vol/vol) β-mercaptoethanol (MCE), respectively. The gel samples were incubated at a 50 °C heater for 20 min and then stored at −20 °C. Another aliquot (approximately 2.5 mg) of myofibrils was added to 750 μl of −20 °C pre-chilled acetone and thoroughly mixed. The samples were then placed in a −20 °C refrigerator for the protein S-nitrosylation detection. The remaining myofibrillar proteins were centrifuged at 3000*g* for 10 min at 4 °C and washed twice with 20 mM phosphate buffer (pH 7.0) to remove the excess GSNO from the myofibrils. The final pellet of each treatment was re-suspended in 20 mM phosphate buffer (pH 7.0). Protein concentration of the suspension was determined using the Biuret assay (Robson et al., 1968). The remaining suspensions were used for the detection of sulfhydryl content, protein surface hydrophobicity and further incubation with purified calpain-1.

2.3. Protein sulfhydryl and protein surface hydrophobicity

The total and free thiol content were determined according to Yongsawatdigul and Park (2003) with following modifications. Myofibrillar protein suspensions (10 mg/ml) of 250 μl were dissolved in 4.75 ml of solution A (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.6 M KCl) and solution B (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.6 M KCl, 8 M urea) for determination of free and total sulfhydryl content respectively. After thorough mixing, 0.4 ml of 0.1% 5, 5′-dinitrobis (2-nitrobenzoic acid) (DTNB) was added and the myofibrils were incubated at 40 °C in a water bath for 25 min. Absorbance (412 nm) of the resulting solution was measured (Ultrospec 3000, Pharmacia Biotech, New York, USA). The extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was used to calculate the SH groups content which was expressed as nmol/mg of protein.

Protein surface hydrophobicity was determined by the method of Chelh, Gatellier, & Santé-Lhoutellier (2006) with a slight modification. An aliquot of 250 μl myofibrillar protein suspension was combined with 750 μl of 20 mM phosphate buffer (pH 7.0). An equal amount of phosphate buffer was used as the control. After adding 50 μl of 1 mg/ml bromophenol blue (BPB), the samples and control were mixed on a rocker with gentle inversion at 20 °C for 10 min. The samples were then clarified (2000 g for 15 min) and the absorbance of the supernatant was assayed at 595 nm. The amount of BPB bound was calculated by multiplying the absorbance difference between the control and sample by 50.

2.4. Myofibril S-nitrosylation detection

A S-nitrosylated protein detection assay kit (No. 10006518, Cayman Chemical Company, Ann Arbor, MI, USA) was used to detect the GSNO-treated myofibrillar proteins with some modifications to the protocol. Acetone-precipitated myofibrils were centrifuged at 3000*g* for 10 min at 4 °C. The pellet was air-dried and dissolved in HENS buffer (250 mM HEPES pH7.7, 1 mM EDTA, 0.1 mM neocupronine and 2.5% (wt/vol) SDS). An equal amount of myofibrils from each treatment were removed to be processed with biotin switch method according to the S-nitrosylation detection kit. The HENS buffer was used to dissolve the blocking, reducing and labeling reagent from the kit according to Liu et al (2016). After the proteins went through the biotin switch method, the pellet was dissolved in 200 μl HENS buffer. The protein concentration was determined by the detergent-compatible DC Protein Assay Kit (Bio-Rad, Laboratories, Hercules, CA, USA). The samples were adjusted to 2 mg/ml with distilled deionized water and gel samples were made as previously described for the reduced myofibrillar protein samples. Western blotting was used to detect the S-nitrosylated protein bands as described in the following section.

2.5. Calpain-1 purification and reaction with GSNO-treated myofibrils

Porcine SM muscle within 45 min postmortem was utilized to purify calpain-1. The successive purification columns for the sarcoplasmic proteins were as follows: Q-Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ USA), Phenyl Sepharose 6 Fast Flow (Amersham Biosciences), Butyl Sepharose 4 Fast Flow (Amersham Biosciences) and DEAE-TSK Toyopearl (Supelco, Bellefonte, PA, USA). as described by Maddock, Huff-Lonergan, Rowe, and Lonergan (2005).

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