Advances in determining signaling mechanisms of ceramide and role in disease

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Running Title: Ceramide signaling in disease

The abbreviations used are: FAO, fatty acid oxidation; ER, endoplasmic reticulum; MC, medium chain LC, long chain; VLC, very-long chain; ULC, ultra-long chain; CerS, ceramide synthase; SMase, sphingomyelinase; CRP, ceramide-rich platform; death-inducing signaling complex, DISC; dSTORM, direct stochastic optical reconstruction microscopy; TEM, transmission electron microscopy; CBP, ceramide-binding protein; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; CAPP, ceramide-activated protein phosphatase; pacCer, photoactivable and clickable ceramide; LD, lipid droplet; LAG1, longevity assurance gene 1; AD, Alzheimer’s Disease; HFD, high fat diet; SkM, skeletal muscle; LCM, lipotoxic cardiomyopathy; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; IBD, inflammatory bowel disease; CHOP, C/EBP homologous protein; DSS, dextran sulfate sodium; RAG1, recombination-activating gene 1

This work was supported by NIH grants GM062887, P01CA097132, and Veterans Affairs Merit Award to LMO.
Abstract

Ceramide is a critical bioactive lipid involved in diverse cellular processes. It has been proposed to regulate cellular processes by influencing membrane properties and by directly interacting with effector proteins. Advances over the past decade have improved our understanding of ceramide as a bioactive lipid. Generation and characterization of ceramide metabolizing enzyme knockout mice, development of specific inhibitors and ceramide-specific antibodies, use of advanced microscopy and mass spectrometry, and design of synthetic ceramide derivatives have all provided insight into the signaling mechanisms of ceramide and its implications in disease. As a result, the role of ceramide in biological functions and disease are now better understood, with promise for development of therapeutic strategies to treat ceramide-regulated diseases.

Supplementary Key Words ● Lipid signaling ● Lipidomics ● Membrane/Fluidity ● Receptors/Plasma membrane ● Animal models ● Cancer ● Aging ● Obesity ● Heart Disease ● Multiple Sclerosis
CERAMIDE STRUCTURE AND METABOLISM

Ceramide is a key member of the sphingolipid family, a subset of bioactive lipids that play critical roles in biology. It has been proposed to regulate diverse cellular processes including apoptosis, autophagy, inflammation, fatty acid oxidation (FAO), senescence and Endoplasmic Reticulum (ER) stress, among others (1). Its ability to regulate these processes is thought to be due to its effects on membranes as well as its direct interactions with target effector proteins. Ceramides constitute a family of closely related molecules that are defined by acyl chain length as medium chain (MC) (C12-C14), long chain (LC) (C16-C18), very-long chain (VLC) (C20-C24) and ultra-long chain (ULC) (≥C26). Ceramides are also distinguished by their sphingoid base and saturation state and can be converted to more complex sphingolipid species by addition of phosphocholine, acyl, phosphate and carbohydrates (Fig. 1). Ceramides are generated by de novo synthesis, salvage of sphingosine, and breakdown of complex sphingolipids, including sphingomyelin. The most well-studied group of enzymes responsible for ceramide generation are the Ceramide Synthases (CerS) and Sphingomyelinases (SMase). CerS are a family of six enzymes (CerS1-6), localized primarily in the ER, with each isoform synthesizing a subset of ceramides with partially distinct acyl chain lengths. CerS are involved in the de novo pathway, by generating dihydroceramide from dihydrospingosine and a fatty acid, as well as the salvage pathway, by generating ceramide from sphingosine and a fatty acid. Dihydroceramide is emerging as an important intermediate in the sphingolipid pathway; however, will not be covered in this review. For additional information on dihydroceramide we refer you to a review by Siddique, et al. (2). SMases are a family of enzymes that generate ceramides by hydrolyzing sphingomyelin and are named after their optimal activity pH conditions (acid, neutral, and alkaline SMase) (Fig. 2). Studies focused on these families of enzymes, coupled with advances in technology and methodology, have provided new insight into the role of ceramide in biology and disease. This review highlights more recent findings of MC, LC and VLC ceramide mechanisms of action and role in disease.

CERAMIDE SIGNALING MECHANISMS

Ceramide membrane dynamics
Ceramides possess unique physical properties that are thought to have important effects on membrane dynamics (3). It has been speculated that they form large ceramide-rich platforms (CRPs), which play a role in several cellular processes, including cell death and immune response, by recruiting proteins and inducing receptor clustering (4). SMase-induced accumulation of ceramide in the plasma membrane and visualization by antibody labeling and direct stochastic optical reconstruction microscopy (dSTORM) found that between 50-60% of all ceramide in the plasma membrane localize in CRPs (5). Ceramide-mediated clustering of Fas receptor (CD95) at the plasma membrane was found to be essential for formation of death-inducing signaling complex (DISC) and subsequent caspase activation (6). Ceramides have also been proposed to influence membrane fluidity, which has been suggested to play a role in regulating cell migration (7, 8). The effects of ceramide on membrane fluidity are complex and depend on acyl chain length (9), saturation (10) and ratio of LC and VLC species present in the membrane (11, 12). Furthermore, ceramides may also regulate membrane permeabilization by formation of channels in mitochondrial and lysosomal membranes. Ceramide channels have been visualized in liposomes (13) and lysosome cell extracts (14) using transmission electron microscopy (TEM) and are a proposed mechanism for activation of cell death; however, the existence of these channels is controversial. By evaluating calcein release from liposomes, a recent study found that the effect of ceramide on membrane permeability may be due to its accumulation in one of the two liposome membrane monolayers resulting in a surface area mismatch. This mismatch then causes membrane defects including collapse of vesicles and content release (15). Although the exact mechanism is still being defined, accumulating evidence suggests ceramide can regulate membrane properties that influence biological responses.

**Ceramide-binding proteins (CBPs)**

Ceramides have been proposed to bind proteins both within and independent of membranes. Some of the most well-characterized CBPs are PP1 and PP2A, known together as ceramide-activated protein phosphatases (CAPPs), as well as PKC zeta and cathepsin D (16). CAPPs mediate diverse cellular processes including apoptosis, mitosis, glycogen metabolism and insulin signaling and play key roles in regulating the phosphorylation status of AKT with implications in cancer (17) and insulin resistance (18). In addition
to the traditional CBPs, there is a growing list of putative binding proteins involved in diverse cellular processes (16). Ceramides can influence several mitochondrial processes by binding to proteins, including mitophagy and electron transport. C18-ceramide in mitochondria has been demonstrated to directly interact with LC3BII, anchoring autophagosomes to mitochondria to induce lethal mitophagy (19). In addition, C16-ceramide has been proposed to disrupt FAO and electron transport through inactivation of complexes II and IV of the respiratory chain (20, 21). Interestingly, the absence of ceramides can also disrupt mitochondrial respiration, suggesting ceramide homeostasis may be important for normal mitochondrial function (22).

Novel CBPs are continually being discovered through the development of new experimental tools, including ceramide derivatives (16). Addition of a photoactivatable diazirine group to ceramide allows for cross-linking to interacting proteins. Furthermore, addition of a terminal alkyne group to ceramide enables the use of ‘click chemistry’ to add functional tags such as biotin or fluorophores. Synthesis of bifunctional ceramide derivatives, with both diazirine and alkyne groups, have become powerful tools used to identify and visualize lipid-protein interactions in living cells. By using a photoactivatable and clickable ceramide (pacCer) derivative, a recent study identified StarD7, a protein critical for transfer of phosphatidylcholine to mitochondria, as a novel CBP from a proteome-wide search (23). In addition, to overcome the extreme hydrophobicity of ceramide, water soluble derivatives have been developed. Biotinylated and water-soluble ceramides were used to determined that C16-ceramide binds to the DNA-binding domain of p53 with high affinity (24). As new methods continue to be developed and novel CBPs are identified, the role of ceramide as an effector molecule in biology continues to expand.

CERAMIDE IN DISEASE

Cancer

Dysregulation of ceramide metabolism is an important effector of cancer cell proliferation, growth and survival. Increasing ceramide levels has proved to be important for activating cell death in cancer cells (25, 26). In many cases, cancer cells maintain low levels of ceramides through different mechanisms, such as increased ceramide turnover (27, 28) or storage of ceramide in lipid droplets (LD) (29). The storage of
ceramide in LD is a novel mechanism involving acylceramide generation carried out by an enzyme complex at the ER-LD interface. Through this pathway, cells can control ceramide/acylceramide levels and modify their response to apoptosis inducers, suggesting that manipulation of this novel pathway may have therapeutic implications. In addition, human glioma tissues have significantly lower levels of C18-ceramide relative to normal tissue \((30)\). Increased C18-ceramide levels, by CerS1 overexpression or exogenous C18-ceramide addition, results in the inhibition of cell viability of glioma cells lines. This decrease in viability is associated with activation of ER stress, induction of autophagy and modulation of the PI3K/AKT pathway, suggesting that decreased levels of C18-ceramide impart a growth advantage to glioma cells. Similarly, it was shown that C18-ceramide is selectively down-regulated in head and neck squamous cell carcinoma compared to normal samples, and that increased generation of C18-ceramide inhibited cell growth through the modulation of telomerase activity and induction of apoptosis by mitochondrial dysfunction \((31)\). These results suggest that the maintenance of low levels of ceramide in certain types of cancer is important for the survival of tumor cells and this could be involved in the resistance against different types of chemotherapies.

**Age-Related Disease**

There is accumulating evidence supporting a role for ceramide in aging and age-related diseases, reviewed by Trayssac et al. \((32)\). Cellular senescence is believed to be a key contributor to aging by reducing the capacity of stem cells to proliferate and differentiate as well as through secretion of pro-inflammatory molecules. Our lab discovered that ceramide levels are significantly elevated in replicative senescent cells and possibly contributes to senescence by inducing cell cycle arrest \((33)\). Ceramide was further linked with aging when CerS was determined to be Longevity Assurance Gene 1 (Lag1) \((34)\), whose genetic knockout results in the extension of yeast lifespan by ~50% \((35)\). The closest homolog to Lag1 in human is CerS1, which synthesizes C18-ceramide, implicating CerS1-derived C18-ceramide as a potential contributor to human aging. In yeast, human homologues of Lag1 have preference to generate C26 and C24-ceramides \((34)\), suggesting these may also play a role in aging. Levels of C24-ceramide were subsequently discovered to increase in brain tissue with normal aging and were also found to be elevated in brain of individuals with
Alzheimer’s Disease (AD) (36). Elevated levels of C16 and C24-ceramides in serum are also correlated with increased risk of AD (37) and interestingly, increased levels of C24:1-ceramide were found in extracellular vesicles isolated from serum of aged individuals, which could induce senescence in bone-derived mesenchymal stem cells (38). In addition to the connection of CerS with aging, SMase activity is also implicated. Magnesium-dependent neutral SMase activity was found to be higher in replicative senescent cells compared to normal cells (33) and increases in VLC-ceramides in aged brain are accompanied by decreases in C24-sphingomyelin (36), implicating SMase activity as a possible source of ceramide accumulation in aging. The mechanism by which ceramide contributes to aging is likely multifactorial (32) and effort towards uncovering its source and role is a promising new area of research that may produce therapeutic opportunities to delay or prevent age-related diseases.

**Obesity & Insulin Resistance**

Ceramides are strongly implicated in obesity through their regulation of FAO and insulin signaling. The development of a potent CerS1-specific inhibitor (P053) has uncovered a novel role for C18-ceramide in the regulation of obesity. Treatment of mice with the inhibitor significantly reduced levels of C18-ceramide in skeletal muscle (SkM) and prevented fat deposition in mice fed a high fat diet (HFD). The decrease in fat deposition correlated with an increase in mitochondrial FAO, implicating C18-ceramide as a regulator of FAO (39). This finding is supported by a recent study demonstrating that targeted reduction of C18-ceramide in SkM by CerS1 knockout significantly improves glucose metabolism and protects against insulin resistance (40). Furthermore, it has been proposed that elevated level of C16-ceramide may also contribute to obesity by impairing FAO and disrupting insulin receptor signaling, possibly by disrupting the electron transport chain and modulating plasma membrane fluidity, respectively (11, 20). In support of this idea, separate studies have shown that the knockout of CerS5 or CerS6 in mice significantly improves insulin sensitivity and prevents diet-induced obesity (41, 42). C16 and C18-ceramides are emerging as important mediators of obesity through their regulation of FAO and insulin signaling with promise for future therapeutic treatments.

**Heart Disease**
Ceramides are a known cardiotoxin, contributing to heart disease by inducing cell death and inflammation. Ceramide activates cardiac cell death by accumulating in mitochondria of cardiomyocytes (43), resulting in either the formation of mitochondrial channels or general permeabilization (44). New evidence suggests CBPs and CRPs may also play a role in cardiac cell death. In a lipotoxic cardiomyopathy (LCM) heart model in Drosophila, Annexin X was identified as a putative CBP and a key mediator of caspase activation in LCM. Annexins interact with ceramide in CRPs and may directly activate mitochondrial-mediated apoptosis by transferring ceramide directly to mitochondria (45). The knockout of Annexin X in Drosophila altered the activation of caspases and mitigated negative effects on the heart caused by ceramide accumulation, suggesting CRPs and CBPs may be important mediators of cell death in LCM (46). In addition, a recent study suggests VLC-ceramides may be important for the formation of atherosclerotic lesions. The inhibition and genetic knockout of neutral SMase2 was demonstrated to significantly reduce levels of VLC-ceramides in plasma, which correlated with reduced macrophage infiltration and prevented the formation of atherosclerotic lesions in atherosclerosis-prone apolipoprotein E-deficient mice. The authors proposed this may be due to PP2A mediated alteration of PI3K/AKT signaling, possibly impacting expression of pro-inflammatory genes necessary for cell adhesion and migration (47). This finding is supported by a recent clinical study identifying an elevated ratio of VLC/LC-ceramides in plasma as a possible biomarker for several heart diseases (48). These studies highlight ceramide metabolism as a potential therapeutic target for the treatment of heart disease.

Multiple Sclerosis (MS)

Elevated levels of ceramides have been observed in individuals with MS and in blood cells of experimental autoimmune encephalomyelitis (EAE) mice, a model for MS (49). Through knockout of CerS2 and CerS6 in EAE mice, it has been proposed that the ratio of C16/C24 ceramide at the plasma membrane of neutrophils may regulate membrane properties and thereby the function of receptors and proteins important for cell migration. Increased levels of C16-ceramide and/or decreased levels of C24-ceramide were demonstrated to reduce the migration and activation of neutrophils, while the opposite changes increased their inflammatory response (12, 50). In addition, the knockout of acid SMase was found
to attenuate clinical symptoms of MS in EAE mice; however, in this case the effects were primarily caused by reduced T lymphocyte adhesion (51). This group has proposed that the observed effects may be due to the prevention of CRP formation at the plasma membrane and subsequent reduction of clustering and activation of proteins important for T lymphocyte adhesion to endothelial cells. The role of ceramide in regulating cell migration is emerging as an important mediator of MS. Continued research in this area may provide valuable therapeutic opportunities.

Inflammatory bowel disease (IBD)

Loss of epithelial barrier integrity in the intestine due to ER stress-mediated apoptosis and subsequent increased immune cell infiltration is a key mechanism of IBD. A new study demonstrates that elevated levels of C14-ceramide, generated by Cers5 or CerS6, may contribute to IBD by inducing chronic ER stress and subsequent activation of CHOP-mediated apoptosis. These results were verified in mice fed a diet high in myristate, finding elevated levels of C14-ceramide in intestines along with increased ER stress markers (52). Ceramide is also implicated in IBD through its regulation of immune cell migration into the intestine. In a DSS mouse model of Colitis, several studies have demonstrated that reduced levels of C16-ceramide, by loss of CerS6 or Acid Ceramidase in mice, enhances neutrophil infiltration into the intestine (7, 53). Interestingly, this regulation appears to be cell type specific as decreasing C16-ceramide in CerS6 KO T-lymphocytes was found to protect against Colitis development in an adoptive transfer model in RAG1-deficient mice. This protection may be due in part to a reduction in the ability of T-lymphocytes to migrate; however, it may also be due to decreased clonal expansion (8). Based on these findings it is possible that ceramide accumulation at the plasma membrane may differentially affect the activation of signaling pathways necessary for migration based on receptor availability in each cell type.

CONCLUSION

Ceramide plays key roles in regulating diverse cellular processes. The mechanisms by which ceramide regulates these processes are speculated to be through modulation of membrane properties and/or direct binding of proteins. Dysregulation of ceramide metabolism plays a key role in many diseases.
Continued efforts to explore and define the role of ceramide in cellular processes and disease will enable the development of novel therapeutic strategies.

The authors declare that there are no conflicts of interest with the contents of this article.
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**Fig. 1. Ceramide structure.** Endogenous ceramides are comprised of a sphingoid base with 18 carbons, a 4,5-trans double bond and an acyl chain that ranges from 12 to greater than 26 carbons in length. Ceramides lacking the 4,5-trans double bond are called dihydroceramides and are an important intermediate during *de novo* synthesis. Both the acyl chain and sphingoid base can contain additional double bonds and can also be hydroxylated. Finally, addition of chemical groups to carbon 1 converts ceramide to more complex sphingolipids. This graphic, in black, represents the chemical structure of C16-ceramide containing an 18-carbon sphingoid base with a 4-5-trans double bond, referred to as d18:1/C16:0 ceramide, most commonly called C16-ceramide.
**Fig. 2. Ceramide metabolism.** Ceramide is the centerpiece of the sphingolipid metabolism and can be synthetized by different pathways. The condensation of serine and palmitoyl-CoA initiates *de novo* synthesis pathway (blue box). Ceramide can be generated through the hydrolysis of sphingomyelin by the action of sphingomyelinases (SMase) (green box) or by hydrolysis of other complex sphingolipids (glucosylceramide and galactosylceramide) (yellow box). Different SMase has been identified according to their cation dependence and pH optima of action. Ceramide can be hydrolyzed to sphingosine and then re-acylated back to ceramide in the salvage pathway (orange box). Both the *de novo* synthesis and the salvage pathways involves the action of Ceramide synthase (CerS), six different CerS have been described, each of them has preference for specific acyl chain length and therefore they synthetize a subset of ceramides. Dihydroceramide desaturase (DES); ceramidase (CDase); sphingomyelin synthase (SMS); glucosylceramide synthase (GCS); glucosylceramidase (GCase); ceramide galactosyltransferase (CGT); galactosylceramidase (GalC).
Graphical Abstract