Effects of sodium salicylate and time postpartum on mammary tissue proliferation, gene transcript profile, and DNA methylation

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ABSTRACT

Previous studies have demonstrated nonsteroidal antiinflammatory drug treatment in early lactation had a positive impact on whole-lactation milk production in older cows. The objective of this study was to evaluate proliferative, transcriptional, and epigenetic changes in the mammary gland that could explain increased production responses due to nonsteroidal antiinflammatory drug treatment. Sodium salicylate (SAL; 125 g/d) or water (CON) were administered via oral drench to multiparous Holstein cows ($n = 8/\text{treatment}$) once daily for 3 d beginning approximately 24 h after parturition, and mammary tissue was collected on d 1, 4, and 45 postpartum. Day 1 tissue was collected immediately preceding the initial drench, and d 4 tissue was collected 24 h following the final drench. Blood was collected twice weekly and analyzed for plasma glucose, insulin, β -hydroxybutyrate, free fatty acids, and prolactin. Cows were milked twice daily until d 7 of lactation, and thrice daily for the remainder of the study. Total RNA extracted from tissue was deep-sequenced and analyzed for differential gene expression using DESeq2. We detected no treatment effect on milk yield or plasma metabolites through 45 d of lactation; additionally, no change in mammary epithelial cell proliferation was detected when assessed by Ki67 labeling. Comparison of SAL versus CON revealed that only 16 of 18,286 genes were differentially expressed (false discovery rate < 0.1) in mammary tissue collected on d 45, whereas no differentially expressed genes due to treatment were detected on d 1 or 4. Analysis of transcriptional differences over time showed downregulation of pathways related to immune cell recruitment and differentiation, and extensive overlap with pathways related to chole-

sterol synthesis and liver X receptor signaling. Global DNA methylation of mammary tissue was decreased for CON compared with SAL. Transcriptome analysis emphasized extensive involvement of immune-related signaling pathways in the switch from lactogenesis to galactopoiesis, and changes in methylation with SAL treatment merit future investigation into epigenetic effects on milk production.

Key words: nonsteroidal antiinflammatory drug, inflammation, RNA-seq, epigenetics

INTRODUCTION

The postpartum period of dairy cows is characterized by a chronic, low-grade inflammatory tone (Bradford and Swartz, 2020). Indeed, inflammatory markers such as acute phase proteins are elevated in even healthy cows during the transition period (Qu et al., 2014; Akbar et al., 2015). Excessive systemic inflammation in early lactation can have short-term implications for risk of metabolic disease as well as detrimental effects on milk production (Yuan et al., 2013). In fact, cows in the upper quartile for inflammatory markers during the first 4 wk of lactation produced 20% less milk than cows in the lowest quartile (Bertoni et al., 2008). Antiinflammatory treatment in early lactation reduced inflammatory markers (Trevisi and Bertoni, 2008), increased milk yield in multiparous cows (Farney et al., 2013b; Carpenter et al., 2016), and decreased SCC (Carpenter et al., 2016; Shock et al., 2018; Barragan et al., 2020).

The effects of inflammatory signaling in the mammary gland can suppress the transcription of milk protein genes (Beaton et al., 2003), reduce cell proliferation, and alter chromatin accessibility near milk protein promoter regions (Vanselow et al., 2006). In mice, inflammatory signaling reduced milk secretion by increasing the rate of apoptosis in the mammary epithelium (Connelly et al., 2010), and conversely, impaired inflamma-

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tory signaling resulted in delayed involution and fewer apoptotic cells (Baxter et al., 2006). As such, one possible explanation for the increase in milk yield following nonsteroidal antiinflammatory drug (**NSAID**) therapy is an increase in mammary epithelial cells (**MEC**) or their activity. Therefore, the objective of this study was to examine mechanistic effects of antiinflammatory treatment on transcriptional and epigenetic changes of the mammary gland in early lactation, with the hypothesis that antiinflammatory treatment increases mammary epithelial proliferation, transcription related to milk component synthesis, or both.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol 3352).

Treatments and Experimental Design

Sixteen Holstein cows in third or greater lactation were enrolled between January and September 2014 and alternately assigned to treatment groups at the time of parturition. Beginning 12 to 36 h after parturition, cows were treated once daily at 0700 h for 3 d with an oral drench containing either 375 mL of water (**CON**; $n = 8$, 3.22 ± 0.44 lactations) or 125 g of sodium salicylate dissolved in water (**SAL**, Wintersun Chemical; final volume 375 mL; $n = 8$, 3.14 ± 0.38 lactations). The sample size based on previous studies of <8 cows per treatment provided sufficient power to detect changes in mammary cell proliferation (Capuco et al., 2001) and transcription profiles (Boutinaud et al., 2012). Investigators were not blinded to treatment allocation.

Animal Management

Cows were housed in tiestalls and offered feed at 110% of ad libitum intake and had ad libitum access to water. The TMR was prepared once daily and cows were fed twice per day at 0630 and 1730 h. Primary dietary ingredients were corn silage, corn gluten feed, alfalfa hay, and corn grain, and TMR contained 18% CP, 33% NDF, and 1.67 Mcal of NE_L /kg of DM (Carpenter et al., 2018). Individual feed intake was recorded daily by subtracting feed refused from feed offered, and daily water intake was recorded via water meters located on each cow's water supply line. Cows were milked twice per day (0700 and 1500 h) until d 7, and then 3 times per day (0700, 1500, and 2300 h) for the duration of the study.

Sample Collection

Daily milk weights were recorded, and milk samples representative of a full milking were collected at all 3 milkings on Tuesdays and Fridays of each week. Milk samples were analyzed for fat, protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc.), MUN (MUN spectrophotometer; Bentley Instruments Inc.), and SCC (SCC 500, Bentley Instruments Inc.) at Heart of America DHIA (Manhattan, KS). The SCC was converted to a linear score [$SCLS = \log_2(SCC/100) + 3$; Shook, 1993]. Energy-corrected milk yield was calculated as $ECM = [(0.327 \times \text{milk yield}) + (12.95 \times \text{fat yield}) + (7.65 \times \text{protein yield})]$, and 3.5% FCM yield was calculated as $FCM = [(0.432 \times \text{milk yield}) + (16.216 \times \text{fat yield})]$.

Blood samples were collected from coccygeal vessels on d 1 (before first treatment), 4, 7, 11, 15, 18, 21, 25, 28, 31, 35, 39, 42, and 45. Samples were collected before morning feeding into evacuated tubes containing potassium oxalate with sodium fluoride or potassium EDTA (Becton, Dickinson and Co.). Blood was centrifuged at $2,800 \times g$ at 20°C for 15 min and isolated plasma was frozen at -20°C until analysis. Using a multispectral plate reader (PowerWave XS spectrophotometer, Biotek Instruments Inc.), plasma was analyzed for glucose (catalog no. 439-90901; Wako Chemicals USA Inc.), insulin (catalog no. 10-1201-01, Mercodia AB), BHB (catalog no. H7587, Pointe Scientific Inc.), and free fatty acids (**FFA**; HR Series kit, Wako Chemicals USA Inc.). Each assay was carried out in duplicate and averaged for analysis. Samples from d 1, 4, and wk 2 (d 7 and 11 pooled), 4 (d 21 and 25), and 6 (d 35 and 39) were analyzed in duplicate for prolactin (Bovine Prolactin ELISA; catalog no. MBS2022462, MyBioSource.com). Detection range was 31.2 to 2,000 pg/mL, spike recovery was 80 to 92%, and intraassay coefficient of variation was <10% with an interassay coefficient of variation <12%.

Mammary biopsies were performed on d 1, 4, and 45 after parturition. One hour before biopsy, the target gland was treated with 50 mg of intramammary prophylactic antibiotic pirlimycin hydrochloride (Pirsue, Zoetis). Biopsies were conducted at 0900 h, approximately 2 h after morning milking. Cows were administered xylazine hydrochloride (0.025 mg/kg BW, Akorn Inc.) intravenously via the coccygeal vessel for partial sedation. The surgical area was scrubbed alternately with Betadine surgical scrub (Purdue Products LP) and ethyl alcohol (70 % vol/vol) 3 times. Local anesthetic (10 to 20 mL of 2% lidocaine-HCl) was administered subcutaneously in a horizontal line just dorsal to the incision site; a 1-cm incision was made with a #22 scalpel blade (Integra York PA Inc.) and mammary tissue

was collected using a Bard Magnum biopsy instrument (catalog no. MG1522; Bard Peripheral Vascular Inc.) with a 12 gauge $\times$ 10 cm tissue biopsy needle (catalog no. MN1210, Bard Peripheral Vascular Inc.). One core of tissue ($\sim$ 100 mg) was placed in 10% formalin for 24 h and stored in 70% (vol/vol) ethyl alcohol at 4°C for later immunohistochemistry. Tissue for RNA extraction ($\sim$ 100 mg) was immersed in Trizol Reagent (Life Technologies Corporation) before storage at -80°C ; tissue samples for global DNA methylation analysis ($\sim$ 100 mg) were snap frozen in liquid nitrogen before storage at -80°C . After the biopsy, pressure was applied until bleeding stopped and the incision was closed with surgical staples. Biopsies on d 1 and 4 were collected from alternate rear mammary glands; d 45 biopsy was conducted on the same gland as d 1 but $\sim$ 3 cm below the site of first incision.

Immunohistochemistry

Formalin-fixed biopsy cores were embedded in paraffin. For this, biopsy cores were removed from storage containers, placed into individually labeled fine-gauge plastic baskets, processed, and embedded into paraffin blocks. Microscope slides were prepared by slicing 5- μm -thick sections from the paraffin-embedded tissue blocks with a microtome. Four to 5 serial tissue sections from each sample were mounted onto Superfrost Plus slides (Fisher Scientific). Approximately 5 microscope slides were prepared from each tissue block. General immunohistochemistry protocol methods are described elsewhere (Daniels et al., 2009). Secondary antibodies for Ki67 [Alexa 532 Goat Anti-rabbit IgG (A1109); 1:200 dilution; Invitrogen], counter-staining, and imaging and quantification methods are described by Tucker et al. (2016). Briefly, MEC were identified based on location (apical ends in contact with lumen) and number of cell layers (a single layer of epithelial cells); all other mammary cell types were excluded from this analysis. The resultant MEC proliferation data are presented as (no. of Ki67 positive MEC/total no. of MEC) $\times$ 100.

RNA Isolation and Sequencing

Total RNA was isolated from tissue using the RNeasy mini kit (Qiagen) and the concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific), whereas quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). Samples with RIN less than 4 were excluded from further analysis, resulting in a total of 43 samples (21 for SAL cows; 22 for CON) submitted for sequencing (RNA integrity number mean: 5.2 ± 0.74).

Libraries were created using a TruSeq RNA Library Prep Kit v2 (Illumina) and sequenced on a HiSeq 2500 at the University of Kansas Medical Center's Genome Sequencing Facility. Average library size was 280.7 bp; samples were run as single read with 100-bp read length. Sequencing generated 27.9 ± 3.8 million reads per sample.

Global DNA Methylation

The DNA was extracted from snap-frozen mammary tissue using the Quick-DNA Miniprep Plus kit (Zymo Research Corp.). Samples were first crushed with mortar and pestle in liquid nitrogen before incubation with Proteinase K, and solid tissue buffer (supplied by kit) for 1 h at 55°C . RNase A (2 $\mu\text{g}/\mu\text{L}$; Zymo Research Corp.) was added and incubated for 2 min, after which samples were centrifuged for 1 min at $10,000 \times g$ at 20°C , and the supernatant was used for the remainder of the kit protocol. The DNA concentrations were determined with a Take3 microvolume plate (Biotek Instruments Inc.). Global DNA methylation was determined (5-methylcytosine Enzyme-Linked Immunosorbent Assay Kit; Zymo Research Corp.) using 100 ng of DNA from each sample in duplicate, and plotted against a standard curve created from *E. coli* methylase-treated gDNA, according to the kit protocol. After horseradish peroxidase substrate was added, the plate was incubated in the dark for 10 min before reading at 450 nm wavelength on a PowerWave XS spectrophotometer (Biotek Instruments).

Data Analysis

Water intake, DMI, milk yield, and milk component data were averaged by week of lactation for analysis. These variables as well as plasma analytes and DNA methylation data were analyzed with SAS (version 9.3, SAS Institute Inc.) using repeated measures with treatment, time, and their interaction as fixed effects and cow as a random effect. Additionally, d 1 values for DNA methylation (before treatment) were used as a covariate in analysis of treatment effects on d 4 and 45. Residuals were checked for normality, and insulin and BHB data were natural log-transformed before analysis. Contrasts between SAL and CON on d 4 and 45 were used to assess treatment effects on DNA methylation (excluding the d 1 pre-treatment time point). Immunohistochemistry data were analyzed with JMP (version 11, SAS Institute Inc.), with fixed effects of treatment, day, and their interaction, and cow as a random effect. Data points with a conditional Studentized residual >4 or <-4 were excluded before final analysis; no more

than 3% of data points were removed as outliers. Significance was declared at $P \leq 0.05$; P -values >0.05 and <0.10 were considered tendencies.

Raw sequencing data were processed using CLC Genomics Workbench 8.0.2 (www.qiagenbioinformatics.com). Sequences were aligned to bovine reference genome UMD 3.1.1 using the methods of Mortazavi et al. (2008). Counts were analyzed in R (version 3.3.2) using the Bioconductor package DESeq2 (Love et al., 2014). Genes with less than 3 total reads across all samples were filtered out, reducing the number of genes included in the analysis from 24,616 to 18,286. Principal component analysis was used to visualize data and identify outliers; based on this assessment, 4 samples (2 from CON, 2 from SAL) were removed from analysis as outliers, leaving 39 samples to be included in analysis. Effects of treatment as well as treatment by time interaction were analyzed. Genes with P -value adjusted for false discovery rate <0.10 were considered differentially expressed.

Fold change and adjusted P -values of differentially expressed (DE) genes were uploaded into Ingenuity Pathway Analysis (IPA) software (Qiagen Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) for functional pathway analysis. Genes with fold changes >1.5 and adjusted P -values <0.05 were used for the following contrasts: d 1 versus 4, d 4 versus 45, and d 1 versus 45. The contrast of CON d 45 versus SAL d 45 only resulted in 16 DE genes, of which only 4 met cutoff conditions for inclusion in IPA, rendering pathway enrichment analysis

infeasible. Evaluation of differences over time utilized both CON and SAL samples.

Data Access

Raw sequencing data files can be accessed at the National Center for Biotechnology Information (NCBI) Sequence Read Archive, under BioProject PRJNA684666 available at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA684666>. Additionally, Supplemental Tables S1–S3 are available at <https://doi.org/10.6084/m9.figshare.13501650.v1> (Bradford, 2020).

RESULTS

Milk Production and Feed Intake

No differences in water intake were detected that could be attributed to antiinflammatory treatment. An overall effect of NSAID treatment on DMI was not detected, but treatment interacted with week ($P = 0.03$; Table 1, Figure 1A), such that those treated with SAL exhibited a less dramatic increase in DMI following parturition (18.9 to 32.0 ± 1.7 kg/d for wk 1 to 7) than CON cows (17.0 to 32.1 ± 1.7 kg/d for wk 1 to 7). No differences between treatments were detected for any individual week.

Milk production results are reported in Table 1 and Figure 1B. Weekly means of milk production parameters revealed no detectable differences for fat content or yield, MUN, SCS, ECM yield, or FCM yield. Milk yield

Table 1. Feed intake and milk production responses to antiinflammatory treatment¹

Item	Treatment (Trt)			P -value		
	CON (n = 8)	SAL (n = 8)	SEM	Trt	Day	Trt $\times$ day
Milk yield, kg/d	45.4	47.8	2.91	0.57	<0.0001	0.10
Fat, %	4.32	4.31	0.15	0.97	<0.0001	0.71
Fat yield, kg/d	1.93	1.99	0.15	0.76	<0.01	0.36
Protein, %	2.90	3.02	0.078	0.28	<0.0001	0.30
Protein yield, kg/d	1.29	1.39	0.070	0.33	<0.0001	0.02
Lactose, %	4.74	4.89	0.051	0.06	<0.0001	0.40
Lactose yield, kg/d	2.18	2.36	0.15	0.42	<0.0001	0.21
MUN, mg/dL	13.05	12.78	0.71	0.79	0.10	0.50
SCLS ²	3.10	2.34	0.83	0.53	<0.0001	0.79
ECM, ³ kg/d	49.7	52.0	3.26	0.62	<0.0001	0.23
3.5% FCM, ⁴ kg/d	50.8	52.9	3.13	0.64	<0.0001	0.19
DMI, kg/d	25.7	26.8	1.4	0.60	<0.0001	0.03
Water intake, L/d	107.9	113.2	6.1	0.55	<0.0001	0.92

¹Multiparous cows (parity 3+, n = 8 per treatment) were treated once daily with an oral drench of sodium salicylate (SAL; 125 g/d) or water (CON) for the first 3 d of lactation. Milk samples representative of a full milking were collected and analyzed twice weekly until d 45 of lactation. Data presented are the overall mean values of the first 45 d of lactation.

²SCLS = somatic cell linear score, $\log_2(\text{SCC}/100) + 3$.

³ECM, calculated as $(0.327 \times \text{milk yield}) + (12.95 \times \text{fat yield}) + (7.65 \times \text{protein yield})$.

⁴FCM, calculated as $(0.432 \times \text{milk yield}) + (16.216 \times \text{fat yield})$.

Figure 1. Feed intake and milk yield over the first 7 wk of lactation. Holstein cows (parity 3+, $n = 8$ per treatment) were administered an oral drench of sodium salicylate (SAL; 125 g/d) or water (CON) for the first 3 d of lactation. Amounts of feed offered and refused were recorded daily to calculate DMI (panel A). Despite significant interaction of treatment and week for overall DMI, no differences were detected on any individual week. Milk yield (B) was recorded at each milking and analyzed by week. Values are LSM $\pm$ SEM.

tended to increase more for SAL (from 26.6 to 55.8 ± 2.6 kg/d) than CON (from 30.7 to 52.3 ± 2.3 kg/d) over time (interaction $P = 0.10$). Treatment interacted with week for protein yield ($P = 0.02$), with SAL cows increasing more (1.07 to 1.49 ± 0.09 kg/d) over the 7 wk of the study than CON (1.19 to 1.37 ± 0.09 kg/d). Lactose content tended to be greater for SAL (4.89%) compared with CON ($4.74 \pm 0.05\%$; $P = 0.06$).

Plasma Analytes

Results for plasma metabolites and hormones are presented in Figure 2; no differences were detected between treatments for plasma glucose, insulin, BHB, FFA, or prolactin. Circulating glucose and insulin both reached a nadir around d 11 after parturition (Figure 2A and B), corresponding with the peak in circulating BHB (Figure 2D) and prolactin (Figure 2E). Circulating FFA levels were greatest at the sampling point

closest to parturition and decreased steadily over time until the end of the study (Figure 2C).

MEC Proliferation

Results of Ki67 labeling are shown in Figure 3. No treatment difference was detected at any of the 3 sampling time points, but the proportion of Ki67-positive cells was greatest on d 1 of lactation and decreased by d 4 and 45 ($P < 0.01$; no difference between d 4 and 45).

Transcriptional Analysis

A total of 24,616 RNA species were detected in mammary biopsy samples via high-throughput sequencing. After removing those with 3 or less reads across all samples, 18,286 remained for differential expression analysis. Analyzing for the main effect of sodium salicylate treatment identified no DE genes. Comparing d 1 to 4, d 1 to 45, and d 4 to 45 samples identified 901, 6,623, and 3,770 DE genes, respectively. Samples clearly grouped within day when plotted by principal component analysis (Figure 4). Additionally, day by treatment interactions were explored such that treatment effects were analyzed within day of lactation; on d 45, 16 genes were DE between SAL and CON, but no DE genes were detected on d 1 or 4 of lactation. The few genes that were DE on d 45 due to antiinflammatory treatment are found in Table 2.

Most Abundant Transcripts

The gene transcripts most abundantly detected are shown in heatmap form (Figure 5). Transcripts involved in milk protein synthesis (*CSN1S1*, *CSN2*, *CSN1S2*, *LGB*, *CSN3*, *LALBA*) collectively accounted for 62% of all transcripts detected. Averaging across all samples, casein α_{S1} was the most abundant mRNA transcript detected, accounting for 20% of all gene transcripts identified. After milk protein genes, functions of the other most abundant gene transcripts related to mitochondrial activity (*COX1*, *COX3*, *MT-CYB*, *ATP6*, *ND1*, *CO2*, and *MT-ND4*), lipid synthesis and oxidation (*FASN*, *FABP3*, and *GPAM*), translation and protein transport (*EEFA1-3*), purine degradation (*XDH*), and cell adhesion (*GLYCAM1*).

Transcriptional Changes Over Time

Data loaded into IPA were analyzed for canonical pathways and upstream regulators that differed over time. Using a comparison analysis, which allows for viewing time-course type data, those outcomes that were affected similarly over multiple time point com-

parisons were identified. Most of the identified pathways were upregulated in early lactation relative to d 45, including dendritic cell maturation, TREM1 signaling, inflammasome pathway, role of NFAT in regulation of the immune response, and CDK5 signaling. Those pathways that were downregulated in early lactation relative to d 45 included IL-1 signaling, STAT3 pathway, PPAR signaling, and GnRH signaling. Pathways

elevated especially on d 4 of lactation relative to d 45 included role of pattern recognition receptor (PRR) in recognition of bacteria and viruses, IL-8 signaling, and acute phase response signaling. A more complete list can be found in Table 3.

Examination of individual contrasts between d 1 and d 45 revealed enrichment of molecules involved in cholesterol synthesis and LPS/IL-1 mediated inhibition

Figure 2. Plasma analytes of multiparous dairy cows over 45 d postpartum. Multiparous Holstein cows ($n = 8$ per treatment; parity 3+) were administered sodium salicylate (SAL; 125 g/d) or water drench (CON) for the first 3 d in lactation. Blood samples were collected at 0600 h twice weekly until d 45 of lactation, and analyzed for plasma glucose (A), insulin (B), free fatty acids (C), BHB (D), and prolactin (E). Values for insulin and BHB were natural-log transformed for statistical analysis and reverse transformed for figures. For prolactin analysis, samples representing wk 2 (d 7 and 11), 4 (d 21 and 25), and 6 (d 35 and 39) were pooled before analysis. Values are LSM $\pm$ SEM.

of RXR function. Prediction of directional changes revealed that pathways of acute phase response signaling, EIF2 signaling, dendritic cell maturation, and HMGB1 signaling were upregulated on d 1, whereas LXR/RXR activation was downregulated. Contrasting d 4 with d 45, significantly enriched canonical pathways included immunodeficiency signaling, T cell differentiation, retinol biosynthesis, and TR/RXR activation. Complement system was upregulated, whereas growth hormone signaling was downregulated on d 4 relative to d 45. Between d 1 and 4, top pathways included cholesterol biosynthesis, FXR/RXR activation, phospholipases, and hematopoiesis. Acute phase response signaling, STAT3 pathway, and cell cycle regulation were downregulated, and ATM signaling, eNOS signaling, and CDK5 signaling were upregulated on d 1 relative to d 4 of lactation. More complete lists can be found in Supplemental Tables S1–S3 available at <https://doi.org/10.6084/m9.figshare.13501650.v1>.

Global DNA Methylation

The SAL treatment increased global DNA methylation in mammary tissue on d 4 and 45 postpartum ($P = 0.02$; Figure 6). Methylation declined numerically between d 1 and d 4 in CON, but showed the opposite pattern in SAL samples, with no apparent shift between d 4 and 45 (day $P = 0.71$; treatment $\times$ day $P = 0.73$).

Figure 3. Proportion of mammary epithelial cells (MEC) positive for Ki67 in early lactation. Cows were administered sodium salicylate (SAL; 125 g/d) or water (CON) once daily on d 1 (after first biopsy), 2, and 3. Mammary biopsies were conducted on d 1, 4, and 45 of lactation. Proliferation of MEC was assessed ($n = 7$ –8 per treatment at each time point) with immunohistochemistry using an antibody for the cell proliferation marker Ki67; data are (no. of Ki67 positive MEC/total no. of MEC) $\times 100$. Values are LSM $\pm$ SEM.

DISCUSSION

Our study is the first to examine the mammary molecular mechanisms behind the milk yield response to SAL treatment in postpartum multiparous dairy cows. In general, minimal responses to SAL treatment were identified; however, differential expression of a few transcripts involved in immune-related signaling pathways were found during the switch from lactogenesis to galactopoiesis, as well as greater global DNA methylation with SAL treatment. More notable were the changes in mammary transcript abundance that occurred over time, particularly in reference to cholesterol synthesis as well as the upregulation of immune-related signaling pathways in early lactation.

Milk Production

Biopsy time points were designed to assess the mammary transcriptome immediately before and following the SAL treatment period, as well as closer to peak lactation. We hypothesized that short-term transcriptional responses would occur during the time of treatment administration, as well as sustained or later occurring transcriptional differences that could help to explain long-term increases in milk production observed in previous studies with early lactation antiinflammatory treatments (Carpenter et al., 2016; Farney et al., 2013b). No overall differences were observed in milk yield in this study over 7 wk of lactation; perhaps more transcriptional changes in mammary tissue would have been evident in studies where milk yield was significantly increased by antiinflammatory treatment. This study was paired with a larger cohort of animals provided the same treatment regimen but without invasive biopsies, which also failed to detect significant effects on milk yield (Carpenter et al., 2018). This pair of studies used essentially the same treatment protocol as that of Carpenter et al. (2016), but a shorter treatment period than that of Farney et al. (2013b). We speculated that the cohort described in Carpenter et al. (2018) may have failed to show a production response to NSAID treatment because of lesser inflammatory status than in previously studied populations.

Plasma Analytes

Lemini et al. (2015) observed that increasing concentrations of $\text{TNF}\alpha$ administration in vitro increased both mRNA and protein levels of prolactin in a rodent GH4C1 pituitary cell line, and also mice fed a high-fat diet displayed elevated circulating $\text{TNF}\alpha$ but reduced circulating prolactin. This led us to wonder if systemic inflammation and elevated inflammatory cytokines

Table 2. Gene transcripts differentially expressed in mammary tissue on d 45 of lactation (n = 7 per treatment)¹

Gene	Mean count ²	Log ₂ fold change	FDR ³ adjusted P-value	Gene name and description
<i>ADAMTSL1</i>	33,838.7	1.62	<0.001	ADAMTS-like-1 (extracellular matrix protein)
<i>ANLN</i>	5,872.6	1.70	<0.001	Anillin actin binding protein (regulates cytoskeleton and promotes cell cycle)
<i>CEP120</i>	28,317.5	1.73	<0.001	Centrosomal protein 120 (promotes centriole elongation during cell division)
<i>ENSBTAG00000047908</i>	99.3	1.66	<0.001	Noncoding signal recognition particle RNA
<i>VAV3</i>	6,779.6	1.53	<0.001	Vav guanine nucleotide exchange factor 3
<i>ENSBTAG00000047264</i>	33,068.5	1.62	<0.001	Uncharacterized
<i>ENSBTAG00000045661</i>	431.3	1.44	0.004	Noncoding signal recognition particle RNA
<i>ENSBTAG00000043250</i>	75.9	1.46	0.004	7SK small nuclear (noncoding) RNA
<i>ENSBTAT00000061860</i>	23.3	1.27	0.037	Nuclear RNase P (ribozyme that processes pre-tRNA)
<i>C3H1orf146</i>	13.8	-1.24	0.069	Chromosome 3 C1orf146 homolog (synaptonemal complex reinforcing element)
<i>C4BPA</i>	99.7	-1.11	0.069	Complement component 4 binding protein α (inhibits Complement)
<i>DICER1</i>	269.5	-0.44	0.069	Ribonuclease 3 (processes microRNA)
<i>H1-3</i>	335.2	0.97	0.074	H1.3 linker histone, cluster member (involved in chromatin structure)
<i>ENSBTAG00000047947</i>	27.5	1.17	0.077	Noncoding signal recognition particle RNA
<i>ENSBTAG00000046912</i>	28.1	1.17	0.077	Noncoding signal recognition particle RNA
<i>BOLA1</i>	107.3	0.41	0.094	BolA family member 1 (mitochondrial redox function)

¹Multiparous Holstein cows were administered sodium salicylate (SAL; 125 g/d) or water drench (CON) for the first 3 d in lactation. Mammary tissue samples were collected on d 1, 4, and 45 of lactation; RNA was sequenced and counts were analyzed for differential expression using the Bioconductor package DESeq2 (Love et al., 2014). Information on genes identified is available at <https://www.ensembl.org>. Note that all 4 noncoding signal recognition particle RNA species listed are paralogues of the gene named *RF00017*.

²Mean count column displays normalized average of all d-45 samples. Normalized counts are calculated by DESeq2 by first dividing raw counts for each gene by the geometric mean of that gene across all samples. Of the resulting ratios, the median value within each sample becomes the normalization factor (for that sample) by which raw counts are divided to produce the normalized counts (Love et al., 2014). Fold change values are for SAL relative to CON; negative fold change values indicate lesser transcript abundance gene expression in SAL relative to CON. No gene transcripts were differentially expressed on d 1 or 4 of lactation.

³FDR = false discovery rate.

Figure 4. Principal component (PC) analysis plot of the log₂-transformed normalized data of RNA sequenced from mammary tissue of dairy cows, obtained on d 1, 4, and 45 of lactation (n = 6–7 per treatment at each time point). Cows were enrolled at parturition and treated with (125 g/d sodium salicylate) or without (control) antiinflammatory over the first 3 d of lactation.

around calving could interfere with prolactin release by the pituitary gland, and that NSAID treatment may affect milk production through increased prolactin levels. However, we did not observe any effects on plasma prolactin to support this, although we acknowledge that plasma sample collection was not timed for optimal detection of differences in prolactin. Similarly, no differences in other metabolites were detected. Previous research in our laboratory observed decreased plasma glucose concentrations and increased insulin sensitivity indices for NSAID-treated cows at d 7 of lactation (Farney et al., 2013a; Montgomery et al., 2019), but the reduced duration of treatment used in this study likely prevented a similar outcome, as was also observed in other studies with 3-d NSAID treatment protocols (Carpenter et al., 2016, 2018).

Transcriptional Analysis

Samples of mammary tissue analyzed likely included not only MEC but also other cell types, including immune cells, fibroblasts, endothelial cells, myoepithelial cells, and adipocytes (Kass et al., 2007). Gene transcripts present may therefore originate from any of these cell types, but histological analysis as well as the overwhelming abundance of milk protein transcripts suggest that MEC account for a large proportion of cells present. Other than milk protein genes, the most abundant gene transcripts detected were all related to either protein synthesis, nutrient metabolism, or mitochondrial function, with the exception of *GLYCAM1*. The prevalence of transcripts related to mitochondrial function reflects the high level of metabolic activity

Figure 5. Heatmap displaying the 20 most abundant genes in mammary tissue. Heatmap showing the top 20 mRNA detected, which were grouped horizontally by similarity between samples (treatment and day of sampling) and vertically by similar expression patterns between genes. Color scale values indicate log₂-transformed normalized mean counts, averaged across all samples. Gene expression was analyzed from mammary tissues sampled on d 1, 4, and 45 of lactation from cows treated with (125 g/d sodium salicylate) or without (control) antiinflammatory treatment over the first 3 d of lactation (n = 6–7 per treatment at each time point).

in the early lactation mammary gland, as was demonstrated by increased ATP synthesis, mitochondrial number, and electron transport chain activity in post-partum murine mammary tissue (Hadsell et al., 2011).

The mucin-like glycoprotein GlyCAM1 is found on endothelial cells and is involved in cell adhesion via interactions with L-selectin; high expression was also detected in other studies of bovine mammary tissue, including one comparing mammary tissue, somatic cells, milk fat globules, and laser-dissected MEC (Cánovas et al., 2015). At least in mice, the GlyCAM detected in MEC and in milk was a different form compared with that found on endothelial cells, lacking the ability to interact with L-selectin, which suggests an alternate function in the mammary gland (Dowbenko et al., 1993; Nishimura, 2003). Evaluation of GlyCAM1 activity is needed to further examine functional differences between endothelial and mammary forms of this glycoprotein.

Treatment Effects

The low number of DE genes detected between CON and SAL corresponds with the histological lack of treatment differences on MEC proliferation. Additionally, many of the genes that were differentially expressed

between CON and SAL on d 45 of lactation have not been previously characterized and functions are currently unknown.

Dicer is an endonuclease enzyme responsible for microRNA processing (Ha and Kim, 2014). Decreased dicer abundance with SAL treatment may have caused a global reduction on microRNA-mediated gene regulation, but it is difficult to know what effects this might have on mammary function around peak lactation. Another endonuclease, nuclear RNase P, was upregulated by NSAID treatment. This ribozyme is involved with transfer RNA processing (Chamberlain et al., 1998); altered activity may have broader effects on amino acid trafficking for ribosomal protein synthesis. Histone 1 D is a member of the histone 1 family, which is involved in formation of chromatin structure, and may regulate epigenetic modifications including DNA methylation (Yang et al., 2013). Its upregulation with SAL treatment could be linked to the increased global methylation observed in SAL-treated cows, though differences in *HIST1H1D* expression were not observed on d 4 of lactation when the greatest difference in DNA methylation occurred. Histones have also been linked to inflammation and immune suppression, especially related to autoimmunity (Kusano et al., 2015). Additionally, there is at least an indirect link between salicylate and

Table 3. Top 25 enriched canonical pathways using time point comparison analysis¹

Canonical pathway ²	d 1 vs. 4	d 1 vs. 45	d 4 vs. 45
Dendritic cell maturation	0.71	3.57*	3.41*
TREM1 signaling	N/A	2.60*	3.05*
Inflammasome pathway	0.00	2.83*	2.24*
Role of NFAT in regulation of the immune response	N/A	2.79	2.24*
CDK5 signaling	1.67*	1.40*	1.90
IL-1 signaling	-2.00	-1.61*	-0.71
Colorectal cancer metastasis signaling	0.00	1.02*	3.27*
STAT3 pathway	-1.41*	-1.00*	-1.73*
Acute myeloid leukemia signaling	2.00	1.80*	0.33
G α i signaling	1.00	2.40*	0.71
PPAR signaling	N/A	-2.40*	-1.67
GnRH signaling	-2.24	-1.18*	0.63
HMGB1 signaling	0.45	1.95*	1.50*
Chemokine signaling	0.00	1.34*	2.53*
Glioma invasiveness signaling	N/A	2.29*	1.51*
G α s signaling	-1.13*	-1.22*	1.41
Glioblastoma multiforme signaling	-0.45	1.48*	1.81
EIF2 signaling	N/A	3.67*	0.00
G α q signaling	0.45	1.26*	1.94
Role of pattern recognition receptors in recognition of bacteria and viruses	-0.82	1.10*	1.73*
Leukocyte extravasation signaling	0.00	1.85*	1.61*
IL-8 signaling	-0.38	1.07*	2.00
Acute phase response signaling	-0.58*	1.48*	1.39*
NF- κ B signaling	1.34	1.85*	0.23*

¹Multiparous Holstein cows were administered sodium salicylate (125 g/d) or water drench (control) for the first 3 d in lactation. Mammary biopsies collected on d 1, 4, and 45 of lactation (n = 6–7 per treatment at each time point) were contrasted to assess differential expression over time, regardless of treatment. N/A indicates that expression of genes in the pathway is not sufficiently consistent to determine direction of regulation.

²Canonical pathways with predicted direction of regulation across multiple time points are displayed. Z-score values indicate direction of regulation for the first day listed relative to the second (i.e., for d 1 relative to d 4).

* $P < 0.05$ for differential pathway enrichment between the 2 d.

decreased histone phosphorylation via cyclin-dependent kinase activity (Dachineni et al., 2016), which may have effects on cell cycle regulation. *C4BPA* is a glycoprotein that inhibits complement by binding to C3 or C4; a downregulation of complement binding protein transcripts is consistent with the activation of complement observed with aspirin treatment in the literature (Lee et al., 2006). Last, it is also possible that the low number of DE genes detected (16 out of 18,286 analyzed) were merely false positives, and overinterpretation of these results without further supporting data should be avoided.

Transcriptional Changes in Mammary Tissue Over Time

Previous time-series analysis of the bovine transcriptome using microarrays demonstrated relative stability of expression profiles between d 30 and 120 of lactation (Bionaz et al., 2012), supporting our use of samples collected at 45 DIM to represent established lactation.

The canonical pathways that appeared to be most enriched in DE genes across time points include pathways involved in cytokine signaling and immune cell development, as well as cholesterol synthesis and intracellular receptor signaling (i.e., LXR/RXR activation). The downregulation of GnRH signaling in early

lactation compared with d 45 agrees with data showing the response to GnRH is decreased postcalving and increases over time (Fernandes et al., 1978; Kadokawa et al., 1998), likely as the cow resumes normal estrous activity. On the other hand, compared with mammary tissue collected 30 d prepartum, GnRH signaling was upregulated during lactation (Bionaz et al., 2012). Although the IL-1 signaling pathway was predicted to be downregulated, *IL1* itself was upregulated in our data, along with other proinflammatory cytokines, which can downregulate GnRH secretion and signaling (Sheldon et al., 2008). The immune pathways enriched in early lactation, as previously reported (Bionaz et al., 2012), are likely involved in mammary tissue remodeling and differentiation that occurs during pregnancy and during the onset of lactation. IL-1 and CDK signaling were shown to mediate TGF β induction of MEC proliferation (Karakas et al., 2006), which may lend insight to the role of elevated mammary IL-1 independent from typical downstream inflammatory signaling. IL-1 and IL-18 are also involved in inflammasome-mediated activation of inflammation; however, a recent review on the role of the inflammasome in the gastrointestinal tract shows its importance in the maintenance of epithelial homeostasis, and that IL-18 can also act to mitigate inflammatory signaling through promotion of regulatory T cells and tolerogenic activity (Rathinam and Chan, 2018).

CDK5 enrichment in early lactation may play a role in tissue organization and membrane trafficking, as has been observed in neural development (Kawauchi, 2014), or differentiation of multiple cell types (Dhavan and Tsai, 2001), though activity of CDK5 differs from other members of the cyclin-dependent kinase family and its functions are not as well understood (Dhavan and Tsai, 2001).

The downregulation of STAT3 signaling in early lactation reflects the role of this transcription factor in the mammary gland, which unlike STAT5, is predominant during involution and promotes apoptosis of MEC (Clarkson et al., 2006). Both STAT and PPAR signaling pathways are crucial components of mammary function and additionally can each be regulated by the other (Shipley and Waxman, 2004); crosstalk between these pathways is likely part of the orchestrated onset of milk production and component secretion (Bionaz et al., 2012).

Pathways related to PRR signaling, the acute phase response, and IL-8 were especially enriched on d 4 of lactation. Murine mammary IL-8 mRNA expression fluctuates by lactation stage (Li et al., 2012); in addition to inflammatory signaling, it regulates angiogenesis and stromal remodeling (Rabot et al., 2007). Our data show that expression of acute phase protein

Figure 6. Global mammary DNA methylation. Mammary DNA was tested for global methylation using a 5-methylcytosine ELISA. Tissue samples were obtained on d 1, 4, and 45 of lactation; cows were administered with (SAL) or without (CON) antiinflammatory treatment tissue for the first 3 d of lactation ($n = 7-8$ per treatment at each time point). Day 1 samples were obtained before the beginning of treatment. Treatment significantly affected methylation on d 4 and on d 45 ($P = 0.02$), with no detectable effect of day ($P = 0.71$) or treatment $\times$ day interaction ($P = 0.73$). Values are LSM $\pm$ SEM.

transcripts downstream of NF-IL6, including haptoglobin, α -1-antitrypsin, and inter- α -trypsin inhibitor, are upregulated on d 1 relative to d 4 or 45, whereas a different group of acute phase mediators, including antichymotrypsin, fibrinogen, C3, LPS-binding protein, fibronectin, serum amyloid A, and C-reactive protein, is predominantly expressed on d 4. Alterations in PRR signaling and acute phase response pathways between d 1 and d 4 likely reflect the nuanced progression of immune signaling in homeostatic mammary tissue.

In many enriched canonical pathways, IPA did not determine whether the pathway was up- or downregulated, because although the molecules involved in the pathway were differentially expressed, the direction of changes were often contradictory within the pathway. For example, NFKB signaling on d 1 relative to d 45 has a positive z-score, indicating predicted upregulation, but a closer look at the pathway (see Figure 7) shows that although the molecules that would activate NFKB are upregulated (*IL-1*, *TNFA*, *LTA*, *CD40*, and *TNFR*) the intermediate signaling molecules are downregulated (*PI3K*, *AKT*), resulting in a predicted downregulation of NFKB activation. Conversely, NFKB also mediates activation of the inflammasome pathway; in this case it is predicted to be upregulated according to downstream molecule expression (Figure 8). This disparity in predicted NFKB activity could be at least partially explained by differences in pathway regulation (i.e., transcriptional vs. posttranslational), as PI3K and AKT activity is regulated more via protein phosphorylation than through transcript abundance. However, signaling pathways involved in acute phase response predicts simultaneous up- and downregulation of *NFKB*, and both up- and downregulation of the downstream inflammatory molecules (Figure 9). This supports the idea that the activation of specific immune signaling pathways may be regulated in multiple ways (i.e., not only through NFKB) and that signaling in the developing mammary gland may differ from conventional activation of inflammation.

The canonical pathways most enriched on d 1 relative to both d 4 and 45 related to cholesterol synthesis, followed by LXR/RXR activation. Directional changes in transcript abundance of those molecules in the cholesterol synthesis pathways collectively indicate downregulation on d 1 compared with subsequent days, although the magnitude of fold changes for these molecules likely prevented IPA from specifying the direction of regulation. It is tempting to attribute changes in cholesterol to milk output by the mammary gland, but studies with other epithelial cell types have observed similar results. Transcriptional analysis of rumen epithelium of beef cattle exposed to acidosis challenge (Steele et al., 2011) or transitioning to a higher grain diet (Zhao et

al., 2017) also reported differences in cholesterol synthesis, suggesting that epithelial tissue charged with adaptation to environment or physiological state may feature changes in cholesterol as an adaptation mechanism. In addition to being an integral part of cellular membranes, changes in cholesterol metabolism can mediate inflammatory response, cellular proliferation, and oxidative stress (Steele et al., 2011). Additionally, the LXR acts as both a mediator of cholesterol signaling in the cell as well as a regulator of the inflammatory response. Studies with T cells have reported that activation and proliferation required for an adaptive immune response require the LXR for regulation of cholesterol synthesis (Bensinger et al., 2008). Similarly, alterations in cholesterol synthesis are linked to toll-like receptor 4 (**TLR4**) signaling in activated macrophages (Carroll et al., 2018). Conversely, in primary bovine MEC, pro-inflammatory cytokine production in response to LPS was inhibited by LXR activation, and this included the inhibition of TLR4 translocation to the plasma membrane (Wang et al., 2018). The similarities in pathway enrichment related to cholesterol synthesis, LXR activity, and immune signaling activity in mammary tissue suggest that increased proliferation of MEC leading up to and continuing through very early lactation (e.g., Figure 3) may involve similar mechanisms as those observed during immune cell activation, and that signaling pathways attributed to immune cell activation are a feature of proliferating cells or tissue remodeling rather than inflammation per se. This concept is supported by a review of the signaling pathways involved in organism development, which includes those acting through NFKB and JAK/STAT signaling (Perrimon et al., 2012). Those authors also described the diverse biological processes that can be carried out through surprisingly few regulatory pathways and stressed the influence of other contributing factors on ultimate cell or tissue response.

Epigenetic Effects of Antiinflammatory Treatment

Global DNA methylation in the mammary gland appeared to decrease for the control cows over our study relative to levels on d 1 ($P = 0.02$), whereas an inverse pattern was observed for SAL. The onset of milk production is marked by chromatin remodeling to allow for the rapid increase in milk protein production (Rijnkels et al., 2013); however, simplistically, the increased methylation observed in SAL cows should indicate less accessible chromatin and decreased transcription of milk component genes, which is not consistent with production responses to salicylate treatment. However, we only measured global methylation and did not examine specific regions of the DNA, and it

Figure 7. NF κ B signaling in mammary tissue on d 1 relative to d 45 of lactation. Molecule color key: green = downregulated (according to gene expression data), blue = predicted inhibition; pink = upregulated; orange = predicted activation. Cows in third or greater lactation were orally administered antiinflammatory (125 g/d sodium salicylate) or control from d 1 to 3 following parturition. Mammary biopsies were obtained on d 1 (before treatment initiation), 4, and 45, and analyzed for gene expression (n = 6–7 per treatment at each time point). Canonical pathway enrichment for NF κ B signaling was obtained through Ingenuity Pathway Analysis of data analyzed by the Bioconductor package DESeq2 (Love et al., 2014).

Figure 8. Inflammasome signaling on d 1 relative to d 45. Molecule color key: green = downregulated, pink = upregulated, blue = predicted inhibition, orange = predicted activation. Cows in third or greater lactation were orally administered antiinflammatory treatment (125 g/d sodium salicylate) or control from d 1 to 3 following parturition. Mammary biopsies were obtained on d 1 (before treatment initiation), 4, and 45, and analyzed for gene expression (n = 6–7 per treatment at each time point). Canonical pathway enrichment for Inflammasome Signaling was obtained through Ingenuity Pathway Analysis of data analyzed by the Bioconductor package DESeq2 (Love et al., 2014).

in a variety of cell types. Suggested mechanisms included direct effects on DNA methyltransferases or induction of epigenetic changes through changes in reactive oxygen species (Yiannakopoulou, 2014), but these have yet to be confirmed. It is unclear whether epigenetic mechanisms are responsible for whole-lactation milk production responses observed in previous studies, but

a more detailed assessment of site-specific epigenetic mechanisms as well as histone modifications seems warranted in light of this global methylation response.

CONCLUSIONS

Sodium salicylate treatment in early lactation did not affect milk production or circulating metabolites over the first 45 d of lactation. Similarly, there were only a few gene transcripts differentially expressed in mammary tissue on d 45, with potential ties to RNA processing, complement regulation, and histone activity. Histological analysis showed decreased MEC proliferation as lactation progressed. Transcriptional changes over time highlighted the involvement of cholesterol synthesis and LXR signaling, as well as the upregulation of immune-related signaling pathways in early lactation. Global DNA methylation was increased in mammary tissue by antiinflammatory treatment; further research may help clarify links between epigenetic alterations and milk production.

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