Food Chemistry 272 (2019) 613–618

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of protein S-nitrosylation on the glycogen metabolism in postmortem pork

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ARTICLE INFO

Keywords:
S-nitrosylation
Glycolysis
Nitric oxide
Glycogen
Lactate
Enzyme

ABSTRACT

The aim of this study was to investigate the effects of protein S-nitrosylation on the glycogen metabolism in postmortem pork. The pork samples were incubated with control (0.9% NaCl), nitric oxide synthase (NOS) inhibitor, or NO donor for 4 and 12 h at 4 °C. Results indicate that NOS inhibitor treatment led to significantly lower level of glycogen and higher lactate content at 24 h compared those of control and NO donor treatments ($P < 0.05$). The pH of NOS inhibitor treatment was significantly lower than other treatments, which indicates the fast glycolysis during postmortem aging ($P < 0.05$). In addition, the activities of glycolytic enzymes including GP, GAPDH and PK were significantly different among three treatments ($P < 0.05$) possibly due to the different modification of protein S-nitrosylation. These results suggest that NO could regulate the glycogen metabolism through modulating the activities of glycolytic enzymes by protein S-nitrosylation.

1. Introduction

As an important gaseous intercellular messenger, nitric oxide (NO) is generated during the transformation of arginine to citrulline, which is catalyzed by NO synthase (NOS) *in vivo*. Protein S-nitrosylation is a ubiquitous posttranslational modification of protein and the reaction is the association of NO moiety with a reactive cysteine thiol of protein (Hess, Matsumoto, Kim, Marshall & Stamler, 2005). Previous studies have shown that NO and NO induced S-nitrosylation possibly are involved in regulating glucose metabolism, calcium homeostasis, muscle contraction and proteolysis (González et al., 2008; Jaffrey, Erdjument-Bromage, Ferris, Tempst, & Snyder, 2001; Stamler & Meissner, 2001; Zhang, Kraus, & Truskey, 2004). Since NO and protein S-nitrosylation have entered the scope of meat science, there are some speculations that NO and NO induced S-nitrosylation could modulate meat quality through influencing μ-calpain activation (Cook, Scott, & Devine, 1998; Cottrell, Ponnampalam, Dunshea, & Warner, 2015; Cottrell, McDonagh, Dunshea, & Warner, 2008; Liu, Warner, Zhou, & Zhang, 2018; Zhang, Marwan, Samaraweera, Lee, & Ahn, 2013). μ-Calpain is a cysteine protease which has five cysteine sites that could be modified by S-nitrosylation *in vitro* (Liu, Li, Wang, Zhou & Zhang, 2016). Li et al. (2014) found that NOS inhibitor treatment of pork LT muscle could significantly increase μ-calpain autolysis, titin and nebulin degradation. It implies that protein S-nitrosylation could negatively regulate μ-calpain activity and decrease the proteolytic ability on myofibrils, putatively playing an important role in water holding capacity and tenderness of meat (Huff-Lonergan, & Lonergan, 2005; Huff-Lonergan, Zhang, & Lonergan, 2010; Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006)

It is clearly known that energy metabolism plays a vital role in meat quality during the conversion of muscle to meat. Previous studies have shown that protein S-nitrosylation could be involved in the regulation of energy metabolism via an insulin-independent pathway (Cook et al., 1998; Deshmukh et al., 2010; Sun, Steenbergen, & Murphy, 2006). For example, NO could activate guanylate cyclase to enhance the cyclic guanosine monophosphate (cGMP) concentration and glucose transport in skeletal muscle (Young, Radda, & Leighton, 1997). NO could regulate the expression of major glucose transport (GLUT4) and the activity of AMP-activated protein kinase (AMPK) which is mainly responsible for balancing the ratio of AMP and ATP in muscle (Lira et al., 2007). Activated AMPK could accelerate the glycolysis rate in postmortem muscle through its phosphorylation and activate the phosphofructokinase-2 (PFK-2) to increase fructose-2, 6-diphosphate content (Shen et al., 2006). In addition, NO and NO induced S-nitrosylation have been reported to regulate the enzyme activity including creatine kinase (CK) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructo kinase (PFK) (Konoev, Kalyanaraman, & Hogg, 2000; Li et al., 2014; Mohr, Stamler, & Brüne, 1996 Wolosker, Panizzutti, & Engelender,1996). Liu et al. (2015) reported that NOS activity

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https://doi.org/10.1016/j.foodchem.2018.08.103
Received 20 December 2017; Received in revised form 20 August 2018; Accepted 22 August 2018
Available online 23 August 2018
0308-8146/

remained within 24 h post-slaughter in pork muscle, indicating that endogenous NO might be produced during postmortem aging. NO was reported to regulate the glycolysis through modifying some cysteines of enzymes to form protein S-nitrosylation in skeletal muscle (Firestein & Bredt, 1999; Mohr et al., 1996; Su et al., 2013; Yan et al., 2011). Therefore, the aim of this study was to explore the effects of NO and NO induced S-nitrosylation on energy metabolism and possible mechanism in postmortem pork.

2. Materials and methods

2.1. Sample preparation

Six crossbred pigs with the weight range from 95 to 105 kg were slaughtered at Sushi Meat Co. Ltd (Huaian, China). The M. longissimus lumborum muscle from 3 to 10 ribs of each right-side carcass was removed immediately within 45 min after exsanguination. Each M. longissimus lumborum was cut into slice (10 * 10 * 1 cm) immediately and randomly assigned to 3 following treatments: control (0.9% NaCl), NOS inhibitor (0.1 M Nω-Nitro-l-arginine methyl ester hydrochloride, N5751, Sigma-Aldrich Corp), and NO donor (200 μM S-nitrosoglutathione, N4148, Sigma-Aldrich Corp). Liu (2015) found that NOS of pork muscle still remained its activity after slaughter in 24 h. Thus we chose adding NOS inhibitor to reduce the activity of NOS to decrease NO in muscle. S-nitrosoglutathione (GSNO) as NO donor could rapidly release NO in water at room temperature and decompose to glutathione disulfide (Wang at al., 2002). To accelerate the penetration of treatment solutions, slices from the same M. longissimus lumborum were punctured with 40 1/2 G needles randomly and soaked into different plates with three treatments. The ratio of meat and solution (w/v) was 1:1. The samples were incubated with different solutions individually for 4 and 12 h at 4 °C. After incubation, the samples were taken out from the solution and frozen immediately in liquid nitrogen for energy metabolism analysis.

2.2. Measurement of S-nitrosothiol content

The S-nitrosothiol (SNO) content was measured using Saville assay (Su et al., 2013). Frozen samples (1 g) were minced and homogenized twice at 5,000 g for 15 s with 5 s break in 4 mL of ice-cold PBS (pH7.4) using a polytron (IKA T25 digital ultraturrox, IKA, German). The mixtures were centrifuged at the speed of 10,000*g* for 10 min at 4 °C. The 50 μL supernatants of each sample were mixed with 50 μL of solution A (1% (w/v) sulfanilamide in 0.5 M HCl) or solution B (0.2% (w/v) $HgCl_2$ in solution A) for 5 min in a 96-well costar clear at room temperature. Then the samples were incubated with 100 μL solution C (0.02% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in 0.5 M HCl) for another 5 min at room temperature. The absorbance value was determined at 540 nm using a microplate reader (Spectramax, MD, USA). The SNO concentration was measured by the different absorbance between solution A and B treatments respectively according to the standard curves of GSNO which was generated by doubled dilution of GSNO ranging from 1.56 to 25 mM.

2.3. pH measurement

The pH was determined following the method of McGeehin, Sheridan and Butler (2001) with some modifications. The 0.5 g samples were minced in a 10 mL centrifuge tube and homogenized twice at 6000*g* for 15 s with interval 5 s break in the ice-bath with 4.5 mL buffer solutions (150 mM KCl, 5 mM miodoacetate, pH = 7.0) by a polytron (IKA T25 digital ultraturrox, IKA, German). The pH of the sample solution was determined by Hanna 211 pH meter (Hanna, Porto, Italy).

2.4. Glycogen and lactate content

Glycogen was measured by a commercial glycogen analysis kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). About 85 mg of minced samples were incubated with 3 vol of concentrated alkali in a test tube and heated in 95 °C water bath for 20 min. Then the samples were diluted with 16 vol distilled water after cooling. Staining solution, distilled water and samples were mixed at a ratio of 20:9:1, heated in boiling water for 5 min and then cooled with flowing water. The absorbance of samples was measured at 620 nm by a microplate reader (Spectramax, MD, USA). Glycogen concentration was determined by referring a glycogen standard solution in the kit.

Lactate content was determined by a commercialized analysis kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The 0.5 g frozen samples were minced and homogenized with 4.5 mL of ice-cold solutions (0.9% NaCl). Homogenates were centrifuged by 3000*g* for 10 min at 4 °C and then 1 mL supernatant was diluted with 4 mL distilled water. The solutions were incubated with 1 mL enzyme reaction mixture compound and 0.2 mL chromogenic agent in 37 °C water bath for 10 min. After adding 2 mL termination solution, the absorbance of samples was measured at 530 nm by a microplate reader (Spectramax, MD, USA). The lactate concentration was calculated by lactate standard curves given by the kit.

2.5. Measurement of ATP, AMP and IMP content

ATP, AMP and IMP contents of sample were measured by high-performance liquid chromatography (HPLC) referring to Xing, Xu, Jiang and Deng (2016). The 1.0 g minced samples were homogenized with 5 mL of 7% ice-cold perchloric acid at 13,500 g (IKA T25 digital ultraturrox, IKA, German) for twice (once for 15 s with 5 s intermittent). The supernatant was further centrifuged at the speed of 15,000*g* for 10 min at 4 °C (Avanti J-E, Beckman, CA, USA). After being neutralized with potassium hydroxide (0.85 mol/L), the supernatant was centrifuged again as mentioned above to remove the extra $KClO_4$. The supernatant was filtrated by a filter (0.22 μm, Nanjing Tapery Equipment Company, Nanjing, China) and then injected into HPLC. The ratio of mobile phases A (2.5 mmol/L tetrabutylammonium hydrogen sulfate, 0.04 mol/L potassium dihydrogen orthophosphate and 0.06 mol/L dipotassium hydrogen orthophosphate, pH 7.0) and B (100% methanol) were 86.5% and 13.5%, respectively. The mobile phase flow velocity was 1.0 mL/min. UV detection was set at 254 nm. Peaks were determined by comparing retention time and peak area through external standards.

2.6. Measurement of the activities of glycogen phosphorolase (GP), glyceraldehydes-3-phosphatedehydrogenase (GAPDH) and pyruvate kinase (PK)

The 1 g frozen samples were well minced and homogenized twice at 5000*g* for 15 s with interval 5 s break in 4 mL of ice-cold PBS (pH7.4) using a polytron (IKA T25 digital ultraturrox, IKA, German). The mixtures were centrifuged at 3000*g* for 15 min at 4 °C. Then the supernatants were used for measuring the activities of GP and GAPDH according to commercially ELISA kits (Shanghai Mlbio Technology Company, Shanghai, China).

The preprocessing of PK was same as the lactate which was mentioned above. The activity of PK was measured by a commercial PK activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Detection the S-nitrosylation of GP, GAPDH and PK

The S-nitrosylated and the total GP, GAPDH and PK were determined by Western Blotting. Briefly, 1 g minced frozen samples were homogenized twice for 15 s with 5 s intermittent with 6 mL HENS buffer