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Dietary ractopamine influences sarcoplasmic proteome profile of pork *Longissimus thoracis*☆

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ABSTRACT

Dietary ractopamine improves pork leanness, whereas its effect on sarcoplasmic proteome has not been characterized. Therefore, the influence of ractopamine on sarcoplasmic proteome of post-mortem pork *Longissimus thoracis* muscle was examined. *Longissimus thoracis* samples were collected from carcasses (24 h post-mortem) of purebred Berkshire barrows (n = 9) managed in mixed-sex pens and fed finishing diets containing ractopamine (RAC; 7.4 mg/kg for 14 days followed by 10.0 mg/kg for 14 days) or without ractopamine for 28 days (CON). Sarcoplasmic proteome was analyzed using two-dimensional electrophoresis and mass spectrometry. Nine protein spots were differentially abundant between RAC and CON groups. Glyceraldehyde-3-phosphate dehydrogenase and phosphoglucomutase-1 were over-abundant in CON, whereas serum albumin, carbonic anhydrase 3, L-lactate dehydrogenase A chain, fructose-bisphosphate aldolase A, and myosin light chain 1/3 were over-abundant in RAC. These results suggest that ractopamine influences the abundance of enzymes involved in glycolytic metabolism, and the differential abundance of glycolytic enzymes could potentially influence the conversion of muscle to meat.

1. Introduction

Dietary strategies have been widely used to improve pork leanness (Dunshea, 2012). Ractopamine is a beta-adrenergic agonist feed additive used in finishing diet to improve growth rate, feed efficiency, carcass yield, and leanness in pigs (Apple et al., 2007). The increase in leanness due to ractopamine is attributed to protein accretion (Bergen et al., 1989) and lipolysis (Mills, Spurlock, & Smith, 2003). Furthermore, ractopamine increases glucose turnover (Dunshea, Leury, Tilbrook, & King, 1998). The mode of action of ractopamine in skeletal muscle is through direct activation of beta-adrenergic receptors resulting in a shift from slow-twitch to fast-twitch muscle fibers and altering the proportion of muscle fiber composition to a fast-contracting glycolytic type (Aalhus, Schaefer, Murray, & Jones, 1992; Depreux, Grant, Anderson, & Gerrard, 2002; Gunawan, Richert, Schinckel, Grant, & Gerrard, 2007).

Fresh meat quality is influenced by muscle source as well as fiber type (Chang et al., 2003; Choe et al., 2008; Lee et al., 2012). Bowker, Grant, Forrest, and Gerrard (2000) reported that the muscles composed mainly of white fibers (type IIB) exhibit greater myofibrillar ATP-ase activity and predominantly anaerobic metabolism than the muscles composed primarily of red fibers (types I and IIA). In pigs, the *Longissimus* muscle is mainly (more than 80%) composed of type IIB white fibers, and, therefore, has predominantly glycolytic metabolism (Larzul et al., 1997). Previous investigations reported that the predominance of type IIB white fibers influences pork quality attributes such as tenderness and water-holding capacity (Kim et al., 2008; Ryu, Lee, Lee, & Kim, 2006).

Several investigations examined the influence of dietary ractopamine on fresh pork quality (Apple et al., 2007; Boler et al., 2011; Kutzler et al., 2011; Lanferdini et al., 2013). The effects of ractopamine on pork quality have been primarily attributed to an increase in myofibrillar protein synthesis and improved carcass yield and cutability (Adeola, Ball, & Young, 1992; Bohrer et al., 2012; Carr et al., 2009; Kutzler et al., 2011). The sarcoplasmic proteome comprises soluble proteins and enzymes, constitutes approximately one-third of the total proteins in skeletal muscles, and governs the biochemical processes influencing muscle metabolism (Scopes, 1970). Furthermore, the conversion of muscle to

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meat involves drastic shifts in metabolism, in which sarcoplasmic proteome plays a critical role (Jia et al., 2006). However, studies have not been undertaken on the effect of ractopamine on sarcoplasmic proteome in pork muscles. Therefore, the objective of this study was to examine the influence of dietary ractopamine on the sarcoplasmic proteome profile of *Longissimus thoracis* muscle in pigs.

2. Materials and methods

2.1. Animal production and carcass fabrication

The animal care protocol for the experiment was reviewed and approved by Institutional Animal Care and Use Committee at The Ohio State University (Columbus, OH, USA). Two-hundred purebred Berkshire pigs (barrows and gilts) with an average initial body weight of 68.9 kg were used as previously described (Bohrer, Kyle, Little, Zerby, & Boler, 2013), and all the animals were raised under similar conditions at The Ohio State University Western Agricultural Research Station (South Charleston, OH, USA). The pigs were stratified over two blocks and housed in mixed-sex pens, and pens served as replicates in this experiment. Each block consisted of ten pens (five pens × 2 dietary treatments). Within each dietary treatment, four pens had six barrows and four gilts, whereas one pen contained five barrows and five gilts. Overall pen size was 16.25 m^2 (including 3.9 m^2 of slatted-floor area); and thus, each pig received approximately 1.63 m^2 of floor space. Each pen had a double nipple water drinker and a 4-hole single-sided box feeder that provided a total of 122 cm of linear feeder space (12.2 cm/pig). Pigs were housed in a curtain-sided, naturally ventilated barn and were provided ad libitum access to feed and water throughout the finishing trial. Pigs were allotted by bodyweight and provided a 14-day allocation period prior to the start of the treatment diets. Within each block, pigs in five pens were finished on a step-up ractopamine diet (RAC; 17.1% crude protein and 1.04% total lysine) with 7.4 mg/kg ractopamine for 14 days followed by 10 mg/kg ractopamine for the last 14 days prior to slaughter, whereas the pigs in the other five pens were finished on a control diet (CON; 13.1% crude protein and 0.76% total lysine) with 0 mg/kg ractopamine. Diets were analyzed to ensure that ractopamine inclusion levels were within acceptable tolerances (75 to 125%) of the claim for each diet.

Costa-Lima et al. (2014) recently reported that the effect of ractopamine on color and textural attributes of pork frankfurters is sex-specific. Therefore, to avoid any potential effect of sex on muscle proteome, only barrows were selected for proteome analysis. At the end of the 28-day feeding period, one barrow (105 kg average live body weight) was randomly selected from nine pens in CON and RAC treatments, and these eighteen pigs were transported to The Ohio State University Meat Science Laboratory. This approach provided nine replicates (n = 9) for proteome analysis. The pigs were kept overnight in lairage with free access to water, but with no access to feed before being humanely slaughtered. The carcasses were chilled for 24 h at 4 °C, and from the right side of the carcasses, a 2.54-cm loin chop (*Longissimus thoracis* muscle) was cut at the 10th rib. The muscle samples were individually vacuum-packaged and frozen at −80 °C. Frozen muscle samples were transported in dry ice to the University of Kentucky (Lexington, KY, USA), where they were stored at −80 °C until proteome analysis.

2.2. Isolation of sarcoplasmic proteome

Sarcoplasmic proteome was extracted according to Joseph, Suman, Rentfrow, Li, and Beach (2012). Five grams of frozen muscle samples was homogenized in 25 ml ice-cold extraction buffer (40 mM Tris, 2 mM EDTA, and pH 8.0). The homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatant (sarcoplasmic proteome) was filtered and utilized.

2.3. Two-dimensional electrophoresis

The protein concentration of the sarcoplasmic proteome extract was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). An aliquot corresponding to 900 μg of protein was mixed with rehydration buffer optimized to 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% Bio-Lyte 5/8 ampholyte (Bio-Rad), and 0.001% Bromophenol blue, and was loaded onto immobilized pH gradient (IPG) strips (pH 5–8, 17 cm). Gels were subjected to passive rehydration for 16 h, and then subjected to first-dimension isoelectric focusing (IEF) in a Protean IEF cell system (Bio-Rad) applying a linear increase in voltage initially and a final rapid voltage ramping to reach a total of 80 kVh. Subsequently, the IPG strips were equilibrated in SDS-containing buffers, first with equilibration buffer I (containing 6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, 2% DTT) for 15 min, followed by equilibration buffer II (containing 6 M urea, 0.375 M Tris–HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% iodoacetamide) for 15 min. The proteins were resolved in the second dimension on 12% SDS-PAGE (38.5:1 ratio of acrylamide to bis-acrylamide) using Protean II XL system (Bio-Rad). The gels were stained using Colloidal Coomassie Blue for 48 h and destained until background was cleared. Samples of both treatments (CON and RAC) were run under the same conditions (two gels/pig), resulting in a total of 36 gels.

2.4. Gel image analysis

Digital images of the stained gels were captured using Versa Doc (Bio-Rad) and analyzed using PDQuest (Bio-Rad). Images were first subjected to automatic spot detection and matching optimized by the aid of landmark protein spots, and the matched spots were normalized (Meunier et al., 2005). For each spot in a given sample, spot quantity values in duplicate gels were averaged for statistical analysis (Joseph et al., 2012). A protein spot was considered to be differentially abundant when it was associated with 1.5-fold intensity difference and 5% significance ($P < 0.05$) level in a pairwise Student's t-test as described by Joseph et al. (2012).

2.5. Protein identification by mass spectrometry

The spots exhibiting differential abundance between the treatments were excised from the gel using pipet tips, placed in microtubes for destaining by two 30-minute washes with 50 mM NH_4HCO_3/50% CH_3CN, vortexed for 10 min, and dried in a vacuum centrifuge. The respective spot was excised from the counterpart treatment to confirm the match. Proteins in the gel fragment were reduced by reaction with 10 mM DTT in 50 mM NH_4HCO_3 solution and incubation at 57 °C for 30 min. The supernatant was discarded, and the proteins (present at the gel piece) were alkylated by the addition of 50 mM NH_4HCO_3 containing 50 mM iodoacetamide and incubated for 30 min at 25 °C without exposure to light. Further, the gel piece was washed twice with 50 mM NH_4HCO_3 and once with CH_3CN, and then partially dried in a vacuum centrifuge. The dried gel piece was rehydrated with a solution of 40 mM NH_4HCO_3/9% CH_3CN, containing 20 ng/μL of proteomic grade trypsin (Sigma, St. Louis, MO, USA) on ice for 1 h. An additional volume of 40 mM NH_4HCO_3/9% CH_3CN was added to cover the sample, and the microtube was incubated for 18 h at 37 °C. Peptides were extracted from the gel piece in 0.1% trifluoroacetic acid by sonication for 10 min followed by vortexing for 10 min, and then the extraction was repeated using a solution on 50% acetonitrile containing 0.1% trifluoroacetic acid. The extracts were combined, and the volume was reduced to remove most of the acetonitrile. Peptide extracts were desalted and concentrated by solid phase extraction using 1 mm of Empore C-18 (3 M, St. Paul, MN, USA) packed in a 0.1 to 10 μL pipet tip (Sarstedt, Newton, NC, USA). The peptides were eluted in 5 μL of 50% CH_3CN/0.1% trifluoroacetic acid.