Invited review: Sphingolipid biology in the dairy cow: The emerging role of ceramide

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ABSTRACT

The physiological control of lactation through coordinated adaptations is of fundamental importance for mammalian neonatal life. The putative actions of reduced insulin sensitivity and responsiveness and enhanced adipose tissue lipolysis spare glucose for the mammary synthesis of milk. However, severe insulin antagonism and body fat mobilization may jeopardize hepatic health and lactation in dairy cattle. Interestingly, lipolysis- and dietary-derived fatty acids may impair insulin sensitivity in cows. The mechanisms are undefined yet have major implications for the development of postpartum fatty liver disease. In nonruminants, the sphingolipid ceramide is a potent mediator of saturated fat-induced insulin resistance that defines in part the mechanisms of type 2 diabetes mellitus and nonalcoholic fatty liver disease. In ruminants including the lactating dairy cow, the functions of ceramide had remained virtually undescribed. Through a series of hypothesis-centered studies, ceramide has emerged as a potential antagonist of insulin-stimulated glucose utilization by adipose and skeletal muscle tissues in dairy cattle. Importantly, bovine data suggest that the ability of ceramide to inhibit insulin action likely depends on the lipolysis-dependent hepatic synthesis and secretion of ceramide during early lactation. Although these mechanisms appear to fade as lactation advances beyond peak milk production, early evidence suggests that palmitic acid feeding is a means to augment ceramide supply. Herein, we review a body of work that focuses on sphingolipid biology and the role of ceramide in the dairy cow within the framework of hepatic and fatty acid metabolism, insulin function, and lactation. The potential involvement of ceramide within the endocrine control of lactation is also considered.

Key words: ceramide, insulin, lactation, liver

INTRODUCTION

The discovery of sphingolipids is attributed to the work of Johannes Ludwig Thudichum, who characterized the chemical composition of brain (Thudichum, 1884). The structure of sphingolipids was described by the existence of an 18-carbon sphingoid base backbone called “sphingosin” and “the many enigmas which it presented to the inquirer.” Indeed, sphingolipids are often described by their amphipathic nature, which explains their “sphinx-like” properties that dictate their bioactive and structural functionality. Major sphingolipid subclasses include dihydroceramides, ceramides (nonglycosylated or glycosylated), sphingomyelins, and gangliosides. The complexity of the sphingolipidome is further complicated by the diverse incorporation of *N*-acylated fatty acids (**FA**) that vary in chain length and degree of saturation. Although the head group of dihydroceramide and ceramide is a single hydroxyl moiety, glycosphingolipids contain polar sugar residues (i.e., glucose and galactose) and sphingomyelins have a phosphocholine head group. In an additive manner, gangliosides are composed of a glycosphingolipid containing one or more sialic acids (e.g., *N*-acetylneuraminic acid).

The properties of sphingolipids remained largely unknown for a century until the recent advancement of analytical lipidomic technologies. Although sphingolipids are structural components of membranes, we now recognize that they are involved in the regulation of cell growth, differentiation, and apoptosis (Hannun and Obeid, 2008). Moreover, ceramide has emerged as a biomarker for metabolic diseases because of its pathogenic role in the development of type 2 diabetes mellitus (**T2DM**), nonalcoholic fatty liver disease (**FLD**), and cardiovascular disease in rodent models and humans (Borodicz et al., 2015). Unfortunately, our understanding of sphingolipid biology and the role of ceramide in dairy cattle was rudimentary and limited to the recognized presence of sphingolipids within the milk fat globule membrane (Lopez et al., 2008), the considered importance of ceramide to promote the hardness of hooves (Higuchi et al., 2005), and the abil-

Received December 3, 2018.

Accepted April 30, 2019.

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ity of the maize-based mycotoxin fumonisin to inhibit sphingolipid synthesis in livestock including dairy calves (Gilchrist, 1997; Mathur et al., 2001). The objective of this review was to summarize a recent body of work that defines sphingolipid biology in dairy cattle. The role of ceramide is considered to refine our understanding of hepatic lipid biology, nutrient partitioning, and lactation physiology. Scientific questions that challenge contemporary theory are proposed with the intent to advance our understanding of dairy cattle biology.

CERAMIDE SYNTHESIS

The unique structural attributes of sphingolipids appear to influence their bioactive properties; therefore,

the network of anabolic and catabolic pathways that converge on sphingolipids may influence their structure and function. Because ceramide is a precursor for all sphingolipids and widely considered the primary bioactive sphingolipid of interest within the context of insulin resistance observed in nonruminants (Chavez and Summers, 2012), research has focused on major metabolic routes that generate ceramide, including the *de novo* synthesis, sphingomyelinase, and salvage pathways (Figure 1). These pathways are tightly controlled and highly conserved in mammals (Pewzner-Jung et al., 2006; Zeidan and Hannun, 2007). The synthesis of ceramide has an important role considering that complex dietary sphingolipids are degraded in the mammalian small intestine (Nilsson and Duan, 2006).

Figure 1. Pathways of ceramide synthesis, degradation, and modification. The synthesis of ceramide is controlled by *de novo* synthesis from primarily saturated fatty acids (FA), the hydrolysis of sphingomyelin, which forms phosphocholine as a byproduct, or the recycling of complex sphingolipids. The acyl-chain composition of ceramide is controlled by dihydroceramide synthase (commonly called ceramide synthase; CerS). The fatty acyl-CoA preferred by each CerS isoform is denoted in brackets (Levy and Futerman, 2010). For illustrative purposes, only C16:0-linked sphingolipids are presented. Glycosphingolipids illustrated include monohexosylceramides (GlcCer; glucosylceramide shown) and lactosylceramides (LacCer). Modified from Rico et al. (2015a) with permission.

De Novo Synthesis Pathway

The coordinated de novo synthesis of ceramide is initiated by serine palmitoyltransferase (**SPT**), which catalyzes a condensation reaction between saturated palmitoyl-CoA and the NEAA serine. The product is 3-ketodihydrosphingosine, which is transformed into dihydrosphingosine by the actions of 3-ketodihydrosphingosine reductase. Dihydrosphingosine is further acylated by the actions of dihydroceramide synthases, which are commonly called longevity assurance genes or ceramide synthases (**CerS**). Located in the cytoplasmic leaflet of the endoplasmic reticulum (Mandon et al., 1992), CerS has an important role because 6 CerS isoforms (CerS1–6) have been discovered (Levy and Futerman, 2010). The type of fatty acyl-CoA selected is influenced by which CerS involved. For instance, CerS2 prefers very-long-chain (**VLC**) fatty acyl-CoA (e.g., C22:0 and C24:0 acyl-CoA), whereas CerS6 predominantly utilizes palmitoyl-CoA (Figure 1). The biochemical activity of CerS and the variable CerS expression profiles across tissues explain why the acyl composition of sphingolipids is not uniform and illuminate the unique biological functions that are attributed to specific ceramide species. Notably, CerS2 primarily controls the synthesis of VLC ceramides in gluconeogenic liver and kidney tissues (Mullen et al., 2012). De novo ceramide synthesis concludes with the NADPH-dependent desaturation of dihydroceramide by dihydroceramide Δ^4 -desaturase. The resulting ceramide may be utilized by glucosylceramide or galactosylceramide synthase to form monohexosylceramide (i.e., **GlcCer** or **GalCer**, respectively) or transformed into sphingomyelin in a reaction controlled by sphingomyelin synthase (Bartke and Hannun, 2009).

Sphingomyelinase Pathway

The hydrolysis of sphingomyelin generates ceramide and phosphocholine in a reaction catalyzed by sphingomyelinase (**SMase**; Figure 1). Major types of SMase that vary in their optimal pH and cellular location include lysosomal and secretory acid SMase (**A-SMase**), and Mg^{2+} -dependent neutral SMase (**N-SMase**; Marchesini and Hannun, 2004). Lysosomal and secretory A-SMase are encoded by the same gene but have unique differences in their oligosaccharide structures. Moreover, lysosomal and secretory A-SMase contain several highly conserved zinc-binding motifs and share a pH optima between 4.5 and 5.0 (Goñi and Alonso, 2002). Only secretory A-SMase requires exogenous Zn^{2+} for functionality (Schissel et al., 1998). Neutral Mg^{2+} -dependent SMase are integral membrane proteins that have a pH optima near 7.4 and require phosphati-

dyserine for catalytic function (Rao and Spence, 1976; Hofmann et al., 2000). The expression of lysosomal A-SMase and Mg^{2+} -dependent N-SMase appears to be ubiquitous in mammalian tissues (Goñi and Alonso, 2002). Two other forms of SMase that have received less attention include Mg^{2+} -independent N-SMase and alkaline SMase (Ghosh et al., 1998; Duan, 2006), the latter being localized to the intestinal mucosa where it may aid in the digestion of dietary sphingomyelin (Cheng et al., 2002).

Salvage Pathway

Otherwise known as the sphingolipid recycling pathway, the salvage pathway involves the catabolic degradation of complex sphingolipids and glycosphingolipids in acidic late endosomes and lysosomes. The biochemical breakdown requires key enzymes including A-SMase, an array of enzymes that remove monosaccharides and sialic acids (e.g., glucosylceramidase and sialidase), and acid ceramidase, which transforms ceramide into sphingosine and free FA. Whereas dihydrosphingosine is primarily generated by the de novo synthesis pathway, sphingosine appears to be solely derived from sphingolipid recycling. Free sphingosine is reutilized to form ceramide by the actions of CerS, or phosphorylated by sphingosine kinase and targeted for degradation (Smith and Merrill, 1995). The regeneration of sphingolipids by the salvage pathway is recognized as energetically favorable and a major contributor to the overall turnover of glycosphingolipid gangliosides (Tettamanti et al., 2003).

CERAMIDE AND INSULIN RESISTANCE IN NONRUMINANTS

The development of lipotoxicity and ectopic lipid deposition causes insulin resistance in nonruminants experiencing metabolic disease caused by overnutrition and obesity (Chavez and Summers, 2012). Although the metabolic fate of FA is either catabolic or anabolic, lipotoxicity favors the partitioning of FA away from mitochondrial β -oxidation and toward FA re-esterification in the form of triacylglycerol (**TAG**). Indeed, mitochondrial FA overload and incomplete FA oxidation contributes to skeletal muscle insulin resistance (Koves et al., 2008; Bell et al., 2010), and TAG accrual in liver and skeletal muscle is apparent in obese individuals experiencing chronic hyperlipidemia and impaired insulin action (Borén et al., 2013). Lipotoxicity also promotes the formation of bioactive signaling molecules called lipid mediators that directly interfere with insulin signaling and peripheral glucose utilization. Ceramide represents a lipid mediator and second messenger that

has been extensively evaluated in biomedical research focused on defining the mechanisms of T2DM, nonalcoholic FLD, and cardiovascular disease (Chavez and Summers, 2012).

Over the past 2 decades, the lipotoxic effects of excess ceramide have been well characterized. Initial work studying diabetic mechanisms in obese *fa/fa* Zucker Diabetic Fatty rats confirmed that the overproduction of ceramide in fat-laden pancreatic islets and the inhibition of SPT within the *de novo* synthesis pathway prevented islet apoptosis in favor of insulin production (Shimabukuro et al., 1998a,b; Unger, 2002). Summers and coworkers (1998) demonstrated that ceramide inhibited the phosphorylation and activation of anti-apoptotic protein kinase B (AKT) within the insulin-signaling cascade to reduce glucose transporter (GLUT)-4 translocation to the plasma membrane in murine 3T3-L1 adipocytes (Figure 2). Subsequent work using C2C12 myotubes confirmed that palmitate and other SFA [i.e., stearate (C18:0), arachidate (C20:0), and lignocerate (C24:0)] blocked insulin signaling via ceramide-dependent mechanisms involving AKT inactivation (Chavez et al., 2003; Chavez and Summers,

2003). These *in vitro* findings were corroborated by the discovery of C16:0- and C24:0-ceramide enrichment in skeletal muscle of obese diabetic patients (Adams et al., 2004). The inhibition of *de novo* ceramide synthesis ameliorated SFA and obesity-induced insulin resistance in rodents (Holland et al., 2007). The current consensus is that ceramide accumulation in skeletal muscle tissue perturbs insulin sensitivity in the presence of SFA oversupply (Adams et al., 2004; Bruce et al., 2013; Summers and Goodpaster, 2016). The focus on skeletal muscle is merited because it represents the primary mammalian tissue for glucose utilization (Vernon et al., 1990; DeFronzo and Tripathy, 2009; De Koster and Opsomer, 2013); however, adipose tissue ceramide accrual also develops with weight gain and high-fat diet consumption (Shah et al., 2008). In addition, ceramide has been shown to promote lipolysis with impaired insulin action in murine adipocytes (Mei et al., 2002).

Although ceramide is involved in the progression of insulin resistance with obesity, it should be mentioned that the mechanisms of the condition are multifaceted, and ceramide may not be the sole mediator of insulin antagonism (Petersen and Jurczak, 2016; Summers and

Figure 2. Mechanisms of ceramide action on insulin signaling. The reduction of insulin sensitivity by ceramide action can be attributed to its action on proteins of the insulin-signaling pathway involved in phosphorylation and dephosphorylation events as follows: (1) lipolytic- or dietary-derived fatty acids (FA) increase hepatic ceramide synthesis and secretion; (2) ceramide aggregates in caveolin-enriched microdomains (CEM); (3) ceramide recruits phosphatase and tensin homolog (PTEN) and protein kinase C zeta (PKCζ) within CEM, and activates phosphatidylinositol 4,5-bisphosphate (PIP2A); (4) phosphatidylinositol (3,4,5)-trisphosphate (PIP3) formation and protein kinase B (AKT) phosphorylation (i.e., activation) are inhibited; (5) insulin-stimulated glucose transporter-4 (GLUT4) translocation and glucose uptake are suppressed. LDL-ceramide = low-density lipoprotein-ceramide; IRS1 = insulin receptor substrate 1, PI3K = phosphatidylinositol-4,5-bisphosphate 3-kinase; PDK1 = phosphoinositide-dependent kinase-1; PIP2 = phosphatidylinositol (4,5)-bisphosphate.

Goodpaster, 2016). Specifically, lipotoxicity and insulin resistance also develop with hepatic and skeletal muscle diacylglycerol accrual (Corcoran et al., 2007). Diacylglycerol is another type of lipid mediator that inhibits insulin-stimulated glucose transport via activation of protein kinase C (**PKC**) and inactivation of insulin receptor substrate 1 (**IRS1**; Itani et al., 2002; Erion and Shulman, 2010). However, the skeletal muscle role of diacylglycerol has been contested, especially considering that muscle diacylglycerol concentrations are elevated in endurance-trained athletes with high insulin sensitivity compared with observations in obese and sedentary individuals (Amati et al., 2011; Summers and Goodpaster, 2016).

The mechanisms that ceramide uses to inhibit insulin signaling are well characterized in nonhuman primates and rodents. First, ceramide downregulates insulin signaling by inhibiting insulin-stimulated AKT serine phosphorylation (i.e., Ser-473; Summers et al., 1998; Schubert et al., 2000). However, the antagonistic behavior of ceramide may not completely rely on AKT suppression. For instance, the ability of ceramide to inhibit insulin-stimulated glucose transport depends on its recruitment within caveolin-enriched microdomains located within the plasma membrane (Blouin et al., 2010). Insulin antagonism by ceramide may also involve the activation of PKC ζ , phosphatase and tensin homolog (**PTEN**), and protein phosphatase 2A (**PP2A**; Figure 2; Hajdich et al., 2008; Blouin et al., 2010; Chavez and Summers, 2012). Ceramide-dependent caveolin-enriched microdomain-recruitment of PKC ζ promotes the phosphorylation of AKT at Thr-34 to inhibit function (Powell et al., 2003). In contrast, the activation of PTEN transforms phosphatidylinositol triphosphate to phosphatidylinositol biphosphate to downregulate phosphatidylinositol 3-kinase (**PI3K**) signal transduction, and PP2A dephosphorylates AKT at Thr-308 and Ser-473. In parallel, ceramide may also deactivate IRS1 via Ser-307 phosphorylation (Hage Hassan et al., 2016).

Although the de novo synthesis of ceramide within tissues is a potential contributor to localized ceramide accrual and insulin resistance, recent work by Boon et al. (2013) and Watt et al. (2012) provides support for the ability of liver-derived lipoprotein ceramide to antagonize insulin action in peripheral tissues. As demonstrated in rats intravenously infused a TAG emulsion or HepG2 liver cells cultured with palmitate, the liver is able to detect and secrete de novo synthesized ceramides in response to FA oversupply (Watt et al., 2012). Hepatic ceramide accumulation is observed in rodent models of FLD and may develop in obese diabetic humans with nonalcoholic FLD (Pagadala et al., 2012). Elevations in hepatic ceramide synthesis and

secretion likely depend on steatosis in the presence of a simultaneous inflammatory insult (Pagadala et al., 2012) and the actions of microsomal triglyceride transfer protein (Iqbal et al., 2015). In human circulation, ceramides are predominantly found within low-density lipoproteins (**LDL**; ~60% of total lipoproteins; Wiesner et al., 2009); although the proportion of ceramide found within liver-secreted very-low-density lipoproteins (**VLDL**) increases with fasting (~39 vs. 48% of ceramide found within VLDL and LDL, respectively; Wiesner et al., 2009). Prior observations suggest that LDL ceramide enrichment occurs within LDL aggregates via the hydrolysis of LDL-sphingomyelin or transfer of pre-existing ceramide from cells (Schissel et al., 1996). Watt and colleagues (2012) hypothesize that hepatocyte de novo synthesis of ceramide may also explain changes in VLDL and LDL ceramide.

The focus on LDL ceramide is important because their concentrations are elevated in diabetic humans (Haus et al., 2009; Boon et al., 2013). The intravenous infusion of LDL containing C24:0-ceramide, the most abundant ceramide in circulation, reduces whole-body and skeletal muscle 2-deoxyglucose (**2DOG**) clearance in mice (Boon et al., 2013). In the same study, C24:0-ceramide concentrations tended to be elevated in fractionated plasma membranes; however, C24:0-ceramide levels were not enriched in whole skeletal muscle. Similar outcomes were observed for mice intravenously infused LDL C16:0-ceramide. The translocation of ceramide from LDL to the plasma membrane may involve LPS receptor CD14, which provokes clustering of co-receptors in membrane microdomain rafts (Pfeiffer et al., 2001). Once in the plasma membrane, ceramide likely accumulates in caveolin-enriched microdomains (Blouin et al., 2010). Subsequent experiments confirmed the inhibition of insulin-stimulated 2DOG uptake, AKT phosphorylation, and GLUT4 translocation in L6 muscle cells incubated with C16:0- or C24:0-ceramide (Boon et al., 2013). Interestingly, the origin of ceramide may not matter when we consider the potential ability of ceramide to “flip-flop” across the phospholipid bilayer of the plasma membrane (Kinnunen and Holopainen, 2002). This action suggests that extracellular- and intracellular-derived ceramide may work in unison to inhibit skeletal muscle insulin sensitivity during lipotoxicity. Interestingly, extracellular ceramide within LDL does to appear to modulate insulin sensitivity in murine white adipose tissue or 3T3-L1 adipocytes (Boon et al., 2013).

In CerS2 haploinsufficient mice (Raichur et al., 2014) or CerS6-deficient mice (Turpin et al., 2014), evidence suggests that C16:0-ceramide promotes obesity-induced insulin resistance. Similarly, mice with CerS1 deficiency

exhibit reduced muscle C18:0-ceramide concentrations and improved systemic glucose homeostasis (Turpin-Nolan et al., 2019), whereas VLC ceramides (i.e., C22:0- and C24:0-ceramide) serve a protective role (Raichur et al., 2014; Tippetts et al., 2018). This argument is complicated in light of evidence in mice demonstrating the ability of C24:0-ceramide within LDL to reduce insulin-stimulated glucose utilization (Boon et al., 2013) and the observation that VLC ceramides play a lipotoxic role in mediating mitochondrial dysfunction and oxidative stress (Law et al., 2018). It seems reasonable to infer that long-chain and VLC ceramides have lipotoxic potential, but their effects on metabolism likely depend on type of pathology, tissue examined, and total ceramide status of the animal.

Chronic inflammation is a key component of insulin resistance caused by overnutrition (Lionetti et al., 2009; Caputo et al., 2017). Ceramide supply and inflammation are tightly intertwined. In detail, the proinflammatory cytokine tumor necrosis factor- α (TNF α) is an inhibitor of insulin signaling (Borst, 2004; Nguyen et al., 2005). Tumor necrosis factor- α inhibits the insulin-dependent tyrosine phosphorylation of insulin receptor and IRS1 (Peraldi et al., 1996). The antagonistic actions of TNF α also involve the hydrolysis of sphingomyelin to form ceramide by A- and N-SMase induction (Peraldi et al., 1996; Peraldi and Spiegelman, 1998; Rozenova et al., 2010). This is of particular interest because endotoxin (i.e., LPS) and saturated palmitate activate SMase via mechanisms that involve toll-like receptor-4 signaling and TNF α (Wong et al., 2000; Holland et al., 2011a). Moreover, inflammation-mediated secretory-SMase activation stimulates LDL-sphingomyelin hydrolysis, which may also contribute to circulating ceramide accrual during insulin resistance (Schissel et al., 1998). In a feedback manner, SMase activation and ceramide may provoke inflammation by stimulating the production of inflammatory cytokines including IL-6 (Ballou et al., 1996). Indeed, ceramide augments TNF α release from macrophages, which has implications for individuals that experience adipose tissue macrophage infiltration with high liver fat content (Kolak et al., 2007; Schilling et al., 2013).

The discovery of ceramide as an insulin antagonist triggered numerous studies to assess sphingolipid as a metabolic disease biomarker in humans. Kremer et al. (1975) first demonstrated the accrual of GlcCer in plasma collected from diabetic patients. Three decades later, Haus and coworkers (2009) demonstrated plasma ceramide (C18:0, C20:0, C24:1, and C24:0) accrual in T2DM patients. They also observed that plasma ceramide levels were inversely related to insulin sensitivity but positively associated with circulating TNF α

levels. Plasma ceramide accumulation is also observed in female children and adolescents with T2DM and suppressed levels of circulating adiponectin (Lopez et al., 2013). As mentioned earlier, plasma LDL ceramide concentrations are elevated in obese diabetic humans (Boon et al., 2013). Suggesting prognostic potential, plasma C16:0-, C22:0-, and C24:0-ceramide concentrations were elevated in prediabetic and diabetic Rhesus Macaque monkeys (Brozinick et al., 2013). In addition, increased plasma levels of C18:0 deoxysphinganine, a signature of SPT activation, were observed before the onset of diabetes. Such relationships have spurred interest in developing nutritional and pharmacological approaches to control ceramide supply. For instance, we have identified a nutritional therapy that reduces circulating ceramides in overweight young adults (Mathews et al., 2017), and the development of effective and safe oral SPT inhibitors is being pursued (Genin et al., 2016; Adachi et al., 2018).

SPHINGOLIPID BIOLOGY IN THE DAIRY COW

Sphingolipids in Milk

Our knowledge of sphingolipids in ruminants has been historically limited to the observation that dairy products are a major source of sphingolipids in the human diet (Vesper et al., 1999). Unlike TAG, which locate predominantly in the core of milk fat globules, sphingolipids co-locate with glycerophospholipids in the milk lipid globule membrane (Lopez et al., 2008). Membrane sphingolipids are believed to prevent globules from coalescing, to act as emulsion stabilizers, and to be critical for the formation and maintenance of the globular membrane structure (Jensen, 2002; Heid and Keenan, 2005). In terms of composition, sphingomyelin predominates (~75% of total sphingolipids; Christie et al., 1987), whereas the concentrations of ceramide, GlcCer, LacCer, and gangliosides are far more limited (Jensen, 2002). Similar to TAG, milk sphingolipids from ruminants are compositionally unique with diverse fatty acyl chains with varying degrees of saturation, hydroxylation, and glycosylation. Comparable to human milk (Bouhours and Bouhours, 1981), the major acyl chains in bovine milk sphingolipids include 16-, 18-, 22-, and 24-carbon units (Morrison and Hay, 1970). The presence of sphingolipids in milk has sparked renewed interest in dairy because of the discovery of anticarcinogenic and anti-inflammatory properties of ceramides and sphingomyelins, respectively (Ogretmen and Hannun, 2004; Norris et al., 2017). Moreover, milk sphingomyelin is a source of dietary phosphocholine, which may benefit neonatal development (Zeisel et al., 1986).

Ceramide Accrual in the Early-Lactation Cow

The transition from gestation to lactation represents a metabolic challenge for high-producing dairy cows. Glucose demand is high for the synthesis of milk lactose, the osmotic regulator of milk volume. To meet this demand, the cow relies on 2 adaptations: hypoinsulinemia and modified peripheral tissue response to insulin (Baumgard et al., 2017). Hypoinsulinemia is caused by reduced glucose-stimulated pancreatic insulin secretion (Rhoads et al., 2004). In addition, insulin reduces whole-body and skeletal muscle glucose uptake in a dose-response manner (Vernon et al., 1990). In adipose tissue, a reduction in insulin action supports lipolysis (Vernon and Taylor, 1988). Although lipolysis serves a purpose to provide FA substrate for oxidative metabolism and milk TAG synthesis, accelerated body fat mobilization provokes hepatic FA uptake, steatosis, ketogenesis, and infertility (Rukkwamsuk et al., 2000; Bobe et al., 2004; Duffield et al., 2009). These outcomes are routinely observed in cows with heightened prepartum adiposity (i.e., elevated BCS) and rampant lipolysis (Roche et al., 2009; Zachut et al., 2013).

Based on biomedical research identifying ceramide as a causal agent of insulin resistance in overweight and diabetic nonruminants experiencing SFA surplus (Summers, 2006; Haus et al., 2009; Chavez and Summers, 2012) and the role of insulin antagonism and lipolysis in the postpartum cow, in our initial work we quantified periparturient changes in plasma ceramides collected from cows classified prepartum as lean or overweight (BCS <3.0 and >4.0, respectively; d -28 through d 4, relative to calving; Rico et al., 2015a). Independent of prepartum adiposity phenotype, we observed a progressive accumulation of plasma ceramides as cows transitioned from pregnancy to lactation. Overweight cows experienced greater body weight loss and circulating free FA and ceramide concentrations postpartum compared with lean cows. The majority of plasma ceramides and GlcCer measured were positively associated with circulating free FA and inversely related to estimated systemic insulin sensitivity (i.e., the Revised Quantitative Insulin Sensitivity Check Index; Holtenius and Holtenius, 2007). The strongest correlations were observed for VLC ceramides including highly abundant C22:0- and C24:0-ceramide. This finding was interesting because the intravenous infusion of C24:0-ceramide within LDL into C57BL/6J mice reduced whole-body and skeletal muscle insulin-stimulated 2DOG uptake (Boon et al., 2013). Our inaugural study supported a role for ceramide in the development of insulin antagonism in postpartum cows that mobilize body fat. Our observations also supported the expected substrate-product relationship that drives de novo ce-

ramide synthesis (Figure 1; Watt et al., 2012), whereby lipolytic-derived palmitic acid is utilized by SPT to form 3-ketosphinganine or elongated by elongases for utilization by CerS to form ceramide (Ohno et al., 2010). Subsequent sample analyses demonstrated that ceramide accrual developed with plasma fatty acylcarnitine accumulation, which might indicate the partitioning of FA away from mitochondrial β -oxidation and toward sphingolipid synthesis (Yang et al., 2009; Rico et al., 2018b).

To independently confirm our findings in a separate cohort and expand our understanding of sphingolipid biology in periparturient cows, we conducted a more complete investigation of the temporal changes in plasma, liver, and skeletal muscle sphingolipid levels compared with hepatic lipid deposition and systemic insulin and glucose tolerance during the totality of the transition period (d -21 through d 21, relative to calving; Rico et al., 2017b). Because of uncertainties associated with the application of insulin sensitivity indices in transition cows (Mann et al., 2016), we chose to utilize more direct measures, including glucose and insulin tolerance testing, to assess systemic glucose utilization. In confirmation of our earlier work (Rico et al., 2015a), plasma ceramides and GlcCer concentrations increased at the onset of lactation, with more pronounced elevations observed in cows with elevated prepartum adiposity in association with plasma free FA concentrations. Plasma ceramide levels were also positively correlated with liver lipid content. Although changes in hepatic ceramide levels were minimal during the transition, hepatic C24:0-ceramide levels did increase as overweight cows transitioned. The hepatic ceramide profile reflected plasma (Figure 3), and positive associations were identified between hepatic and plasma ceramide levels, which further supported the role of the liver as a primary source of circulating ceramides.

Because we were cognizant that enhanced de novo ceramide synthesis may not be the only explanation for plasma ceramide accrual, we investigated the possibility that sphingomyelin hydrolysis by SMase activation may be at play. This inquiry was warranted because inflammatory cytokines activate SMase in nonruminants and the transition cow experiences inflammation (Peraldi et al., 1996; Bertoni et al., 2008; Sordillo and Raphael, 2013). In our investigations (Rico et al., 2017b, 2018b), circulating phosphocholine-containing sphingomyelins, including C16:0-, C22:0-, and C24:0-sphingomyelin, were lowest at parturition in all cows. Plasma reductions in C18:1- and C20:1-sphingomyelin were greater for overweight cows compared with lean cows. Although these findings support the possibility that inflammation-mediated SMase activation may also promote ceramide accrual in postpartum cows,

especially in overweight cows that may preferentially experience inflammation (O'Boyle et al., 2006), the apparent reduction in circulating sphingomyelin may be an artifact of reduced DMI and low dietary choline bioavailability (Neill et al., 1979; Sharma and Erdman, 1989; Rico et al., 2017b). Nevertheless, plasma and hepatic sphingolipidome remodeling developed with C16:0-ceramide accrual in skeletal muscle postpartum independent of late-gestation adiposity status. This is of potential significance because C16:0- and C18:0-ceramide are believed to mediate skeletal muscle insulin resistance in nonruminants (Boon et al., 2013; Tonks et al., 2016). Although we did not evaluate skeletal muscle insulin action, plasma C24:0-ceramide and skeletal muscle C16:0-ceramide levels were inversely related to glucose clearance rate and insulin-stimulated reductions in glucose following an insulin challenge postpartum, respectively (Rico et al., 2017b; Figure 4A). Similar results were observed for plasma C18:0-ceramide (Figure 4B). Additionally, plasma C16:0-GlcCer concentrations were inversely related to glucose-stimulated reductions of free FA following a glucose challenge. Although data from Rico et al. (2015a, 2017b) supported the possi-

bility that ceramides contribute to insulin antagonism during early lactation, controlled trials were needed to assess the effects of hyperlipidemia on ceramide supply.

Lessons Learned from the Controlled Induction of Hyperlipidemia

The oversupply of free FA enhances hepatic de novo ceramide synthesis and secretion in nonruminants (Watt et al., 2012). To mimic hyperlipidemia during obesity, lean humans and rats underwent an acute intravenous infusion of plant-based TAG emulsion, which resulted in increased circulating FA and ceramides in both groups and promoted hepatic ceramide accumulation in rats (Watt et al., 2012). Considering that palmitic acid is the principal substrate for de novo ceramide synthesis (Figure 1), Watt and coworkers (2012) confirmed that palmitate incorporation into intracellular and extracellular ceramide is dose- and time-dependent in HepG2 liver cells. Considering these findings, we sought to confirm the importance of hyperlipidemia for promoting hepatic and plasma ceramide accrual using nonpregnant and nonlactating dairy cows continuously infused with

Figure 3. Ceramide compartment profiles in Holstein dairy cows. Fatty acyl chain profiles of ceramides in liver, skeletal muscle, adipose tissue, plasma, low-density lipoprotein (LDL), and triacylglycerol (TAG)-rich lipoproteins.

an identical TAG emulsion delivered intravenously over 16 h (Caixeta et al., 2017; Rico et al., 2018c). Intravenous TAG infusion raised circulating free FA and fibroblast growth factor 21 (FGF21) concentrations and hepatic TAG levels and robustly elevated circulating and hepatic ceramides and GlcCer. Fibroblast growth factor 21 can induce adiponectin secretion to suppress ceramide production (Holland et al., 2013); however, TAG infusion does not increase circulating adiponectin concentrations in anabolic cows (Staiger et al., 2002; Krumm et al., 2017). In nonpregnant, nonlactating cows, the inability of adiponectin secretion to respond

to increases in FGF21 and prevent ceramide accrual is interesting. It is likely that the increase in circulating FA concentrations supported de novo ceramide synthesis solely because of increased substrate supply. Indeed, we observed marked increases in C22:0- and C24:0-dihydroceramide concentrations. Additionally, hepatic CerS2 mRNA expression was enhanced by TAG infusion, which is significant because CerS2 controls the de novo synthesis of VLC ceramides (Figure 1) and TAG infusion did not modify plasma sphingomyelin levels. In clinically healthy dairy cows in positive energy balance and challenged by surplus FA, we concluded that hepatic de novo ceramide synthesis—not sphingomyelin hydrolysis—was the predominant driver of ceramide accrual. In support, the supplementation of bovine neonatal hepatocytes with palmitic acid increased intracellular ceramide concentrations, and this effect was completely reversed by the inhibition of SPT with myriocin (McFadden et al., 2018).

To explore the effects of hyperlipidemia in dairy cows experiencing negative energy balance, nonpregnant, nonlactating dairy cows were fed ad libitum or nutrient-restricted (i.e., straw feeding only) for 32 h (Davis et al., 2017a). We anticipated that the upregulation of adipose tissue lipolysis would increase hepatic saturated palmitic acid uptake and increase ceramide synthesis (Douglas et al., 2007). As expected, nutrient restriction dramatically elevated circulating free FA and enhanced liver lipid content. Simultaneously, serum and hepatic ceramide accumulation developed with nutrient restriction. Comparable to the effect in the transition cow, serum and liver C22:0- and C24:0-ceramide were inversely related to systemic changes in insulin-stimulated glucose disposal in nutrient-restricted cows challenged by insulin (i.e., glucose clearance rate and area under the curve following an intravenous glucose challenge) which was not observed for C16:0-, 18:0-, or C20:0-ceramide. In contrast to that in the transition cow, serum and liver C16:0-ceramide increased with the induction of negative energy balance and contributed more to the ceramide pool. These findings suggest that physiological stage may govern hepatic CerS isoform expression and activation, and thus influence the ceramide profile of liver and plasma. Unfortunately, circulating or hepatic dihydroceramides or sphingomyelins were not measured in Davis et al. (2017a), which means that the contributions of de novo ceramide synthesis versus sphingomyelin hydrolysis could not be distinguished. Alternatively, CerS2 regulates FA elongase-1 to ensure coordinated VLC ceramide synthesis (Ohno et al., 2010). We do not know whether the functionality of elongase isoforms changes with stage of physiology. Perhaps an acute feed restriction fails to induce palmitic acid elongation in the nonlactating, nongestating

Figure 4. Ceramide concentrations are inversely related to insulin-stimulated glucose disposal in lactating dairy cows. (A) Regression analysis of plasma C18:0-ceramide and (B) skeletal muscle C16:0-ceramide against insulin-stimulated reductions in plasma glucose (ISRG) in Holstein cows during postpartum (d 5 of lactation; $n = 14$).

cow. Although the physiological differences in ceramide production require further study, our findings suggest that ceramide may represent a link between FA surplus and peripheral insulin resistance observed in Holstein cows (Pires et al., 2007).

Circulating Sphingolipids

The liver is a major contributor of circulating sphingolipids, as suggested by the close overlap in the compositional profiles of liver and plasma ceramides (Haus et al., 2009; Rico et al., 2018c; Figure 3) and by the rapid increase in plasma ceramides in response to hepatic free FA influx (Watt et al., 2012; Rico et al., 2018c). Indeed, estimates suggest that approximately 75 to 97% of circulating ceramides are contained within the apolipoprotein B100-containing VLDL and LDL of hepatic origin, with the remainder associated with high-density lipoproteins (Lightle et al., 2003; Wiesner et al., 2009; Hammad et al., 2010). These ceramides are primarily C16:0-, C22:0-, and C24:0-ceramide in the dairy cow. Interestingly, although ceramide and sphingomyelins are largely contained in secreted VLDL, these sphingolipids do not seem necessary for VLDL secretion (Merrill et al., 1995). Ceramide incorporation into nascent lipoprotein is mediated by microsomal triglyceride transfer protein via a mechanism similar to that used to incorporate TAG into VLDL (Iqbal et al., 2015). Furthermore, palmitate increases cellular microsomal triglyceride transfer protein levels, which suggests that elevated SFA availability not only up-regulates ceramide synthesis but also induces its secretion from liver (Konstantynowicz-Nowicka et al., 2015).

Although the liver represents the major source of circulating ceramides, not all ceramides in plasma can be accounted for by lipoproteins. Indeed, lipoprotein-deficient serum contains ~15% of serum ceramides (Lightle et al., 2003). One possible alternative source is adipose tissue, which has been hypothesized to release ceramides and modulate insulin sensitivity in liver (Holland et al., 2007; Cowart, 2009). A second possibility is that ceramides are shed as cargo from cells within extracellular vesicles (i.e., exosomes) that bud from the plasma membrane as part of intercellular communication mechanisms operating during cell stress conditions such as during lipotoxicity (Trajkovic et al., 2008; Kornek et al., 2012; Yoon et al., 2014).

Dietary Fatty Acids and Ceramide Synthesis

If ceramide is able to control insulin signaling and glucose utilization by nonmammary tissues, then dietary approaches that modulate ceramide production would be of commercial interest to control lactation.

Certainly, the rumen biohydrogenation of UFA to form nonesterified SFA (i.e., ~65% of free FA reaching the duodenum are C16:0 and C18:0; Lock et al., 2006) represents a potential substrate for SPT. Although biohydrogenation serves to detoxify UFA (Maia et al., 2007, 2010), it is intriguing to consider whether the magnitude of rumen biohydrogenation is correlated with ceramide production. Such a relationship would provide the cow with a glucose-sparing mechanism to support milk production when feeding on grasses that contain UFA. Although this remains to be defined, we recognize that rumen-inert fat supplementation (i.e., SFA products) is common on dairy farms, and the dietary supplementation of SFA-enriched fat supplements may favor ceramide synthesis and accumulation. To assess this possibility, a series of *in vivo* studies were performed to evaluate the effects of palmitic acid feeding compared with no added fat, stearic acid, medium-chain TAG (**MCT**), palmitoleic acid, and α -linolenic acid supplementation.

In mid-lactation low-producing Holstein dairy cows fed a sorghum silage-based TMR, we investigated the effects of feeding a diet containing supplemental palmitic acid at ~4% of ration DM for 7 wk relative to cows supplemented with soyhulls (Rico et al., 2016). Similar to previous observations (Wang et al., 2010; Piantoni et al., 2013), palmitic acid feeding increased milk and milk fat yields without modifying DMI (Mathews et al., 2016). In support of adipose tissue insulin antagonism, the magnitude of plasma free FA disappearance following an intravenous glucose challenge progressively decreased with palmitic acid supplementation in the absence of changes in systemic glucose tolerance. These outcomes that accompanied palmitic acid supplementation occurred with pronounced elevations in circulating ceramides and GlcCer, whereas lactosylceramide (**LacCer**) levels were not overtly modified (Rico et al., 2016). Increases were greatest for VLC ceramides including C22:0- and C24:0-ceramide but no changes were observed for C16:0-ceramide. Palmitic acid feeding also increased hepatic ceramide concentrations (e.g., C20:0, C24:0, and total), although the response was less pronounced relative to plasma. Although we describe that CerS2 and CerS6 are likely the predominant controls of hepatic *de novo* ceramide synthesis in cows (Rico et al., 2018c), palmitic acid feeding reduced CerS2 mRNA expression in liver, perhaps reflecting a compensatory mechanisms to maintain ceramide homeostasis. Alternatively, palmitic acid feeding may not have induced *de novo* ceramide synthesis but rather activated A-SMase (Jin et al., 2013). Nevertheless, removal of supplemental palmitic acid from the diet immediately restored ceramide levels to those observed in control cows (Rico et al., 2016).

Of particular interest, palmitic acid feeding prevented the gradual decline in circulating ceramides that was detected in control cows advancing toward late lactation (Figure 5A; Rico et al., 2016). This gradual reduction in circulating ceramides developed concomitantly with a steady decrease in circulating FA levels and increase in plasma insulin concentrations. The observed increase in circulating ceramides during early lactation (Rico et al., 2015a, 2017b) and their decline beyond peak milk production (Rico et al., 2016) suggests that ceramide supply responds in a reciprocal manner as energy balance and insulin sensitivity improve with the progression of lactation. The ability of palmitic acid to augment ceramide synthesis may be a means to maintain glucose-sparing mechanisms to drive lactation after peak milk yield in low-production cows. In support, circulating ceramides were positively correlated with plasma FA, FA area under the curve following a glucose challenge, and milk yield (Rico et al., 2016). Increased nutrient partitioning toward milk with high palmitic acid feeding has also been independently observed in mid-lactation Holstein cows (de Souza et al., 2018) relative to cows fed in the absence of supplemental fat.

Although the restoration of insulin antagonism and nutrient (i.e., glucose and FA) partitioning to support milk and milk fat production later in lactation is desirable, such an outcome during early lactation may exacerbate insulin antagonism to a point where body fat mobilization is further accelerated. Indeed, the cow may produce more milk with a milk FA composition that reflects the lipolytic profile but she might be at increased risk for developing a metabolic disease such as FLD or ketosis. These possibilities require further study; however, recent evidence suggests that palmitic acid feeding (1.5% of ration DM) enhances body fat mobilization during early lactation (de Souza et al., 2019) compared with a control diet containing no supplemental fat. These palmitic acid-fed early-lactation cows exhibited greater circulating ceramide concentrations (e.g., total and C24:0) in a positive association with circulating plasma total FA and milk yield (Figure 5B; Davis et al., 2017b). Interestingly, recent work suggests that the partial substitution of dietary palmitic acid with oleic acid increases plasma insulin levels and limits body weight loss during the postpartum (de Souza et al., 2018), relative to feeding palmitic acid alone. The inclusion of oleic acid decreased and increased energy partitioning toward milk and body tissue gain, respectively. Requiring confirmation in cows, these responses may involve the ability of oleic acid to lower ceramide (Jin et al., 2018). Considering that dietary FA feeding appears to modulate lipolysis by using mechanisms that may involve ceramide, it is intriguing to consider whether ceramide might indirectly influence the milk

FA profile. Palmitic acid feeding routinely increases yields of milk fat and 3.5% FCM (de Souza et al., 2016), and some studies have shown increases in palmitic acid in milk fat but also preformed *cis*-9 oleic acid (de Souza and Lock, 2018). Palmitic, stearic, and oleic acids are abundant FA found in adipose tissue and a potential source for milk fat production (Rukkwamsuk et al.,

Figure 5. Plasma very-long-chain ceramide concentrations are increased during palmitic acid feeding and are positively associated with milk yield in dairy cows. (A) Plasma concentration of abundant C24:0-ceramide during palmitic acid feeding in mid-lactation Holstein cows (~4% of ration DM; palm fat composed of 98% palmitic acid) during (d 0 to 49) and after (d 49 to 63; dotted arrow) treatment, relative to no supplemental fat. Data are presented as least squares means and their standard errors; * $P < 0.05$. From Rico et al. (2016). (B) Regression analysis between plasma C24:0-ceramide concentrations and daily milk yield in multiparous Holstein cows during early lactation. Data represent measurements between d 7 and 54 postpartum. Adapted from Davis et al. (2017b).

2000). Their proportions in milk fat are controlled to maintain fluidity (Chilliard et al., 2000).

Although palmitic acid is the principal substrate for de novo ceramide synthesis, palmitic acid or other FA (most often longer chain SFA) are incorporated by CerS. Moreover, the consumption of PUFA has been shown to decrease ceramide accrual compared with the consumption of SFA (Blachnio-Zabielska et al., 2010). To evaluate, we quantified circulating ceramides in mid-lactation Holstein cows abomasally infused with SFA in the form of palmitic acid, MCT (C8:0/C:10), and stearic acid (Rico et al., 2017a). Although milk yield was not influenced by these treatments, plasma ceramide, GlcCer, and LacCer concentrations were positively associated with plasma total FA and yields of milk and FCM. Even though total FA digestibility was higher for cows infused MCT (Rico et al., 2015b), palmitic acid was more effective at raising plasma palmitic acid and ceramide concentrations relative to MCT and stearic acid (Rico et al., 2017a). We note that the reduced ability of stearic acid to raise plasma ceramides may be explained by lower FA digestibility observed with the infusion of this FA. Moreover, the importance of hepatic elongases to convert stearic acid to VLC FA to generate VLC ceramides in ruminants is unknown. Although this could not be discerned, a positive relationship between circulating free FA and ceramide and elevations in GlcCer concentrations were inversely associated with a lower hepatic expression of apolipoprotein B100 and carnitine palmitoyltransferase-1A (Rico et al., 2017a). These observations suggest that plasma ceramide accrual develops with the downregulation of hepatic lipoprotein assembly and mitochondrial FA oxidation. Such conditions are typical of the postpartum dairy cow experiencing plasma ceramide accumulation (Rico et al., 2015a, 2017b), and ceramide has been proposed to mediate nonalcoholic FLD in nonruminants (Pagadala et al., 2012; Kasumov et al., 2015). Interestingly, PUFA may lower ceramide synthesis (Blachnio-Zabielska et al., 2010; Jin et al., 2018). In support, Duckett et al. (2019) demonstrated that the dietary supplementation of insulin-sensitizing palmitoleic acid decreased circulating C24:0- and C24:1-ceramide concentrations in juvenile lambs, relative to no-lipid or α -linolenic acid supplementation.

Although we have shown palmitic acid to be an inducer of ceramide synthesis in dairy cows, an important consideration is that dietary palmitic acid supplementation does not always increase milk yield in cows (Loften et al., 2014; de Souza et al., 2016). One possibility is that ceramide and localized insulin sensitivity were not modified in studies that did not report a change in milk yield with palm feeding. In addition, other SFA besides palmitic acid may increase ceramide

synthesis via their incorporation by CerS (Figure 1); therefore, it may prove difficult to observe an effect on milk yield in studies evaluating palmitic acid feeding versus stearic acid. The functionality of stearoyl-CoA desaturase that transforms palmitic and stearic acids to their monounsaturated forms, and the effects of dietary nonlipid nutrients (e.g., starch) on ceramide synthesis deserve consideration within the context of changes in milk yield. Stage of lactation, changes in intake, body condition status, the complete lipolytic and dietary FA profile, and production level of the cow may also be influential factors.

Ceramide Inhibits Adipocyte Insulin Signaling

Ceramide may inhibit insulin signaling in dairy cows by downregulating AKT activation and GLUT4 translocation to the plasma membrane (Rico et al., 2018a). In primary bovine adipocytes, we evaluated the ability of ceramide to modulate AKT phosphorylation status (i.e., activation) and insulin-stimulated 2DOG uptake. First, we demonstrated that the inhibition of SPT and de novo ceramide synthesis by myriocin decreased intracellular ceramide and GlcCer concentrations. Additionally, the ceramide-lowering ability of myriocin developed with elevations in Ser-473 phosphorylation of AKT and 2DOG uptake in the presence of insulin. Second, primary bovine adipocytes were cultured with exogenous C2:0-ceramide, which is a hydrophilic ceramide that can freely pass through the plasma membrane. In this experiment, C2:0-ceramide impaired AKT phosphorylation and insulin-stimulated 2DOG uptake. Of importance, these findings were observed in differentiated bovine adipocytes cultured in nutrient excess. Upon reflection, the ability of palmitic acid feeding to increase ceramide supply and elevate circulating FA following a glucose challenge in mid-lactation cows may involve similar mechanisms. Indeed, the ability of ceramide to inhibit insulin action and promote lipolysis has been observed in nonruminants (Summers et al., 1998; Mei et al., 2002).

Although reductions in insulin sensitivity may contribute to lipolysis in the dairy cow (Pires et al., 2007), it is uncertain whether ceramide inhibits insulin-stimulated AKT phosphorylation in adipose tissue of the early-lactation cow experiencing catabolism. It is expected that adipocyte FA oxidation increases with postpartum lipolysis; therefore, FA substrate for localized ceramide synthesis may be limiting. When FA release exceeds oxidative capacity of the adipocyte, then FA may be used to generate ceramide and inhibit insulin signaling in an autocrine manner. We also consider the possibility that ceramide does not inhibit the adipose tissue insulin signaling cascade immediately

postpartum but rather serves as a repository of substrate (i.e., FA) for hepatic de novo ceramide synthesis. Based on work by Boon et al. (2013), hepatic-derived lipoprotein ceramide may antagonize AKT activation in skeletal muscle or promote macrophage inflammation. Because lipolytic-derived FA may be used to stimulate de novo ceramide synthesis in bovine skeletal muscle, intracellular ceramide may also inhibit insulin signaling in skeletal muscle. Although these outcomes are often observed in obese and insulin-resistant nonruminants experiencing myocellular lipid deposition (Corcoran et al., 2007), we do recognize the ability of diacylglycerol to also reduce insulin-stimulated glucose uptake via PKC-dependent mechanisms. In the dairy cow, the ability of ceramide or diacylglycerol to inhibit insulin signaling in skeletal muscle or myotubes has not been studied and requires further investigation.

Summary

A summary of established and potential mechanisms in the transition dairy cow is provided in Figure 6. First, the magnitude of hepatic de novo ceramide synthesis is contingent on the state of energy balance, dietary- or lipolytic-derived FA supply, and the mitochondrial capacity to oxidize FA in liver. Periparturient cows with greater prepartum body fat reserves that mobilize more FA or those fed palmitic acid will experience greater elevations in de novo ceramide synthesis. Hepatic ceramides that are responsive to increased FA supply include C20:0- to C26:0-linked ceramides as well as C16:0-, C22:0-, and C24:0-GlcCer. It is likely that hepatic elongase, CerS, and glucosylceramide synthase play a critical role in regulating the production of ceramides. Once synthesized, ceramides are packaged within VLDL for secretion. Transition cows that experience inflammation may also experience activation of secretory A-SMase to transform LDL sphingomyelin into ceramide. Macrophage inflammation or palmitic acid (diet- or lipolytic-derived) may induce intracellular de novo ceramide synthesis in bovine adipose and skeletal muscle tissues. These ceramides may include C16:0- and C24:0-ceramide. Collectively, extracellular lipoprotein or intracellular ceramide may inhibit adipose and skeletal muscle insulin sensitivity via AKT-dependent mechanisms to reduce glucose uptake. In adipose, ceramide-mediated insulin antagonism may also accelerate adipose tissue lipolysis. One outcome is the partitioning of glucose and FA toward milk and milk fat production, respectively. Another is the acceleration of hepatic FA uptake, which may predispose the transition cow to developing FLD. The ability of ceramide to partition nutrients may influence lactation and health.

FUTURE DIRECTIONS

Does Ceramide Influence AKT Activation in the Bovine Mammary Gland?

The serine/threonine kinase AKT has 2 major roles in mammary epithelial cells. First, the PI3K/AKT signaling pathway inhibits programmed cell death (i.e., apoptosis) to promote survival (Kennedy et al., 1997; Schwertfeger et al., 2001). Specifically, AKT inactivates cell death effector proteins BAD and caspase-9, and increases the abundance of anti-apoptotic protein Bcl-2 (Pettus et al., 2002; Bratton et al., 2010). Second, AKT orchestrates developmental changes that allow mammary nutrient uptake and synthesis for milk production (Boxer et al., 2006). Activation of AKT regulates the expression of the lipogenic transcription factor sterol regulatory element binding protein, as well as its proteolytic processing, to increase concentrations of cellular FA and glycerophospholipids (Porstmann et al., 2005; Zhang et al., 2018). Additionally, AKT-dependent activation of mammalian target of rapamycin (mTOR) stimulates protein translation via S6 kinase and eukaryotic elongation factor 2 (Hassan et al., 2013; Zhang et al., 2018). In the lactating goat, AKT expression is greatest in mid lactation followed by peak lactation, and lowest during the dry period and late lactation (Zhang et al., 2018). Unfortunately, our understanding of the role of sphingolipids in the mammary epithelial cell or their ability to regulate AKT-dependent cellular proliferation, survival, or lipid and protein synthesis is inadequately defined.

In alignment with milk sphingolipid levels (Bitman and Wood, 1990), the mammary expression of SPT1 and CerS2 is highest during peak lactation and declines gradually until dry-off (Bionaz and Loor, 2008). As described by Bionaz and Loor (2008), elevated ceramide synthesis may serve a regulatory role to maintain milk lipid synthesis (Worgall, 2007). Because ceramide is a potent inducer of apoptosis (Pettus et al., 2002), we also consider the possibilities that (1) the suppressed mammary expression of SPT1 and CerS2 during the dry period and early lactation ensures cellular proliferation, and (2) the heightened SPT1 and CerS2 expression of peak lactation initiates mammary gland regression through the process of apoptotic cell death. In support, unsaturated oleic acid activates AKT and induce cell proliferation in bovine mammary epithelial cells (Yonezawa et al., 2008), whereas saturated ceramide precursors palmitic and stearic acids induce apoptosis without inducing cellular proliferation (Hardy et al., 2000; Yonezawa et al., 2008). Recalling that ceramide activates PTEN (Hajdich et al., 2008), the overexpression of PTEN inhibits proliferation and differentiation,

and milk yield while provoking apoptosis in mammary epithelial cells (Dupont et al., 2002; Wang et al., 2014). Upregulated apoptosis by ceramide may also explain observed decreases in mammary epithelial cells during chronic nutrient restriction in lactating cows (Dessaige et al., 2011), a signal that may be suppressed by the mammogenic and lactogenic roles of prolactin and somatotropin (Tucker, 1981; Capuco et al., 2001). Collectively, the role of ceramide in modulating mammary AKT function, apoptosis, and milk fat and protein synthesis represents a realm of dairy biology open for exploration.

Are Ceramide and Inflammation Related in the Transition Cow?

Chronic low-grade inflammation is a major promoter of obesity-associated insulin resistance (Hotamisligil, 2006; Osborn and Olefsky, 2012). In dairy cows, a high degree of inflammation is observed during the peripartum (Bertoni et al., 2008), which is exacerbated

in overweight cows and those with FLD (Ohtsuka et al., 2001; O'Boyle et al., 2006). Two prominent mechanisms linking inflammation to insulin resistance include the activation of the toll-like receptors, which induce the transcription of inflammatory cytokines such as TNF α and IL-6 (Senn, 2006; Shi et al., 2006) as well as activation of sphingomyelinase (Peraldi et al., 1996). These inflammatory outcomes drive ceramide synthesis (Peraldi et al., 1996; Holland et al., 2011a). This is a potential concern if we consider that ceramide induces inflammation by activating nuclear factor- κ B signaling to promote the expression of TNF α , as demonstrated in RAW 264.7 macrophages (Boon et al., 2013). In the dairy cow, we need to determine whether inflammation promotes ceramide accrual and vice versa. The relative activities and contributions of the various sphingomyelinases (e.g., neutral and acid types in cell membranes and circulation) to postpartum ceramide accruals need to be evaluated within the context of TNF α status. We may need to ensure that approaches that enhance ceramide supply, as a potential means to restore glucose

Figure 6. Summary of ceramide biology. The diagram represents a summary of knowledge in mammals including dairy cattle, rodents, and humans. AKT = protein kinase B; CerS1–6 = ceramide synthases 1 to 6; GLUT4 = glucose transporter-4; LDL = low-density lipoprotein; SMase = sphingomyelinase; SPT = serine palmitoyltransferase; TAG = triacylglycerol; VLDL = very-low-density lipoprotein; FA = fatty acid.

partitioning for milk production (e.g., palmitic acid feeding), do not inadvertently compromise health by provoking inflammation.

Is the FGF21–Adiponectin–Ceramide Axis Uncoupled in the Early-Lactation Dairy Cow?

Fibroblast growth factor 21 and adiponectin are 2 endocrine signals that promote energy expenditure (Steinberg and Kemp, 2007; Owen et al., 2014). As stated previously, the ability of FGF21 to improve glucose homeostasis during obesity involves the stimulation of adiponectin secretion (Holland et al., 2013). In diet-induced obese mice, the insulin-sensitizing effects of FGF21 are likely due to the ability of adiponectin to decrease ceramide accumulation (Holland et al., 2011b, 2013). This was not observed in ad libitum-fed non-pregnant, nonlactating dairy cows intravenously infused a TAG emulsion (Caixeta et al., 2017; Krumm et al., 2017; Rico et al., 2018c). In the early-lactation dairy cow experiencing negative energy balance, circulating FGF21 concentrations are high, whereas adiponectin levels are low (Schoenberg et al., 2011; Giesy et al., 2012). We postulate that suppressed adiponectin secretion during early lactation may contribute to ceramide accrual. Such findings would suggest that FGF21 does not modulate ceramide supply or improve insulin action in early-lactation cows because of unresponsive adiponectin secretion and upregulated ceramide synthesis.

Are Ceramides Predictors of Postpartum Metabolic Disease?

Common periparturient disorders such as FLD, ketosis, retained placenta, and displaced abomasum are associated with exacerbated negative energy balance and uncontrolled lipolysis, which are likely related to the severity of insulin antagonism (Herdt, 1988; Bobe et al., 2004; Roche et al., 2009). Consequently, circulating free FA and BHB concentrations are elevated in Holstein cows that develop periparturient diseases. However, the ability of these metabolites to predict for periparturient diseases and reproductive or lactation success may not be optimal. For example, Ospina et al. (2010) reported a low ability of prepartum serum free FA concentrations to predict postpartum displaced abomasum (sensitivity = 0.57, specificity = 0.62 for a serum free FA threshold of 0.27 mEq/L). The power to predict clinical ketosis and metritis was even lower. Although the predictive power of both free FA and BHB improved when cows were sampled postpartum (i.e., higher sensitivity and specificity), its prognostic usefulness is obviously limited to a very small time window before the manifestation of disease. Although

other studies have explored the use of different lipid biomarkers, including sphingomyelins, for the prediction of periparturient diseases (Hailemariam et al., 2014), limitations in sample size preclude extrapolation and generalization. Importantly, the efficacy of ceramides as predictors for disease, relative to free FA and BHB, has not been studied in dairy cows. This new focus is justified because ceramide has emerged as a novel biomarker for FLD, T2DM, and cardiovascular disease in humans (Laaksonen et al., 2016; Alonso et al., 2017; Hilvo et al., 2018). Although cow-side or laboratory ceramide monitoring may not become a reality, the identification of ceramide as a strong predictor for disease would likely trigger the development of nutritional or pharmacological therapies that modulate ceramide synthesis as a preventive measure to enhance insulin sensitivity, control lipolysis, and support health and longevity.

Does Ceramide Mediate Somatotropin Action in Dairy Cattle?

A key endocrine hormone and homeostatic control of lactation is somatotropin (Bauman and Currie, 1980; Bell and Bauman, 1997). Circulating somatotropin levels are greatest during the copious milk production of early lactation (Koprowski and Tucker, 1973; Rhoads et al., 2004; Carcangiu et al., 2017). Somatotropin enhances basal and epinephrine-stimulated rates of lipolysis by inhibiting the antilipolytic actions of adenosine (Sechen et al., 1990; Lanna et al., 1995; Lanna and Bauman, 1999). The corresponding elevation in circulating free FA develops with the inactivation of skeletal muscle insulin signaling downstream of insulin receptor binding and the reduction in peripheral glucose disposal (Vernon et al., 1990; Wilson et al., 1996; Møller and Jørgensen, 2009). In detail, the ability of somatotropin to inhibit muscle glucose utilization likely involves the inactivation of IRS1, PI3K, and AKT to downregulate the translocation of GLUT4 to the plasma membrane (Rizza et al., 1982; Balage et al., 1997; Thirone et al., 1997). Importantly, these early-lactation outcomes develop with the uncoupling of the somatotropic axis during a catabolic state of negative energy balance (Bauman and Currie, 1980; Gluckman et al., 1987; Rhoads et al., 2004). Specifically, lactation initiates with a decrease in the release of the insulin-sensitizer insulin-like growth factor-I from the liver. Because the ability of somatotropin to promote lipolysis would provide substrate for de novo ceramide synthesis, we hypothesize that somatotropin downregulates peripheral insulin signaling via ceramide-dependent mechanisms in a coordinated adipose–liver–muscle axis. Such a mechanism would link adipose energy reserve

status with the magnitude of skeletal muscle insulin antagonism via the liver. Dietary therapies that decrease ceramide production could be developed as a means to improve insulin sensitivity during early lactation for optimum health. Alternatively, dietary approaches that increase ceramide synthesis could be used to enhance milk production efficiency later in lactation.

CONCLUSIONS

The discussion of lipolysis and nutrient partitioning within the context of lactation now includes a new chapter focused on ceramide. Although our comprehension of sphingolipid biology in the dairy cow was previously limited, scientific breakthroughs support the continued investigation of ceramide. The presumed ability of ceramide to regulate glucose utilization by modulating insulin signaling has major implications for maintaining postpartum health and milk production in dairy cattle. Additionally, the ability to utilize nutrition to modulate ceramide synthesis is a promising approach to control lipolysis, energy partitioning, and milk production in ruminants. The interplay between ceramide with regard to inflammation and endocrine function is also exciting to consider. Because of the growing accessibility of lipidomics technologies that can measure sphingolipids, we should expect the study of sphingolipid biology in the dairy cow to continue. In doing so, discoveries will continue to emerge that may challenge our understanding of bovine lipid biology, metabolic disease, and lactation.

ACKNOWLEDGMENTS

The research described was performed with major support from the Agriculture and Food Research Initiative Competitive Grants 2014-67016-21611 and 2016-67015-24582 from the US Department of Agriculture (USDA) National Institute of Food and Agriculture (Washington, DC), the USDA Northeast Sustainable Agriculture Research and Education Program (GNE13-067), and Hatch Project WVA00661. The authors also express appreciation to F. Brad Hillgartner (West Virginia University, Morgantown) for impactful mentorship and intellectual insight.

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