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Epigenetic regulation of pyruvate carboxylase gene expression in the postpartum liver

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ABSTRACT

Hepatic gluconeogenesis is essential for maintenance of whole body glucose homeostasis and glucose supply for mammary lactose synthesis in the dairy cow. Upregulation of the gluconeogenic enzyme pyruvate carboxylase (PC) during the transition period is vital in the adaptation to the greater glucose demands associated with peripartum lactogenesis. The objective of this study was to determine if PC transcription in hepatocytes is regulated by DNA methylation and if treatment with a nonsteroidal anti-inflammatory drug (NSAID) alters methylation of an upstream DNA sequence defined as promoter 1. Dairy cows were left untreated (n = 20), or treated with a NSAID during the first 5 d postcalving (n = 20). Liver was biopsied at d 7 precalving and d 7, 14, and 28 postcalving. Total *PC* and transcript specific gene expression was quantified using quantitative PCR and DNA methylation of promoter 1 was quantified using bisulfite Sanger sequencing. Expression of *PC* changed over the transition period, with increased expression postcalving occurring concurrently with increased circulating concentration of nonesterified fatty acids. The DNA methylation percentage was variable at all sites quantified and ranged from 21 to 54% across the 15 CpG dinucleotides within promoter 1. The DNA methylation at wk 1 postcalving, however, was not correlated with gene expression of promoter 1-regulated transcripts and we did not detect an effect of NSAID treatment on DNA methylation or *PC* gene expression. Our results do not support a role for DNA methylation in regulating promoter 1-driven gene expression of *PC* at wk 1 postcalving. Further research is required to determine the mechanisms regulating increased *PC* expression over the transition period.
Key words: epigenetic, gluconeogenesis, transcription

INTRODUCTION

Hepatic gluconeogenesis is essential for maintenance of whole-body glucose homeostasis and glucose supply to the mammary gland for lactose synthesis in the dairy cow (Drackley et al., 2001). The major precursor for gluconeogenesis is propionate, which is derived from microbial fermentation of NSC in the rumen. During the transition from pregnancy into lactation, the demand for glucose increases 3- to 4-fold (Bell, 1995; Reynolds et al., 2003). Pyruvate carboxylase (***PC***) plays an essential role in the production of glucose in the liver and its expression is increased during the periparturient period as an adaptation to increased glucose requirements (Greenfield et al., 2000; Velez and Donkin, 2005; White et al., 2011a,b). Pyruvate carboxylase supplies carbon for gluconeogenesis and oxaloacetate for the tricarboxcylic acid cycle (White, 2015). Several transcript variants of *PC* are driven by 3 promoters (Agca et al., 2004; Hazelton et al., 2008). Increased *PC* expression during the periparturient period and during feed restriction is the result of increased promoter 1 activity (White et al., 2011a).

Dairy cows experience a degree of inflammation during early lactation that is linked to lipid mobilization, insulin resistance, and depressed milk production (Bertoni et al., 2008a). Inflammation can induce changes in DNA methylation in response to oxidative stress and pro-inflammatory cytokines, and inhibition of inflammation can affect DNA methylation of CpG dinucleotides and the expression of DNA methyltrans-

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ferases (Niwa et al., 2010; Hur et al., 2011; Kominsky et al., 2011). Methylation of *PC* promoter DNA in human liver is negatively associated with *PC* gene expression (Ahrens et al., 2013). Further, a positive effect of nonsteroidal anti-inflammatory drug (**NSAID**) treatment on hepatic gluconeogenesis has been reported (Vailati Riboni et al., 2015). We therefore, hypothesized that bovine *PC* gene expression is regulated by promoter DNA methylation and that DNA methylation of promoter 1 is positively influenced by NSAID treatment.

MATERIALS AND METHODS

Animals

Six hundred thirty-nine cows (n = 134 primiparous and n = 505 multiparous) calving between July 4 and September 5, 2012, in 2 herds (herd 1: n = 228; herd 2: n = 411) were enrolled. Using a randomized block design, cows were allocated to 1 of 3 treatment groups as they calved: no treatment (control; n = 221), NSAID administered on d 1, 3, and 5 postcalving (early; n = 214), or NSAID administered on d 19, 21, and 23 postcalving (late; n = 204). Detailed methods and production data for the cows used in this study have been published previously (Meier et al., 2014). A subset of 20 cows from the control group and 20 cows from the early NSAID treatment group were selected for biopsy.

Blood Metabolites

Blood was sampled 7 (±3.3) d precalving, and at 7 (±1.0), 14 (±1.3), and 28 (±2.1) d postcalving. Blood was collected into evacuated blood tubes containing lithium heparin (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ), placed in iced water immediately, and centrifuged (1,500 × *g* for 12 min at 4°C). Aspirated plasma was stored at −20°C before analysis for nonesterified fatty acids (**NEFA**). Analyses for NEFA were performed on a Modular P800 analyzer (Roche, Basel, Switzerland) at 37°C by Gribbles Veterinary Pathology Ltd. (Hamilton, New Zealand) and NEFA were measured (mmol/L) by the acyl Co-A synthetase, acyl-CoA oxidase colorimetric method using the NEFA C Kit from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The intra- and interassay coefficients of variation were <5%.

Liver Biopsy

Liver tissue was biopsied 7 d precalving and 7, 14, and 28 d postcalving. Briefly, the skin was shaved and disinfected and the area through the skin and body wall was anesthetized with 7 mL of 2% lignocaine (Lopaine 2%, lignocaine hydrochloride 20 mg/mL, Ethical Agents, South Auckland, New Zealand). A stab incision was made through the skin in the right 11th intercostal space at the level of the greater trochanter, through which a 12-gauge × 20 cm biopsy needle was inserted into the liver and approximately 1 g (wet weight) of liver tissue was collected. Liver samples were snap-frozen in liquid nitrogen and stored at −80°C.

Nucleic Acid Extraction

The DNA and RNA were extracted from liver tissue using the Qiagen All-Prep mini kit Plus (Qiagen GmbH, Hilden, Germany). Briefly, approximately 30 mg of tissue was homogenized using a TissueLyser II (Qiagen) for 2 min with 3 ball bearings of 1/8 inch diameter (Farrell Bearings, Hamilton, NZ) in a tube containing RLT Plus lysis buffer (Qiagen). The DNA was purified using the Qiagen All-prep column and the flow through was used to isolate total RNA using a Qiagen RNeasy column (Qiagen). All RNA samples were DNase treated using the Ambion DNA-free kit (Ambion, Austin, TX) to remove any contaminating DNA as previously described (Grala et al., 2010, 2013; Crookenden et al., 2015). The quantity of DNA and RNA was determined using Qubit fluorometric quantification. The DNA and RNA purity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). The DNA integrity was assessed on a 0.8% agarose gel and RNA integrity was assessed with a RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All DNA samples were of high molecular weight (single high molecular weight band) and all RNA samples had an integrity number above 7. Final sample numbers used in gene expression analysis for each time point were as follows: precalving d 7 (±3.2), control: n = 15, NSAID: n = 10; and postcalving d 7 (±1.4), control: n = 19, NSAID: n = 19; d 14 (±1.1), control: n = 18, NSAID: n = 19; and d 28 (±1.7), control: n = 17, NSAID: n = 19. The wk-1 time point was used for DNA methylation analyses. Precalving samples were excluded if they had been taken within 4 d of calving.

cDNA Synthesis

One microgram of each RNA sample (final volume = 20 μL) was used for cDNA synthesis using the Invitrogen Superscript III Supermix kit (Invitrogen Corporation, Carlsbad, CA). Total RNA was transcribed according to the manufacturer's instructions using 27 μ*M* of random pentadecamers. Briefly, RNA and random pentadecamers were mixed and denatured at 65°C