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Short communication

Gene expression of beta-adrenergic receptors and myosin heavy chain isoforms induced by ractopamine feeding duration in pigs not carrying the ryanodine receptor mutation

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ABSTRACT

To evaluate the effects of ractopamine hydrochloride (RAC) feeding duration on mRNA abundance of beta-adrenergic receptors (β-AR) and myosin heavy chain (MyHC) isoforms in the *Longissimus dorsi* (LD) muscle of pigs not carrying the ryanodine receptor type I (*RyR1*) mutation, 80 finishing barrows (initial body weight = 69.4 ± 7.87 kg) were randomly assigned to 1 of 5 treatments with 8 replicate pens per treatment and 2 pigs per pen. Pigs were fed a corn–soybean meal-based diet with no added RAC (control) or with 10 mg/kg RAC fed for 7, 14, 21, or 28 d before slaughter. On d 28, hair root samples were collected for *RyR1* genotyping and LD muscle samples were collected to measure β-AR (β_1- and β_2-subtypes) and MyHC isoforms (Iβ, IIa, IIx/d, and IIb) gene expression using the quantitative real-time PCR method. Six heterozygous negative pigs for the *RyR1* mutation were detected, and therefore not considered for statistical analysis. Increasing RAC feeding duration did not affect the abundance of β_1-AR mRNA, but resulted in a tendency ($P=0.073$) towards a linear decrease in β_2-AR mRNA abundance. Even though MyHC Iβ mRNA abundance was linearly decreased ($P=0.002$) with increasing RAC feeding duration, both MyHC IIa and IIx/d gene expressions were not affected by the RAC treatment. There was a linear increase ($P=0.029$) in MyHC IIb mRNA abundance as RAC feeding duration was increased. In conclusion, RAC feeding alters gene expression of β_2-AR and MyHC isoforms in the LD muscle of pigs not carrying the *RyR1* mutation in a time-dependent manner.

1. Introduction

Ractopamine hydrochloride (RAC) is a beta-adrenergic agonist (β-AA) that triggers the downstream signaling cascade by binding to the beta-adrenergic receptors (β-AR) in the cell membranes, thus stimulating muscle growth at the expense of fat deposition in finishing pigs. Three β-AR subtypes have been described in mammalian species, including β_1-AR, β_2-AR, and β_3-AR (Mills, 2002). Although the β_2-AR are predominantly expressed in porcine *Longissimus dorsi* (LD) muscle (Sillence et al., 2005), RAC exhibits a greater affinity for the β_1-AR population (Moody et al., 2000). However, β-AR are down-regulated in response to chronic

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RAC feeding, which may reduce the effectiveness of RAC (Mills, 2002).

Four myosin heavy chain (MyHC) isoforms, including 1 slow (MyHC Iβ) and 3 fast isoforms (MyHC IIa, MyHC IIx/d, and MyHC IIb), which correspond to the histochemically-defined fiber types I, IIA, IIX/D, and IIB, respectively, are expressed in adult mammalian skeletal muscle (Pette and Staron, 2001). Interestingly, muscle fibers display a great adaptive potential in response to β-AA feeding (Lefaucheur and Gerrard, 2000). In pigs, RAC feeding was observed to alter muscle fiber type composition towards a faster-contracting phenotype (Depreux et al., 2002) and induce changes in MyHC gene expression in the LD muscle (Gunawan et al., 2007). However, the insight into the molecular mechanisms mediating muscle growth in pigs fed a continuous RAC feeding program over longer periods is still limited.

Because a mutation in the gene encoding the skeletal muscle ryanodine receptor type I (*RyR1*) increases carcass leanness (Oliver et al., 1993), removing the *RyR1* mutation effects from RAC studies is essential to ensure that the animal response is defined only by the β-AA. We hypothesized that longer RAC feeding periods would down-regulate β-AR expression and increase fast MyHC isoform expression in the muscle of *RyR1*-normal pigs. Thus, our objective was to determine the effects of RAC feeding duration on mRNA abundance of β-AR (β_1- and β_2-subtypes) and MyHC isoforms (I, IIa, IIx/d, and IIb) in the LD muscle of pigs not carrying the *RyR1* mutation.

2. Materials and methods

2.1. Animals, experimental design, and diets

All procedures involving animals were conducted in accordance with the experimental animal guidelines approved by the "Luiz de Queiroz" College of Agriculture (University of São Paulo, Piracicaba, Brazil) Animal Care and Use Committee. The pigs evaluated in this experiment were also used in another study and additional information on animal housing and experimental diets has been previously described (Almeida et al., 2013). Eighty crossbred (Large White × Landrace dams × Pietrain sires) finishing barrows were blocked by initial body weight (BW; 69.4 ± 7.87 kg) and allotted to a randomized complete block design with 5 treatments, 8 replicate pens per treatment, and 2 pigs per pen. The treatments consisted of a basal corn–soybean meal-based diet containing either no RAC (control) or 10 mg/kg RAC (Ractosuin, Ourofino Animal Health, Cravinhos, Brazil) fed for 7, 14, 21, or 28 d before slaughter. Experimental diets were formulated (as-fed basis) to contain 0.88% standardized ileal digestible lysine and 3.23 Mcal/kg metabolizable energy.

2.2. Slaughter and sampling procedures

At the end of the 28-d study, all pigs were slaughtered (102.5 ± 9.13 kg final BW) according to humanitarian approved methods by electrical stunning followed by exsanguination. Immediately after sticking, at least 15 hair roots were plucked from each animal for subsequent DNA analysis for the *RyR1* genotype. After exsanguination, approximately 2 g of LD muscle was obtained between the 10th and 11th ribs from the right side of the carcasses, immediately snap-frozen in liquid nitrogen, and then stored at −80 °C until analysis.

2.3. Determination of RyR1 genotype

Genomic DNA was isolated from hair root samples using the protocol described by Graffy and Foran (2005), with modifications. Briefly, 50 μL of 0.2 M NaOH was added to microcentrifuge tubes containing 1–5 hair roots with visible bulbs. Tubes were vortexed for 10 s, incubated at 95 °C for 10 min, and the solution was then neutralized using 50 μL of an equal mixture of HCl (0.2 M) and Tris–HCl (0.1 M; pH 8.5). The *RyR1* genotype (*NN*, homozygous negative *RyR1* mutant; *Nn*, heterozygous negative *RyR1* mutant; *nn*, homozygous positive *RyR1* mutant) of all pigs was determined by PCR amplification and digestion with restriction enzymes as described by Fujii et al. (1991).

2.4. RNA isolation and quantitative real-time PCR

Total RNA was isolated from LD muscle samples using Trizol reagent (Invitrogen Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's guidelines. Total RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), and integrity was examined on a 1.5% agarose gel stained with GelRed (Biotium Inc., Hayward, CA, USA). Total RNA samples were treated with DNAse I RNase-free (MBI Fermentas Inc., Burlington, ON, Canada) before being reverse transcribed into cDNA with an ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to standard procedures.

Quantitative real-time PCR was performed using a Light-Cycler 480 real-time PCR System (Roche Applied Science, Mannheim, Germany). Each reaction was processed in a 10- μL volume containing template cDNA (2 μL of 1:20 cDNA), 0.4 μM of forward and reverse primers, and 5 μL of SYBR Green I Master 2X (Roche Diagnostics, Pleasanton, CA, USA). Primers sequences, except for MyHC IIa, MyHC IIx/d, and MyHC IIb (Da Costa et al., 2002), were designed using the Primer3 software (Table 1). For each gene, all samples were run in a 96-well plate, alongside a non-template control (RNAse-free water). Cycling conditions were 95 °C for 2 min; then 45 cycles of 95 °C for 10 s, 58–60 °C (depending on primer set) for 10 s, and 72 °C for 11 s. Melting curve analysis was conducted to confirm the specificity of the primers. Expression of β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 18S ribosomal RNA (18S rRNA) was evaluated as housekeeping genes. All gene candidates were unaffected by the treatments, and when comparing the standard error of the mean (SEM) on their respective threshold cycle value, the following sequence could be observed: 18S rRNA (0.310) < GAPDH (0.363) < β-actin (0.508). The housekeeping gene with the smallest SEM was chosen. The relative gene expression was calculated as described by Pfaffl (2001) after normalization to 18S rRNA. The PCR efficiency was estimated using LinReg software (Ramakers et al., 2003).