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Modification-Specific Proteomic Analysis Reveals Cysteine S-Nitrosylation Mediated the Effect of Preslaughter Transport Stress on Pork Quality Development

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ABSTRACT: This study aimed to explore the effects of preslaughter transport stress on protein S-nitrosylation levels and S-nitrosylated proteome in post-mortem pork *longissimus thoracis* (LT) muscle. Pigs (N= 16) were randomly divided into 3 h transport (high-stress group, HS) and 3 h transport followed by 3 h resting treatments (low-stress control group, LS). Results demonstrated that high transport stress levels induced nitric oxide (NO) overproduction by promoting NO synthase (NOS) activity and neuronal NOS (nNOS) expression, which thereby notably increased protein S-nitrosylation levels in post-mortem muscle ($p < 0.05$). Proteomic analysis indicated that 133 S-nitrosylation-modified cysteines belonging to 85 proteins were significantly differential, of which 101 cysteines of 63 proteins were higher in the HS group ($p < 0.05$). Differential proteins including cytoskeletal and calcium-handling proteins, glycolytic enzymes, and oxidoreductase were mainly involved in the regulation of muscle contraction and energy metabolism that might together mediate meat quality development. Overall, this study provided direct evidence for changes in S-nitrosylation levels and proteome in post-mortem muscle in response to preslaughter transport stress and revealed the potential impact of S-nitrosylated proteins on meat quality.

KEYWORDS: *preslaughter transport stress, S-nitrosylation, proteomic, pork quality*

INTRODUCTION

Meat quality attributes primarily include color, texture, and water holding capacity (WHC), which not only affect the purchasing behavior of consumers but also determine the further processing potential of meat. However, the problems of high purge loss and incidence of pale, soft, and exudative (PSE) pork triggered by preslaughter stressors lead to enormous economic losses and consequently limit the development of the pork industry. Preslaughter stressors primarily include repelling, loading, transit, improper lairage, and others.[1] Among these, preslaughter transport is common and inevitable in modern husbandry practices and is considered a critical determinant affecting animal welfare and carcass quality.[2] It has been extensively discussed in the past decades that preslaughter transport-induced acute stress could impair meat quality via mediating the processes of energy metabolism, ionic equilibrium, proteolysis, protein oxidation, and apoptosis of post-mortem muscle.[1,3] Despite these observations, it still remains largely undefined about the involved molecular mechanisms of meat quality change as a result of preslaughter transport stress.

Nitric oxide (NO) acts as a second messenger in skeletal muscle and is considered to be a crucial regulator of muscle metabolism and contraction. Particularly, the reaction of NO binding to the thiol group of cysteine residues to form S-nitrosothiol is known as protein S-nitrosylation, which is the typical and main pathway by which NO exerts its biological effects in post-mortem muscle.[4] As a widespread protein post-translational modification, protein S-nitrosylation, similar to phosphorylation and acetylation, can regulate protein activity and function in organisms by changing the structure and stability of proteins.[5] In pork LT muscle, many proteins involved in the regulation of glycolysis, calcium (Ca^{2+}) release, stress response, and redox status have been identified to be modified by S-nitrosylation.[6] Furthermore, our lab has shown that S-nitrosylation of glycolytic enzymes and Ca^{2+}-handling proteins, such as glycogen phosphorylase (GP), pyruvate kinase (PK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sarcoplasmic reticulum calcium ATPase (SERCA), and ryanodine receptor 1 (RyR1) could regulate their activities via S-nitrosylation-advanced conformational changes,[7−9] thus controlling glycolysis rate and ultimate pH of post-mortem muscle. S-Nitrosylation of calpain inhibited its proteolytic activity, thereby reducing the hydrolysis degree of cytoskeletal proteins such as desmin in post-mortem muscle.[10,11] Recent studies from our lab have discovered that protein S-nitrosylation could inhibit the apoptosis process of post-mortem muscle cells through the mitochondrial pathway, which subsequently delayed the tenderization process of beef.[12] More importantly, it has been reported that exercise training or acute stress could induce NO overproduction in human and mouse skeletal muscle via promoting nitric oxide

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synthase (NOS) activity.[13,14] Cottrell et al.[15] also demonstrated that the modulation of ovine skeletal muscle metabolism by the treatment of NOS activity inhibition with *Nω*-nitro-L-argininemethyl ester hydrochloride was dependent on the degree of NOS stimulation by exercise or absence of exercise. Furthermore, neuronal NOS (nNOS) is known as the major isoform of NOS in skeletal muscle, and its abundance and localization contribute distinctly to NOS activity and the resulting NO production and function. Our recent studies have shown that nNOS was not only localized in the pork LT muscle membrane but also partially distributed in the cytoplasm, and importantly, this distribution was observed to be intensified by preslaughter transport stress.[16] Based on the above facts, we speculate that preslaughter transport stress could increase protein S-nitrosylation levels and then change S-nitrosylated proteome in post-mortem muscle by promoting NO synthesis, which might in turn affect meat quality by mediating various biological processes involved during the conversion of muscle to meat.

Recently, modification-specific proteomics such as phosphoproteome and acetylated-proteome have greatly contributed to discovering protein biomarkers with various meat quality and exploring the molecular mechanism of impaired meat quality in response to preslaughter stress.[17,18] However, there are no investigations regarding the changes in overall protein S-nitrosylation levels induced by preslaughter stress in post-mortem muscle and the specific S-nitrosylated sites and S-nitrosylated proteins mediating the effect of preslaughter stress on meat quality development. Thus, this study first assessed the level of preslaughter stress in pigs by means of a blood stress index. Subsequently, the overall protein S-nitrosylation levels of pork *longissimus thoracis* (LT) muscle at 1 h post-mortem was evaluated by NOS activity, nNOS expression, NO content, and abundance of S-nitrosylated protein. The changed S-nitrosylated proteome was determined using the biotin S-nitrosylated-peptide enrichment method in combination with tandem mass tag (TMT)-based quantitative proteomic technology. It is anticipated that the results of this study will contribute a novel insight into the change of post-mortem meat quality in pigs subjected to preslaughter transport stress.

MATERIALS AND METHODS

Experimental Design and Sampling Preparation. A total of 16 castrated crossed (Duroc × Landrace × Yorkshire) with an age of 6 months (110 ± 10 kg) obtained from a local commercial farm (Zhengda Pig Industry Co., Ltd., Suqian, China) were used for the present transport trial. The pigs were randomly assigned to two treatments (n = 8): 3 h transport without resting (transport-induced high-stress, HS) and 3 h transport followed by 3 h resting (low-stress control, LS). In the current study, we chose 3 h transport followed by 3 h rest as a lower-stress control group, as previously stated.[19,20] The transport vehicle was driven on a flat road (60 km/h) without stop. During transport, the average ambient temperature was about 30 °C. Pigs were slaughtered at a commercial slaughter factory (Sushi Meat Products Co., Ltd., Huai'an, China) according to the Chinese national slaughtering standards (GB/T 19479-2019). Blood samples were immediately collected in 5 mL heparinized tubes (Jiancheng Bioengineering Institute, Nanjing, China), while pigs were exsanguinated and centrifuged at 2500g at 4 °C for 10 min. The collected supernatant was then stored at −80 °C for determining the blood stress indicator. At 1 h post-mortem, the LT muscle between the sixth and 10th thoracic vertebrae was used to measure pH and then excised from the left and right side of carcasses. At 1, 12, and 24 h post-mortem, the right LT muscle was taken, quickly frozen in liquid nitrogen, and then stored at −80 °C for the determination of glycogen content and lactate concentration. The overall protein S-nitrosylation levels and the changed S-nitrosylated proteome were determined using samples taken at 1 h post-mortem. The left LT muscles were stored at 4 °C and used to measure meat quality at 12 and 24 h post-mortem.

Plasma Stress Indicators. The activity of plasma creatine kinase (CK) was detected by commercial kits (Jiancheng Bioengineering Institute, Nanjing, China), as previously stated.[18]

Meat Quality. Meat quality was evaluated by referring to the procedures of Wang et al.[9] with some modifications. Briefly, the pH of the meat sample was detected at the corresponding time points using a portable pH meter (205-PH1, Testo Fisher, Germany). At 12 h post-mortem, the lightness (L^*) value was determined by a colorimeter (CR-400, Konica Minolta, Inc., Tokyo, Japan) equipped with 8 mm diameter, D65 illuminant, 10° observer angle, and 11 mm opening aperture. For the drip loss, the meat sample of 1 h post-mortem was trimmed into 2 × 2 × 2 cm^3 pieces along the myofibril direction and weighed as M1, and then suspended in a sealed incubator at 4 °C for 48 h. Next, the sample was weighed again as M2. The drip loss was obtained by the equation: drip loss (%) = (M1 − M2)/M1 × 100%. For the shear force, the meat sample of 24 h post-mortem was trimmed into 2 × 3 × 5 cm^3 pieces, packaged in a boiling bag, and immersed in a water bath at 72 °C until the center temperature reached 70 °C. After cooling, the sample was trimmed into 1 × 1 × 3 cm^3 columns along the myofibril direction. The shear force was determined by using a tenderizer (XL 1155, Tenovo, Haerbing, China) with a shear rate of 5 mm/s. Three meat samples from each LT muscle were analyzed, and an average value was obtained as one replication for statistical analysis.

Glycogen Content and Lactate Concentration. Glycogen content and lactate concentration were determined in accordance with the procedures of Wang et al.[8] using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

NOS Activity. The NOS activity was detected in accordance with an assay kit (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 0.5 g of muscle was mixed with 4.5 mL of prechilled standard PBS (0.01 M, pH 7.2) and homogenized at 10 000 rpm (3 × 15 s, 4 °C). The homogenate was centrifuged at 4000g for 15 min (4 °C). The supernatant protein concentration was measured by using a Coomassie Brilliant Blue kit (Jiancheng Institute, Nanjing, China). The 50 μL of supernatant or PBS was mixed with 100 μL of substrate solution, 5 μL of accelerator, and 50 μL of chromogenic solution and incubated at 37 °C for 15 min. Then, 100 μL of clearing agent and 2 mL of terminator were added to the reaction solutions, and the absorbance was measured at 530 nm (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA). The NOS activity is expressed as nmol/mg protein/min.

nNOS Content. The nNOS content was detected referring to Ma et al.[16] Briefly, the LT muscle (0.5 g) was added to 4.5 mL of prechilled buffer (50 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, and 5 mM dithiothreitol, pH 7.4) and homogenized at 10 000 rpm (3 × 15 s, 4 °C). The homogenate was then centrifuged at 10 000g for 15 min (4 °C). The supernatant protein concentration was detected by a bicinchoninic acid (BCA) kit (Solarbio, Beijing, China) and diluted to 6 mg/mL. Next, the loading buffer (JBS-0654, GenScript, China) was mixed with the supernatant in a 1:1 ratio (v/v) and then boiled at 95 °C for 5 min. Afterward, 40 μg of proteins was loaded into the 8% precast gel and separated using an electrophoresis system (80 V for 30 min and 120 V for 90 min, Bio-Rad, CA, USA). After the protein was transferred to the poly(vinylidene difluoride) (PVDF) membrane (90 V for 90 min at 4 °C), the 5% skim milk dissolved in Tris-buffered saline with Tween-20 (TBST) buffer (137 mM NaCl, 5 mM KCl, 20 mM Tris-base, and 0.05% Tween 20) was used to block the PVDF membrane at 25 °C for 2 h. Next, the membrane was washed (3 × 10 min) with TBST buffer and then incubated for 12 h with the antibody of nNOS (sc-5302, Santa Cruz, USA) and GAPDH (A19056, Abclonal, China) diluted in TBST buffer (1:1000 and 1:80 000), respectively. After that, the membrane was rewashed (3 × 10 min) and subsequently incubated at 25 °C for 2 h using the antibody of goat-antimouse/rabbit IgG-HRP (CW0102S/CW0103S,

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Kangwei Century, China) diluted in TBST buffer (1:5000). After washing, the membrane was incubated with ECL reagent (34580, Thermofisher Scientific, USA) in the dark for 5 min and imaged with the Image Quant LAS4000 (GE, CT, USA). Finally, the band intensity was analyzed with Image J software (NIH, Bethesda, MD).

NO Content. NO content was determined by a NO content test kit (Beyotime Co., Ltd., Shanghai, China). Briefly, 0.2 g of muscle was mixed with 1.8 mL of NO special lysate (Beyotime, Shanghai, China) and homogenized at 3000 rpm for 20 s (4 °C). The homogenate was centrifuged at 4000*g* for 10 min, and then the supernatant was collected. In the dark, 50 μL of Griess reagent I and 50 μL of Griess reagent II were sequentially added to 50 μL of the supernatant. The absorbance was measured at 540 nm. The NO content was calculated from the standard curve and expressed as μM.

Overall Protein S-Nitrosylation Levels. The overall protein S-nitrosylation levels were measured using a pierce S-nitrosylation Western blot kit (90105, Thermo Scientific, USA). The specific experimental procedures are consistent with one of our previously published studies.[16]

S-Nitrosylated Peptide Enrichment and TMT Labeling. Briefly, the LT muscle was ground into powder using liquid nitrogen, and then 50 mg of powders were dissolved in 200 μL of PBS buffer A (4% SDS and 150 mM Tris-HCl, pH 8.0) and sonicated for 2 min under ice water bath. Protein was extracted by centrifugation, and the concentration was detected by a BCA kit. The collected protein (1 mg) was incubated with 5 mL of PBS buffer B (0.5% SDS, 1% Triton X-100, protease inhibitor, 25 mM NEM, and 5 mM EDTA) for 12 h at 4 °C, and the excess NEM was removed by precooled acetone precipitation. Then, the protein was resuspended in 1 mL of PBS buffer C (4% SDS, protease inhibitor, and 5 mM EDTA, pH 7.4), mixed with sodium ascorbate (final concentration: 20 mM) and 500 μL of 4 mM Biotin-HPDP, and then incubated at 25 °C for 1.5 h. After that, the protein was again precipitated with acetone, resuspended in 6 M urea, and diluted 6-fold with 50 mM ammonium bicarbonate. The protein was further digested (protein:enzyme (m/m) = 25:1) with trypsin (Madison, WI, USA) at 37 °C for 14 h. Next, the peptide was desalted with a C18 spin column, resuspended with 200 μL of PBS buffer D (0.2% SDS, 0.2% Triton X-100, and 500 mM NaCl), and then incubated with 100 μL of high-capacity streptavidin beads for 1 h at 25 °C. After being cleaned five times in PBS buffer E (0.2% SDS and 500 mM NaCl), the bead was suspended with 200 mM HEPES, and the peptide was labeled with TMT reagents. After the mixture was reacted at 25 °C for 1 h, the mixed labeled peptides were incubated with 0.2 mL of eluent (50 mM NH_4HCO_3 and 10 mM TCEP, pH 8.2) for 1 h at 25 °C. The eluted peptides were then collected, mixed with iodoacetamide (final concentration: 20 mM), and incubated in the dark for 1 h. Finally, the TMT labeled peptide was fractionated with a high-pH reversed-phase using a C18 column, and six fractions were desalted with C18 Stage Tips and prepared for further high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Some special reagents and materials in this section, including a protease inhibitor, NEM, sodium ascorbate, biotin-HPDP, HEPES, TMT reagent, C18 spin column, high-capacity streptavidin bead, and C18 Stage Tips were purchased from Thermo Fisher (ThermoFisher Scientific, Massachusetts, USA).

LC-MS/MS Identification, Database Searching, and Bioinformatics Analysis. LC-MS/MS was conducted with a Q Exactive HF-X (ThermoFisher Scientific, Massachusetts, USA) coupled with an Easy nLC 1200 system for chromatographic separation. The column C18 column (75 μm × 200 mm, 3 μm, Dr. Maisch GmbH) was equilibrated in buffer A consisting of 0.1% formic acid, and samples were separated (300 nL/min) with a linear gradient using buffer B consisting of 85% acetonitrile and 0.1% formic acid. The linear gradient was set as follows: 0−2 min, 2−8% buffer B; 2−45 min, 8−28% buffer B; 45−50 min, 28−40% buffer B; 50−52 min, 40−100% buffer B; 52−60 min, buffer B maintained at 100%. The separated peptides were analyzed by mass spectrometry. The specific parameters were set according to the study of Liu et al.[21] The original LC-MS/MS data were imported into the Proteome Discoverer 2.4 software (Thermo Fisher Scientific, IL, USA) for database searching against the porcine protein database from UniProt-Sus scrofa (Pig) [9823]-329592−20221021.fasta. The search was executed with tryptic cleavage specificity with up to two miscleavages. The primary search segment and MS/MS tolerances were set to 10 ppm and 0.02 Da, respectively. The false discovery rate of peptide spectral matches and protein was set to below 0.01. The $p < 0.05$ and the fold-change (FC) >1.30 or <0.77 (HS vs LS groups) were utilized as cutoff values to screen the S-nitrosylated site and protein variously determined between HS and LS groups. Gene Ontology (GO) terms (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/) of differential proteins were carried out with the Fisher's exact test and the enriched GO and KEGG pathways were significant at the $p < 0.05$ level. Additionally, the String database (https://string-db.org/) was performed for the construction of protein−protein interaction (PPI) networks.

Statistical Analysis. All data were analyzed by the SPSS 22.0 software (SPSS, Inc., USA), and the results were shown as mean ± standard deviation. A univariate model with the Fisher's Least Significant Difference test was conducted to analyze the differences among the individual groups for the indexes of pH, glycogen, and lactic acid. For other indicators, a *t* test was employed to compare the differences between the two groups. The significant difference was set at $p < 0.05$.

RESULT AND DISCUSSION

Plasma Stress Indicator and Meat Quality. Previous research has indicated that preslaughter stress could cause the destruction of muscle cells, thus leading to the release of CK into the blood. Therefore, the activity of CK in blood, as a stress-sensitive indicator, is widely employed to assess the preslaughter stress level of livestock and poultry.[22] As shown in Figure 1, the plasma CK activity in the HS group was remarkably higher than that in the LS group ($p < 0.05$), implying that the pigs of the HS group had a higher preslaughter stress level.

Meat quality attributes are exhibited in Figure 2D−H. The samples from the HS group showed a higher drip loss and L^* compared to the LS group ($p < 0.05$), which was possibly related to the increased incidence of PSE pork.[21] This result is

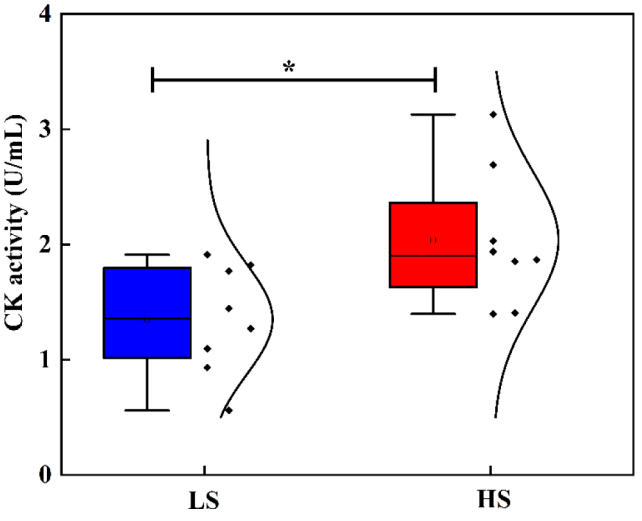

Figure 1. Change in plasma stress indicator from pigs with different preslaughter management methods. * indicates a significant difference between the two groups ($n = 8$), where ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. HS represents the transport-induced high-stress group. LS represents the low-stress control group.

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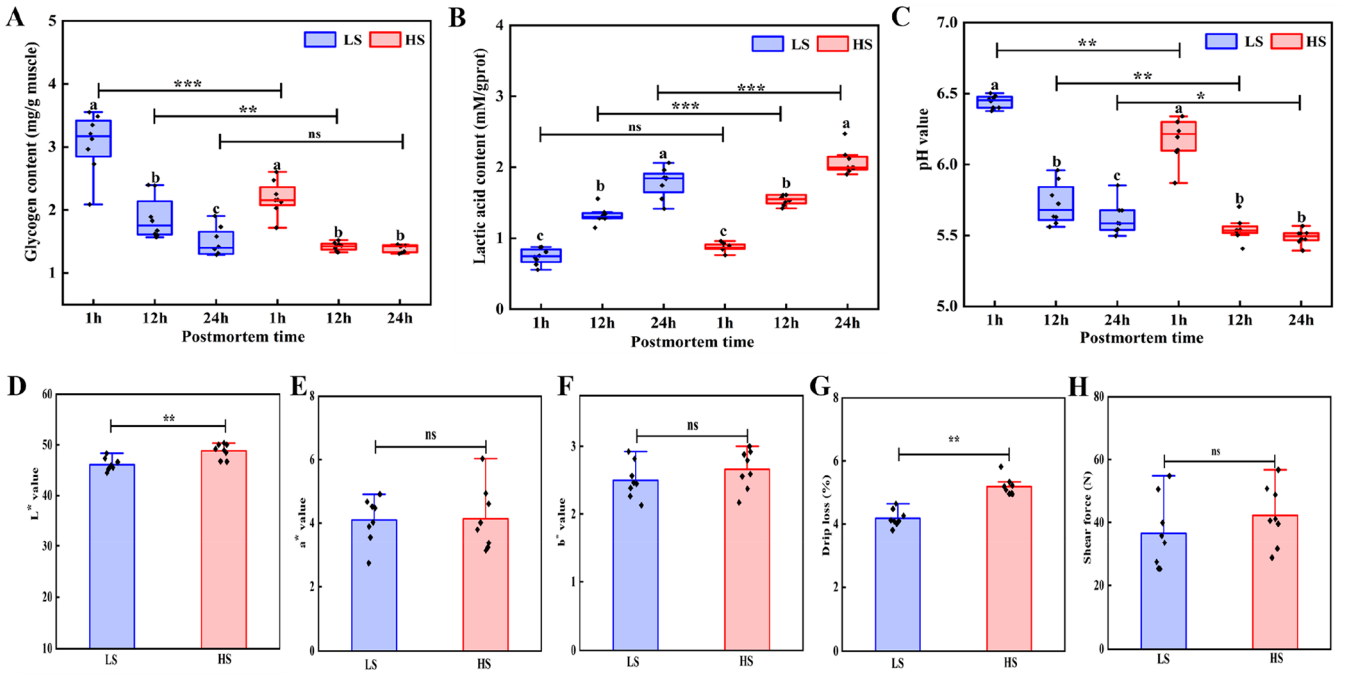

Figure 2. Changes in muscle glycogen content, lactic acid content, and meat qualities from pigs with different preslaughter transport stress levels, (A) muscle glycogen content, (B) muscle lactic acid content, and (C−H) meat quality indicators. * indicates a significant difference between the two groups (n = 8), where ns indicates $p > 0.05$, * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$. HS represents the transport-induced high-stress group; LS represents the low-stress control group.

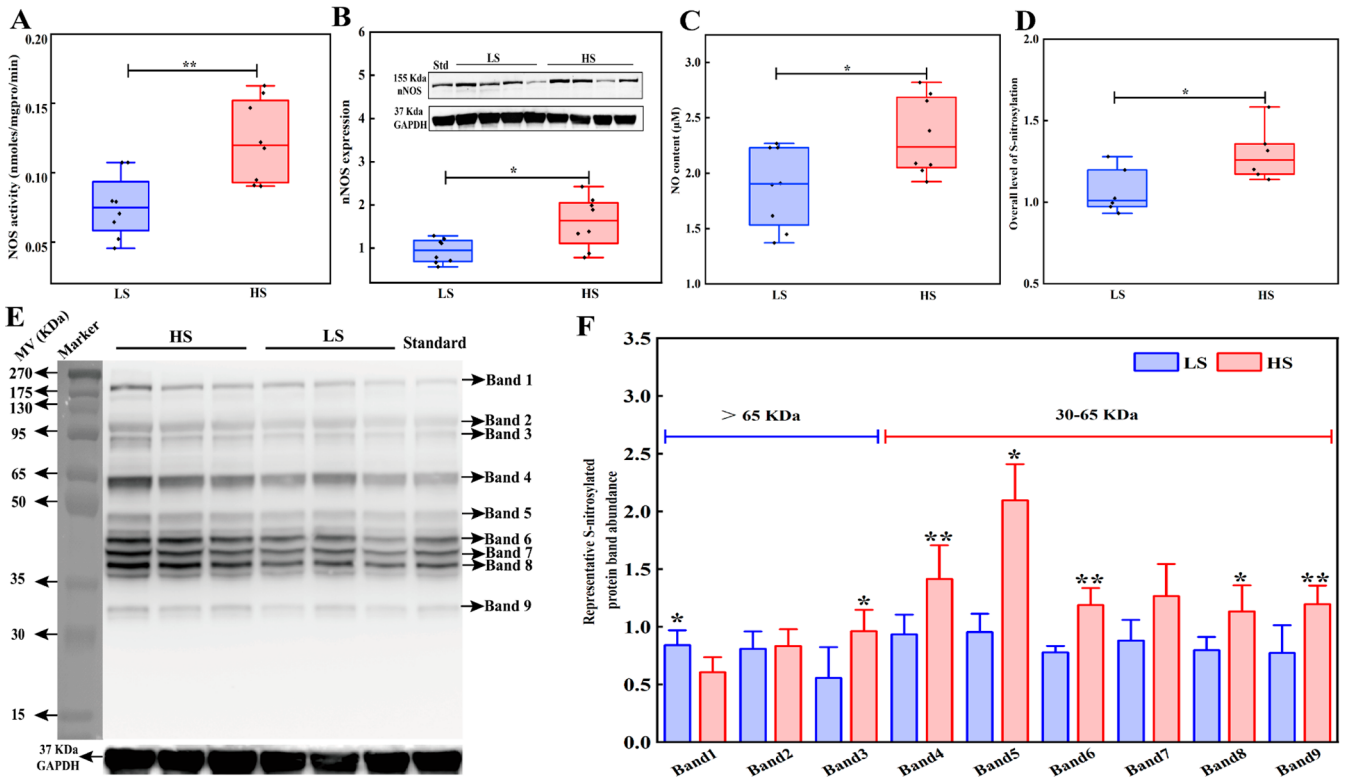

Figure 3. Changes in protein S-nitrosylation levels of post-mortem LT muscle from pigs with different preslaughter transport stress levels, (A) NOS activity, (B) nNOS content, (C) NO content, (D) abundance of overall S-nitrosylated protein, (E) images of Western blotting of overall S-nitrosylated protein, and (F) abundance of representative protein bands. * indicates a significant difference between two groups (n = 8), where * indicates $p < 0.05$, and ** indicates $p < 0.01$. Std indicates the standard sample selected from post-mortem muscle for comparative analysis in different batches of experiments. HS represents the transport-induced high-stress group; LS represents the low-stress control group.

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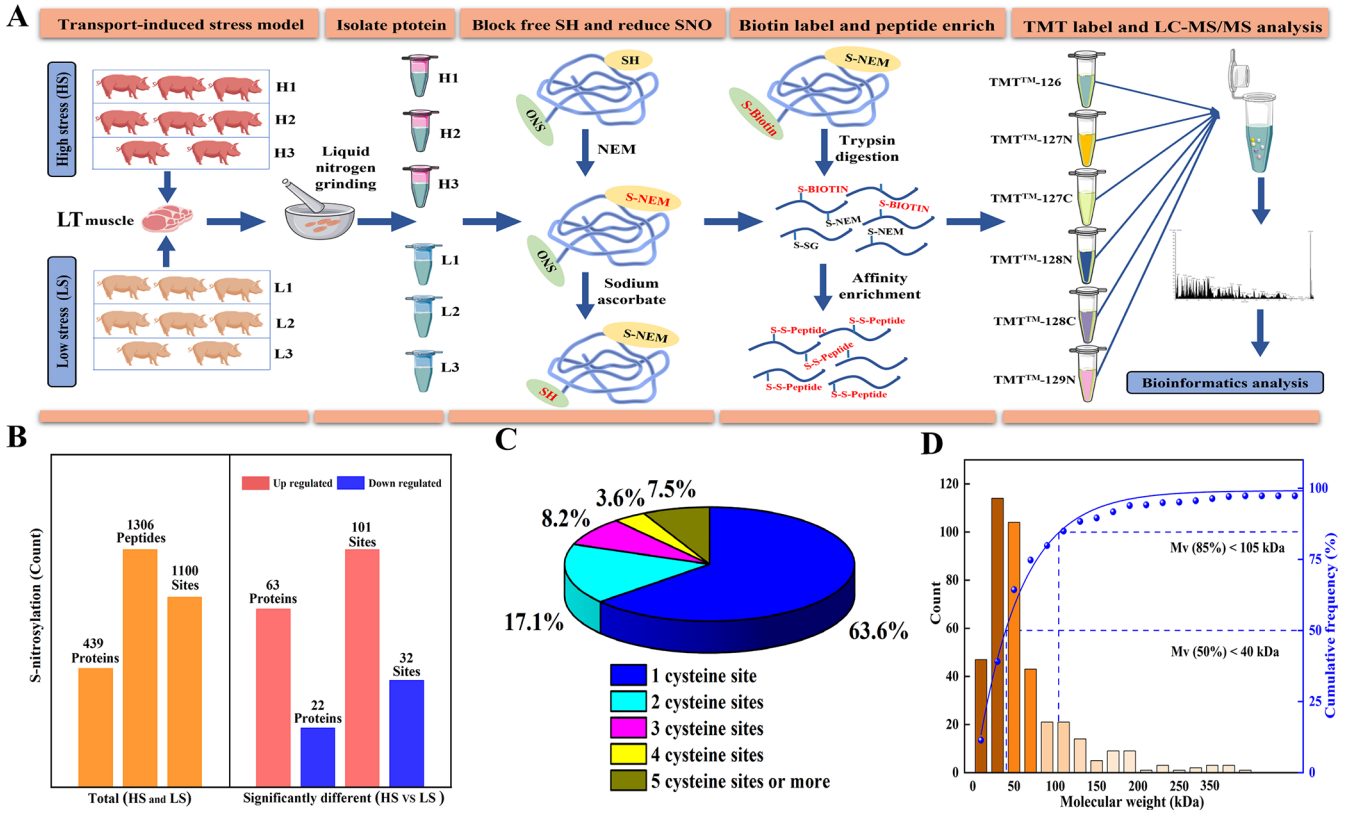

Figure 4. Modification-specific proteomics identification of S-nitrosylated proteins and sites in post-mortem LT muscle from pigs with different preslaughter transport stress levels, (A) workflow for biotin enrichment of S-nitrosylated peptides and TMT-labeling based quantitative proteomic analysis, (B) number of total and differential S-nitrosylated proteins and sites, (C) distribution of cysteine modification site count, (D) molecular mass distribution of S-nitrosylated proteins. HS represents the transport-induced high-stress group. LS represents the low-stress control group.

in line with the investigation of Zheng et al.,[20] who found that the muscles from pigs without resting after transport exhibited an increased L^* and a decreased WHC. However, no significant variation was found in shear force, a^*, and b^* between the HS and LS groups ($p > 0.05$). Additionally, a lower muscle glycogen content from 1 to 12 h, a higher lactic acid concentration from 12 to 24 h, and a lower muscle pH during the whole post-mortem period were determined in the HS group in comparison to the LS group (Figure 2A−C, $p < 0.05$). The results could be explained by the fact that preslaughter transport stress could promote glycogenolysis via activating adenosine 5′-monophosphate-activated protein kinase (AMPK) signaling pathway in the early post-mortem muscle and thus cause an increased pH drop rate and low ultimate pH.[23] Abnormally high glycolytic rate at the early post-mortem period is directly responsible for the formation of PSE meat.[24] In summary, the above results demonstrate that the muscle samples from the HS group exhibited noticeable changes in the metabolic rate of post-mortem muscle and meat color and water holding capacity relative to the LS group.

NOS Activity, nNOS Expression, and NO Content. Endogenous NO, as a critical mediator of protein S-nitrosylation, is mainly synthesized during the transformation of L-arginine to L-citrulline catalyzed by NOS. In mammals, three isoforms of NOS including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) have been identified. Among these, nNOS is the primary isoform in skeletal muscle, and its expression contributes to a pronounced effect on NOS activity and resulting NO production.[25] As shown in Figure 3A,B, a relatively higher NOS activity and an increased nNOS expression were determined in the HS group compared to the LS group ($p < 0.05$), indicating that higher preslaughter transport stress levels positively contributed to the NOS activity and the nNOS expression of post-mortem muscle. This result appears to be partly inconsistent with the study of Wang et al.,[9] where higher nNOS expression and lower NOS activity were found in PSE pork than that of normal pork at 1 h post-mortem. This might be attributed to the fact that PSE meat possessed higher carcass temperature and lower pH in the early post-mortem compared with the HS group samples, which together induced severe denaturation of NOS and thus inhibited its activity.[26] Moreover, several investigations have shown that different protein interactions, post-translational modification levels (e.g., oxidation and S-nitrosylation), and myofiber contraction degree could be associated with NOS activity and nNOS expression.[8,16] Additionally, the NO content in the HS group corresponding to the changes in NOS activity and nNOS content was higher than that of the LS group (Figure 3C, $p < 0.05$). In summary, diverse nNOS content, NOS activity, and NO levels between the HS and LS groups presumably imply different protein S-nitrosylation levels in post-mortem muscle. For confirming the speculation, the iodoTMT switch method combined with immunoblotting was further used to evaluate the overall protein S-nitrosylation levels in subsequent experiments.

Overall Protein S-Nitrosylation Levels. As shown in Figure 3E, the S-nitrosylated proteins (SNO-Pr) in pork LT muscle exhibited multiple bands on the immunoblot and were primarily distributed in 35−65 kDa, suggesting that a considerable quantity of proteins was modified by S-nitro-

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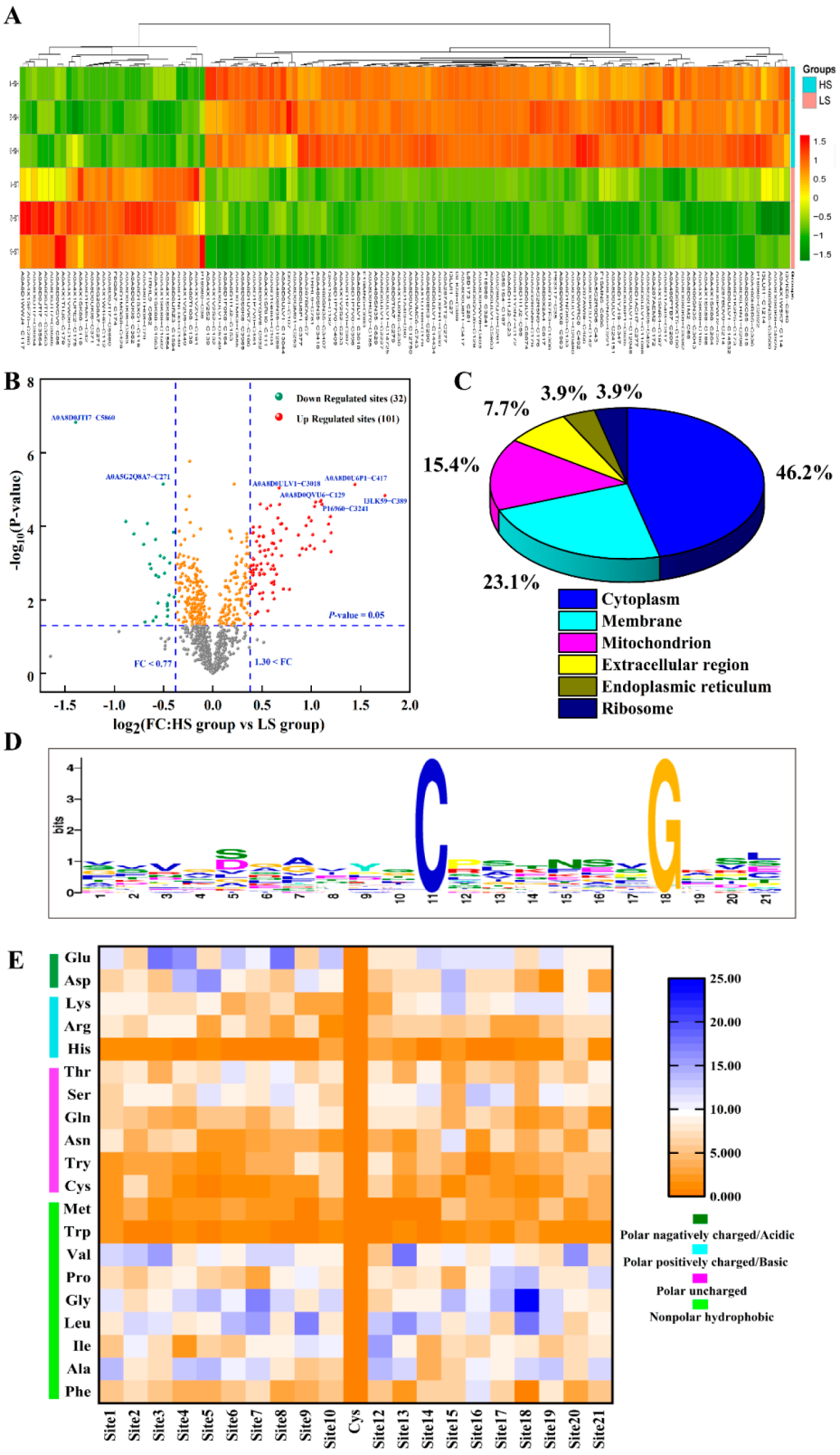

Figure 5. Modification-specific proteomics identification of S-nitrosylated proteins and sites in post-mortem LT muscle from pigs with different preslaughter transport stress levels, (A) heatmap of differentially S-nitrosylated sites. (B) volcano plot of differentially S-nitrosylated sites, (C) subcellular localization of differential S-nitrosylated proteins, (D) primary sequence motifs of ±10 amino acids from differential S-nitrosylated cysteine sites (the size of the amino acid character indicates the likelihood of its appearance in the SNO motif), (E) heat map of the frequency distribution of amino acids flanking differential S-nitrosylated cysteine sites. HS represents the transport-induced high-stress group. LS represents the low-stress control group.

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sylation. The overall intensity analysis of band showed that the HS group was much higher than that of the LS group (Figure 3D, $p < 0.05$), indicating that the preslaughter transport stress increased protein S-nitrosylation levels in post-mortem muscle. In addition, there was no significant discrepancy in the number of SNO-Pr bands between the two groups. However, the intensity analysis of individual bands showed a high variability. Compared with the LS group, the HS group exhibited a higher intensity in bands 3, 4, 5, 6, 8, and 9, while a lower intensity in band 1(Figure 3F, $p < 0.05$). The rest of the protein bands were not remarkably different between the two groups ($p > 0.05$). As an essential reaction substrate, the NO synthesis contributes substantially to protein S-nitrosylation levels in post-mortem muscle. The result here supports that preslaughter higher transport stress levels could induce NO overproduction by promoting the NOS activity and nNOS expression, which ultimately caused a higher protein S-nitrosylation level in post-mortem muscle. Several studies have investigated the relationship between other modification levels (e.g., phosphorylation and acetylation) and meat quality development under different preslaughter stress levels and differential phosphorylated and acetylated proteins involved in muscle contraction and glycolysis have been identified.[17,18] Likewise, this work also proved that protein S-nitrosylation levels in post-mortem muscle from pigs with different preslaughter transport stress levels were notably diverse, implying that NO and its induced protein S-nitrosylation might be the mediators of meat quality changes induced by preslaughter transport stress.

Identification of SNO-Pr. To further excavate the S-nitrosylated proteome alterations in pork LT muscle at 1 h post-mortem in response to preslaughter transport stress, we applied a biotin switch enrichment method in combination with a TMT-based quantitative proteomic method for determining the protein profiles (Figure 4A). In total, 1100 S-nitrosylated cysteine-sites (SNO-Cys) from 439 proteins were detected and accurately quantified across all samples (Figure 4B). According to Figure 4C, it could be found that 63.6% of identified SNO-Pr was mono-S-nitrosylated, about 17% was di-S-nitrosylated, and about 8% was detected to possess 3 SNO-Cys. The highest number of SNO-Cys was identified in titin isoform X6 (55), followed by fibrillin-1 (27) and filamin C (21). Besides, 85% of identified SNO-Pr had a molecular weight below 105 kDa, including 50% below 40 kDa (Figure 4D). This outcome is in agreement with the analysis of the Western blot showing that most SNO-Pr was concentrated below 100 kDa, mainly in the range of 25−55 kDa (Figure 3E), indicating that proteins with a small molecular weight might be more easily modified by S-nitrosylation. In addition, 133 SNO-Cys with remarkable differences corresponding to 85 proteins between HS and LS groups were identified and presented in Supporting Information Data 1. Compared with the LS group, the HS group possessed 101 significantly up-regulated SNO-Cys belonging to 63 proteins as well as 32 significantly down-regulated SNO-Cys from 22 proteins (Figure 4B and Figure 5B, $p < 0.05$). To visualize the differential SNO-Cys between the two groups, a hierarchical clustering analysis was executed in the current study. As displayed in Figure 5A, the similarity of SNO-Cys abundance within groups was high, whereas it was extremely poor between groups, indicating diverse response mechanisms of post-mortem biochemical in pork LT muscle from pigs with different preslaughter transport stress levels.

Subcellular Localization of Differential SNO-Pr and Primary Sequence Motif of Differential SNO-Cys. Exploring the subcellular localization of differential SNO-Pr would further provide insight into how preslaughter transport stress-induced S-nitrosylation affected meat quality development. As shown in Figure 5C, differential SNO-Pr was mainly localized in the cytoplasm, membrane, mitochondrion, and extracellular regions, with the highest ratio in the cytoplasm (46.2%). It indicates that S-nitrosylation might be implicated in a broad range of cellular structures and actively participate in various biochemical regulatory pathways occurring in the cytoplasm (e.g., glycolysis, Ca^{2+} release, and apoptosis), which in turn possibly mediate the development of post-mortem meat quality. In addition, discovering the structural commonality of differential SNO-Cys could provide clues to elucidate the selectivity and specificity of S-nitrosylation modification on the target cysteine.[27] In this study, the regularity of the primary sequence motif with a total of 21 amino acid residues using the cysteine site as the central point was calculated by Motif-X. As presented in Figure 5D, only one underlying motif (xxxxxxxxxCxxxxxGxxx) was identified in differential SNO-Cys, suggesting that the glycine (G) was typically flanked at the +7 position of SNO-Cys. Similar results were previously demonstrated by Zhu et al.,[26] who reported that the G was flanked through the SNO-Cys at sites −3, −6, and +6 separately and suggested that these motifs might facilitate NO to accommodate the surrounding biological mediators. In addition, this study also showed the distribution pattern of 20 common amino acids on both sides of SNO-Cys by drawing the frequency heat map of amino acids flanking SNO-Cys (±10 positions) (Figure 5E). The results demonstrate that there were a large number of charged acidic amino acids (aspartate (Asp) and glutamate (Glu)) and hydrophobic amino acids (e.g., valine (Val), proline (Pro), and leucine (Leu)) in the flank of SNO-Cys. Moreover, the high frequency of hydrophobic amino acids surrounding SNO-Cys suggested the presence of a stronger hydrophobic region around it. Those motifs in the present study are basically comparable to the majority recognized motif which was characterized by partially charged amino acid residues (acid/base motifs) surroundings and hydrophobic domain.[27,28] Charged amino acid residues typically play an essential role in proteins. In addition to their involvement in direct acid−base motif-dependent Cys activation, it is also common for them to have a strong effect on the electrostatic potential distribution of proteins and consequently are essential for protein−protein interactions and protein modification.[29] Another feature of the structural environment of the NO-Cys site that is frequently identified is the hydrophobicity around modified Cys. Previously, it has been indicated that S-nitrosylated cysteines were located at hydrophobic pockets of proteins, which could sequester or stabilize NO radicals and thus enhance the possibility of forming S-nitrosylation species.[30] Synthesizing the results of the current study, it is further confirmed that the typical and main distribution pattern of SNO-Cys flanking amino acids was charged amino acids surrounded by hydrophobic domains, which might further favor NO to adapt to the surrounding biological media and exert its signal transduction through the S-nitrosylation pathway.

Bioinformatics Analysis of Differential SNO-Pr. The enriched GO terms, KEGG pathway, and PPI network were employed to explore the functional terms of differential SNO-Pr. The differential SNO-Pr was classified by biological

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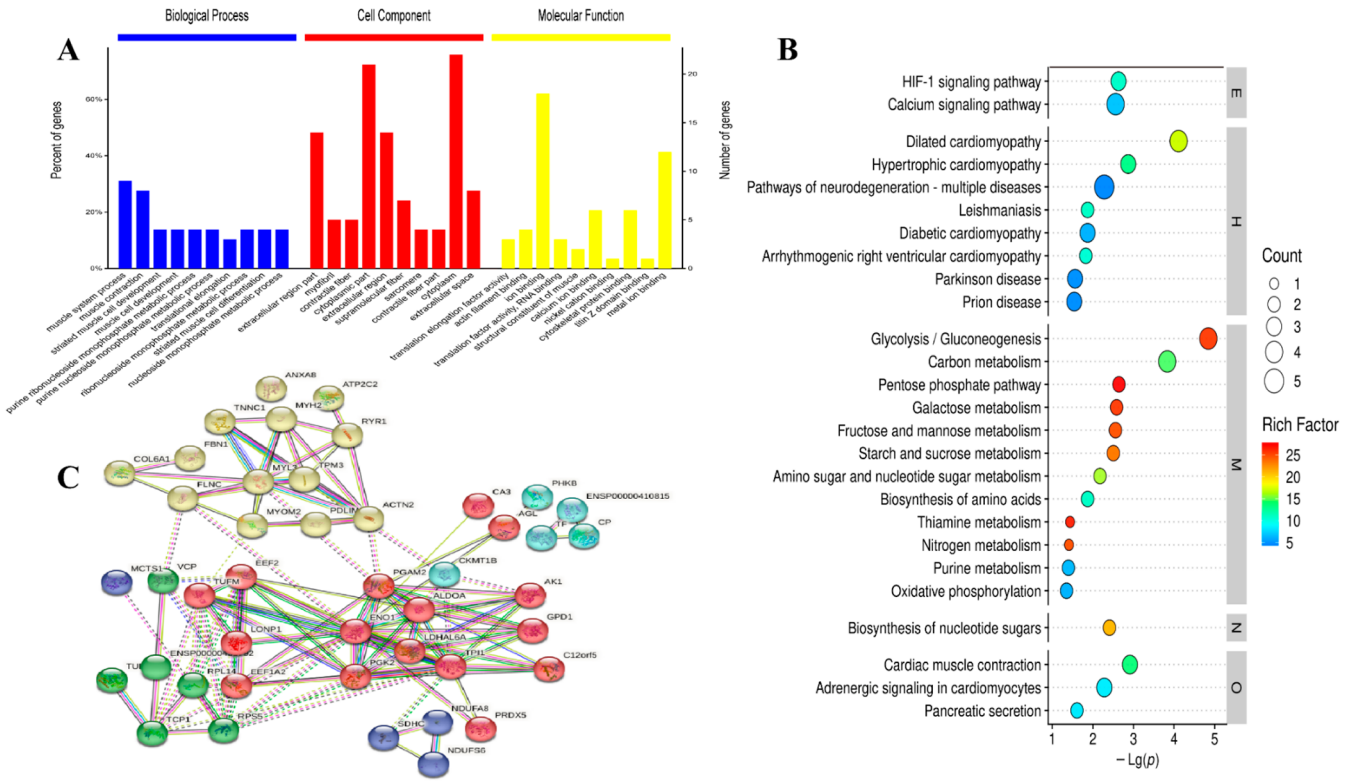

Figure 6. GO functional classifications (A), KEGG pathway (B), and Protein−protein interaction networks (C) of differentially S-nitrosylated proteins in post-mortem LT muscle from pigs with different preslaughter transport stress levels.

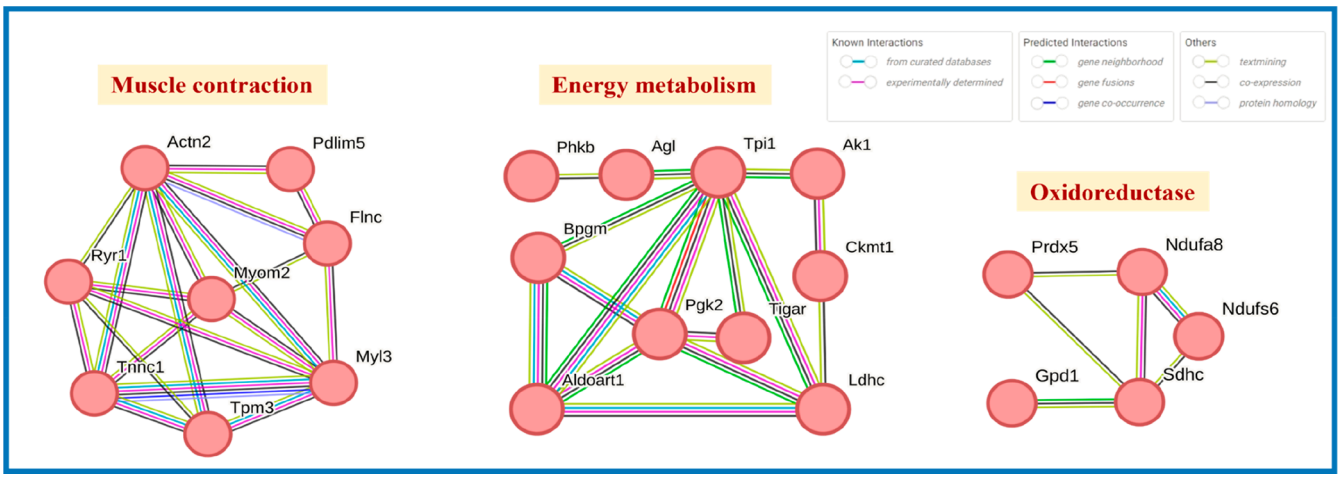

Figure 7. Representative protein−protein interaction networks of differentially S-nitrosylated proteins in post-mortem LT muscle from pigs with different preslaughter transport stress levels.

processes, molecular functions, and cellular components (Figure 6A). For biological processes, the differential SNO-Pr were primarily involved in muscular biological processes (muscle system process, muscle contraction, muscle cell development, and muscle cell differentiation) and metabolic processes (purine ribonucleoside monophosphate, purine nucleoside monophosphate, and nucleoside monophosphate). According to molecular function, numerous differential SNO-Pr presented a binding function including actin filament binding, ion binding, calcium ion binding, and cytoskeletal protein binding. Besides, the molecular functions of translation elongation factor activity and structural constituent of muscle were also enriched. The primary cellular components of these proteins were associated with cytoplasm, extracellular region, and myofibrils (such as contractile fiber, contractile fiber par, sarcomere, and supramolecular fiber). Additionally, the diverse metabolic pathways related to differential SNO-Pr were extracted by using KEGG pathway analysis. As shown in Figure 6B, the remarkable pathways for environmental information processing were found as Ca^{2+} and HIF-1 signaling pathways. The enrichment of pathways participated in post-mortem muscle metabolism was glycolysis/gluconeogenesis, carbon metabolism, oxidative phosphorylation (OXPHOS), and biosynthesis of amino acid. Besides, the remarkable pathway involved in organismal systems was identified as cardiac muscle contraction. Additionally, PPI

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Table 1. Candidates of Differential S-Nitrosylated Proteins and Cysteine Sites Identified from Pork LT Muscle between HS and LS Groups[a]

protein name	accession	annotated sequence	Cys-site	FC	*p*-value	type
Glycolysis						
fructose-bisphosphate aldolase	A0A4X1U0N5	[R].YASICQQNGIVPIVEPEILPDGDHDLK.[R]	C230	1.54	**	↑
L-lactate dehydrogenase	A0A8D0PWV6	[K].EDVFLSVPCILGQNGISDVVK.[V]	C403	2.14	**	↑
phosphoglucomutase	A0A8D0QVU6	[R].LVIGQNGILSTPAVSCIIR.[K]	C129	2.13	**	↑
triosephosphate isomerase	A0A8D1F0K8	[K].VAHALAEGLGVIACIGEK.[L]	C164	1.42	**	↑
phosphoglycerate mutase	A0A8D1I1J2	[R].YAGLKPGELPTCESLK.[D]	C153	1.45	**	↑
		[R].FCGWFDAELSEK.[G]	C23	2.02	**	↑
		[K].MEFDICYTSVLK.[R]	C55	2.29	**	↑
fructose-bisphosphatase	A0A8D1XFH1	[R].VPLILGSPDDVQEYLTCVQK.[N]	C300	1.46	**	↑
creatine kinase	A0A8D1A4I3	[R].LGYILTCPSNLGTGLR.[A]	C317	1.52	**	↑
		[K].VTPNGYTLDQCIQTGVDNPGHPFIK.[T]	C90	1.39	**	↑
Calcium Handling						
calcium-transporting ATPase	A0A8D0U6P1	[K].VEGDICLLNEFSITGSTYAPEGEVLK.[N]	C377	1.57	**	↑
		[R].AGQYDGLVELATICALCNDSSLDFNEAK.[G]	C417	2.72	**	↑
		[R].VIMITGDNKGTAIAICR.[R]	C635	1.54	**	↑
	A0A8D1MGS8	[K].VGEATETALTCLVEK.[M]	C429	0.76	**	↓
ryanodine receptor 1	P16960	[K].DGEDEEDCPLPDEIR.[Q]	C2022	1.71	**	↑
		[R].AILGLPNSVEEMCPDIPVLER.[L]	C3241	2.15	**	↑
four and a half LIM domains 1	A0A4X1SFZ0	[K].GEDFYCVTCHETK.[F]	C280	0.73	*	↓
annexin	A0A8D0TIA7	[R].VLIEILCTR.[T]	C279	1.39	**	↑
Oxidative Phosphorylation						
NDUFA8	A0A8D0PCU7	[K].LVNQCALDFFR.[Q]	C142	1.44	**	↑
NDUFS6	A0A8D0PCU7	[R].VISCDGGGGALGHPR.[V]	C86	0.72	*	↓
SDHC	D0VWV4	[K].SLCLGPTLIYTAK.[F]	C107	1.33	**	↑
Cell Structure						
myosin-2	A0A8D0X2A4	[R].ESIFCIQYNIR.[A]	C817	2.19	**	↑
myomesin 2	A0A8D1S7E3	[R].IGGSEEMAWLQICEPTEK.[D]	C1306	1.53	**	↑
titin isoform X6	A0A480SN35	[K].DTVGVLGTSCILECK.[V]	C1288	1.50	**	↑
		[R].NDAGMQECFATVSILEPAAIVEKPESIR.[V]	C3043	1.47	**	↑
		[R].SAILEIPSSTVEDAGQYNCYIENASGK.[D]	C3407	1.33	**	↑
		[K].DSCSAQILILEPPYFVK.[Q]	C3418	1.36	**	↑
		[K].SVDVTEKDPVTLECVVAGTPELR.[V]	C629	1.39	**	↑
tubulin alpha chain	A0A8D1YJ19	[R].SIQFVDWCPTGFK.[VG]	C347	1.54	**	↑
actinin alpha 2	F1RHL9	[R].ACLISMGYDLGEAEFAR.[I]	C781	1.33	**	↑
		[R].ELPPDQAQYCIK.[R]	C862	0.74	**	↓
tropomyosin alpha-3 chain	A0A4X1VZV4	[K].CSELEEELK.[N]	C191	0.68	**	↓
filamin C	F1SMN5	[K].AFGPGLEPTGCIVDKPAEFTIDAR.[A]	C683	1.61	**	↑
		[K].CAPGVVGPAEADIDFDIIK.[N]	C806	1.35	**	↑
troponin C	P63317	[K].AAFDIFVLGAEDGCISTK.[E]	C35	1.43	**	↑

[a]Note: Name and accession numbers of the proteins were obtained from the UniProt database (http://www.uniprot.org/). Significant S-nitrosylated cysteine sites in the table were filtered by abundance change (FC) >1.3-fold or <0.77 and FDR-adjusted *p*-value <0.05. The *p*-values are indicated as $*p < 0.05$ and $**p < 0.01$. NDUFA8, NADH dehydrogenase 1 alpha subcomplex subunit 8; SDHC, succinate dehydrogenase cytochrome b560 subunit; NDUFS6, NADH dehydrogenase iron−sulfur protein 6; Troponin C, troponin C, slow skeletal and cardiac muscles. HS represents the high-stress treatment group. LS represents the low-stress control group.

networks were constructed to investigate the interactions between differential SNO-Pr (Figure 6C). The first and largest cluster was closely associated with Ca^{2+} homeostasis (gene name: ATP2C2, and RYR1) and muscle shrinkage (gene name: TNNC1, MYH2, MYL3, FBN1, FLNC, MYOM2, TPM3, PDLIM, and ACTN2), while the second and third

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clusters were glycolytic enzymes (gene name: PGAM2, ALDOA, PGK2, TPI1, and LDHAL6A) and oxidoreductases (gene name: SDHC, NDUFA8, NDUFA6, and PRDX5), which were mainly implicated in the energy metabolism of post-mortem muscle (Figure 7).

According to Horenberg et al.,[31] a host of cytoskeletal proteins in striated muscle could be directly modified by NO-mediated protein S-nitrosylation. Likewise, Liu et al.[21] recently concluded that there were numerous cytoskeletal proteins modified by S-nitrosylation in the LT muscle myofibrillar protein and suggested that these SNO-Pr could be involved in the formation of PSE meat via mediating muscle contraction, cell development, and myofibril assembly. Moreover, previous reports from our laboratory have suggested that S-nitrosylation of Ca^{2+} channel proteins,[9] glycolytic enzymes,[8] and oxidoreductase[27,32] had crucial effects on the muscle contraction, energy metabolism, and muscle cell apoptosis, which were in turn involved in the regulation of meat quality (especially tenderness and WHC). All in all, bioinformatic analysis of differential SNO-Pr implied that cytoskeletal and Ca^{2+} homeostasis-related proteins mainly engaged in muscle shrinkage were interactive with glycolytic enzymes and oxidoreductase involved in energy metabolism, which might together serve as crucial roles in meat quality changes in response to preslaughter transport stress.

Biological Implication of Differential SNO-Pr in Post-Mortem Muscle. As shown in Table 1, the representatives of differential SNO-Pr were grouped into four functional categories by referring to Xing et al.[22] including glycolysis, Ca^{2+} handling, OXPHOS, and cell structure. It is noteworthy that many of these differential proteins have been demonstrated to serve as vital protein biomarkers to predict meat quality,[33] implying the potential role of S-nitrosylation in regulating the development of meat quality. Glycolysis is a critical multistep enzymatic reaction during the conversion of post-mortem muscle to meat, and the activity of glycolytic enzymes largely determines the level and speed of pH drop and consequently affects meat quality.[24] Extensive research has shown that triosephosphate isomerase (TPI), fructose bisphosphatase (FBP), L-LDH, phosphoglycerate kinase (PGK), and phosphoglycerate mutase (PGM) were closely associated with WHC, color, shear force, and ultimate pH of fresh meat.[33] In this study, the S-nitrosylation levels of seven enzymes catalyzing glycolytic reaction, including Cys129 of PGM, Cys300 of FBP, Cys164 of TPI, Cys296 of PGK, Cys430 of L-LDH, Cys230 of fructose-bisphosphate aldolase (ALDOA), and Cys129 of phosphoglucomutase (PGAM), were identified to be remarkably upregulated in the HS group compared with the LS group ($p < 0.05$). As S-nitrosylation modification could alter the activity, conformation, and function of proteins, the initial biological implication of these SNO-Pr might be partially altered with the increased S-nitrosylation levels. Previously, Liu et al.[4] demonstrated that the 11 glycolytic enzymes in post-mortem muscle could be modified by S-nitrosylation, especially, of which the activity of three pivotal rate-limiting enzymes (PK, GP, and phosphofructokinase) and GAPDH was inhibited by S-nitrosylation modification.[8,34] Although the current study was unable to identify significant alterations in S-nitrosylation levels of GAPDH and three rate-limiting enzymes that directly regulate the glycolytic process, some enzymes that play a key function in catalyzing the production of glycolytic metabolic intermediates were discovered to be remarkably altered. For instance, ALDOA affects fructose 1,6-diphosphate conversion levels and its S-nitrosylation modification was noted to induce dysregulation of the glycolytic pathway and adenosine triphosphate (ATP) depletion.[35] TPI acts as a critical part in the catalysis of dihydroxyacetone phosphate to glyceraldehyde-3 phosphate, and the report has revealed that the V_{max} of this catalytic reaction could be depressed by 30% once the TPI was S-nitrosylated.[36] PGK is an enzyme that drives the first energy-producing reaction (ATP synthesis) in the glycolysis process. Similar to our result, Liu et al.[21] also identified a higher S-nitrosylation level of PGK in PSE meat as compared with normal meat. L-LDH can convert pyruvate (the end product of muscle glycolysis) to lactate, which was responsible for the reduced pH in post-mortem muscle. According to Lu et al.,[7] NOR-3 (NO donor) treatment could increase the S-nitrosylation level of L-LDH while improving L-LDH activity, inducing an increased rate of glycolysis metabolism in an *in vitro* model. More importantly, the present study also identified that creatine kinase (CK) was modified by S-nitrosylation, in which Cys90 and Cys317 were significantly up-regulated in the HS group ($p < 0.05$). CK can catalyze the phosphocreatine conversion system to phosphorylate adenosine diphosphate into ATP, and a higher CK activity could contribute to maintaining ATP content in post-mortem muscle, thereby delaying the post-mortem glycolytic process.[37] However, it is clear that S-nitrosylation of CK could produce a strong inhibitory effect on its activity.[38] Thus, an interesting conjecture of this outcome is that the increased S-nitrosylation levels of CK inhibited its activity, disrupted the phosphocreatine conversion system, and further led to an imbalance between ATP supply and demand in the early post-mortem. As a result, the anaerobic glycolysis was prematurely activated, ultimately inducing a rapid reduction in pH of post-mortem muscle. In addition, the process of decomposing creatine phosphocreatine to produce ATP can consume H^+, and the inhibition of this conversion system could induce an excessive accumulation of H^+ and thus promote the drop of muscle pH. Together, these unearthed differential SNO-Pr might be the mechanisms by which transport-induced stress regulated the glycolysis process of post-mortem muscle and subsequently affected meat quality development.

Although anaerobic glycolysis is recognized as the primary pathway of post-mortem muscle energy metabolism, recent studies have demonstrated that the oxygen in post-mortem pork gradually decreased within 2 h post-mortem rather than immediately depleted.[39] This emerging data indicated that the mitochondrial OXPHOS could be carried out in the early post-mortem stage and might be a critical way to provide ATP for muscle cells. The OXPHOS is composed of five mitochondrial respiratory enzymes: complexes I, II, III, IV, and V.[40] In the current study, significant changes were observed in the S-nitrosylation levels of two subunits of complex I and one subunit of complex II. For complex I, NADH dehydrogenase iron−sulfur protein 6 (Cys86) in the HS group was down-regulated while NADH dehydrogenase 1 alpha subcomplex subunit 8 (Cys142) was up-regulated as compared with the LS group ($p < 0.05$). It has been revealed that NO could modify complexes I−IV through the S-nitrosylation pathway, inhibiting their activities, destroying the electron transport chain (ETC), and ultimately exerting a negative regulatory effect on OXPHOS.[41] When the ECT of OXPHOS is disrupted, mitochondrial ATP synthase will hydrolyze the ATP derived from glycolysis to stabilize the membrane potential ($ATP + H_2O \rightarrow ADP + P_i + H^+$), which in turn facilitates glycolysis to

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produce ATP and simultaneously results in proton load, thereby exacerbating the rate and extent of pH drop in post-mortem muscle.[42] In addition, this study also showed that the S-nitrosylation level of succinate dehydrogenase (SDH) cytochrome b560 subunit (Cys107) was higher in the HS group ($p < 0.05$). According to previous studies, SDH, forming complex II of the respiratory chain, was closely associated with metabolic events of post-mortem muscle and meat quality development.[43] Its activity could be suppressed by NO, which could be mainly attributed to the production of NO oxides such as peroxynitrite.[44] Inhibition of SDH not only led to a destruction of OXPHOS but also induced a dysregulation of the citric acid cycle,[45] which might together result in the premature activation of anaerobic glycolysis and a rapid drop of pH in post-mortem muscle. England et al.[46] suggested that one molecule of pyruvic acid could generate 15 molecules of ATP through a functioning mitochondrion. Thus, even minimal changes in mitochondria enzyme activity could also exert dramatic effects on post-mortem energy metabolism and resulting meat quality development.

In addition, the four proteins related to Ca^{2+} signaling were also detected to be remarkably changed in the S-nitrosylation levels. The S-nitrosylation abundance of Ca^{2+}-transporting ATPase (Cys377, Cys417, and Cys635), RyR1 (Cys2022 and Cys3241), and annexin (Cys279) in the HS group was remarkably higher, but lower for Ca^{2+}-transporting ATPase (Cys429), RyR1 (Cys36), and four and a half LIM domains 1 (FHL1, Cys280) when compared to the LS group ($p < 0.05$). As previously stated, RyR1 and SERCA play pivotal roles in keeping Ca^{2+} homeostasis in sarcoplasm.[24] Yet, S-nitrosylation modification would make their activity and function dysregulate, thus causing the excessive accumulation of sarcoplasmic Ca^{2+}, intense muscle contraction, and consequently rapid glucose metabolism.[4] This view is further supported by Wang et al.,[9] who demonstrated that S-nitrosylation of RyR1 and SERCA could induce Ca^{2+} overload of sarcoplasm in post-mortem muscle by inhibiting SERCA and activating RyR1, which was largely responsible for the formation of PSE meat. In addition, FHL1, a novel mediator of Ca^{2+} homeostasis, was strongly associated with muscular dystrophy and muscle wasting,[47] and it has been shown to be involved in the development of post-mortem meat quality.[48] Zhu et al.[32] recently reported that S-nitrosylation of Cys114 on FHL1 was up-regulated while Cys178 was down-regulated in beef, with high ultimate pH relative to normal beef. Moreover, it is interesting to find that S-nitrosylation of annexin (Cys279), a Ca^{2+} regulatory protein that contributes to the formation of the Ca^{2+} channel, was overexpressed in the HS group compared with the LS group. According to Ouali et al.,[43] annexin could affect meat tenderization via regulating the post-mortem apoptosis process. Thus, a diverse S-nitrosylation level of annexin between the HS and LS groups could regulate the Ca^{2+} channel stability and thereby affect the apoptosis of muscle cells after slaughter. To date, numerous studies have shown that sarcoplasmic Ca^{2+} could be involved in the regulation of energy metabolism, proteolysis, and apoptosis processes of post-mortem muscle via mediating glycolysis, calpain-1 autolysis, and mitochondrial integrity.[49,50] Overall, it is conceivable that preslaughter transport stress-induced changes in S-nitrosylation levels of these proteins might lead to abnormal alternations in sarcoplasmic Ca^{2+} concentration and its mediated many biochemical pathways, which could further mediate the meat quality development.

Previous studies have demonstrated that a host of cytoskeletal proteins (e.g., myosin, actin, desmin, and filamin C) could be modified by S-nitrosylation.[31] Likewise, differential S-nitrosylated cytoskeletal proteins in large numbers, including myosin, myomesin, titin, tubulin, actinin, tropomyosin, filamin C, troponin C, and PDZ and LIM domain 5 were also identified in the current study. Among them, Cys817 in myosin 2, Cys1306 in myomesin 2, Cys347 in α-tubulin, Cys781 in actinin alpha 2, Cys683 and Cys806 in filamin C, and Cys35 in troponin C were overexpressed in the HS group, while Cys862 in actinin alpha 2 and Cys191 in tubulin alpha chain were lower-abundant when compared to the LS group ($p < 0.05$). In addition, the giant protein titin was discovered with several cysteines (Cys629, Cys1288, Cys3043, Cys3407, Cys3418, and Cys3500) modified by S-nitrosylation, and all showed a higher abundance in HS group compared to LS group ($p < 0.05$). Myosin is the unit of thick filaments of myofibrils, which is involved in numerous cellular processes. The S-nitrosylation of myosin could regulate its hydrolysis cycle and inhibit the sliding between myosin and actin, thereby affecting muscle shrinkage function.[31,21] The α-tubulin can polymerize with β-tubulin into dynamic microtubules, which are one of the main components of the cytoskeleton. Horenberg et al.[31] previously proposed that the S-nitrosylation of tubulin could reduce the free sulfhydryl group of tubulins, prevent its disulfide cross-linking production, and thus improve the polymerization of tubulin. Titin, the largest known protein, ranks the third place in content after myosin and actin and serves as a critical part in the structural stability, elasticity, and integrity of myofibrils.[51] In the present study, the S-nitrosylation levels of identified SNO-Cys of titin isoform X6 were all remarkably up-regulated in the HS group rather than the LS group. According to Figueiredo-Freitas et al.,[52] the S-nitrosylation of titin has been demonstrated to be closely associated with impaired Ca^{2+} sensitivity and relaxation of muscle fibers. Moreover, the S-nitrosylated actinin alpha 2, filamin C, and troponin C were also identified in the studies of Figueiredo-Freitas et al.[52] and Ulrich et al.[53] Meanwhile, these studies suggested that lower S-nitrosylation levels in sarcomeric proteins contributed to a slight reduction in Ca^{2+} sensitivity to the downregulation of shrinkage function. However, there was a subsequent inhibitory effect on muscle fiber relaxation as the accumulation of S-nitrosylated sarcomeric proteins and the increase of their modification levels. Investigating the effect of NO and its induced S-nitrosylation on the degradation of myofibrillar proteins using *in vitro* experiments, Liu et al.[54] stated that they could alter the sensitivity of the myofibrillar protein to calpain-1, improving the degradation of desmin and titin while inhibiting troponin-T. Based on the above facts, we herein speculate that the overall increased S-nitrosylation levels of cytoskeletal proteins in response to preslaughter transport stress might potentially participate in the development of meat quality via regulating muscle contraction.

In conclusion, this research provided direct evidence for S-nitrosylation levels and S-nitrosylated proteome variations in pork LT muscle in response to preslaughter transport stress. It was found that the HS group showed a level of S-nitrosylation modification that was higher than that of the LS group. In addition, the differential SNO-Pr were primarily involved in Ca^{2+} handling, muscle system processes (myofiber contraction and myocyte development), and post-mortem muscle energy metabolism (OXPHOS, phosphocreatine conversion, and

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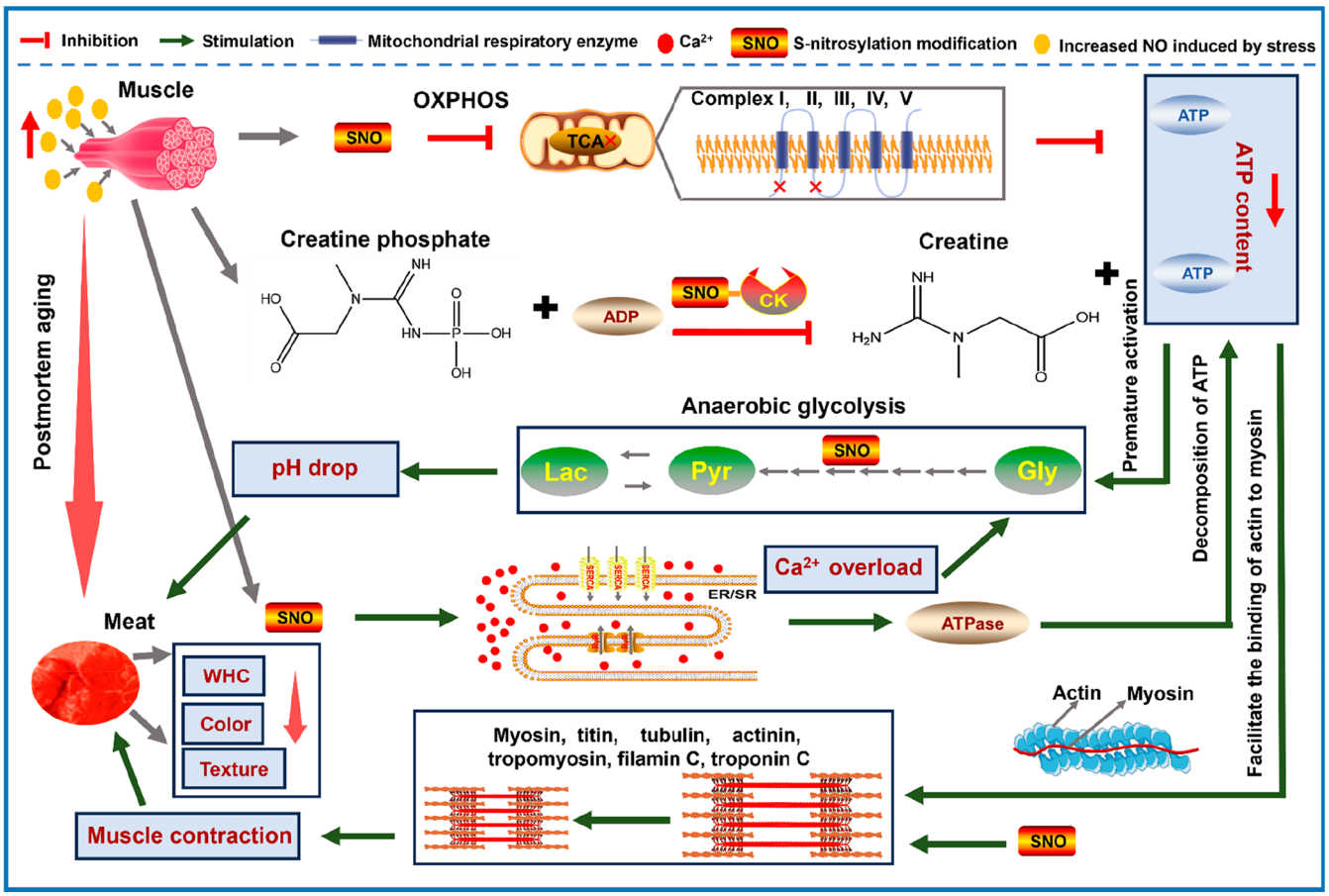

Figure 8. Schematic diagram of protein cysteine S-nitrosylation mediated the effect of preslaughter transport stress on pork quality development.

glycolysis), indicating their roles in mediating post-mortem muscle contraction, pH decline, and resulting meat quality development (Figure 8). Many differential SNO-Pr in the preslaughter transport stress-induced quality-deficient meat (HS group) presented a higher abundance, and some of them (such as PGK, L-LDH, TPI, CK, RyR1, FHL1, and SDHC) can be indicated as potential biomarkers for predicting the fresh pork quality and meat grading. This investigation from the perspective of NO-induced protein S-nitrosylation modification contributes to a deeper understanding of the biochemical mechanisms involved in meat quality deterioration in response to preslaughter transport stress. In the future, the function of specific Cys sites modified by S-nitrosylation will be further revealed by an *in vitro* model. In addition, we will also manipulate the level of S-nitrosylation by injecting the corresponding NO donor/inhibitor *in vivo* before slaughter to further clarify the regulatory mechanism of protein S-nitrosylation on meat quality development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c05254.

Detailed information for differential S-nitrosylated proteins and cysteine sites identified from pork LT muscle between HS and LS groups (XLSX)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HS, high-stress; LS, low-stress control; WHC, water holding capacity; PSE, pale, soft, and exudative; LT muscle, *longissimus thoracis* muscle; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; GP, glycogen phosphorylase; PK, pyruvate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SERCA, sarcoplasmic reticulum calcium ATPase; RyR1, ryanodine receptor 1; LDH, lactate dehydrogenase; CK, creatine kinase; SNO-Pr, S-nitrosylated proteins; SNO-Cys, S-nitrosylated cysteine-sites; OXPHOS, oxidative phosphorylation; TMT, tandem mass tags; ATP, triphosphate; SDH, succinate dehydrogenase; ETC, electron transport chain

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