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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 methyltransferases (Niwa et al., 2010; Hur et al., 2011; Kominsky et

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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 for 5 min, followed by 1 min on ice. Annealing buffer

Table 1. Pyruvate carboxylase (*PC*) transcript variant assays[1]

Target	Accession	Probe	Primer sequence	PCR efficiency
PC coding	AY185595	Probe - FAM	GCTTCCCCATCATCTTCAAG	2
		Forward primer	CCACGAGTTCTCCAACACCT	
		Reverse primer	TTCTCCTCCAGCTCCTCGTA	
5′UTR_D	AY185598	Probe - HEX	GCCGACAAAACACTGAGGAT	2.1
		Forward primer	ATAGTGCCTGCCTCCCAAG	
		Reverse primer	CCCCGGACAGTCTGAAACTT	
5′UTR_E	AY185599	Probe - Cy3	AATGCAGCTGTATGTTGGCA	1.9
		Forward primer	CCAAGAGCCGACAAAACACT	
		Reverse primer	GTTTCAAGCAGGTCACAGCA	
5′UTR_F	AY185600	Probe - Cy5	ACTTATAAACAGGCAGCGGG	1.9
		Forward primer	CAGAGGCTTAACCCAGAACG	
		Reverse primer	AAACTTCAGCATCGCAGCA	

[1]Primer sequences and PCR efficiency of quantitative real-time-PCR assays targeting pyruvate carboxylase (White et al. 2011a). The PC coding assay targets the coding region of pyruvate carboxylase and amplifies all variants of the gene. Three transcript specific assays were also utilized. One of the promoter 1-regulated transcripts (5′UTR_F) was amplified with the UTR_F assay. The promoter 2-regulated transcript (5′UTR_D), was amplified with UTR_D assay. The promoter 3-regulated transcript (5′UTR_E), was amplified with the UTR_E assay.

and Superscript/RNase was added to samples and incubated for 10 min at 25°C (primer annealing), followed by 50 min at 50°C and 5 min at 85°C to inactivate the enzyme. Reverse transcription controls were performed, whereby the above process was completed without the addition of superscript enzyme. No amplification products were detected for any genes in the reverse transcriptase control samples.

Quantitative Real-Time PCR

Four endogenous control genes were tested for suitability for normalizing gene expression (*COX4I1*, *EIF3K*, *RPS9*, and *YWHAZ*). Analysis with GeNorm and Normfinder revealed *COX4I1* had the most stable expression with a stability value of 0.250 and 0.038, respectively. Normfinder identified *RPS9* and *COX4I1* as the best combination of 2 genes (0.042 stability value). Analysis with GeNorm did not indicate any increase in stability with the addition of a third endogenous control gene. As a result of this analysis, *RPS9* and *COX4I1* were chosen as the 2 endogenous control genes for this study. Advanced relative quantification of the *PC* transcription was analyzed using Roche LightCycler480 software. The *PC* coding assay quantified all transcript variants. Three calibrator samples were used on all plates to normalize expression over multiple quantitative real time-PCR plates. Additionally, transcript-specific expression of *PC* variants (D, E, and F) was determined using assays developed by White et al., 2011b (Table 1; Supplemental Figure S1, http://dx.doi.org/10.3168/jds.2015-10331). Promoter 1 of *PC* regulates expression of transcripts A, B, C, and F, promoter 2 regulates expression of transcript D, and promoter 3 regulates expression of transcript E. Total promoter 1 transcript expression is calculated as *PC* coding (all transcripts) minus variants D and E. All primers and probes were synthesized commercially (Integrated DNA Technologies, Singapore).

Bisulfite Conversion and PCR

The DNA was bisulfite converted and purified using the Zymo EZ DNA methylation kit Gold (Zymo, Irvine, CA) per the manufacturer's instructions. Two assays were optimized to amplify the defined promoter 1 region (Table 2; Supplemental Figure S1, http://dx.doi.org/10.3168/jds.2015-10331). The KAPA HiFi Uracil+ Ready Mix (KAPA Biosystems, Wilmington, MA) was used for amplification per the manufacturer's instructions. Annealing temperatures of 57.4 or 58.2°C were used for assay 1 and 2, respectively. Following

Table 2. Primer design for bisulfite PCR[1]

Assay	Primer name	Primer sequence	Position
1	Forward primer	TGGGTTTTTTGTTYGTTGTTTGTT	Chr29: 45563659–45564009
	Reverse primer	AAAATCCTCTACCTCTTTTCTTA	
2	Forward primer	TTTAAGAAAAGAGGTAGAGGAT	Chr29: 45563986–45564315
	Reverse primer	ACCTTACTAAAAAAATTCCC	

[1]Two assays were designed to target promoter 1 of pyruvate carboxylase as defined in Hazelton et al. (2008) (Chr29: 45563769–45564378, Bos taurus UMD 3.1: http://genome.ucsc.edu).

amplification, PCR products were sequenced by Sanger sequencing to confirm the correct product was generated and to detect variation in the methylation status.

A-Tailing, Cloning, and Sequencing

A-Tailing of the PCR products was conducted using Roche Taq DNA polymerase before cloning. Briefly, 4 μL of each PCR product was poly-A tailed with 1 μL of 10 × buffer + Mg (Roche Diagnostics), 2 μL of 1 m*M* dATP, 1 μL of Taq polymerase (Roche Diagnostics), and 2 μL of DNase-free water for 20 min at 72°C. For cloning, 4 μL of fresh A-tailed DNA was ligated into pCR4-TOPO vector (Life Technologies, Carlsbad, CA) using the TOPO TA cloning kit for sequencing (Life Technologies) as per the manufacturer's instructions using 1 μL of salt solution and 1 μL of vector per reaction. Ligated vector was then transformed into Top10 cells (Life Technologies) as per manufacturer's instructions, and the cells plated onto Luria-Bertani agar plates containing ampicillin (50 μg/mL), X-gal (20 μg/mL), and isopropyl β-D-1-thiogalactopyranoside (100 μ*M*). The following day, 20 white colonies per sample were isolated, placed into Luria-Bertani broth containing ampicillin (50 μg/mL) and incubated on shaking incubators at 180 rpm at 37°C overnight. Plasmid DNA and was then harvested from the cells using Macherey-Nagel miniprep kits (Bethlehem, PA) as per the manufacturer's instructions. Plasmid DNA was aliquoted into 96-well MLtraFlux semi-skirted ABI-style plates (SSIBIO) and quantified by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies). Plasmid DNA was present in all 20 isolates for most samples. Samples that did not have sufficient plasmid DNA were repeated. The plasmid DNA was then diluted to 100 ng/μL. Applied Biosystems BigDye version 3.1 (Foster City, CA) terminator chemistry was used to generate dye-terminated PCR products for Sanger sequencing. Each reaction consisted of 2 μL of BigDye, 2 μL of M13 −20 forward primer (5 pmol/μL), 3 μL of 5 × buffer, 11 μL of DNase-free water, and 2 μL of plasmid DNA (200 ng) in a 96-well plate. The PCR was performed on Applied Biosystems 9700 Gold Block thermal cyclers with an initial denaturation of 1 min at 96°, followed by 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Dye-terminator removal was performed immediately before sequencing using Agencourt CleanSeq beads (Beckman Coulter, Fullerton, CA) as per the manufacturer's instructions. Briefly, 10 μL of CleanSeq reagent and 60 μL of 85% ethanol were transferred to each well, a foil seal (4titude, Dorking, UK) was placed on the plate, and mixed by inversion. The plate was then placed on a white Agencourt SPRIPlate 96R (Beckman Coulter) for 3 min and the cleared supernatant removed. The beads were washed twice with 100 μL of 85% ethanol and allowed to air dry in the dark. Finally, 40 μL of DNase-free water was added to each well, mixed by pipetting, and placed on a white SPRIPlate 96R for 5 min, and then 28 μL of supernatant was transferred to a fresh 96-well plate. Sanger sequencing was performed on an ABI Prism 3130XL Genetic Analyzer by the Centre of Genomics, Proteomics, and Metabolomics at the School of Biological Sciences, The University of Auckland. Sequence data were analyzed using Geneious R6.1.7 (Biomatters Ltd., Auckland, New Zealand). All samples analyzed had at least 20 DNA isolates that mapped to the promoter region. Any sequencing reactions that failed to produce high-quality sequence were repeated.

Statistical Analysis

The DNA methylation analysis was based on the percentage methylated for each CpG dinucleotide and the percentage methylated across all CpG dinucleotides within each cow. The model used was a one-way ANOVA for the effect of treatment (control vs. NSAID). The relationship between promoter 1 DNA methylation and gene expression (PC coding and promoter 1-regulated transcripts) was investigated using Pearson correlations between percentage methylation for each dinucleotide and gene expression. Results are presented as Pearson correlation coefficient and P-value. Gene expression and NEFA concentrations were subjected to repeated measures ANOVA using a linear mixed effects model. Group, sample day relative to calving date, and their interactions were included as fixed factors and cow (intercept) as a random factor. All results are presented as least squares means and standard errors of the mean for predetermined times. Significance was declared if $P < 0.05$.

RESULTS AND DISCUSSION

Promoter 1-Regulated PC Transcripts Increased with the Transition to Lactation

Promoter 1-regulated *PC* transcripts increased with the transition to lactation coincident with increased demand for glucose for mammary lactose synthesis. Promoter 1-driven transcripts increased 1.5-fold from d 7 precalving to d 7 postcalving, then decreased back to precalving expression by d 28 postcalving ($P < 0.05$, Figure 1, Table 3). In contrast, promoter 2- and 3-regulated transcripts did not increase immediately postcalving and decreased 1.2-fold at d 28 postcalving ($P < 0.05$, Table 3). Promoter 1-regulated transcripts had a similar profile to NEFA concentrations, with NEFA in-

Table 3. Nonesterified fatty acids (NEFA) and gene expression of pyruvate carboxylase (*PC*) transcripts[1]

Fixed effect	*P*-value	Day	LSM	SE
PC coding		−7	0.137	0.0175
Day	0.047	7	0.177	0.0139
Treatment	0.284	14	0.155	0.0141
Day:Treatment	0.556	28	0.122	0.0143
PC_ABCF		−7	0.085	0.0140
Day	0.041	7	0.124	0.0111
Treatment	0.336	14	0.105	0.0113
Day:Treatment	0.468	28	0.081	0.0115
UTR_D		−7	0.005	0.0004
Day	0.049	7	0.005	0.0003
Treatment	0.122	14	0.005	0.0003
Day:Treatment	0.985	28	0.004	0.0003
UTR_E		−7	0.005	0.0004
Day	0.052	7	0.005	0.0003
Treatment	0.317	14	0.005	0.0003
Day:Treatment	0.804	28	0.004	0.0003
NEFA (mmol/L)		−7	0.321	0.0351
Day	0.013	7	0.499	0.0408
Treatment	0.420	14	0.401	0.0406
Day:Treatment	0.596	28	0.375	0.0393

[1]The *PC* coding assays amplified all transcripts of *PC*. UTR_D amplifies promoter 2-regulated transcript, UTR_E amplifies promoter 3-regulated transcripts and UTR_F amplifies 1 of the 4 transcripts regulated by promoter 1. Expression of PC_ABCF was calculated by taking *PC* coding minus UTR_D and UTR_E.

creasing 1.6-fold with the transition to lactation (Figure 1). This is consistent with previous reports that fatty acid concentration increases *PC* transcript expression, particularly promoter 1-regulated transcripts both in vivo and in vitro (White et al., 2011a).

The *PC* expression increases during the periparturient period and during feed restriction to increase hepatic glucose production coincident with decreased intake and increased mobilization of body reserves (Greenfield et al., 2000; Velez and Donkin, 2005; White et al., 2011b). As requirements for glucose increase with the transition to lactation, body reserves are mobilized and the gluconeogenic capacity of the liver is increased to maintain milk production. The results from this study support the hypothesis that transition cows achieve this through an increase in expression of promoter 1-regulated *PC* transcripts.

DNA Methylation of PC Was Not Correlated with Gene Expression

The DNA methylation of CpG dinucleotides in promoter 1 of *PC* was not correlated with *PC* gene expression at 1 week postcalving ($P = 0.83$, Table 4). Average methylation status across all CpG was 43% and 42% in control and NSAID-treated cows, respectively, and ranged from 20% (CpG 1) to 56% (CpG 5) in control and 21% (CpG 1) to 52% (CpG 5) in treated cows. No correlation was found between average DNA methylation and total *PC* transcript expression ($P = 0.4$). Additionally, no correlation was found among DNA methylation at specific CpG sites and total *PC* transcript expression ($P > 0.05$).

Promoter 1 drives expression of the main variants of *PC* that are expressed at wk 1 postcalving. The precise mechanism regulating promoter 1 activity is unknown;

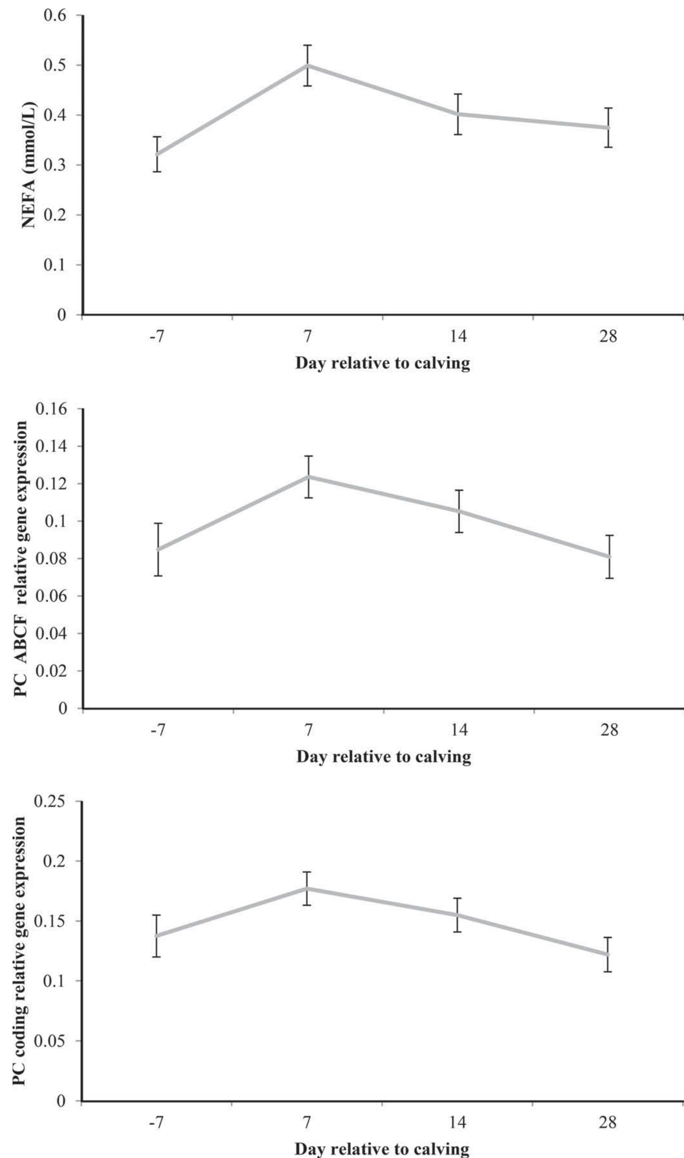

Figure 1. Pyruvate carboxylase (*PC*) gene expression and nonesterified fatty acids (NEFA) over the transition period. Data are presented as least squares means, and error bars are the standard errors of the mean. Postcalving d 7 was greater than 7 d precalving for NEFA, *PC* coding, and *PC* ABCF ($P < 0.05$). Expression of PC_ABCF was calculated by taking *PC* coding minus UTR_D (promoter 2-regulated transcript expression) and UTR_E (promoter 3-regulated transcript expression).

Table 4. Correlation of DNA methylation and pyruvate carboxylase (*PC*) gene expression[1]

CpG	PC coding		PC_ABCF	
	Correlation	*P*-value	Correlation	*P*-value
AllCpG	−0.04	0.83	−0.04	0.81
CpG1	−0.06	0.73	−0.07	0.66
CpG2	−0.05	0.75	−0.05	0.75
CpG3	−0.19	0.26	−0.17	0.32
CpG4	−0.20	0.24	−0.18	0.27
CpG5	−0.12	0.47	−0.12	0.47
CpG6	−0.12	0.46	−0.14	0.40
CpG7	0.07	0.67	0.06	0.70
CpG8	−0.20	0.22	−0.19	0.25
CpG9	0.14	0.42	0.10	0.55
CpG10	0.09	0.60	0.08	0.63
CpG11	0.07	0.66	0.06	0.71
CpG12	0.12	0.48	0.10	0.56
CpG13	−0.03	0.85	−0.05	0.79
CpG14	0.11	0.52	0.10	0.56
CpG15	0.12	0.48	0.12	0.47

[1]Percentage DNA methylation was correlated with gene expression of the promoter 1-regulated transcripts (PC_ABCF) and PC coding, which measures all PC transcripts.

however, it has been hypothesized that binding of transcription factors that are upregulated in response to metabolic status, such as NEFA concentration, increases promoter 1 activity and transcript expression (White et al., 2011a). The DNA methylation is generally associated with inhibition of promoter activity, as it can prevent the binding of transcription factors and is associated with an indirect repression of chromatin state, through the recruitment of methyl-CpG-binding proteins (Wolffe and Matzke, 1999). The DNA methylation may, therefore, prevent binding of transcription factors to promoter 1, so that it is unresponsive to homeorhetic signals. In human liver, *PC* gene expression is negatively correlated with promoter DNA methylation (Ahrens et al., 2013). Results presented here, however, do not support a role for DNA methylation of promoter 1 in regulating *PC* transcript expression during early lactation. However, analysis of other time points may have revealed a role for DNA methylation in regulating the expression of *PC* within an animal over time. Alternative epigenetic mechanisms, such as histone modification and miRNA transcriptional repression, warrant further investigation. For example, reduced *PC* gene expression in pigs has been associated with downregulation of miRNA that are predicted to target *PC* (Cai et al., 2014).

NSAID Treatment Did Not Alter DNA Methylation or PC Gene Expression

No difference was found between control and NSAID treated cows in average DNA methylation across the 14 CpG sites investigated at 7 d postcalving ($P = 0.9$, Table 5). Further, no differences were found between control and NSAID-treated cows in any of the CpG dinucleotides quantified, and NSAID treatment had no effect on *PC* gene expression.

Dairy cows experience a degree of inflammation in early lactation that is linked to lipid mobilization, insulin resistance, and depressed milk production. Despite this, it has been suggested that inflammation-induced insulin resistance is an adaptive rather than a pathological condition in the dairy cow (Farney et al., 2013a) and low-grade inflammation during early lactation is probably a normal part of homeorhesis in the dairy cow. However, inflammation is also linked to increased incidence of subclinical diseases and reduced milk production (Bertoni et al., 2008b). This has led many researchers to investigate the efficacy of anti-inflammatory drugs to reduce inflammation and improve milk production (Bertoni et al., 2004; Farney et al., 2013b; Priest et al., 2013; Meier et al., 2014).

Studies in other species have revealed that inflammatory processes can cause aberrant DNA methylation and that NSAID treatment can cause promoter demethylation (Hahn et al., 2008; Pan et al., 2008; Niwa et al., 2010; Hur et al., 2011; Kominsky et al., 2011). Our results do not support a role for NSAID in regulating DNA methylation of promoter 1 of the *PC* gene. This does not preclude the possibility that the NSAID used in this study regulated DNA methylation at other sites in the genome or that a different type of NSAID may have positive effects on DNA methylation and *PC* gene expression in the cow.

Table 5. DNA methylation in nonsteroidal anti-inflammatory drug (NSAID) and control cows[1]

CpG	Position	Control	NSAID[2]	SEM	*P*-value
All CpG		0.43	0.42	0.020	0.89
CpG1	Chr29:45563672	0.20	0.21	0.021	0.75
CpG2	Chr29:45563692	0.53	0.48	0.035	0.35
CpG3	Chr29:45563790	0.47	0.43	0.031	0.47
CpG4	Chr29:45563865	0.55	0.48	0.031	0.13
CpG5	Chr29:45563876	0.56	0.52	0.032	0.34
CpG6	Chr29:45563883	0.48	0.46	0.032	0.63
CpG7[3]	Chr29:45563895	0.52	0.51	0.036	0.81
CpG8	Chr29:45563936	0.44	0.42	0.044	0.76
CpG9	Chr29:45564012	0.37	0.40	0.029	0.47
CpG10	Chr29:45564064	0.42	0.43	0.032	0.72
CpG11	Chr29:45564127	0.40	0.43	0.032	0.54
CpG12	Chr29:45564148	0.30	0.34	0.027	0.36
CpG13	Chr29:45564159	0.38	0.38	0.028	0.99
CpG14	Chr29:45564219	0.39	0.43	0.030	0.41
CpG15	Chr29:45564234	0.41	0.44	0.032	0.48

[1]Fifteen CpG dinucleotides were quantified in promoter 1 of pyruvate carboxylase.

[2]The DNA methylation was measured as the percentage of DNA that was methylated at each site. Position is based on *Bos taurus* UMD 3.1 (http://genome.ucsc.edu/).

[3]A mutation was present at position 45563895 from CA to CG, creating a new CpG. A CG > CA mutation was also present at position 45564273 that resulted in a loss of a CpG dinucleotide. All animals were homozygous for both mutations.

CONCLUSIONS

Our data support the hypothesis that transition dairy cows increase liver gluconeogenic capacity through an increase in expression of promoter 1-regulated *PC* transcripts during the transition to lactation. The results presented here, however, do not support a role for DNA methylation of promoter 1 in regulating *PC* transcript expression at 1 wk postcalving. Our study was limited to analyzing DNA methylation at wk 1 postcalving. Therefore, a more comprehensive analysis of DNA methylation across the transition period is warranted to determine if DNA methylation regulates transcription of *PC* at different time points. Furthermore, our results do not support a role for NSAID in regulating DNA methylation of promoter 1 of the *PC* gene. Further research is required to determine the effect of NSAID treatment on epigenetics in the dairy cow and how *PC* gene expression is regulated in early lactation.

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REFERENCES

Agca, C., C. A. Bidwell, and S. S. Donkin. 2004. Cloning of bovine pyruvate carboxylase and 5′ untranslated region variants. Anim. Biotechnol. 15:47–66. http://dx.doi.org/10.1081/ABIO-120037897.

Ahrens, M., O. Ammerpohl, W. von Schönfels, J. Kolarova, S. Bens, T. Itzel, A. Teufel, A. Herrmann, M. Brosch, H. Hinrichsen, W. Erhart, J. Egberts, B. Sipos, S. Schreiber, R. Häsler, F. Stickel, T. Becker, M. Krawczak, C. Röcken, R. Siebert, C. Schafmayer, and J. Hampe. 2013. DNA methylation analysis in nonalcoholic fatty liver disease suggests distinct disease-specific and remodeling signatures after bariatric surgery. Cell Metab. 18:296–302. http://dx.doi.org/10.1016/j.cmet.2013.07.004.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. 73:2804–2819.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008a. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J. Dairy Sci. 91:3300–3310. http://dx.doi.org/10.3168/jds.2008-0995.

Bertoni, G., E. Trevisi, X. Han, and M. Bionaz. 2008b. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J. Dairy Sci. 91:3300–3310. http://dx.doi.org/10.3168/jds.2008-0995.

Bertoni, G., E. Trevisi, and F. Piccioli-Cappelli. 2004. Effects of acetyl-salicylate used in post-calving of dairy cows. Vet. Res. Commun. 28:217–219. http://dx.doi.org/10.1023/B:VERC.0000045410.86004.03.

Cai, D., Y. Jia, H. Song, S. Sui, J. Lu, Z. Jiang, and R. Zhao. 2014. Betaine supplementation in maternal diet modulates the epigenetic regulation of hepatic gluconeogenic genes in neonatal piglets. PLoS ONE 9:e105504 http://dx.doi.org/10.1371/journal.pone.0105504.

Crookenden, M. A., K. S. Mandok, T. M. Grala, C. V. C. Phyn, J. K. Kay, S. L. Greenwood, and J. R. Roche. 2015. Source of metabo-

lizable energy affects gene transcription in metabolic pathways in adipose and liver tissue of nonlactating, pregnant dairy cows. J. Anim. Sci. 93:685–698. http://dx.doi.org/10.2527/jas.2014-7978.

Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. J. Dairy Sci. 84:E100–E112. http://dx.doi.org/10.3168/jds.S0022-0302(01)70204-4.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, B. KuKanich, L. M. Sordillo, S. K. Stoakes, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013a. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305:R110–R117. http://dx.doi.org/10.1152/ajpregu.00152.2013.

Farney, J. K., L. K. Mamedova, J. F. Coetzee, J. E. Minton, L. C. Hollis, and B. J. Bradford. 2013b. Sodium salicylate treatment in early lactation increases whole-lactation milk and milk fat yield in mature dairy cows. J. Dairy Sci. 96:7709–7718. http://dx.doi.org/10.3168/jds.2013-7088.

Grala, T. M., J. K. Kay, C. V. C. Phyn, M. Bionaz, C. G. Walker, A. G. Rius, R. G. Snell, and J. R. Roche. 2013. Reducing milking frequency during nutrient restriction has no effect on the hepatic transcriptome of lactating dairy cattle. Physiol. Genomics 45:1157–1167. http://dx.doi.org/10.1152/physiolgenomics.00134.2013.

Grala, T. M., J. K. Kay, C. G. Walker, A. J. Sheahan, M. D. Littlejohn, M. C. Lucy, and J. R. Roche. 2010. Expression analysis of key somatotropic axis and liporegulatory genes in ghrelin- and obestatin-infused dairy cows. Domest. Anim. Endocrinol. 39:76–83. http://dx.doi.org/10.1016/j.domaniend.2010.02.004.

Greenfield, R. B., M. J. Cecava, and S. S. Donkin. 2000. Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. J. Dairy Sci. 83:1228–1236. http://dx.doi.org/10.3168/jds.S0022-0302(00)74989-7.

Hahn, M. A., T. Hahn, D.-H. Lee, R. S. Esworthy, B.-W. Kim, A. D. Riggs, F.-F. Chu, and G. P. Pfeifer. 2008. Methylation of polycomb target genes in intestinal cancer is mediated by inflammation. Cancer Res. 68:10280–10289. http://dx.doi.org/10.1158/0008-5472.CAN-08-1957.

Hazelton, S. R., D. M. Spurlock, C. A. Bidwell, and S. S. Donkin. 2008. Cloning the genomic sequence and identification of promoter regions of bovine pyruvate carboxylase. J. Dairy Sci. 91:91–99. http://dx.doi.org/10.3168/jds.2007-0542.

Hur, K., T. Niwa, T. Toyoda, T. Tsukamoto, M. Tatematsu, H.-K. Yang, and T. Ushijima. 2011. Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation. Carcinogenesis 32:35–41. http://dx.doi.org/10.1093/carcin/bgq219.

Kominsky, D. J., S. Keely, C. F. MacManus, L. E. Glover, M. Scully, C. B. Collins, B. E. Bowers, E. L. Campbell, and S. P. Colgan. 2011. An endogenously anti-inflammatory role for methylation in mucosal inflammation identified through metabolite profiling. J. Immunol. 186:6505–6514. http://dx.doi.org/10.4049/jimmunol.1002805.

Meier, S., N. V. Priest, C. R. Burke, J. K. Kay, S. McDougall, M. D. Mitchell, C. G. Walker, A. Heiser, J. J. Loor, and J. R. Roche. 2014. Treatment with a nonsteroidal antiinflammatory drug after calving did not improve milk production, health, or reproduction parameters in pasture-grazed dairy cows. J. Dairy Sci. 97:2932–2943. http://dx.doi.org/10.3168/jds.2013-7838.

Niwa, T., T. Tsukamoto, T. Toyoda, A. Mori, H. Tanaka, T. Maekita, M. Ichinose, M. Tatematsu, and T. Ushijima. 2010. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. Cancer Res. 70:1430–1440. http://dx.doi.org/10.1158/0008-5472.CAN-09-2755.

Pan, M.-R., H.-C. Chang, L.-Y. Chuang, and W.-C. Hung. 2008. The nonsteroidal anti-inflammatory drug NS398 reactivates SPARC expression via promoter demethylation to attenuate invasiveness of lung cancer cells. Exp. Biol. Med. (Maywood) 233:456–462. http://dx.doi.org/10.3181/0709-RM-257.

Priest, N. V., S. McDougall, C. R. Burke, J. R. Roche, M. Mitchell, K. L. McLeod, S. L. Greenwood, and S. Meier. 2013. The responsiveness of subclinical endometritis to a nonsteroidal antiinflammatory drug in pasture-grazed dairy cows. J. Dairy Sci. 96:4323–4332. http://dx.doi.org/10.3168/jds.2012-6266.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. J. Dairy Sci. 86:1201–1217. http://dx.doi.org/10.3168/jds.S0022-0302(03)73704-7.

Vailati Riboni, M., S. Meier, N. V. Priest, C. R. Burke, J. K. Kay, S. McDougall, M. D. Mitchell, C. G. Walker, M. Crookenden, A. Heiser, J. R. Roche, and J. J. Loor. 2015. Adipose and liver gene expression profiles in response to treatment with a nonsteroidal antiinflammatory drug after calving in grazing dairy cows. J. Dairy Sci. 98:3079–3085. http://dx.doi.org/10.3168/jds.2014-8579.

Velez, J. C., and S. S. Donkin. 2005. Feed restriction induces pyruvate carboxylase but not phosphoenolpyruvate carboxykinase in dairy cows. J. Dairy Sci. 88:2938–2948. http://dx.doi.org/10.3168/jds.S0022-0302(05)72974-X.

White, H. M. 2015. The role of TCA cycle anaplerosis in ketosis and fatty liver in periparturient dairy cows. Animals (Basel) 5:793–802. http://dx.doi.org/10.3390/ani5030384.

White, H. M., S. L. Koser, and S. S. Donkin. 2011a. Characterization of bovine pyruvate carboxylase promoter 1 responsiveness to serum from control and feed-restricted cows. J. Anim. Sci. 89:1763–1768. http://dx.doi.org/10.2527/jas.2010-3407.

White, H. M., S. L. Koser, and S. S. Donkin. 2011b. Bovine pyruvate carboxylase 5′ untranslated region variant expression during transition to lactation and feed restriction in dairy cows. J. Anim. Sci. 89:1881–1892. http://dx.doi.org/10.2527/jas.2010-3697.

Wolffe, A. P., and M. A. Matzke. 1999. Epigenetics: Regulation through repression. Science 286:481–486.