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Regulation of calpain-1 activity and protein proteolysis by protein nitrosylation in postmortem beef

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ABSTRACT

The effects of nitric oxide (NO) and its induced protein nitrosylation on calpain-1 activation and protein proteolysis in beef during postmortem aging were investigated. Five semimembranosus muscles were removed from beef cattle carcass. Beef samples were incubated with one of following treatments for 24 h at 4 °C: control (normal saline), NO donor (100, 200 and 400 μM S-nitrosoglutathione (GSNO)) or nitric oxide synthase (NOS) inhibitor (0.05, 0.1 and 0.15 M Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)). After incubation, the beef samples were vacuum-packaged and aged at 4 °C for 1, 4, and 7 days. Results showed that GSNO decreased and L-NAME increased the extent of calpain-1 autolysis at d 1. Degradation of desmin and troponin-T was increased by L-NAME while decreased by GSNO. These results suggest that NO could regulate calpain-1 autolysis and its proteolysis activity during postmortem aging in beef SM muscle.

1. Introduction

NO, as a signal molecule that widely exists in the skeletal muscles, is generated by the reaction of L-arginine to L-citrulline catalyzed by nitric oxide synthase (NOS) (Stamler & Meissner, 2001). NO and NO-induced protein S-nitrosylation could be involved in diverse physiological processes and affect protein structure and function in biological systems (Stamler & Meissner, 2001). NO could regulate the activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and ryanodine receptor (RyR) that are responsible for controlling the calcium concentration of muscle cells (Bellinger et al., 2008; Stamler, Sun, & Hess, 2008). The calpain system is known as the key protease system during the transformation of muscle to meat while the calpain-1 is a predominant isoform contributing to the meat quality changes during postmortem aging (Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006). In the presence of calcium, autolysis of calpain-1 from 80 to 76 kDa signifies its activation during postmortem aging (Zhang et al., 2006). Samengo et al. (2012) showed that the loss of neuronal nitric oxide synthase (nNOS, the major isoform of NOS in skeletal muscle) decreased calpain S-nitrosylation and increased myofibril degradation in mice skeletal muscle. However, whether NO and NO-induced nitrosylation could be involved in variation of fresh meat quality remains illusive.

Few studies have been conducted to investigate the effects of NO on meat quality during postmortem aging. Cook, Scott, and Devine (1998) suggested that the tenderness of beef longissimus thoracis (LT) muscle increased by infusing NOS inhibitor and decreased by infusing NO donor during postmortem aging for 6 days. However, Cottrell, Ponnampalam, Dunshea, and Warner (2015) observed that the injection of NOS inhibitor into live lambs reduced tenderness and proteolysis in lamb semimembranosus (SM) muscle. The different results across studies may be due to the differences in species, treatments and muscle types.

Liu, Li, et al. (2015) found that pork muscles had a long duration of NOS activity within 24 h during postmortem aging while pork SM showed higher and more sustained NOS activity than LT and psoas major. In our recent research, we found that NOS inhibitor L-NAME increased the calpain-1 autolysis and proteolysis in postmortem pork after 1 day storage at 4 °C (Li et al., 2014). Liu, Li, Wang, Zhou, and Zhang (2016) found that the autolysis and the catalytic ability of calpain-1 were decreased by incubating with NO donor GSNO in vitro. However, no studies have been conducted to explore the possible contribution of protein nitrosylation to the calpain activation and protein degradation in postmortem beef. Thus, in current study, the effects of NO on the autolysis of calpain and the proteolytic activity of myofibrillar proteins of beef SM muscle during postmortem aging were investigated.

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2. Materials and methods

2.1. Sample preparation

Five 18-month-old Luxi cattle with live weight 260 ± 10 kg were slaughtered at Haoyue Co. Ltd. (Dehui, Jilin, China). Semimembranosus (SM) muscles were obtained from right side of each carcasses within 1 h after slaughter. Muscles were cut into 1 × 1 × 3 cm strips and needled with 20 Gauge needles to facilitate the penetration of solutions. Strips from same SM muscle were randomly assigned to one of seven treatments: control (normal saline), 100 μM, 200 μM and 400 μM S-nitrosoglutathione solubilized in normal saline (GSNO, N4148, Sigma-Aldrich Corp., St. Louis, MO, USA) or 0.05 M, 0.1 M and 0.15 M Nω-Nitro-L-arginine methyl ester hydrochloride solubilized in normal saline (L-NAME, N5751, Sigma-Aldrich Corp., St. Louis, MO, USA). The ratio of muscle strips and solution was 1:1 (m/v). After incubation for 24 h at 4 °C, samples were filter paper dried and then stored at 4 °C with vacuum-packaged for 1, 4 and 7 days. The samples were stored at −80 °C for the biochemical analysis.

2.2. Sarcoplasmic protein extraction

Sarcoplasmic proteins were prepared using the method described by Fu et al. (2015) with slight modification. One gram of well minced beef samples was homogenized twice using a Polytron (IKA T25 digital ultraturrox Made in Staufen, Germany) at 15000 rpm for 15 s with 5 volume cold buffer (100 mM Tris-base, pH 8.3 and 10 mM EDTA-Na_2). The homogenate was then centrifuged at 20,000 ×*g* for 15 min at 4 °C. The protein concentration of supernatant was measured by BCA Protein Assay Kit (Thermo, RD, USA) and then adjusted to 6 mg/ml. One volume of diluted solutions was combined with one volume sarcoplasmic loading buffer (10 mM Tris–HCl, 2.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01% (v/v) bromophenol blue, pH 6.8). The samples were heated at 95 °C for 5 min and then stored at −20 °C.

2.3. Myofibrillar protein preparation

Myofibrillar proteins were prepared using the method of Li et al. (2014) with slight modification. Half gram of well minced beef was homogenized twice at 15000 rpm for 30 s with 7.5 volume PRB solution (2 mM $Na_4P_2O_7$, 1 mM NaN_3, 2 mM $MgCl_2$, 2 mM ethylene glycol-bis (β-aminoethyl ether)-*N, N, N′, N′*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 100 mM KCl, and 10 mM Tris-maleate, pH 6.8). The homogenate was centrifuged at 1000 ×*g* for 10 min at 4 °C. The supernatant was discarded and precipitate was resuspended with 7.5 volume LSB solution (1 mM NaN_3, 2 mM $MgCl_2$, 2 mM ethylene glycol-bis (β-aminoethyl ether)-*N, N, N′, N′*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 100 mM KCl, and 10 mM Tris-maleate, pH 6.8) for 7 times. The precipitate was mixed with 4 volume myofibrillar loading buffer (20% glycerol, 125 mM Tris-HCl, 4% SDS, pH 6.8) and then deposited in 50 °C water bath for 20 min. After the samples were centrifuged at 20,000 ×*g* for 30 min, the protein concentration of supernatant was measured by Protein Assay Kit (Thermo, RD, USA) and adjusted to 8 mg/ml with distilled water. One volume of diluted solutions was combined with one volume myofibrillar loading buffer. After heated for 5 min at 95 °C, the samples were stored at −20 °C until further analysis.

2.4. Protein solubility

Protein solubility measured and referred to the method of Chen, Zhou, and Zhang (2015) with a slight adjustment. To measure the solubility of sarcoplasmic proteins, 1 g of well minced beef meat was homogenized with 10 volume of ice-cold 25 mM potassium phosphate buffer (pH 7.2) and gentle agitate in a rocker at 4 °C overnight, then the homogenate was centrifuged at 2000 ×*g* for 30 min and the protein concentration of supernatant was measured using Biuret method. The total protein solubility was acquired similarly by replacing extraction buffer as 1.1 M potassium iodide in 0.1 M phosphate buffer (pH 7.2). Thus, myofibrillar protein solubility was calculated by the difference between the solubility of total protein and sarcoplasmic protein.

2.5. S-nitrosothiol (SNO)

SNO measurement was referred to the method of Su et al. (2013) with slight adjustment. One gram well minced beef was homogenized twice in 5 mL solutions containing 50 mM NaCl and 50 mM NH_4HCO_3 (pH 7.8) at 15000 rpm. After centrifugation at 8000 ×g for 10 min at 4 °C, the supernatant was collected and the protein concentration was determined by BCA Protein Assay Kit (Thermo, RD, USA). The protein concentration was adjusted to 1 mg/ml with distilled water. Diluted samples of 50 μL were incubated with 50 μL solution A (1% (w/v) sulfanilamide in 0.5 M HCl) or 50 μL solution B (0.2% (w/v) $HgCl_2$ in solution A) in wells of a 96-well plate for 5 min at room temperature. Then, 100 μL solution C (0.02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in 0.5 M HCl) was added to each well and incubated for another 5 min at room temperature. The absorbance was measured immediately at 540 nm. To generate a standard curve, two fold serial dilutions of GSNO ranging from 1.56 to 150 μM were employed. The SNO contents were calculated by the difference between absorbance B and absorbance A according to the GSNO standard curves.

2.6. SDS-PAGE and western blotting

To analyze the calpain-1 autolysis, 60 μg sarcoplasmic proteins were loaded using 4% stacking gel and 10% separating gel. Twenty microgram of myofibrillar proteins were loaded for analyzing troponin-T degradation using 4% stacking gel and 12.5% separating gel (Bio-Rad Laboratories, Hercules, CA, USA). Thirty microgram of myofibrillar proteins were loaded to analyze desmin degradation using 4% stacking gel and 10% separating gel. Twenty microgram of myofibrillar proteins were loaded for the detection of protein degradation using 4–15% gel. All the gels were run at 120 V using a Mini-Protean II system (Bio-Rad Laboratories) until the samples reached the bottom. The gels of 4–15% were stained by using coomassie staining buffer containing 7% (v/v) glacial acetic acid, 40% (v/v) ethanol and 0.1% (w/v) coomassie brilliant blue R-250, and were then scanned using the scanner GT-800 F (Epson, Nagano, Japan).

After the electrophoresis was completed, the proteins of calpain-1, troponin-T and desmin were transferred to nitrocellulose (NC) membranes running at 90 V for 1.5 h. The membranes were then blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris-base, 137 mM NaCl, 5 mM KCl and 0.05% (v/v) Tween-20) for 90 min at room temperature. The membranes were incubated with primary antibody of calpain-1 (MA3–940 Thermo Scientific, Bremen, Germany), desmin (ab8976 Abcam, Cambridge, Cambridge shire, UK) or troponin-T (T6277 Sigma-Aldrich, Darmstadt, Germany) at a dilution of 1:1000 in TBST and gently agitated overnight at 4 °C. After being washed five times (5 min per wash) with TBST, the membranes were incubated with the second antibody (goat anti-mouse, Bioworld, Atlanta, USA) diluted with TBST in a ratio of 1:5000. After five washes, the membranes were stained with ECL reagents for 5 min and scanned by Image Quant LAS4000 (GE, Fairfield, CT, USA). Bands densities of all lanes were quantified using Quality One software (Version 4.6.2, Bio-Rad, USA).

2.7. Statistical analysis

The experimental data was analyzed by analysis of variance (ANOVA) (SAS, version 9.2). A Mixed Model was applied to evaluate the main effect of the treatment (L-NAME, control, GSNO), aging time and cross effect of treatment × aging time (TM × AT) which chose the