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Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle

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Farney JK, Mamedova LK, Coetzee JF, KuKanich B, Sordillo LM, Stoakes SK, Minton JE, Hollis LC, Bradford BJ. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. *Am J Physiol Regul Integr Comp Physiol* 305: R110–R117, 2013. First published May 15, 2013; doi:10.1152/ajpregu.00152.2013.—Adapting to the lactating state requires metabolic adjustments in multiple tissues, especially in the dairy cow, which must meet glucose demands that can exceed 5 kg/day in the face of negligible gastrointestinal glucose absorption. These challenges are met through the process of homeorhesis, the alteration of metabolic setpoints to adapt to a shift in physiological state. To investigate the role of inflammation-associated pathways in these homeorhetic adaptations, we treated cows with the nonsteroidal anti-inflammatory drug sodium salicylate (SS) for the first 7 days of lactation. Administration of SS decreased liver TNF-α mRNA and marginally decreased plasma TNF-α concentration, but plasma eicosanoids and liver NF-κB activity were unaltered during treatment. Despite the mild impact on these inflammatory markers, SS clearly altered metabolic function. Plasma glucose concentration was decreased by SS, but this was not explained by a shift in hepatic gluconeogenic gene expression or by altered milk lactose secretion. Insulin concentrations decreased in SS-treated cows on *day 7* compared with controls, which was consistent with the decline in plasma glucose concentration. The revised quantitative insulin sensitivity check index (RQUICKI) was then used to assess whether altered insulin sensitivity may have influenced glucose utilization rate with SS. The RQUICKI estimate of insulin sensitivity was significantly elevated by SS on *day 7*, coincident with the decline in plasma glucose concentration. Salicylate prevented postpartum insulin resistance, likely causing excessive glucose utilization in peripheral tissues and hypoglycemia. These results represent the first evidence that inflammation-associated pathways are involved in homeorhetic adaptations to lactation.

homeorhesis; salicylate; metabolism

THE TRANSITION FROM LATE PREGNANCY to lactation is a time of great physiological stress, especially for the dairy cow. The decline in feed intake that accompanies parturition, coupled with the rapid increase in energy requirements during lactogenesis, requires a dramatic shift in nutrient fluxes to release stored nutrients and direct them to the mammary gland. This programmed shift in metabolic setpoints is an archetypal example of homeorhesis, defined as the "coordinated changes in metabolism of body tissues necessary to support a physiological state" (4).

Mechanisms underlying homeorhetic adaptions to lactation have been described to some extent. The somatotropic axis is decoupled during this time, resulting in dramatic elevations of plasma growth hormone concentrations without the expected rise in insulin-like growth factor 1 secretion (11, 51). Likewise, insulin sensitivity declines substantially from late gestation (5, 48). These endocrine shifts are critical for promoting the mobilization of stored nutrients and sparing glucose for use by the mammary gland. This conservation of glucose is particularly important in ruminants. The microbes that inhabit the rumen ferment most dietary carbohydrate to volatile fatty acids, leaving very little glucose to be absorbed in the small intestine. As a result, lactating cows absorb almost no glucose from the gastrointestinal tract and must synthesize as much as 5 kg of glucose in the liver daily (2).

The homeorhetic adaptations that allow cows to increase milk production to 40 kg/day within days after parturition can stress the metabolic system. Rapid lipolysis can increase plasma nonesterified fatty acid (NEFA) concentrations by as much as 10-fold within a few days after parturition (21), and both hypoglycemia and hypocalcemia are common, as nutrients are drawn into the mammary gland. Ketosis and fatty liver (FL) are common metabolic diseases that result during this time; in fact, nearly 90% of all metabolic diseases in dairy cattle occur during the first 4 wk of the 305-day lactation (24).

Despite their reliance on mobilized lipid as an energy source, dairy cattle entering lactation with greater adipose mass are at greater risk of developing metabolic diseases (34). It has become clear in the past decade that animals with excessive adiposity exhibit a low-grade inflammation (23), suggesting that perhaps inflammation underlies metabolic disturbances in obese dairy cows. In support of this hypothesis, cows with moderate or severe FL have increased levels of the inflammatory cytokine TNF-α (41). Inflammatory cytokines cause myriad metabolic changes in dairy cattle, including anorexia, lipomobilization, impaired insulin sensitivity, and reduced milk yield (7, 26, 27), all of which are associated with FL and ketosis. Furthermore, daily injection of TNF-α for 7 days increased liver triglyceride content independent of effects on feed intake, and this effect was accompanied by changes in

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hepatic gene expression consistent with both inflammation and a shift from fatty acid oxidation to triglyceride synthesis (8).

These recent findings suggest that exogenous inflammatory agents are sufficient to induce metabolic dysfunction. Whether inflammation is a necessary causative factor in the natural progression of bovine FL and ketosis, however, remains unclear. To address this broad question, we used the nonsteroidal anti-inflammatory drug (NSAID) sodium salicylate (SS). Sodium salicylate is a weak inhibitor of cyclooxygenase (COX)-1 and COX-2 (31), and its probable mode of action is that it inhibits phosphorylation of the NF-κB inhibitor IκB-α (53). Phosphorylation of IκB results in its degradation, allowing NF-κB to be released for translocation into the nucleus and subsequent activation of an inflammatory transcription program (3). The specific hypothesis for this study was that SS would slow liver triglyceride accumulation, promote gluconeogenesis, and limit metabolic disease in dairy cows entering lactation. In contrast, our findings suggest that inflammatory signals may contribute to homeorhetic adaptations to lactation, especially regulation of glucose metabolism and modulation of lipolysis and ketogenesis as animals return to positive energy balance.

MATERIALS AND METHODS

All experimental procedures were approved by the Kansas State University Institutional Animal Care and Use Committee (protocol no. 2880).

Design, treatments, data, and sample collection. A total of 78 Holstein cows were blocked by parity ($n = 39$ first-parity, $n = 24$ second-parity, and $n = 15 \geq$ third-parity) and alternately assigned to treatment within block at parturition. Salicylate has a half-life of ~30 min in cattle (18). Therefore, to deliver a therapeutic dose and to maintain relatively consistent plasma concentrations throughout the day, SS was delivered in drinking water at a concentration of 1.95 g/l beginning on *day 1* postpartum and continuing through *day 7*. A preliminary study indicated that SS decreased water consumption by cows, but this problem was avoided when the flavor of SS was masked with 221 mg/l molasses. Water with either molasses alone (control) or with molasses and SS (1.95 g/l) was offered at 6:00 PM to initiate treatment; treatment began at least 6 h and no more than 30 h after parturition. On *day 8* of lactation, all cows were provided untreated water for the remainder of the experiment.

Cows were housed in individual tie-stalls, milked 3 times daily (2:00 AM, 10:00 AM, and 6:00 PM) and fed twice daily (6:30 AM and 6:00 PM) for ad libitum intake. Feed intake was recorded, and feeding behavior was analyzed, as described previously (36). The diet composition was similar to that reported in Bradford et al. (8), was fed at 53.2% dry matter, and supplied 186 g crude protein, 305 g neutral detergent fiber, 381 g nonfiber carbohydrate, 52 g crude fat, and 79 g ash/kg of dry matter. The net energy for lactation content was estimated at 1.72 Mcal/kg dry matter (37).

Blood samples (14 ml) were collected from coccygeal vessels (4:00 PM) on *days 1*, *7*, *14*, and *21* postpartum and processed, as described previously (33). Liver samples were taken on *days 4* and *21,* as described previously (33). Body condition score (scale of 1 to 5 with 1 = emaciated and 5 = severely obese) was evaluated by three trained personnel on *days 1*, *7*, *14*, and *21*. Mean body condition score on *day 1* was 3.20 ± 0.028 (mean ± SE).

Plasma analyses. Nonesterified fatty acids, glucose, β-hydroxybutyric acid (BHBA), and insulin were analyzed as previously described (36). Plasma was analyzed using enzymatic colorimetric procedures to determine concentrations of NEFA (NEFA-HR; Wako Chemicals, Richmond, VA), glucose (kit no. 439–90901, Wako Chemicals), and BHBA (kit no. H7587–58; Pointe Scientific, Canton, MI). Plasma insulin was determined by a bovine-specific sandwich ELISA (no. 10-1201-01; Mercodia AB, Uppsala, Sweden) with a detection limit of 0.025 pg/μl. The revised quantitative insulin sensitivity check index was calculated according the following equation (22): $\text{RQUICKI} = 1/[\log(G_b) + \log(I_b) + \log(\text{NEFA}_b)]$, where G_b is basal glucose (mg/dl), I_b is basal insulin (μU/ml), and NEFA_b is basal NEFA (mmol/l), such that a higher RQUICKI value indicates greater insulin sensitivity. Plasma TNF-α was determined with an ELISA, as previously described (15), using bovine-specific antibodies (Thermo Fisher Scientific, Rockford, IL); the detection limit was 0.50 pg/ml. Coefficients of variation were 3.8, 4.5, 3.5, 7.2, and 13.0% for NEFA, glucose, BHBA, insulin, and TNF-α assays, respectively. Plasma cortisol was measured using radioimmunoassay (Cortisol Coated Tube Kit, catalog #07/221106, MP Biomedical LLC, Eschwege, Germany).

Plasma salicylate concentrations were determined with liquid chromatography (Shimadzu Prominence, Shimadzu Scientific Instruments, Columbia, MD) and triple quadrupole mass spectrometry (API 2000; Applied Biosystems, Carlsbad, CA). The ions monitored were salicylate (*m/z* 136.91→93.00) and the internal standard acetaminophen (*m/z* 152.17→110.0). The mobile phase consisted of A: acetonitrile and B: 0.1% formic acid. The mobile phase gradient was 75% B from 0 to 0.5 min, 75% B to 50% B from 0.5 to 4.0 min, and 50% B to 75% B from 4.0 to 5.5 min, with a total run time of 6.5 min. A C8 column (Supelco Discovery, 2.1×150 mm, 5 μM; Sigma-Aldrich, St. Louis, MO) achieved separation. Sample processing consisted of adding 0.1 ml plasma to 0.4 ml methanol with 0.1% formic acid containing 1 μg/ml acetaminophen. The samples were vortexed, centrifuged for 5 min at 15,000 *g*, the supernatant transferred to an injection vial, and 0.01 ml was the injection volume. The accuracy of the assay was 100, 96, 104, 103, 105, and 97% at 1, 5, 10, 25, 50, and 100 μg/ml on replicates of three for each concentration, respectively. The coefficients of variation were 5, 9, 2, 10, 1, and 12% at 1, 5, 10, 25, 50, and 100 μg/ml on replicates of three for each concentration, respectively. A similar method was used to determine salicylate concentration in water samples to determine the amount of salicylate delivered.

Eicosanoid concentrations were determined in the lipid fraction of plasma samples from parity-3+ cows using LC-MS. Plasma samples (500 μl) were mixed with 500 μl of HPLC-grade water, 100 μl methanol, 4 μl antioxidant/reducing agent containing EDTA, butylhydroxy toluene, triphenylphosphine, and indomethacin (4 μl/ml), 100 μl of a mixture of internal standards, and 1 μl of formic acid. The internal standards mixture contained the following deuterated eicosanoids (0.1 ng/μl, 10 ng total): leukotriene (LT) B_4-d4, thromboxane (Tx) B_2-d4, prostaglandin (PG) $F_{2\alpha}$-d4, PGE_2-d4, PGD_2-d4, 13(*S*)-hydroxyoctadecadienoic acid (HODE)-d4, 6-keto $PGF_{1\alpha}$-d4, 9(*S*)-HODE-d4, LTD_4-d5, 12(*S*)-hydroxyeicosatetraenoic acid (HETE)-d8, and 15(*S*)-HETE-d8. Sample mixtures were centrifuged at 3,200 *g* for 10 min at 4°C. Lipids were isolated from the sample supernatant by solid phase extraction using a Phenomenex Strata-X 33-μm Polymeric Reversed Phase 60 mg/3 ml columns, catalog no. 8B-S100-UBJ SPE (Phenomenex, Torrance, CA). Columns were first conditioned with 3 ml methanol (MeOH), then 3 ml water. The samples were passed through the columns; 3 ml of 40% MeOH was passed through afterward as the wash. After a 4-min vacuum-drying step, samples were eluted in 2 ml MeOH/acetonitrile (50:50 vol/vol), dried in a Savant SVD121P SpeedVac (Thermo Scientific, Waltham, MA), and resuspended in 100 μl acetonitrile/water/formic acid (37:63:0.02 vol/vol/vol).

Eicosanoids were analyzed using two distinct LC-MS methods. Both used reverse-phase LC on a Waters Acquity UPLC BEH C18 1.7-μm column (2.1 × 100 mm) at a flow rate of 0.6 ml/min at 35°C and a quadrupole mass spectrometer (SQD H-Class, Waters Acquity; Waters, Milford, MA) in electrospray-negative ionization mode. The electrospray voltage was −3 kV, and the turbo ion spray source temperature was 450°C. Nitrogen was used as the drying gas. For each method, 10-μl samples were injected in triplicate. An isocratic mobile phase consisting of acetonitrile:water:0.1% formic acid (45:55:10; vol/vol/vol) with an

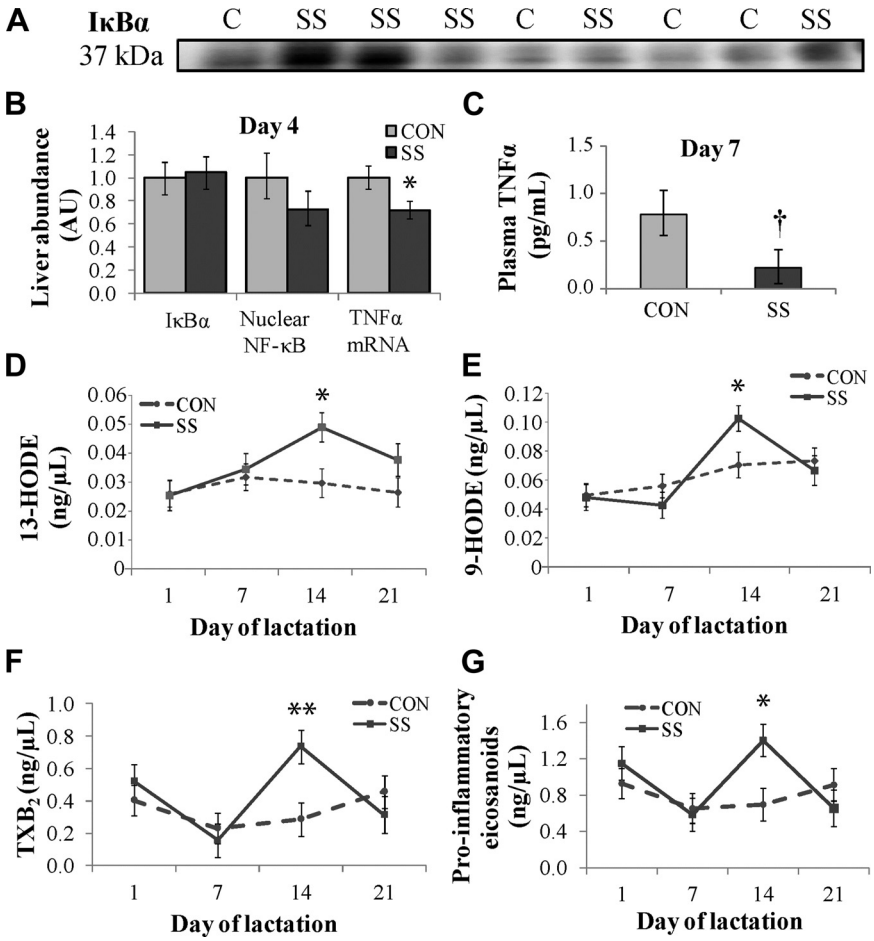

Fig. 1. Sodium salicylate suppresses inflammatory signals, but removal causes a rebound in inflammatory eicosanoids. Treatments (C, CON: control, SS: sodium salicylate) were applied from *days 1* through *7* of lactation. *A*: total IκBα protein was detected by Western blot in liver biopsies taken on *day 4*. *B*: liver samples were collected on *day 4* and were used to assess relative abundance of IκBα by Western blot densitometry ($n = 18$), nuclear NF-κB by DNA hybridization assay ($n = 36$), and TNF-α mRNA by quantitative PCR ($n = 39$). *C*: plasma TNF-α concentrations of parity-2+ cows were determined on *day 7* of lactation by bovine-specific ELISA ($n = 19$). *D–F*: plasma concentrations of 13-hydroxyoctadecadienoic acid (13-HODE) (*D*), 9-HODE (*E*), and thromboxane B_2 (TXB_2; *F*) were determined for parity 3+ cows ($n = 5–6$). *G*: 14 eicosanoids were determined in plasma from parity-3+ cows ($n = 5$), and the sum concentration of 11 proinflammatory eicosanoids is shown. Values are expressed as least square means ± SE. †$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

analysis time of 15 min was used to analyze LTB_4, PGE_2, PGD_2, 5(S),6(R)-lipoxin A4, $PGF_{2\alpha}$, TxB_2, 6-keto $PGF_{1\alpha}$, resolvin D_1, and resolvin D_2. The second method used an isocratic mobile phase of acetonitrile:methanol:water:0.1% formic acid (47.4:15.8:26.8:10; vol/vol/vol/vol) and an analysis time of 10 min to analyze 9(*S*)-HODE, 13(*S*)-HODE, 15-deoxy-$^{\Delta 12,14}$-PGJ_2, 15-oxo-eicosatetraenoic acid (oxoETE), 5-oxoETE, 5(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 7(*S*)-maresin 1, 7(*S*),17(*S*)-dihydroxy-8(*E*),10(*Z*),13(*Z*),15(*E*),19(*Z*)-docosapentaenoic acid, and LTD_4. Eicosanoids were identified in samples by matching their deprotonated (i.e., [M-H]-) *m/z* values and LC retention times with those of a pure standard. Extraction and ionization efficiencies were measured for the internal standards by comparing the intensity of the samples with the ion intensity of the extraction controls. The efficiency range was 18 to 84%. The combined proinflammatory eicosanoid data (Fig. 1*G*) were calculated as the sum of plasma concentrations of TxB_2, 13-HODE, 9-HODE, 15-HETE, 12-HETE, 5-HETE, 15-oxoETE, LTD_4, PGD_2, PGE_2, and $PGF_{2\alpha}$.

Liver analyses. Triglyceride (TG) content was measured, as described previously (33), from liver biopsies collected on *days 4* and *21*. Variation in water content of liver samples was accounted for by normalizing with protein concentration of the sample (17). Transcript abundance of genes involved in glucose and lipid metabolism and inflammation were determined by quantitative real-time PCR, as previously described (36), using gene-specific primers detailed in Table 1. The geometric mean of control genes β-actin, GAPDH, and RPS9 was used to determine the relative abundance of target genes.

Protein abundance of IκBα was determined by Western blot for liver biopsies collected on *day 4* in parity 2+ cows. Briefly, ~20 mg of liver tissue was homogenized at 4°C in RIPA lysis buffer containing a broad-spectrum protease inhibitor cocktail (Protease Inhibitor Cocktail I; Calbiochem, Gibbstown, NJ). The homogenate was centrifuged, and protein content of the supernatant was measured by the Bradford method (9). Forty micrograms of total protein were diluted in Laemmli sample buffer, heated at 90°C for 5 min, cooled, vortexed,

Table 1. *Primers used for quantitative real-time PCR detection of transcripts in liver tissue*

Gene[1]	Accession No.[2]	Forward Primer (5′–3′) Reverse Primer (5′–3′)
β-actin	NM_173979.3	ACGACATGGAGAAGATCTGG ATCTGGGTCATCTTCTCACG
GAPDH	NW_0031039231.1	TCAACGGGAAGCTCACTGG CCCCAGCATCGAAGGTAGA
RPS9	DT860044.1	GAACAAACGTGAGGTCTGGAGG ATTACCTTCGAACAGACGCCG
TNFα	NM_173966.1	AAGTAACAAGCCGGTAGCCCA CTTCCAGCTTCACACCGTTG
G6PC	NM_001076124.1	TGAGGATGGAGAAGGGAATG AACCAAATGGGGAAGAGGAC
PCK1	NM_174737.2	CGAGAGCAAAGAGATACGGTGC TGACATACATGGTGCGACCCT
PC	NM_177946.4	CTTCAAGGACTTCACTGCCACC GCCAAGGCTTTGATGTGCA

[1]*GAPDH,* glyceraldehyde 3-phosphate dehydrogenase; *RPS9,* ribosomal protein subunit 9; *TNFα*, tumor necrosis factor-α; *G6PC,* catalytic subunit of glucose-6-phosphatase; *PCK1,* cytosolic phosphoenolpyruvate carboxykinase; *PC*, pyruvate carboxylase. [2]From NCBI Entrez Nucleotide Database (http://www.ncbi.nlm.gov/sites/entrez?db=nucleotide).

separated by SDS-PAGE on a 4 to 12% Tris·HCl gel, and dry-transferred onto nitrocellulose membranes (iBlot; Invitrogen, Carlsbad, CA). Membranes were blocked in Tris buffer with 5% dry milk powder for 2 h at room temperature with the primary anti-IκBα antibody (diluted 1:500; catalog no. sc-847; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, a secondary antibody (diluted 1:10,000; anti-rabbit sc2313; Santa Cruz Biotechnology) was incubated for 1 h at room temperature. Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA), and bands were quantified by scanning densitometry (Chemi-Doc-It Imaging System; UVP, Upland, CA). ImageJ software (National Institutes of Health) was used for densitometry.

Nuclear extracts were collected from *day 4* liver samples, according to manufacturer's guidelines (kit no. SK-0001; Signosis, Sunnyvale, CA). Briefly, 20 mg of liver was homogenized using lysis buffer and centrifuged, the pellet was reconstituted with a second lysis buffer and centrifuged, and supernatant was collected. This supernatant was the nuclear extract. Total protein was measured using the Bradford method (9), and a total of 0.7 mg of nuclear extract was used in the collection plate for evaluation of NF-κB content. Extract was stored at −20°C until used in an NF-κB filter plate assay (no. FA-0001; Signosis, Sunnyvale, CA). The extract was incubated with an NF-κB probe for 30 min at 18°C and then washed through a filter plate in which NF-κB-bound probes are retained. Bound NF-κB was eluted, denatured, and hybridized overnight at 45°C. The captured DNA was then detected with streptavidin-horseradish peroxidase and measured using luminescence on a microplate luminometer.

Statistical Analysis

Statistical analyses were carried out using the MIXED procedure of SAS (version 9.2; SAS Institute, Cary, NC) to assess the fixed effects of parity, time, treatment, and all two- and three-way interactions; cow was included as a random effect. Repeated measures over time were modeled with autoregressive covariance structures, and denominator degrees of freedom were estimated using the Kenward-Rogers method. Values were deemed outliers and omitted from analysis when Studentized residuals were > 3.5 or < -3.5. Interactions were investigated when $P < 0.15$ using the slice option, and slices were declared significant at $P < 0.05$.

RESULTS

Sodium salicylate mildly inhibits inflammatory cascades in the liver. Sodium salicylate, which has proven effective at inhibiting metabolic inflammation (54), was used to block endogenous inflammation in postpartum dairy cows. Daily intake of salicylate was 123.3 ± 5.5 g/cow, and plasma salicylate concentrations on *day 7* were 34.4 ± 15.0 μg/ml, near the therapeutic target of 30 μg/ml (1, 13, 18), but well below concentrations recently reported to activate AMP-activated protein kinase (20). Plasma SS concentration did not differ by parity ($P > 0.50$). There was no effect of treatment on water intake ($P = 0.81$; 72.4 vs. 73.2 ± 3.5 l/day for control and SS, respectively) or feed intake ($P = 0.85$; 14.8 vs. 15.0 ± 0.6 kg dry matter/day) during the 7-day treatment period.

To assess whether SS effectively inhibited IKK-β signaling in the liver (53), the abundance of IκB-α was determined (Fig. 1*A*). Total IκB-α protein was not significantly altered ($P = 0.82$), nor was a treatment effect on nuclear NF-κB abundance observed ($P = 0.26$, Fig. 1*B*). However, transcript abundance of the inflammatory cytokine TNF-α was decreased 28% in SS-treated cows on *day 4* of lactation ($P = 0.03$, Fig. 1*B*), and SS tended to decrease plasma TNF on *day 7* ($P = 0.06$, Fig. 1*C*).

Salicylate withdrawal results in elevated inflammatory eicosanoids in plasma. A panel of 14 eicosanoids were determined in plasma to assess treatment effects on these key lipid mediators. Although few effects were detected during the 7-day treatment period, numerous proinflammatory eicosanoids were elevated on *day 14*, including TxB_2, 13-HODE, and 9-HODE (all $P < 0.05$; Fig. 1, *D–F*). These patterns were summarized with a combined concentration of 11 proinflammatory eicosanoids, which was significantly elevated on *day 14*, after treatments ended ($P = 0.01$; Fig. 1*G*).

Salicylate increases liver TG content and causes hypoglycemia in multiparous cows. In contrast to the hypothesis that blocking endogenous inflammation during the transition period would decrease liver TG content, liver TG concentrations were increased 29% by SS on *day 4* of lactation ($P < 0.01$), although no differences were observed by *day 21* ($P = 0.47$, Fig. 2*A*). Glucose metabolism was likewise altered, as evidenced by decreased ($P < 0.05$) plasma glucose concentration on *day 7* (Fig. 2*B*). Interestingly, SS induced hypoglycemia on *day 7* in second-parity (45.4 vs. 53.5 ± 2.4 mg/dl; $P = 0.05$) and third-parity (36.0 vs. 50.2 ± 3.5 mg/dl; $P < 0.01$) cows, but not in first-parity cows (55.5 vs. 53.5 ± 2.1 mg/dl for SS vs. control, $P = 0.50$).

Given the negligible absorption of glucose from the ruminant gastrointestinal tract, SS-induced hypoglycemia must have been caused by some combination of decreased gluconeogenesis and increased clearance of circulating glucose. Transcript abundance for key rate-determining enzymes in gluconeogenesis was measured to assess potential mechanisms underlying these responses. No differences in hepatic expression of glucose-6-phosphatase (catalytic subunit, *G6PC*), cytosolic phosphoenolpyruvate carboxykinase (*PCK1*), or pyruvate carboxylase (*PC*) were observed during treatment (all $P > 0.10$, Fig. 2*C*). Targeted analysis of hepatic transcripts failed to identify a clear shift in gene expression to explain the observed hypoglycemia.

Milk lactose synthesis requires more than 70% of total glucose supply in lactating dairy cattle (45). Conserving glucose for such use by the mammary gland is accomplished by a combination of hypoinsulinemia and decreased insulin sensitivity in early lactation (6). Insulin concentrations were marginally decreased in SS-treated cows on *day 7* compared with control ($P = 0.07$; Fig. 2*D*), which is consistent with the decline in plasma glucose concentration. Given that insulin concentration alone did not point to an increase in insulin-dependent glucose uptake, we next used the revised quantitative insulin sensitivity check index (RQUICKI; Ref. 22) to assess whether altered insulin sensitivity may have influenced glucose utilization rate with SS. The RQUICKI estimate of insulin sensitivity was significantly elevated by SS on *day 7* ($P = 0.02$, Fig. 2*E*), coincident with the decline in plasma glucose concentration, whereas controls showed suppressed RQUICKI values on *days 1* and *7*, which is consistent with a homeorhetic suppression of insulin signaling during this adaptation phase of lactation. Salicylate has been shown to enhance insulin sensitivity through interruption of glucocorticoid signaling (38), and salicylate can attenuate cortisol release in cattle during periods of stress (13); however, no significant treatment effects on plasma cortisol concentration were detected on *day 7* (1.58 vs. 2.03 ± 0.46 ng/ml for control vs. SS; $P = 0.49$). Salicylate prevented early lactation insulin resistance, but likely not by blocking cortisol release.

Salicylate withdrawal causes an elevation in plasma BHBA and NEFA concentrations. Enhanced hepatic ketogenesis is another well-described adaptation used by dairy cattle to compensate for energy and glucose deficiencies in the first several weeks of lactation. Surprisingly, although SS did not alter plasma

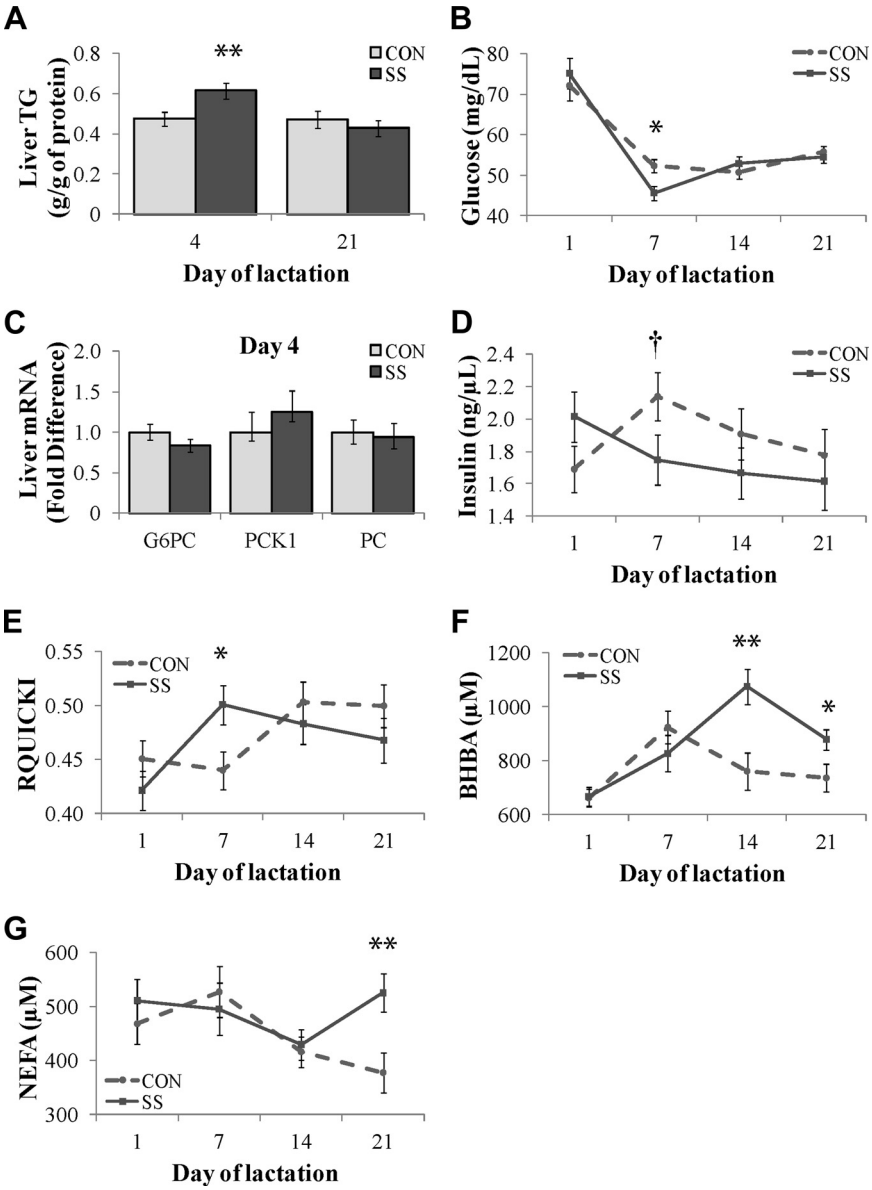

Fig. 2. Salicylate alters carbohydrate and lipid metabolism in early lactation. Treatments (CON: control, SS: sodium salicylate) were applied from *days 1* through *7* of lactation. *A*: liver biopsies collected on *days 4* and *21* postpartum were analyzed for triglyceride (TG) content. *B*: plasma glucose concentrations were determined immediately prior to treatment initiation (*day 1*) and on *days 7*, *14*, and *21* postpartum. *C*: liver biopsies from *day 4* postpartum were analyzed to determine relative transcript abundance for rate-determining gluconeogenic enzymes. *D–G*: plasma insulin, β-hydroxybutyric acid (BHBA), and nonesterified fatty acid (NEFA) concentrations were determined immediately prior to treatment initiation (*day 1*) and on *days 7*, *14*, and *21* postpartum; the revised quantitative insulin sensitivity index (RQUICKI) was calculated from plasma glucose, insulin, and NEFA concentrations. Values are least square means ± SE; $n = 39$. *$P < 0.05$; **$P < 0.01$; †$P < 0.10$.

BHBA concentration during treatment (*day 7*), we observed increased BHBA levels in SS-treated cows on *days 14* and *21* of lactation, well after treatments ended on *day 7* (both $P < 0.05$, Fig. 2*F*). NEFA concentrations were increased by SS, but again, this response was observed only on *day 21*, 2 wk after treatment ended ($P < 0.01$, Fig. 2*G*).

Salicylate accelerates the early lactation increase in milk fat secretion, leading to exacerbated negative energy balance. Net energy balance is negative for most species at the onset of lactation (40). Accordingly, cows on both treatments experienced energy deficits through the first 3 wk of lactation, but control cows had increased energy balance compared with SS in *weeks 2* ($P = 0.08$) and *3* ($P = 0.02$, Fig. 3*A*). As expected for cows in negative energy balance, body condition score (a measure of body fat on a scale of 1 to 5, with 1 = thin and 5 = obese) decreased through the 3-wk trial ($P < 0.01$), but loss of body condition was greater for SS in *week 2* of lactation ($P < 0.01$, Fig. 3*B*), consistent with treatment effects on measures of lipolysis and ketogenesis. Energy balance is primarily a function of energy intake (which was not altered by treatment) and energy expenditure, 75% of which is devoted to milk production in early lactation cows (37). Therefore, milk component yields were investigated to assess the impact of SS treatment on energy requirements for mammary biosynthesis of milk nutrients. Yields of milk lactose (Fig. 3*C*) and protein (Fig. 3*D*) increased as expected during the study (both $P < 0.001$), but no treatment effects were observed. In contrast, milk fat yield was significantly increased by SS treatment in *week 3* ($P = 0.02$, Fig. 3*E*).

Fatty acids used for milk TG synthesis are derived either from preformed plasma lipids or are synthesized de novo in the mammary gland. Milk fatty acid profiles were analyzed to determine the source of the additional fatty acids; de novo fatty acid synthesis in the bovine mammary gland produces carbon chains no longer than 16 carbons (32). In *week 3* of lactation, short- and medium-chain fatty acid yield was increased by 13% ($P = 0.14$), 16-carbon fatty acid yield by 22% ($P < 0.01$), and long-chain fatty acid yield by 17% ($P = 0.01$) following SS

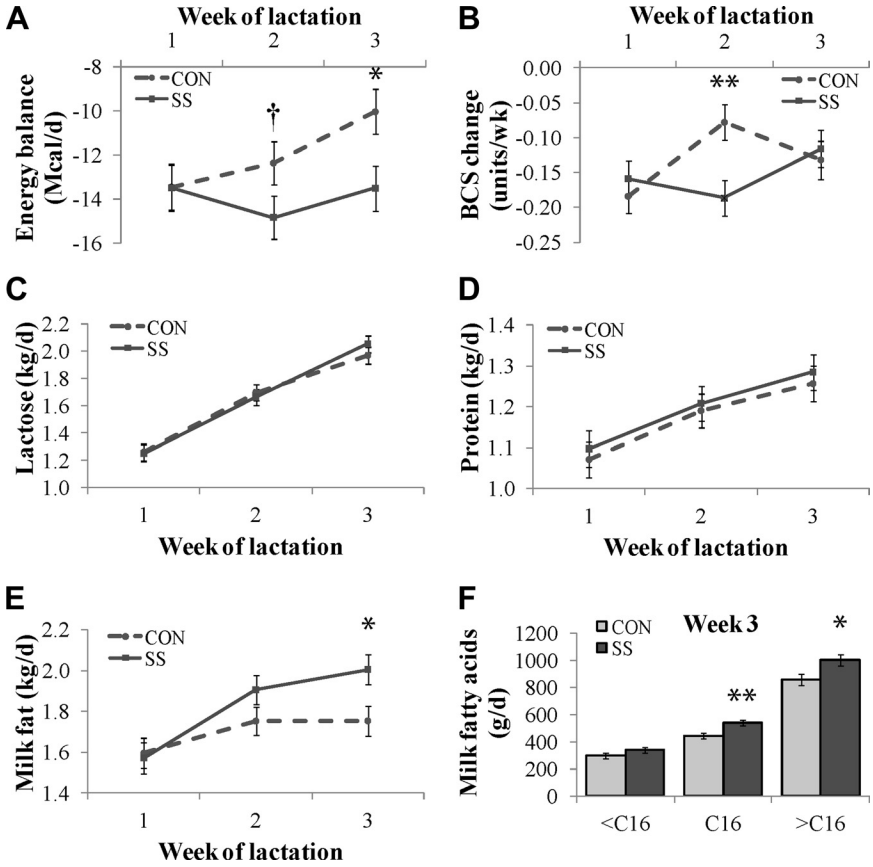

Fig. 3. Early lactation salicylate administration induces a delayed decrease in energy balance through increased milk fat secretion. Treatments (CON, control; SS, sodium salicylate) were applied during *week* 1 of lactation. *A*: net energy balance was calculated as energy intake minus energy required for maintenance and milk energy output. *B*: body condition score, a measure of relative adiposity ranging from 1 (thin) to 5 (obese), was determined weekly by three investigators, and the mean change over the course of a week was analyzed. *C–E*: milk lactose, protein, and fat secretion were determined from daily milk yields and twice weekly composition analyses. *F*: milk fatty acid profiles were determined for *week 3* and grouped into de novo synthesized (<C16), serum-derived (>C16), and mixed-source (C16) fatty acid yields. Values are expressed as least square means ± SE; $n = 27$ for panel *F*, while $n = 39$ for other panels. †$P < 0.10$; *$P < 0.05$; **$P < 0.01$.

treatment (Fig. 3*F*), suggesting that the enhancement of TG secretion did not reflect either preformed or newly synthesized fatty acids exclusively. The proportion of C14 fatty acids desaturated to C14:1 (an index of mammary stearoyl-CoA desaturase activity) was also increased by SS (7.6 vs. 6.6 ± 0.34%; $P = 0.04$), which is consistent with an increase in mammary lipogenesis. The 250 g/day increase in milk fat secretion represented an energy drain of 2.3 Mcal/day, accounting for 66% of the difference in energy balance between treatments in *week 3*.

DISCUSSION

A wide variety of physiological set points are altered to support lactation, involving tissues as diverse as adipose tissue, liver, the gastrointestinal tract, and bone. These adaptations collectively helped crystalize the concept of homeorhesis (4). Although endocrine factors are known to contribute to homeorhesis during the transition to lactation, many gaps remain in our understanding of the mechanisms underlying these changes in nutrient flux. The results presented here represent the first evidence that inflammation-associated pathways are involved in homeorhetic adaptations to lactation.

Eicosanoids such as prostaglandins, prostacyclins, leukotrienes, lipoxins, and thromboxanes have been identified as key regulators of both acute and chronic inflammatory reactions and their resolution (47). Our observation that proinflammatory eicosanoids in blood plasma dramatically increased after cessation of SS treatment suggests that despite the lack of observed suppression of systemic inflammatory mediators during treatment, our treatment regime did alter inflammatory signaling. Rebounds in inflammatory mediators have been observed after cessation of NSAID treatment in humans (44, 50), but to our knowledge, this is the first evidence that the rebound results in concentrations of inflammatory mediators that exceed those in untreated controls. This finding suggests the existence of a homeostatic target for inflammatory signals such that chronic treatment with an NSAID results in a compensatory increase in endogenous inflammation. Such a mechanism could also explain why no treatment effects on proinflammatory eicosanoids were observed during the treatment period. Inflammatory mediators may have been suppressed for a portion of the week of treatment before a compensatory response returned their concentrations to control levels by *day 7*; unfortunately, samples were not collected to assess this hypothesis. It is also worth noting that cows in this study generally did not display the hallmarks of metabolic disease, such as dramatically elevated NEFA and BHBA concentration (12), nor was there evidence of an abnormal degree of inflammation compared with previous findings (46). These findings may, therefore, be more relevant for understanding the normal homeorhetic processes in early lactation than the etiology of early lactation disorders.

Several observations from this study suggest that SS treatment altered metabolic setpoints in early lactation. One key adaptation to lactation in the ruminant animal is peripheral insulin resistance, leading to decreased utilization of glucose by muscle and adipose tissue, thus sparing glucose for use by the mammary gland (6). The ability of inflammatory mediators, such as TNF-α to induce

insulin resistance is now well established (42), and SS increases glucose uptake in diverse models ranging from conscious dogs (30) to cultured adipocytes (43). Studies in mice (38) and humans (19) have suggested that salicylate can improve insulin sensitivity, resulting in increased glucose utilization rates and decreased fasting glucose concentrations. The increase in insulin sensitivity induced by SS in this study, as quantified by RQUICKI, likely caused the hypoglycemia observed on the final day of SS treatment.

Sodium salicylate treatment also dramatically affected lipid metabolism in early-lactation cows. Following SS treatment, plasma NEFA and BHBA concentrations were elevated; these changes coincided with increased milk fat secretion, greater net energy deficit, and a more pronounced loss of body condition in SS-treated cows. These responses are all components of the adaptive transfer of energy from dam to offspring in early lactation (40), but these processes typically recede by *week 3* in dairy cattle (33), in contrast to the resurgence we observed following SS treatment. Whether this response was driven first by changes in liver and adipose tissue lipid metabolism, followed by a mammary response to substrate availability, or by a greater demand from the mammary gland (potentially communicated to other tissues by endocrine changes) is unclear. Indices of mammary fatty acid synthesis and desaturase activity, however, suggest that the mammary gland was not simply a passive recipient of more lipogenic substrate. Another intriguing question posed by our results is whether the responses that only appeared following withdrawal of SS were induced by inflammatory blockade during *week 1* of lactation or by the rebound inflammation in *week 2* after SS treatment ended; unfortunately, there is no way to isolate these two possibilities. Nevertheless, the existing literature is more consistent with the idea that the rebound inflammation altered lipid metabolism. Inflammatory agents, such as lipopolysaccharide (52), TNF-α (25), and interferons (29), can stimulate adipose tissue lipolysis, increasing plasma NEFA concentrations and providing substrate for hepatic ketogenesis.

Inflammation of muscle and adipose tissue is tightly linked to insulin resistance in many metabolic scenarios (39), and these findings are the first to suggest the adaptive role of this phenomenon during early lactation. Although the dairy cow is unique in the degree of genetic selection for milk yield, postpartum insulin resistance has been documented across a broad array of wild and domestic mammals, including northern elephant seals (16), goats (14), pigs (35), and rats (10, 49). We suggest that inflammatory signals underlie this conserved postpartum insulin resistance.

Inflammatory mediators play critical roles in determining rates of lipolysis, glucose transport, protein synthesis, and other metabolic processes, in many cases via alterations in insulin sensitivity. Current hypotheses suggest that inflammatory mediators and metabolic pathways are tightly intertwined because both are derived from a common, multifunctional organ, where mechanisms have emerged to provide for both immunity and homeostatic control of the nutritional environment (23). However, the myriad negative health consequences of metabolic inflammation, as manifested in the metabolic syndrome, suggest that inflammation and metabolism should have dissociated through evolutionary selection as independent organ systems evolved (28). The role of inflammation in promoting a temporary insulin resistance that aids the homeorhetic shift to a new metabolic state may provide one clue to the evolutionary benefit of such links.

Perspectives and Significance

The lactating state requires a shift in nutrient utilization that depends on adaptations in the liver, gut, adipose tissue, muscle, and bone in addition to the mammary gland. This study improves our understanding of this reprioritization process by demonstrating that anti-inflammatory treatment prevents the temporary insulin resistance that occurs in early-lactation dairy cows, leading to hypoglycemia as mammary glucose demands increase. These findings suggest that inflammation-induced insulin resistance is, in some cases, an adaptive, rather than pathological, phenomenon, and may help clarify why the links between inflammation and metabolism are evolutionarily conserved.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.K.F., L.K.M., J.F.C., B.K., and L.M.S. performed experiments; J.K.F., L.K.M., S.K.S., and B.J.B. analyzed data; J.K.F., J.F.C., B.K., L.M.S., J.E.M., and B.J.B. interpreted results of experiments; J.K.F. drafted manuscript; J.K.F., L.K.M., J.F.C., B.K., L.M.S., S.K.S., J.E.M., L.C.H., and B.J.B. approved final version of manuscript; J.F.C., J.E.M., L.C.H., and B.J.B. conception and design of research; L.M.S., J.E.M., and B.J.B. edited and revised manuscript.

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