Palmitic acid feeding increases ceramide supply in association with increased milk yield, circulating nonesterified fatty acids, and adipose tissue responsiveness to a glucose challenge

J. E. Rico,* A. T. Mathews,* J. Lovett,† N. J. Haughey,† and J. W. McFadden*¹

*Division of Animal and Nutritional Sciences, West Virginia University, Morgantown 26506

†Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD 21287

ABSTRACT

Reduced insulin action is a key adaptation that facilitates glucose partitioning to the mammary gland for milk synthesis and enhances adipose tissue lipolysis during early lactation. The progressive recovery of insulin sensitivity as cows advance toward late lactation is accompanied by reductions in circulating nonesterified fatty acids (NEFA) and milk yield. Because palmitic acid can promote insulin resistance in monogastrics through sphingolipid ceramide-dependent mechanisms, palmitic acid (C16:0) feeding may enhance milk production by restoring homeorhetic responses. We hypothesized that feeding C16:0 to mid-lactation cows would enhance ceramide supply and ceramide would be positively associated with milk yield. Twenty multiparous mid-lactation Holstein cows were enrolled in a study consisting of a 5-d covariate, 49-d treatment, and 14-d posttreatment period. All cows were randomly assigned to a sorghum silage-based diet containing no supplemental fat (control; $n = 10$; 138 ± 45 d in milk) or C16:0 at 4% of ration dry matter (PALM; 98% C16:0; $n = 10$; 136 ± 44 d in milk). Blood and milk were collected at routine intervals. Liver and skeletal muscle tissue were biopsied at d 47 of treatment. Intravenous glucose tolerance tests (300 mg/kg of body weight) were performed at d -1, 24, and 49 relative to start of treatment. The plasma and tissue concentrations of ceramide and glycosylated ceramide were determined using liquid chromatography coupled with tandem mass spectrometry. Data were analyzed as repeated measures using a mixed model with fixed effects of treatment and time, and milk yield served as a covariate. The PALM treatment increased milk yield, energy-corrected milk, and milk fat yield. The most abundant plasma and tissue sphingolipids detected were C24:0-ceramide, C24:0-mono-hexosylceramide (GlcCer), and C16:0-lactosylceramide. Plasma

concentrations of total ceramide and GlcCer decreased as lactation advanced, and ceramide and GlcCer were elevated in cows fed PALM. Palmitic acid feeding increased hepatic ceramide levels, a response not observed in skeletal muscle tissue. Plasma ceramides (e.g., C24:0-ceramide) were positively correlated with plasma NEFA and milk yield, and positively correlated with NEFA levels following a glucose challenge. Our data demonstrate a remodeled plasma and hepatic sphingolipidome in mid-lactation dairy cows fed PALM. The potential involvement in ceramide in homeorhetic nutrient partitioning to support lactation requires further consideration.

Key words: ceramide, insulin resistance, lactation

INTRODUCTION

The development of insulin resistance in adipose and skeletal muscle tissues enables the dairy cow to partition glucose toward the mammary gland during early lactation (Bell, 1995; Bell and Bauman, 1997). As a consequence, a decrease in insulin sensitivity can enhance the mobilization of nonesterified fatty acids (NEFA) from adipose tissue (Contreras et al., 2010; Zachut et al., 2013). Nonesterified fatty acids can undergo mitochondrial β -oxidation in peripheral tissues, and re-esterification in the mammary gland to contribute to milk fat synthesis. These coordinated metabolic adaptations support energy demand as milk production rapidly increases following parturition. Beyond peak milk yield, insulin sensitivity improves and the lipolytic release of NEFA decreases as the cow progresses toward late lactation and milk production steadily declines (McNamara and Hillers, 1986; Bell and Bauman, 1997).

A causal relationship between enhanced fatty acid supply and the development of insulin resistance in dairy cattle has been reported (Pires et al., 2007a). Specifically, the intravenous infusion of tallow emulsion can improve insulin responsiveness (Pires et al., 2007a). In support, suppressing adipose tissue lipolysis during feed restriction can improve insulin sensitivity (Pires

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¹Corresponding author: JWMcFadden@mail.wvu.edu

et al., 2007b). In type 2 diabetic monogastric animal models, sustained hyperlipidemia shifts fatty acid processing toward the hepatic synthesis and lipoprotein secretion of sphingolipid ceramide (Watt et al., 2012; Boon et al., 2013). Interestingly, de novo ceramide synthesis is controlled by the availability of saturated palmitoyl-CoA and the activation of serine palmitoyltransferase-1 (**SPT1**; Figure 1; Watt et al., 2012). The accrual of ceramide can antagonize insulin-stimulated glucose uptake by inhibiting the phosphorylation of protein kinase B (Summers et al., 1998; Chavez et al., 2003). With the advent of mass spectrometry-based lipidomics, sphingolipid ceramide and monohexosylceramide (glucosylceramide or galactosylceramide; **GlcCer**) have emerged as predictive biomarkers for the development of insulin resistance (Chavez et al., 2014). Similar to the progression of the diabetic state in humans (Kautzky-Willer et al., 1997), we recently discovered that the decline in insulin sensitivity with the onset of lactation occurs concomitantly with the accumulation of ceramide in plasma (Rico et al., 2015). For example, we and others have observed a positive relationship between the concentration of NEFA and the sphingolipid C24:0-ceramide in plasma, and the severity of insulin resistance (Haus et al., 2009; Rico et al., 2015); however, the relationships between NEFA, ceramide supply, and milk production in context of homeorhetic nutrient partitioning have not been evaluated in the mid-lactation dairy cow.

Supplementing diets with SFA is a common nutritional approach on farm to increase energy intake. Although production responses to SFA feeding are inconsistent (Loften et al., 2014), some evidence supports increased milk yield or conversion of feed to milk with SFA supplementation (Mosley et al., 2007; Lock, et al., 2013; Piantoni et al., 2013). Highly enriched SFA supplements are used by dairy producers because of their minimal effects on rumen microbial activity (Palmquist and Jenkins, 1980; Jenkins, 1993; Maia et al., 2010) and because of their beneficial milk production response as compared with unsaturated fatty acid supplementation (Christensen et al., 1994; Relling and Reynolds, 2007), particularly in high-producing dairy cows (Rico et al., 2014a). Although SFA feeding increases dietary energy, favorable lactation outcomes may not completely depend on the energy content of the SFA. For instance, feeding palmitic acid (C16:0) increases ECM and milk fat yield when compared with C18:0 supplementation (Rico et al., 2014b). It is clear that long-chain SFA are antagonists of insulin action in monogastric animals (Boden, 1997; Funaki, 2009), a response that is mediated by ceramide-dependent protein kinase B inactivation (Summers et al., 1998; Chavez et al., 2003). Because SPT1 upregulation is dependent

Figure 1. De novo ceramide synthesis initiated by the condensation of palmitoyl-CoA and serine to form 3-ketosphinganine. For simplicity, C24:0-linked sphingolipids and glycosphingolipids are illustrated. Monohexosylceramides (GlcCer) consist of a single sugar residue, either glucose or galactose (galactosylceramide is not shown).

upon palmitoyl-CoA availability (Figure 1), feeding mid-lactation dairy cows C16:0 may upregulate de novo ceramide synthesis. Humans consuming a diet high in C16:0, relative to oleic acid, display increased circulating levels of ceramide (Kien et al., 2013), resembling upregulated ceramide synthesis observed in subjects diagnosed with type 2 diabetes (Haus et al., 2009). Although we and others have not observed changes in systemic glucose tolerance in dairy cows fed C16:0 when compared with unsupplemented control cows (Piantoni et al., 2013; Mathews et al., 2016), we have observed a decrease in glucose-stimulated NEFA disappearance in mid-lactation cows fed C16:0 by wk 7 (Mathews et al., 2016), suggesting the possibility of localized adipose tissue insulin resistance with prolonged C16:0 feeding. The unexplored metabolic fates for absorbed C16:0, and the associative and functional role of ceramide to modify insulin sensitivity in response to C16:0 supplementation merits attention.

The objective for the current study was to employ a targeted lipidomics approach to quantify ceramides, GlcCer, and lactosylceramides (**LacCer**) in plasma, and liver and skeletal muscle tissues collected from mid-

lactation dairy cows supplemented with high purity C16:0 for an extended duration, in comparison with a non-added-fat control. We hypothesized that increasing dietary C16:0 would enhance ceramide synthesis, and ceramide supply would be positively associated with milk yield. Because insulin sensitivity improves and lipolysis is suppressed as lactation advances toward late gestation (McNamara and Hillers, 1986; Bell and Bauman, 1997), we expected control cows to exhibit a decline in circulating ceramide as lactation progressed.

MATERIALS AND METHODS

Experimental Design

Experimental procedures were approved by the West Virginia University Institutional Animal Care and Use Committee at West Virginia University (Morgantown). Twenty multiparous, mid-lactation Holstein cows were housed in a freestall barn and trained to access feed by using Calan gate feeders (American Calan Inc., Norwood, NH) at the West Virginia University Animal Science Farm. Diets were mixed and offered twice daily. Access to feed was blocked from 0800 to 1000 h and 1600 to 1700 h to allow for orts collection and feeding. Cows were fed at 110% of expected intake, adjusted daily, and provided free access to water. Cows were milked twice daily at 0800 and 1800 h, and milk yields were recorded.

All cows received a common TMR for 14 d before experimentation. Following a 5-d covariate period, cows were grouped by DIM and milk yield (136 ± 43 DIM, 24.8 ± 4.9 kg of milk/d), and offered a similar TMR consisting of no added fat (control; soyhull pellets; $n = 10$) or supplemented with palmitic acid (PALM; 98% C16:0; Palmit 98; Global Agri-Trade, Long Beach, CA; $n = 10$) provided at 4% ration DM for 49 d. Posttreatment, supplemental C16:0 was removed and all cows were fed the control diet for 14 d. Diets provided were composed of sorghum silage and alfalfa haylage as the main forage components, and were formulated to meet or exceed nutrient recommendations (Table 1; NRC, 2001). Forages and concentrates were collected weekly, stored at -20°C , and composited at trial completion.

Basal blood collection (10 mL) by coccygeal venipuncture occurred at 0600 h on d -3 , -2 , and 0 of the covariate period, and d 4, 6, 8, 14, 17, 21, 22, 24, 31, 38, 47, and 49 of the treatment period. Although treatment ceased on d 49, additional basal blood samples were collected at 0600 h on d 52, 56, 62, and 63, relative to the initiation of treatment to characterize the post-treatment period. Blood was kept on ice for 30 min until centrifugation at $3,400 \times g$ for 10 min. Following

centrifugation, EDTA-preserved plasma was removed and stored at -80°C until further analysis. Liver and skeletal muscle biopsies were performed on d -3 and 47 relative to the start of treatment using aseptic techniques. For tissue biopsies, cows were anesthetized with xylazine (0.04 mL/kg of BW) delivered via the coccygeal vein. After the hair was clipped, biopsy sites were sanitized with iodine scrub and anesthetized with a 5 mL of lidocaine HCl (Vedco Inc., Saint Joseph, MO) delivered subcutaneously. For liver biopsies, the 11th intercostal space was prepared by making a 0.5-cm incision through the skin, and a fabricated trocar was used to collect approximately 1 g of liver tissue (Hughes, 1962). Skeletal muscle biopsies were performed by making a 0.5-cm incision in the skin adjacent to the semitendinosus muscle. Approximately 500 mg of muscle was collected using a Bard Magnum biopsy instrument mounted with a 12-gauge needle (Bard Biopsy Systems, Tempe, AZ). Following the collection of tissue, biopsy

Table 1. Ingredients and nutrient composition (% of DM unless otherwise noted) of experimental diets supplemented with palmitic acid (PALM; C16:0) and control (no added fat)

Item	Treatment	
	Control	PALM
Ingredient (% of DM)		
Sorghum-sudangrass silage	30.3	30.1
Ground corn	29.1	28.9
Alfalfa haylage	15.7	15.5
Soybean meal	11.4	11.3
Soyhulls	6.60	3.60
Beet pulp pellets	2.90	2.80
Vitamin and mineral premix ¹	0.63	0.60
Protein blend ²	1.60	1.60
Sodium bicarbonate	0.97	0.94
Zeolite	0.48	0.47
Limestone	0.32	0.29
C16:0-enriched fat supplement ³	0.00	3.90
Nutrient composition		
DM, %	59.6	59.8
CP, % of DM	17.8	17.3
NDF, % of DM	33.6	31.6
Forage NDF, % of DM	24.8	24.8
Starch, % of DM	21.7	21.6
Ether extract, % of DM	2.80	6.70
Ash, % of DM	12.6	13.1
NE _L , Mcal/kg of DM	1.40	1.50

¹Vitamin-mineral mix contained 14% Ca, 9.5% P, 6.3% Mg, 0.13% K, 0.78% S, 4% Na, 17.6% C, and (per kg) 4,282 mg of Fe, 3,000 mg of Zn, 590 mg of Cu, 1,600 mg of Mn, 62 mg of Se, 53 mg of Co, and 31 mg of I, as well as 507 kIU of vitamin A, 69 kIU of vitamin D, and 1,997.6 kIU of vitamin E.

²Protein blend contained 30.2% canola meal solvent, 1.1% rumen-protected methionine (Smartamine, Adisseo, Antony, France), 11.2% corn gluten meal, 45.2% rumen bypass protein (SoyPlus, Dairy Nutrition Plus, Ralston, IA), and 12.3% blood meal.

³Palmitic acid supplement contained 98% C16:0 (Palmit 98, Global Agri-Trade, Long Beach, CA).

sites were stapled and sprayed with antiseptic, and Excenel RTU (0.68 mL/kg of BW; Zoetis, Florham Park, NJ) was delivered once by intramuscular injection. Tissue for ceramide profiling was snap-frozen in liquid nitrogen and stored at -80°C . Tissue for PCR analysis was stabilized using RNeasy Lysis Buffer (Qiagen, Germantown, MD) and refrigerated at 4°C for 24 h, then stored at -20°C until RNA isolation was performed.

Glucose Tolerance Tests

Glucose tolerance tests (GTT) were performed on d -1 , 24, and 49, relative to the start of treatment using previously described methods (Pires et al., 2007a; Schoenberg et al., 2012). Briefly, a jugular catheter was inserted 24 h before the GTT, and patency was maintained by flushing with heparinized saline every 12 h. Following the removal of feed at 0800 h, cows were intravenously infused with 300 mg of glucose (dextrose, 50% wt/vol) per kg of BW at 1000 h. Glucose infusion occurred within 8 ± 0.88 min, and was followed by a 10-mL saline flush. Blood samples (10 mL) were collected at -10 , 0, 10, 20, 30, 40, 60, 90, 120, 150, and 180 min relative to initiation of glucose infusion. Blood was processed as described above. Feed was provided immediately following the completion of the GTT. The area under the curve (AUC) for NEFA during GTT was calculated using the trapezoidal method as previously described by Pires et al. (2007a).

Sample Analyses

Individually composited feed ingredients were analyzed for NDF with heat-stable α -amylase and sodium sulfite (Van Soest et al., 1991), CP (AOAC International, 2000; method 990.03), and starch (Hall, 2009) by Cumberland Valley Analytical Services Inc. (Cumberland, MD).

To evaluate the relationship between circulating ceramide and NEFA, we measured the concentrations of NEFA in plasma using an enzymatic method (HR series NEFA-HR; Wako Chemicals USA Inc., Richmond, VA). Spectrophotometric measurements were conducted using a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA). Intra- and interassay CV were 3.7 and 3.7% for plasma NEFA, respectively.

Plasma ceramide extraction was conducted using previously established methods that employ a modified Bligh and Dyer procedure including C12:0-ceramide as an internal standard (Avanti Polar Lipids, Alabaster, AL; Haughey et al., 2004; Rico et al., 2015). The organic layers from plasma extracts were dried in a

nitrogen evaporator (Organomation Associates Inc., Berlin, MA) and re-suspended in pure methanol before analysis. Ceramides were detected by multiple reaction monitoring using a liquid chromatography coupled electrospray ionization tandem mass spectrometry (API3000, AB Sciex Inc., Thornhill, ON, Canada) operated in positive mode. Liquid chromatography and tandem mass spectrometry parameters have been previously described (Mielke et al., 2015). Slight differences in extraction efficiency and fluctuations in mass spectrometer efficiency were normalized to the C12:0-ceramide internal standard. Instrument efficiency was monitored daily and individual plasma extracts were re-analyzed if the internal standard deviated more than 30% from the overall median internal standard value. The original or re-run data with internal standard concentrations closest to the median C12:0-ceramide value were included in the final analysis. Plasma ceramide concentrations were determined by fitting the identified ceramide species to standard curves based on acyl-chain length. Ceramide standards were purchased from Sigma-Aldrich (C16:0, C18:0, and C18:1; St. Louis, MO) or Avanti Polar Lipids (C20:0, C22:0, C24:0, and C24:1). Glycosylated ceramides were purchased from Matreya Inc. (C24:0-GlcCer, C16:0-LacCer, C18:0-LacCer; Pleasant Gap, PA). Instrument control and quantitation were performed using Analyst 1.4.2 and MultiQuant software (AB Sciex Inc.), respectively.

To evaluate changes in hepatic gene expression, RNA isolation and real-time PCR were performed. The RNA was extracted from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Pellets of RNA were resuspended in RNase-free water and quantified at 260 nm using a spectrophotometer. Total RNA (1 μg) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Real-time PCR reactions were performed using the SYBR Green Master Mix qPCR kit (Bio-Rad) and a CFX96 Real-time PCR machine (Bio-Rad). Quantification of gene transcripts for SPT1, ceramide synthases 1–6, and acid sphingomyelinase (SMase) was completed using gene-specific primers (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2016-11296>). We used expression of 18S ribosomal RNA (18S-rRNA) as our endogenous control, which was not modified by treatment ($P = 0.66$). Reaction conditions were as follows: 50 cycles of 95°C for 3 min, 95°C for 30 s, variable temperature for 30 s (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2016-11296>), and 72°C for 30 s, followed by 95°C for 1 min, 60°C for 1 min, and a temperature gradient from 60°C to 95°C in increments of 5°C .

Statistical Analysis

All plasma data were analyzed as repeated measures over time relative to start of treatment under the MIXED procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC). The statistical model included the random effect of cow, and the fixed effects of treatment and day relative to treatment start, and their interaction. The most appropriate covariance structure for the repeated measures analysis was selected for each variable after evaluating 5 different covariance structures (variance components, first-order autoregressive, heterogeneous first-order autoregressive, compound symmetry, and heterogeneous compound symmetry), and the structure with the smallest Akaike's information criterion coefficient was selected for analysis. Modeling of the covariance structure allowed the identification of patterns that best describe relationships between the repeated measures in the model. The method of Kenward-Rogers was used for calculation of denominator degrees of freedom. Normality of the residuals was evaluated with normal probability and box plots and homogeneity of variances with plots of residual versus predicted values. When necessary, data were transformed and presented in the original scale. The SLICE option of SAS was used to compare treatment differences at individual time points. Differences between treatments for tissue data were analyzed using the GLM procedure of SAS. Studentized residual values >3.0 or <-3.0 were considered outliers and removed from the analysis (typically 1 per response variable). Parametric Pearson correlations were performed to determine associations between plasma ceramides, and milk yield, plasma NEFA, and NEFA disappearance following a GTT. All results are expressed as least squares means and their standard errors, unless stated otherwise. To reduce risk of type I error for repeated measures analysis, the significance level was decreased to $P \leq 0.05$, and trends toward significance were considered at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Detailed production and metabolic responses are described by Mathews et al. (2016). Briefly, PALM elevated energy intake, milk yield, ECM, milk fat and protein yield, and increased C16:0 incorporation in milk fat, relative to control. Estimated insulin sensitivity was lower during the first week of PALM treatment; however, glucose disposal following GTT was not modified at wk 3 or 7 of treatment. Although plasma NEFA declined with the progression of lactation, we did observe enhanced basal plasma NEFA and reduced glucose-stimulated NEFA disappearance by wk 7 of PALM treatment, suggesting localized adipose tissue

insulin resistance and enhanced lipolysis in mid-lactation cows fed PALM for an extended duration.

The acyl chain diversity of sphingolipid ceramide is attributed to the action of 6 CerS genes (Figure 1; Park and Pewzner-Jung, 2013). Using a targeted lipidomics approach, we quantified 21 ceramides, GlcCer, and LacCer in plasma, and liver and skeletal muscle tissues. Comparable to diabetic humans (Haus et al., 2009) and our previous evaluation of insulin resistant periparturient dairy cows (Rico et al., 2015), C24:0-ceramide, C24:0-GlcCer, and C16:0-LacCer represented the major plasma and liver sphingolipids in each class (Figure 2). For instance, C24:0-ceramide represented approximately 70% of total ceramide measured in plasma. Lignoceroyl sphingosine (C24:0-ceramide) is a bioactive lipid mediator that can antagonize insulin-stimulated glucose uptake in mice and is elevated in type 2 diabetic humans (Haus et al., 2009; Boon et al., 2013). Because approximately 98% of plasma ceramides are found within liver-derived lipoprotein subfractions (i.e., high-, low-, and very low density lipoproteins; Lightle et al., 2003; Boon et al., 2013), we measured the concentrations of ceramides in liver (Figure 3). As expected, our results demonstrate that C24:0-ceramide is the predominant hepatic ceramide. Recent evidence demonstrates that ceramides contained in low-density lipoproteins are elevated in a type 2 diabetic state, promote macrophage inflammation, and reduce insulin-stimulated glucose uptake in cultured myocytes (Boon et al., 2013). Boon et al. (2013) further demonstrated that ceramide is elevated in plasma membrane-enriched fractions of skeletal muscle obtained from mice infused with low-density lipoprotein-ceramide, a response accompanied by a reduction in Akt phosphorylation and insulin-stimulated glucose disposal. Although we did not measure adipose tissue ceramide levels, we did quantify the concentrations of ceramides and glycosylated ceramides in skeletal muscle (Figure 3). Our results reveal that C24:0-ceramide is the most abundant ceramide in skeletal muscle tissue, similar to observed plasma profile and skeletal muscle measurements made in monogastrics (Haus et al., 2009; Boon et al., 2013). Relative to other ceramide subspecies, the biological importance of C24:0-ceramide in plasma and tissues remains to be delineated in dairy cows.

In agreement with our hypothesis, PALM increased the concentrations of total ceramide in plasma by d 7 of treatment (Figure 4; $P < 0.01$). The increase in circulating total ceramide was primarily due to C24:0-ceramide accumulation. Specifically, the concentration of C24:0-ceramide in plasma increased by d 7 and remained elevated for the duration of PALM feeding (80% increase on average; Figure 5; $P < 0.001$). Likewise, concentrations of C16:1-ceramide, C22:0-ceramide,

C22:1-ceramide, C24:1-ceramide, and C26:0-ceramide were greater in PALM-fed cows (Figure 5; $P < 0.05$). Interestingly, PALM did not modify C16:0-ceramide levels in plasma. Because C16:0 is required for the de novo synthesis of all ceramides and glycosylated ceramides, we recently proposed that an increase in ceramide

synthesis in dairy cows may limit C16:0 availability for C16:0-ceramide synthesis (Rico et al., 2015). Following the removal of PALM, C16:1-ceramide, C22:0-ceramide, C22:1-ceramide, C24:0-ceramide, C24:1-ceramide, and C26:0-ceramide concentrations in plasma returned to control levels, although total ceramide tended to remain elevated ($P = 0.09$). We previously demonstrated that plasma concentrations of total ceramide and C24:0-ceramide increase concomitantly with NEFA mobilization and the development of insulin resistance as dairy cattle transition from gestation to lactation (Rico et al., 2015). Beyond peak milk production, insulin sensitivity improves and lipogenesis is favored as cows progress toward late lactation (McNamara and Hillers, 1986). In the present study, we observed a gradual decline in the concentration of many circulating ceramides as control cows progressed through lactation, a response that was delayed with PALM. Interestingly, our observed decline in plasma ceramide occurred concurrently with a steady decrease in NEFA concentration (Mathews et al., 2016). In humans and rodents, increased levels of NEFA in circulation can promote insulin resistance by promoting ceramide accumulation in plasma and peripheral tissues (Adams et al., 2004; Holland et al., 2007; Watt et al., 2012). Our data support a decrease in ceramide synthesis as mid-lactation cows advance toward late gestation, a response that is suppressed with continuous PALM feeding. Because ceramide synthesis is controlled by palmitoyl-CoA availability, the efficacy of other energy sources (i.e., other SFA or carbohydrates) to promote ceramide synthesis is uncertain.

Glycosylated ceramides include GlcCer and LacCer, glycosphingolipids synthesized from ceramide and precursors for monosialodihexosylganglioside synthesis (GM3, a ganglioside; Figure 1). Similar to ceramide, GlcCer levels are elevated in plasma collected from insulin-resistant rodents (Chavez et al., 2014), and feeding a diet high in saturated fat can increase GlcCer levels (Aerts et al., 2007). In our experiment, PALM feeding increased total GlcCer and C24:0-GlcCer levels in plasma 32 and 33% by wk 3 and 7, respectively (Figures 4 and 6; $P < 0.001$), a response that was not observed posttreatment. The PALM also increased C16:0-GlcCer, C22:0-GlcCer, C22:1-GlcCer, and C24:1-GlcCer in plasma (Figure 6); however, PALM did not change total or individual LacCer levels in plasma (Figure 4 and Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2016-11296>). Comparable to our observed changes in ceramide with time, we measured a gradual decline in the majority of GlcCer and LacCer as lactation progressed. For example, C24:0-GlcCer and C24:0-LacCer decreased 28 and 25% by wk 9 of experimentation, relative to the start of the study (Figure 6 and Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2016-11296>).

Figure 2. The C24:0-ceramide is the most abundant ceramide in plasma, and liver and skeletal muscle tissue of lactating dairy cows fed control or palmitic acid-supplemented diets (PALM). Ceramide profile of (A) plasma, (B) liver, and (C) skeletal muscle tissue at d 47 of treatment. The C16:0-dihydro-lactosylceramide is not included. Data are represented as LSM and their SE. * $P < 0.05$; † $P < 0.10$ compared with control.

Figure 3. Palmitic acid-feeding (PALM) increases hepatic ceramide concentrations (ng/mg). Liver concentration of (A) ceramide, (B) mono-hexosylceramide (GlcCer), and (C) lactosylceramide (LacCer), and skeletal muscle concentration of (D) ceramide, (E) GlcCer, and (F) LacCer in control and PALM-fed cows at d 47 of treatment. Data are represented as LSM and their SE. * $P < 0.05$ compared with control.

dx.doi.org/10.3168/jds.2016-11296; $P < 0.05$). Similar to our previous work investigating ceramide metabolism in periparturient dairy cows (Rico et al., 2015), we observed a strong positive association between C24:0-ceramide and C24:0-GlcCer across all sampled cows (Supplemental Figure S2; <http://dx.doi.org/10.3168/jds.2016-11296>). These results continue to support a coordinated regulation of synthesis for these sphingolipids containing the C24:0 acyl moiety in dairy cows (Rico et al., 2015).

Evaluating the relationship between plasma and liver ceramides is a means to study sphingolipid metabolism because circulating ceramides are incorporated within lipoproteins (Lightle et al., 2003; Watt et al., 2012; Boon et al., 2013). Coinciding with the elevation in circulating ceramide with PALM treatment, we detected an increase in total ceramide, C22:0-ceramide, C22:1-ceramide, C24:0-ceramide, C24:1-ceramide, and C26:0-ceramide within liver tissue collected at wk 7 (Figure 3; $P < 0.05$). Moreover, changes in hepatic total ceramide

Figure 4. Palmitic acid-feeding (PALM) increases total ceramide and monohexosylceramide (GlcCer) concentrations (ng/mL) in plasma. Plasma concentration of total (A) ceramide, (B) GlcCer, and (C) lactosylceramide (LacCer) in control and PALM-fed cows. Dotted arrows denote the end of treatment period. Data are represented as LSM and their SE. * $P < 0.05$; † $P < 0.10$ compared with control.

and abundant C24:0-ceramide were positively associated with plasma total ceramide and C24:0-ceramide ($r = 0.63$ and 0.58 , respectively; $P < 0.05$). We propose

that feeding mid-lactation dairy cows C16:0 may increase hepatic de novo ceramide synthesis and in turn increase lipoprotein-ceramide packaging and secretion, a mechanism described by others (Watt et al., 2012; Boon et al., 2013). A response that also likely develops during early lactation in response to adipose tissue lipolysis (Rico et al., 2015). To investigate de novo synthesis, we measured the hepatic mRNA expression of genes that control ceramide supply. Although feeding mid-lactation dairy cows PALM did not change the mRNA expression of SPT1 at d 47 of treatment (Figure 7), CerS2 and CerS5 mRNA were lower with PALM treatment. Because each isoform may control the synthesis of specific ceramides, we characterized the isoform distribution in liver (Figure 7). Whereas CerS6 was the predominant isoform, CerS2 and CerS5 mRNA were also expressed. Interestingly, CerS2 and CerS6 can control the synthesis of abundant C24:0-ceramide and C16:0-ceramide, respectively (Levy and Futerman, 2010). An alternative route for ceramide synthesis requires the activation of SMase to catalyze the hydrolysis of sphingomyelin, a response that is often triggered by pro-inflammatory tumor necrosis factor- α (Kolesnick and Golde, 1994; Peraldi et al., 1996). In our investigation, PALM decreased hepatic SMase mRNA expression (Figure 7). Considering that ceramide levels were elevated in liver and serum, reduced CerS and SMase mRNA expression may be a negative feedback mechanism to lower ceramide synthesis with extended PALM feeding. Considering that ceramide can induce apoptosis (Pettus et al., 2002), decreasing ceramide synthesis during PALM treatment may serve as a protective response. In addition to our observed changes with PALM feeding, our data also suggest that the decline in NEFA from mid to late lactation is accompanied by a decrease in hepatic ceramide synthesis and lipoprotein ceramide secretion, a response that is reversed during the acceleration of NEFA mobilization observed during early lactation (McNamara, 1991; Rico et al., 2015). Although the mechanisms involved in the transport of lipoprotein sphingolipid from the liver to circulation are unknown in monogastrics and ruminants, current evidence suggests a role for microsomal triglyceride transfer protein (Iqbal et al., 2015).

We did not observe changes in skeletal muscle concentrations of total ceramide, GlcCer, or LacCer by wk 7 in PALM-fed cows (Figure 3). With the exception of C18:0-ceramide (23% decrease; $P < 0.05$), PALM feeding did not change skeletal muscle concentrations of individual ceramides or glycosylated ceramides (Figure 3). Intriguingly, infusing mice with lipoprotein-ceramide did not increase total ceramide levels in skeletal muscle (Boon et al., 2013); however, insulin resistance caused by lipoprotein-ceramide was accompanied by

Figure 5. Plasma concentration (ng/mL) of C24:0-ceramide is elevated in palmitic acid-fed (PALM) cows. Plasma concentration of (A) C16:0-ceramide, (B) C16:1-ceramide, (C) C20:0-ceramide, (D) C22:0-ceramide, (E) C22:1-ceramide, (F) C24:0-ceramide, (G) C24:1-ceramide, and (H) C26:0-ceramide. Dotted arrows denote the end of treatment period. Data are represented as LSM and their SE. * $P < 0.05$; † $P < 0.10$ compared with control.

Figure 6. Plasma concentration (ng/mL) of C24:0-monohexosylceramide (GlcCer) is elevated in palmitic acid-fed (PALM) cows. Plasma concentration of (A) C16:0-GlcCer, (B) C16:1-GlcCer, (C) C18:0-GlcCer, (D) C22:0-GlcCer, (E) C22:1-GlcCer, (F) C24:0-GlcCer, and (G) C24:1-GlcCer. Dotted arrows denote the end of the treatment period. Data are represented as LSM and their SE. * $P < 0.05$; † $P < 0.10$ compared with control.

Figure 7. Hepatic expression of ceramides synthase (CerS) and acid sphingomyelinase (SMase) is lower in cows fed palmitic acid (PALM) for an extended duration, relative to control cows. Effect of PALM on the hepatic mRNA expression of (A) serine palmitoyltransferase-1 (SPT1) and (B) CerS, relative to control cows. (C) Hepatic distribution of ceramide synthases 1–6 mRNA. (D) Change in SMase mRNA expression. The 18S ribosomal RNA was used as the endogenous control gene and control CerS1 was used as the calibrator for making relative comparisons across isoforms. Data are represented as LSM, and their SE bars were calculated using the $2^{-\Delta\Delta C_t}$. *An asterisk indicates a significant difference in ΔC_t from control ($P < 0.05$). ND = not detected.

plasma membrane ceramide accrual. In the context of homeorhetic glucose partitioning toward the mammary gland, the ability of intracellular or extracellular ceramide to antagonize insulin action in skeletal muscle requires further investigation in dairy cows.

We have previously demonstrated in periparturient dairy cows that ceramide is positively correlated with plasma NEFA concentration (Rico et al., 2015). To validate this relationship in our current investigation, we performed a Pearson correlation analysis to identify significant associations between individual sphingolipid species and plasma NEFA levels across the duration of our 9-wk evaluation. With the exception of C16:0-ceramide, all circulating ceramides, GlcCer, and LacCer were positively correlated with plasma NEFA concentrations ($P < 0.05$; Table 2). The strongest correlations were observed for C22:0-, C24:0-, and C26:0-ceramide

(0.41–0.53; $P < 0.001$). Comparably, our previous work has demonstrated a remarkably similar relationship in periparturient dairy cows (Rico et al., 2015). Most notably, C16:0-ceramide plasma concentration is not linked with NEFA levels. Because we observed reduced glucose-stimulated NEFA disappearance at wk 7 of PALM treatment (Mathews et al., 2016), indicative of adipose tissue insulin resistance, we analyzed the correlative relationship between circulating ceramide concentration and NEFA AUC during a GTT (Table 2). Circulating ceramide and glycosylated ceramide were positively correlated with NEFA AUC ($P < 0.05$). For instance, total ceramide and C24:0-ceramide were positively associated with NEFA AUC (0.51 and 0.57, respectively; $P < 0.001$). Although the effect of long-chain plasma ceramide on lipolysis has not been characterized in dairy cows, culturing 3T3-L1 adipocytes

Table 2. Plasma ceramides are positively correlated with circulating nonesterified fatty acids (NEFA) and milk yield, and negatively associated with NEFA area under the curve (AUC) during a glucose challenge in dairy cows during mid lactation¹

Item ²	Milk yield		NEFA		NEFA AUC 180	
	r	P-value	r	P-value	r	P-value
Ceramide						
C16:0	0.19	<0.10	0.10	0.26	0.19	0.16
C16:1	−0.01	0.91	0.23	<0.01	0.05	0.72
C20:0	0.37	<0.01	0.24	<0.01	0.31	<0.05
C22:0	0.43	<0.001	0.53	<0.001	0.39	<0.01
C22:1	0.14	0.24	0.41	<0.001	0.34	<0.01
C24:0	0.44	<0.001	0.52	<0.001	0.57	<0.001
C24:1	0.15	0.18	0.38	<0.001	0.19	0.14
C26:0	0.51	<0.001	0.41	<0.001	0.55	<0.001
Total	0.39	<0.001	0.53	<0.001	0.51	<0.001
GlcCer						
C16:0	0.00	0.99	0.35	<0.001	0.17	0.19
C16:1	−0.08	0.49	0.30	0.001	0.08	0.57
C18:0	−0.22	<0.10	−0.23	<0.01	0.13	0.34
C22:0	0.42	<0.001	0.35	<0.001	0.53	<0.001
C22:1	−0.11	0.33	0.19	<0.05	0.03	0.80
C24:0	0.35	<0.01	0.29	<0.001	0.39	<0.01
C24:1	−0.09	0.44	0.24	<0.01	−0.01	0.94
Total	0.08	0.46	0.34	<0.001	0.24	<0.10
LacCer						
C16:0	0.25	<0.05	0.31	<0.001	0.46	<0.001
C18:1	0.06	0.62	0.36	<0.001	0.44	<0.001
C22:0	0.31	<0.01	0.40	<0.001	0.55	<0.001
C24:0	0.36	<0.01	0.45	<0.001	0.59	<0.001
C24:1	−0.10	0.38	0.41	<0.001	0.41	<0.01
Total	0.24	<0.05	0.33	<0.001	0.47	<0.01
C16:0-DH-LacCer	0.14	0.23	0.32	<0.001	−0.28	<0.05

¹Pearson correlations (r) of circulating ceramides to plasma NEFA, milk yield, and NEFA area under curve (AUC) for 180 min following a glucose challenge. Previous 24 h milk yield or basal NEFA measurements were correlated with plasma ceramides measured on d 0, 6, 8, 21, 47, 56, and 63, relative to the start of treatment. Basal NEFA AUC 180 measurements for d −1, 24, and 49 were correlated with plasma ceramides measured on d 0, 21, and 47, relative to the start of treatment, respectively.

²DH = dihydro; GlcCer = monohexosylceramide; LacCer = lactosylceramide.

with exogenous hydrophilic C2:0-ceramide can increase lipolysis by suppressing insulin-stimulated activation of cyclic nucleotide phosphodiesterase 3B (Mei et al., 2002). A limitation of our current work is that we have not established the origin of circulating NEFA. It is conceivable that under conditions of elevated lipid supply, such as those induced with elevated dietary fat supplementation, the rates of peripheral tissue fatty acid uptake may be limited relative to the rate of delivery upon triacylglycerol hydrolysis by lipoprotein lipase in the blood capillaries. It is also possible, as we posit here, that circulating NEFA may also originate from adipose tissue lipolysis, as a direct consequence of reduced insulin action.

Considering that ceramide induces insulin resistance in monogastrics (Summers et al., 1998), and lower insulin sensitivity in skeletal muscle and adipose tissue facilitates glucose partitioning to the mammary gland

for milk synthesis (Bell, 1995; Spachmann et al., 2013), we hypothesized that milk yield would be associated with the level of circulating ceramide. As expected, significant positive correlation coefficients between milk yields and the majority of ceramides and glycosylated ceramides were detected ranging from 0.25 to 0.51 ($P < 0.05$; Table 2), with the strongest correlations observed for C22:0-ceramide, C24:0-ceramide, and C26:0-ceramide ($P < 0.001$). It should be noted that these strong relationships between circulating ceramides and milk yields were observed in mid-lactation cows experiencing positive energy balance. Whether a similar observation occurs in early-lactation cows experiencing a greater magnitude of insulin resistance and lipolysis is uncertain.

The development of insulin resistance and lipolysis represent coordinated shifts in metabolism to support the onset of lactation, adaptations that fade with

the progression of lactation. Although ceramides are known effectors of insulin resistance in monogastrics, the ability of ceramide to promote glucose partitioning toward the mammary gland is unknown. In our study, we were able to demonstrate a delayed decline in plasma ceramide concentrations with the advancement of lactation during PALM feeding. In future research, we will need to determine whether ceramide mediates insulin resistance in dairy cows. If it is established that ceramide can antagonize insulin action, then promoting C16:0 intake in dairy cattle may be a nutritional approach for cows to induce a homeorhetic response beyond milk yield. We recognize that insulin resistance and NEFA mobilization during early lactation is accompanied by ceramide accrual (Rico et al., 2015); therefore, we cautiously contemplate whether C16:0 supplementation could augment ceramide supply and insulin resistance during early lactation, and inadvertently challenge metabolic health. These uncertainties will need to be addressed with further investigation.

CONCLUSIONS

Our targeted lipidomics approach has revealed that long-term feeding of C16:0 to mid-lactation cows increased hepatic synthesis and plasma accrual of ceramide. We can conclude that increased C16:0 intake in mid-lactation dairy cows can slow the decline in circulating ceramide observed with the progression of lactation. Furthermore, an increase in plasma ceramide is associated with elevated basal NEFA, reduced adipose tissue NEFA disappearance to a glucose challenge, and milk yield. In support of our previous work (Rico et al., 2015), our data suggest that ceramides may be intrinsically involved in the homeorhetic adaptation to lactation. To confirm this possibility, further studies should investigate whether ceramide mediates the development of systemic or localized insulin resistance in dairy cattle, and how these relationships are influenced by stage of lactation. Specific attention should be focused on highly abundant C24:0-ceramide.

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