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Exploring the Effects of S-Nitrosylation on Caspase-3 Modification and Myofibril Degradation of Beef In Vitro

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ABSTRACT: This study aimed to explore the effects of S-nitrosylation on caspase-3 modification and its subsequent effects on beef myofibril degradation in vitro. Recombinant caspase-3 was reacted with different concentrations of S-nitrosoglutathione (GSNO, nitric oxide donor) at 37 °C for 30 min and subsequently incubated with purified myofibrillar protein from bovine *semimembranosus* muscle. Results indicated that the activity of caspase-3 was significantly reduced after GSNO treatments ($P < 0.05$) and showed a dose-dependent inhibitory effect, which was attributed to the increased S-nitrosylation extent of caspase-3. LC−MS/MS analysis revealed that caspase-3 was S-nitrosylated at cysteine sites 116, 170, 184, 220, and 264. Moreover, the degradation of desmin and troponin-T was notably suppressed by S-nitrosylated caspase-3 ($P < 0.05$). To conclude, protein S-nitrosylation could modify the cysteine residues of caspase-3, which accounts for the reduced caspase-3 activity and further represses its proteolytic ability on beef myofibrillar protein.

KEYWORDS: *protein S-nitrosylation, caspase-3, modified cysteine sites, proteolytic ability, beef muscle*

INTRODUCTION

Post-mortem tenderization of meat is primarily attributed to the disruption of myofibrillar structures resulting from the degradation of myofibrillar and cytoskeletal proteins by endogenous proteolytic enzymes.[1] The calpain system is primarily responsible for post-mortem proteolysis;[2,3] however, the incubation of myofibrils with the calpain inhibitor still resulted in an obvious cleavage of myofibrillar proteins,[4] indicating that meat tenderization might be a multienzymatic process.[5] It was supported by Ouali et al.[6] and Herrera-Mendez et al.[7] that other proteolytic enzymes including proteasome and caspases also contributed to meat aging. Unlike calpain, which has been a focus of attention for several decades, caspase is recently emerging as a topic of interest. As a family of cysteine proteases, caspases play a crucial part in the execution phase of apoptosis.[8] Generally, caspases are classified into two categories, featuring caspases-3, -6, and -7 as effector caspases and caspases-8, -9, and -10 as initiator caspases. Besides, the initiator caspases seem to be highly selective, cleaving only their precursors and the effector caspases.[9] Consequently, most of the proteolysis that occurs during the proceeding of apoptosis is executed by effector caspases.[10] Caspase-3, as a vital effector caspase for apoptosis, was found to make a significant contribution to post-mortem aging through degrading skeletal proteins.[11−15] However, Underwood[16] reported that the activation of caspase-3 was not triggered and thus was unlikely to be involved in post-mortem beef proteolysis. As noted above, it seems that the roles of caspase-3 in post-mortem proteolysis are multifaceted and complex, hence warranting further research.

Protein S-nitrosylation is a significant signaling pathway for nitric oxide (NO) that acts in biological systems.[17] Accumulating evidence supports the importance of S-nitrosylation as a regulatory post-translational modification with functional consequences for proteins, such as regulation of protein activity, localization, stability, and interactions.[18] Over the past decade, efforts have been made to verify the roles of S-nitrosylation in post-mortem muscle. It was suggested that S-nitrosylation was implicated in regulating meat quality, including tenderness and water-holding capacity, through mediating calcium release, energy metabolism, protein degradation, and apoptosis.[19−22] Recently, our research demonstrated that S-nitrosylation could downregulate the mitochondria-dependent caspase-3 cascade reaction, possibly accounting for the variations in muscle apoptosis and beef quality.[22] Caspase-3 has been investigated focusing on its involvement in apoptosis; however, few studies have been conducted to explore the mechanism of S-nitrosylation in regulating caspase-3. Further exploration is needed to clarify the impacts of S-nitrosylation on the structure and function of caspase-3. As caspase-3 exerts potential influences on muscle proteolysis, it is tempting to speculate that NO could mediate S-nitrosylation of caspase-3, which could affect caspase-3 activity as well as its proteolytic ability on myofibrillar proteins, thereby affecting beef quality.

Besides, it has been reported from our lab that endogenous NO synthesis and its induced protein S-nitrosylation could be positively modulated by preslaughter stress, which in turn affected meat quality development by mediating various

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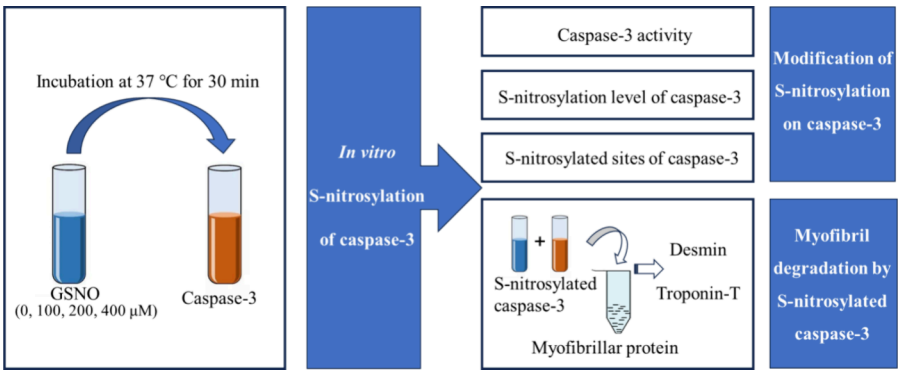

Figure 1. Schematic diagram of the experimental design for in vitro S-nitrosylation of caspase-3.

biological processes involved during the conversion of muscle to meat.[23,24] In order to further clarify the S-nitrosylation-based post-mortem muscle biochemistry theory for the regulation of fresh meat quality, an in vitro experimental model was specially designed here to explore the modification effect of protein S-nitrosylation on caspase-3 and its subsequent impact on beef myofibril degradation. To the best of our knowledge, this is the first report of the investigation of the proteolytic activity of S-nitrosylated caspase-3 on beef myofibrillar protein in vitro. In addition, specific S-nitrosylation modification sites of caspase-3 were also identified in this paper. This investigation verifies the regulatory effect of S-nitrosylation on caspase-3 and provides a new insight into elucidating the regulatory mechanism of protein S-nitrosylation on beef quality from the perspective of caspase-3-mediated myofibril proteolysis.

MATERIALS AND METHODS

Muscle Sample Preparation. Five crossbred cattle (Simmental × Luxi yellow cattle, 20 months old, 480 ± 20 kg) with the same feeding and management conditions were slaughtered in an abattoir (Guangfu Animal Products Company, Yangxin, China) in accordance with the guidelines of GB/T 19477-2018. Immediately after slaughter, beef *semimembranosus* (SM, only right side) was collected. Subsequently, pH values and color of samples were assessed to avoid abnormal beef, as stated in our previous study.[25] After the removal of visible connective tissue and fat, approximately 20 g pieces were collected from each SM muscle. Collected samples were chopped and put into cryostorage tubes followed by quick freezing in liquid nitrogen. Finally, muscle samples were preserved at −80 °C for subsequent myofibrillar protein extraction.

Preparation of the Myofibrillar Protein. A myofibrillar protein (MP) was purified referring to the report of Feng et al.[26] with slight modification. One gram of muscle sample was minced and then homogenized with 10 mL of precooled phosphate buffer (0.1 M KCl, 10 mM K_2HPO_4, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.0) twice at 8000 rpm (15 s for each homogenization). The homogenate was collected for centrifugation at 1000 × *g* and 4 °C for 10 min. The obtained pellet was washed with 8 mL of phosphate buffer four times. After washing, centrifugation was conducted again. The protein pellet was collected and washed twice with 8 mL of 0.1 M KCl. The final pellet was resuspended in 8 volumes of incubation buffer (0.1 M NaCl, 5 mM HEPES, and 0.1% CHAPS, pH 6.5) to obtain the MP extract. The BCA protein assay was performed to determine the protein concentration of MP.

In Vitro S-Nitrosylation of Caspase-3. An in vitro model was established in the current study to clarify the impacts of protein S-nitrosylation on caspase-3. Specifically, 1 mg/mL of recombinant caspase-3 (Sigma-Aldrich Corp., MO, USA) was reacted with 0 μM (control, ultrapure water), 100 μM, 200 μM, or 400 μM GSNO (NO donor, Sigma-Aldrich Corp., MO, USA) to induce S-nitrosylation of caspase-3 in vitro. Many studies have been conducted to investigate the association between protein S-nitrosylation and fresh meat quality by managing levels of NO,[21,27−29] and no harmful substances were detected. Therefore, it is safe to utilize GSNO to induce the S-nitrosylation of caspase-3 in this study. The concentrations of GSNO were chosen according to the report of Zhang et al.[30] The reaction was allowed to proceed at 37 °C for 30 min.[31] After that, a portion of the samples was collected to determine the modification sites, activity, and S-nitrosylated extent of caspase-3, whereas the resulting samples were incubated with purified myofibrillar protein from beef SM to investigate the proteolytic ability of S-nitrosylated caspase-3 on the myofibrillar protein. The specific experimental design is displayed in Figure 1.

Determination of Caspase-3 Activity. The activity of caspase-3 was determined according to the procedures of the Caspase-3 Fluorescence Kit (KeyGEN Biotech, Nanjing, China). To be specific, 30 μL of sample solution from the Preparation of the Myofibrillar Protein section was subjected to black 96-well plates (Microfluor, VA, USA). Blank control was generated by adding 30 μL of 10 mM PBS instead of the reaction mixture. After that, 50 μL of 2× reaction buffer was reacted with 0.5 μL of dithiothreitol (DTT) and subsequently added to the 96-well plates together with 10 μL of ultrapure water. Then, the caspase-3 reaction solution was prepared by adding 0.5 μL of caspase-3 substrate to 19.5 μL of 2× reaction buffer. Following this, 10 μL of the caspase-3 reaction solution was added to the reaction system. After the reaction at 37 °C for 90 min, the fluorescence intensity of samples at 485/535 nm (excitation/emission) was read on a microplate reader (SpectraMax iD5, Molecular Devices, CA, USA). Caspase-3 activity was presented as the fluorescence intensity ratio of the treatments to the blank control.

Detection of S-Nitrosylated Caspase-3 Extent. S-Nitrosylation extent of caspase-3 was detected based on the protocol of the S-nitrosylation kit (Thermo Scientific, IL, USA). The assay process principally comprised four steps, including protein preparation, blocking, S-nitrosocysteine labeling, and Western blotting. Briefly, the sample solution of GSNO-treated caspase-3 was reacted with 1 M methyl methanethiosulfonate (MMTS) at 25 °C for 30 min. Following that, precooled acetone was added to precipitate the protein and remove excess MMTS. The precipitation was allowed to proceed for 60 min. After centrifugation at 10,000 × *g* and 4 °C for 10 min, the acetone was decanted by carefully inverting the tubes without disturbing the pellet. The obtained pellet was subjected to drying for 10 min. Subsequently, HENS buffer was added to dissolve the pellet. Afterward, the samples were reacted with 1 μL of labeling reagent and 2 μL of 1 M sodium ascorbate in the dark at 25 °C for 60 min. The labeled protein was enriched with Anti-TMT resin (Thermo Scientific, IL, USA). In order to prepare gel samples for blot analysis, protein samples were incubated with loading buffer (GenScript, Nanjing, China) at 95 °C for 5 min. After that, the prepared samples were subjected to −80 °C for a subsequent investigation.

Identification of S-Nitrosylated Caspase-3 Sites by LC−MS/MS. To identify the S-nitrosylated sites of caspase-3, 400 μM GSNO-treated caspase-3 was mixed with 6 volumes of precooled acetone for

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incubation overnight at −20 °C. Then, centrifugation was conducted at 16,000 × *g* and 4 °C for 10 min. The protein precipitate was collected, washed twice with acetone, and then placed in a fume hood to allow the acetone to volatilize completely. Subsequently, the protein precipitate was dissolved in 100 μL of 50 mM NH_4HCO_3 and then incubated with 2 μL of 1 μg/μL trypsin overnight at 37 °C for digestion. After digestion, the peptides were desalted using a C18 StageTip (Millipore, CA, USA) followed by vacuum concentration and drying. Then, peptides were redissolved in 0.1% formic acid for subsequent LC−MS/MS analysis, which was performed through the Easy nLC 1200 system (Thermo Scientific, IL, USA) coupled to an Orbitrap Exploris 480 MS. The sample was loaded onto a reversed-phase trap column and separated by a reversed-phase analytical column. Elution was performed with 8−25% acetonitrile in 0.1% formic acid for 70 min. The peptides were separated, followed by MS identification through the mass spectrometer (Thermo Scientific, IL, USA). MS data were subjected to MaxQuant Software (Max-Planck Institute for Biochemistry, Germany) for the analysis of S-nitrosylated caspase-3 sites. The parameter settings of MaxQuant analysis are shown in Supplementary Table 1.

Incubation of the Myofibrillar Protein with S-Nitrosylated Caspase-3. The extracted MP (600 μg) was mixed with different concentrations (0, 100, 200, and 400 μM) of GSNO-treated caspase-3 to further evaluate the impacts of S-nitrosylated caspase-3 on myofibrillar protein degradation. The mixtures were subjected to a shaker at 200 rpm and 37 °C for 30 min.[26] After that, the mixtures were immediately subjected to ice to terminate the reaction. Then, 0.5 volume of loading buffer was added to the reaction solution, followed by a water bath for 5 min. The prepared gel samples were preserved at −80 °C for further Western blotting analysis.

Western Blotting. Gel samples from the Detection of S-Nitrosylated Caspase-3 Extent section were separated by 12% precast gels (Bio-Rad Laboratories, CA, USA) at 90 V for 90 min. After that, transmembranes were conducted through the Trans-Blot Equipment (Bio-Rad Laboratories, CA, USA) running at 90 V and 4 °C for 90 min. After blocking with 5% skim milk in TBST for 90 min, membranes were immunoblotted with the primary anticaspase-3 antibody (ab4051, Abcam, Cambridge, UK) or primary β-actin antibody (a reference protein, Cell Signaling Technology, MA, USA) with a dilution of 1:1000 in TBST. After gentle agitation by a shaker at 4 °C for 14 h, membranes were washed using TBST (10 min each wash, a total of three times). Thereafter, an antimouse IgG-HRP conjugate solution (Bioworld, Atlanta, USA) with a 1:5000 dilution in TBST was utilized to incubate with membranes for 120 min. After washing again, membranes were reacted with chemiluminescent substrates (Thermo Scientific, IL, USA) for 5 min in the dark. Immediately after the reaction, the membranes were exposed to a Gel Imager System (ImageQuant LAS4000, CT, USA) to detect the corresponding protein bands. Following this, Quantity-one software (Bio-Rad Laboratories, CA, USA) was utilized for densitometric analysis of S-nitrosylated caspase-3.

In addition, gel samples from the Incubation of the Myofibrillar Protein with S-Nitrosylated Caspase-3 section were subjected to 10% precast gels to detect the degradation of desmin and troponin-T by S-nitrosylated caspase-3. Electrophoresis, transmembrane, blocking, marking secondary antibody, and protein band detection were carried out as above, except for the primary antibody incubation. Herein, primary antibodies against desmin (ab8976, Abcam, Cambridge, UK) and troponin-T (T6277, Sigma-Aldrich, Darmstadt, Germany) diluted with TBST buffer at 1:1000 were employed instead of the anticaspase-3 antibody.

Statistical Analysis. The experiment was performed in five replicates. SPSS Statistics Software was employed in the analysis of the experimental data. ANOVA analysis was conducted for comparison in each group. The differences among the four treatment groups were assessed using Duncan's multiple test. Finally, results were displayed as means ± standard error (SE) with a significant difference level being set as $P < 0.05$.

RESULTS AND DISCUSSION

Caspase-3 Activity. Caspase-3, as a crucial effector enzyme during the execution of apoptosis, was considered a potential contributor to meat tenderization.[10] Recently, the inhibition of NO on caspase-3 activity has been detected in post-mortem beef.[22] However, it remains unknown whether the inhibitory impact of NO on caspase-3 was attributed to NO-mediated S-nitrosylation. To verify the impact of S-nitrosylation on caspase-3, an in vitro experiment was designed in this study by incubating different concentrations of the NO donor with recombinant caspase-3. GSNO was commonly recognized as a NO donor and functioned as the equilibrium with a pool of S-nitrosothiol (SNO) proteins,[32] and thus different concentration gradients of GSNO were selected for exogenous NO supply to induce in vitro S-nitrosylation of caspase-3 in the current study. The impact of in vitro S-nitrosylation on caspase-3 activity is illustrated in Figure 2.

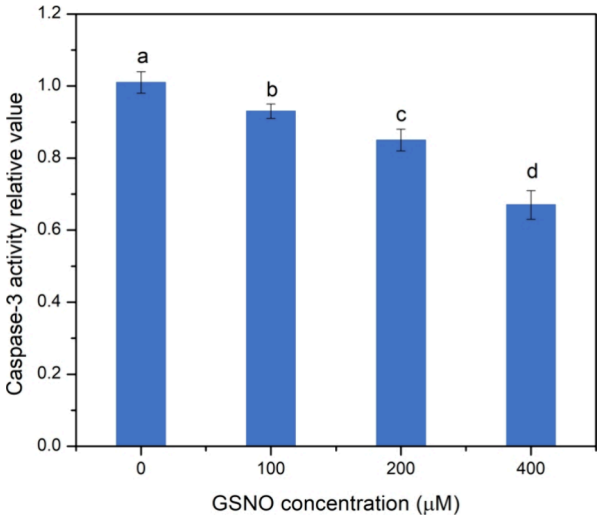

Figure 2. Effect of different concentrations of GSNO treatments on the caspase-3 activity. Different letters indicate significant difference ($P < 0.05$, $n = 5$). GSNO: S-nitrosoglutathione.

Results indicated that compared to the group in the absence of GSNO, the activities of caspase-3 were remarkably decreased in groups of 100, 200, and 400 μM GSNO ($P < 0.05$), revealing that the addition of GSNO effectively repressed the activity of caspase-3. Furthermore, caspase-3 activity was increasingly restrained with stepwise increments of GSNO concentrations. This revealed that the restraint effect of S-nitrosylation on caspase-3 activity was dose-dependent. This was confirmed by Ascenzi et al.[33] that the NO donor dose-dependently blocked the activity of effector caspases including caspases 3, 6, and 7. Notably, GSNO- and SNAP-inhibited caspase activities could be recovered by DTT,[33] suggesting that NO-induced R-SNO formation, that is, protein S-nitrosylation modification was reversible. This was consistent with the report of Hess et al.[34] that protein S-nitrosylation was recognized as a reversible post-translational modification. Similarly, Zhang[35] found that a part of the S-nitrosylated μ-calpain was reversed by DTT, which resulted in enhanced μ-calpain activity in the reducing condition. However, the activities of caspases inhibited by peroxynitrite and SIN-1 could not be reactivated by DTT, indicating thiol oxidation was beyond sulfenic, sulfinic, or disulfide formation.[33] The

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underlying reason for the decreased caspase-3 activity by GSNO treatments remains to be elucidated.

S-Nitrosylated Extent of Caspase-3. The extent of S-nitrosylation of caspase-3 was investigated to clarify the reactivity of GSNO to caspase-3. Figure 3 shows that a considerable S-nitrosylated band intensity of caspase-3 was detected after treatments with GSNO. Besides, higher S-nitrosylated caspase-3 was found in the groups treated with GSNO at 100, 200, and 400 μM compared to the untreated group ($P < 0.05$). Similar findings were reported in our earlier in vivo investigation, in which beef SM muscle treated with GSNO had increased levels of S-nitrosylated caspase-3.[22] Notably, gradual increases in the S-nitrosylation extent of caspase-3 were observed with stepwise increments of GSNO ($P < 0.05$), and the 400 μM GSNO group possessed the highest modification intensity. This was possibly attributed to the elevated NO level released by the increased GSNO concentrations. These findings indicate that exogenous GSNO incubation could successfully induce the in vitro S-nitrosylation of caspase-3. Surprisingly, a weak S-nitrosylation band was detected in the control group without the presence of GSNO, suggesting that caspase-3 might undergo endogenous S-nitrosylation.

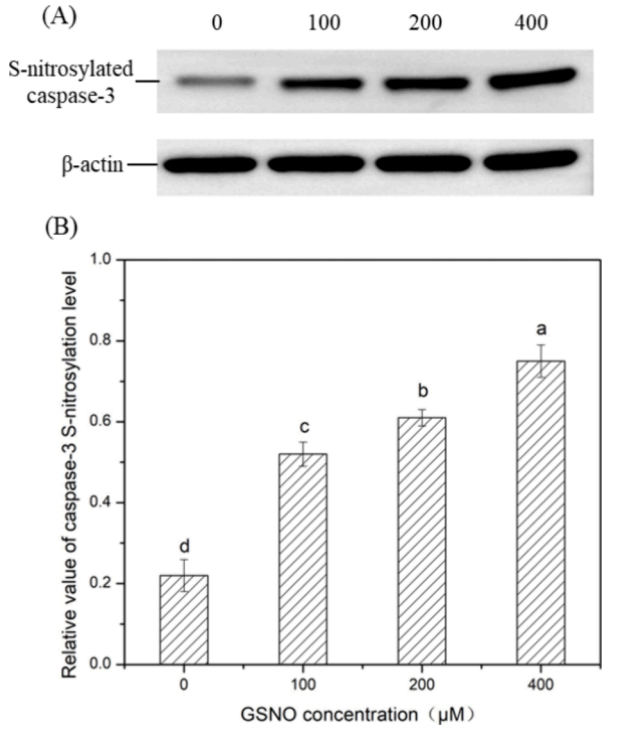

Figure 3. Effects of GSNO treatments on the S-nitrosylation level of caspase-3. (A) Representative Western blot of S-nitrosylated caspase-3 under different concentrations of GSNO (0, 100, 200, and 400 μM). (B) Relative value of S-nitrosylated caspase-3. The values for each group were presented as percentages calculated from the intensities of individual bands relative to the intensity of β-actin (the loading control protein). Different letters indicate significant difference ($P < 0.05$, $n = 5$). GSNO: S-nitrosoglutathione.

Additionally, a higher S-nitrosylation extent of caspase-3 corresponded to the reduced activity of caspase-3 in this study, which is in accordance with a previous study that lower caspase-3 activity was detected in post-mortem bovine SM with a higher S-nitrosylation extent.[22] It is convincible that caspase-3 mediated by S-nitrosylation could be responsible for the decreased activity of caspase-3. In addition, all three NOS isoforms colocalized with caspase-3, enabling a significant amount of NO from NOS catalysis to quickly attach to the catalytic sites of caspase-3.[36] Accumulating evidence suggested that S-nitrosylation restrained the activities of caspases through the modification on catalytic cysteine sites.[37] However, the specific S-nitrosylation modification sites of caspase-3 remain unclear, and further exploration is required.

S-Nitrosylated Sites of Caspase-3. To further investigate the mechanism of S-nitrosylation on caspase-3, it is essential to identify the S-nitrosylated sites of caspase-3 by LC−MS/MS. The sequence of caspase-3 with the modification site positions is presented in Figure 4. Results indicated that a total of 5 cysteine residues from 4 peptides on caspase-3 were modified by protein S-nitrosylation. The S-nitrosylated cysteines and sites are summarized in Table 1. It was found that S-nitrosylated cysteines were located in the peptides of SSFVCVLLSHGEEGIIFGTNGPVDLK, GTELDC-GIETDSGVDDDMACHKIPVEADFLYAYSTAPGYYSWR, DGSWFIQSLCAMLK, and KQIPCIVSMLTK at positions 116, 170, 184, 220, and 264, respectively. These observations confirm the modification effect of S-nitrosylation on caspase-3 after incubation with GSNO. In addition, MS1 and MS2 spectra for S-nitrosylated caspase-3 peptides are shown in Figure 5. Caspase-3 is a cysteine protease with 8 cysteine residues, among which 5 cysteines were found to be S-nitrosylated, suggesting that caspase-3 is susceptible to S-nitrosylation modification. Normally, caspase-3 exists in the form of a zymogen and is activated proteolytically into the heterodimer with a large subunit and a small subunit.[38−40] The five identified modification cysteine sites in this study contain two residues (Cys 116 and 170) in the large subunit and three residues (Cys 184, 220, and 264) in the small subunit. It has been previously shown that a sequence located in the N-terminus of its large subunit was responsible for the activation of caspase-3.[39] In addition, it was reported by Mittl et al.[41] that the catalytic residue of caspase-3 was S-nitrosylated at Cys 163, which differs from the results of this study. Differences in the abundance of S-nitrosylated caspase-3, the modification level of the sites, and the sensitivity of the detection method possibly contributed to the inconsistent results. As noted above, it is plausible to clarify that the S-nitrosylation modification of caspase-3 cysteine residues, including Cys 116, 170, 184, 220, and 264, might account for the decreased

```
          10         20         30         40         50
MENTENSVDS KSIKNLEPKI IHGSESMDSG ISLDNSYKMD YPEMGLCIII
          60         70         80         90        100
NNKNFHKSTG MTSRSGTDVD AANLRETFRN LKYEVRNKND LTREEIVELM
         110        120        130        140        150
RDVSKEDHSK RSSFVCVLLS HGEEGIIFGT NGPVDLKKIT NFFRGDRCRS
         160        170        180        190        200
LTGKPKLFII QACRGTELDC GIETDSGVDD DMACHKIPVE ADFLYAYSTA
         210        220        230        240        250
PGYYSWRNSK DGSWFIQSLC AMLKQYADKL EFMHILTRVN RKVATEFESF
         260        270
SFDATFHAKK QIPCIVSMLT KELYFYH
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Figure 4. Sequences of caspase-3. Sequences marked in blue indicate S-nitrosylated peptides, and C marked in red represents S-nitrosylated cysteine.

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Table 1. Summary of S-Nitrosylated Sequences and Modifications Sites of Caspase-3

S-nitrosylated sequences	S-nitrosylated sites
SSFVCVLLSHGEEGIIFGTNGPVDLK	Cys 116
GTELDCGIETDSGVDDDMACHKIPVEADFLYAYSTAPGYYSWR	Cys 170, Cys 184
DGSWFIQSLCAMLK	Cys 220
KQIPCIVSMLTK	Cys 264

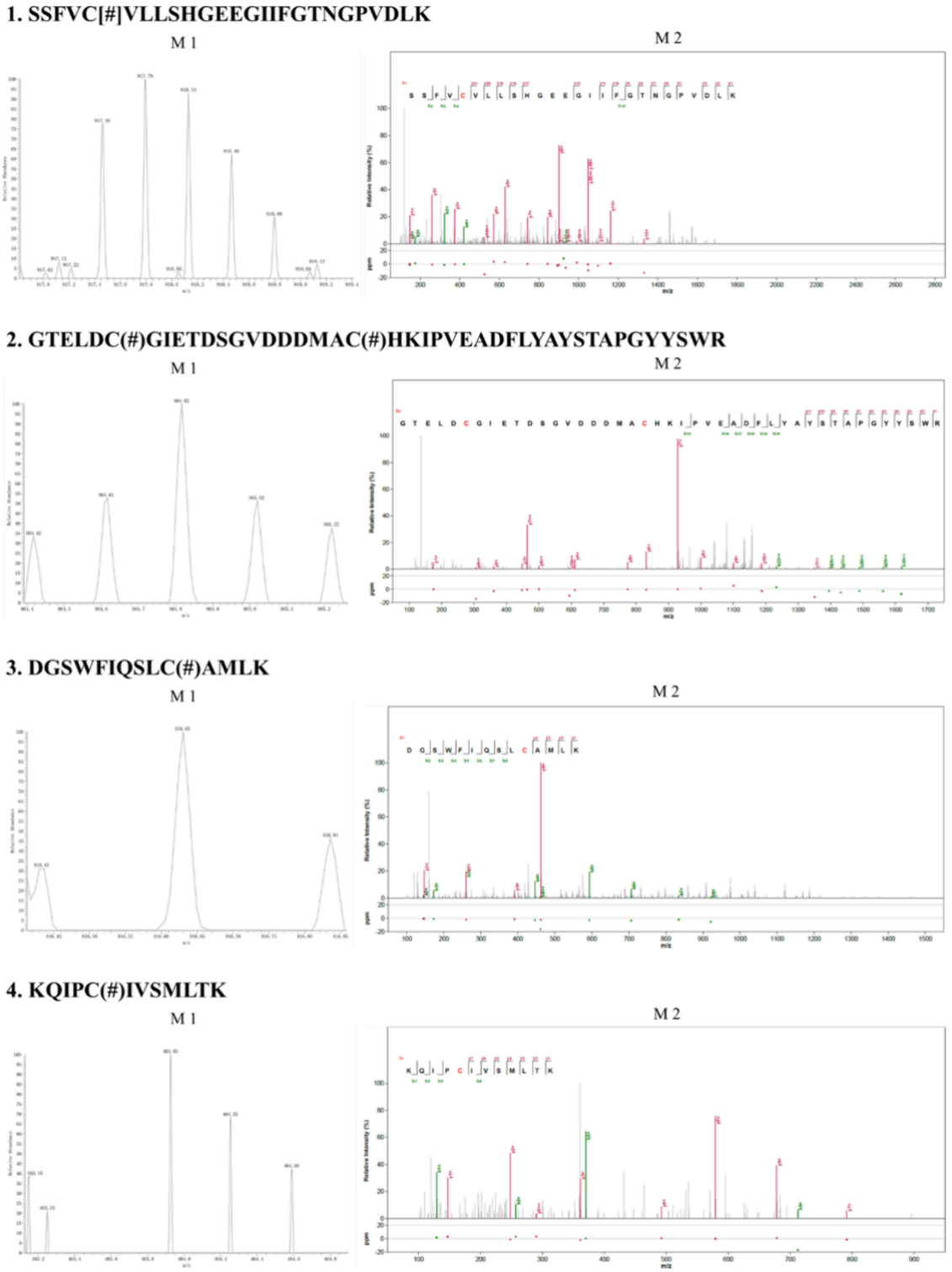

Figure 5. MS1 and MS2 spectra for S-nitrosylated peptides and sites of caspase-3.

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caspase-3 activity after GSNO treatments. In addition to caspase, NO could also inhibit the activity of calpain and cathepsin by binding to cysteine sites,[33] suggesting that NO may be a common regulator of cysteine proteases. It can be inferred that the modulation of cysteine protease activity may be mediated by NO and protein S-nitrosylation, which could impact protein degradation and contribute to post-mortem aging. The influence of SNO-modified caspase-3 on the myofibrillar protein was further explored and will be discussed later.

Desmin Degradation by S-Nitrosylated Caspase-3. The action of caspase-3 on the cytoskeleton and myofibrillar protein is acknowledged as a potential participant during post-mortem aging.[14,42] Desmin, as a crucial cytoskeleton protein, plays a significant role in maintaining the structural integrity and contractility of muscle cells.[43,44] Physiologically, the degradation of desmin could promote the tenderization of meat during aging.[45] Thus, it is of great significance in investigating the sensitivity of S-nitrosylated caspase-3 to desmin degradation. Primary MP without incubation with caspase-3 and GSNO was considered the standard (Std) for desmin analysis. Compared to the Std group, the content of intact desmin in the 0 μM GSNO group (incubation of myofibrils and caspase-3 without GSNO) was remarkably decreased (Figure 6), suggesting the potential proteolytic ability of caspase-3 on desmin. An in vitro myofibrillar protein model carried out by Ding et al.[46] proved that recombinant caspase-3 could promote the proteolysis of desmin. However, Huang et al.[47] reported that caspase-3 induced only minor degradation of desmin, implying that caspase-3 might be involved in muscle protein proteolysis together with other proteases. This was probably due to the different levels of caspase-3 in the reaction system. In addition, the content of intact desmin was noticeably increased after GSNO incubation ($P < 0.05$). Moreover, the content of intact desmin was consistently upregulated when the GSNO concentration was elevated ($P < 0.05$). This finding implies that SNO-modified caspase-3 inhibited desmin degradation in a level-dependent manner. Regulation of caspase activity was demonstrated to influence myofibril degradation.[14,48] The reduction in the proteolytic ability of S-nitrosylated caspase-3 on desmin in this study might be explained by its downregulated activity resulting from S-nitrosylation.

Troponin-T Degradation by S-Nitrosylated Caspase-3. Troponin-T (TnT) is an essential myofibrillar protein for tropomyosin to regulate the filament that is responsible for muscle contraction. The degradation of TnT was characterized by the detection of 28 and 30 kDa TnT, which can disrupt its interaction with the filament component. Besides, the degradation product content of intact TnT in post-mortem muscle was demonstrated to be closely linked to meat tenderness.[49] The degradation of TnT by caspase-3 under S-nitrosylation conditions is indicated in Figure 7. The degradation of TnT was detected with the appearance of a

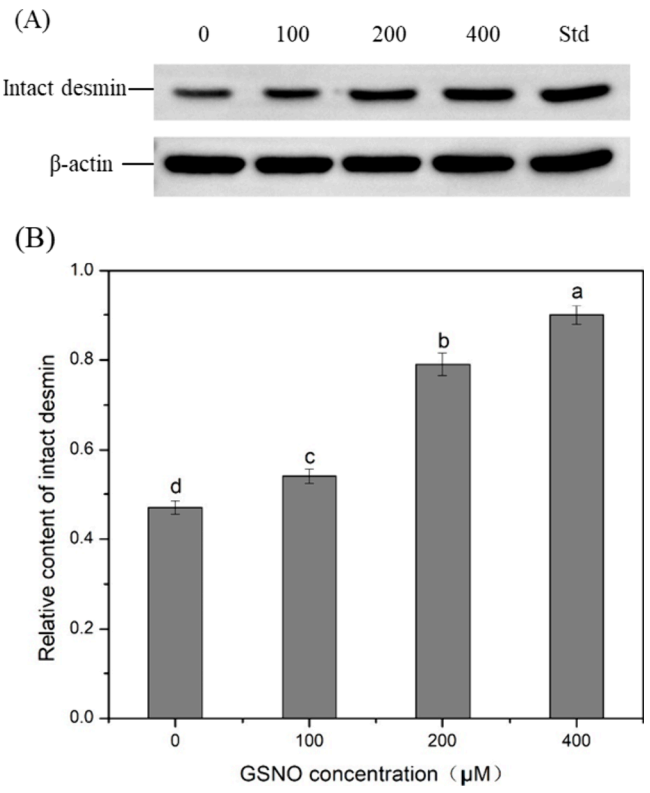

Figure 6. (A, B) Degradation of desmin by S-nitrosylated caspase-3. Std: standard (primary myofibril without GSNO and caspase-3). 0, 100, 200, and 400 μM represent different concentrations of GSNO. The values for each group were presented as percentages calculated from the intensities of individual bands relative to the intensity of the standard band. β-actin: the loading control protein. Different letters indicate significant difference ($P < 0.05$, $n = 5$). GSNO: S-nitrosoglutathione.

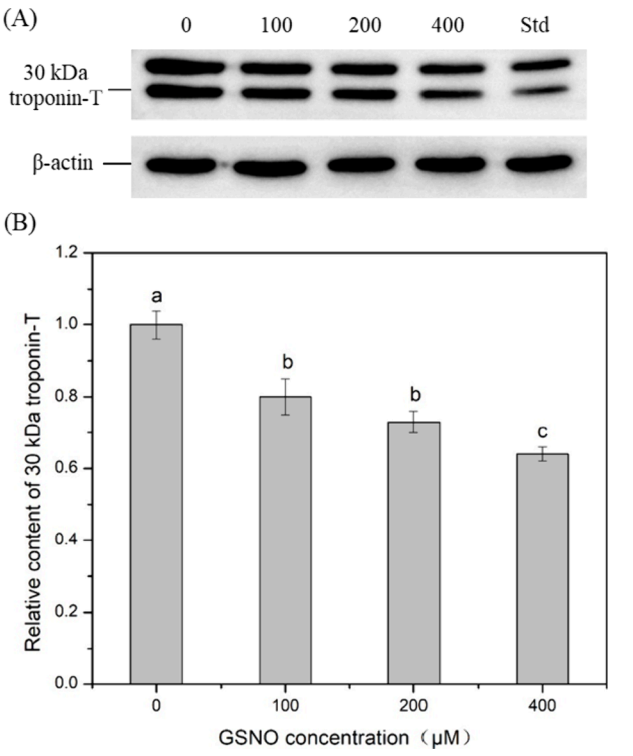

Figure 7. (A, B) Degradation of troponin-T by S-nitrosylated caspase-3. Std: standard (primary myofibril without GSNO and caspase-3). 0, 100, 200, and 400 μM represent different concentrations of GSNO. The relative values of the 30 kDa fragment of troponin-T were calculated as blot intensities in each gel relative to the fragment intensity in the 0 μM GSNO-treated caspase-3 group. β-actin: the loading control protein. Different letters indicate significant difference ($P < 0.05$, $n = 5$). GSNO: S-nitrosoglutathione.

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30 kDa fragment in all groups. Besides, compared to the Std group (myofibrils without caspase-3 and GSNO), a notable increase in 30 kDa TnT fragment was detected in the groups with caspase-3, indicating that caspase-3 was involved in the degradation of TnT. In agreement, some reports demonstrated that the addition of caspase-3 caused a significant decline of intact TnT.[50−52] Moreover, the incubation of caspase-3 with GSNO resulted in a decrease of 30 kDa TnT. No overt difference was detected in the degradation fragment between 100 and 200 μM GSNO groups ($P > 0.05$), whereas the fragment significantly decreased when the concentration of GSNO reached 400 μM ($P < 0.05$). These findings demonstrated the prohibitive effect of SNO-modified caspase-3 on TnT degradation. Taken together, it reveals that desmin and TnT were susceptible substrates for caspase-3, and the decreased activity of caspase-3 by GSNO treatments could result in a lower degree of myofibrillar protein degradation. Collectively, these observations support that NO-mediated S-nitrosylation may modify cysteine sites of caspase-3, which suppressed the activity and proteolytic ability of caspase-3 on the myofibrillar protein, validating the roles of S-nitrosylation and caspase-3 in post-mortem aging. In addition, GSNO-treated μ-calpain showed decreased proteolytic ability on desmin and TnT.[31] It is reasonable to infer that the degradation of desmin and TnT may be the result of the synergistic effects of caspase-3 and μ-calpain. This was evidenced by Kemp,[10] who found an interaction between the calpain and caspase systems that were involved in muscle proteolysis and meat tenderization. The interaction of S-nitrosylated caspase-3 and calpain remains to be further studied. As noted above, the incubation of S-nitrosylated caspase-3 with purified myofibrillar protein notably inhibited the degradation of desmin and TnT. This is the first report of the investigation of the proteolytic activity of S-nitrosylated caspase-3 on beef myofibrillar protein in vitro. To conclude, NO-induced S-nitrosylation could regulate the activity of caspase-3 by modifying the cysteine residues, which could further affect its proteolytic ability on desmin and TnT, thus exerting impacts on beef quality. This study provides a new perspective to explain the regulatory mechanism of protein S-nitrosylation on meat quality.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c06663.

Supplementary Table 1: Parameter settings of MaxQuant analysis (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GSNO, S-nitrosoglutathione; NO, nitric oxide; SM, semimembranosus; MP, myofibrillar protein; DTT, dithiothreitol; MMTS, methyl methanethiosulfonate; LC−MS/MS, liquid chromatography-tandem mass spectrometry; SNO, S-nitrosothiol; Cys, cysteine; TnT, troponin-T

■ REFERENCES

(1) Koohmaraie, M.; Geesink, G. H. Contribution of postmortem muscle biochemistry to the delivery of consistent meat quality with particular focus on the calpain system. *Meat Sci.* **2006**, *74*, 34−43.

(2) Huff-Lonergan, E.; Zhang, W.; Lonergan, S. M. Biochemistry of postmortem muscle-Lessons on mechanisms of meat tenderization. *Meat Sci.* **2010**, *86*, 184−195.

(3) Geesink, G. H.; Kuchay, S.; Chishti, A. H.; Koohmaraie, M. μ-Calpain is essential for postmortem proteolysis of muscle proteins. *J. Anim. Sci.* **2006**, *84*, 2834−2840.

(4) Hopkins, D. L.; Thompson, J. M. Factors contributing to proteolysis and disruption of myofibrillar proteins and the impact on tenderisation in beef and sheep meat. *Aust. J. Agr. Res.* **2002**, *53*, 149−166.

(5) Warner, R. D.; Wheeler, T. L.; Ha, M.; Li, X.; Bekhit, A. E. D.; Morton, J.; Vaskoska, R.; Dunshea, F. R.; Liu, R.; Purslow, P.; Zhang, W. G. Meat tenderness: advances in biology, biochemistry, molecular mechanisms and new technologies. *Meat Sci.* **2022**, *185*, No. 108657.

(6) Ouali, A.; Herrera-Mendez, C. H.; Coulis, G.; Becila, S.; Boudjellal, A.; Aubry, L.; Sentandreu, M. A. Revisiting the conversion of muscle into meat and the underlying mechanisms. *Meat Sci.* **2006**, *74*, 44−58.

(7) Herrera-Mendez, C. H.; Becila, S.; Boudjellal, A.; Ouali, A. Meat ageing: Reconsideration of the current concept. *Trends Food Sci. Technol.* **2006**, *17*, 394−405.
(8) Kesavardhana, S.; Malireddi, R. K. S.; Kanneganti, T. D. Caspases in Cell Death, Inflammation, and Pyroptosis. *Annu. Rev. Immunol.* **2020**, *38*, 567−595.
(9) Walsh, J. G.; Cullen, S. P.; Sheridan, C.; Luthi, A. U.; Gerner, C.; Martin, S. J. Executioner caspase-3 and caspase-7 are functionally distinct proteases. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12815−12819.
(10) Kemp, C. M.; Parr, T. Advances in apoptotic mediated proteolysis in meat tenderisation. *Meat Sci.* **2012**, *92*, 252−259.
(11) Huang, F.; Huang, M.; Zhou, G. H.; Xu, X.; Xue, M. *In vitro* proteolysis of myofibrillar proteins from beef skeletal muscle by caspase-3 and caspase-6. *J. Agr. Food Chem.* **2011**, *59*, 9658−9663.
(12) Kemp, C. M.; Bardsley, R. G.; Parr, T. Changes in caspase activity during the postmortem conditioning period and its relationship to shear force in porcine longissimus muscle. *J. Anim. Sci.* **2006**, *84*, 2841−2846.
(13) Cao, J. X.; Ou, C. R.; Zou, Y. F.; Ye, K. P.; Zhang, Q. Q.; Khan, M. A.; Pan, D. D.; Zhou, G. H. Activation of caspase-3 and its correlation with shear force in bovine skeletal muscles during postmortem conditioning. *J. Anim. Sci.* **2013**, *91*, 4547−4552.
(14) Chen, L.; Feng, X. C.; Lu, F.; Xu, X. L.; Zhou, G. H.; Li, Q. Y.; Guo, X. Y. Effects of camptothecin, etoposide and Ca^{2+} on caspase-3 activity and myofibrillar disruption of chicken during postmortem ageing. *Meat Sci.* **2011**, *87*, 165−174.
(15) Yang, Y. Y.; Han, L.; Yu, Q. L.; Gao, Y. F.; Shi, H. M. Effects of caspase activity of yak meat and internal environment changing during aging. *J. Food Sci. Technol.* **2022**, *59*, 1362−1371.
(16) Underwood, K. R.; Means, W. J.; Du, M. Caspase 3 is not likely involved in the postmortem tenderization of beef muscle. *J. Anim. Sci.* **2008**, *86*, 960−966.
(17) Horenberg, A. L.; Houghton, A. M.; Pandey, S.; Seshadri, V.; Guilford, W. H. S-nitrosylation of cytoskeletal proteins. *Cytoskeleton* **2019**, *76*, 243−253.
(18) Stomberski, C. T.; Hess, D. T.; Stamler, J. Protein S-nitrosylation: determinants of specificity and enzymatic regulation of S-nitrosothiol-based signaling. *Antioxid. Redox Signaling* **2019**, *30*, 1331.
(19) Wang, Y. Y.; Liu, R.; Tian, X. N.; Fan, X. Q.; Shi, Y. W.; Zhang, W. G.; Hou, Q.; Zhou, G. H. Comparison of activity, expression, and S-nitrosylation of calcium transfer proteins between pale, soft, and exudative and red, firm, and non-exudative pork during post-mortem aging. *J. Agric. Food Chem.* **2019**, *67*, 3242−3248.
(20) Zhang, L. L.; Liu, R.; Cheng, Y. P.; Xing, L. J.; Zhou, G. H.; Zhang, W. G. Effects of protein S-nitrosylation on the glycogen metabolism in postmortem pork. *Food Chem.* **2019**, *272*, 613−618.
(21) Li, Y. P.; Liu, R.; Zhang, W. G.; Fu, Q. Q.; Liu, N.; Zhou, G. H. Effect of nitric oxide on μ-calpain activation, protein proteolysis, and protein oxidation of pork during post-mortem aging. *J. Agric. Food Chem.* **2014**, *62*, 5972−5977.
(22) Hou, Q.; Zhu, Q.; Lu, W.; Zhang, W. G. Protein S-nitrosylation regulates postmortem beef apoptosis through the intrinsic mitochondrial pathway. *J. Agric. Food Chem.* **2022**, *70*, 1252−1260.
(23) Ma, C.; Zhang, W. G.; Zhang, J.; Du, T. Y. Modification-specific proteomic analysis reveals cysteine S-nitrosylation mediated the effect of preslaughter transport stress on pork quality development. *J. Agr. Food Chem.* **2023**, *71*, 20260−20273.
(24) Ma, C.; Zhang, W. G.; Zhou, L.; Feng, F. Effect of pre-Slaughter transport stress on protein S-nitrosylation levels of pork during postmortem aging. *J. Agric. Food Chem.* **2023**, *71*, 11150−11157.
(25) Hou, Q.; Zhang, C. Y.; Zhang, W. G.; Liu, R.; Tang, H. Q.; Zhou, G. H. Role of protein S-nitrosylation in regulating beef tenderness. *Food Chem.* **2020**, *306*, No. 125616.
(26) Feng, F.; Yin, Y. T.; Zhou, L.; Ma, C.; Zhang, W. G. Effect of nitric oxide and its induced protein S-nitrosylation on the structures and in vitro digestion properties of beef myofibrillar protein. *J. Agr. Food Chem.* **2023**, *71*, 2532−2540.
(27) Cook, C. J.; Scott, S. M.; Devine, C. E. Measurement of nitric oxide and the effect of enhancing or inhibiting it on tenderness changes of meat. *Meat Sci.* **1998**, *48*, 85−89.
(28) Cottrell, J. J.; McDonagh, M. B.; Dunshea, F. R.; Warner, R. D. Inhibition of nitric oxide release pre-slaughter increases post-mortem glycolysis and improves tenderness in ovine muscles. *Meat Sci.* **2008**, *80*, 511−521.
(29) Zhang, W. G.; Marwan, A. H.; Samaraweera, H.; Lee, E. J.; Ahn, D. U. Breast meat quality of broiler chickens can be affected by managing the level of nitric oxide. *Poultry Sci.* **2013**, *92*, 3044−3049.
(30) Zhang, C. Y.; Liu, R.; Wang, A. R.; Kang, D. C.; Zhou, G. H.; Zhang, W. G. Regulation of calpain-1 activity and protein proteolysis by protein nitrosylation in postmortem beef. *Meat Sci.* **2018**, *141*, 44−49.
(31) Liu, R.; Li, Y. P.; Wang, M. Q.; Zhou, G. H.; Zhang, W. G. Effect of protein S-nitrosylation on autolysis and catalytic ability of μ-calpain. *Food Chem.* **2016**, *213*, 470−477.
(32) Weerapana, E.; Wang, C.; Simon, G. M.; Richter, F.; Khare, S.; Dillon, M. B.; Cravatt, B. F. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature* **2010**, *468* (7325), 790−795.
(33) Ascenzi, P.; Salvati, L.; Bolognesi, M.; Colasanti, M.; Polticelli, F.; Venturini, G. Inhibition of cysteine protease activity by NO donors. *Curr. Protein Pept. Sci.* **2001**, *2*, 137−153.
(34) Hess, D. T.; Matsumoto, A.; Kim, S.-O.; Marshall, H. E.; Stamler, J. S. Protein S-nitrosylation: Purview and parameters. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 150−166.
(35) Zhang, W. G. Involvement of protein degradation, calpain autolysis and protein nitrosylation in fresh meat quality during early postmortem refrigerated storage. In *Dissertations & Theses-Gradworks*; Iowa State University: Ames, Iowa, USA, 2009.
(36) Matsumoto, A.; Comatas, K. E.; Liu, L. M.; Stamler, J. S. Screening for nitric oxide-dependent protein-protein interactions. *Science* **2003**, *301*, 657−661.
(37) Maejima, Y.; Adachi, S.; Morikawa, K.; Ito, H.; Isobe, M. Nitric oxide inhibits myocardial apoptosis by preventing caspase-3 activity via S-nitrosylation. *J. Mol. Cell. Cardiol.* **2005**, *38*, 163−174.
(38) Zou, H.; Henzel, W. J.; Liu, X.; Lutschg, A.; Wang, X. Apaf-1, a human protein homologous to c. elegans ced-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* **1997**, *90*, 405−413.
(39) Pelletier, M.; Cartron, P. F.; Delaval, F.; Meflah, K.; Vallette, F. M.; Oliver, L. Caspase 3 activation is controlled by a sequence located in the n-terminus of its large subunit. *Biochem. Biophys. Res. Commun.* **2004**, *316*, 93−99.
(40) Liu, H.; Chang, D. W.; Yang, X. Interdimer processing and linearity of procaspase-3 activation. A unifying mechanism for the activation of initiator and effector caspases. *J. Biol. Chem.* **2005**, *280*, 11578−11582.
(41) Mittl, P. R. E.; Di Marco, S.; Krebs, J. F.; Bai, X.; Karanewsky, D. S.; Priestle, J. P.; Tomaselli, K. J.; Grutter, M. G. Structure of Recombinant Human CPP32 in Complex with the Tetrapeptide Acetyl-Asp-Val-Ala-Asp Fluoromethyl Ketone. *J. Biol. Chem.* **1997**, *272*, 6539−6547.
(42) Bernard, C.; Cassar-Malek, I.; Le Cunff, M.; Dubroeucq, H.; Renand, G.; Hocquette, J. New indicators of beef sensory quality revealed by expression of specific genes. *J. Agric. Food Chem.* **2007**, *55*, 5229−5237.
(43) Kristensen, L.; Purslow, P. P. The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. *Meat Sci.* **2001**, *58*, 17−23.
(44) Melody, J. L.; Lonergan, S. M.; Rowe, L. J.; Huiatt, T. W.; Mayes, M. S.; Huff-Lonergan, E. Early postmortem biochemical factors influence tenderness and water-holding capacity of three porcine muscles. *J. Anim. Sci.* **2004**, *82*, 1195−1205.
(45) Zakrys-Waliwander, P. I.; O'Sullivan, M. G.; O'Neill, E. E.; Kerry, J. P. The effects of high oxygen modified atmosphere packaging

https://doi.org/10.1021/acs.jafc.4c06663
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

on protein oxidation of bovine M. Longissimus dorsi muscle during chilled storage. *Food Chem.* **2012**, *131*, 527−532.
(46) Ding, Z.; Huang, F.; Zhang, C.; Zhang, L.; Sun, H.; Zhang, H. Effect of heat shock protein 27 on the in vitro degradation of myofibrils by caspase-3 and μ-calpain. *Int. J. Food Sci. Technol.* **2018**, *53*, 121−128.
(47) Huang, M.; Huang, F.; Xu, X.; Zhou, G. Influence of caspase-3 selective inhibitor on proteolysis of chicken skeletal muscle proteins during post mortem aging. *Food Chem.* **2009**, *115*, 181−186.
(48) Huang, F.; Huang, M.; Zhang, H.; Guo, B.; Zhang, D.; Zhou, G. Cleavage of the calpain inhibitor, calpastatin, during postmortem ageing of beef skeletal muscle. *Food Chem.* **2014**, *148*, 1−6.
(49) Bowker, B. C.; Fahrenholz, T. M.; Paroczay, E. W.; Eastridge, J. S.; Solomon, M. B. Effect of hydrodynamic pressure processing and aging on the tenderness and myofibrillar proteins of beef strip loins. *J. Funct. Foods* **2008**, *19* (1), 74−97.
(50) Cramer, T.; Penick, M. L.; Waddell, J. N.; Bidwell, C. A.; Kim, Y. H. B. A new insight into meat toughness of callipyge lamb loins-the relevance of anti-apoptotic systems to decreased proteolysis. *Meat Sci.* **2018**, *140*, 66.
(51) Dang, D. S.; Stafford, C. D.; Taylor, M. J.; Buhler, J. F.; Thornton, K. J.; Matarneh, S. K. Ultrasonication of beef improves calpain-1 autolysis and caspase-3 activity by elevating cytosolic calcium and inducing mitochondrial dysfunction. *Meat Sci.* **2022**, *183*, No. 108646.
(52) Mohrhauser, D. A.; Underwood, K. R.; Weaver, A. D. In vitro degradation of bovine myofibrils is caused by μ-calpain, not caspase-3. *J. Anim. Sci.* **2011**, *89*, 798−808.

https://doi.org/10.1021/acs.jafc.4c06663
J. Agric. Food Chem. XXXX, XXX, XXX−XXX