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Effect of protein S-nitrosylation on autolysis and catalytic ability of μ-calpain

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ABSTRACT

The effect of S-nitrosylation on the autolysis and catalytic ability of μ-calpain *in vitro* in the presence of 50 μM Ca^{2+} was investigated. μ-Calpain was incubated with different concentrations of nitric oxide donor S-nitrosoglutathione (GSNO) and subsequently reacted with purified myofibrils. Results showed that the amount of 80 kDa μ-calpain subunit significantly decreased as GSNO increased from 0 to 300 μM, but increases of GSNO to 300, 500 and 1000 μM did not result in further inhibition. The catalytic ability of nitrosylated μ-calpain to degrade titin, nebulin, troponin-T and desmin was significantly reduced when the GSNO concentration was higher than 300 μM. The cysteine residues of μ-calpain at positions 49, 351, 384, and 592 in the catalytic subunit and at 142 in small subunit were S-nitrosylated, which could be responsible for decreased μ-calpain activity. Thus, S-nitrosylation can negatively regulate the activation of μ-calpain resulting in decreased proteolytic ability on myofibrils.

1. Introduction

Nitric oxide (NO) plays a critical role in signal transduction of biological systems by coupling with reactive cysteine thiols to form S-nitrosylation (SNO) products that have been shown to regulate protein conformation, activity and function (Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). In skeletal muscle, NO and NO induced S-nitrosylation could be involved in glucose uptake, muscle contraction and muscular dystrophies by regulating cyclic guanosine monophosphate (cGMP) and ryanodine receptor 1 (Stamler & Meissner, 2001). Managing the NO content in postmortem muscles could possibly affect fresh meat quality, including tenderness and water holding capacity, although reports appear to be inconsistent across different species and muscle types (Cook, Scott, & Devine, 1998; Cottrell, Nagh, Dunshea, & Warner, 2008; Cottrell, Ponnampalam, Dunshea, & Warner, 2015; Zhang, Marwan, Samaraweera, Lee, & Ahn, 2013). For example, injection of nitric oxide synthase inhibitor (L-NAME) and NO donors into excised muscles or pre-slaughtered livestock were employed to manage the NO content in postmortem muscles. Beef longissimus lumborum was observed to become tough in the NO enhanced groups compared to nitric oxide synthase (NOS) inhibitor group (Cook et al., 1998). In addition, others have shown that tenderness of ovine longissimus thoracis et lumborum (LTL) muscle was improved by treatment with NOS inhibitor (Cottrell et al., 2008). However, there have been few investigations of the mechanisms involved in the role of NO and S-nitrosylation in regulating of meat quality during postmortem aging.

The meat quality traits of water holding capacity and tenderness largely depend on the overall integrity of muscle cells which can be disrupted by the degradation of key myofibrillar and cytoskeletal proteins (Hughes, Oiseth, Purslow, & Warner, 2014). The well-known calpain system is responsible for the proteolysis of cytoskeletal proteins (titin and nebulin) and intermediate filaments (desmin) (Huff-Lonergan et al., 1996). Among the calpain system, μ-calpain shows a predominant effect on catalysis of proteins during postmortem aging (Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006). μ-Calpain possesses a proteolytic ability which is accompanied with its autolysis in the presence of calcium in postmortem muscle (Huff-Lonergan, Zhang, & Lonergan, 2010). Apart from calpastatin, the specific endogenous inhibitor of the calpains, modification of cysteine residues in its active site by oxidation and S-nitrosylation can inhibit its autolysis and proteolytic activities (Ascenzi et al., 2001; Lametsch, Lonergan, & Huff-longergan, 2008). They reported that 80% of calpain catalytic activity was inhibited by the incubation with NO enhancer at pH of 5.5 (Michetti, Salamino, & Pontremoli, 1995).

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Recently, Samengo et al. (2012) reported that the decreased expression of neuronal nitric oxide synthase (nNOS, predominant form of NOSs in skeletal muscle which catalyzes L-arginine to generate NO) caused reduction in calpain S-nitrosylation and increased myofibril degradation. In our recent research, the inhibition of NOS promoted μ-calpain autolysis after 1 d of refrigerated storage and resulted in increased degradation of titin and nebulin (Li et al., 2014). However, whether the inhibition of μ-calpain activity caused by NO was through S-nitrosylation modification in pork muscle during postmortem aging still remains unknown. Therefore, the objective of this study was to further investigate the effects of NO and NO-induced nitrosylation on the autolysis and catalytic activity of μ-calpain *in vitro*. The nitrosylated cysteine sites of μ-calpain were also identified.

2. Materials and methods

2.1. Sample preparation

Five six-month-old crossbred pigs with live weight 100 ± 10 kg were slaughtered at a commercial meat processing company (Sushi Meat Co. Ltd., Huaian, China). The *M. longissimus lumborum* was removed from the right side of each porcine carcass within 45 min of slaughter and then frozen rapidly in liquid nitrogen. These meat samples were used for the purification of myofibrils.

2.2. Incubation of μ-calpain with S-nitrosoglutathione (GSNO)

Pure μ-calpain (Merck, Darmstadt, Germany), at a final concentration of 1 mg/mL, was incubated at 37 °C for 30 min with one of the following treatments: 1) control: μ-calpain + distilled water; 2) μ-calpain + 100 μM GSNO (Sigma-Aldrich, Darmstadt, Germany); 3) μ-calpain + 300 μM GSNO; 4) μ-calpain + 500 μM GSNO; 5) μ-calpain + 1000 μM GSNO. At 30 min, $CaCl_2$ was added to each preparation to give a final concentration of 50 μM and then incubated at 37 °C for another 30 min. Reaction was stopped by adding 0.5 volume loading buffer (3 mM ethylenediaminetetraacetic acid (EDTA), 3% (w/v) sodium dodecyl sulphate (SDS), 30% (v/v) glycerol, 0.001% (w/v) pyronin Y and 30 mM Tris-HCl, pH 8.0) and 0.1 volume β-mercaptoethanol (MCE). After heating at 95 °C, the samples were frozen at −80 °C for the later detection of calpain autolysis.

2.3. Purification of myofibrillar protein

Myofibrils were prepared at 4 °C according to method described by Goll, Young, and Stromer (1974) with minor modifications. Two grams of finely minced meat samples were homogenized in 10 volumes (w/v) of extraction buffer (20 mM potassium phosphate, 0.1 M KCl, 2 mM $MgCl_2$, and 2 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), pH 6.8). The muscle homogenate was centrifuged at 1000*g* for 10 min to discard the supernatant. The pellet was washed with 8 volumes of extraction buffer and followed by 8 volumes of 0.1 M KCl for two times. Finally, the myofibril was suspended in incubation buffer (5 mM 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulphonic acid (HEPES), 100 mM NaCl, 0.1% (w/v) 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulphonate (CHAPS), and 5 mM NaN_3, pH 6.5). The protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and each was adjusted to 8 mg/mL with distilled water.

2.4. Incubation myofibril with S-nitrosylated μ-calpain

To assess the proteolytic ability of μ-calpain after the reaction with GSNO, 1200 μg myofibril preparations, 15 μL μ-calpain and 50 μM $CaCl_2$ were mixed together for 30 min at 37 °C. The reaction was terminated by adding 0.5 volume loading buffer and 0.1 volume of MCE. After heated at 95 °C for 5 min, the samples were stored at −80 °C for subsequent SDS-PAGE and western blotting analysis.

2.5. SDS-PAGE and western blotting of myofibrils degradation

For SDS-PAGE of titin and nebulin, 70 μg proteins were loaded onto 5% continuous gels (8 cm wide × 7.3 cm tall × 1.5 mm thick) and then run on a Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA, USA) at a constant setting of 5 mA/gel for 18 h. For SDS-PAGE of μ-calpain autolysis, 60 μg proteins was loaded in each well and then run at 60 V of 30 min for the 4% stacking gel and 110 V of 75 min for 10% separating gel. The running buffer constituted 25 mM Tris-base, 192 mM glycine, and 0.1% SDS. Staining with coomassie brilliant blue R-250 was applied for the visualization of titin and nebulin and staining of silver was employed to detect the bands of μ-calpain. All the gels were scanned with the scanner GT-800 F (Epson, Nagano, Japan) and then the densities of bands were quantified by Quality One software (Version 4.6, Bio-Rad).

Gels for desmin and troponin-T (10, 12.5% for separating gels, respectively) were transferred to polyvinylidene difluoride (PVDF) membranes using a Mini-Protean II system (Bio-Rad Laboratories) at 4 °C for 1.5 h at a constant voltage of 90 V. The transfer buffer consisted of 25 mM Tris-base, 192 mM glycine, 2 mM EDTA, and 15% (v/v) methanol. The membranes were blocked with 5% (w/v) non-fat dry milk powder in TBST buffer (20 mM Tris-base, 137 mM NaCl and 5 mM KCl, 0.05% Tween-20) for 1.5 h at room temperature. After blocking, membranes were incubated with the primary antibody of desmin (Abcam, Cambridge, Cambridgeshire, UK) and troponin-T (Sigma-Aldrich, Darmstadt, Germany) at a dilution of 1:500 in TBST buffer. Blots were placed on a rocker with gentle agitation overnight at 4 °C. After five washes (6 min/wash) with TBST, the membranes were then incubated with the second antibody (1:5000, goat anti rabbit, Chemicon, Temecula, CA, USA) for 1.5 h at room temperature. After the last washing of membranes for 30 min, the blots were visualized by the reaction with ECL reagent and scanned with Image Quant LAS4000 (GE, Fairfield, CT, USA).

2.6. Detection of S-nitrosylated μ-calpain

S-nitrosylated μ-calpain was detected by the S-Nitrosylated Protein Detection Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). For this procedure, samples were first subjected to the procedure described in the company's instruction manual, using an 8% polyacrylamide separating gel and a 4% polyacrylamide stacking gel. The gels were run at 120 V and then transferred to PVDF membranes at a constant voltage of 90 V for 90 min at 4 °C. Membranes were blocked with 5% bovine serum albumin (BSA) in TBST for 30 min at room temperature. The blots were incubated with S-nitrosylation detection reagent I using a dilution of 1:75 (v/v) in TBST and then placed on a rocker with gentle agitation overnight at 4 °C. The subsequent steps were performed as described above.

2.7. Detection of S-nitrosylated sites of μ-calpain

Biotin switch method (BSM) coupled with LC-MS/MS was utilized to detect the S-nitrosylated sites of μ-calpain as described by Jaffrey, Erdjument-Bromage, Ferris, Tempst, and Snyder (2001) with minor modification. Acetone precipitated μ-calpain with GSNO treatment was fully suspended in HENS buffer (250 mM HEPES, pH 7.7, 1 mM EDTA, 1 mM neocuproine and 1% SDS)