Lipolysis modulates the biosynthesis of inflammatory lipid mediators derived from linoleic acid in adipose tissue of periparturient dairy cows

G. Andres Contreras,^{1*} Jenne De Koster,¹ Jonas de Souza,² Juliana Laguna,^{1,2} Vengai Mavangira,¹ Rahul K. Nelli,¹ Jeff Gandy,¹ Adam L. Lock,² and Lorraine M. Sordillo¹

¹Department of Large Animal Clinical Sciences, Michigan State University, East Lansing 48824

²Department of Animal Science, Michigan State University, East Lansing 48824

ABSTRACT

Oxidized linoleic acid metabolites (OXLAM) are products of adipocyte lipolysis with the potential to modulate adipose tissue (AT) lipid metabolism and inflammation. In periparturient cows, linoleic acid is preferentially mobilized from AT during lipolysis by hormone-sensitive lipase (HSL) compared with other polyunsaturated fatty acids. Enzymatic and nonenzymatic reactions generate OXLAM from linoleic acid. Among OXLAM, 9-, 10-, and 12-hydroxy-octadecadienoic acids (HODE) are associated with pro-inflammatory responses, whereas 9- and 13-oxo-octadecadienoic acids (oxoODE) and 13-HODE can facilitate inflammation resolution and promote lipogenesis. This study evaluated the effect of HSL activity on OXLAM biosynthesis using subcutaneous AT explants collected from multiparous dairy cows at 10 d before and again at 10 and 24 d after calving. Explants were treated for 3 h without or with the β -adrenergic agonist isoproterenol (ISO; 1 μ M; MilliporeSigma, Burlington, MA) to induce HSL activity. The contribution of HSL to OXLAM biosynthesis was determined by inhibiting its activity with CAY10499 (2 μ M; Cayman Chemical, Ann Arbor, MI). After treatments, media and explants were collected for lipidomic analysis using HPLC–tandem mass spectroscopy. Results indicated that ISO increased the biosynthesis of 9-, 12-, and 13-HODE and 9-oxoODE, and this effect was reduced at 24 d after calving. Inhibiting HSL activity partially reversed ISO effects on HODE and 9-oxoODE. Our ex vivo model demonstrated for the first time a direct effect of HSL activity on the biosynthesis of OXLAM in AT, especially at 10 d before and 10 d after calving. The biosynthesis of anti-inflammatory OXLAM is limited during the first weeks after parturition and may pro-

mote AT inflammation and lipolytic responses to negative energy balance. These results indicate that HSL activity releases linoleic acid for OXLAM biosynthesis in concentrations of a magnitude that may bypass the need for the activation of phospholipases linked with the inflammatory cascade and thus supports, in part, lipolysis-driven inflammation within AT of periparturient cows.

Key words: periparturient period, lipolysis, oxidized linoleic acid metabolite, oxylipid

INTRODUCTION

From around 3 wk before parturition and up to 5 to 7 wk postpartum, the rate of lipolysis exceeds that of lipogenesis in adipose tissues (AT) of dairy cows (Santos et al., 2016). During this period, lipolysis is driven by hormonal changes that are associated with parturition and the onset of lactation and by energy deficits caused by high milk production and limited DMI (Bradford et al., 2015). As lactation progresses, the rate of lipolysis normally decreases, and lipogenesis begins to replenish the triacylglyceride stores of the adipocytes. However, when AT function becomes dysregulated, lipolysis rates remain high, rendering cows more susceptible to inflammatory and metabolic diseases (Hachenberg et al., 2007; Chapinal et al., 2011). The development of an excessive inflammatory response within the adipose organ is among the mechanisms that lead to AT dysregulation in periparturient cows. Inflammation in AT is characterized by macrophage infiltration and has been linked with reduced insulin sensitivity of adipocytes and high rates of free fatty acid (FFA) release into circulation (Contreras et al., 2017b; Newman et al., 2019). However, the stimulatory and inhibitory cues modulating inflammation within AT are not well characterized.

During lipolysis, FFA are released from adipocyte lipid droplets into the adipose milieu and into circulation by the sequential action of adipose triglyceride lipase, hormone-sensitive lipase (HSL), and monoac-

Received July 12, 2019.

Accepted October 2, 2019.

*Corresponding author: contre28@msu.edu

ylglycerol lipase (Arner and Langin, 2014). Whereas adipose triglyceride lipase is the regulator of basal lipolysis, HSL is the rate-limiting enzyme of demand lipolysis and therefore has the highest activity during states of negative energy balance (Koltes and Spurlock, 2011).

Among PUFA released by lipolysis, linoleic acid [**LA**; (9Z,12Z)-octadeca-dienoic acid] is the most abundant in AT, especially in dairy cows fed corn-based diets (Rego et al., 2016). Remarkably, LA is preferentially mobilized during the periparturient period compared with other PUFA such as arachidonic acid (Rukkwamsuk et al., 2000; Douglas et al., 2007; Contreras et al., 2010). Like other PUFA, LA is highly susceptible to oxidation by cyclooxygenases (**COX**) and lipoxygenases (**LOX**; Niki, 2009; Sordillo, 2018). Nonenzymatic reactions triggered by the presence of reactive oxygen and nitrogen species also can oxidize LA (Schieber and Chandel, 2014). During periods of pro-oxidant challenge, such as the periparturient period, reactive oxygen and nitrogen species production is dramatically increased, leading to oxidative stress and thus higher synthesis of oxidized LA metabolites (**OXLAM**; Sordillo, 2018). Isoprostanes are another category of lipid metabolites derived from the nonenzymatic oxidation of arachidonic acid during times of oxidative stress. Indeed, isoprostanes are considered gold standard biomarkers of oxidative stress

in humans, and the quantification of these molecules was recently validated in bovine mastitis and metritis (Mavangira and Sordillo, 2018). Thus, the biosynthesis of certain OXLAM and isoprostanes may be used as biomarkers of nonenzymatic oxidation of PUFA during inflammation.

Upon release, LA is initially oxidized to produce hydroperoxyl-octadecadienoic acids that are rapidly reduced by glutathione peroxidases to hydroxyoctadecadienoic acids (**HODE**; Figure 1). The most abundant HODE in dairy cattle are 9-HODE and 13-HODE (Kuhn et al., 2018; Putman et al., 2019). The quantitative 9-HODE:13-HODE ratio may be indicative of the enzymes preferentially oxidizing LA. Cyclooxygenase enzyme reactions favor 9-HODE biosynthesis, whereas LOX enzymes produce more 13-HODE (Kaduce et al., 1989). The downstream metabolites of the HODE are 9- and 13-oxo-octadecadienoic acids (**oxoODE**). These compounds result from the action of hydroxyl fatty acid dehydrogenases on HODE (Figure 1). Of these dehydrogenases, only 13-HODE dehydrogenase activity has been described, but its structure and transcription remain to be established (Silverman et al., 1996). Although oxidized products of other PUFA also act as proinflammatory mediators, evidence from rodent models of lipolysis supports a role for OXLAM as specific mediators of pro- and anti-inflammatory responses in

Figure 1. Linoleic acid is oxidized by enzymatic and nonenzymatic pathways. Lipoxygenases (LOX), cyclooxygenases (COX), and nonenzymatic (NE) pathways synthesize regioisomers of hydroxy-peroxy-octadecadienoic acid (HPODE) that are reduced to hydroxy-octadecadienoic acid (HODE) by glutathione peroxidases (GPx) and then dehydrogenated to oxo-octadecadienoic acid (oxoODE) by hydroxy fatty acid dehydrogenase (FADH).

AT and as modulators of lipogenic and lipolytic activity in adipocytes (Kwon et al., 2016).

Hydroxyoctadecadienoic acids can modulate inflammation within AT and other organs with high triacylglyceride content. For example, 13-HODE triggers murine macrophage anti-inflammatory polarization during lipolysis and acts as a peroxisome proliferator-activated receptor (PPAR)- γ ligand that promotes adipogenesis and lipogenesis (Lee et al., 2016). Hydroxyoctadecadienoic acids derived from nonenzymatic oxidation of LA, such as 10-HODE and 12-HODE, are associated with AT inflammation and were identified as early biomarkers of lipolysis dysregulation in diabetic humans (Umeno et al., 2013). Another regioisomer, 9-HODE, triggers proinflammatory polarization in lipid-rich environments such as atherosclerotic arteries (Vangaveti et al., 2010). In contrast to HODE, oxoODE have anti-inflammatory properties. For example, 9-oxoODE is an endogenous ligand to PPAR- γ and downregulates inflammatory responses in macrophages and other innate immune cells (Altmann et al., 2007; Mattmiller et al., 2014). Despite the potency of OXLAM to modulate the metabolic function of adipocytes and the inflammatory process within AT, the effect of periparturient AT lipolysis on the biosynthesis of these lipid mediators in dairy cows is unknown.

In rodents, lipolysis significantly increases the content of HODE in adipocytes (Gartung et al., 2016). In dairy cows, periparturient lipolytic activity was associated with changes in the AT content of 13-HODE (Contreras et al., 2017a). However, it is currently unknown whether the activity of HSL enhances the synthesis of LA-derived lipid mediators of inflammation in dairy cows. In addition, the oxidative stress status of AT may promote the production of nonenzymatically derived OXLAM and reduce the production of anti-inflammatory metabolites that also enhance lipogenesis and may reduce FFA release into circulation. Therefore, the hypothesis of the present study was that lipolysis enhances the biosynthesis of OXLAM in AT and that this stimulatory effect is dependent on the activity of HSL. The objective of this study was to determine the effect of HSL lipolytic activity on the synthesis of AT-derived OXLAM at different stages of the periparturient period.

Using an ex vivo AT explant model, we demonstrated that the activity of HSL promotes the biosynthesis of OXLAM. However, generation of OXLAM in response to HSL activation is reduced as lactation progresses. Within AT, the abundance of isoprostanes is higher after parturition and coincides with a reduction in the synthesis of anti-inflammatory and lipogenic OXLAM.

MATERIALS AND METHODS

Animals and Sample Collection

All animal procedures were approved by the Michigan State University Animal Care and Use Committee (07/14-117-00). Six healthy, mature, nonlactating and pregnant (>235 d of gestation) Holstein cows in their second (n = 4), third (n = 1), and fourth (n = 1) lactation were selected from the Michigan State University Dairy Teaching and Research Center. These animals were part of a larger study described in De Koster et al. (2018) and de Souza et al. (2018). Animals were housed in tiestalls and monitored daily by experienced farm personnel throughout the periparturient period and into early lactation. Body weight and BCS were determined weekly starting 2 wk before calving and until the end of the study. Animals were fed a close-up diet from 3 wk before expected parturition and were then transitioned to a lactation diet after calving as described in De Koster et al. (2018). Subcutaneous AT samples were collected at 11 ± 1 d before expected parturition date (close-up; **CU**) and 11 ± 0.2 d (fresh; **FR**) and 24 ± 0.4 d (lactation; **LC**) after calving. Blood samples were collected weekly by coccygeal venipuncture in EDTA and serum separator tubes at 0730 h immediately before feeding and before AT specimen collection. The EDTA blood samples were kept on wet ice until centrifugation, which was performed for 20 min at $3,000 \times g$ at 15°C . Next, plasma and serum fractions were collected, aliquoted, and stored at -80°C until further analyses. A subsample of plasma was used to quantify FFA and BHB as previously described (Kabara et al., 2014). None of the 6 cows had a health event reported during the study.

Subcutaneous AT samples were obtained from the right flank using a surgical procedure (De Koster et al., 2018). Five grams of AT was collected. A part of the adipose sample was snap frozen in liquid nitrogen and stored at -80°C until further analyses, and part was used for the in vitro lipolysis assay and for adipocyte volume determination. The skin was closed using a continuous interlocking suture with Braunamid (USP1, Aesculap, Center Valley, PA). Sutures were removed after 14 d.

The diameter of 100 adipocytes was determined in cells obtained by collagenase digestion of fresh AT using an Olympus BX-40 microscope (Olympus America, Lombard, IL) and ImageJ software (De Koster et al., 2018). Mean and standard deviation of the diameter were used to calculate the volume of the adipocytes using the formula of Goldrick (1967).

In Vitro Lipolysis Assay

The effect of the lipolytic activity of HSL on the biosynthesis of OXLAM was determined using a short-term in vitro explant culture as described by De Koster et al. (2018). Adipose tissue explants (~100 mg) were incubated in 6-well plates containing 3 mL of Krebs Ringer bicarbonate HEPES buffer (pH 7.4) + 3% BSA on a shaker at 38°C. After 20 min of preincubation, AT explants were incubated with the following treatments: (1) medium alone (**BAS**), (2) medium + (–)-isoproterenol (**ISO**; I6504; MilliporeSigma, Burlington, MA) at a concentration of 1 μ M, (3) medium + CAY10499 (**CAY**; no. 10007875; Cayman Chemical, Ann Arbor, MI; 2 μ M), and (4) medium + ISO + CAY (**ISOCAY**). Isoproterenol is a potent β -adrenergic agonist that induces lipolysis, and CAY10499 inhibits HSL activity (Iglesias et al., 2016). All conditions were performed in duplicate. Statistical analyses were performed using the average of the duplicates. After 3 h of incubation, supernatant samples were collected, snap-frozen in liquid nitrogen, and stored at –80°C until liquid chromatography–tandem MS (**LC-MS/MS**) analyses. After collection of supernatants, explants were weighted snap-frozen in liquid nitrogen and stored at –80°C for further analysis.

Gene Expression Analysis

Ribonucleic acid was extracted from AT samples using a Maxwell RSC simplyRNA tissue kit (cat. no. AS1390) and a Maxwell RSC instrument according to the manufacturer's instructions (Promega, Madison, WI) and as described in Contreras et al. (2017a). Purity, concentration, and integrity of mRNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). All samples had a 260:280-nm ratio between 1.9 and 2.1; RNA integrity number values were 7.84 ± 0.83 (mean $\pm$ SD), and the median was 7.8. A qScript cDNA Super Mix kit (QuantaBio, Beverly, MA) was used for cDNA synthesis. TaqMan gene expression primers were used for qPCR assays and were either commercially available or designed from bovine sequences and synthesized by Applied Biosystems Inc. (Foster City, CA; Contreras et al., 2017a). Samples were assayed in triplicate on a high-throughput qPCR instrument (Wafergen Smartchip System; WaferGen Biosystems, Fremont, CA) as described in Contreras et al. (2017a). The housekeeping genes *EIF3K*, *B2M*, and *RPS9* were selected using GeNorm from a pool of genes that also included *ACTB*, *GAPDH*, *PPIA*, and *RPL0* (Vandesompele et al., 2002). Expression of genes of

interest was normalized against the geometric mean of selected housekeeping genes as described by Hellemans et al. (2007).

Lipidomics

Targeted oxylipids in AT were quantified using LC-MS/MS as described in Contreras et al. (2017a).

Lipid Extraction. Adipose tissue was collected, extracted, and analyzed using methods published previously (Contreras et al., 2017a). Briefly, flash-frozen AT was weighed and then pulverized in 0.5 mL of 4% formic acid acetonitrile. Subsequently added were an additional 3.5 mL of 4% formic acid acetonitrile; 16 μ L of antioxidant reducing agent consisting of 50% methanol, 25% ethanol, and 25% water with 0.9 mM 2,6-di-tert-butyl-4-methylphenol, 0.54 mM EDTA, 3.2 mM triphenylphosphine, and 5.6 mM indomethacin; and 15 μ L of a mixture of internal standards containing 0.25 μ M 5(S)-hydroxyeicosatetraenoic acid-*d*₈, 0.25 μ M 15(S)-hydroxyeicosatetraenoic acid-*d*₈, 0.5 μ M 8(9)-epoxy-eicosatrienoic acid-*d*₁₁, 0.5 μ M 9-oxo-11 α ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid-*d*₉, and 0.25 μ M 8,9-dihydroxy-eicosatetraenoic acid-*d*₁₁. Samples were vortexed for 2 min and centrifuged at $4,816 \times g$ for 15 min at 4°C. The supernatant was pushed through Waters Oasis Prime HLB 3 cm³ (150 mg) solid-phase extraction columns (cat. no. 186008717; Waters, Milford, MA). The flow through was collected and the volatile solvents were removed using a Savant Speed-Vac (Thermo Fisher, Waltham, MA). Residues were reconstituted in 150 μ L of methanol, mixed at a 2:1 ratio with HPLC water, and stored in chromatography vials at –80°C until analysis. A 6-point standard curve was created with a mix of standards and the internal standards mentioned above for quantification.

LC-MS/MS Analyses. Details of LC-MS/MS analysis can be found in Putman et al. (2019). In short, metabolites were quantified using a Waters Xevo-TQ-D tandem quadrupole mass spectrometer using multiple reaction monitoring. Chromatography separation was performed with an Ascentis Express C18 HPLC column (Supelco, Bellefonte, PA) held at 50°C and an autosampler held at 10°C (Waters, Medford, MA). Mobile phase bottle A was water containing 0.1% formic acid, and mobile phase bottle B was acetonitrile; the flow rate was 0.3 mL/min. Liquid chromatography separation took 15 min with linear gradient steps programmed as follows (A:B ratio): time 0 to 0.5 min, 99:1; to 60:40 at 2.0 min; to 20:80 at 8.0 min; to 1:99 at 9.0 min; 0.5 min held at 1:99 until min 13.0; returning to 99:1 at 13 min 1 s; and holding at this condition until 15.0 min. All oxylipids were detected using electrospray ionization in negative-ion mode. Cone voltages and collision

voltages were optimized for each analyte using Waters QuanOptimize software, and data analysis was carried out with Waters MassLynx software.

LC-MS/MS Analyses of Isoprostanes. Isoprostanes were quantified with a Waters Xevo TQ-S tandem quadrupole mass spectrometer using multiple reaction monitoring. Chromatography separation was performed with a Waters Acquity UPLC using a BEH C18 1.7 μ M (2.1 $\times$ 150 mm) column held at 50°C and an autosampler held at 10°C. Mobile phase bottle A was 0.1% acetic acid, mobile phase bottle B was acetonitrile, and mobile phase bottle C was methanol; the flow rate was 0.3 mL/min. The gradient initial phase was A:B 80:20 to 1 min; changing to A:B:C 50:30:20 to 7 min; changing to A:B:C 1:80:19 to 7 min 1 s; changing back to initial mobile phase and holding until 10 min. All isoprostanes were detected using electrospray ionization in negative-ion mode. Cone voltages and collision voltages were optimized for each analyte using Waters QuanOptimize software, and data analysis was carried out with Waters MassLynx software.

Statistical Analysis

Statistical analyses were performed using R (R Development Core Team, 2013). Normality of the variables was checked using the Kolmogorov-Smirnov test ($P <$

0.05). Non-normally distributed variables (serum BHB and FFA concentrations, gene expression data) were ln-transformed. For reporting, these variables were back-transformed and geometric means and 95% confidence intervals are presented. The effect of independent variables on the dependent variables was checked using a linear mixed effect model. Parity of the animals was included as a covariate in all models. Period or week relative to the calving date was included as a repeated observation within the random factor cow. Two-way interactions were removed from the model if nonsignificant ($P > 0.05$). Pairwise comparisons were done using Tukey's post hoc test. Residuals of the models were checked and found to be normally distributed. Significance and tendency were declared at $P < 0.05$ and $0.05 < P < 0.10$, respectively. Results are presented as least squares means $\pm$ standard error of the mean unless otherwise stated.

RESULTS

Lipolytic Response During the Periparturient Period

As expected, parturition and the onset of lactation triggered a lipolytic response that decreased BW as lactation progressed (Figure 2A). Concurrently, concentrations of serum FFA increased in the weeks following

Figure 2. The onset of lactation coincided with changes in biomarkers of lipolysis. (A) BW, (B) serum free fatty acid (FFA), (C) BHB, and (D) insulin concentrations were measured at 2 and 1 wk before and 1, 2, and 3 wk after parturition. (E) Adipocyte volume in subcutaneous adipose tissue samples collected at close-up (CU; -11 ± 1 d relative to calving), fresh (FR; $+11 \pm 0.2$ d relative to calving), and lactation (LC; $+24 \pm 0.4$ d relative to calving). Values in panels A, D, and E are LSM, and error bars represent SEM. Values in panels B and C are back-transformed geometric means and 95% CI. Time points with different letters (a, b) or an asterisk differ significantly ($P < 0.05$).

parturition compared with precalving values (Figure 2B). Circulating BHB was increased only during wk 3 after calving (Figure 2C). Insulin concentrations in blood were reduced at wk 1 postpartum compared with values 1 wk before calving (Figure 2D). Reflecting the release of FFA from AT, the volume of adipocytes was reduced at the LC sample compared with CU and FR (Figure 2E).

Lipolysis Activity Enhances AT Enzymatic Biosynthesis of OXLAM in a Time-Dependent Manner

The most abundant OXLAM in AT were 9- and 13-HODE. The AT content of 9-HODE was affected by time, treatment (i.e., stimulation of lipolysis with ISO), and their interaction (Table 1). The ISO treatment increased 9-HODE concentrations at all time points. The highest concentrations of 9-HODE were detected at CU during ISO stimulation and the lowest were detected at FR under BAS conditions (Table 1). Time did not affect 9-HODE content at BAS conditions. The biosynthesis patterns of 13-HODE in AT explants followed dynamics similar to those of 9-HODE. Compared with BAS, ISO enhanced the production of 13-HODE at CU, FR, and LC (Table 1). During ISO stimulation, the highest abundance of 13-HODE was observed at CU compared with FR and LC. At BAS conditions, no changes were observed at any of the sampling time points (Table 1). The AT content of 9-oxoODE, the downstream product of 9-HODE, was affected by treatment with ISO increasing its biosynthesis compared with BAS at all time points (Table 1). There was no effect of sampling and treatment on the biosynthesis of the ketone 13-oxoODE (Table 1).

The 9-HODE:13-HODE ratio was affected by time and treatment. Values for this marker were higher at LC in BAS and ISO conditions (Table 1). In this study, the 9-HODE:9-oxoODE ratio was not affected by time, treatment, or their interaction (Table 1). As for the 13-HODE:13-oxoODE ratio, there was an effect of time and its interaction with treatment. Explants treated with ISO at CU exhibited the higher values for this ratio, whereas the lowest values were detected in LC samples (Table 1).

OXLAM Enzymes Transcription During the Periparturient Period

The expression of *ALOX5*, the gene encoding 5-LOX, was upregulated at LC compared with CU and FR (Table 2). Transcription of *PTGS1* and *PTGS2*, encoding COX-1 and COX-2, respectively, was increased at FR and remained high at LC (Table 2).

Table 1. Enzymatic biosynthesis of oxidized linoleic acid metabolites (OXLAM) in adipose tissues from dairy cows is affected by lipolysis activity and time relative to parturition¹

Linoleic acid-derived oxylipid ²	CU		FR		LC		P-value	
	BAS	ISO	BAS	ISO	BAS	ISO	SEM	Time × treatment
9-HODE	0.227 ^{cd}	1.421 ^a	0.159 ^d	0.571 ^{bc}	0.231 ^{cd}	0.825 ^b	0.14	<0.01
9-oxoODE	0.047	0.367	0.043	0.199	0.178	0.240	0.089	NS
13-HODE	0.286 ^c	1.679 ^a	0.231 ^c	0.728 ^b	0.276 ^c	0.659 ^b	0.120	<0.001
13-oxoODE	0.011	0.026	0.015	0.022	0.048	0.088	0.036	NS
9-HODE:13-HODE ratio	0.747 ^a	0.820 ^a	0.703 ^a	0.772 ^a	0.895 ^b	1.194 ^c	0.093	NS
9-HODE:9-oxoODE ratio	4.82	3.76	8.21	3.01	6.17	12.84	4.62	NS
13-HODE:13-oxoODE ratio	25.590 ^a	62.100 ^b	43.030 ^{ab}	35.400 ^{ab}	20.420 ^a	26.250 ^a	7.580	NS

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

¹Adipose samples were collected at 11 ± 1 d before lactation (close-up; CU) and 11 ± 0.2 d (fresh; FR) and 24 ± 0.4 d (lactation; LC) after calving. OXLAM biosynthesis was evaluated after 3 h of incubation at basal conditions (BAS) or stimulation with the β -adrenergic agonist isoproterenol (ISO).

²Values are LSM of OXLAM concentrations (nmol/ μ g) in adipose tissue as measured in HPLC MS/MS. HODE = hydroxy-octadecadienoic acid; oxoODE = oxo-octadecadienoic acid.

³ $P > 0.10$.

Lipolysis and Time Relative to Parturition Affect Nonenzymatic Biosynthesis of OXLAM and Production of Isoprostanes

The biosynthesis of the nonenzymatically derived 10-HODE was not affected by time, treatment, or their interaction (Table 3). There was an effect of treatment and time $\times$ treatment on AT 12-HODE concentrations (Table 3). The ISO treatment increased the content of 12-HODE at all time points. Multiple comparisons indicated that 12-HODE concentrations were higher at CU ISO compared with other treatments and time points. In BAS conditions, 12-HODE tended to be higher at the LC sample compared with CU and FR. The concentrations (mmol/mg of AT) of the isoprostanes 8-iso-PGE₂ and 12-iso-PGF_{2 α} were affected by time, with higher concentrations at LC compared with FR (Table 3). There was no effect of treatment or time $\times$ treatment on isoprostane content.

Inhibition of HSL Activity Alters OXLAM Biosynthesis

To evaluate the effect of HSL activity on the production of the different OXLAM, we exposed AT explants to the HSL-specific inhibitor CAY alone or in the presence of adrenergic stimulation (ISOCAY) and compared results relative to the BAS and ISO conditions described in Table 1. Exposing AT explants to ISOCAY reduced the biosynthesis of 9-HODE and 13-HODE at all time points compared with ISO, but the effect was more pronounced at CU, where concentrations were reduced by more than 50% (Figure 3A and D). A similar treatment effect was observed for 9-oxoODE, where ISOCAY reduced the content of this OXLAM by at least 30% (Figure 3B). The effect of ISOCAY on the reduction of 12-HODE biosynthesis was more pronounced at CU and was reduced at LC (Figure 3C). No treatment effects were observed on the content of 10-HODE and 13-oxoODE (Figure 3E and F). Exposure of AT explants to CAY did not induce any changes

in the content of the OXLAM evaluated compared with BAS conditions (Figure 3G–L).

DISCUSSION

The time of highest susceptibility to inflammatory and metabolic diseases in periparturient dairy cows coincides with the peak of AT lipolysis and inflammation. In fact, cows with ketosis and displaced abomasum exhibit increased infiltration of macrophages into AT compared with healthy periparturient cows (Contreras et al., 2015). Cows that loose excessive BW have more AT macrophages than cows with minimal changes in BW around parturition (Newman et al., 2019). However, the mechanisms by which AT inflammation develops during lipolysis are poorly understood. Here we demonstrated, using an AT explant model, that HSL lipolytic activity in AT of periparturient cows drives the biosynthesis of the potent mediators of inflammation OXLAM.

In the present study, cows lost around 10% of their BW between CU and LC sampling time points. This reduction in BW was expected and is similar to observations in periparturient multiparous Holstein cows in commercial and research settings worldwide (Roche et al., 2007; van Straten et al., 2008). Postpartum BW loss in cows in our current study is explained, in part, by a reduction in AT mass that was reflected in increased concentrations of lipolysis biomarkers FFA and BHB and the reduction in adipocyte volume. Previously, Akter et al. (2011) demonstrated that BW losses in periparturient cows are driven by reductions in AT mass that can reach more than 25% in both visceral and subcutaneous depots. Studies in rodents demonstrate that when AT mass is rapidly reduced (i.e., days compared with weeks), an inflammatory response is triggered within AT that is driven in part by products of lipolysis and cytokines secreted by adipocytes (Kosteli et al., 2010; Mottillo et al., 2010).

During lipolysis, fatty acids are released, and these can be exported out of the adipocyte, re-esterified and

Table 2. Gene expression of oxylipid enzymes in adipose tissue is affected by time relative to parturition¹

Gene ²	CU	FR	LC	P-value
<i>ALOX5</i>	0.94 ^a (0.80–1.48)	1.19 ^a (0.19–2.58)	2.15 ^b (0.88–4.05)	0.05
<i>ALOX15</i>	0.82 (0.16–1.05)	0.98 (0.63–1.23)	1.76 (0.56–1.89)	NS ³
<i>PTGS1</i>	0.45 ^a (0.23–0.87)	1.08 ^b (0.88–2.18)	1.67 ^b (0.94–2.94)	0.04
<i>PTGS2</i>	0.48 ^a (0.28–0.81)	1.98 ^b (1.01–3.52)	1.47 ^b (0.96–3.97)	0.04

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Relative abundance of mRNA in subcutaneous adipose tissue collected at 11 $\pm$ 1 d before lactation (close-up; CU) and 11 $\pm$ 0.2 d (fresh; FR) and 24 $\pm$ 0.4 d (lactation; LC) after calving.

²Values are back-transformed geometric means (95% CI in parentheses) of genes *ALOX5* (encoding 5-lipoxygenase), *ALOX15* (15-lipoxygenase), *PTGS1* (cyclooxygenase 1), and *PTGS2* (cyclooxygenase 2).

³ $P > 0.10$.

Table 3. Biosynthesis of nonenzymatic oxidized linoleic acid metabolites and oxidative stress marker isoprostanes in adipose tissue from dairy cows is affected by lipolysis activity and time relative to parturition¹

Oxidative stress marker ¹	CU		FR		LC		P-value	
	BAS	ISO	BAS	ISO	BAS	ISO	Time	Time × treatment
10-HODE	0.011	0.022	0.010	0.018	0.030	0.045	NS ³	NS
12-HODE	0.010 ^b	0.058 ^a	0.013 ^b	0.027 ^b	0.016 ^b	0.015 ^b	0.08	<0.05
8-iso-PGE ₂	0.490 ^a	0.534 ^a	0.463 ^a	0.236 ^a	0.860 ^b	0.870 ^b	0.05	NS
12-iso-PGF _{2α}	0.040 ^a	0.059 ^a	0.043 ^a	0.028 ^a	0.065 ^b	0.063 ^b	0.05	NS

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).¹Adipose samples were collected at 11 ± 1 d before lactation (close-up; CU) and 11 ± 0.2 d (fresh; FR) and 24 ± 0.4 d (lactation; LC) after calving. Markers were evaluated after 3 h of incubation at basal conditions (BAS) or stimulation with the β-adrenergic agonist isoproterenol (ISO).²Values are LSM of 10-HODE, 12-HODE, 8-iso-PGE₂, and 2-iso-PGF_{2α} concentrations (nmol/μg) in adipose tissue as measured in HPLC MS/MS. HODE = hydroxy-octadecadienoic acid.³ $P > 0.10$.

stored as triacylglycerides, used as an energy substrate by the mitochondria, or oxidized to synthesize oxylipids, including OXLAM (Nielsen et al., 2014). The abundance of intracellular fatty acids may alter their fate. In our current study, activation of HSL by ISO induced lipolysis, which then increased the concentrations of FFA in AT and the biosynthesis of 9- and 13-HODE and 9-oxODE. Similar dynamics in the generation of OXLAM were reported in murine AT upon HSL activation by an adrenergic agonist (Gartung et al., 2016). However, the biosynthesis of 9- and 13-HODE upon HSL activation was reduced at FR and LC compared with CU. This may be caused by a reduction in the availability of LA for oxidation due to the continuous export of FFA to circulation during the first weeks of lactation (Douglas et al., 2007; Contreras et al., 2010). Thus, HSL activation in adipocytes enhances the biosynthesis of HODE that, in turn, may be limited by the availability of LA.

In our study, the expression of genes encoding COX-1, COX-2, and 5-LOX was upregulated as lactation progressed; therefore, these enzymes were one of the potential sources of OXLAM. Both COX and LOX enzymes are capable of oxidizing LA and produce HODE (Daret et al., 1989; Schneider et al., 2007); however, COX enzymes have affinity for arachidonic acid, whereas LOX enzymes are more selective for LA. In rodents, the activation of COX-2 during lipolysis increases the biosynthesis of 9- and 13-HODE and 9-oxODE (Gartung et al., 2016). The activation of enzymatic pathways that oxidize LA to produce 9- and 13-HODE can be beneficial to the metabolic function of AT during periods of intense lipolysis. Both 9- and 13-HODE are the natural covalent ligands of the nuclear receptor PPAR-γ with the strongest stability and the highest potency compared with other biologically relevant ligands (Itoh et al., 2008). Production of 9-oxODE was also triggered by lipolysis. This ketone is a potent ligand of the nuclear receptors PPAR-α and PPAR-γ that can activate the lipogenic machinery of adipocytes (Goto et al., 2011; Fuentes et al., 2014). At the same time, 9-oxODE can inhibit LOX and nuclear factor-κB activity and therefore mitigate inflammatory responses induced by lipolysis (Armstrong et al., 2014; Mattmiller et al., 2014). Our data and those from rodent models of lipolysis may indicate that the biosynthesis of 9- and 13-HODE by the COX and LOX enzymes and the production of 9-oxODE during lipolysis are part of a negative feedback loop that prevents excessive catabolism of triglyceride reserves that minimizes the lipotoxicity generated by lipolysis by-products.

This is the first study to detect and quantify 10- and 12-HODE as products of lipolysis in mammals and to report their presence in AT from ruminants. Unlike 9-

Figure 3. Inhibition of hormone-sensitive lipase (HSL) during lipolysis reduces the biosynthesis of oxidized linoleic acid metabolites (OXLAM). Adipose samples were collected at 11 ± 1 d before lactation (close-up; CU) and 11 ± 0.2 d (fresh; FR) and 24 ± 0.4 d (lactation; LC) after calving. OXLAM biosynthesis was evaluated after 3 h of incubation in the presence of the HSL-specific inhibitor CAY10499 alone (CAY) or in the presence of adrenergic stimulation with isoproterenol (ISOCAY). Values are LSM of the percentage reduction of the biosynthesis of each OXLAM in explants treated with ISOCAY and CAY compared with basal conditions (BAS) or stimulation with the β -adrenergic agonist isoproterenol (ISO), respectively. Error bars represent SEM. Time points with different letters (a, b) differ ($P < 0.05$). HODE = hydroxy-octadecadienoic acid; oxoODE = oxo-octadecadienoic acid.

and 13-HODE, both 10- and 12-HODE are synthesized exclusively through nonenzymatic oxidation of LA by reactive oxygen species (Minami et al., 2008; Niki, 2009). In our experiments, the content of 12-HODE was increased upon activation of lipolysis and tended to increase after parturition. This finding may be associated with the heightened pro-oxidant status reflected in the increased production of isoprostanes after parturition (Kuhn et al., 2018). This is the first study to report changes in concentrations of isoprostanes in AT of periparturient cows that may be indicative of oxidative stress within the organ. In our study, the concentrations of the isoprostanes 8-iso-PGE₂ and 12-iso-PGF_{2α} were increased in the postpartum samples. Other studies found a closely related arachidonic acid-derived isoprostane, 15-F_{2t}-isoprostane, in early postpartum cows, suggesting an alteration in redox status (Kuhn et al., 2018). Our results and those of Sun et al. (2019) provide evidence for increased oxidative stress status in AT of cows during lipolysis. Previous studies demonstrate that during lipolysis, reactive oxygen species are generated in the adipocyte by excessive mitochondrial oxidation and by nicotinamide adenine dinucleotide phosphate oxidases that produce superoxide and hydrogen peroxide (Han, 2016). Adipose tissue macrophages, especially those of the M1 phenotype, also generate the highly reactive singlet oxygen by activation of their myeloperoxidases (Rodrigues et al., 2002). During the first 2 to 3 wk after parturition, the antioxidant defenses of periparturient cows can be rapidly depleted. It is possible that in cows with intense and protracted lipolysis, the generation of reactive oxygen species by adipocytes and macrophages within AT may exacerbate the production of 12-HODE, a potent proinflammatory product of the nonenzymatic oxidation of LA (Umeno et al., 2013).

Because HSL is the most active lipase during the periparturient period, we determined the effect of its activity on OXLAM biosynthesis using CAY10499, a selective inhibitor of HSL whose activity in bovine AT was recently characterized by our group (De Koster et al., 2018). Inhibiting the lipolytic activity of HSL drastically reduced the biosynthesis of all OXLAM during adrenergic stimulation of AT explants. These findings are similar to those reported by Gartung et al. (2016) in murine AT. In their study, lipolysis was stimulated by a β₃ adrenergic agonist, and HSL was inhibited by a specific inhibitor. Results from our study and that of Gartung et al. (2016) demonstrate that, in AT, lipolysis releases LA that can be used as a substrate for OXLAM biosynthesis and that the concentrations released by adipocytes via HSL are of a magnitude that bypasses the need for the activation of phospholipases. This contrasts with other tissues where the release of

LA and other PUFA by phospholipases is required for production of OXLAM and other lipid mediators (Muraakami et al., 2017).

The present study focused on OXLAM that were characterized as potent ligands of PPAR-γ and mediators of inflammation (Ramsden et al., 2012). However, other OXLAM generated by cytochrome P450 enzymes could also exert similar functions (Sordillo, 2018); therefore, future research should evaluate the effect of these mediators on adipocyte function in dairy cattle. In addition, we evaluated only the effect of HSL activity on the production of OXLAM by AT and did not assess the effect of adipose triglyceride lipase function. Hence, evaluating the role that the later enzyme plays in OXLAM synthesis, especially in lactation stages where basal lipolysis can be altered, should be a priority for future research work. Finally, it is important to note that OXLAM can be esterified into phospholipids and neutral lipids (i.e., cholesterol esters and triglycerides) by endothelial cells and leukocytes (Shearer and Newman, 2009). However, it is currently unknown whether adipocytes esterify OXLAM efficiently into triacylglycerides and whether their storage capacity has physiological relevance.

CONCLUSIONS

Hormone-sensitive lipase activity enhances the biosynthesis of 9-, 12-, and 13-HODE and their downstream metabolite 9-oxoODE. This effect is higher at CU and is reduced as lactation progresses, likely reflecting a reduction in the pool of LA available for oxidation. However, the generation of nonenzymatic OXLAM may be exacerbated in early lactation coinciding with changes in the redox state of AT.

ACKNOWLEDGMENTS

This research was supported by USDA National Institute of Food and Agriculture (Washington, DC) competitive grant 2019-67015-29443; Department of Large Animal Clinical Sciences, Michigan State University (East Lansing); Office of the Associate Dean for Research and Graduate Studies of the College of Veterinary Medicine, Michigan State University (East Lansing); Michigan Alliance for Animal Agriculture (East Lansing, award AA18-003); Discretionary Funding Initiative, Michigan State University (East Lansing); and Perdue Agribusiness (Salisbury, MD). The authors are grateful to the personnel at the Mass Spectrometry and Metabolomics Core Facility, Michigan State University (East Lansing); Clarissa Strieder-Barboza in the Department of Large Animal Clinical Sciences, Michigan State University (East Lansing); Lynn Worden in the

Department of Animal Science, Michigan State University (East Lansing); and the staff at the Michigan State University Dairy Teaching and Research Center (East Lansing).

REFERENCES

- Akter, S. H., S. Haussler, S. Danicke, U. Muller, D. von Soosten, J. Rehage, and H. Sauerwein. 2011. Physiological and conjugated linoleic acid-induced changes of adipocyte size in different fat depots of dairy cows during early lactation. *J. Dairy Sci.* 94:2871–2882. <https://doi.org/10.3168/jds.2010-3868>.
- Altmann, R., M. Hausmann, T. Spottl, M. Gruber, A. W. Bull, K. Menzel, D. Vogl, H. Herfarth, J. Scholmerich, W. Falk, and G. Rogler. 2007. 13-Oxo-ODE is an endogenous ligand for PPARgamma in human colonic epithelial cells. *Biochem. Pharmacol.* 74:612–622. <https://doi.org/10.1016/j.bcp.2007.05.027>.
- Armstrong, M. M., G. Diaz, V. Kenyon, and T. R. Holman. 2014. Inhibitory and mechanistic investigations of oxo-lipids with human lipoxigenase isozymes. *Bioorg. Med. Chem.* 22:4293–4297. <https://doi.org/10.1016/j.bmc.2014.05.025>.
- Arner, P., and D. Langin. 2014. Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance. *Trends Endocrinol. Metab.* 25:255–262. <https://doi.org/10.1016/j.tem.2014.03.002>.
- Bradford, B. J., K. Yuan, J. K. Farney, L. K. Mamedova, and A. J. Carpenter. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. *J. Dairy Sci.* 98:6631–6650. <https://doi.org/10.3168/jds.2015-9683>.
- Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. P. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. *J. Dairy Sci.* 94:4897–4903. <https://doi.org/10.3168/jds.2010-4075>.
- Contreras, G. A., E. Kabara, J. Brester, L. Neuder, and M. Kiupel. 2015. Macrophage infiltration in the omental and subcutaneous adipose tissues of dairy cows with displaced abomasum. *J. Dairy Sci.* 98:6176–6187. <https://doi.org/10.3168/jds.2015-9370>.
- Contreras, G. A., N. J. O'Boyle, T. H. Herdt, and L. M. Sordillo. 2010. Lipomobilization in periparturient dairy cows influences the composition of plasma nonesterified fatty acids and leukocyte phospholipid fatty acids. *J. Dairy Sci.* 93:2508–2516. <https://doi.org/10.3168/jds.2009-2876>.
- Contreras, G. A., C. Strieder-Barboza, J. de Souza, J. Gandy, V. Mavangira, A. L. Lock, and L. M. Sordillo. 2017a. Periparturient lipolysis and oxylipid biosynthesis in bovine adipose tissues. *PLoS One* 12:e0188621. <https://doi.org/10.1371/journal.pone.0188621>.
- Contreras, G. A., C. Strieder-Barboza, and W. Raphael. 2017b. Adipose tissue lipolysis and remodeling during the transition period of dairy cows. *J. Anim. Sci. Biotechnol.* 8:41. <https://doi.org/10.1186/s40104-017-0174-4>.
- Daret, D., P. Blin, and J. Larrue. 1989. Synthesis of hydroxy fatty acids from linoleic acid by human blood platelets. *Prostaglandins* 38:203–214. [https://doi.org/10.1016/0090-6980\(89\)90083-X](https://doi.org/10.1016/0090-6980(89)90083-X).
- De Koster, J., R. K. Nelli, C. Strieder-Barboza, J. de Souza, A. L. Lock, and G. A. Contreras. 2018. The contribution of hormone sensitive lipase to adipose tissue lipolysis and its regulation by insulin in periparturient dairy cows. *Sci. Rep.* 8:13378. <https://doi.org/10.1038/s41598-018-31582-4>.
- de Souza, J., C. Prom, and A. L. Lock. 2018. Altering the ratio of dietary palmitic and oleic acids impacts production and metabolic responses during the immediate postpartum and carryover period in dairy cows. Page 114 in *Proc. ADSA Annual Meeting*, Knoxville, TN. American Dairy Science Association, Champaign, IL.
- Douglas, G. N., J. Rehage, A. D. Beaulieu, A. O. Bahaa, and J. K. Drackley. 2007. Prepartum nutrition alters fatty acid composition in plasma, adipose tissue, and liver lipids of periparturient dairy cows. *J. Dairy Sci.* 90:2941–2959. <https://doi.org/10.3168/jds.2006-225>.
- Fuentes, E., F. Fuentes, and I. Palomo. 2014. Mechanism of the anti-platelet effect of natural bioactive compounds: Role of peroxisome proliferator-activated receptors activation. *Platelets* 25:471–479. <https://doi.org/10.3109/09537104.2013.849334>.
- Gartung, A., J. Zhao, S. Chen, E. Mottilo, G. C. VanHecke, Y. H. Ahn, K. R. Maddipati, A. Sorokin, J. Granneman, and M. J. Lee. 2016. Characterization of eicosanoids produced by adipocyte lipolysis: Implication of cyclooxygenase-2 in adipose inflammation. *J. Biol. Chem.* 291:16001–16010. <https://doi.org/10.1074/jbc.M116.725937>.
- Goldrick, R. B. 1967. Morphological changes in the adipocyte during fat deposition and mobilization. *Am. J. Physiol.* 212:777–782. <https://doi.org/10.1152/ajplegacy.1967.212.4.777>.
- Goto, T., J.-Y. Lee, A. Teraminami, Y.-I. Kim, S. Hirai, T. Uemura, H. Inoue, N. Takahashi, and T. Kawada. 2011. Activation of peroxisome proliferator-activated receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. *J. Lipid Res.* 52:873–884. <https://doi.org/10.1194/jlr.M011320>.
- Hachenberg, S., C. Weinkauff, S. Hiss, and H. Sauerwein. 2007. Evaluation of classification modes potentially suitable to identify metabolic stress in healthy dairy cows during the periparturient period. *J. Anim. Sci.* 85:1923–1932. <https://doi.org/10.2527/jas.2006-480>.
- Han, C. Y. 2016. Roles of reactive oxygen species on insulin resistance in adipose tissue. *Diabetes Metab. J.* 40:272–279. <https://doi.org/10.4093/dmj.2016.40.4.272>.
- Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19. <https://doi.org/10.1186/gb-2007-8-2-r19>.
- Iglesias, J., J. Lamontagne, H. Erb, S. Gezzar, S. Zhao, E. Joly, V. L. Truong, K. Skorey, S. Crane, S. R. M. Madiraju, and M. Prentki. 2016. Simplified assays of lipolysis enzymes for drug discovery and specificity assessment of known inhibitors. *J. Lipid Res.* 57:131–141. <https://doi.org/10.1194/jlr.D058438>.
- Itoh, T., L. Fairall, K. Amin, Y. Inaba, A. Szanto, B. L. Balint, L. Nagy, K. Yamamoto, and J. W. Schwabe. 2008. Structural basis for the activation of PPARgamma by oxidized fatty acids. *Nat. Struct. Mol. Biol.* 15:924–931. <https://doi.org/10.1038/nsmb.1474>.
- Kabara, E., L. M. Sordillo, S. Holcombe, and G. A. Contreras. 2014. Adiponectin links adipose tissue function and monocyte inflammatory responses during bovine metabolic stress. *Comp. Immunol. Microbiol. Infect. Dis.* 37:49–58. <https://doi.org/10.1016/j.cimid.2013.10.007>.
- Kaduce, T. L., P. H. Figard, R. Leifur, and A. A. Spector. 1989. Formation of 9-hydroxyoctadecadienoic acid from linoleic acid in endothelial cells. *J. Biol. Chem.* 264:6823–6830.
- Koltes, D. A., and D. M. Spurlock. 2011. Coordination of lipid droplet-associated proteins during the transition period of Holstein dairy cows. *J. Dairy Sci.* 94:1839–1848. <https://doi.org/10.3168/jds.2010-3769>.
- Kosteli, A., E. Sugaru, G. Haemmerle, J. F. Martin, J. Lei, R. Zechner, and A. W. Ferrante Jr.. 2010. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *J. Clin. Invest.* 120:3466–3479. <https://doi.org/10.1172/JCI42845>.
- Kuhn, M. J., V. Mavangira, J. C. Gandy, and L. M. Sordillo. 2018. Production of 15-F2t-isoprostane as an assessment of oxidative stress in dairy cows at different stages of lactation. *J. Dairy Sci.* 101:9287–9295. <https://doi.org/10.3168/jds.2018-14669>.
- Kwon, H. J., S. N. Kim, Y. A. Kim, and Y. H. Lee. 2016. The contribution of arachidonate 15-lipoxygenase in tissue macrophages to adipose tissue remodeling. *Cell Death Dis.* 7:e2285. <https://doi.org/10.1038/cddis.2016.190>.
- Lee, Y.-H., S.-N. Kim, H.-J. Kwon, K. R. Maddipati, and J. G. Granneman. 2016. Adipogenic role of alternatively activated macrophages in β -adrenergic remodeling of white adipose tissue. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 310:R55–R65. <https://doi.org/10.1152/ajpregu.00355.2015>.
- Mattmiller, S. A., B. A. Carlson, J. C. Gandy, and L. M. Sordillo. 2014. Reduced macrophage selenoprotein expression alters oxi-

- dized lipid metabolite biosynthesis from arachidonic and linoleic acid. *J. Nutr. Biochem.* 25:647–654. <https://doi.org/10.1016/j.jnutbio.2014.02.005>.
- Mavangira, V., and L. M. Sordillo. 2018. Role of lipid mediators in the regulation of oxidative stress and inflammatory responses in dairy cattle. *Res. Vet. Sci.* 116:4–14. <https://doi.org/10.1016/j.rvsc.2017.08.002>.
- Minami, Y., K. Yokoyama, N. Bando, Y. Kawai, and J. Terao. 2008. Occurrence of singlet oxygen oxygenation of oleic acid and linoleic acid in the skin of live mice. *Free Radic. Res.* 42:197–204. <https://doi.org/10.1080/10715760801948088>.
- Mottillo, E. P., X. J. Shen, and J. G. Granneman. 2010. β 3-adrenergic receptor induction of adipocyte inflammation requires lipolytic activation of stress kinases p38 and JNK. *Biochim. Biophys. Acta* 1801:1048–1055.
- Murakami, M., Y. Nakatani, G. I. Atsumi, K. Inoue, and I. Kudo. 2017. Regulatory functions of phospholipase A2. *Crit. Rev. Immunol.* 37:121–179. <https://doi.org/10.1615/CritRevImmunol.v37.i2-6.20>.
- Newman, A. W., A. Miller, F. A. Leal Yepes, E. Bitsko, D. Nydam, and S. Mann. 2019. The effect of the transition period and postpartum body weight loss on macrophage infiltrates in bovine subcutaneous adipose tissue. *J. Dairy Sci.* 102:1693–1701. <https://doi.org/10.3168/jds.2018-15362>.
- Nielsen, T. S., N. Jessen, J. O. Jorgensen, N. Moller, and S. Lund. 2014. Dissecting adipose tissue lipolysis: Molecular regulation and implications for metabolic disease. *J. Mol. Endocrinol.* 52:R199–R222. <https://doi.org/10.1530/JME-13-0277>.
- Niki, E. 2009. Lipid peroxidation: Physiological levels and dual biological effects. *Free Radic. Biol. Med.* 47:469–484. <https://doi.org/10.1016/j.freeradbiomed.2009.05.032>.
- Putman, A. K., J. L. Brown, J. C. Gandy, A. Abuelo, and L. M. Sordillo. 2019. Oxylipid profiles of dairy cattle vary throughout the transition into early mammary gland involution. *J. Dairy Sci.* 102:2481–2491. <https://doi.org/10.3168/jds.2018-15158>.
- R Development Core Team. 2013. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
- Ramsden, C. E., A. Ringel, A. E. Feldstein, A. Y. Taha, B. A. MacIntosh, J. R. Hibbeln, S. F. Majchrzak-Hong, K. R. Fautot, S. I. Rapoport, Y. Cheon, Y. M. Chung, M. Berk, and J. D. Mann. 2012. Lowering dietary linoleic acid reduces bioactive oxidized linoleic acid metabolites in humans. *Prostaglandins Leukot. Essent. Fatty Acids* 87:135–141. <https://doi.org/10.1016/j.plefa.2012.08.004>.
- Rego, O. A., A. R. J. Cabrita, H. J. D. Rosa, S. P. Alves, V. Duarte, A. J. M. Fonseca, C. F. M. Vouzela, F. R. Pires, and R. J. B. Bessa. 2016. Changes in milk production and milk fatty acid composition of cows switched from pasture to a total mixed ration diet and back to pasture. *Ital. J. Anim. Sci.* 15:76–86. <https://doi.org/10.1080/1828051X.2016.1141330>.
- Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. *J. Dairy Sci.* 90:3802–3815. <https://doi.org/10.3168/jds.2006-740>.
- Rodrigues, M. R., D. Rodriguez, M. Russo, and A. Campa. 2002. Macrophage activation includes high intracellular myeloperoxidase activity. *Biochem. Biophys. Res. Commun.* 292:869–873. <https://doi.org/10.1006/bbrc.2002.6724>.
- Rukkwamsuk, T., M. J. H. Geelen, T. A. M. Kruip, and T. Wensing. 2000. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. *J. Dairy Sci.* 83:52–59. [https://doi.org/10.3168/jds.S0022-0302\(00\)74854-5](https://doi.org/10.3168/jds.S0022-0302(00)74854-5).
- Santos, J. E., R. S. Bisinotto, and E. S. Ribeiro. 2016. Mechanisms underlying reduced fertility in anovular dairy cows. *Theriogenology* 86:254–262. <https://doi.org/10.1016/j.theriogenology.2016.04.038>.
- Schieber, M., and N. S. Chandel. 2014. ROS function in redox signaling and oxidative stress. *Curr. Biol.* 24:R453–R462. <https://doi.org/10.1016/j.cub.2014.03.034>.
- Schneider, C., D. A. Pratt, N. A. Porter, and A. R. Brash. 2007. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem. Biol.* 14:473–488. <https://doi.org/10.1016/j.chembiol.2007.04.007>.
- Shearer, G. C., and J. W. Newman. 2009. Impact of circulating esterified eicosanoids and other oxylipins on endothelial function. *Curr. Atheroscler. Rep.* 11:403–410. <https://doi.org/10.1007/s11883-009-0061-3>.
- Silverman, A. L., J. C. Bronstein, S. Krymgold, D. Kahlon, and A. W. Bull. 1996. Decreased levels of 13-hydroxyoctadecadienoic acid (13-HODE) dehydrogenase in neoplastic tissue of human colon biopsies. *Cancer Epidemiol. Biomarkers Prev.* 5:53–56.
- Sordillo, L. M. 2018. Symposium review: Oxylipids and the regulation of bovine mammary inflammatory responses. *J. Dairy Sci.* 101:5629–5641. <https://doi.org/10.3168/jds.2017-13855>.
- Sun, X., X. Li, H. Jia, J. J. Loo, R. Bucktrout, Q. Xu, Y. Wang, X. Shu, J. Dong, R. Zuo, L. Yang, G. Liu, and X. Li. 2019. Effect of heat-shock protein B7 on oxidative stress in adipocytes from preruminant calves. *J. Dairy Sci.* 102:5673–5685. <https://doi.org/10.3168/jds.2018-15726>.
- Umeno, A., M. Shichiri, N. Ishida, Y. Hashimoto, K. Abe, M. Kataoka, K. Yoshino, Y. Hagihara, N. Aki, M. Funaki, Y. Asada, and Y. Yoshida. 2013. Singlet oxygen induced products of linoleates, 10- and 12-(Z,E)-hydroxyoctadecadienoic acids (HODE), can be potential biomarkers for early detection of type 2 diabetes. *PLoS One* 8:e63542. <https://doi.org/10.1371/journal.pone.0063542>.
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.1.
- Vangaveti, V., B. T. Baune, and R. L. Kennedy. 2010. Hydroxyoctadecadienoic acids: Novel regulators of macrophage differentiation and atherogenesis. *Ther. Adv. Endocrinol. Metab.* 1:51–60. <https://doi.org/10.1177/2042018810375656>.
- van Straten, M., N. Y. Shpigel, and M. Friger. 2008. Analysis of daily body weight of high-producing dairy cows in the first one hundred twenty days of lactation and associations with ovarian inactivity. *J. Dairy Sci.* 91:3353–3362. <https://doi.org/10.3168/jds.2008-1020>.