Meat Science 124 (2017) 25–33

Contents lists available at ScienceDirect

Meat Science

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

Long term betaine supplementation regulates genes involved in lipid and cholesterol metabolism of two muscles from an obese pig breed

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ARTICLE INFO

Article history:
Received 7 March 2016
Received in revised form 20 October 2016
Accepted 24 October 2016
Available online 27 October 2016

Keywords:
Betaine
Longissimus lumborum
Biceps femoris
Intramuscular fat and cholesterol content
Gene expression
Alentejano pig

ABSTRACT

This study evaluates the effects of betaine supplementation (1 g kg^{-1} for 20 weeks) on the regulation of genes involved in lipid and cholesterol metabolism of *Longissimus lumborum* and *Biceps femoris* from obese Alentejano pigs. Betaine supplementation led to an increase in total cholesterol in both muscles, complementing results previously published indicating a significant increase on the intramuscular lipid content. The expression of twelve genes involved in lipogenesis, lipolysis/FA oxidation, FA transport, and cholesterol metabolism, as well as two transcription factors were also evaluated. Genes related to lipid and cholesterol synthesis plus FA transport were consistently up-regulated in both muscles of betaine fed pigs. On the other hand, genes related to lipolysis/FA oxidation were not affected or down-regulated by betaine supplementation.
Our data suggest that the underlying mechanism regulating IMF and cholesterol accumulation in Alentejano pigs supplemented with betaine is associated with the up-regulation of genes involved in lipid synthesis, FA transport, and cholesterol synthesis.

1. Introduction

Betaine, *i.e.*, *N*,*N*,*N*-trimethylglycine, is used by mammals primarily as an osmolyte (Lang, 2007), and as a methyl donor for the remethylation of homocysteine to methionine (Schwab et al., 2006). This nontoxic and chemically stable product has been used as a dietary supplement in pig nutrition to reduce fat deposition and increase lean muscle mass with inconsistent results so far. In lean pigs, betaine supplementation reduces (Cadogan, Campbell, Harrison, & Edwards, 1993), increases (Haydon, Campbell, & Prince, 1995) or has no effect on body fat (Matthews, Southern, Pontif, Higbie, & Bidner, 1998; Overland, Rorvik, & Skrede, 1999). On the other hand, methionine obtained through betaine remethylation of homocysteine can be converted to S-adenosylmethionine. This compound is further converted to S-adenosylhomocysteine by glycine *N*-methyltransferase, thereby donating the methyl group for DNA and protein methylation (Bestor, 2000; Cai et al., 2014). Consequently, betaine may modulate gene expression through modifying epigenetic marks such as DNA and histone methylation (Bestor, 2000).

Following years of intensive selection for leaner pigs, resulting in a reduction in meat eating quality, in the last decade the market has shifted to an increasing demand for better quality pork (Cánovas et al., 2010; Gao et al., 2010). In fact, although suspicious of the subcutaneous or abdominal fat depots, European consumers prefer meat with a minimal amount of intramuscular fat (IMF) which is known to contribute to eating quality of pork (Doran et al., 2006; Wood et al., 2008). Meanwhile, the contribution of several lipogenic enzymes to the tissue-specific regulation of fat deposition in pigs remains unclear, as well as the mechanisms regulating IMF and subcutaneous fat deposition (Li, Li, et al., 2011; Shan, Wu, Reng, & Wang, 2009). Knowledge of these mechanisms could contribute to the identification of physiological candidate genes used for evaluation of effectiveness of genetic selection or dietary manipulations (Cánovas et al., 2010; Gao et al., 2010; Liu et al., 2015).

The Alentejano (AL) pig, reared in the south of Portugal, is characterized by slow growth rates, high lipogenic activity at early stages of development, and a higher monounsaturated fatty acid (MUFA)

Abbreviations: *ACC*, *Acetyl-CoA carboxylase*; *ADPN*, *adiponectin*; AL, Alentejano; BW, body weight; C, commercial; CB, C diet supplemented with betaine; FA, fatty acid; *FASN*, *Fatty acid synthase*; *H-FABP*, *Heart-type fatty acid binding protein*; *HMGCR*, *3-Hydroxy-3-methyl-glutaryl-CoA reductase*; *HPRT1*, *hypoxanthine phosphoribosyltransferase 1*; *HSL*, *Hormone-sensitive lipase*; IMF, intramuscular fat; *LDLr*, *Low-Density Lipoprotein Receptor*; *LPL*, *Lipoprotein lipase*; *MCPT1*, *Muscle-type carnitine palmitoyltransferase 1*; MUFA, monounsaturated fatty acid; *PPARα*, *Peroxisome proliferator-activated receptor alpha*; *PPARγ*, *Peroxisome proliferator-activated receptor gamma*; PUFA, polyunsaturated fatty acid; *SCD*, *Stearoyl-CoA desaturase*; SFA, saturated fatty acid; TAG, triacylglycerol.

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http://dx.doi.org/10.1016/j.meatsci.2016.10.012
0309-1740/

synthesis when compared to lean breeds (López-Bote, 1998; Neves, Sabio, Freitas, & Almeida, 1996). This obese breed genetically similar to the Iberian pig is traditionally finished under free-range conditions during fall/winter months, with acorns and grass, and slaughtered at heavy weights (~150 kg) for the manufacture of high quality dry cured products (López-Bote, 1998). Nowadays, the all-year production of high quality fresh meat is been increasingly used as a quality alternative for the lean pig meat, commonly consumed in Europe. These animals are fed balanced mixed diets and natural resources (pasture grazing) and slaughtered at lower body weights (90–110 kg) than those traditionally reared.

Metabolic modifiers such as betaine should supposedly be more effective in decreasing body fat in obese animals (Fernández-Fígares, Conde-Aguilera, Nieto, Lachica, & Aguilera, 2008) and therefore have a beneficial impact on commercial value of AL carcasses, pork quality, and consumer's health. Acting as a methyl donor, betaine can enhance the synthesis of methylated compounds such as phosphatidylcholine and carnitine. Thus, it may be integrally involved in lipid metabolism *via* its role on phosphatidylcholine synthesis and in FA oxidation because carnitine is required for transport of long chain FA into mitochondria for β-oxidation (Huang et al., 2009). On the other hand, meat quality depends on physiological processes which could involve a large pattern of genes associated with both muscle structural and metabolic features (Damon, Wyszynska-Koko, Vincent, Hérault, & Lebret, 2012). Another important factor for the consumers and their increasing knowledge of the link between diet and health is meat cholesterol content. Still, the impact of betaine on the expression of genes important for the regulation of IMF and cholesterol metabolism in obese pigs has not yet been defined.

A previous study from our team showed that long-term betaine supplementation at a moderate dose selectively increased IMF while not affecting body fat deposition and other chemical and physical characteristics of the AL pig muscles studied. The purpose of the present study was to evaluate the effects of betaine on mechanisms regulating IMF and cholesterol deposition through the expression of twelve genes involved in lipid and cholesterol metabolism of *Longissimus lumborum* and *Biceps femoris* in this obese pig breed. The relative expression of these genes was analyzed and this information was complemented with muscles chemical characteristics and FA profiles previously reported (Martins, Neves, Freitas, & Tirapicos, 2012).

2. Materials and methods

2.1. Animals, diets, and experimental design

This study was carried out in accordance with the regulations and ethical guidelines set by the Portuguese Animal Nutrition and Welfare Commission (DGAV, Lisboa, Portugal) following the 2010/63/EU Directive. Staff members involved in animal trials had licenses for conducting experiments on live animals from the Directorate of Animal Protection (DSPA, DGAV, Lisboa, Portugal).

The design of this study is detailed elsewhere (Martins et al., 2012). Briefly, fourteen purebred AL pigs surgically castrated, with an initial body weight (BW) of 36.7 ± 0.9 kg (mean ± SEM) were allocated to open-air individual pens (3 m^2) and fed a commercial (C) diet (Proibérico 2, Provimi, Alverca, Portugal). Feed analysis of the experimental diet showed a dry matter (DM) content of 89.5 g 100 g^{-1}, which included 7.04 g 100 g^{-1} ash, 16.01 g 100 g^{-1} crude protein, 26.05 g 100 g^{-1} neutral detergent fiber and 3.32 g 100 g^{-1} total lipids. The major FA composition of the feed (expressed as g per 100 g total FA methyl esters (FAME) identified) was as follows: lauric acid (C12:0) 10.4, palmitic acid (C16:0) 18.4, oleic acid (C18:1*n*-9) 26.7, and linoleic acid (C18:2*n*-6) 30.7. Pigs had free access to water and were individually fed in a single daily meal (09:00 h), at 85% estimated *ad libitum* consumption, and at a weekly-adjusted daily rate. Diet refusals and spillage were measured daily.

Pigs were randomly assigned into two experimental groups, Group C ($n = 6$), consuming the C diet, and Group CB ($n = 8$), consuming the C diet supplemented with betaine (1 g kg^{-1}) (Betafin® S1, Danisco Animal Nutrition). Animals were killed at 100 kg BW by electronarcosis and bleeding at an industrial slaughterhouse, following 20 weeks of trial. Carcasses were split longitudinally and after a 24 h chilling process, the left side of each carcass was submitted to commercial cuts as described (Martins et al., 2012). The weights of major commercial cuts were also recorded. *Longissimus* and backfat thicknesses were measured with a caliper rule by an average of three measurements (3rd–4th lumbar vertebrae, 10th–11th ribs, and last rib) on the chilled left half carcass. Finally, *Longissimus lumborum* (*L. lumborum*), and *Biceps femoris* (*B. femoris*) samples, freed from visible fat, were vacuum packed and frozen for biochemical (−30 °C) and molecular biology (−80 °C) analyses.

2.2. Diet and muscle composition

Diet dry matter, total ashes, crude protein (N × 6.25), neutral detergent fiber, total lipids and their major FA determination were performed as previously described (Martins et al., 2012). FA methyl esters (FAME) were identified based on the retention time of reference compounds (Supelco cat. n. 47801 and 47885-U) and FA composition of the diet was expressed as g 100 g^{-1} of the total FAME identified.

L. lumborum and *B. femoris* muscles were chosen as representing meat cuts of greatest mass and economic value (loin and leg). Neutral and polar lipids were obtained from muscle samples (10 g each) as described in Martins et al. (2012) and total intramuscular lipids calculated as the sum of neutral and polar lipid fractions analyzed. The FA composition of both lipid fractions was analyzed and identified as described (Martins et al., 2012), and expressed as g 100 g^{-1} of the total FAME identified. Total lipids from *L. lumborum* and *B. femoris* samples (4 g each) were extracted (Folch, Lees, & Stanley, 1957), and total cholesterol was measured in propanol-2 extracts, using enzymatic kits (Roche Diagnostics), and following manufacturer's instructions.

2.3. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR

Tissue samples from *L. lumborum* and *B. femoris* were collected at the slaughterhouse, snap frozen in liquid nitrogen and stored at −80 °C until analysis. Total RNA was extracted with TRIzol® Plus RNA Purification Kit (Invitrogen, USA) from each muscle tissue (1 mL of TRIzol reagent per ~150 mg of tissue sample), according to the manufacturer's instructions and stored at −80 °C. Assessment of total RNA integrity (NanoDrop® 2000 UV–vis Spectrophotometer, Thermo Scientific) was performed by the OD_{260}/OD_{280} nm absorption ratio calculation and was >1.9, indicating highly pure RNA. For reverse transcription, 1 μg of total RNA was used in a 20 μL reaction following Maxima® First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, USA) manufacturer's instructions.

Expression of genes involved in lipogenesis (*ACC, Acetyl-CoA carboxylase; FASN, Fatty acid synthase; SCD, Stearoyl-CoA desaturase*), lipolysis/FA oxidation (*LPL, Lipoprotein lipase; HSL, Hormone-sensitive lipase, MCPT1, Muscle-type carnitine palmitoyltransferase 1; ADPN, adiponectin*), FA transport (*H-FABP, Heart-type fatty acid binding protein*), and in cholesterol metabolism (*HMGCR, 3-Hydroxy-3-methyl-glutaryl-CoA reductase; LDLr, Low-Density Lipoprotein Receptor*), as well as two transcription factors (*PPARα, Peroxisome proliferator-activated receptor alpha; PPARγ, Peroxisome proliferator-activated receptor gamma*) and two housekeeping genes, *hypoxanthine phosphoribosyltransferase 1 (HPRT1)* and *β-actin* (Supplementary Table ***1***) was investigated by real-time quantitative reverse transcription-PCR. Amplification mixtures containing 12.5 μL of 2 × SYBR Green PCR Master Mix, 0.3 μM of each primer and 12.5 ng of cDNA per sample were prepared in 96-well plates and run using a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, USA) for 40 amplification cycles comprising a