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Johnny Stiban *Editor*

Bioactive Ceramides in Health and Disease

Intertwined Roles of Enigmatic Lipids

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Johnny Stiban
Editor

Bioactive Ceramides in Health and Disease

Intertwined Roles of Enigmatic Lipids

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To my sons, Pierre and Nizar, who have the ability and passion to be future scientists, I dedicate this book. I also dedicate it to my loving wife, Razan, for her continuous and unwavering support of my various "adventures" in editing books, writing other chapters, performing research, and teaching. It has been, and will always be, a tough ride, but worthwhile indeed. Finally, I like to dedicate this book to my beloved institution, Birzeit University, to my colleagues at the Department of Biology Biochemistry, and especially to my past, current, and future students whom I envisage great endeavors coming out of them.

Preface

After a very successful meeting in Puerto Vallarta, Mexico, in October 2017, I was offered to edit a book covering bioactive ceramides. I was thrilled by this opportunity because ceramides and other sphingolipids deserve all the recognition they are receiving due to their multifaceted roles in cellular physiology and pathophysiology. I gathered world-renowned experts in the field of sphingolipid biology to contribute chapters to this book. Thus, different sphingolipids are discussed, and different roles of these lipids are presented. The authors present their ideas clearly and concisely as they give intricate details of recent scientific findings pertaining this subject. This volume is directed to experts in the field as well as newcomers and interested students who would like to venture into this exciting realm of inquisition and discovery. The authors and I have genuinely tried to present our ideas in a direct and vivid manner, and we hope we succeeded in this endeavor.

I would like to acknowledge the extraordinary help I received from my former undergraduate student at the Department of Biology and Biochemistry, and my research team member, Yara Khodour, for her constant and unconditional help she offered to allow this book to come to fruition. She is also the first author on another book chapter I am currently writing, and I could not have done it without her! I am adamant that she will excel in her career in the near future and would become a colleague, with a doctorate soon. My other research team members, Leen Humos, Ruba Shaheen, Tanya Mitwasi, Zainab Hmedan, Muna Abedrabbo, Noor Qasem and Mutaz Alayan, have been extremely helpful in restricting my presence in the lab to focus on writing. I am blessed to have such talented and really dedicated undergraduate students running my lab with wonderful results and impeccability. Last but not least, I would like to express my sincere gratitude to our star technicians at the department, Mr. Rateb Hussein and Mr. Munther Metani, for their steady support to everyone in the department, especially to my research team. Their presence is always helpful and required for the smooth operation of the labs.

Even though they were not actually directly involved in this project, I would also acknowledge the receipt of several university grants to support my ongoing research in the fields of sphingolipid biochemistry. The university has been exemplary in supporting ideas and applications, and for that reason, grants have been awarded to the faculty members to excel and publish. I wish more funding can continue and increase in the coming years.

Ramallah, Palestine — Johnny Stiban

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About the Editor

Johnny Stiban is an associate professor of cell biology and biochemistry at the Department of Biology and Biochemistry, Birzeit University, West Bank, Palestine. He served as the chairperson of the department and the director of the master's program in environmental biology from February 2016 until August 2019. He obtained both his undergraduate degree in biochemistry and his Ph.D. in biology from the University of Maryland, College Park. During his doctoral studies, he was mentored by the highly rated Prof. Marco Colombini. Back in 2006, he was part of the research team that discovered and characterized the ceramide channel, following the seminal work performed by the legendary Dr. Leah Siskind. Together with Prof. Colombini, he published three papers on ceramide metabolism and ceramide channel formation. He later received a prestigious postdoctoral fellowship from the Department of Biological Chemistry at the Weizmann Institute of Science to work under the guidance of world-renowned Prof. Anthony H. Futerman. During his postdoctoral tenure, he worked mostly on ceramide synthase enzymology and published a review article and a book chapter together, as well as five papers where he had contributed. In 2009, he joined the Department of Biology and Biochemistry at Birzeit University as an assistant professor. In 2016, he was promoted to associate professor and then in 2017 was awarded tenure.

Dr. Stiban is still running a respected research lab focusing on ceramide metabolism and apoptosis. His lab currently consists of eight undergraduate students and one graduate student who are working on a variety of projects.

Dr. Stiban was awarded four prestigious awards. In 2012, he received university support to spend the academic year 2012–2013 as a visiting scholar at Michigan State University (MSU) on a career developmental grant. In 2015 and 2017, he received two Zamalah fellowships and spent the summer as a visiting scholar at MSU working with Prof. Laurie S. Kaguni on the biochemistry of mitochondrial DNA helicase. In 2017, he was awarded the Distinguished University Teacher Award from the university president for his teaching excellence and creativity. He was also a recipient of two Erasmus+ Teaching Mobility Awards to teach an advanced course for Ph.D. students at Sapienza University of Rome, Italy, and undergraduate metabolic biochemistry at Aristotle University in Thessaloniki, Greece.

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1 Introduction: Enigmas of Sphingolipids

Johnny Stiban

Abstract

Sphingolipid biology has enjoyed a remarkable rise to fame over the last two decades. Various molecules from this lipid family have been implicated in a variety of cellular functions in health and disease. Ceramides, which constitute the hub of sphingolipid metabolism, are apoptogenic molecules that have many proposed mechanisms of actions. Enigmas revolving around this area of research are slowly being cleared with the advent of better laboratory techniques and data analyses. In this chapter, a general introduction of the topics presented in this book is undertaken highlighting the main ideas of each chapter.

Keywords

Ceramide · Ceramide 1-phosphate · Ceramide channels · Ceramide microdomains · CerS · Disease biomarkers · Inflammation · Ocular health · Sphingolipids

When Thudichum coined the term "sphingolipids" near the end of the nineteenth century, the mysterious Sphinx of the Greek mythology was referenced (Thudichum 1962). Indeed, back then, and for many years to come, sphingolipids were so enigmatic that limited functions were assigned to them. They were merely considered structural components of membranes that complement the glycerophospholipid bilayer. Not until the late 1970s and early 1980s of the twentieth century did researchers start to assign some biological roles to a subset of glycosphingolipids, gangliosides (Aloj et al. 1979; Tsuji et al. 1983; Yavin et al. 1981). Structurally, sphingolipids are diverse; nevertheless they all contain the amino alcohol sphingoid backbone. Either sphingosine or dihydrosphingosine (sphinganine) constitute the base, which is N-acylated to form ceramides or dihydroceramides, respectively. The hydroxyl group on C-1 of the base can be further modified to generate ceramide 1-phosphate, globosides, gangliosides and sphingomyelins (Fahy et al. 2005). Various members of the sphingolipid family possess strong biologic functions in the cell. Ceramide, the parent molecule of sphingolipids, has been shown to induce a plethora of cellular functions. The bioactivity of this group of biomolecules has been implicated in cellular growth, metabolism, senescence, and death (Hannun et al. 1993). Ceramide 1-phosphate and sphingosine 1-phosphate also have important functions in the cell and extracellularly. Sphingosine 1-phosphate has G protein coupled receptors (GPCRs) on the surface of target cells and is a mitogenic substance (Lee et al. 1998).

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In the first part of this book, we focus on the parent sphingolipid, ceramide, and its phosphorylated derivative, ceramide 1-phosphate and discuss their biological activities. Bioactive ceramides function as second messengers (Hannun 1996), protein modulators (Becker et al. 2005) and channel formers (Colombini 2013; Siskind and Colombini 2000). Ceramide 1-phosphate, on the other hand, was shown to be involved in the regulation of adipogenesis (Ordonez et al. 2018), the control of immunological response (Gomez-Munoz et al. 2016) and migration of mesenchymal stem cells to the sites of injury (Yu et al. 2019).

These molecules are discussed in the following chapters and are the main topic of this specialized book. World experts in the field of ceramide and sphingolipid biochemistry and biology have been assembled to discuss specific facets of bioactive ceramide biology. In the final chapters of this book, a global picture of bioactive sphingolipids in health and disease is presented in a concise, yet thorough manner.

In the second chapter of this book, a survey of similar evolutionary origins between mitochondrial lipids, especially ceramides, and the membranes of bacteria is undertaken. The processes of mitochondrial and bacterial apoptosis are discussed in relation to sphingolipid content and utilization. The mechanistic details of ceramide- induced permeabilization of mitochondrial outer membranes to initiate intrinsic apoptosis is discussed in Chap. 3, in which the discoverer of the ceramide channel, Marco Colombini, reviews the accumulating data on these surprising biological structures and presents new exciting findings. In Chap. 4, Anthony H. Futerman and colleagues excellently describe the enzymology of the mammalian ceramide synthase enzyme family and review the evidence that link the structure and function of the various ceramide synthase proteins. The most recent literature on the topic is presented in this chapter along with new figures and analysis. In the following chapter, Charles Chalfant, a world expert in ceramide 1-phosphate biology, outlines and discusses the various roles this molecule plays in inflammation, cellular proliferation, and wound healing. Interesting new findings and enigmatic functions are assigned to ceramide 1-phosphate.

Moving into the biophysics of structured ceramide microdomains in membranes, Silva and colleagues expertly discuss, in details, the varying roles of sphingolipid structured domains in health and disease in Chap. 6. The biophysical nature of ceramide microdomains and their proposed biochemical functions are detailed in this chapter.

Moreover, in the last two chapters, the roles of sphingolipids in diseases, especially in ocular health are discussed. In Chap. 7, the group of Samar Hammad outlines the remarkable prevalence of sphingolipids as biomarkers of disease in general noting different metabolic routes that are encountered in diseased states. New sets of data are presented in this chapter and the authors present them in an elaborate manner. Finally, in Chap. 8, the specific effects of sphingolipids in the various diseases of the eye are presented by Mandal and coworkers.

In summary, whereas sphingolipid research has surged in the last two decades, questions remain and intensify with more knowledge of the subject. This book is intended to illuminate the field of bioactive sphingolipids with new information for both the novice and expert reader. As editor of this book, I extend my sincere gratitude to all authors who contributed chapters and the reviewers for the time spent to write and to edit and critique the work diligently and accurately.

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2 Prokaryotic and Mitochondrial Lipids: A Survey of Evolutionary Origins

Emilia Rappocciolo and Johnny Stiban

Abstract

Mitochondria and bacteria share a myriad of properties since it is believed that the powerhouses of the eukaryotic cell have evolved from a prokaryotic origin. Ribosomal RNA sequences, DNA architecture and metabolism are strikingly similar in these two entities. Proteins and nucleic acids have been a hallmark for comparison between mitochondria and prokaryotes. In this chapter, similarities (and differences) between mitochondrial and prokaryotic membranes are addressed with a focus on structure-function relationship of different lipid classes. In order to be suitable for the theme of the book, a special emphasis is reserved to the effects of bioactive sphingolipids, mainly ceramide, on mitochondrial membranes and their roles in initiating programmed cell death.

Keywords

Membrane lipids · Sphingolipids · Mitochondria · Prokaryotes · Evolution

Abbreviations

BCFA	branched-chain fatty acids
Cer	ceramide
CL	cardiolipin
DAG	diacyl glycerol
DES	DHCer desaturase
DHCer	dihydroceramide
DMPE	dimethyl-PE
ER	endoplasmic reticulum
GDGT	glycerol dibiphytanyl glycerol tetraether
HIF-1α	hypoxia inducible factor
LPS	lipopolysaccharide
MAM	mitochondria-associated membranes
MDOs	membrane-derived oligosaccharides
MICOS	mitochondrial contact site and cristae organizing system
MIM	mitochondria inner membrane
MMPE	monomethyl-PE
MOM	Mitochondrial outer membrane
MOMP	MOM permeabilization
mtDNA	mitochondrial DNA
MUFA	monounsaturated fatty acids
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLs	phospholipids
PUFA	polyunsaturated fatty acids
SLs	sphingolipids
SPT	serine palmitoyltransferase

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2.1 Introduction

Life is a complex phenomenon. Starting from very simple prokaryotic organisms, life has evolved to be more complicated and advanced (Lane 2011). The exact evolutionary origins of eukaryotic cells remain elusive despite mounting evidence that links organelles, particularly mitochondria and chloroplasts, to a prokaryotic origin (Allen 2015; Ku et al. 2015; Muller et al. 2012; Stefano et al. 2015). The links between prokaryotic cells and mitochondria have been postulated as endosymbiotic (Douglas 2014; Lopez-Garcia et al. 2017); a proto-eukaryotic cell engulfing a bacterium which found a safe haven to procreate and thrive while giving the newly-formed cell ample energy in the form of ATP from oxidative phosphorylation (Pittis and Gabaldon 2016). This view was recently modified to what is known as the "*inside out origin*" hypothesis (Baum and Baum 2014) that postulates that membranes were made to cover the prokaryotic cell and make a new cell. Whether each hypothesis is acceptable and correct is beyond the scope of this chapter. The common theme between both theories is the membrane.

Membranes are made of lipids and proteins that are in a continuous dynamic motion. Lipids are enigmatic molecules in cells (Fujimoto and Parton 2011), as they serve a multitude of functions in cellular architecture and physiology. Lipids are characterized by their hydrophobicity. Yet many amphipathic lipids are absolutely essential for the cell, as membranes are made from them. Many lipids were considered to be merely structural with no actual biological role in the cell. This view has changed considerably in the past three decades as more lipids have been proven to be "bioactive" (Bazan et al. 1997; Chiurchiu et al. 2018; Dahinden et al. 1990; Hannun and Obeid 2018; Ioan-Facsinay and Kloppenburg 2018; Mene et al. 1989). Indeed, lipids do have a very important structural role in cells; nevertheless one cannot only categorize their biological functions as such. Lipids have been implicated as signaling molecules in a variety of cellular functions: from regulation of receptor proteins (Coskun et al. 2011) to cell division (Negishi et al. 2016), senescence (Hannun 1996), apoptosis (Abou-Ghali and Stiban 2015; Mathias et al. 1998; Mulkidjanian et al. 2018; Praharaj et al. 2018), mobilization of stem cells (Klyachkin et al. 2014; Nagareddy et al. 2014), and ageing (Das 2018), among a plethora of other important cellular functions (Balla 2013; Magtanong et al. 2016).

Both prokaryotes and mitochondria share similar membranes and compartments. Both mitochondria and bacteria may secrete outer membrane vesicles into the surrounding environments, and this was postulated to be the evolutionary origin of the eukaryotic endomembrane system (Gould et al. 2016). Interactions between lipids forming the plasma membrane in bacteria and eukaryotes have been revealed indicating the evolutionary origins of the eukaryotic cell from bacterial lipids. Both mitochondrial and chloroplast membrane lipids share significant similarities with bacterial membrane lipids (Bansal and Mittal 2015). It is not farfetched to envisage that lipids did move throughout evolution from a type of cell to another, as movement of lipids in the cell from one organelle to another have been implicated in a variety of lipid metabolic routes (Petrungaro and Kornmann 2018; Vance 2014, 2018) and functions (Stiban et al. 2008a). In this chapter, a comparison between lipid content in prokaryotes and mitochondria will be undertaken trying to underline the evolutionary relationships between prokaryotic and eukaryotic cells. Roles of different lipids in mitochondria and prokaryotes will also be addressed to identify possible converging physiological mechanisms within the tree of life.

2.2 Bacterial and Mitochondrial Membranes

The ability of mitochondria to generate ATP for the cell is strictly correlated with their structure (Mannella et al. 2001). Mitochondrial cristae localize proton gradients, concentrate metabolite, and prevent the release of signaling molecules (as in apoptosis) (Munoz-Gomez et al. 2017). The mitochondrial contact site and cristae organizing system (MICOS) is a multi protein complex in

charge of creating both cristae junctions and cristae contact sites at the mitochondrial envelope by combining the functions of its six different subunits. The similarity between the intracytoplasmic membranes of photosynthetic bacteria and cristae was the basis for the homology hypothesis proposed by Stewart and Mattox that suggested that cristae were derived from purple non-sulfur bacteria and had a pre-endosymbiotic origin (Stewart and Mattox 1984). Mic60 is the central and largest protein in MICOS, and together with Mic10, constitutes the core of the complex (John et al. 2005). The major components of MICOS are conserved amongst animal and fungi, Mic60 and Mic10 are consistently identified outside these groups, and a homolog of Mic60 has been isolated in α-proteobacteria confirming the endosymbiotic origin of cristae (Munoz-Gomez et al. 2015).

Photosynthetic bacteria also show an interesting lipid composition. The purple bacterium *Rhodobacter sphaeroides* membrane contains betaine lipid and a glycolipid, while the membrane of the green sulfur bacterium *Chlorobium limicola* contains sphingolipids (Benning 1998).

2.3 Bacterial Lipids

Like all cells, prokaryotic cells are bounded by membranes. Prokaryotic cell membranes carry out the usual function as selective barriers for the exchange of material between the cytoplasm and the environment. In addition, they are the location of proteins involved in communication (receptors) and interaction with other cells and the environment, and, in many cases, also carry out the role of being the site of cellular metabolism (Dowhan and Bogdanov 2002; Huffer et al. 2011). According to the chemiosmotic theory, the production of ATP during respiration and photosynthesis requires the presence of a membrane to generate the gradient of protons necessary for the proton motive force that drives ATP synthesis through the action of ATP synthase (Lane 2017). As the vast majority of prokaryotic cells do not contain internal membranes or organelles that can be dedicated to these functions, cellular respiration and photosynthesis take place on the prokaryotic plasma membrane. It is worth noting that in some cases a number of rare and uncommon organelles have been described in prokaryotic cells (Murat et al. 2010). In particular, the chromatophores of purple photosynthetic bacteria and the thylakoid membranes of cyanobacteria can be considered compartmentalized structures with defined functions (Liu 2016; Noble et al. 2017). The only phylum presenting extensive compartmentalization of the cytoplasmic space is, however, *Planctomycetes* (Fuerst and Sagulenko 2013; Lindsay et al. 2001). In this phylum it is possible to observe the separation of the chromosome through a double lipid-bilayer membrane to form a nuclear body reminiscent of the eukaryotic nucleus (Liu 2016; Murat et al. 2010).

Bacteria and archaea are both prokaryotic cells and they share the characteristic of not having a defined nucleus or a system of internal membranes. These two types of cells belong to two separate domains and there are substantial differences between them. While bacterial membranes are composed mainly of a phospholipid bilayer, archaeal membranes present more variability and unique characteristics (Jain et al. 2014). One major difference between the lipids of archaea and bacteria is the absence of ester bonds between glycerol and fatty acids: Archaea fats contain exclusively ether bonds. Moreover, phospholipids (PLs) are not the only major component of membranes, and a unique type of tetraether monolayer can be found in some archaeal cells, most commonly in those that are adapted to living in extreme environments (Albers et al. 2000; Siliakus et al. 2017).

Classically bacteria are classified as either Gram-positive or Gram-negative according to their coloring after applying the Gram staining technique (Coico 2005). This classification reflects structural differences between the cell walls of the two major types. The Gram-positive bacterial cell wall presents a plasma membrane and a thick peptidoglycan layer that can range between 20 and 80 nm in thickness; on the other hand, the Gram-negative cell wall consists of a plasma membrane, a thin (1.5–10 nm) peptidoglycan layer and an outer membrane

which is in direct contact with the environment (Mai-Prochnow et al. 2016). The outer membrane contains lipids that are exclusive to the Gram-negatives such as Lipid A and lipopolysaccharide (LPS). Another major group of Bacteria, the Mycobacteria, has a cell wall that does not fit into the Gram-positive or negative characterization and contains mycolic acid (Jankute et al. 2015).

For many years it was thought that bacterial lipids were limited in kind and metabolism. In addition to the well known and understood lipids of *Escherichia coli*, it is now evident that bacterial membrane composition is not only variable within species, but also depends on environmental conditions to which cells are exposed (Sohlenkamp and Geiger 2016).

E. coli has been regarded as the model organism for the study of the Gram-negative proteobacteria and much is known about lipid metabolism and membrane composition of this organism. The predominant lipid in *E. coli* membranes is phosphatidylethanolamine (PE) which constitutes about 75% of membrane lipids composition. In addition about 20% of the membrane is composed of phosphatidylglycerol (PG) and cardiolipin (CL) (Sohlenkamp and Geiger 2016). When compared with other bacteria it is now accepted that many variations in lipid composition exist. In many cases the variations are limited to modifications of PE like monomethyl-PE (MMPE) or dimethyl-PE (DMPE) (Sohlenkamp and Geiger 2016).

Moreover, the Gram-positive and Gram-negative bacteria membrane contains several enzymes including aminoacyl-phosphatidylglycerol synthases that can add amino acids to the polar heads of PG within the membrane using aminoacetylated tRNA as donor molecules (Roy et al. 2009). In some cases the amino acid modification changes the overall negative charge of the phosphate head to a positive charge with implications regarding the ability to resist antimicrobial peptides. Lysine (LysPG), alanine (AlaPG) or arginine (ArgPG) have been described (Sohlenkamp and Geiger 2016). In *Listeria monocytogenes* (a foodborne Gram-positive gastrointestinal pathogen) concentrations of LysPG are correlated to osmotic stress, growth temperature, and growth phase (Dare et al. 2014). These findings suggest a physiological role for these modifications beyond conferring antimicrobial peptide resistance. Modifications of the lipid composition and overall membrane charge have the potential of affecting the activity of membrane-associated proteins (Roy 2009; Roy et al. 2009). In *Staphylococcus aureus* LysPG may act as regulator of the cell cycle by controlling the number of replication origins per cell (Ichihashi et al. 2003).

Some bacteria are able to synthesize phosphatidylinositol (PI) which, in some cases can be modified with mannose residues and fatty acid residues to form acyl-phosphatidylinositol mannosides that can be further metabolized to form lipomannan and lipoarabinomannan. PI is an essential lipid at least in *Mycobacterium tuberculosis* (Jackson et al. 2000). In addition, bacteria produce lipids that lack phosphorus such as ornithine lipids (OL) and sulfoquinovosyl diacylglycerol (Sohlenkamp and Geiger 2016). An extensive analysis of bacterial lipids composition and metabolism can be found in the 2016 review by Sohlenkamp and Geiger (2016). Table 2.1 summarizes the findings of several research groups on the lipid composition of prokaryotic cells.

2.3.1 Environmental Factors

It is generally accepted that environmental conditions can influence lipid composition of membranes and that the concentration of PLs can also vary in response to growth phase; environmental factors such as pH, osmolarity, salinity, and the presence of organic solvents can have an impact on the relative amount of PLs found in the membrane (T. Y. Lin and Weibel 2016). In particular, CL seems to be strictly regulated according to the phase of growth and can increase by 200% as cells enter the stationary phase compared to cells in the logarithmic phase. CL content is regulated by the activity of CL synthase (Cls) with a feedback regulation mechanism during cellular growth in the absence of stress (T. Y. Lin and Weibel 2016).

Table 2.1 Plasma lipid composition in representative prokaryotic cells

Type of prokaryotic cell	Predominant lipid(s)	Reference
Gram-positive		
S. aureus	PG, CL, LPG, GPL	Sohlenkamp and Geiger (2016)
B. subtilis	PG, CL, PE, LPG, GL, GPL	Sohlenkamp and Geiger (2016)
L. monocytogenes	PG, CL, LPG, LCL, PI	Sohlenkamp and Geiger (2016)
Nocardia sp.	CL, PE, PI, PIM, OL, SQD, HOP	Sohlenkamp and Geiger (2016)
Gram-negative		
E. coli	PG, CL, PE	Sohlenkamp and Geiger (2016)
P. aeruginosa	PG, CL, PE, PC, OL, APG	Sohlenkamp and Geiger (2016)
R. tropici	PG, CL, PE, MMPE, DMPE, PC, OL, S2, P1, P2, DGTS, LPG	Sohlenkamp and Geiger (2016)
Mycobacteria		
M. tuberculosis	PG, CL, PE, PI, PIM, OL	Sohlenkamp and Geiger (2016)
M. leprae	PDIM, PGL, LAM	Kaur and Kaur (2017)
Archaea		
Halobacteriales	Archaeol, Extended archaeol	Villanueva et al. (2014)
Thermoplasmatales	GDGT-0, GDGT-1–4 GDGT-5–8	Villanueva et al. (2014)
Thermoproteales	GDGT-0, GDGT-1–4 GDGT-5–8	Villanueva et al. (2014)

Abbreviations used: *PG* phosphatidylglycerol, *CL* cardiolipin, *PE* phosphatidylethanolamine, *GPL* glycophospholipid, *LPG* lysyl-phosphatidylglycerol, *OL* ornithine lipid, *PI* phosphatidylinositol, *GL* glycolipid, *LCL* lysyl-cardiolipin, *APG* alanyl-phosphatidylglycerol, *MMPE* monomethyl PE, *DMPE* dimethyl PE, *S2* OL hydroxylated in ornithine headgroup, *P1* OL hydroxylated in the 2-position of the ester-bound fatty acid, *P2* OL hydroxylated in ornithine headgroup and in 2-position of ester-bound fatty acid, *DGTS* diacylglyceryl-N,N,N-trimethylhomoserine, *PIM* phosphatidylinositol mannoside, *SQD* sulfolipid sulfoquinovosyl diacylglycerol, *HOP* hopanoid, *PDIM* phthiocerol dimycocerosat, *LAM* lipoarabinomannan, *PGL* Phenolic glycolipid, GDGT glycerol dibiphytanyl glycerol tetraether (with 0 to 8 0–8 cyclopentane moieties)

Lipid metabolism and membrane lipids relative composition can change in bacteria in response to rapid changes in the environment. A molecular thermosensor of *Bacillus subtilis* can induce the expression of a PL acyl desaturase that inserts a *cis* double bond in pre-existing PLs as a response to decreased temperatures (Aguilar et al. 2001), thus preserving membrane fluidity. Response to acid stress in *E. coli* results in the transformation of pre-existing unsaturated fatty acids to cyclopropane fatty acids. This change is observed also as a physiological change during the growth phase. This modification starts at the beginning of stationary phase and is carried out until all the unsaturated fatty acids are modified. Such change is irreversible and if the cell re-enters a logarithmic growth phase the content of cyclopropane fatty acids needs to be diluted by *de novo* synthesis of *cis*-unsaturated fatty acids (Y. M. Zhang and Rock 2008).

Bacteria that have adapted to living in environments in which the majority of organisms would die (extreme environments), have permanent modifications of their membrane lipids and in some cases, require the presence of unique lipids. These lipids can vary from long polyunsaturated fatty acids (PUFAs) to tetraester or tetraether lipids. The following sections describe adaptations to such environmental conditions.

2.3.1.1 Pressure

Extreme barophilic bacteria (bacteria that require pressures of at least 50 MPa for growth and that are able to grow well at 100 MPa) have been shown to synthesize long PUFAs C22:6 (DHA) and C20:5 (EPA) as a mechanism to adapt to the low temperature and high hydrostatic pressure of deep sea environments (Fang et al. 2000). PUFAs could reduce the transition temperature and maintain membrane fluidity under low temperature and high pressure conditions (DeLong and Yayanos 1985; Fang et al. 2000). The bacterial origin of long PUFAs under high pressure conditions suggests that bacteria can be an important

source of C 20:5 and C22:6 to deep sea sediments.

2.3.1.2 Temperature

Hyperthermophilic bacteria also need the synthesis of special lipids to support growth at high temperatures. The bacterium *Thermotoga maritima* grows at temperatures as high as 90 °C and contains a novel glycerol ether lipid containing 15,16-dimethyltriacontanedioic acid (Damste et al. 2007). It is interesting to note that at high temperatures bacteria show the presence of ether bonding which is usually considered an exclusive of archaeal lipids. In fact, it has been postulated that above 80 °C membranes that are composed exclusively of ester lipids are not stable, and that ether bonds are essential for hyperthermophilic growth (Siliakus et al. 2017).

Often extremophiles that grow at high temperatures present a monolayer rather than bilayer membrane (Siliakus et al. 2017); the tetraester and tetraether lipids found in bacteria adapted to life at high temperatures are similar to the tetraether lipids found in archaea and they are believed to be the result of a tail-to-tail condensation between fatty acids that originally belonged to bilayers (Fitz and Arigoni 1992). Tetraester lipids are found in *Thermoanaerobacter ethanolicus* (T_{max} = 78 °C) amongst others, while diether fatty acids are found in *Aquifex pyrophilus* (T_{max} = 95 °C) and *Thermodesulfobacterium commune* (T_{max} = 85 °C) (Siliakus et al. 2017).

Membrane proteins depend on their surrounding lipids to maintain correct folding and functionality (Findlay and Booth 2006). Considering the broad variation in membrane lipids and the relative stability of membrane proteins it is interesting to note that even profound variations as the ones seen in hyperthermophiles allow for protein based membrane physiological processes to be maintained. In the archeaon *Archaeoglobus fulgidus* the modifications to the membrane as adaptation to high temperatures are considerable and the membrane of this organism is mainly composed of ether-linked diglycerides of either conventional (diether) or cyclical tetraether architecture (Lai et al. 2008); comparison between the ammonium transporter Amt-1 of this archaeon with the one of *E .coli* shows remarkable similarity at the structural level. The same can be said for an aquaporin protein which shares 30% sequence similarity with aquaporin AqpZ of *E. coli* but presents very similar three dimensional structure (Sanders and Mittendorf 2011). When comparing crystal structures rather than amino acid or nucleotide sequences, in the hyperthermophilic bacterium *Thermus thermophilus* HB27, the three dimensional shape of a beta-barrel outer membrane protein TtoA, shows no obvious structural differences from mesophilic beta-barrel outer membrane proteins (Sanders and Mittendorf 2011) indicating once more that the modified surrounding lipids still retain the ability to allow correct folding and functionality of membrane proteins.

Archaea are mostly better adapted at living in high temperature environments compared to bacteria. The highest recorded maximum temperature for growth in bacteria is 100 °C for *Geothermobacterium ferrireducens* (Kashefi et al. 2002); the record for archaea is 122 °C in *Methanopyrus kandleri* when subjected to hydrostatic pressure of 40 MPa (Takai et al. 2008). It is therefore interesting to explore the reasons behind this difference and what it is that makes archaea that much better at maintaining their membrane in the liquid crystalline phase at temperatures significantly higher than the temperature of boiling water. Rather than assigning specific single lipids an intrinsic ability to resist high temperatures and therefore confer hypothermophilic characteristics to the organism, it has been proposed that archaeal lipids (isoprenoid ether lipids) in the context of the membrane provide the cell with a membrane that is in a low permeability liquid crystalline state throughout the possible growth temperature range (0–100 °C) and therefore allow these cells to grow and divide at all possible range of temperature without having to regulate lipid composition or concentration (Koga 2012).

Similarly psychrophiles (bacteria that grow at low temperatures, in some cases below the temperature at which water freezes) need to modify their membrane to adapt, and the modifications that are commonly observed are membranes with unsaturated fatty acids, short

chain fatty acids, branched-chain fatty acids (BCFAs), carotenoids, and glycolipids (Siliakus et al. 2017). The most prevalent modification found in the psychrophiles is the presence of monounsaturated fatty acids (MUFAs) that result from the activity of desaturases. In some Gram-positive (e.g. *B. subtilis*) as well as in some Gram-negative bacteria, the iso configuration of BCFAs (iso-BCFA) is modified to anteiso-BCFAs. The anteiso position of methyl groups in the latter causes greater fluidity of the membrane (Siliakus et al. 2017).

2.3.1.3 Hypersalinity

Halophiles and extreme halophiles grow at concentrations of salt that would kill the majority of microorganisms. The general principle that solute concentrations in the cytoplasm should be at least isoosmotic with the environment applies to these cells, but, like other microorganisms, the ability to increase cytoplasmic solute concentration to reach hypertonicity when compared to the environment, is the preferred option (Gunde-Cimerman et al. 2018). To maintain high osmotic pressure, prokaryotic cells can follow a number of strategies such as accumulation of inorganic salts (mainly KCl), or synthesis of organic compounds (compatible solutes) including polyols, sugars, amino acids, and betaines among others (Gunde-Cimerman et al. 2018).

Soil microorganisms are often facing dramatic changes in osmolarity due to fluctuations of water in soil during different seasons. The soil bacterium *B. subtilis* can tolerate changes in osmotic pressure by synthesizing the compatible solute glycine betaine. The precursor for glycine betaine is choline, which must be obtained from exogenous sources (Nau-Wagner et al. 2012; Wood et al. 2001). OpuB and OpuC are two high affinity choline transporters that are regulated by osmosis. In addition to synthesis of glycine betaine *B. subtilis* membranes contain multiple transport systems that have overlapping substrate specificity which can take up these osmoprotectants (Wood et al. 2001).

Salinibacter ruber on the other hand, is an extreme halophilic bacterium that grows optimally at salt concentrations between 200 and 300 g/L and requires a minimum concentration of salt of 150 g/L for growth. This bacterium prefers the salt-in option to the synthesis of compatible solutes and uses KCl for osmotic stabilization. The energy required for the pumps that move ions across the membrane is provided by the proton motive force generated by a bacteriorhodopsin-like retinal pigment that uses the energy provided by light to pump H^+ ions across the membrane (Oren 2013). CL is present in high concentrations (20%) in what might be an adaptation to high salt concentrations. In addition to increased concentrations of CL *Salinibacter* contains unusual sulfonolipids named acylhalocapnines (Baronio et al. 2010; Oren 2013).

In general, it is safe to say that the lipid composition of halophilic bacteria is not the most important factor for establishing adaptation. In *E. coli* (a normosmotic bacterium) stabilization of the cell against changes in osmolarity appears to be a function of membrane-derived oligosaccharides (MDOs) that changes the osmolarity of the periplasm (Kennedy 1982). The lipid byproduct of MDO synthesis is diacylglycerol (DAG) which could be involved in a signaling pathway analogous to that of PI in eukaryotes that, in turn, could regulate the response to changes in osmolarity by transcription of the genes encoding other cell wall proteins, including OsmB, OsmC, OsmE, and OsmY. Both OsmB and OsmE are believed to be lipoproteins. These proteins may be involved in osmoregulatory cell wall remodeling as structural elements and/or as enzymes (Wood 1999).

2.3.1.4 pH

Prokaryotic cells have also evolved mechanisms that allow them to grow in environments with pH values above or below neutrality. Some cells can simply tolerate and acclimatize to changes in pH. In other cases, conditions of low or high pH values are required for growth (Lund et al. 2014; X. Zhang et al. 2017). It is worth mentioning that prokaryotes often use proton gradients as a source of energy and protons are pumped across the cell membrane either as part of respiratory metabolism or using light in photosynthetic metabolism.

A bacterium that gained notoriety for growing in the low pH environment of the stomach is *Helicobacter pylori*. This bacterium is well adapted to the low acidity of the stomach through the secretion of urease enzyme that produces large amounts of ammonia, which is then protonated to create ammonium, thus creating a neutral bacterial microniche (Goodwin et al. 1986; Abadi 2017). Another important strategy that allows this bacterium to establish itself in the mucosa of the stomach is its helical shape that allows it to penetrate the mucous layers covering the stomach (Abadi 2017). However, so far there are no reports linking specific lipids to the ability of this bacterium to adapt to the stomach low pH.

Acidophiles (cells that require relatively low pH values for growth) alter the expression of certain membrane proteins (proton pumps) to maintain a cytoplasmic neutral pH, which results in reversal of their membrane potential. In addition to this strategy, changes in the membrane lipids can be put into place to modify membrane permeability (Siliakus et al. 2017). In a study of acid resistance in pathogenic *E. coli* O157:H7 growth at low pH induced an increase in palmitic acid (C16:0) and a decrease in *cis*-vaccenic acid (C18:1 Δ^{11}) indicating a preference to saturated rather than unsaturated fatty acids in response to acidic environment (Yuk and Marshall 2004). This adaptation allows *E. coli* to establish itself in the digestive tract where it can cause serious diseases such as diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans (Nguyen and Sperandio 2012). Changes in membrane lipids composition in response to acidic stress seems to be variable in nature. *Streptococcus mutans* (one of the major causes of caries and the major component of plaque in the oral cavity) increases the proportion of MUFAs in the plasma membrane together with a shift to longer carbon chains in the membrane fatty acids, in response to growth in acidic pH (Baker et al. 2017). CL also seems to play a critical role in the ability of *S. mutans* to respond to acid stress and deletion of Cls produces a more acid-sensitive strain with lower percentage of unsaturated fatty acids (Baker et al. 2017).

Enterococcus faecalis (a commensal human bacterium) has the ability to obtain exogenous fatty acids from external sources like bile and serum. The incorporation in the plasma membrane of oleic acid (C18:1 Δ^{9}) and linoleic acid (C18:2 $\Delta^{9,12}$) could confer resistance to stress (Saito et al. 2014). It is worth mentioning that even though this bacterium has been considered for a long time a harmless commensal member of the intestinal microbiome, it is quickly becoming a serious concern for human disease as well (Fisher and Phillips 2009). A further adaptation example is the one of *Listeria monocytogenes* which adapts to acidity stress by increasing anteiso- and decreasing iso-BCFAs (Siliakus et al. 2017).

Extreme acidophilic bacteria (bacteria that tolerate pH lower than 2.5) of which *Alicyclobacillus* (*Bacillus*) *acidocaldarius* is an example, show a more consistent pattern of lipid membrane adaptations. MUFAs and saturated fatty acids, high levels of iso- and anteiso-BCFAs, uncommon β-hydroxy, ω-hydroxy, and cyclopropane fatty acids are standard modifications in these bacteria. A widespread presence of iso-diabolic acid lipids was also detected in species of the phylum *Acidobacteria*. So far, no bacteria have been isolated that grow at pH lower than 1, while there are examples of archaea with optimum pH of 0.7 (Siliakus et al. 2017). The archaeon *Picrophilus torridus* showed enrichment of glycerol dibiphytanyl glycerol tetraether (GDGT) with 4, 5, and 6 cyclopentyl rings and depletion of GDGT with 1, 2, and 3 cyclopentyl rings in cells undergoing pH or thermal stress relative to those grown under optimal conditions suggesting that GDGT composition may be a physiological response to acidic and temperature stress (Feyhl-Buska et al. 2016).

Organic acids, generally considered weak acids, play an important role in food microbiology as preservatives and are found in fruits and vegetables where they play a protective role against bacterial contamination and spoilage. However, it is possible for bacteria to become accustomed to the presence of these acids and to acquire tolerance and the ability to grow in their presence (Hirshfield et al. 2003; H. Yu et al. 2010). Both *E. coli* and *Salmonella enterica* show the ability to grow in acidic media if they are pre-exposed to mildly acidic conditions. This

phenomenon is referred to as the Acid Tolerance Response and it results in new protein synthesis as well as modifications of the membrane similar to the ones described above (Baik et al. 1996; Hirshfield et al. 2003). In particular, an increase in cyclopropane fatty acids with an accumulation of C17 and C19 odd-chain fatty acids can be observed after habituation to acidic conditions (Hirshfield et al. 2003).

On the other hand, alkaliphiles present bis-monoacylglycerophosphate lipids and CL in high concentrations. Their membranes can also contain large amounts of both iso- and anteiso-BCFAs; and it is often possible to identify the presence of MUFAs. Characteristically these cells present squalene, tetrahydrosqualenes and other polyisoprenes (Siliakus et al. 2017).

2.3.2 The Gram-Negative Outer Membrane

Gram-negative outer membrane is a bilayer consisting of PLs (innermost layer) and LPS forming the outermost layer (Beveridge 1999). The Lipid A moiety of LPS forms the hydrophobic core; Lipid A is bound through 2-keto-deoxyoctanoate to a core polysaccharide (Ladner-Keay et al. 2016). The outer polysaccharide is known as O antigen and consists of up to 25 repeating units of 3–5 sugars. The combination of Lipid A and the polysaccharides confers the molecule amphipathic properties. While lipid A is required for growth, the O antigen and core sugars are not but afford the bacteria protection from antibiotics and complement-mediated lysis (Bos et al. 2007; Raetz et al. 2007). Lipid A is a unique and distinctive phosphoglycolipid. It contains glucosamine residues, which are present as β(1 → 6)-linked dimers. The disaccharide contains α-glycosidic and non-glycosidic phosphoryl groups in the 1 and 4′ positions, and (*R*)-3-hydroxy fatty acids at positions O-2, O-3, O-2′ and O-3′ in ester and amide linkages, of which two are usually further acylated at their 3-hydroxyl group. Changes in growth conditions and environmental stresses can induce a response that results in the modification of Lipid A (Raetz et al. 2007).

Cationic antimicrobial peptides can induce the modification of lipid A either by acylation or addition of an aminoarabinose moiety to increase resistance in *Salmonella typhimurium* (Bader et al. 2005; Otto 2009). Hexa acylated *E. coli* Lipid A with side chains of 12–14 carbons in length induces a robust immune response in humans, while a change to the length of the attached fatty acids can reduce the immune response considerably. Lipid A chains with only four or five acyl chains confer the bacteria the ability to hide from the immune system increasing their virulence and pathogenicity (Miller et al. 2005).

H. pylori is a Gram-negative bacterium that colonizes the gastric mucosa of humans, can cause recurrent gastroduodenal inflammatory disease, and is the primary cause of chronic gastritis and peptic ulcers in humans (Blaser 1993). *H. pylori* persists in the gastric mucosa thanks to modifications to its LPS that allow it to minimize host defenses. In particular, *H. pylori* presents tetracylated Lipid A with 16-carbon and 18-carbon fatty acids that is poorly recognized by Toll-Like Receptors, rendering the innate immunity response much weaker than required to remove the pathogen (Miller et al. 2005). The O side chain of some *H. pylori* strains have been shown to mimic Lewis^{X} and Lewis^{Y} blood groups antigen giving the bacteria the ability to camouflage further diminishing the immune response (Moran et al. 1997). Interestingly key enzymes in Lipid A biosynthesis were found to be targeted to mitochondria in the plant *Arabidopsis thaliana* although the structure of the final Lipid A molecule remains undetermined (C. Li et al. 2011).

In the case of *Pseudomonas aeruginosa* colonization of patients with cystic fibrosis, a fixed mutation induces highly modified Lipid A with aminoarabinose and fatty acids side chains (Maldonado et al. 2016; Miller et al. 2005). Besides having a role in pathogenicity and virulence, modifications of LPS have also been linked to the ability of extremophiles to adapt to extreme environmental conditions. A novel type of Lipid A that contains D-galacturonic acid instead of phosphate residues has been isolated from *Aquifex pyrophilus*, a hyperthermophile that

grows at temperatures as high as 95 °C (Plotz et al. 2000).

2.3.3 Common, and Uncommon, Lipids in Bacteria

2.3.3.1 Hopanoids and Sterols

Membranes fluidity is highly influenced by the ratio of saturated to unsaturated fatty acids, and the molecular order of membrane lipids influences functionality (Pinto et al. 2014; Stiban et al. 2008b). Besides PLs and their variants, sterols also play a fundamental role in maintaining this order, and in eukaryotes, they are also involved in intra- and intercellular signaling (Desmond and Gribaldo 2009). Bacteria contain hopanoids which provide a similar structure and function in this domain.

Both steroids and hopanoids derive from the universal precursor isopentyl diphosphate that is metabolized to squalene and its cyclization products. For the synthesis of sterols the metabolism of squalene is very demanding in oxygen and up to 11 molecules of oxygen are required for the synthesis of one molecule of cholesterol (Summons et al. 2006). Bacterial squalene-hopene cyclases, on the other hand, produce hopanoids without a requirement for molecular oxygen. It has been proposed that hopanoids are the ancestor molecules of sterols and that the pathway of sterol biosynthesis appeared after the emergence of oxygenation of the atmosphere and oceans (Summons et al. 2006). Hopanoids are one of the most common lipids in sedimentary rocks and can be used to predict the presence of microbial ancient life (Ourisson and Albrecht 1992), which provides a solid foundation that hopanoids are molecular predecessors of sterols. The function of hopanoids as molecular stabilizers of membrane *in vivo* has been shown in the Gram-negative bacterium *Methylobacterium extorquens*, a plant associated hopanoid producer. In this bacterium, hopanoids have been shown to interact in a preferential way with glycolipids of the outer membrane to produce highly ordered membrane domains (Saenz et al. 2015). Stress like high temperatures, low pH, high osmotic pressures, and antibiotic or detergent treatments have an influence on the relative amounts of hopanoids in the bacterial membrane; the 2-methyl-hopanoids seem to be the modification of preference for pH stress. High temperature adaptations seem to require hopanoids with elongated side chains (Poalla et al. 1984). The cyanobacterium *Nostoc punctiforme* forms spore-like structures called akinetes under low light or phosphate starvation, allowing it to survive cold and desiccation. These spores, like the spores of *Streptomyces coelicolor*, have an increased concentration of hopanoids (Belin et al. 2018).

Nevertheless, the generally made assumption that bacteria do not produce sterols is somehow misguided. Even though the presence of sterols in bacteria seems to be relatively limited, sterols have been reported in bacterial membranes as early as 1976 in *Methylococcus capsulatus* (Bouvier et al. 1976).

M. capsulatus produces modified lanosterol products and the presence of similar sterols has been demonstrated in other aerobic methanotrophs of the *Methylococcales* order within the γ-proteobacteria. In addition, sterol biosynthesis has also been observed in a few myxobacteria of the δ-proteobacteria and the planctomycete *Gemmata obscuriglobus* (Wei et al. 2016). A bioinformatics-based research on the presence of oxidosqualene cyclase, a key enzyme in the biosynthesis of sterols, predicts its presence in 34 bacterial genomes from 5 phyla and in 176 metagenomes, indicating that the presence of sterols in bacteria might be much more widespread than previously predicted (Wei et al. 2016).

2.3.3.2 Sphingolipids and Lipid Rafts

Sphingolipids (SLs) are structural components of the membrane and play significant roles in eukaryotic cell signaling (Hannun 1996; Hannun et al. 1993). They are acylated derivatives of the amino alcohol sphingosine (Hannun et al. 1993; Olsen 2001; Stiban et al. 2008b). SLs are present in bacterial membranes and their role and

characterization is still poorly understood. SLs occurrence seems to be more common in anaerobes (LaBach and White 1969), even though their presence is not restricted to this group of bacteria (A. S. B. Olsen and Faergeman 2017; Olsen 2001). The Gram-negative genus *Sphingomonas* contains glycosphingolipids rather than LPS in its outer membrane (Kawasaki et al. 1994). The genus *Bacteroides* show heterogeneity in the composition of SLs and contains ceramides (Cer), Cer phosphoethanolamines, glucosyl sphinganine and Cer phosphoinositols (I. J. Olsen 2001). The list of bacteria containing SLs is growing and not restricted to the phylum *Bacteroidetes* (Kato et al. 1995; Miyagawa et al. 1979; Nichols et al. 2004).

In eukaryotic cells lipid rafts are defined as membrane regions with rich content of sterols and SLs that play specific roles including adhesion, migration, and membrane transport (Stiban et al. 2008b). In bacteria, the presence of functional membrane microdomains was unexpectedly discovered in *B. subtilis* while studying the ability of this bacterium to form biofilms (Lopez et al. 2009). These membrane regions are particularly compact and contain high concentrations of hopanoids which make protein movement more difficult and result in the trapping of proteins with specific functions. These proteins are normally defined as cargo proteins. There is convincing evidence that the lipids are associated with bacterial flotillins in strong similarity with the structure of lipid rafts in eukaryotic cells (Bramkamp and Lopez 2015). In *B. subtilis* lipid rafts have been associated with signaling that can result in cell division and cell differentiation (Bramkamp and Lopez 2015) similar to the roles of lipid rafts in eukaryotes.

Some bacteria have the SL biosynthetic machinery, yet others that do not synthesize SLs may scavenge these lipids from the host to increase their virulence (An et al. 2011). In the case of *Chlamidya trachomatis* (the causal agent of trachoma) the acquisition of SLs from the host prevents fusion of the endosome of macrophages with the lysosome protecting the bacterium from the lytic activity of hydrolytic enzymes (van Ooij et al. 2000). *Mycobacterium tuberculosis* also uses host SLs to influence the level of Ca^{2+} in the cytoplasm to control the maturation of the endosome (Heung et al. 2006).

2.3.3.3 Ladderanes

Planctomycetes are a relatively recent discovery in microbiology (Kuenen 2008). Even though the presence of a microbe able to oxidize ammonia under anaerobic conditions was predicted as early as 1977 (Broda 1977), the actual isolation of this group of organisms took place in the nineties (Kuenen 2008). This group of bacteria is quite unique from several perspectives:

Recent revisions of the tree of life based on ribosomal RNA sequences place the *Planctomycetales* deep at the base of the tree as the first branching bacterial group (Brochier and Philippe 2002) even though it has been argued that *Planctomycetes* belong to a super phylum that includes the *Verrucomicrobia* and the *Chlamydia* (Wagner and Horn 2006).

The cell wall lacks peptidoglycan, a defining component of bacterial cell walls. It appears to be more similar to the cell wall of archaea rather than bacterial cells (Fuerst 2005). Despite the absence of peptidoglycan, recent sequencing of the genome of *Kuenenia stuttgartiensis* revealed the presence of a gene cluster for proteins involved in peptidoglycan synthesis even though they might not be active (Kartal et al. 2013).

Possibly the most interesting feature unique to these bacteria is the lipid composition of the anammoxosome compartment. This organelle is dedicated to the metabolism of ammonia that generates highly toxic intermediates such as hydrazine (N_2H_4) and hydroxylamine (NH_2OH) (Sinninghe Damste et al. 2005). The anammoxosome membrane consists mostly of the highly unusual lipids known as ladderanes. Ladderanes contain one or both of two different ring systems: either three cyclobutane moieties and one cyclobutane moiety substituted with an octyl chain, or five linearly concatenated cyclobutane rings substituted with a heptyl chain. In both systems the rings are fused by *cis*-ring junctions which gives the structure an overall staircase-like arrangement (van Niftrik and Jetten

2012). This is a unique set of lipids that is not seen anywhere else to our knowledge.

2.4 Lipids in Mitochondria

In contrast to most prokaryotes, eukaryotic cells contain internal membranes, including the energy-producing cellular powerhouses, the mitochondria. The uniqueness of mitochondria in cellular physiology is a result of the complex roles they perform in cells. Mitochondria are implicated in cell survival and cell death, among other vital cellular processes (Birbes et al. 2002). They contain two independent genetic systems, as most of their components are synthesized outside and imported into mitochondria (Sickmann et al. 2003). Mitochondrial DNA (mtDNA) is responsible for the synthesis of very specific subset of proteins and RNA molecules in the organelle (Stiban et al. 2016). The roles that mtDNA (which is mostly maternally inherited) play in mitochondrial physiology are well documented and several diseases have been identified due to aberrant mitochondrial genetics (El-Hattab et al. 2017; Stiban et al. 2014, 2016; Viscomi and Zeviani 2017). mtDNA is located in the matrix which is shielded by the cristae of the mitochondrial inner membrane (MIM). Mitochondria are surrounded by two membranes, which are structurally and functionally very different. While the mitochondrial outer membrane (MOM) is permeable to solutes under 5 kDa in size (Mannella 1992; Vander Heiden et al. 2000), the MIM is mostly impermeable (Carbonera and Azzone 1988). The protein:lipid ratio of the MIM is the largest of all cellular membranes (Schenkel and Bakovic 2014), thus supporting the impermeability of the membrane. The MOM is in close proximity of other organelles, particularly the endoplasmic reticulum (ER) and this proximity is necessary for several important processes in the cell (such as PL translocation across membranes and apoptosis induction) (Ardail et al. 1990; Daum and Vance 1997; Fiorini et al. 2019; Stiban et al. 2008a; Vance 2014).

The lipids in mitochondrial membranes have been studied extensively (for excellent reviews (Horvath and Daum 2013; Schenkel and Bakovic 2014)), mainly focusing on the unique mitochondrial PL, CL, which are also prevalent in bacteria (see previous). CL is located primarily in the MIM and is essential for respiration (Chabi et al. 2018), protein clustering and cell death (Mulkidjanian et al. 2018). Yet, other lipid components of mitochondria have been identified as key regulators of cell function and homeostasis. Traffic across and within mitochondrial membranes remains a hot topic in cellular biochemistry, as it is an important aspect in energy metabolism and also cell death. Transport of fatty acids into the matrix via the carnitine-acylcarnitine shuttle initiates β-oxidation (Rubio-Gozalbo et al. 2004), while transport of pro-apoptotic proteins from the intermembrane space into the cytosol initiates apoptosis (Matassa et al. 2001). Hence, the lipid (and protein) composition of mitochondrial membranes is of paramount significance.

Differences in lipid composition between mitochondria from various organs are minimal. Cardiac mitochondria, for instance, contains plasmalogens which are ether lipids involved in membrane fusion and transmembrane protein functions. Lipid composition between the MOM and MIM varies greatly (Fig. 2.1). Generally, mitochondrial membranes show a characteristic abundance of CL, particularly in the MIM. Whereas the MOM is smooth, the MIM is convoluted with cristae that increase the surface area to accommodate higher respiration rates. This entails the MIM to be more flexible and accommodating for protein complexes to reside and function properly. Additionally, compared to other membranes, SL content is lower, as is the ratio of sterols, with the MIM being devoid of sterols. Protein:lipid ratio is higher than other membranes. Eighty percent of mitochondrial PL consists of PC and PE. PE, which is the abundant lipid in *E.* coli (see above), together with the presence of CL hint of a potential bacterial evolutionary origin of the MIM. Comparing the lipids in the membranes of

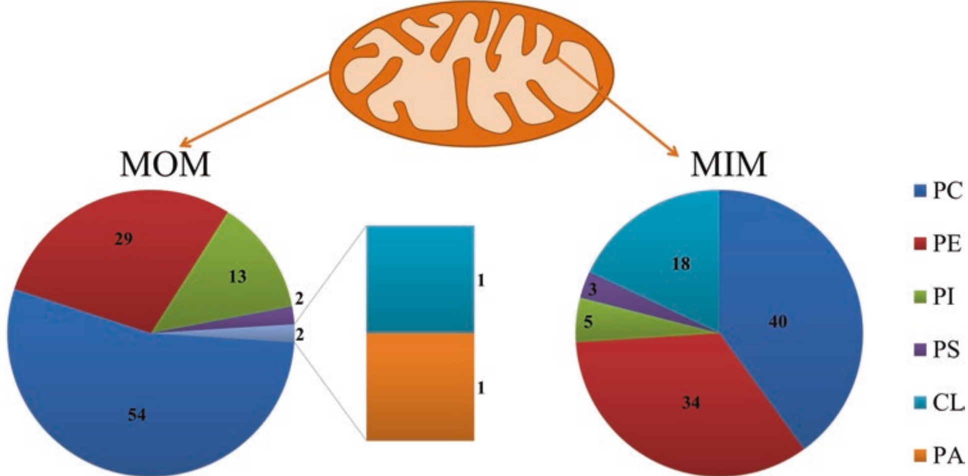

Fig. 2.1 Differences in MOM and MIM lipid composition. The data are obtained from (Daum and Vance 1997; Horvath and Daum 2013) and the numbers represent the percentage of total phospholipids in rat liver mitochondria. Abbreviations used: *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *CL* cardiolipin, *PA* phosphatidic acid

Table 2.2 Comparison between lipid content of MIM from rat liver and bacterial membranes

Lipid	MIM[c]	Gram negative *E. coli* plasma membrane[a,b]	Gram negative *E. coli* outer membrane[a,b]	Gram positive *B. subtilis*[a,b]
	% total PL			
PC	44	0	0	0
PE	34	60	61	13
PI	5	0	0	0
PS	1	0	0	0
CL	14	20	1	4
PA	<1	NA	NA	NA
SM	1	0	0	0
Sterols	0.003 mg/mg protein	0	0	0

[a]Data from: (Sohlenkamp and Geiger 2016)
[b]Data from: (Warschawski et al. 2011)
[c]Data from: (Daum and Vance 1997)

different bacteria and the MOM, the similarity is evident (Table 2.2).

Like their bacterial counterparts, mitochondrial lipids play important roles in cellular homeostasis. Mitochondria are organelles that are very dynamic in structure. A plethora of proteins aid the double membranes to fuse with other mitochondria, or one mitochondrion to split by fission. In addition to proteins, fusion and fission of mitochondria are mediated by key membrane lipids (mainly, CL, PE, DAG and phosphatidic acid (PA)) (reviewed in (Frohman 2015)). In short, CL is required for the function of the GTPase Opa1 that induces mitochondrial fusion (DeVay et al. 2009). CL was shown to be important for the recruitment and dimerization of Opa1, which is a key event for its GTPase activity, and hence, fusion of the membrane (Ban et al. 2010). Interaction between the negatively charged CL head group with the positively charged lysine residues in Mgm1 (yeast homolog of Opa1) was necessary for the activity of the protein (Rujiviphat et al. 2009). Similarly, PE stimulates mitochondrial fusion as yeast lacking the decar-

boxylase that produces PE have shown to present fragmented mitochondria (Chan and McQuibban 2012). On the other hand, CL was also implicated in mitochondrial fission, via similar mechanisms. Recruitment by CL of Drp1, a key GTPase involved in fission, was required for the activity of the protein and for fission to occur (Macdonald et al. 2014; Nakamura et al. 2011; Ugarte-Uribe et al. 2014). Similar conclusions were made about CL interaction with other fission-inducing proteins such as alpha-synuclein (Guardia-Laguarta et al. 2014).

Interestingly, both PE and CL are implicated in the respiratory function of mitochondria oppositely. Whereas CL was shown to induce respiratory complex III and IV oligomerization and stability, and enhanced complex IV activity, PE favored the destabilization of the supercomplexes (Bottinger et al. 2012). Ubiquinone is another lipid directly involved in respiration by moving electrons from complexes I or II to complex III. Ubiquinone is present in both prokaryotic cells and mitochondria further indicating possible evolutionary connections (Degli Esposti 2017).

Unlike bacteria, which are free-living organisms, mitochondrial lipids are rarely affected by environmental conditions, obviously. The endosymbiotic nature of mitochondria within the eukaryotic cell obliterates the direct effects of the environment on mitochondria as the eukaryotic cell bears them instead. Additionally, eukaryotes seldom thrive in extreme conditions (except perhaps some species in deep oceans or hydrothermal vents), like many bacterial species. Hence, the effect of extreme environments on mitochondrial lipid composition is not established compared to that in prokaryotes.

2.4.1 Mitochondrial Cholesterol and Other Sterols

Cholesterol import into mitochondria, its metabolism and its egress seems to play vital roles in mitochondrial physiology (F. Li et al. 2015). Similar to bacteria and other eukaryotic membranes, cholesterol and other sterols modulate the fluidity of the membranes and impart structural and functional restrictions to membranes. As sterols are mostly planar with small hydrophilic head groups, fluidity is mediated differentially at different temperatures. Overall, cholesterol in eukaryotic membranes is distributed primarily to plasma membranes, Golgi, and ER (Table 2.2).

Compared to other subcellular organelles, however, mitochondria have significantly lower cholesterol levels in both membranes (Horvath and Daum 2013). Cholesterol is important for the function of mitochondria; it is needed for membrane biogenesis and maintenance and for the synthesis of other sterols (Martin et al. 2016). The low abundance of cholesterol in mitochondria implies that even small changes in cholesterol concentrations can influence mitochondrial physiology greatly. The levels of mitochondrial cholesterol are regulated by the transfer of cholesterol from the ER to MOM (possibly via mitochondria-associated membranes (MAM)), and transfer from the MOM to the MIM, and its metabolism in the matrix. Modulation of mitochondrial cholesterol content has been implicated in several pathological conditions. Higher mitochondrial cholesterol levels have been described in human hepatocellular carcinoma (Montero et al. 2008). Interestingly, knockout mice for a cholesterol-transferring protein (CAV1) have been shown to accumulate cholesterol in mitochondria leading to respiratory chain dysfunctions and susceptibility to apoptosis and predisposing mice to steatohepatitis and neurodegeneration (Bosch et al. 2011). Conversely, lowering cholesterol level in mitochondria following exercise, helped mitochondria to be resistant to calcium-induced swelling, therefore increasing mitochondrial health (Ziolkowski et al. 2013). In A549, THP-1 and U937 cultured cells, (human lung epithelial adenocarcinoma, monocytic leukemia, and histiocytic lymphoma, respectively) however, initiation of apoptosis by the steroid saponin Rh2 is exacerbated by removal of cholesterol from mitochondrial membranes (Verstraeten et al. 2018). Increase of mitochondrial PL and reduction of cholesterol have been shown to be directly correlated with the preventative effects of S-allyl cysteine sulfoxide on the onset of myocardial infarction in Wistar rats

(Sangeetha and Darlin Quine 2009). In Alzheimer's disease, cholesterol accumulation in mitochondria as a result of amyloid beta-induced ER stress cause neurotoxicity. Protection against mitochondrial cholesterol loading ameliorated these effects (Barbero-Camps et al. 2014). Thus higher or lower mitochondrial cholesterol levels may serve several complex roles in different body systems under differing conditions.

Mitochondrial cholesterol has also been implicated in redox signaling mediated by hypoxia inducible factor (HIF-1α). Cholesterol was shown to induce HIF-1α activation under normoxic conditions in the liver (Anavi et al. 2014). HIF-1α, which is a nuclear transcription factor induced under low oxygen, promoted transcription of several proteins in mitochondria including some electron transport chain proteins such as complex IV (Hwang et al. 2015). Both mitochondrial lipids and proteins, therefore, are modulated by hypoxic conditions that affect mitochondrial health, with cholesterol an important factor in such adaptability.

2.4.2 Mitochondrial Sphingolipids

The concentration of Ceramides (Cer) and other SLs in mitochondrial membranes are minimal, but once imported, detrimental effects to the mitochondria and the cell ensue (Hannun and Obeid 2008). Cer is the parent SL consisting of sphingosine backbone *N*-acylated with a variety of fatty acyl-CoAs. Sphingosine is an amino alcohol long chain base synthesized from condensation and reduction of the amino acid serine, and palmitoyl-CoA (Jenkins et al. 2002). The head group of Cer consists of a hydroxyl functional group (Fig. 2.2). Cer is a highly bioactive lipid; it is involved in a variety of cellular processes, particularly programmed cell death, or apoptosis (Bartke and Hannun 2009). While being a minority of mitochondrial lipids, Cer accumulation in mitochondria leads to permeabilization of the MOM (Siskind et al. 2006), mitochondrial dysfunction (J. Yu et al. 2007) and cell death. Cer biogenesis is highly compartmentalized in eukaryotic cells (Hernandez-Corbacho et al. 2017). Cer lies in the center of all SL metabolism (Fig. 2.3). The majority of Cer is produced in the ER by the *de novo* synthesis pathway (Reynolds et al. 2004), or in the plasma membrane via the sphingomyelin hydrolysis pathway (Mathias et al. 1998). Alternatively, the salvage pathway of Cer biogenesis involves the recycling of complex SLs into Cer again in the ER (Becker et al. 2005).

In the *de novo* pathway, serine palmitoyltransferase (SPT) condenses serine with palmitoyl-CoA using the pyridoxal 5′-phosphate cofactor to form ketosphinganine, which is subsequently reduced by a reductase and NADPH to sphinganine (which is also named dihydrosphingosine). A family of 6 mammalian Cer synthases (CerS) acylates the amino group of sphinganine with different length of fatty acyl-CoAs forming dihydroceramides (DHCer) (Stiban et al. 2010). Cer is later produced by oxidation and desaturation of the 4–5 bond in DHCer using molecular oxygen and the enzyme DHCer desaturase (DES). The compartmentalization of Cer metabolism ensures that Cer is produced in certain places where it can have specific functions. In MCF-7 cells, it was shown that generation of Cer directly in mitochondria, but not in other cellular locations, induced apoptosis (Birbes et al. 2001). Hence, mitochondria are organelles that are affected severely by Cer, yet not all Cer biosynthetic machinery was found there. Interestingly some enzymes (such as CerS) were located in MAM and mitochondria (Bionda et al. 2004), yet others (such as DES) were only located in MAM but not

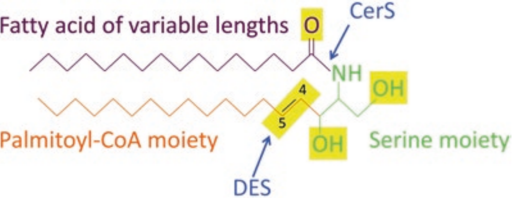

Fig. 2.2 The structure of C_{16}-Cer. The functional groups and key groups for the biological activity of Cer are highlighted in yellow. Bonds added by important *de novo* synthetic enzymes, CerS and DES, are indicated by blue arrows. Serine moiety is indicated in green and palmitoyl-CoA in orange. CerS *N*-acylate sphinganine with a fatty acids of variable chain lengths (purple)

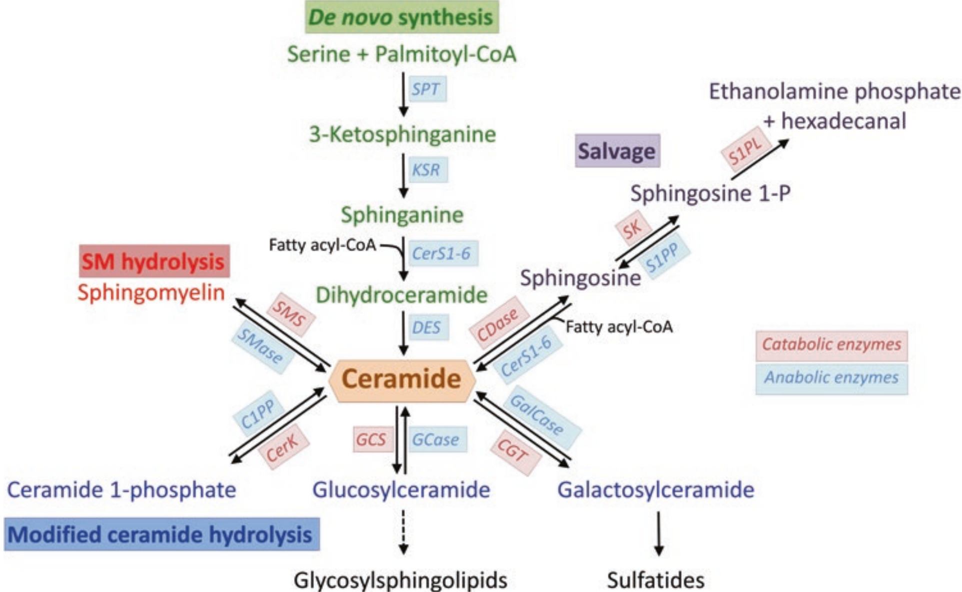

Fig. 2.3 Centrality of Cer in SL metabolism (adjusted from (Abou-Ghali and Stiban 2015)). Four different metabolic pathways can lead to Cer production in cells. The *de novo* pathway (green) employs four enzymes to produce Cer from the amino acid serine and palmitoyl-CoA. In the salvage (or recycling) pathway (purple), sphingosine, which is a direct metabolite of Cer, can be acylated back to Cer by CerS. S1PL breaks down sphingosine 1-phosphate and thus is the exit point of SL metabolism. The hydrolysis of complex SL (e.g. SM (red) and glycosphingolipids (blue)) represents the final pathways of Cer generation. Anabolic enzymes are in blue boxes whereas catabolic enzymes are in red boxes. Enzyme abbreviations used are as follows: *SPT* Serine palmitoyltransferase, *KSR* 3-ketosphinganine reductase, *CerS1-6* ceramide synthase, *DES* dihydroceramide desaturase, *CDase* ceramidase, *SK* sphingosine kinase, *S1PL* sphongosine 1-phosphate lyase, *S1PP* sphingosine 1-phosphate phosphatases, *SMase* sphingomyelinases, *SMS* sphingomyelin synthase, *GCS* glucosylceramide synthase, *GCase* glucosylceramidase, *CGT* ceramide galactosyltransferase, *GalCase* galactosylceramidase, *CerK* ceramide kinase, *C1PP* ceramide 1-phosphate phosphatase

mitochondria (Stiban et al. 2008a). Nevertheless, other enzymes in SL metabolism were also found in mitochondria (e.g., CDases (El Bawab et al. 2000) and nSMase (Wu et al. 2010)). Irrespective to whether the whole machinery of Cer generation exists in mitochondria, Cer generation in (or transport to) this organelle is sufficient to induce MOM permeabilization, cytochrome *c* release and the onset of apoptosis (Birbes et al. 2002). Ionizing radiation-induced apoptosis in *C. elegans* was abolished in cells lacking functional CerS. This was later corrected by the exogenous addition of Cer signifying the role of Cer in mitochondria inducing apoptosis (Deng et al. 2008).

2.5 Programmed Cell Death

Programmed cell death, or apoptosis, is a characteristic of cells that allows unneeded and/or damaged cells to be removed without causing an inflammatory response in the surrounding tissue. In eukaryotic cells, mitochondria are at the center of intrinsic apoptosis. Interestingly, prokaryotes,

despite being single-celled, can undergo apoptosis to benefit the community.

2.5.1 Mitochondria and Apoptosis

Mitochondrial outer membrane permeabilization (MOMP) is a hallmark of the intrinsic apoptotic pathway (Stiban et al. 2006). Compromising the intactness of the MOM allows inter membrane space proteins (e.g. cytochrome *c*) to be released into the cytosol where they work in tandem with other cytosolic proteins to induce a cascade of events mediated by caspases to result in apoptosis (Birbes et al. 2002). MOMP is mediated by a variety of cellular components, from Bcl-2 family members (some of which are pro-, while others are anti-apoptotic) (Ganesan et al. 2010; Ganesan et al. 2012; S. H. Lin et al. 2011; Perera et al. 2012), to Cer (Abou-Ghali and Stiban 2015; Hannun 1996; Senkal et al. 2007) or combinations of Cer and Bax (Ganesan et al. 2010), to other possible channels (mitochondrial permeability transition pore) (Dhingra et al. 2019; Parks et al. 2019), or the mitochondria apoptosis channel (Pavlov et al. 2001). Regardless of the pathway of inter membrane space protein egress, MOM lipids play a crucial role in dictating when and how apoptosis is initiated. Stress-induced oxidized PLs induce apoptosis at the MOM by facilitating Bax translocation and oligomerization (Dingeldein et al. 2017). Oxidation of CL can trigger both pro-apoptotic (pore formation) and anti-apoptotic (membrane potential drop and reduction of reactive oxygen species production) reactions (Mulkidjanian et al. 2018). Membrane lipids and proteins, therefore, dictate key apoptotic functions in mitochondria.

Despite its low abundance, Cer is an important lipid for mitochondria. Cer levels increase in mitochondrial membranes prior to the initiation of apoptosis (Birbes et al. 2001; El Bawab et al. 2000; Zeidan et al. 2008). The ability of Cer to form barrel-stave channels in membranes was a key finding in the field of lipid biochemistry (Colombini 2013, 2017). Cer channels have been previously visualized by transmission electron microscopy in asolectin liposomes (Samanta et al. 2011) as well as liposomes derived from lysosomal membranes (Yamane et al. 2017). MOM permeabilization by Cer channel formation has been demonstrated in many studies (Shao et al. 2012; Siskind et al. 2002, 2005, 2006; Stiban et al. 2006; Stiban and Perera 2015). The ability of Cer to form channels in membranes is dependent on the lipid composition of the membrane (Perera et al. 2016). For instance, Cer is unable to permeabilize plasma membranes, as indicated by the lack of lysis of red blood cells in response to Cer treatment (Siskind et al. 2005). Within mitochondria, while the MOM is susceptible to Cer channel permeabilization, the MIM is very resistant to such channel formation activity (Siskind et al. 2002). Cer is produced in the ER and no channel forming activity was reported in that organelle to our knowledge (unpublished results). In lysosomes, the production of Cer was required to cause permeabilization and channel formation in liposomes derived from lysosomal membranes (Yamane et al. 2017). In all, the formation of Cer channels, and their regulation, is widely dependent on other lipids in the membrane. Thus the specificity of Cer action on the MOM and its role in permeabilization and apoptosis is lipid-specific. This deserves further studies on the lipid-lipid and protein-lipid interaction in mitochondria.

2.5.2 Bacterial Apoptosis

Even though it seems illogical for a unicellular organism to kill itself, bacteria do have a programmed cell death process that is activated when the cells are living in communities such as

biofilms (Bayles 2014). Multicellular biofilm communities consist of cells with differentiated structures that serve specialized functions resembling the relationships found in multicellular organisms.

The evolution of multicellular organisms is the result of selective pressures that gave multicellularity a selective advantage. Multicellular entities such as biofilms are provided with several advantages amongst which resistance to environmental stresses seems to be the most common. Living within a biofilm can protect cells from changes in temperature, pH, osmotic pressure, oxygen availability, desiccation, metals, and other compounds toxicity including antibiotics (Lyons and Kolter 2015). It is generally acknowledged that cells in a biofilm will have better access to nutrients as well, and there are some striking examples of how life in a multicellular entity provides considerable energy gains. One of such examples is found in deep sea sediments where filamentous bacteria of the *Desulfobulbaceae* family form multicellular cable-like structures that oxidize sulfur at one end and transport the harvested electrons to the other through internal, insulated wires to be used for oxygen reduction. The competitive advantage of such system is the ability of these cells to separate soluble electron acceptors and donors in space, enabling them to monopolize major energy sources (Pfeffer et al. 2012).

Bacteria populations that live as multicellular organisms use programmed cell death to sacrifice part of the colony to protect other cells (Allocati et al. 2015), a similar function to eukaryotic apoptosis. In biofilms, DNA released in the matrix by dying cells (extracellular DNA) is an important adhesion molecule for the establishment and maintenance of the biofilm (Conover et al. 2011) and is also a source of genes that can be picked up by other living cells in a horizontal gene transfer manner (Lewis 2000).

CidA and LgrA are bacterial proteins that were originally isolated from *Staphylococcus aureus*, but are widely conserved in bacteria (Ranjit et al. 2011). These proteins are holin-like molecules and it has been postulated that they work in an analogous manner to the Bcl-2 family proteins in eukaryotic cells (Rice and Bayles 2008). The CidA gene encodes a putative holin, with a positive effect on cell death, while LgrA encodes an anti-holin with inhibitory effect on the process (Ranjit et al. 2011). It is remarkable to note that, both Bcl-2-family proteins and holins cause depolarization of membranes (MOM and cytoplasmic membrane, respectively) and both are regulated by homologous proteins that oppose their function. MOM depolarization leads to the activation of caspases and cell death in eukaryotic cells, cytoplasmic membrane depolarization in bacteria leads to the activation of peptidoglycan hydrolases and bacterial cell death (Lewis 2000).

Experimental evidence in *E. coli* demonstrated that Bcl 2-family proteins are functional in these cells. Bax and Bak were found to polymerize on *E. coli* plasma membrane and induce cell death and lysis (Pang et al. 2011). Moreover, coexpression of Bax in *E. coli* with the anti-apoptotic protein Bcl-X_L resulted in the inhibition of cell death and lysis (Bayles 2014). These experiments clearly show that the function of these proteins is conserved between bacteria and eukaryotic cells and it is therefore possible to hypothesize that apoptotic proteins were transferred to the eukaryotic cell during the endosymbiosis process that resulted in the formation of mitochondria (Allocati et al. 2015; Bayles 2014). Interestingly, in *Arabidopsis thaliana* an LrgAB-like gene product, which is involved in the control of plant programmed cell death, is found in the chloroplast and when absent causes chlorosis and prematurely necrotic leaves, suggesting it has an anti-apoptotic role in plants (Yang et al. 2012).

2.6 Conclusions and Future Directions

While there may be many pieces of evidence implicating the evolutionary connections between mitochondria and prokaryotes, such as rRNA sequences, DNA structure, or ribosomes, the lipid content cannot be underestimated. Prominent MIM lipids such as CL and PE have been found only in prokaryotic cells and very rarely in other eukaryotic membranes. The enzymatic machinery that produces these lipids is also presented in these membranes, establishing further evidence of commonality in origin. Importantly, the relative low abundance of sterols and sphingolipids in mitochondria and prokaryotes indicate closeness of the membranes in ancestral trees. Whether the original protoeukaryotic cell "engulfed" a bacterium that eventually evaded degradation and remained as an integral component of the cell, or membranes were formed from a protonucleus to surround a prokaryotic cell remains to be elucidated. Regardless of the mechanism, it is evident that membrane lipids play central roles in mitochondrial and prokaryotic physiology and pathophysiology, roles that mitochondria may have inherited from their ancestors. It is worth noting that the lack of PC in bacteria and its high concentration in MIM represents a puzzle for evolutionary biologists. PC is made using proteins encoded in nuclear genes and imported into mitochondria to make the bulk of the PL membrane. It is of great importance to identify this discrepancy in the structure of the mitochondrial membrane compared to its ancestral prokaryotic membrane. In addition, there is now mounting evidence that programmed cell death is also a feature of prokaryotic cell death and it is possible to conceive of research in this field unveiling a universal mechanism underlying apoptosis. The holin-antiholin class of proteins were originally discovered in bacteriophages, where they modulate host cell lysis during lytic infection (van den Esker et al. 2017a). The transfer of genes from the phage to the bacterium through integration in the genome, could have resulted into a permanent feature of the bacterium. These bacteria delivered the genes to eukaryotes with the endosymbiotic event that created mitochondria and chloroplasts (van den Esker et al. 2017a). In some bacteria, like *B. subtilis*, the function of the holin-antiholin system seems to be essential in metabolism rather than cell death regulation (van den Esker et al. 2017b). Understanding the evolutionary origin of these proteins could shed light on the origin of apoptosis in eukaryotic cells and open new investigations on the potential of using these proteins for antimicrobial therapy in the age of antibiotic resistance.

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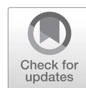

Ceramide Channels

3

Marco Colombini

Abstract

Are ceramide molecules capable of self-assembling in biological and phospholipid membranes to form ceramide channels: membrane channels capable to translocating proteins through said membranes? A number of papers have been published which support the conclusion that ceramide forms these large channels in membranes. The evidence is extensive and consisting of: flux studies using isolated mitochondria, liposomes and planar membranes; visualization by electron microscopy; elastic deformation studies; and regulation by Bcl-2 family proteins. The evidence supports a structural model of the channel shown to be stable by molecular dynamic simulations and having structural and mechanical properties consistent with multiple experiments. Yet the novelty of this claim raises legitimate questions. Indeed, a recent report questions the existence of ceramide channels based on liposome experiments. This review presents both a comprehensive description of the major observations supporting the case that ceramide channels do exist and addresses the issues raised in the skeptical report.

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Keywords

Ceramide · Sphingolipid · Membrane channel · Mitochondria · Electron microscopy · Lipid channel · Apoptosis

Abbreviations

MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
SLs	sphingolipids

3.1 Introduction

The interactions between components in a membrane environment are complex and poorly understood. There is a tendency to seek the simplest interpretation (Ockham's razor) but with complex systems especially in Biology, the simplest interpretation is almost always wrong. Unlike the situation in aqueous solutions, we almost always have no idea of the "activity" of membrane components in the liquid phase of the membrane. We resort to expressing concentrations as mole fractions, tacitly assuming that all components are not interacting with other components in the membrane. Of course interactions are the rule, not the exception. In addition, when considering lipid components of membranes, these can take many

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conformations resulting in many possible interactions with other membrane components. Of course lipid polymorphism is well described as is the temperature dependence of lipid phases and phase formation. These gross changes are easy to study with a variety of techniques. The problem is much more difficult with nanoscopic structures formed by membrane components. The formation of ceramide channels is a case in point. A recent publication (Artetxe et al. 2017) has questioned whether such structures exist or whether the membrane permeabilization can be explained by mismatch between adjacent lipid phases or mismatch in local curvature or lipid packing. This alternative hypothesis fails to explain the large majority of the experimental observations attributed to ceramide channels. These are very nicely described in a review article published 2 years earlier (Abou-Ghali and Stiban 2015). In this review article, I will summarize the experimental results that together make a compelling case for the existence of ceramide channels and just touch, "en passant", on the biological importance of such structures. I will also address the criticisms of the paper in question in the appropriate sections. This review will also present compelling results that were conveniently ignored by the critical paper, results that cannot be explained by the simplest interpretation.

3.1.1 Membrane Channels

There is a diversity of opinions as to what constitutes a membrane channel. Thus I will briefly begin with the basics. Membranes are quasi-two-dimensional structures and yet their primary role is to separate aqueous spaces and maintain differences in the composition of those spaces. They form a thin (3–3.5 nm) hydrophobic layer that dramatically reduces the transmembrane flux of most solutes found in the aqueous compartments. Solutes dissolve in water by interacting strongly with water's polar structure. This strong interaction must be broken in order for solutes to traverse the thin hydrophobic layer within the membrane. Thermal energy is not quite great enough to make this process rapid; hence membranes are effective barriers to solute flow. Membrane channels form polar, typically aqueous pathways through membranes thus reducing the need for solutes to break their bonds with water. This generally results in a continuous flow of solutes through the membrane and is distinguished from a carrier mechanism. This view of a membrane channels encompasses many possible structures and thus is not limited to a cylindrical transmembrane pore.

When considering membrane channels, one often thinks of proteins as being the channel-formers. Cells typically use proteins to form membrane channels but do not do so exclusively. Indeed, some cells produce small, non-protein molecules that form membrane channels. *Streptomyces noursei* produces nystatin, an antifungal channel-forming antibiotic (Holz and Finkelstein 1970). *Streptomyces nodosus* produces amphotericin B which has a similar function and mode of action (Holz and Finkelstein 1970). *Streptomyces mashuensis* produces monazomycin, a voltage-dependent channel former (Muller and Finkelstein 1972). Thus, cells generate whatever structure is appropriate to achieve the desired purpose.

3.1.2 Membrane Channels Formed by Cellular Lipids

Cellular glycerolipids are lipids consisting of glycerol esterified to one or more fatty acids. These are used to store energy, form membranes, and in cell signaling. The latter two functions are primarily formed by glycerophospholipids often referred to simply as phospholipids. For simplicity this review will use the term phospholipids. Sphingolipids (SLs), based on the molecule sphingosine, rather than glycerol, have a wide range of functions, from cell development to cell death (Hannun and Obeid 2017). One of the SLs, ceramide, is well known to favor cell death although it has also other housekeeping functions in the cell. Indeed, there are typically as many as 50 different kinds of ceramides in cells likely performing different functions in different membrane compartments (Hannun and Obeid 2011).

From a functional perspective, cellular lipids are generally viewed as either forming structural units such as membranes or modifying the function of proteins. Yet lipids are known to be able to self-organize not only as membranes but also as rafts (Simons and Ikonen, 1997) and lipidic pores during the fusion process (Chanturiya et al. 1997). In the case of ceramide, it can self-assemble to form large channels capable of translocating proteins through membranes. This review will examine the experimental evidence for the conclusion that ceramide channels exist and are stable cylindrical structures with restorative mechanical properties.

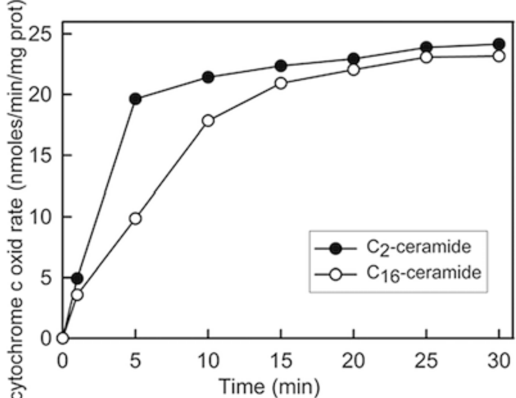

Fig. 3.1 Time dependence of the development of the permeability of the MOM to cytochrome *c*. Rat liver mitochondria were incubated for the indicated time with either C_2- or C_{16}-ceramide. Then reduced cytochrome *c* was added and the absorbance monitored at 550 nm. The initial rate of oxidation of the cytochrome *c* is plotted. It is a measure of the MOM permeabilization induced by ceramide. The results were published in Siskind et al. (2002)

3.2 Evidence for the Existence of Ceramide Channels

3.2.1 Ceramide-Induced Permeabilization of the Mitochondrial Outer Membrane to Proteins

Mitochondria isolated from both mammalian and yeast cells are readily permeabilized to exogenously-added cytochrome *c* by the addition of ceramide, if ceramide is delivered in such a way as to be able to partition into the mitochondrial outer membrane (MOM) (Siskind et al. 2002). An example of the time-dependence of the MOM permeabilization is shown in Fig. 3.1. Notice that both the ceramide with the typical physiological length of the acyl group, C_{16}-ceramide (16 carbon), and the very short chain acyl group, C_2-ceramide (2 carbon) permeabilize the MOM to roughly the same extent and with very similar kinetics. Short-chain ceramides are more likely to insert into the MOM because of significant solubility in aqueous solutions (CMC 5–6 μM for C_2-ceramide (Sot et al. 2005)). However ceramides relevant in the initiation of the apoptotic process, such as C_{16}-ceramide, are highly insoluble and unlikely to be able to transfer from dispersed micelles to mitochondrial membranes at rates compatible with the useful lifetime of isolated mitochondria (or the patience of the investigator). In both cases, to affect sufficient transfer, dispersal of ceramide can be augmented by dissolving ceramide in a water-compatible solvent and dispersing a small quantity into a mitochondrial suspension under vigorous mixing. Individual ceramide molecules may collide with mitochondrial membranes prior to colliding with other ceramide molecules and thus forming micelles. Obviously the kinetics is both complex and critical and thus effectiveness of transfer is best determined experimentally. Of the solvents tested in my lab: DMSO, DMSO/DMF solutions, ethanol, and isopropanol; the latter was most effective at producing reproducible results. When conditions were kept constant (e.g. the quantity of ceramide solution added) the major variable that influenced the degree of ceramide insertion into the MOM was the rate and extent of mixing using a vortex mixer. Gentle mixing resulted in poor insertion. Isolated mitochondria are not damaged by vigorous vortexing for 30 s. With isolated mitochondria, as expected, the more ceramide that was dispersed, the more ceramide inserted but the smaller the % of the added ceramide that actually inserted into the MOM (Fig. 3.2; Siskind et al. 2006). There was more opportunity for ceramide molecules not

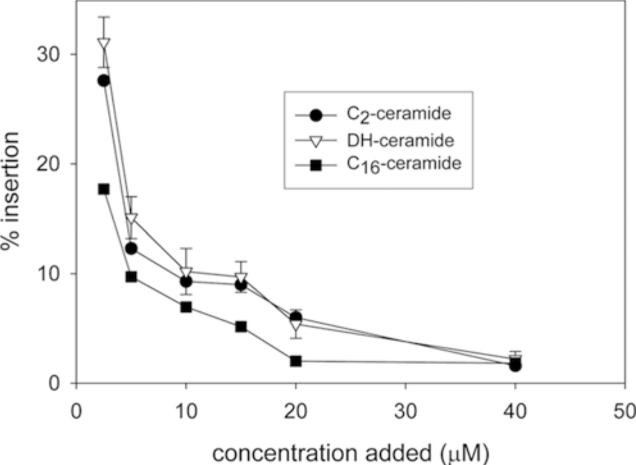

Fig. 3.2 Ceramide delivery to rat liver mitochondria as a function of the absolute concentration added to the solution. Radiolabeled ceramide was used to determine how much of the added ceramide inserted into the mitochondria. The percent of the total ceramide added that actually inserted is plotted as a function of the amount added. The concentration shown assumes homogeneous dispersion of ceramide in the mitochondrial suspension. The results were published in Siskind et al. (2006)

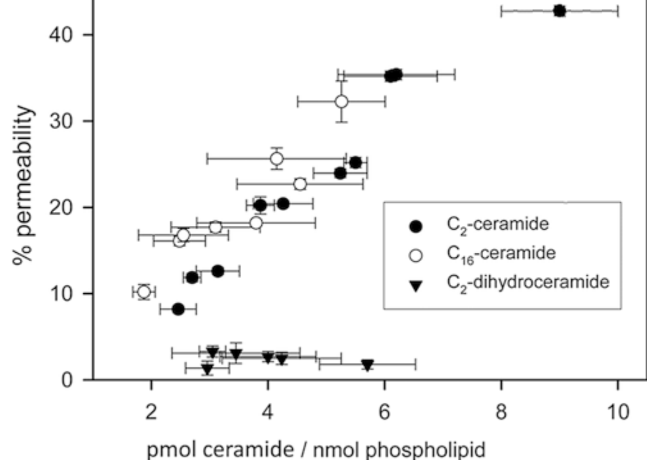

Fig. 3.3 The permeabilization of the MOM, as measured by the initial rate of oxidation of cytochrome *c* (as in Fig. 3.1), is plotted against the mole fraction of ceramide in the mitochondria assessed as in Fig. 3.2. The results were published in Siskind et al. (2006)

only to collide with the MOM but also with each other to form micelles. To achieve 30% MOM permeabilization to added cytochrome *c*, an amount of ceramide was added that would have resulted in a 20 μM final concentration if the ceramide were in free solution (Fig. 3.3). Under this condition only 2% of the added ceramide actually inserted in the MOM resulting in a molar ratio of ceramide to phospholipid of 6 pmoles/nmole (Siskind et al. 2006). Interestingly, the addition of TNF to induce apoptosis resulted in an elevation of ceramide in mitochondria to a ratio of 12–15 pmoles/nmole of phospholipid (García-Ruiz et al. 1997; Birbes et al. 2005). This is consistent with the conclusion that ceramide permeabilization of the MOM to cytochrome *c* is sufficient to account for the initiation of the execution phase of the apoptotic process.

The permeabilization of the MOM by ceramide was not assessed solely by measuring protein release because that could be attributed to a transient, perhaps non-specific permeabilization associated with the method used to deliver the ceramide to the mitochondria despite negative results with

solvent controls. Permeabilization was assessed by measuring the rate of oxidation of exogenously added reduced cytochrome *c* by cytochrome oxidase in the outer surface of the mitochondrial inner membrane (MIM) (Siskind et al. 2002). For such oxidation to continue for minutes and obey first order kinetics requires a constant pathway for the bidirectional movement of cytochrome *c*. This is prima facie evidence for a channel in the MOM. Still one could argue that the pathway is merely damage of the MOM. This possibility was effectively eliminated by using C_2-ceramide to produce the permeabilization and then reversing this permeabilization by removing the ceramide using fatty acid free albumin to bind ceramide (Siskind et al. 2002, 2006). The albumin itself did not somehow heal a damaged MOM because the same experiment using C_{16}-ceramide failed to reverse the permeabilization, as the albumin failed to remove the C_{16}-ceramide from the MOM.

Artetxe et al. (2017) did not address these findings in their critique. They were satisfied to assume that their findings using liposomes with an overall composition similar to the lipids in mitochondria would suffice to justify their conclusions. In fact, the details of the permeabilization of mitochondria by ceramide provide important insights that make the simple mechanistic model proposed by these investigators to be quite unlikely. Further results described in Sect. 3.2.6 on the regulation of ceramide-mediated MOM permeabilization by Bcl-2 family proteins make the case for ceramide channels even stronger.

3.2.2 Ceramide Addition Permeabilizes Phospholipid Membranes

Ceramide permeabilization of the MOM of isolated mitochondria could be interpreted as ceramide activating some process by which the MOM is permeabilized to proteins via a machinery that has nothing to do with ceramide. In this case ceramide would not form channels but simply be an activator of an existing permeabilizing machinery. This, after all, is the expected action of lipids. Being relatively simple structures they work by binding to and activating or inhibiting the action of proteins. However, if ceramide itself forms large channels in membranes it should be able to do so in the absence of proteins, in a defined system.

Indeed, the dispersal of ceramide into an aqueous solution containing single-walled phospholipid vesicles results in the release of fluorescent solutes from these vesicles (Stiban et al. 2006). A dose-dependent release of carboxyfluorescein was demonstrated (see also Fig. 3.4) and it was found that this release was inhibited if dihydroceramide was also added along with the ceramide. Dihydroceramide is a precursor of ceramide in the biosynthetic pathway and lacks the *trans* double bond at the 4–5 position of the sphingoid base. It does not permeabilize membranes but interferes with the self-assembly of ceramide into membrane channels. Sphingosine acts in a similar way (Elrick et al. 2006). The nature of the membrane-permeabilization pathway is undefined by experiments on liposomes but a great deal of insight was gained by performing experiments on planar phospholipid membranes.

The dispersal of ceramide into the aqueous solution bathing a planar phospholipid membrane results in the formation of an ion-conducting pathway in that membrane (Fig. 3.5; Siskind and Colombini 2000; Siskind et al. 2006). The permeability of the membrane to ions, measured as a conductance, increases in a seemingly erratic way until it reaches a fluctuating steady state. The structure of the conductance increase is reminiscent of the formation of many permeability pathways of varying conductance but, in fact, it is the growth of one large channel. Some evidence in support of this conclusion comes from the occasional observation of small step-wise increases in conductance followed by the complete loss of conductance in one step (Siskind et al. 2003). Hence, all the conductance increases were part of the same structure. However, compelling evidence for the growth of one large channel was obtained from observing channel disassembly following the addition of lanthanum chloride (La^{3+}) to the solution. If a population of channels were responsible for the overall permeability then these

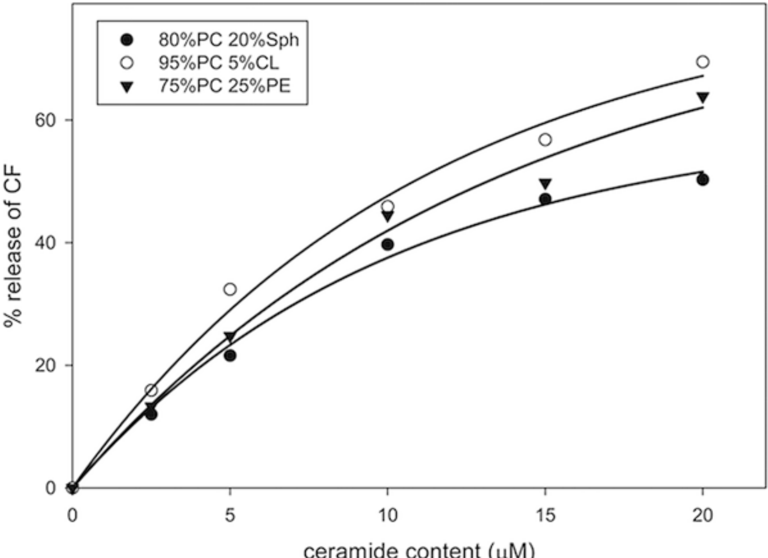

Fig. 3.4 Single-walled phospholipid vesicles (lipid composition indicated) containing carboxyfluorescein and a quenching agent were permeabilized by the addition of C_2-ceramide. The amount of fluorescence released at steady state is expressed as a percent of the total released in the presence of detergent. This percent release is a measure of the percent of liposomes permeabilized by the added ceramide. These are previously unpublished data but the methodology used is as described in Stiban et al. (2006)

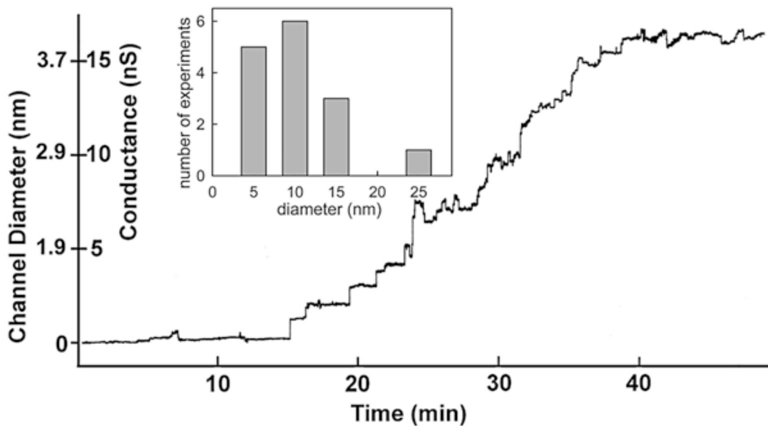

Fig. 3.5 Formation of a single ceramide channel in a planar phospholipid membrane. The channel diameter is calculated from the conductance assuming a cylindrical channel. The steady state conductance indicates that the channel has achieved a steady size. The inset shows a histogram of channel diameters calculated from a set of independent experiments. The results were published in Samanta et al. (2011)

would be responding to the lanthanum ions independently resulting in an exponential decay in conductance. However, what was observed is a delay followed by a structured disassembly. The critical observation was that the delay was variable from one experiment to another. A delay is expected because there is an unstirred layer (about 50 μm (Negrete et al. 1996)) next to the membrane when the solution is stirred using magnetic bars. Then La^{3+} needs to bind to the negatively-charged lipids resulting in some cross-linking. The whole process is expected to take 5 s to 10 s. However the observed delay lasts from about 10 s to 60s or more (Siskind et al. 2003). Indeed, the

variable, stochastic, delay is diagnostic of a single entity overcoming an energy barrier using thermal energy. By pooling the data from more than 30 separate experiments one observed a delay of about 8 s and then an exponential decay with a time constant of 17 s (Fig. 3.3 in Colombini 2010; Siskind et al. shows an incorrect fit to the data). The pooling of many experiments does produce the expected exponential decay because the single channel in each experiment undergoes disassembly with a time dependence that is independent of the channel observed in other experiments. An unlikely alternative interpretation is that there are many small channels that close in synchrony.

A careful examination of conductance decrements (Siskind et al. 2003), either observed spontaneously or induced by La^{3+} addition, revealed an unexpected observation. Small conductance decrements were very heterogeneous in magnitude but conductance decrements greater than about 16 nS showed a marked preference for conductance drops that were multiples of 4 nS. This could be explained if the decrements were due to losses of multiple staves from a cylindrical barrel-stave channel. The staves would be uniform in size for an organized structure and for a channel that is large compared to the width of a stave the conductance drop would be proportional to the number of staves lost at one time. The results obtained were consistent with a single stave of width 1.1–1.2 nm. The working model of the ceramide channel proposes that the channel is formed by columns of ceramide molecules that span the membrane as staves in a barrel. The width of such a column would be half that of the stave width above. Thus the experimental result would indicate that the ceramide channel tends to disassemble in pieces that are a multiple of 2 ceramide columns. This observation is consistent with adjacent columns being organized in an anti-parallel fashion and their interaction stabilized by a dipole-dipole attraction.

Artetxe et al. (2017) report very slow ceramide induced release of fluorescent solute from lipid vesicles (6 h) when ceramide is added to the preformed vesicles and none from C_2-ceramide additions. Stiban et al. (2006), Elrick et al. (2006), Perera et al. (2012a) and Stiban and Perera (2015) reported that the release that would take place occurred mostly within a minute. Both the rate of carboxyfluorescein release and the steady state level of release increased with increasing amounts of ceramide added (Fig. 3.4; Stiban et al. 2006) indicating that the population of permeabilized vesicles increased with ceramide dose. Thus the permeabilized vesicles released their contents whereas the other did not. This is very different from the observations reported by Artetxe and coworkers. Not only is the time scale very different but they report a gradual decrease in fluorescence from liposomes based on a calculated fluorescence lifetime. What could be the reason for this great difference? Perhaps their statement: “Because of its high hydrophobicity, it can be safely assumed that all of the added ceramide was readily incorporated into the lipid bilayers.” In fact, using radiolabeled ceramide, Siskind et al. (2006) showed that even with vigorous mixing during addition only a small fraction of the ceramide actually becomes inserted (Fig. 3.2). The rest is likely located in micelles. Exchange of ceramide between micelles and the vesicles seems to be very slow and it is unclear in which phase ceramide reaches a lower energy level and thus which direction the net movement would take place however slowly. The quoted statement indicates that the authors were not concerned about the effectiveness of delivering ceramide to the liposomes. Experiments in my lab demonstrated that the mode of addition was critical to the success in permeabilizing both phospholipid bilayers and mitochondrial membranes. The vehicle was important and DMSO (used by Artetxe and coworkers) is not very effective at delivering ceramide. Isopropanol is much more effective. In addition, the rate and effectiveness of rapid mixing is critical. Thus failure to deliver sufficient ceramide to the liposomes readily explains the lack of permeabilization reported by Artetxe and coworkers. However, these researchers reported much greater success at releasing fluorescent solutes from the vesicles when they use sphingomyelinase to convert sphingomyelin in the vesicle membrane to ceramide. With that method, delivery is not an issue. However, the

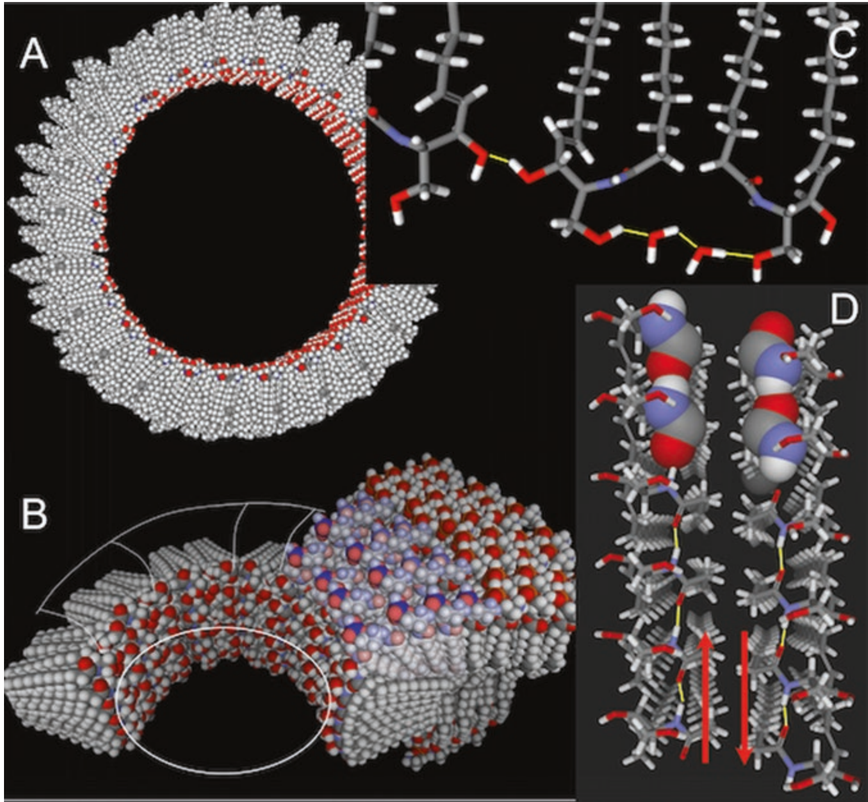

Fig. 3.6 The working model of a ceramide channel. (**a**) A slightly tilted top view of a channel of the most frequently observed size (10 nm in diameter). 48 columns of ceramides are arranged in an antiparallel fashion. (**b**) A partial channel is shown along with a portion of the phospholipid membrane in order to illustrate how the channel could interface with the bilayer membrane. The lighter colored phospholipids are proposed to curve so as to cover the aliphatic chains of ceramide. The channel takes on an hourglass type of shape so as to share a portion of the interfacial strain. (**c**) Illustration of how adjacent ceramide columns are linked together in the polar areas by a hydrogen-bonded network that includes water. (**d**) Two anti-parallel ceramide columns are shown, each containing 6 ceramide molecules. Hydrogen bonding (yellow lines) between adjacent amide linkages is believed to be the primary organizing interaction. Space-filling amide groups bonded together are shown. The anti-parallel orientation results in favorable dipole-dipole interactions (red arrows)

mole fraction of ceramide they generated is very high (10 mole percent). At that level one would expect the formation of different phases in the membrane and thus the process of permeabilization would be quite different. When quantitated properly, the mole fraction of ceramide needed to permeabilize membranes is less than 1 mole percent (Siskind et al. 2006). The two different experimental regimes naturally explain the different results observed.

3.2.3 Model of the Ceramide Channel

The structure of ceramide includes an amide linkage between the sphingoid base and the fatty acyl group. Amide linkages are organizing centers responsible for the secondary structure of proteins. The working model of the ceramide channel (Siskind and Colombini 2000; Siskind et al. 2003) uses the hydrogen bonding between amide linkages of adjacent ceramides to generate columns of ceramides that would span the apolar membrane region (Fig. 3.6). The model uses columns con-

sisting of 6 ceramide molecules but that number is somewhat arbitrary. The aliphatic chains of ceramide lying parallel to the plane of the membrane would abut the phospholipid tails aligned perpendicular to the plane of the membrane. The polar head would form the polar lining of the channel. However both ends of the ceramide column would expose a hydrophobic surface so that surface would need to be covered by curvature of the membrane surface (Fig. 3.6b). The channel would also have a somewhat hourglass shape in order for the ends of the column to meet the phospholipids (Anishkin et al. 2006). This structure limits the length of the ceramide column. Six ceramides seems to be a happy medium between being too short or too long from a structural and energetic point of view. The column of ceramides requires that all the amide linkages are in the same direction (Fig. 3.6d) and this results in a strong dipole moment. Thus adjacent columns should be oriented in an anti-parallel fashion to further stabilize the structure. The inner walls of the channel consist of a hydroxyl-hydrogen-bonded network formed by the twin hydroxyls on ceramide and water bound to these hydroxyls (Fig. 3.6c). Molecular dynamic simulations indicate that this ceramide channel structure is stable (Anishkin et al. 2006). Experiments indicate that it is a dynamic structure with individual ceramides and ceramide columns coming on and off the channel. Channel disassembly of C_2-ceramide can be induced by reducing the concentration of ceramide monomers in solution by the addition of fatty acid free albumin (Siskind et al. 2003). Thus, for C_2-ceramide, the ceramide molecules in the channel are in equilibrium with those in the membrane and also this in aqueous solution so that binding ceramide in free solution by albumin results in channel disassembly.

3.2.4 Visualization of the Ceramide Channel by Electron Microscopy

The conclusion from electrophysiological studies that ceramide forms large cylindrical channels in phospholipid membranes was strengthened by observing such channels formed in single-walled vesicles made from phospholipids. Negative stain was used to visualize the pores formed in the vesicles (Fig. 3.7; Samanta et al. 2011). The vesicles containing the channels were dried in a thin layer of sample on a microscope grid in the presence of the negative stain. This results in a 2-dimensional image. The third dimension is the thickness of the material at any point in the image and this is critical to the interpretation of the image. The negative stain fills in the aqueous spaces, such as the space around the flattened vesicle and the pores of the channels. The uranium atoms in the negative stain are by far the major electron scatters and thus produce the dark areas in the electron microscope image (the only source of contrast). The darker the intensity the greater the amount of negative stain present at any point in the image. By densitometry the thickness of the stain was quantitated. The vesicles are visualized as “footprints”, areas of lack of stain with a shape consistent with a flattened vesicle. In some cases the sample preparation resulted in a stain of fairly uniform thickness except for where the vesicle was located and stain was displaced. In other cases, the vesicle had stain surrounding it but the stain thickness decayed with distance as stain was washed off (Fig. 3.7a). However, the focus is on the dark circles in the vesicle itself. Densitometry indicates that the stain depth is consistent with a stain-filled pore as opposed to a surface stain (Fig. 3.7b). It is highly unlikely that the stain would form a little hill on top of the vesicle of a height comparable to the membrane thickness and a width consistent with the diameter of a large channel. What would hold that hill together? In addition, if the resolution of the images is considered the shape of the densitometry image of the stained pores is consistent with pores that are right cylinders. In addition, the size distribution of the pores observed by electron microscopy is essentially the same as the size distribution of the pores as calculated from electrophysiological experiments. Thus alternative interpretations of the electron microscopic images are very unlikely to be correct.

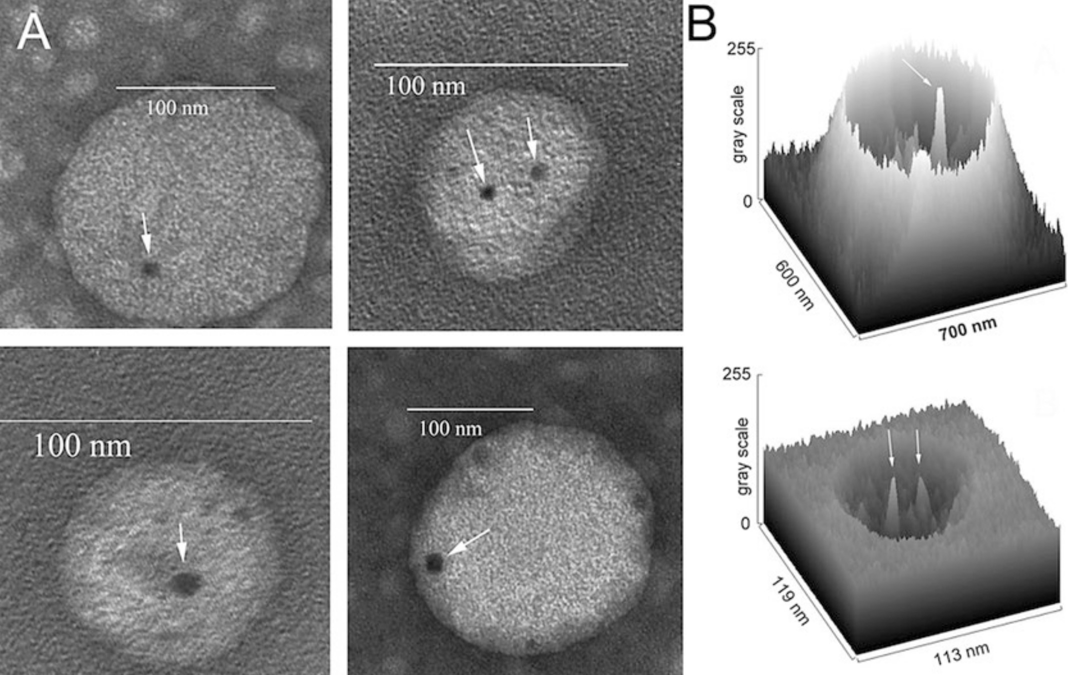

Fig. 3.7 Visualization of pores formed by ceramide channels using electron microscopy. (**a**) The illustration shows 4 examples of negatively-stained flattened phospholipid vesicles containing ceramide channels (arrows) that were negatively stained. The stain is present around the vesicles and in the pore formed by the channel. (**b**) Densitometry of two electron micrographs of such vesicles showing the thickness of the stain around the vesicle and in the channel (arrows). The results were published in Samanta et al. (2011)

Ceramide channels were also visualized in lysosomes by negative stain electron microscopy following treatments that resulted in endogenous C_{16}-ceramide accumulation (Yamane et al. 2017). Stain-filled circular pores 10–20 nm in diameter were observed. These are consistent with the report of Samanta and coworkers but formed under relevant physiological conditions.

Artetxe and coworkers published an electron micrograph of liposomes treated with ceramide using cryo transmission electron microscopy. They did not use any stain so the contrast arises from differences in electron scattering by the mass of the atoms present. They speculate on the nature of the observed changes from controls but do not do any rigorous analysis. Clearly their speculation arises from a biased perspective. From what is reported it difficult to make any conclusion regarding the presence of channels.

3.2.5 Dynamics of Ceramide Channels

Unlike the well-studied membrane channels formed by proteins, ceramide channels, as described in Sect. 3.2.3, would be thin-walled structures held together by a flexible, hydrogen-bonded network. Such structures might be expected to be deformable. As described in Sect. 3.2.2, the addition of La^{3+} results in total disassembly of ceramide channels formed in solvent-free membranes made from monolayers. However, when these membranes were made in such a way as to include hexadecane, the effect of La^{3+} addition was very different (Shao et al. 2012). The use of a microfluidic system (Fig. 3.8 inset), which allows easy perfusion of the solution on one side of a planar phospholipid membrane, required the inclusion of hexadecane to form a planar membrane. When La^{3+} was perfused into one side of the membrane, rather than

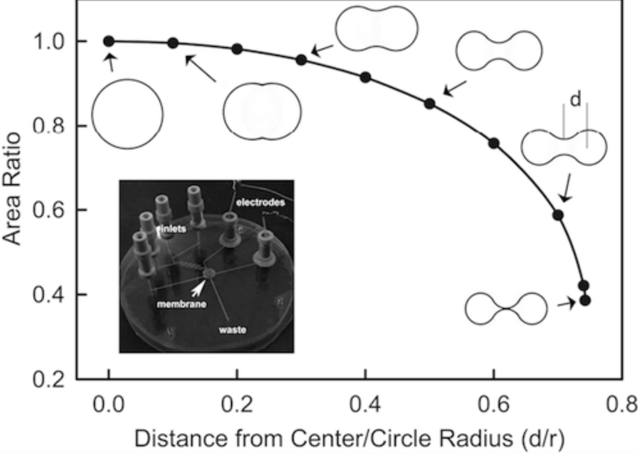

Fig. 3.8 Area changes expected from the deformation of a right cylindrical channel. The calculated ratios of the area of the deformed cylinder to that of the undeformed are plotted as a function of the ratios of the deformation parameter, *d*, to the radius of the undeformed cylinder. The final area ratio matches the conductance drop observed when the membrane was exposed to La^{3+}. The inset shows the microfluidic system. The results were published in Shao et al. (2012)

observing ceramide channel disassembly, one observed a reduction in conductance to a value that was about 40% of that of the original conductance regardless of the magnitude of the original conductance. Upon removal of the La^{3+} by perfusion with the chelator, EDTA, the original conductance was restored. Reperfusion with La^{3+} resulted in the same conductance drop as before and, once again, perfusion with EDTA restored the original conductance. This process was repeated many times on the same membrane and the result was the same. Rather than interpreting the result of La^{3+} addition as a partial channel disassembly, it was interpreted as a distortion of the channel by the addition of La^{3+} and a restoration of the cylindrical channel by removal of the La^{3+}. The distortion interpretation was preferable for a number of reasons: (1) It would be difficult to understand why channel disassembly would stop at a channel size that would result in a conductance that is 40% of the original regardless of the magnitude of the original conductance. If the conductance of the membrane after La^{3+} addition were roughly the same regardless of the starting conductance then one could conceive of the possibility that in the presence of La^{3+} a form of the channel was stabilized that resulted in the measured conductance. However, the final conductance in the presence of La^{3+} was very different but about 40% of the conductance before La^{3+} addition. (2) The restoration of the original conductance after La^{3+} removal would be hard to explain with a disassembly model. (3) The distortion model not only explains the conductance changes but explains the drop to 40% of the original conductance. The formation of a biconcave structure that stops collapsing when the opposite membranes touch, results in the observed conductance drop (Fig. 3.8). The distortion model allows one to consider the ceramide channel as a distortable structure depending on the lateral pressure of the membrane. The structure also has an elastic restoring force that restores the cylindrical structure once the lateral pressure is reduced. This restoring force would be due to the structure seeking the optimal hydrogen-bonding configuration of the ceramides. Channel distortion would be expected to force the hydrogen-bonding to a less favorable configuration. The initial and final structures would seem to be the only stable configurations under the conditions tested. The intermediate structures are part of an energy barrier to structural change.

Artetxe and coworkers do not comment on these findings. They do not try to interpret these results using an alternative hypothesis.

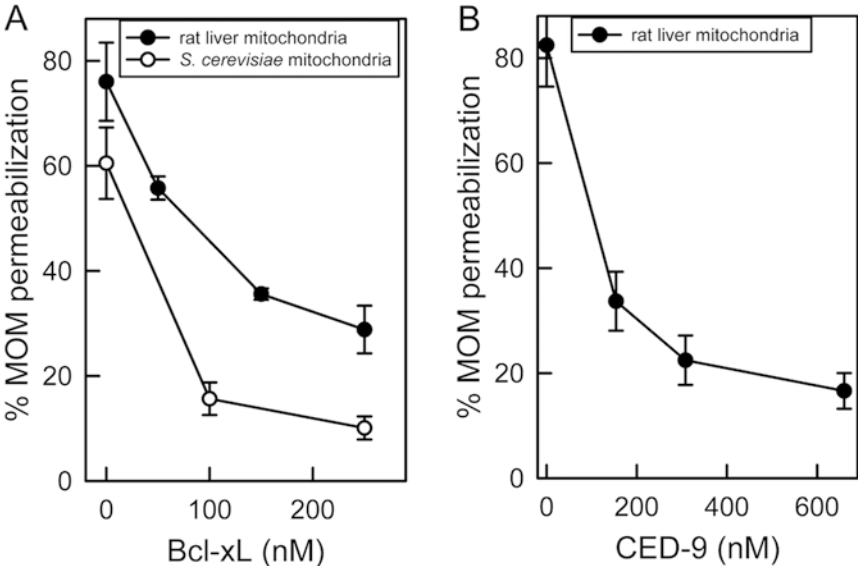

Fig. 3.9 Anti-apoptotic proteins destabilize C_{16}-ceramide channels formed in both rat liver and *S. cerevisiae* MOM. The results were published in Siskind et al. (2008) © the American Society for Biochemistry and Molecular Biology

3.2.6 Ceramide Channel Destabilization by Anti-apoptotic Bcl-2 Family Proteins

Anti-apoptotic Bcl-2 family proteins destabilize ceramide channels (Siskind et al. 2008; Perera et al. 2012b; Chang et al. 2015). Most of the published work is focused on Bcl-xL but work with Bcl-2 and CED-9 also supports this statement (Siskind et al. 2008). The addition of either Bcl-xL or CED-9 inhibits ceramide permeabilization of the MOM of isolated mitochondria (Fig. 3.9). Each of these also inhibits channel formation by ceramide in planar membranes where the possibility of indirect action by anti-apoptotic proteins is virtually eliminated. These findings are consistent with the generally accepted conclusion that elevation of cellular ceramide levels favor apoptosis and anti-apoptotic proteins inhibit apoptosis but, in addition, they identify at least one mechanism by which this antagonism takes place.

Detailed studies with Bcl-xL show that the mechanism of inhibition is highly specific (Perera et al. 2012b). Although channel formation by ceramide takes place whether the acyl chain has anywhere from 2 carbons to 24 carbons, Bcl-xL inhibition favors 16–20 carbon acyl chains (Fig. 3.10). No inhibition of C_2-ceramide channels was detected. Reducing the length of the alkyl chain of the sphingoid base also abrogates the ability of Bcl-xL to inhibit channel formation. Introducing an unsaturation in the chain was also observed to reduce the ability of Bcl-xL to inhibit channel formation in isolated mitochondria. This specificity for the structure of ceramide is matched by sensitivity to mutations in Bcl-xL's hydrophobic grove. Small molecules known to bind to the hydrophobic grove of Bcl-xL (ABT-263, ABT-737, 2-methoxyantimycin A3) and thus inhibit its anti-apoptotic function also inhibit Bcl-xL's ability to destabilize ceramide channels. This inhibition arises from the ability of ceramide to bind to the hydrophobic grove of Bcl-xL. Point mutations that interfere with this binding also reduce the ability of Bcl-xL to destabilize ceramide channels (Chang et al. 2015). Most but not all of these point mutations also result in a reduction in the ability of Bcl-xL to inhibit the formation of Bax channels. Thus the binding pocket on Bcl-xL for these two channels formers has a great deal of overlap.

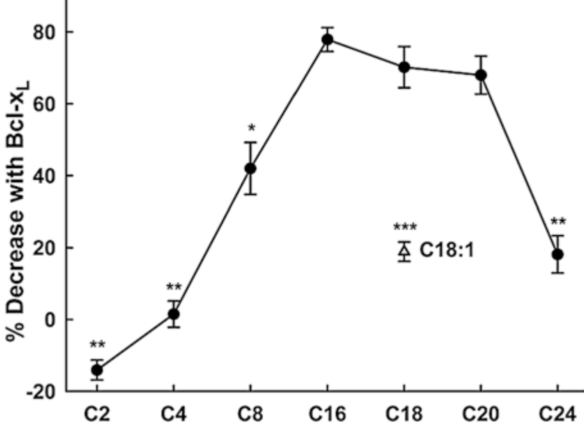

Fig. 3.10 The ability of Bcl-xL to destabilize ceramide channels depends strongly on the length of the aliphatic chains on the ceramide. The figure illustrates the variation of the inhibition when the acyl group is varied from that present in C_2-ceramide to C_{24}-ceramide. Introducing one double bond in the middle of the acyl group in C_{18}-ceramide forming $C_{18:1}$-ceramide also reduced the ability of Bcl-xL to destabilize that channel. The results were published in Perera et al. (2012b)

Thus the ability of Bcl-xL to destabilize ceramide channels with a hand-in-glove specificity argues for a highly organized channel structure that can be destabilized by the binding of a single Bcl-xL molecule (Siskind et al. 2008). The most frequent size of the observed ceramide channels is 10 nm in diameter with many much larger (Samanta et al. 2011). Thus the channel is also much larger that a Bcl-xL molecule. The binding is proposed to elicit a structural change that is propagated throughout the structure via the hydrogen-bonded network, much like an allosteric process. This propagated structural change would favor channel disassembly over the assembly process resulting in channel diminishing in size or being eliminated altogether.

Artetxe and coworkers do not comment on these findings. They do not try to interpret these results using an alternative hypothesis.

3.2.7 Synergy Between Ceramide and the Pro-apoptotic Protein Bax

Activated Bax or a combination of Bid and Bax is well known to form channels in the MOM of isolated mitochondria. The same is true for ceramide addition. However, when small amounts of either of these that would result in only a small amount of permeabilization in the mitochondrial population are now added to the same mitochondrial suspension the permeabilization observed was greater than the sum of the effects of either agent alone (Ganesan et al. 2010; Perera et al. 2012b). This synergy can be interpreted in different ways but it indicates a cooperative interaction between these agents. This interaction is very sensitive to the nature of the polar region of ceramide, even to the stereochemistry (Perera et al. 2012b). It is quite insensitive to changes in the apolar chains. The difference in structural specificity of the Bcl-2 family protein for the structure of ceramide is interesting and may indicate that by acting on different portions of the ceramide channel structure they are either stabilizing or destabilizing the ceramide channel. Alternatively one could argue that ceramide merely binds to Bax favoring its ability to self-assemble into channels. In that case it would be unexpected that this binding would be insensitive to the size of the hydrophobic region of ceramide. One would expect the hydrophobic interaction to dominate the energetics of ceramide binding to Bax. Further research is needed to distinguish between these two possibilities.

Artetxe and coworkers do not comment on these findings. They do not try to interpret these results using an alternative hypothesis.

3.2.8 Membrane Specificity of Ceramide Channel Formation

Although the addition of ceramide to liposomes with a variety of lipid compositions still forms channels (Fig. 3.4), results with erythrocytes show a remarkable resistance to ceramide channel formation (Siskind et al. 2002, 2006). Erythrocytes were used as models of plasma membranes and thus the results indicate that the environment in the plasma membrane is not conducive to ceramide channel formation. Physiologically this is important as ceramide levels are elevated in the plasma membrane under a variety of conditions (e.g. Grassme et al. 2001; Corrotte et al. 2013). Thus, the question arises as to what prevents channel formation in the plasma membrane of at least erythrocytes? Since channel formation takes place in membranes with a variety of lipid compositions (e.g. Fig. 3.4), the lipids used seem not to be the major factor. This was recently confirmed by the findings of Artetxe and coworkers although their conclusion is wrong because it ignores other components in the plasma membrane. The observed selective membrane targeting of ceramide channels was further examined by Perera et al. (2016) using a panel of ceramide analogs. They found that many rather small changes in structure resulted in permeabilization of erythrocyte membranes. Indeed even stereochemical changes resulted in channel formation in erythrocytes. Thus, some specific interaction between ceramide and another component of the erythrocyte plasma membrane is responsible for inhibiting channel formation. That component does not have to be a protein because other SLs interfere with ceramide channel formation (e.g. dihydroceramide (Stiban et al. 2006) and sphingosine (Elrick et al. 2006)). Indeed, ceramides of different chain lengths can interfere with each other's ability to form channels. A beautiful demonstration of this was published by Stiban and Perera (2015). They demonstrated in a variety of ways how ceramide with a 16-carbon acyl chain interfered with channel formation by ceramides with either a 22-carbon or a 24 carbon acyl chain and vice versa. This interference was observed not only when these ceramides were added to isolated mitochondria but also when ceramide synthases with different acyl chain specificities were overexpressed in cells and mitochondria were isolated from those cells. These results can be understood easily if one considers the packing required to form an organized channel through the membrane. Ceramides with different acyl chain lengths may not pack properly, forming a less stable structure requiring a higher concentration of free ceramide in the membrane to favor channel formation (recall that ceramide channels are in dynamic equilibrium with ceramide monomers in the membrane).

3.3 Conclusions

Elevation of ceramide levels in a number of membrane systems results in an increase in the permeability of those membranes to both small molecules and proteins. This is not controversial. What has been questioned by Artetxe and coworkers is the nature of the release pathway. Both proposals would result in polar pathways through membranes allowing a continuous flow of water and solute. Thus both proposed mechanisms feature channels formed by ceramide (i.e., ceramide channels). What is controversial is the formation of cylindrical water-filled pores formed by hundreds of ceramide molecules. Negative-stain electron microscopy by two different labs using two very different systems have observed cylindrical pores in membranes with elevated levels of ceramide. Detailed electrophysiological studies provide strong support for such channels. The mechanical fluctuation of these channels was studied and is consistent with deformation of cylindrical structures. The destabilization of these channels in a very specific way by anti-apoptotic proteins is inconsistent with the vague mechanistic model of Artetxe and coworkers. The synergy

between ceramide and Bax in permeabilizing MOM indicates that ceramide and Bax may form more complex structures than the simple cylindrical pores but the results are also consistent with Bax enhancing the formation of the simple ceramide channel. Thus the published work provides compelling evidence for cylindrical ceramide channels under conditions where the mole fraction of ceramide in the membrane is low, less than 1 mole percent. At high mole fractions and under conditions where separate ceramide phases exist, the permeabilizing entity may be quite different. In addition, complications may arise wherein the activity of ceramide in membranes is influenced by other components. These may interfere with the natural ability of ceramide to self-assemble.

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A Stroll Down the CerS Lane

4

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and Anthony H. Futerman

Abstract

The majority of enzymes in the sphingolipid (SL) biosynthetic pathway have been identified over the past couple of decades. Despite significant work, and despite their crucial and central roles in SL synthesis, significant information is still lacking concerning the enzymes that catalyze the *N*-acylation of sphingoid long chain bases, namely the ceramide synthases (CerS), a family of six mammalian genes originally named longevity assurance (Lass) genes. Each of these six endoplasmic reticulum (ER) membrane-bound enzymes utilizes a relatively restricted sub-set of fatty acyl-CoAs for *N*-acylation, but are far more promiscuous about the use of long chain bases. The reason that mammals and other species have multiple CerS, generating a specific subset of ceramides, is not yet known, but implies an important role for ceramides containing specific fatty acids in cell physiology. In this brief chapter, we will stroll down the CerS lane and discuss what is known, and what is not known, about this important enzyme family.

Keywords

Ceramide synthase · Ceramide · Sphingolipids · *N*-acylation · *N*-acyl transferase

Abbreviations

CerS	(dihydro)ceramide synthases
CK2	casein kinase 2
ER	endoplasmic reticulum
FB1	fumonisin B1
FTY720	fingolimod
FTY720-P	FTY720 phosphate
HEK	human embryonic kidney
IMPs	integral membrane proteins
Lass	longevity assurance
LCB	long chain base
miRs	micro-RNAs
S1P	sphingosine 1-phosphate
SLs	sphingolipids
SPT	serine palmitoyl transferase
TLC	TRAM-Lag-CLN8
TMD	transmembrane domain
TRAM	translocating chain-associating membrane
YFP	yellow fluorescent protein

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Table 4.1 Phylogeny of CerS

Phylogeny[a]	Name	Amino acid seq.	Protein size (kD)	Acyl-CoA specificity	Main mRNA tissue distribution[b]
	CerS1	337	39.5	C18	Brain, Skeletal muscle, Lymph nodes, Testis
	CerS2	380	44.9	C22-C24	Bone marrow, Intestine, Kidney, Liver
	CerS3	383	46.2	C26 and higher	Prostate, Skin, Testis
	CerS4	394	46.4	C18-C20	Heart, Leukocytes, Liver, Skin
	CerS5	392	45.7	C16	Heart, Liver, Skeletal muscle, Testis, Kidney
	CerS6	384	44.9	C14-C16	Intestine, Lymph nodes, Spleen, Kidney

CerS phylogeny and primary structure characteristics are shown along with the fatty acyl-CoAs that they each utilize for ceramides synthesis. The four mouse tissues displaying highest mRNA levels for each specific CerS are indicated. The data is based on Refs. [a]Tidhar and Futerman (2013) and [b]Laviad et al. (2008)

4.1 A Stroll Through the CerS Sequence, from N- to C-Terminus

Ceramide, the backbone of all sphingolipids (SLs), is a bioactive second messenger, regulating many vital biological processes (Hannun and Obeid 2018). Ceramide can be produced by two pathways, either by the hydrolysis of complex SLs such as sphingomyelin (SM) (Clarke et al. 2006), or by *de novo* synthesis. The latter begins with the condensation of palmitoyl-CoA and serine by serine palmitoyl transferase (SPT), forming 3-ketosphinganine, which by subsequent reduction generates sphinganine via 3-ketosphinganine reductase. This step is followed by the *N*-acylation of sphinganine via sphinganine *N*-acyl transferase, also known as (dihydro)ceramide synthase (CerS), to form dihydroceramide. Dihydroceramide is converted to ceramide via dihydroceramide desaturase, which forms a *trans* double bond at the 4-5 position. Ceramide is the structural unit of all SLs, and is further used for the synthesis of more complex SLs, by addition of different head groups. The formation of ceramide takes place on the cytosolic leaflet of the endoplasmic reticulum (ER) (Futerman and Riezman 2005).

In mammals, (dihydro)ceramides are synthesized by a family of six CerS, transmembrane proteins located in the ER, with each using fatty acyl-CoAs of defined chain length for ceramide synthesis (Pewzner-Jung et al. 2006) (Table 4.1). Despite their crucial roles in SL synthesis, the three-dimensional structure of CerS is not available, likely due to difficulties in purifying and crystallizing multi-membrane-spanning proteins, which require extraction from the ER membrane in such a way that they retain their enzymatic activity (Lewinson et al. 2008), which is a notoriously difficult proposition. As a result, current information about their membrane topology, substrate binding sites and modes of substrate selectivity is currently inadequate.

Although no 3D structures are currently available, in silico studies, such as use of software to predict the number and topology of transmembrane domains (TMDs), suggest six TMDs (Fig. 4.1a). In a study using 19 different programs (Tidhar et al. 2018), an amino acid residue was considered part of a TMD if at least 10 of the 19 prediction programs identified it as such. Previous data suggested the N- and C- termini of CerS reside on opposite sides of the ER membrane (Mizutani et al. 2005; Laviad et al. 2012), and current data is consistent with the notion that the fourth TMD is unlikely to completely cross the ER membrane (Tidhar et al. 2018) (Fig. 4.1b). Importantly, the majority of the programs gave similar predictions for the first two and last two TMDs although there was little consensus regarding the intermediate TMDs which are likely to contain the active site.

In this chapter, we will walk through the CerS sequence and discuss structural and molecular features of the CerS, strolling from the N- to the C-terminus.

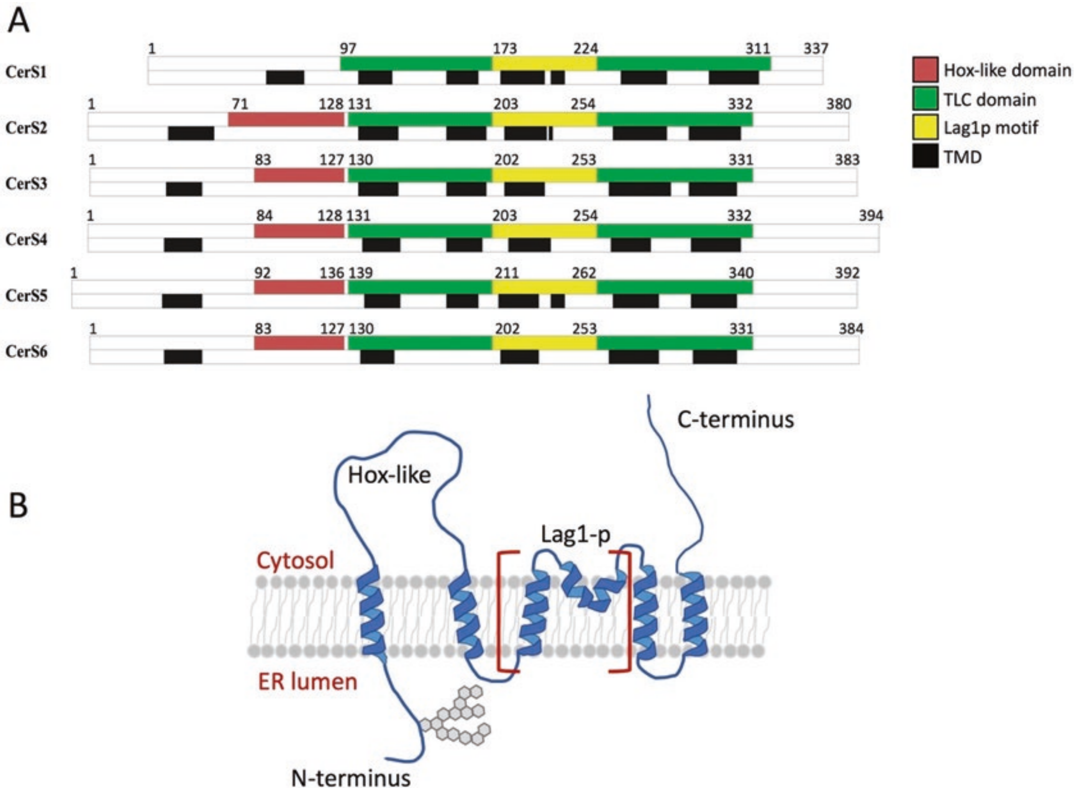

Fig. 4.1 CerS domains and putative topology. (**a**) Schematic representation of human CerS1-6 including the location of three main domains and TMDs. Residues were considered part of a TMD if the majority of the TMD prediction programs (>10/19) predict a TMD. (**b**) Putative topology of the Hox-like containing CerS (i.e. Cer2-6). Red brackets indicate the area where TMD prediction programs are in significant disagreement. Hexagons indicate glycosylation site

4.1.1 The CerS N-Terminus Faces the ER Lumen and Contains a Glycosylation Site

Sequence alignment of the N-termini of the human CerS (Fig. 4.2a) reveals high similarity between CerS2-6 (~50–70%) with substantial identity (~40–50%) (Fig. 4.2b). CerS1, however, shows no similarity to the other five CerS and cannot be aligned with the other CerS in this region (Fig. 4.2a). This is not surprising since phylogeny analysis has shown CerS1 to be in a separate lineage compared to CerS2-6, which forms a separate branch (Pewzner-Jung et al. 2006) (Table 4.1).

A conserved *N*-glycosylation motif (NXT, NXS) is found in CerS2 and CerS4-CerS6 (Fig. 4.2a). CerS2, CerS5 and CerS6 have been shown experimentally to be modified by *N*-glycosylation at Asn-19, Asn-26 and Asn-18, respectively (Mizutani et al. 2005). It was recently shown that, in contrast to CerS3, which is not modified by *N*-glycosylation, CerS4 also undergoes *N*-glycosylation at Asn-19 (Tidhar et al. 2018). Since the initial enzymatic steps of *N*-glycosylation are facilitated by enzymes restricted to the lumen of the ER (Breitling and Aebi 2013), it was inferred that the N-termini of CerS2, and CerS4-6 are located in the ER lumen (Mizutani et al. 2005; Tidhar et al. 2018) (Fig. 4.1b). Due to the high similarity between the CerS in the N-terminal region, with the exception of CerS1, (Fig. 4.2a) it is reasonable to assume that the N-terminus of all of the mammalian CerS reside in the ER lumen. Interestingly, *N*-glycosylation is not essential for the ceramide synthesis activity of CerS; indeed,

A

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CerS1           MAAAGPAAGPTGPEPMPSYAQLVQRGWGSALAAARG

CerS2           MLQTLYDYFWWERLWLPVNLTWADLEDR-DGRVYAK
CerS3           MFWTFKEWFWLERFWLPPTIKWSDLEDH-DGLVFVK
CerS4           MLSSFNEWFWQDRFWLPPNVTWTELEDR-DGRVYPH
CerS5     MATAAQGPLSLLWGWLWSERFWLPENVSWADLEGPADGYGYPR
CerS6            MAGILAWFWNERFWLPHNVTWADLKNTEEAT-FPQ
                        °  *  °*  ***  °    *  °*         °°
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B

	CerS2	CerS3	CerS4	CerS5	CerS6	**Identity**
CerS2	----	50	55.3	43.6	39.5	CerS2
CerS3	63.2	----	52.6	38.1	34.2	CerS3
CerS4	71.1	68.4	----	46.2	39.5	CerS4
CerS5	56.4	50	56.4	----	38.5	CerS5
CerS6	63.2	50	63.2	61.5	----	CerS6
Similarity	CerS2	CerS3	CerS4	CerS5	CerS6	

Fig. 4.2 Sequence similarity of the N-termini of CerS. (**a**) A comparison of the N-termini of human CerS1-6 by ClustalW multiple alignments. Asterisks (∗) indicate identity in CerS2–6. Circles (°) indicate similarity between CerS2-6. Arrowheads indicate the conserved glycosylation sites. (**b**) Percent identity (light grey) and similarity (dark grey) of human CerS2-6 in the N-terminal region analyzed using EMBOSS Needle

CerS5 activity increases upon de-glycosylation *in vitro* and a possible role for *N*-glycosylation in protein folding was suggested (Tidhar et al. 2018).

4.1.2 The First TMD Targets CerS to the ER

Integral membrane proteins (IMPs) such as CerS are normally inserted into the ER membrane by one of two parallel pathways. The classical co-translational pathway involves targeting by the signal recognition particle followed by membrane insertion through an ER-bound translocon (Rapoport et al. 2004). In rare cases, IMP TMDs are post-translationally inserted into the membrane (Borgese and Fasana 2011).

Since the CerS sequence does not encode a classical signal peptide, we recently studied its mode of insertion into the ER membrane. Chimeric proteins were constructed, composed of the N-terminal sequence of CerS5 fused to yellow fluorescent protein (YFP). We examined two CerS5 sequences, the first including the N-terminus lumenal portion (residues 1-43) and the second consisting of the N-terminus including the first putative TMD (residues 1-66) (Fig. 4.3a). The expression of these constructs clearly showed that the first TMD of CerS5 is essential for insertion of the protein into the ER membrane (Fig. 4.3b). These results were further verified by examining glycosylation of the chimera, confirming that the N-terminus of CerS5$^{(1\text{-}66)}$-YFP chimera indeed resides in the ER lumen (Fig. 4.3c). Since the CerS5$^{(1\text{-}43)}$-YFP chimera failed to co-localize in the ER, along with the fact that expression of a partial CerS sequence was sufficient for targeting of the chimera to the ER, we suggest CerS are inserted into the ER membrane co-translationally via the first TMD.

4.1.3 A Conserved, Enigmatic Homeobox-Like Domain

Approximately two decades ago, the first ceramide synthases, Lag1 and Lac1, were described in yeast (Guillas et al. 2001). The mammalian homolog, *uog1*, now known as CerS1, complemented the lethality of a *lag1*Δ*lac1*Δ double deletion in yeast (Jiang et al. 1998) due to its ability to recover ceramide synthase activity (Venkataraman et al. 2002). An extensive database search revealed that a large

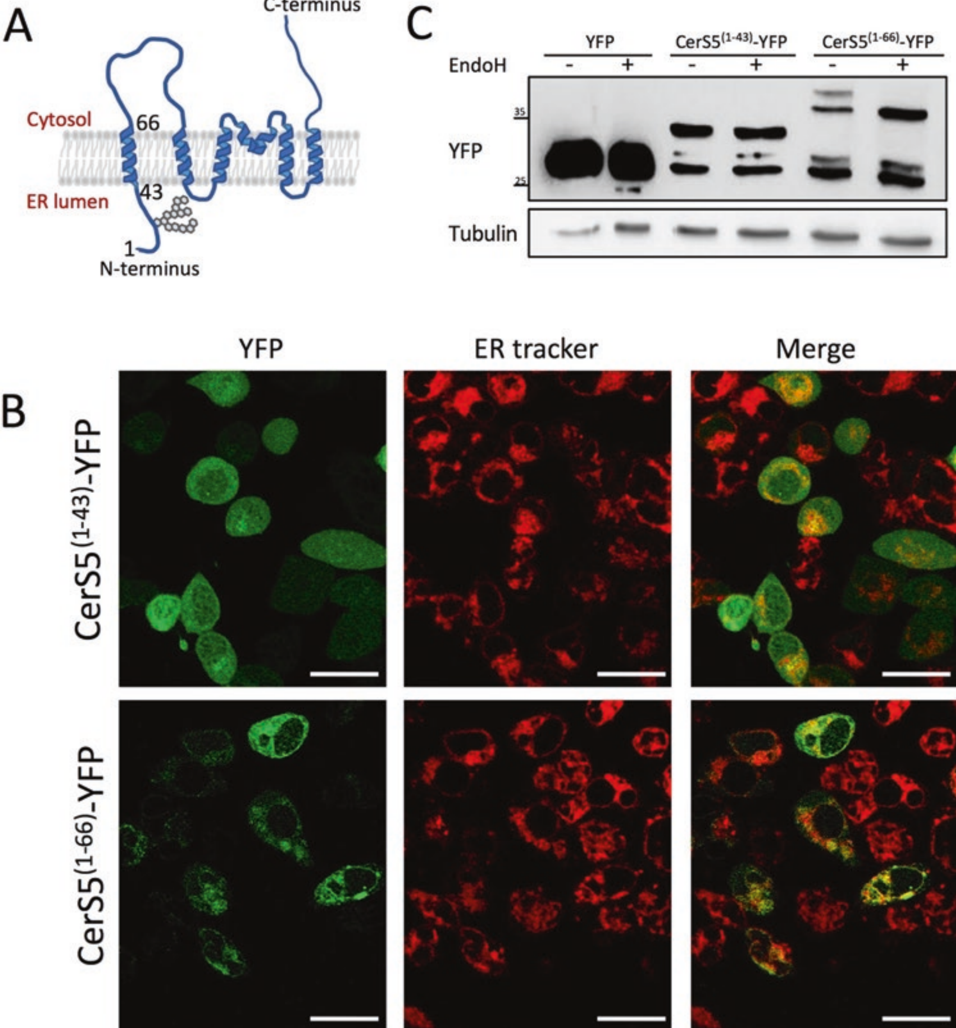

Fig. 4.3 The first TMD is necessary for CerS translocation to the ER membrane. (**a**) The location of residues 1, 43 and 66 in CerS5 are indicated along with the glycosylation site, based on the putative topology designated in Fig. 4.1b. (**b**) Localization of 43 or 66 residues from the N-terminus of CerS5 conjugated to YFP (green) in human embryonic kidney (HEK) cells. ER tracker was used as control (red). Scale bar 20 µm. (**c**) Cell lysates from HEK cells overexpressing the indicated CerS5-YFP construct were incubated with or without endoH prior to Western blotting using an anti-YFP antibody. Molecular weight markers are indicated. Tubulin was used as a loading control

subset of *lag1* homologs in higher organisms contain a Hox-like domain (Venkataraman and Futerman 2002), including all mammalian CerS, with the exception of CerS1. The Hox-like domain is conserved in insects and vertebrates, and in mammals is located after the first TMD in a loop which is predicted to face the cytosol (Venkataraman and Futerman 2002) (Fig.4.1b). The Hox-like domain is homologous to the classic Hox domain derived from homeobox proteins, sequence specific transcription factors important in development (Gehring 1994). These 60-amino-acid long domains are involved in sequence specific recognition and DNA-binding.

Although it has been proposed that the Hox-like domain of CerS may be involved in transcriptional regulation directly linked to ceramide levels, or to rates of ceramide synthesis, there is only one study that supports this suggestion. Schlank, a Drosophila CerS ortholog, downregu-

lates gene expression of *lip3* and *magro* by binding to their promotor regions via the Hox-like domain (Sociale et al. 2018). In the same study it was also shown that CerS2 alters the expression of *lip3*, however no other mammalian CerS were examined. Since the Hox-like domain of CerS2 is the longest of all the Hox-like domains (Fig. 4.1a) and is closest to that of homeobox proteins which act as transcription factors, it will be crucial to study whether other CerS can alter transcription. Additionally, it will be necessary to demonstrate a direct interaction of the CerS with the promotors. The 3D structures of the Hox-like domain of mouse CerS5 and CerS6 have been solved (2CQX and 1X2M, respectively) as part of a larger study of Hox domain structures.

The majority of the Hox-like domain is not required for CerS activity, since upon its deletion in CerS5 and CerS6, there was no alteration in CerS activity assayed *in vitro*. However, a conserved motif of 12 amino acid residues that flanks the end of the Hox-like domain is essential for activity (Mesika et al. 2007). More specifically, two positively charged residues within this 12-residue motif, Lys-134 and Lys-140 in CerS5, are essential for CerS activity. Site-directed mutagenesis of either residue to Ala resulted in 50% loss of activity, whereas mutation of both lead to complete loss of activity. This region is conserved across species, and this therefore suggests an as yet unknown and critical role of the Hox-like domain in CerS structure-function. That being said, even though CerS1 and its yeast homologs, Lag1 and Lac1, lack this domain, they still have CerS activity. Many unresolved questions remain regarding the role of this domain in regulating either CerS activity or in regulating other cellular processes.

4.1.4 The TLC Domain Contains the Active Site and Determines Substrate Specificity

All CerS share a common domain, namely the TRAM-Lag-CLN8 (TLC) domain, a region of ~200 residues also found in other protein families including the translocating chain-associating membrane (TRAM) protein, and CLN8, a protein that is mutated in the human genetic disease, neuronal ceroid lipofuscinoses (Winter and Ponting 2002). However, when carefully examining the TLC domain sequence in CerS, significant differences from that of TRAM and CLN8 become apparent, which suggests that a new classification may be required.

4.1.4.1 The Lag1p Motif May Contain the *N*-Acylation Site

A key example of this distinction is found when examining the Lag1p domain, a stretch of 52 amino acids residues within the TLC domain (Fig. 4.1), and a highly conserved region shared only by the CerS homologs (Jiang et al. 1998). Critical and conserved residues within the Lag1p motif, including two adjacent histidine residues, have been identified as essential for the activity of mouse and human CerS, as well as the yeast homolog, Lag1 (Kageyama-Yahara and Riezman 2006; Spassieva et al. 2006). Although no direct evidence has yet been obtained to support this suggestion, it is generally assumed that these highly conserved histidine residues are involved in catalysis and/or substrate binding (Winter and Ponting 2002). A number of studies demonstrated that *de novo* ceramide formation occurs on the cytosolic leaflet of the ER membrane (Mandon et al. 1992; Hirschberg et al. 1993) (Fig. 4.1b), although these studies, performed before molecular identification of the CerS and using techniques such as proteolytic cleavage of microsomes, cannot be taken as definitive proof that the active site residues of the CerS are located near to the cytosolic leaflet of the ER membrane.

4.1.4.2 Long Chain Base Specificity

Ceramides consist of a sphingoid long chain base (LCB) to which a fatty acid is linked by an amide bond at C-2. The LCB contains two chiral carbon atoms (carbons 2 and 3). Natural SLs occur in the *D*-erythro (2S, 3R) configuration, but three additional stereoisomers exist, *L*-erythro (2R, 3S) (the enantiomer of *D*-erythro-), *D*-threo (2R, 3R) and *L*-threo (2S, 3S). In a study which evaluated the stereospecificity of CerS by *in vitro* analysis

in subcellular fractions as well as in cultured cells, it was shown that the *L*-erythro enantiomers of sphinganine do not act as substrates for the CerS, even though the diastereoisomer, *L*-threo sphinganine, was rapidly metabolized (Venkataraman and Futerman 2001).

SPT, the first enzyme in the SL biosynthesis pathway, determines the chain length of the LCBs. Various LCBs occur in SLs, although for many years the most abundant species in most tissues, d18:1, was considered the main LCB in most tissues. With rapid advances in lipidomics, it is now apparent that there is much more variety in the structure of LCBs than previously appreciated. For instance, in human plasma, d16-LCBs comprise as much as 15% of total circulating sphingoid bases and have been suggested as potential biomarkers for type 2 diabetes (Hornemann et al. 2009; Othman et al. 2012). A study to test the LCB specificity of the CerS isoforms indicated that while CerS2, CerS4, CerS5 and CerS6 all utilize d18-sphinganine more readily than d16-sphinganine, CerS1 displays higher activity towards d16-sphinganine (Russo et al. 2013). The role of d16-ceramides remains unknown. In general, most SL researchers have largely overlooked the role of non-canonical LCBs, but recent work suggests that more studies should be focused on these non-canonical LCBs, not least a systematic analysis of the specificity of the CerS towards LCB variants, so as to determine how their incorporation into ceramides and complex SLs is regulated.

4.1.4.3 Acyl-CoA Specificity Is Determined by the Last Loop of the TLC Domain

The first mammalian CerS was identified when the CerS1 gene (*uog1*) was over-expressed in cultured cells, resulting in an increase in C18-ceramide synthesis and in C18-ceramide (and C18-SL) levels (Venkataraman et al. 2002). Later, it was demonstrated that overexpression of each CerS led to an increase in a specific subset of ceramides containing a unique fatty acid composition (Table 4.1). Thus, CerS1 uses mostly C18 acyl-CoA (C18-CoA) (Venkataraman et al. 2002), CerS4 uses C18- and C20-CoAs (Riebeling et al. 2003), CerS5 and CerS6 use mostly C16-CoA (Mizutani et al. 2005; Lahiri and Futerman 2005), CerS3 uses very-long chain acyl-CoAs (C26 and higher) (Mizutani et al. 2006); CerS2 can utilize a wider range of fatty acyl-CoAs, from C20 to C24 (Laviad et al. 2008). In order to investigate CerS acyl-CoA specificity, chimeric proteins, which combined sequences from CerS2 and CerS5, were generated (Tidhar et al. 2012). A chimeric CerS5/2 protein containing the first 158 residues and the last 83 residues of CerS2 displayed specificity toward C16-CoA similar to that of CerS5. Likewise, a chimeric CerS2/5 protein containing the first 150 residues and the last 79 residues of CerS5 displayed specificity toward C22-CoA similar to that of CerS2. These and additional results demonstrate that a 150-residue region within the TLC domain is sufficient for determining CerS acyl-CoA specificity.

More recently, the region involved in determining specificity was narrowed-down significantly to an 11-amino acid sequence in a loop putatively located between the last two TMDs of the CerS (Tidhar et al. 2018). The specificity of a chimeric protein based on the backbone of CerS5 (which produces C16-ceramide), but containing 11 residues from CerS2 (which synthesizes C22–C24-ceramides), was altered such that it generated C22–C24 ceramides. Moreover, chimeric CerS4 proteins with either CerS2 or CerS5 sequences in the same region, displayed a significant elevation in activity towards C24:1-CoA and C16-CoA respectively. Structurally, it was suggested that this short loop may restrict the movement of adjacent transmembrane domains, which may cause a conformational change in the membrane. Further examination of this loop indicated that the loop in CerS5 and CerS6 consists of 15 and 16 residues, in CerS1 and CerS4, 21 and 20 residues respectively, and in CerS2 and CerS3, only 11 and 9 residues (Fig. 4.1a). Hence, CerS that utilize the longest acyl-CoAs have the shortest number of residues in this loop. This finding led to the hypothesis that CerS which utilize shorter acyl-CoAs may have a longer and more flexible loop, permitting transmembrane flexibil-

ity and proximity, which constricts them to using shorter length acyl-CoAs (Tidhar et al. 2018).

4.1.4.4 CerS2 Contains a S1P Receptor-Like Motif

A sphingosine 1-phosphate (S1P) receptor-like motif has been identified within the TLC domain of CerS2. S1P, a bioactive SL, binds this motif and thus regulates the activity of CerS2. S1P can therefore be classified as a low affinity, non-competitive inhibitor of CerS2. Two charged residues (Arg-230 and Arg-325 in both human and mouse CerS2) were reported to regulate this inhibition (Laviad et al. 2008), perhaps suggesting an important interplay between two SLs that could be relevant to the regulation of SL metabolism, and relevant to the opposing functions that these lipids play in signaling pathways such as cell proliferation, migration, and survival.

4.1.5 The CerS C-Terminus Faces the Cytosol and Contains Phosphorylation Sites

The sequence of CerS1 indicates that there are several putative phosphorylation sites, and when tested *in vivo*, CerS1 was phosphorylated by protein kinase C (Sridevi et al. 2009). Furthermore, in an additional study, CerS2-6 were phosphorylated at the C-terminus (Sassa et al. 2016). Most of the phosphorylated residues were part of a consensus motif for phosphorylation by casein kinase 2 (CK2), and treatment of cells with the CK2-specific inhibitor, CX-4945, lowered the phosphorylation levels of CerS2, 4, 5, and 6. Phosphorylation of CerS2 was especially important for its catalytic activity, acting mainly by increasing its V_{max}. Dephosphorylation of endogenous CerS in the mouse brain led to reduced activity toward the CerS2 substrates, C22:0/C24:0-CoAs. This suggests that the phosphorylation of CerS may be a key regulatory point in the control of the distribution and levels of SLs of various acyl-chain lengths. Interestingly, no potential phosphorylation sites were predicted in the C-terminal region of CerS1. Since CK2 is located in the cytoplasm (Ahmad et al. 2008), these findings are consistent with an earlier report suggesting that the C-terminus of CerS6 faces the cytosol (Mizutani et al. 2005) (Fig. 4.1b).

4.2 CerS Regulation

In addition to using different length acyl-CoAs as substrates, CerS differ in their tissue distribution, which presumably regulates the formation of ceramides and complex SLs with defined acyl chain lengths in these different tissues. CerS mRNA distribution in mouse tissues shows significant variability (Laviad et al. 2008) (Table 4.1). Unfortunately, high quality antibodies against CerS are for the most part not commercially available, and therefore systematic studies of CerS protein distribution have not been performed. Such studies are critical because there is little correlation between CerS mRNA levels and the SL distribution within specific tissues, as shown over 10 years ago (Laviad et al. 2008). qPCR analysis demonstrated that CerS2 mRNA was found at the highest level of all CerS and has the broadest tissue distribution. CerS2 displays wide acyl-CoA specificity, showing no activity using C16:0-CoA and very low activity using C18:0, rather using longer acyl-chain-CoAs (C20–C24) for ceramide synthesis. Although CerS2 mRNA levels are high, this does not always correspond to CerS2 activity levels, suggesting post translational regulation of CerS. This regulation remains poorly understood, although since the study in 2008, various modes of post-translational regulation of CerS activity have been demonstrated experimentally, such as phosphorylation, glycosylation, and homo- and hetero-dimerization. While glycosylation and phosphorylation have been shown to take place at the N- and C-termini of the various CerS (see Sects. 4.1.1 and 4.1.5), there is no empiric data defining the localization of the dimerization sites or inhibition sites of CerS (with the exception of S1P inhibition of CerS2, see Sect. 4.1.4.4). Below, we summarize what is currently known about the mechanisms of CerS regulation.

4.2.1 CerS Dimerization

It has been proposed that CerS exist as hetero-complexes and can form both homo- and heterodimers. Upon over-expression of CerS2 with either CerS5 or CerS6, dimers are formed and CerS2 activity is up-regulated (Laviad et al. 2012). Moreover, it was shown that both CerS2 and CerS5 activity was inhibited when co-expressed with a catalytically-inactive CerS5, implying that activity of one member of a heterodimer depends upon, and can be modulated by the activity of the other members. This supports a potentially rapid and reversible mode of regulation of ceramide synthesis. To further examine this, the activity of each CerS was assayed upon co-expression with other CerS (Table 4.2). CerS1 and CerS2 activities were up-regulated by CerS4-6 while CerS3 activity was unaffected upon co-expression of other CerS. CerS4 activity was upregulated by CerS2-3. When co-expressing CerS5 with CerS6, an elevation in activity was observed. However, since CerS5 and CerS6 utilize the same acyl-CoA, it is not possible to distinguish between their activities. While CerS5 activity was not influenced by other CerS, CerS6 was up-regulated upon co-expression with CerS3 and CerS4. These results indicate that most CerS can be regulated by dimerization.

Of all the mammalian CerS, CerS2 activity is the most significantly up-regulated. Based on *in vitro* studies, CerS2 is less active enzymatically, requiring a longer reaction time and more protein to obtain similar levels of enzymatic activity to the other CerS (as discussed in (Tidhar et al. 2018)). This is somewhat surprising, since C22-C24:1-SLs are found at high levels in many tissues, even though enzyme activity, at least when assayed *in vitro*, is much lower than the other CerS. We suggest that CerS2 might be rapidly regulated by dimerization *in vivo* to up-regulate its activity. Thus, in cases where large quantities of C22-C24:1-ceramides are required, dimerization could play an important role in the activation of CerS2 and the generation of very-long-chain SLs. Studies are currently ongoing to determine dimerization sites and to determine whether formation of higher complexes, such as trimers, quatromers and even complexes of higher orders, may also exist. In fact, IMPs, which span the membrane multiple times, are often organized in functional complexes and form homo- or hetero-oligomeric assemblies (Cymer and Schneider 2012).

Table 4.2 Dimerization affects enzymatic activity of CerS1-6

CerS	1	2	3	4	5	6
1 (C18)	–	1.4 ± 1.0	4.8 ± 2.8	8.5 ± 0.3***	9.3 ± 1.3***	9 ± 1.1***
2 (C24:1)	0.8 ± 0.8	–	5.1 ± 4.7	8 ± 2*	11.8 ± 8.7***	16.3 ± 9.1***
3 (C26)	0.7 ± 0.2	0.9 ± 0.7	–	0.7 ± 0.7	1 ± 0.6	0.9 ± 0.3
4 (C20)	1.2 ± 0.8	5.5 ± 3.3*	9.8 ± 7.9*	–	6.1 ± 7.0	6.9 ± 6.4
5 (C16)	1.1 ± 1.2	0.4 ± 0.4	1.8 ± 1.1	1.2 ± 1.6	–	2.2 ± 0.5
6 (C16)	1.2 ± 0.8	1.9 ± 0.7	3.5 ± 0.5*	3 ± 0.7*	3.2 ± 0.3*	–

CerS activity was assayed using the indicated acyl-CoA for each of the human CerS upon co-expression with other CerS. Data is represented as fold-change compared to the activity of the monomeric CerS ± SD. ∗, $p < 0.05$; ∗∗∗, $p < 0.001$

4.2.2 CerS Inhibition

Over the years, a variety of inhibitors of the CerS have been described (Table 4.3). The first inhibitor identified, Fumonisin B1 (FB1), was initially described in 1988 (Bezuidenhout et al. 1988) after two decades of research aimed to explain the high incidence of esophageal cancer in certain villages in South Africa (Merrill et al. 1996). Fumonisins are a class of mycotoxins produced by fungi that are common contaminants of maize (*Zea mays*), sorghum and related grains throughout the world. FB1, the most prevalent species, was shown to act as a natural, potent competitive inhibitor of the CerS enzymes, inhibiting the *N*-acylation of both sphinganine and sphingosine (Wang et al. 1991; Merrill et al. 1993). Inhibition of ceramide biosynthesis by FB1 differentially affects the relative formation of different SLs downstream to ceramide production (Merrill et al. 1993) and causes sphinganine to accumulate (Wang et al. 1991), thus increasing the formation of sphinganine l-phosphate and cleavage of the sphingoid base backbone to fatty aldehydes and ethanolamine l-phosphate (Smith and Merrill 1995). FB1, as well as its hydrolyzed form, HFB1, can act as LCB substrates and be *N*-acylated by different CerS to form acylated metabolites of various chain lengths (Humpf et al. 1998; Seiferlein et al. 2007). Interestingly, HFB1 acts as a better substrate of CerS compared to the non-hydrolyzed form (Harrer et al. 2013). This might be due to the lack of the tricarboxylic acid moieties in HFB1, which may allow better access to the active site. Surprisingly, the acylation of HFB1 does not detoxify hydrolyzed fumonisins (Seiferlein et al. 2007). Moreover, the acylated FB1 is significantly more cytotoxic than the non-acylated precursor, suggesting an important contribution to the cytotoxicity of FB1 (Harrer et al. 2013).

The sphingosine analog, FTY720, is a multi-target inhibitor, affecting the activity of S1P lyase (Bandhuvula et al. 2005), cytosolic phospholipase A2 (Payne et al. 2007) as well as CerS (Lahiri et al. 2009). It acts as an uncompetitive inhibitor towards the sphinganine substrate of CerS, suggesting there may be two sphinganine binding sites that act allosterically with respect to one another, or that CerS dimers interact allosterically (Lahiri et al. 2009). *In vivo*, FTY720 is rapidly phosphorylated by sphingosine kinase 2 (Billich et al. 2003; Kharel et al. 2005) to form FTY720 phosphate (FTY720-P), an analog of S1P. FTY720-P binds the S1P receptor (Mandala et al. 2002; Brinkmann et al. 2002) and induces a variety of occurrences including T-lymphocyte

Table 4.3 CerS inhibitors

Name	Chemical formula	Structure	CerS inhibited	Mode of inhibition	Acylated by CerS	References
FB1	$C_{34}H_{59}NO_{15}$		CerS1-6	Competitive	+	Wang (1991) and Merrill (1993)
FTY720	$C_{19}H_{33}NO_2$		CerS1, CerS2, CerS4, CerS5	Noncompetitive toward acyl-CoA and uncompetitive inhibition toward LCB	–	Lahiri (2009)
Jaspine B	$C_{18}H_{37}NO_2$		CerS1-2, CerS4-6	Competitive	+	Cingolani (2017)
P053	$C_{18}H_{21}Cl_2NO_2$		CerS1	Non competitive	N/A	Turner (2018)
S1P	$C_{18}H_{38}NO_5P$		CerS2	Non competitive	N/A	Laviad (2008)

A list of known CerS inhibitors including their chemical formula, structure, inhibition mode as well as their ability to be acylated by CerS. FB1, fumonisin B1; FTY720, Fingolimod; S1P, sphingosine1-phosphate

migration (Zhang and Schluesener 2007; Kihara and Igarashi 2008). While S1P only inhibits the activity of CerS2, FTY720 inhibits most CerS.

A variety of ceramide species are implicated in numerous pathologies including cystic fibrosis (Grassmé et al. 2013), cardiovascular pathologies (Yu et al. 2015; Laaksonen et al. 2016), cancer (Saddoughi and Ogretmen 2013; Jensen et al. 2014) and epilepsy (Mosbech et al. 2014), hence, a number of laboratories are attempting to identify inhibitors of individual CerS. Recently, a non-phosphorylatable analog of FTY720 was characterized (Turner et al. 2018). Unlike FTY720, P053 selectively downregulated CerS1 activity resulting in a reduction of C18-ceramide levels in cultured cells and mouse skeletal muscle, making it the first potent, isoform-selective CerS inhibitor. Although CerS1 is highly expressed in the brain (Table 4.1), P053 failed to show any effect on brain ceramide levels in mice, presumably due to its inability to cross the brain blood barrier, or possibly due to a lower rate of SL turnover in the brain.

An additional sphinganine analog, Jaspine B, a cyclic anhydrophytosphingosine naturally found in marine sponges, also acts as a significant inhibitor of all CerS studied (Cingolani et al. 2017). This inhibition results in the accumulation of free LCBs and alteration of the cellular lipidome. Interestingly, Jaspine B was shown to induce bulk cellular vacuolation and cell death in a non-apoptotic and non-autophagic manner. This vacuolation process was shown to occur in additional cancer cell models, suggesting the generality of this effect.

4.2.3 Transcriptional Regulation of CerS

As discussed above, mRNA levels do not always correspond to levels of CerS activity (Laviad et al. 2008), and moreover, little is known about the regulation of mRNA levels *in vivo*. In a preliminary study, we examined the role of micro-RNAs (miRs) on CerS expression. miRs are small non-coding RNAs that bind to target mRNAs and act as gene repressors, regulating expression in cells and tissues. miR-124, which is known to have a significant impact on neuronal differentiation (Maiorano and Mallamaci 2010), as well as being a tumor-suppressor (Izzotti et al. 2009), was predicted to target CerS2 by homology to a 3′-untranslated region. Indeed, when expressing miR-124 in HEK cells, down-regulation of CerS2 mRNA and protein levels was observed (Fig. 4.4a, b). This effect was specific to CerS2 and was not observed with any of the other CerS. The specificity of miR-124 suggests CerS2 has a unique site which allows this negative regulation.

Since most mammalian miRs silence their target genes by preventing translation rather than altering transcript levels, it is somewhat surprising that mRNA levels of CerS2 were altered, which may imply a possible mechanism of silencing by destabilization of the CerS2 mRNA via cleavage of the poly-A tail, which targets the mRNA for degradation. miR-124 is crucial for cell fate in differentiation to either neuronal or glial cells (Maiorano and Mallamaci 2010) and CerS2 expression is negatively correlated to miR-124 expression in these two cell types (Becker et al. 2008), reinforcing our finding and the importance of this regulation *in vivo*. Unexpectedly, despite the reduction in mRNA levels, CerS2 *in vitro* enzymatic activity was elevated twofold subsequent to miR-124 regulation (Fig. 4.4c). miR-124 is known to downregulate the activity of sphingosine kinase 1 (Xia et al. 2012), the key enzyme in S1P synthesis, and S1P has been shown to inhibit CerS2 (Sect. 4.1.4.4). The broad tissue distribution of CerS2, along with studies from CerS2 null mice (Ben-David et al. 2011; Silva et al. 2012; Park et al. 2013), suggest that CerS2 requires tight regulation, and maintenance of its activity might be crucial for cell viability.

4.3 Summary and Conclusions

CerS are essential enzymes in the SL biosynthetic pathway. Studying SLs, their metabolism, and their biological and physiological signifi-

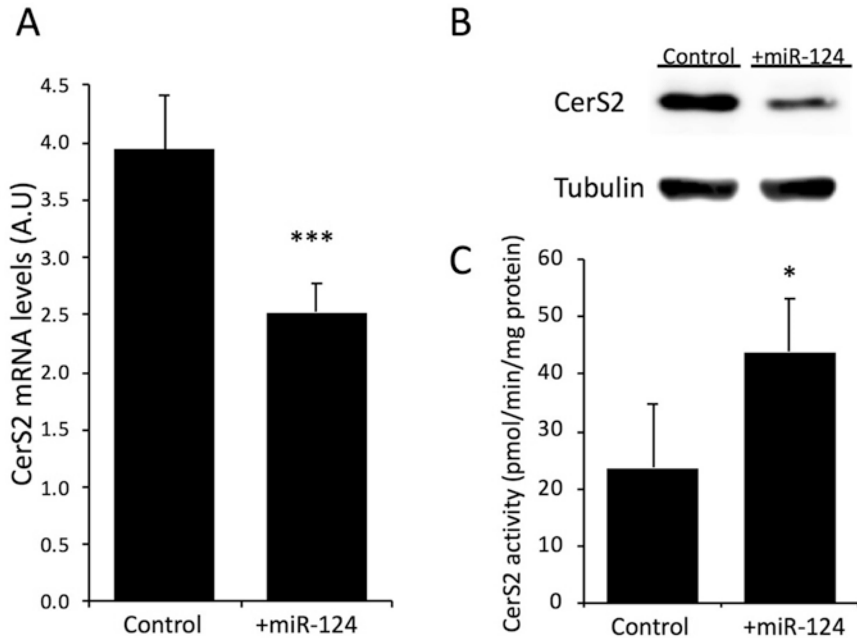

Fig. 4.4 CerS2 is regulated by miR-124. (**a**) CerS2 mRNA levels in HEK cells transfected with miR-124 or a vector control. The results were normalized to TBP levels. Values are means ± SD n = 4. ∗∗∗, $p < 0.001$. (**b**) CerS2 expression was ascertained by Western blotting using an anti-CerS2 antibody. An anti-tubulin antibody was used as a loading control. (**c**) *In vitro* assay of CerS2 activity in HEK cells transfected with miR-124 or an empty vector. Values are means ± SD n = 3. ∗, $p < 0.05$

cance is crucial for answering key questions regarding many pathologies. However, studies examining the structural features of CerS are few and far between. In this chapter, we have documented what is known about CerS structure and function, and have attempted to compile a global picture of the known domains, motifs, topology as well as regulating mechanisms of the CerS enzymes. Although different CerS act on distinct substrates, they all share the same enzymatic activity and exhibit high sequence similarity as well as shared domains. This implies structural similarity within this enzyme family. Resolving the three-dimensional structure of the CerS enzymes is imperative in order to develop a more thorough understanding of CerS activity and regulation. Recent studies have made it clear that a substantial and basic understanding of the CerS enzymatic properties is key to cracking the enigma of the significance of ceramides and other SLs in metabolism and pathology.

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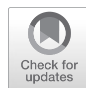

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The Role of Ceramide 1-Phosphate in Inflammation, Cellular Proliferation, and Wound Healing

Melissa L. Berwick, Brittany A. Dudley, Kenneth Maus, and Charles E. Chalfant

Abstract

The phospho-sphingolipid, ceramide 1-phosphate (C1P), has long been implicated as a dynamic bioactive agent. Over two decades of research has begun to characterize various regulatory roles for C1P from mammalian inflammatory response and wound healing to cellular proliferation and survival. As a metabolite of the intricately balanced "sphingolipid rheostat", C1P stands as a crucial physiological regulator of both upstream and downstream mechanisms. This chapter serves as an overview of established and implicated roles for C1P in cellular processes vital to diseases and mammalian physiology. Additionally, we discuss potential clinical roles for C1P in cancer treatment, wound therapy, and pre-disease diagnosis. While many questions remain regarding C1P metabolism and the extent of signaling factors targeted by this bioactive lipid, new technologies and methodologies show great promise to discern key targets, signaling pathways, and physiologies regulated by C1P.

Keywords

Ceramide 1-phosphate · Proliferation · Wound healing · Cell signaling · Cell survival · Sphingolipids · Phospholipases · Inflammation

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Abbreviations

AA	arachidonic acid
A-SMase	acid sphingomyelinase
BMDMs	bone marrow-derived macrophages
C1P	ceramide 1-phosphate
CaLB domain	Ca^{2+}-dependent lipid binding domain
CerK	ceramide kinase
CERT	ceramide transport protein

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cPLA2α	cytosolic phospholipase A2 alpha
CPTP	C1P transport protein
COX	cyclooxygenases
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERK1/2	extracellularly regulated kinases 1 and 2
IL-1β	interleukin-1 beta cytokine
LPPs	lipid phosphate phosphatases
LTB	leukotriene B
NF-kB	nuclear factor kB
NSAIDs	non-steroidal anti-inflammatory drugs
PC	phosphatidylcholine
PCERA-1	a synthetic C1P analog
PGE2	prostaglandin E2
PI3-K	phosphatidylinositol 3-kinase
PRP	platelet-rich plasma
S1P	sphingosine 1-phosphate
SM	sphingomyelin
SMase	sphingomyelinase
SphKs	sphingosine kinases
SPT	serine palmitoyl transferase
TGN	*trans*-Golgi network
TNFα	tumor necrosis factor alpha

5.1 Introduction

5.1.1 Ceramide 1-Phosphate

Ceramide 1-phosphate (C1P), a sphingoid analog of phosphatidic acid, is one of the metabolites in the sphingomyelin (SM) cycle (Fig. 5.1). C1P is formed from ceramide by the action of a specific ceramide kinase (CerK), which is related to, but distinct from the sphingosine kinases (SphKs) that synthesize sphingosine 1-phosphate (S1P) (Sugiura et al. 2002; Wijesinghe et al. 2007). Indeed, CerK was found to contain the five conserved domains (C1–C5) previously identified for Sphk1 and 2, but CerK showed complete substrate specificity for ceramide with no enzymatic activity observed for sphingosine. In animal tissues, the substrate ceramide is derived primarily from the hydrolysis of SM and the *de novo* sphingolipid biosynthesis pathway, which begins via the initial condensation of serine and palmitoyl-CoA (Chalfant and Spiegel 2005). Due to the interconvertibility of ceramide, C1P, sphingosine and S1P, the SM cycle is also referred to as a "sphingolipid rheostat" that can regulate immune cell function, cell survival, and inflammatory response (Maceyka et al. 2002).

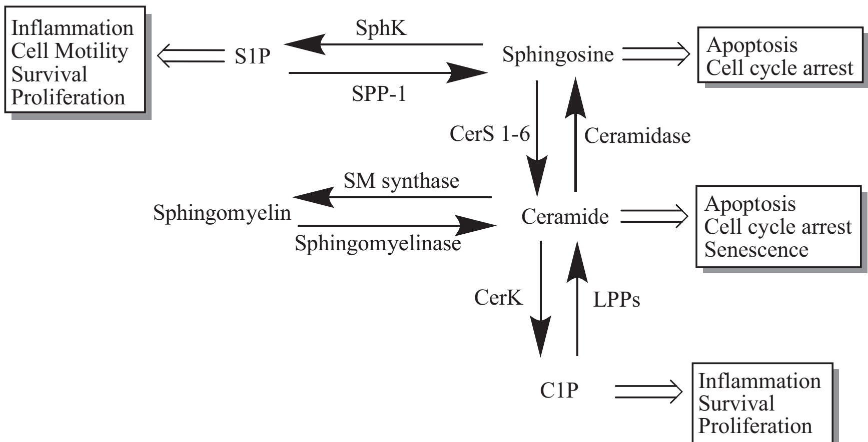

Fig. 5.1 Simplified schema demonstrating the interconvertibility of ceramide, C1P, sphingosine, and S1P in the sphingomyelin cycle, creating the "sphingolipid rheostat"

C1P has been explicitly implicated in propagation and regulation of the wound healing and inflammatory processes (Stephenson et al. 2017).

In regard to inflammatory processes, the production of arachidonic acid (AA) is an early response to various inflammatory agonists such as mechanical trauma associated with wounding. AA liberation by phospholipases is the initial rate-limiting step in the biosynthesis of eicosanoids (Clark et al. 1995), which are well-established mediators of inflammation (Chalfant and Spiegel 2005). The major phospholipase that regulates eicosanoid synthesis in response to inflammatory cytokines is group IVA cytosolic phospholipase A_2 alpha (cPLA$_2$α).

A decade of research from our laboratory has demonstrated that C1P produced by CerK is a proximal mediator of PLA$_2$ activation and subsequent AA release in response to inflammatory cytokines, interleukin-1 beta cytokine (IL-1β) and tumor necrosis factor alpha (TNFα), calcium ionophore, and ATP. Activation and translocation of cPLA$_2$α in cells requires the association of the enzyme with membranes in a calcium-dependent manner via a Ca^{2+}-dependent lipid binding (CaLB) domain located near the N-terminus. Published findings from our laboratory demonstrated that C1P is a direct activator of cPLA$_2$α through specific interaction with the C2/CaLB domain (Pettus et al. 2004; Lamour et al. 2009), which enhances the membrane affinity for cPLA$_2$α via decreasing the dissociation rate of the enzyme from phosphatidylcholine (PC)-rich membranes (Subramanian et al. 2007). These results coupled with the previous findings that CerK/C1P pathway is required for cPLA$_2$α activation in response to calcium ionophore and inflammatory cytokines (Pettus et al. 2003) demonstrated that C1P was a "missing link" in the eicosanoid biosynthetic and regulatory pathways (Lamour and Chalfant 2005).

5.1.2 The Biosynthesis of Ceramide 1-Phosphate in Mammalian Cells

In mammalian cells, the precursor molecule of C1P, ceramide, is synthesized in the endoplasmic reticulum (ER) and transferred to the Golgi apparatus by either ceramide transport protein (CERT) in an active manner or passive vesicular trafficking (Lamour et al. 2007; Boath et al. 2008; Wijesinghe et al. 2010). Like activated cPLA$_2$α, CerK is primarily associated with membranes such as the cytosolic surface of the *trans*-Golgi network (TGN) as well as early and late endosomes (Lamour et al. 2007). This allows C1P to be generated in the appropriate cellular compartment for recruitment of cPLA$_2$α in response to inflammatory agonists (Carre et al. 2004). CerK utilizes ATP as the phosphate donor and requires calcium ion interaction via a calmodulin-binding motif (Sugiura et al. 2002). In mammals, the only established pathway for C1P production is through phosphorylation of ceramide by CerK. CerK recognizes ceramide in a stereospecific manner, requiring a minimum of a 4-carbon acyl chain and a 4-5 *trans* double bond (Wijesinghe et al. 2005). Additionally, the free hydrogen of the secondary amide group and the sphingoid chain are necessary for substrate recognition by CerK. While the saturation state of the fatty acyl chain appears not to affect specificity, methylation of the primary or secondary hydroxyl groups decreased the phosphorylative activity of CerK. Combined, these factors indicate a very high specificity for substrate recognition by CerK for ceramide and not chemically similar compounds such as sphingosine, diacylglycerol, and dihydroceramide.

A membrane-bound C1P transport protein designated "CPTP" is also an essential component in the metabolism of C1P (Fig. 5.2). CPTP operates in a C1P concentration-dependent manner to maintain a constant level of C1P in the Golgi membrane and transfers the lipid to the plasma membrane or other cellular compartments as required (Simanshu et al. 2013). Our laboratory in collaboration with Brown, Patel, and colleagues demonstrated that C1P is in constant flux within the cell and is not catabolized locally requiring CPTP to move C1P to specific cellular locations for rapid catabolism. For example, in CPTP-depleted cells, marked increases in AA levels and their downstream metabolites indicated C1P accumulation at the Golgi/TGN, which was confirmed by sphingo-

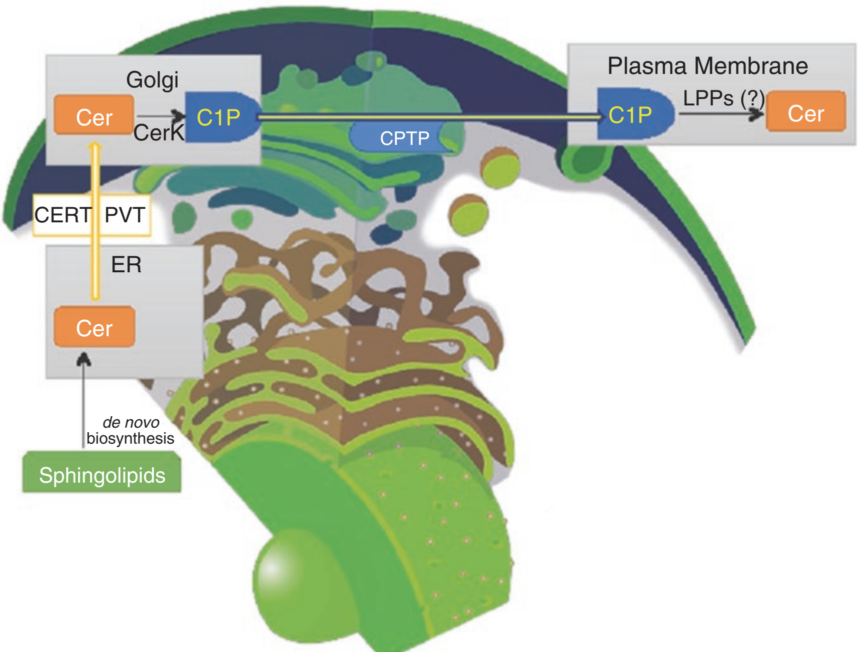

Fig. 5.2 Ceramide 1-phosphate metabolism. Sphingolipids are synthesized to produce ceramide in the endoplasmic reticulum (ER) via *de novo* biosynthesis. Ceramide (Cer) is transported from the ER throughout the cell, but primarily to the Golgi apparatus by either passive vesicular trafficking (PVT) or actively via the ceramide transport protein (CERT). Ceramide is then phosphorylated by CerK to create C1P, which is then transported to the plasma membrane and additional cellular membranes by CPTP. C1P may then be hydrolyzed to ceramide by lipid phospholipid phosphatases (LPPs)

lipidomics (Mishra et al. 2018). Additionally, siRNA-induced knockdown of CerK, and hence C1P production, restored the levels of C1P, AA, and eicosanoids to normal cells that were initially elevated by CPTP depletion (Simanshu et al. 2013). Conversely, AA and eicosanoid levels decreased upon overexpression of wild-type CPTP signifying rapid transport of C1P from the Golgi to other areas of the cell, which also led to a concomitant decrease in the total intracellular levels of C1P. The complete picture of C1P catabolism is not fully understood, but the current hypothesis suggests that lipid phosphate phosphatases (LPPs), specifically LPPs 1-3, may catabolize C1P to ceramide following transport of the lipid by CPTP to the plasma membrane (Figs. 5.1 and 5.2). However, the roles of other enzymes such as ceramidases remain possible contributors in the possible conversion of C1P to S1P.

In regard to the anabolism of C1P, research suggests that at least one additional anabolic pathway for C1P generation exists in mammalian cells as the genetic ablation of CerK only reduces the cellular context of C1P by approximately 50% (Mietla et al. 2014). One possible explanation for a significant amount of C1P remaining in these cells is the reported generation of C1P via acylation of sphingosine 1-phosphate (S1P) by an unknown lipid acylase (Binoda et al. 2004). Indeed, unpublished findings from our laboratory have shown that the exogenous addition of ^{32}P-labeled S1P to cells leads to the production of C1P, but the percentage of total cellular C1P attributed to this anabolic pathway is currently unknown and understudied.

Another possible route of C1P anabolism is via a sphingomyelinase D (SMase D), which is found in the venom of the brown recluse spider and related species. Indeed, this form of SMase D

hydrolyzes SM to produce C1P and cyclic C1P (Hoeferlin et al. 2013), which is associated with an inflammatory response mediated by prostaglandins and AA following the bite from this species of spider. Although a SMase D activity has not been described in mammalian cells, endogenous C1P production by an enzyme with SMase D activity is possible. To our knowledge, neither PLD1 nor PLD2 have been extensively studied for enzymatic activity towards SM, and even minimal activity of these enzymes toward SM would produce significant amounts of C1P as the levels of this bioactive lipid are very low in most cellular contexts. As with the examination of an S1P acylase, this research area of lipid metabolism is also understudied.

5.2 The Generation of Eicosanoids and Induction of the Inflammatory Response by Ceramide 1-Phosphate

At the membrane, bioactive lipids serve to anchor cPLA$_2$α, and the enzyme then proceeds to release sn2-unsaturated fatty acids (AA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA)) from membrane phospholipids. Through interactions with cyclooxygenases, cytochrome P450, or lipoxygenases, AA is then converted into pro-inflammatory mediators (Fig. 5.3) while released EPA and DHA are converted to primarily anti-inflammatory lipid mediators (Stephenson et al. 2017). Research in over a dozen different cell

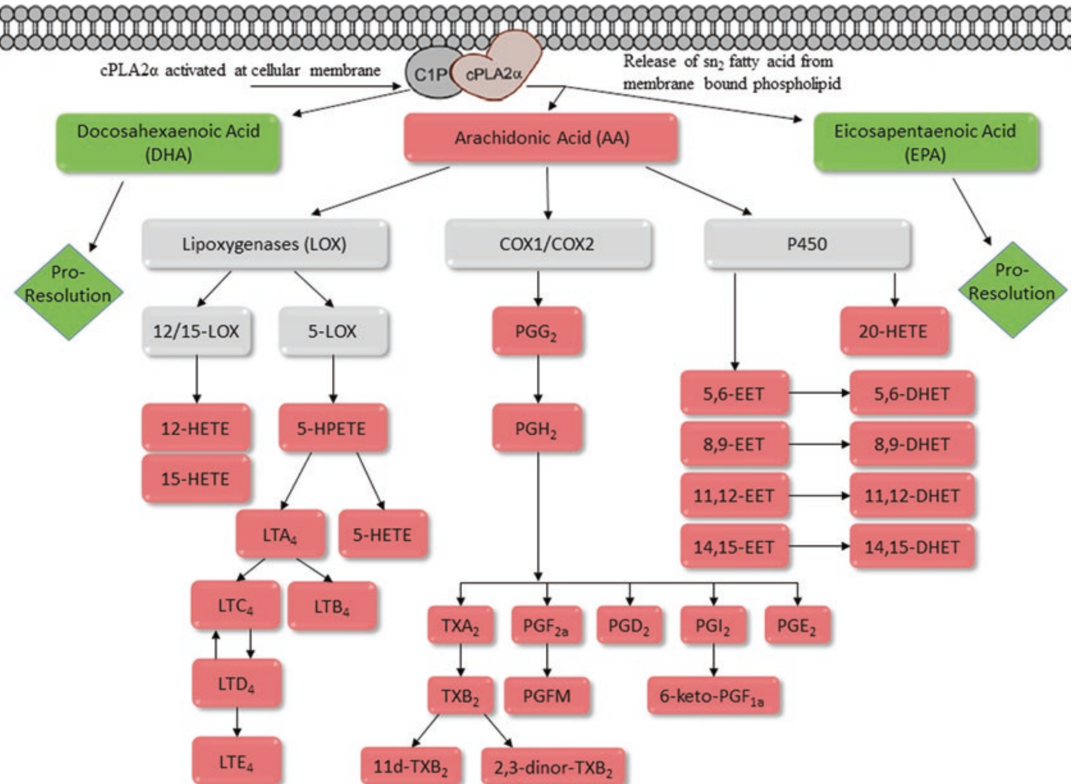

Fig. 5.3 Localization of C1P-cPLA$_2$α interaction at the cellular membrane generates AA-derived eicosanoids that tend to be pro-inflammatory mediators (indicated in red). Downstream products of DHA and EPA-derived eicosanoids are anti-inflammatory/pro-resolution (not shown)

types has shown that nanomolar concentrations of C1P induce AA release and the synthesis of eicosanoids (Stephenson et al. 2017, Pettus et al. 2003). Further exploration demonstrated that this effect was also lipid-specific as the closely related lipids, phosphatidic acid, ceramide, diacylglycerol, and S1P had either minimal or no effects on AA release and prostanoid synthesis. Experimentation with siRNA downregulation of cPLA$_2$α demonstrated that the induction of AA release by C1P was strictly dependent on cPLA$_2$α activation (Pettus et al. 2004). Indeed, the colocalization of cPLA$_2$α and C1P to the cellular membrane is required for the production of eicosanoids linked to inflammation (Lamour et al. 2007).

Inflammation is a localized immune response as a consequence to infection or injury. The inflammatory response is achieved in part by eicosanoid synthesis, resulting in the formation of prostaglandins and leukotrienes. Common non-steroidal anti-inflammatory drugs, such as ibuprofen, target these prostaglandin biosynthetic enzymes known as cyclooxygenases (COX-1 and -2). Inhibition of these enzymes blocks the conversion of AA to inflammatory prostaglandins (Botting and Botting 2004; Pettus et al. 2005). As first noted earlier in this chapter, the production of these eicosanoids is initially mediated via cPLA$_2$α, which is activated by translocation of the enzyme to the cell membranes requiring direct association with C1P (Fig. 5.3). Indeed, cPLA$_2$α is translocated to the membrane of the Golgi and binds to PC in a calcium-dependent manner. C1P directly interacts with cPLA$_2$α and decreases the dissociation rate of the enzyme from PC-rich membranes, thereby increasing the residence time of the enzyme and subsequent hydrolysis of zwitterionic phospholipids at the sn-2 position producing AA and driving eicosanoid biosynthesis (Lamour and Chalfant, 2008). Our laboratory showed the high specificity of the physical interaction between C1P and the C2 domain of cPLA$_2$α. For example, other structurally similar lipids (e.g., ceramide and S1P) were incapable of activating cPLA$_2$α *in vitro* or in cells as well as bind to the enzyme with substantial affinity (Hoeferlin et al. 2013).

The requirement for C1P association with cPLA$_2$α to activate eicosanoid synthesis was further explored by our laboratory, and the allosteric activity of C1P was substantiated to regulate cPLA$_2$α association with PC-rich vesicles by the novel β-groove of cPLA$_2$α. Specific amino acids in this region were identified to be required for bioactive sphingolipid interaction with C1P. This assertion was demonstrated by inducing mutations in the basic amino acids in the β-groove (Stahelin et al. 2007). Indeed, mutations in Arg57, Lys58, and Arg59 within this domain resulted in inhibition of the activation of cPLA$_2$α by C1P. Further exploration by Stahelin and colleagues revealed that cPLA$_2$α/C1P interaction also required Arg61, and His62 (Ward et al. 2013). These studies were the first to assess a specific interaction site for C1P interaction with a specific target protein.

C1P also regulates the inflammatory response via modulation of cytokine maturation/secretion, specifically TNFα. Excessive TNFα is found in sepsis, thereby resulting in an accumulation of downstream inflammatory components and subsequent uncontrolled immune responses. Our laboratory showed that CerK through the production of C1P acts as a negative regulator in the production of TNFα by blocking the proteolytic conversion of pro-TNFα to the active form of the cytokine (Lamour et al. 2011). More specifically, isolates of BMDMs from CerK knockout mice presented with elevated levels of TNFα and the activity of its proteolytic converting enzyme, TACE, in comparison to wild type cells when challenged with the inflammatory agonist, lipopolysaccharide (Goldsmith et al. 2011). The reduced TNFα secretion observed in the cells from the CerK knockout mice could be reverted to wild type levels via re-expression of CerK (Lamour et al. 2011). In mechanistic studies, our laboratory showed that C1P served to directly bind and inhibit TACE, and thus, the maturation of TNFα, but it is currently unknown as to the exact location of these C1P binding sites although a consensus amino acid sequence similar to the known C1P binding site in cPLA$_2$α was apparent. This seemingly opposing role for C1P in the inflammatory response is logical in which C1P

both induces the inflammatory response via activating cPLA$_2$α and inducing eicosanoids, and also limits the inflammation from cascading out of control by blocking the maturation and secretion of TNFα.

5.3 Ceramide 1-Phosphate and Cell Survival

5.3.1 The Role of Ceramide 1-Phosphate in Cellular Proliferation

Of the many roles C1P plays in the regulation of cellular processes, major findings promote the idea that survival, proliferation, and apoptosis are among those that have been shown to impact human disease (Gómez-Muñoz et al. 2005; Mishra et al. 2019). Where ceramide is involved in inducing apoptosis, C1P provides the opposite effect and inhibits apoptosis. Indeed, our laboratory in collaboration with Spiegel and co-workers showed that at low concentrations C1P had a positive impact on cellular proliferation although high concentrations of C1P were cytotoxic to A549 lung carcinoma cells (Mitra et al. 2007). Another study by Gómez-Muñoz and co-workers (2004) revealed the same phenomenon in macrophages. This observed biphasic effect points to a "balancing system" inside the cell as high levels of C1P induced apoptosis due to catabolism by dephosphorylation creating increased levels of the apoptogenic sphingolipid, ceramide (Mitra et al. 2007). Hence, if there is any deviation from this balance between C1P and ceramide, the functioning metabolism of the cell may be compromised and lead to lethality (Arana et al. 2010). This metabolic balancing act within the cell as well as the differential partitioning of lipids added exogenously to cells makes definitive conclusions of cellular function for a specific sphingolipid, in many cases, elusive. Indeed, C1P and ceramides are implicated in a multitude of pathways that make use of many different components depending on concentration, location, and the type of cell being studied. This biochemical conundrum likely explains many of the discrepancies and overlap in activity and functions ascribed to these two bioactive lipid mediators in a number of published studies. Regardless, specific and possibly overlapping signal transduction pathways ascribed as regulated directly by C1P are discussed further in subsequent sections (Fig. 5.4).

Additionally, Gómez-Muñoz and co-workers have reported the possibility of cell surface receptors recognizing and mediating C1P interaction. Indeed, many of the effects of C1P on cell function (i.e., cellular proliferation) have been shown via exogenous C1P as discussed previously in this chapter. By applying C1P exogenously in cell culture, Gómez-Muñoz and co-workers observed stimulation of cell migration, a phenomenon which is independent of the production of intracellular C1P. These findings suggested a specific receptor for this biological function of C1P. Indeed, PKB and MAP kinases such as extracellularly regulated kinase (ERK) play a large role in C1P-mediated cellular migration, which may be linked to receptor activation (Granado et al. 2009). Thus, the effects on cellular signaling pathways may be receptor mediated, and Gómez-Muñoz and colleagues have provided strong evidence in this regard. In further investigations, a synthetic C1P analog known as PCERA-1 was utilized to identify the specific receptors that mediate C1P activity. Unfortunately, PCERA-1 was unable to interfere with C1P binding to the cell surface receptor, alluding to the possibility that C1P and PCERA-1 bind to separate cell surface receptors (Katz et al. 2016). Overall, the role of C1P in mediating cellular effects via activation of cell surface receptors is an area of intense investigation, but the lack of identifying the receptor has not yet allowed for wide-spread acceptance of the model.

5.3.2 Acid Sphingomyelinase

Acid sphingomyelinase (A-SMase) is a lipid hydrolase that converts SM to ceramide and phosphocholine (Wang et al. 2015). Deficiencies in

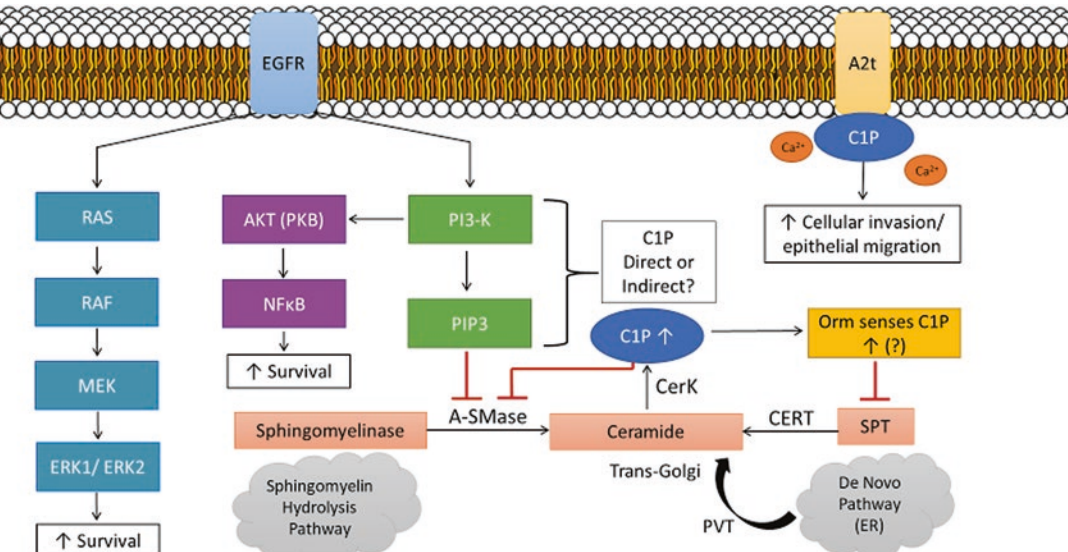

Fig. 5.4 C1P induced cell proliferation and survival pathways. Ceramide from the *de novo* and sphingomyelin hydrolysis pathways is converted into ceramide 1-phosphate (C1P) via phosphorylation by CerK. Increased concentrations of synthesized C1P can directly inhibit acid sphingomyelinase (A-SMase) and possibly serine palmitoyl transferase (SPT) via Orm sensors that detect increased C1P levels. Exogenous and endogenous C1P also influence cell proliferation and survival in a number of ways: (1) C1P activates EGFR signaling and the phosphatidylinositol 3-kinase (PI3-K) pathway either directly or indirectly, which induces cell survival and has also been shown to have an inhibitory effect on A-SMase; (2) Annexin A2t binds directly to C1P and drives cellular invasion and endothelial migration in the presence of calcium; and (3) C1P directly or indirectly interacts with and activates ERK1/2 to increase cell survival by enhancing the MAP kinase pathway. Individually, any one of these pathways result in proliferation and survival, and co-activation of combinations of these survival pathways could induce a heightened response

this catabolic sphingolipid enzyme are connected to a number of human diseases, from cardiovascular and neurodegenerative to cancers. In regard to C1P signaling and A-SMase, a previous study by Gómez-Muñoz and co-workers showed that apoptosis of primary bone marrow-derived macrophages (BMDMs) was impeded in the presence of natural C1P. Apoptotic BMDMs, a frequently used model to explore apoptosis, are known to have high levels of A-SMase as well as ceramide, which blocks survival pathways in favor of apoptosis. In opposition, the direct inhibition of A-SMase by C1P without the presence of growth factors, and in conjunction with the activation of the PI3-K/AKT signaling pathway, stimulates cell survival (Bornancin 2011). Indeed, the addition of C1P to the cell culture medium proved to increase cell viability when compared to sham controls (Gómez-Muñoz et al. 2005). C1P of differing chain lengths were also tested and showed increased viability, however not to the extent that of natural C1P (Gómez-Muñoz et al. 2005). Of note, the anabolism of C1P from specific ceramide species stemming from the six known ceramide synthases has not been examined, but interestingly, different chain lengths of ceramide derived from specific ceramide synthases have been attributed to specific and differing cell responses. For example, Ogretmen and co-workers have shown that D-e-$C_{16:0}$-ceramides produced by ceramide synthases 5 or 6 have anti-apoptotic properties, but D-e-$C_{18:0}$-ceramides, produced by ceramide synthases 1 and 4 are involved with cellular death (Ponnusamy et al. 2010). Stiban and Perera reported interference of very long chain ceramides ($C_{22:0}$ and $C_{24:1}$) on $C_{16:0}$-induced channel formation and vice versa (2015). Hence, one can hypothesize that different chain lengths of C1P may also be responsible for specific cellular physiologies.

As stated above, A-SMase showed high activity in BMDMs (Gómez-Muñoz et al. 2005). In mechanistic studies, Gómez-Muñoz and co-workers demonstrated that in the presence of C1P, A-SMase activity was inhibited. Furthermore, decreased levels of C1P were observed in apoptotic cells giving credence to the idea that A-SMase was no longer being inhibited by C1P, and C1P was an inhibitory factor for this pathway (Arana et al. 2010). Additional mechanistic studies demonstrated that inhibition of A-SMase occurs via a direct physical interaction with C1P that prevents the accumulation of ceramide, and thereby, contributes to the anti-apoptotic effect of C1P observed in mammalian cells. A later study also found that serine palmitoyltransferase (SPT), which is the initial and rate-limiting enzyme in the generation of ceramide via the *de novo* sphingolipid biosynthetic pathway, followed this pattern of anti-apoptosis through inhibition by C1P causing decreased levels of ceramide (Arana et al. 2010). To date, the mechanism by which C1P modulates SPT activity is unknown, but Orm proteins, known regulators of SPT activity and sphingolipid feedback sensors, may play a role. Indeed, Orm proteins may act as sensors for elevated C1P levels, and thereby, induce the inhibition of SPT to preserve the sphingolipid rheostat, but how Orms are regulating the sphingolipid biosynthesis pathways is still under intense investigation (Siow and Wattenberg 2012).

5.3.3 The Association of Ceramide 1-Phosphate with the PI3-K/AKT, NF-kB, and Other Survival Pathways

Another commonly studied cell survival mechanism involves the activation of the PI3-K pathway by various growth factors through tyrosine kinase receptors. This lipid kinase pathway plays major roles in regulating adhesion, motility, and survival. Furthermore, mutations or amplifications of the PI3-K gene to produce constitutive activation of the enzyme has shown the PI3-K pathway as oncogenic in many types of cancers via apoptosis inhibition (Larsen and Minna 2011). Interestingly, C1P has been reported to trigger mitogenesis through phosphorylation and activation of the PI3-K/AKT (also known as PKB) pathway, whose downstream targets includes the stimulation of the DNA binding activity of the transcription factor, nuclear factor κB (NF-κB) (Arana et al. 2010). Whether this is a direct or indirect interaction remains largely unknown. NF-κB, like PI3-K, regulates cellular processes implicated in human cancers when constitutively activated making it a possible target for cancer treatment (Dolcet et al. 2005). More specifically, C1P stimulates binding of NF-κB to DNA through phosphorylation of IκB to increase expression of cell proliferation marker regulators (e.g. Bcl-xL and GSK-3B). These are downstream targets of NF-κB that impact mitogenic proteins such as cyclin D1 and Myc, and thus, encourage cell proliferation (Arana et al. 2010).

Interestingly, there is a reported connection on the activation of PI3-K to the A-SMase activity via the synthesis of phosphatidylinositol triphosphate (PIP3), a main product of PI3-K. Specifically, PIP3 has an inhibitory effect on A-SMase. This secondary inhibition may increase the effect of the direct physical interaction and subsequent inactivation of A-SMase through C1P (Arana et al. 2010). Additionally, the effect of C1P on proliferation stems from activation of the extracellularly regulated kinases 1 and 2 (ERK1/2). A likely mechanism of promoting cell survival through C1P could be its ability to activate AKT and ERK1/2 paired with the ability of CerK to enhance epithelial growth factor (EGF) effects via the EGF receptor in the activation of AKT and ERK1/2 phosphorylation (Mitra et al. 2007). In support of this hypothesis, when CerK is downregulated in A549 lung adenocarcinoma cells, EGF-induced proliferation is blocked, and the ability of EGF to induce the phosphorylation of AKT and ERK1/2 is also reduced (Mitra et al. 2007). These data, combined with data showing that exogenous C1P can activate AKT and ERK1/2, support the hypothesis that these additional kinases as well as additional survival pathways outside of simple inhibition of

A-SMase are involved in C1P-induced proliferation (Mitra et al. 2007).

One possible additional pathway for C1P-induced proliferation was recently reported. Specifically, Kester and co-workers showed that C1P also plays a role in cell proliferation and invasion when bound to the annexin a2/protein p11 extracellular heterotetrameric complex (A2t). Annexins are scaffolding proteins that regulate phospholipid binding in the presence of calcium. The A2t complex acts as a "receptor platform" for proteins, and when bound to exogenous C1P assists with endothelial cell migration (Hankins et al. 2013). Interestingly, when endothelial invasion was tested in the presence of either ceramide or S1P, there was only moderate migration in contrast to the much more dramatic invasion induced by C1P. It was hypothesized that that C1P was more effective at stimulating invasion due to selective binding with A2t, specifically the p11 protein component. Indeed, C1P was demonstrated to bind directly to A2t with high specificity while no interaction between A2t and S1P was detected. Ultimately, the ability of the cell to invade was shown to require both p11 and annexin proteins bound to exogenous C1P in a calcium-dependent manner (Hankins et al. 2013).

5.4 The Role of Ceramide 1-Phosphate in Wound Healing

Wound healing is a dynamic process involving four main phases: hemostasis, inflammation, proliferation, and remodeling. During hemostasis, clotting factors work in tandem with constricting blood vessels to decrease bleeding. As the hemostasis phase ends, the wound transitions into the inflammatory phase, where vasodilation, increased vascular permeability, and neutrophil and macrophage infiltration into the wound site occur. After the wound has been cleared of foreign debris and prepped by the cells involved in inflammation, the proliferation phase introduces fibroblasts and keratinocytes into the wound to initiate wound closure and angiogenesis. The final phase of wound healing is remodeling, where collagen is deposited and crosslinked, allowing new epithelial layers to form (Clark 1988).

The role of individual lipids and their regulation relevant to wound healing has been studied for many decades. However, evaluation of lipids using lipidomics approaches has only recently been possible and widely available to the research community, which is now causing a resurgence of research into bioactive lipids as key mediators of underlying mechanisms related to chronic wound development and progression. Lipidomics also opens new avenues of risk assessment and appraisal of targeted therapeutics by detecting "lipid fingerprints" associated with impaired wound healing before clinical manifestations are evident. Indeed, a characteristic of impaired wound healing is an imbalance between pro- and anti-inflammatory lipids (Dhall et al. 2015). For example, prostaglandin E_2 (PGE_2) has been implicated in the inhibition of cell migration and meandering during the acute wound healing process (White et al. 2005). Leukotrienes are another class of eicosanoid that play a major role in the wound healing process. For example, leukotriene B_4 (LTB_4) recruits neutrophils to areas containing damaged tissue to initiate the inflammatory phase of the wound healing process, and the stalling of wound healing at this stage is linked to chronic neutrophilia in the wound environment (Luo et al. 2017). Additionally, the impaired healing of pressure ulcers correlates with high levels of pro-inflammatory eicosanoids such as LTD_4, LTE_4, and PGE_2 which lead to excess inflammation (Dhall et al. 2015), and the reduction of COX-2-derived eicosanoids (e.g., PGE_2) has also been shown to improve the healing of pressure ulcers in mice (Romana-Souza et al. 2016). Furthermore, localized excess of PGE_2 has been linked to delayed wound healing and impaired fibroblast function (White et al. 2005). Lastly, PGE_1, an anti-inflammatory prostaglandin acting in opposition to the pro-inflammatory PGE_2, was approved in 2010 to treat human wounds.

Additional reports indicate that the bioactive fraction in platelet-rich plasma (PRP) for autologous wound therapy is the lipid component

rather than the protein component and is responsible for driving specific wound healing mechanisms at the cellular level. Further, nutritional preparations containing pro-resolution lipids decrease the progression of pressure ulcers (Hoeferlin et al. 2015). These studies present the possibility of altering the lipid profile of PRP via diet or exogenous pathway manipulation of the SM cycle to obtain a better healing outcome. Taken together, the accumulating evidence provides a strong foundation for the premise that bioactive lipid mediators play key roles in the wound healing process, and further study into multicentric and temporal approaches to treatment may be warranted in manipulating these biosynthetic pathways for a positive clinical outcome.

Recent studies have also shown C1P as a key regulator of bioactive eicosanoids involved in wound healing. For example, a number of direct downstream products of C1P/cPLA$_2$α interaction were found to be increased in PRP from healthy individuals. In particular, 5-, 12-, and 15-HETE, which have been shown to be mitogenic toward fibroblasts, and PGF$_2$α, which could be a contributing factor to the observed increase in cell proliferation and improved wound healing. Overall, the scientific literature supports key and distinct roles for AA-derived eicosanoids regulated by C1P in the wound healing process and in its dysregulation and resultant "stalling" in the inflammatory phase. In this pursuit, Gómez-Muñoz and co-workers are seeking to identify distinct macrophage receptors that could be exploited to design sphingolipid mimics for exogenous use in the treatment of inflammatory diseases (Katz et al. 2016). Further, our laboratory reported that C1P derived from CerK negatively regulates the migration of fibroblasts. Importantly, C1P levels were elevated in the inflammatory phase, and subsequently decreased during the proliferation and remodeling stages in human wound healing correlating with the timing of enhanced fibroblast migration (Wijesinghe et al. 2014). Since the C1P/cPLA$_2$α interaction is the "master regulator" of these downstream eicosanoids, research into the specific timings and signaling mechanisms characteristic of optimal wound healing is vital in the context of C1P production in the wound environment. Similarly, formation of distinct lipid profiles (e.g., C1P, eicosanoids) may prove to be a critical method of early detection and treatment of patients prone to impaired wound healing. Thus, the hypothesis as to a role for the C1P/cPLA$_2$α interaction during the wound healing process provides "food for thought" as to targeting this unique and specific interaction for the development of future therapeutics to enhance healing outcomes.

5.5 Future Scientific Questions for Ceramide 1-Phosphate

Going forward, a number of unanswered questions remain for this understudied, but important bioactive lipid mediator. For example, little is known about how C1P anabolism is regulated. Is CerK activated in some manner, post-translational modifications or activating cofactors? Is the inhibition of CPTP a regulating step, which is known to block C1P catabolism by unknown means. In that regard, nothing is known about the catabolism for C1P, which must be highly active to keep the levels of C1P very low in most cell types. Additionally, what other cellular physiologies are regulated by C1P. Indeed, C1P is found in mitochondria, and CerK is specifically activated by cardiolipin *in vitro*, but no physiology has been elucidated in regard to these findings. As to some of the findings ascribed to C1P in this chapter, whether the association of C1P with cPLA$_2$α regulates the biosynthesis of specific eicosanoids versus a blanket activation of the pathway is unknown. Further, are there additional direct targets of C1P outside of cPLA$_2$α, annexins, A-SMase, and TACE? Fortunately, new proteomics and lipidomics technologies may now allow us to find the elusive answers to these questions.

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6 Ceramide Domains in Health and Disease: A Biophysical Perspective

Ana E. Ventura, Beatriz Mestre, and Liana C. Silva

Abstract

Ceramides are the central molecules in sphingolipid metabolism. In addition, they are recognized as important modulators of cell function, playing key roles in several cellular processes that range from cell proliferation to cell death. Moreover, ceramides were implicated in multiple diseases, including cancer, neurodegenerative and metabolic diseases, and also in infection by different pathogens. The mechanisms underlying the diverse biological and pathological actions of ceramides are yet to be fully elucidated. Several lines of evidence suggest that the structural features of ceramides, namely their high hydrophobicity and ability to establish strong H-bond network, are responsible for changes in the biophysical properties of biological membranes that can affect the activity of proteins and activate signaling pathways. Ceramide-induced alterations in membrane biophysical properties might also influence the internalization, trafficking and sorting of lipids, proteins, drugs and even pathogens contributing to cell pathophysiology. In this chapter, we critically discuss the ability of ceramides to form lipid domains with atypical biophysical properties and how these domains can be involved in those processes.

Keywords

Ceramide domains · Membrane biophysical properties · Membrane fluidity · Membrane lateral organization

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Abbreviations

ACDase	Acid Ceramidase
AFM	Atomic Force Microscopy
ASAH1	N-Acylsphingosine Amidohydrolase 1
aSMase	Acid Sphingomyelinase
C1P	Ceramide 1-Phosphate
C1PP	Phosphatase C1P
CAPK	Ceramide-activated Protein Kinase
CAPP	Ceramide-activated Protein Phosphatase
CD95	Cluster of Differentiation 95
CDase	Ceramidase

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Cer	Ceramide
CerS	Ceramide Synthase
CERT	Ceramide Transfer Protein
Chol	Cholesterol
CerK	Ceramide Kinase
CPTP	C1P-specific Transfer Protein
DEPE	1,2-Dielaidoyl-sn-glycero-3-phosphoethanolamine
DES	Dihydroceramide Desaturase
dhCer	Dihydroceramide
DISC	Death-inducing Signaling Complex
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DOPC	1,2-dioleyol-sn-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSC	Differential Scanning Calorimetry
ER	Endoplasmic Reticulum
FAPP2	Phosphatidylinositol-4-phosphate Adaptor Protein 2
FCS	Fluorescence Correlation Spectroscopy
FD	Farber Disease
FTIR	Fourier-Transform Infrared Spectroscopy
GCS	Glucosylceramide Synthase
GlcCer	Glucosylceramide
GSLs	Glycosphingolipids
H-bonds	Hydrogen Bonds
HIV	Human Immunodeficiency Virus
hTERT	human Telomerase Reverse Transcriptase
KDS	3-ketosphinganine reductase
l_o	Liquid Ordered
LSD	Lysosomal Storage Disease
MAMs	Mitochondria Associated Endoplasmic Reticulum Membranes
MAPK	Mitogen-activated Protein Kinase
MD	Molecular Dynamic
NMR	Nuclear Magnetic Resonance
nSMase	Neutral Sphingomyelinase
PC	Phosphatidylcholine
PD	Parkinson's disease
PKCζ	Protein Kinase Cζ
PM	Plasma Membrane
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl phosphatidylcholine
ROS	Reactive Oxygen Species
S1P	Sphingosine 1-Phosphate
SK	Sphingosine Kinase
SL	Sphingolipid
SM	Sphingomyelin
SMase	Sphingomyelinase
SMS	Sphingomyelin Synthase
Sph	Sphingosine
SPL	S1P lyase
SPT	serine palmitoyltransferase
TEM	Transmission Electron Microscopy
Tm	Melting Temperature
TRAIL	TNF-Related Apoptosis Inducing Ligand
UV	Ultraviolet Radiation

6.1 Introduction

Over the last decades, ceramides (Cer) - the building block of complex sphingolipids - have received special attention due to their key role in a myriad of cellular and physiological processes such as regulation of cell growth (Senkal et al. 2010), differentiation (Geilen et al. 1997), senescence (Venable and Yin 2009), vesicular trafficking (Trajkovic et al. 2008), apoptosis (Hannun and Obeid 1995; Henry et al. 2013; Ordoñez et al. 2015) and stress responses (Hannun and Luberto 2000; Nikolova-Karakashian and Rozenova 2010). Moreover, ceramides have been implicated in many pathological processes including, cancer (Henry et al. 2013; Nagahashi et al. 2016), inflammation (Seitz et al. 2015; Gomez-Munoz et al. 2016), diabetes (Galadari et al. 2013) and neurodegenerative diseases (Jana et al. 2009; Filippov et al. 2012; Czubowicz and Strosznajder 2014), among others (Becker et al. 2008; Teichgraber et al. 2008; Baker et al. 2018; Koike et al. 2018; Poolman et al. 2019).

Ceramides are comprised of a sphingoid backbone linked via an amide bond to a fatty acid of varying chain length and degree of saturation. The biological activity of ceramides seems to depend on the type of linked fatty acyl group

(Karahatay et al. 2007; Senkal et al. 2010). Different moieties can attach to the polar C1 hydroxyl group of ceramides. The few polar groups of ceramide, including the C1 and C3 hydroxyl group and the amino group, and the hydrophobic character of the hydrocarbon chains, confer an amphipathic nature to ceramides, which can justify their tendency to aggregate into membranous structures. Nonetheless, ceramides extreme hydrophobicity makes them virtually insoluble in aqueous biological fluids. This suggests that the biological actions of ceramides need to take place at the membrane. Therefore, it has been suggested that the molecular mechanisms underlying ceramides action involve changes in the biophysical properties of the membranes that might subsequently affect the function and/or distribution of proteins in the membrane, causing the activation of signaling pathways (reviewed in (Castro et al. 2014; Alonso and Goñi 2018)). In fact, ceramides affect membrane fluidity and lateral organization (Hsueh et al. 2002; Silva et al. 2006; Dutagaci et al. 2014), permeability (Ruiz-Arguello et al. 1996; Montes et al. 2002), curvature (Veiga et al. 1999; Jesús et al. 2005), induce the transition to non-lamellar phases (Ruiz-Arguello et al. 2002; Doroudgar and Lafleur 2017). In addition they were shown to form tubule-like structures (Pinto et al. 2011; Varela et al. 2014), and to generate ceramide channels (Colombini 2017). All of these changes are dependent on the lipid composition of the membrane and on the structure of ceramides (Jiménez-Rojo et al. 2014; Pinto et al. 2014; Maula et al. 2015). This suggests that very specific alterations in the biophysical properties of the membranes might occur in response to changes in ceramide levels, which can potentially function as a sensing mechanism to trigger specific cellular responses.

A vast number of studies has been performed to investigate the biological roles of ceramides, and an equally long list of publications has been dedicated to the understanding of the biophysical properties of ceramides. Nonetheless, it remains to be elucidated how ceramide-induced changes in the biophysical properties of the membranes contribute to their mechanisms of action. Advances in the field have mainly been obtained through studies performed in artificial membrane systems that, although much less complex than biological membranes, allow elucidating the interactions established between ceramides and other lipid species. In this chapter, we critically address the literature data concerning the effects of ceramides on membrane biophysical properties, focusing on their ability to form lipid domains with specific biophysical properties. We then discuss literature evidence that implicates these domains in the development of different pathologies.

6.2 Ceramide Metabolism

The sphingolipid metabolic pathway is an extremely coordinated system linking several pathways. Ceramide acts as a metabolic hub because it occupies a central position in sphingolipid *de novo* biosynthesis and catabolism (Hannun and Obeid 2008). The enzymes involved in ceramide generation are distributed throughout different subcellular compartments, and their location selectively activates complex effectors (Jenkins et al. 2009). It is therefore considered that the response to diverse stimuli depends on which pathway is triggered for ceramide synthesis, as well as, when and where in the cell this takes place (Kitatani et al. 2008).

De novo biosynthesis of sphingolipids (SLs) produce ceramides with different acyl chain lengths from non-sphingolipid precursors (Gault et al. 2010). This process starts at the cytosolic leaflet of the endoplasmic reticulum (ER) (Norris et al. 2017) and probably involves ER-associated membranes, like the perinuclear membrane and mitochondria-associated membranes (Bartke and Hannun 2009). The first step is the condensation of amino acid serine and palmitoyl-CoA catalyzed by the rate-limiting enzyme, serine palmitoyltransferase (SPT) (Fig. 6.1). The resulting product, the unstable molecule 3-keto-dihydrosphingosine, is rapidly reduced into dihydrosphingosine through the 3-ketosphinganine reductase (KDS) (Menaldino et al. 2003). Although to a minor extent, SPT also catalyzes

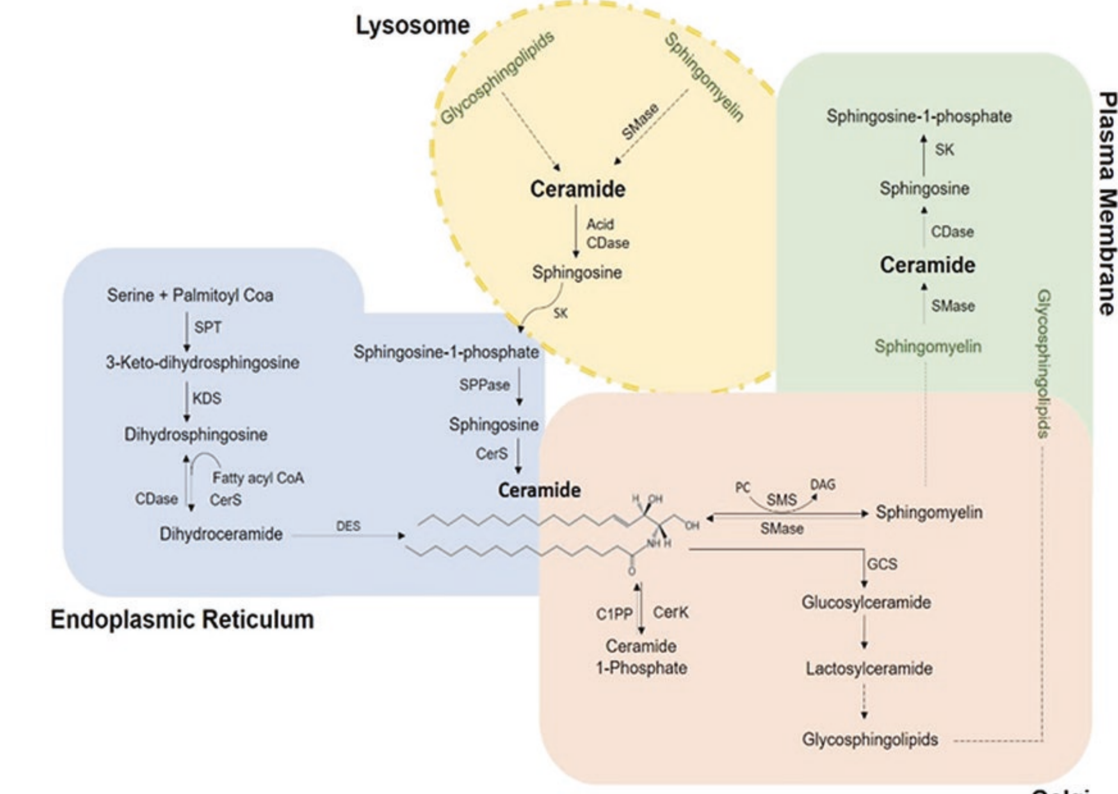

Fig. 6.1 Brief overview of ceramides metabolism and their subcellular compartmentalization. Schematic representation of the metabolic pathways that contribute to the generation of ceramides: the *de novo* biosynthesis in the endoplasmic reticulum, the sphingomyelin hydrolysis in the plasma membrane, and the salvage pathway in the Golgi, which includes acylation of sphingosine and dephosphorylation of ceramide 1-phosphate. See text for further details. *C1PP* ceramide 1-phosphate phosphatase, *CDase* ceramidase, *CerS* ceramide synthase, *CerK* ceramide kinase, *DAG* diacylglycerol, *DES* dihydroceramide desaturase, *GCS* glucosylceramide synthase, *KDS* 3- ketosphinganine reductase, *PC* phosphatidylcholine, *SK* sphingosine kinase, *SMase* sphingomyelinase, *SMS* sphingomyelin synthase, *SPPase* sphingosine phosphate phosphatase, *SPT* serine palmitoyltransferase

the formation of SLs with atypical long chain base length and/or structurally different sphingoid bases, which result from the use of, respectively, other acyl-CoA or amino acids. For example, 1-deoxy-sphingolipids, which lack the C1-OH group, are formed by condensation of alanine with palmitoyl-CoA. The metabolism and properties of these atypical sphingolipids have been recently reviewed in (Lone et al. 2019). The canonical SLs *de novo* pathway proceeds with the N-acylation of dihydrosphingosine into dihydroceramide (dhCer) by six different enzymes of the ceramide synthase (CerS) family. These enzymes exhibit preference for specific acyl-CoAs with different chain-lengths, hence they generate distinct ceramides with unique N-linked fatty acids (Lahiri and Futerman 2007; Hannun and Obeid 2018). dhCer is then desaturated by dihydroceramide desaturase (DES), which introduces a 4,5-*trans*-double bond into the sphinganine backbone yielding ceramide (Michel et al. 1997).

The metabolism of ceramides to complex SLs, like sphingomyelin (SM) and glycosphingolipids (GSLs), occurs in the Golgi complex (Maceyka and Spiegel 2014). Since ceramides are highly hydrophobic they cannot freely move from one membrane to another. Therefore the cell employs two specific pathways to mobilize ceramides from the ER to the Golgi: vesicular transport for glucosylceramide (GlcCer) synthesis, or ATP-dependent transport mediated by ceramide transfer protein (CERT) for SM synthesis (Hanada et al. 2003). Synthesis of SM occurs in the lumenal leaflet of the Golgi. Sphingomyelin synthase 1 (SMS1) catalyzes the transfer of phosphocholine from phosphatidylcholine (PC) into ceramides yielding SM. Ceramides can also be converted to SM at the plasma membrane (PM) through the action of SMS2 (Huitema et al. 2004; Tafesse et al. 2007). On the cytosolic leaflet of the *cis*-Golgi, ceramides are glycosylated to GlcCer by glucosylceramide synthase (GCS), which catalyzes the transfer of glucose from UDP-glucose to ceramides. GlcCer is then transported by phosphatidylinositol-4-phosphate adaptor protein 2 (FAPP2) to the *trans*-Golgi network for the synthesis of lactosylceramide and thereafter complex GSLs (D'Angelo et al. 2007). In addition, ceramides may also be phosphorylated to ceramide 1-phosphate (C1P) by ceramide kinase (CerK) at the Golgi or PM. In turn, the C1P can be recycled by a phosphatase (C1PP) forming ceramides (Bartke and Hannun 2009; Mullen et al. 2012). The complex SLs, SM and GSLs, are transported to the PM by vesicular transport and C1P by a C1P-specific transfer protein (CPTP) (Yamaji and Hanada 2015).

Alternatively to the *de novo* biosynthesis pathway, ceramides can be generated in the PM through hydrolysis of SM by sphingomyelinases (SMases) (Marchesini and Hannun 2004). There are 5 different SMases, which are distinguished according to their subcellular location and optimal pH and/or presence of specific ions for the reaction (reviewed in (Marchesini and Hannun 2004)). The neutral and the acid isoforms of SMase have been implicated in the generation of ceramides at the PM, particularly in response to stress stimuli (Henry et al. 2013; Beckmann et al. 2014; Simonis et al. 2014). The ceramides can then be deacylated into sphingosine (Sph) by neutral ceramidases (CDases). The Sph can be recycled or undergo phosphorylation by one of two Sph kinases (SK1 and SK2) localized in the cytosol or peripherally associated with specific membrane compartments (Wattenberg 2010). This phosphorylation results in sphingosine 1-phosphate (S1P), which can be dephosphorylated by specific S1P phosphatases, regenerating Sph, or irreversibly degraded by S1P lyase (SPL) resulting in two non-SL products, phosphoethanolamine and hexadecenal. However, phosphoethanolamine and hexadecenal can be reduced to palmitate and posteriorly reintegrated into lipid metabolic pathways (Aguilar and Saba 2012).

Another important source of ceramides is the sphingolipid salvage pathway, which consists of the catabolism of SM and GSLs within the lysosomes (Kitatani et al. 2008). In lysosomes, the breakdown of GSLs proceeds through the action of specific hydrolases, which cleave the sugar residues leading to the formation of GlcCer and galactosylceramide. Subsequently, these products are hydrolyzed by specific β-glucosidases and galactosidases to produce ceramides (Kolter

and Sandhoff 2005). On the other hand, acid SMase mediates sphingomyelin hydrolysis into ceramides. The resulting ceramides are later deacylated by an acid CDase to generate Sph (Kitatani et al. 2008; Gault et al. 2010). The Sph thus produced can further be salvaged in ER into ceramides, which accounts for 50% to 90% of total sphingolipid biosynthesis, relying on the cell life stage (Régnier et al. 2019).

6.3 Physical and Chemical Properties of Ceramides

Ceramides are among the most studied SLs in different fields of research. Over the last two decades, ceramides gained the attention of the biophysical community due to the evidence that these highly hydrophobic lipid species could regulate cellular events through mechanisms that would involve changes in the biophysical properties of biological membranes (Cremesti et al. 2002; Grassmé et al. 2007). Indeed, several studies unraveled the physiochemical properties of these lipids that make them unique. This subject has been comprehensively reviewed in (Castro et al. 2014; Alonso and Goñi 2018). As mentioned above, ceramides are composed of a fatty acid with an acyl chain of variable length bound to an amino group of a sphingoid base, typically sphingosine or sphinganine (Fahy et al. 2005), although other sphingoid backbones can be found (Lone et al. 2019). The fatty acyl chain can be saturated or contain a double bond, which influences the physiochemical properties of ceramides (Shah et al. 1995; Pinto et al. 2011; Jiménez-Rojo et al. 2014), as well as their interaction with neighboring lipids (Massey 2001; Silva et al. 2009; Pinto et al. 2013; Jiménez-Rojo et al. 2014). In mammalian cells, the hydrocarbon chain can vary from 14 to 36 or more carbons (Sandhoff 2010). However, the most abundant ones are the long (C16-C20) and very long (C22-C24) acyl chain ceramides. These structural features, i.e. long hydrocarbon chains and very few polar hydroxy and amino groups, confer very low polarity and high hydrophobicity to ceramides, making this lipid very unlikely to be in solution in aqueous biological fluids or in the cytosol (Goñi and Alonso 2006).

Because of these features, ceramides are poorly hydrated and display an extremely complex thermotropic behavior (Shah et al. 1995). One of the most remarking characteristics of ceramides, particularly those presenting long and saturated acyl chains, are the high gel-to-fluid transition temperature or melting temperature (*Tm*). The *Tm* of ceramides, which depends on the structure of their acyl chain (Jesús et al. 2005; Pinto et al. 2011; Jiménez-Rojo et al. 2014) and sphingoid base backbone (Jimenez-Rojo et al. 2014; Maula et al. 2015), is well above the range of physiological temperatures, and is attributed not only to their hydrophobic characteristics, but also to the facility that ceramides have to establish strong intra and intermolecular hydrogen bonds (H-bonds) (Pascher 1976; Gillams et al. 2015). Several studies report on the thermotropic behavior of ceramides, highlighting the importance of hydration (Shah et al. 1995) and chain length (Pinto et al. 2011; Jiménez-Rojo et al. 2014; Maula et al. 2015). The *Tm* of ceramides with C16 to C24 acyl chains are within the same range, ap. 92–94 °C, yet the presence of an unsaturation in the acyl chain of ceramides decreases significantly the *Tm* (Shah et al. 1995; Pinto et al. 2008; Jiménez-Rojo et al. 2014). As an example, a double bond in the C15 position of C24 acyl chain, as is the case of nervonoyl-ceramide (C24:1-ceramide) decreases the *Tm* by more than 20 °C compared to its saturated counterpart (Pinto et al. 2011; Jiménez-Rojo et al. 2014). Moreover in the case of oleoyl-ceramide (C18:1) the *Tm* is close to the physiological temperature (Pinto et al. 2011; Jiménez-Rojo et al. 2014; Maula et al. 2015).

Due to their structure, ceramides also present a high tendency to form non-lamellar phases (Veiga et al. 1999; Jiménez-Rojo et al. 2014), which cause stress curvature in the membrane and enhance membrane packing defects (Ruiz-Arguello et al. 1996; Ruiz-Argüello et al. 1998; van Blitterswijk et al. 2003), that might affect membrane permeability. Moreover, due to the inverted cone structure of ceramides, characterized by the small polar head together with the

long hydrocarbon chains, ceramides are prone to induce negative curvature in membranes (Veiga et al. 1999; Sot et al. 2005), suggesting that ceramides might have a key role in the formation of vesicles.

To summarize, the key to most of the physiochemical properties of ceramides is their ability to form an extensive network of H-bonds. It confers, together with the high hydrophobicity of ceramides, an elevated *Tm* and the propensity to form highly stable and condensed membranes. Furthermore, when in mixtures with other lipids ceramides have the ability to drive lateral phase separation leading to the formation of ceramide-enriched domains with atypical ordered properties (Veiga et al. 1999; Silva et al. 2006; Sot et al. 2006). In addition, ceramides are able to increase the packing of the fluid phase, as discussed below.

6.4 Structure and Properties of Ceramide Domains

An extensive array of biophysical techniques, including atomic force microscopy (AFM) (Carrer and Maggio 1999; Fidorra et al. 2006; Chiantia et al. 2008; Ira et al. 2009), differential scanning calorimetry (DSC) (Carrer and Maggio 1999; Pinto et al. 2008; Maula et al. 2015), monolayer studies and Brewster angle microscopy (Maggio 2004; Dupuy et al. 2011), nuclear magnetic resonance (NMR) (Hsueh et al. 2002; Al Sazzad et al. 2017), transmission electron microscopy (TEM) (Silva et al. 2006), X-ray diffraction (Holopainen et al. 2000a, b; Pinto et al. 2008), Fourier-transform infrared spectroscopy (FTIR) (Veiga et al. 1999; Fidorra et al. 2006), fluorescence spectroscopy (Fidorra et al. 2006; Silva et al. 2006; Castro et al. 2007; Varela et al. 2014), fluorescence and super resolution microscopy (Fidorra et al. 2006; Castro et al. 2009; Staneva et al. 2009; Pinto et al. 2011, 2013; Varela et al. 2014; Burgert et al. 2017) and fluorescence correlation spectroscopy (FCS) (Chiantia et al. 2007; Carrer et al. 2009; Pinto et al. 2011) were used to characterize the alterations promoted by ceramides in the biophysical properties of artificial (Veiga et al. 1999; Silva et al. 2006; Pinto et al. 2008; Garcia-Arribas et al. 2016) and, to a minor extent, cellular membranes (Pinto et al. 2014). In fact, most of the current knowledge on ceramides biophysical properties derives from studies in synthetic membranes that display different lipid composition, regarding both the type and number of lipids present in the model membranes (Pinto et al. 2011; Jiménez-Rojo et al. 2014; Varela et al. 2014; Maula et al. 2015; Catapano et al. 2017; Cebecauer et al. 2018; Wang and Klauda 2018). Even though these membranes do not mimic the complexity of biological membranes, they provide important insights regarding the interactions established between ceramide molecules and specific lipid species. The use of model systems also allows a deeper understanding on the molecular and structural features determining ceramides ability to drive alterations in the biophysical properties of the membranes, such as fluidity and ability to change membrane lateral organization (Chiantia et al. 2006; Silva et al. 2006; Dutagaci et al. 2014; Slotte et al. 2017), formation of non-lamellar phases (Ruiz-Arguello et al. 2002; Pinto et al. 2008; Catapano et al. 2011; Busto et al. 2014; Al Sazzad et al. 2017; Doroudgar and Lafleur 2017), membrane fusion (Basáñez et al. 1997; Montes et al. 2002; Ibarguren et al. 2010; Schneider-Schaulies and Schneider-Schaulies 2015), among others (Barbosa-Barros et al. 2008; Chiantia et al. 2008; Carrer and Maggio 2009; Colombini 2010; Samanta et al. 2011). The simpler model systems contain only two lipid species: a ceramide and typically a biologically relevant phospholipid, such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (Hsueh et al. 2002; Contreras et al. 2003; Silva et al. 2006; Pinto et al. 2008), 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) (Doroudgar and Lafleur 2017), 1,2-Dielaidoyl-sn-glycero-3-phosphoethanolamine (DEPE) (Sot et al. 2005), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Holopainen et al. 2000a, b; Dutagaci et al. 2014), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Carrer and Maggio 1999) or 1,2-dioleyol-sn-glycero-3-phosphocholine (DOPC). More complex membrane model systems are used to investigate the ability of ceramides to change the biophysical

properties of membranes already displaying complex organization patterns and phase behavior properties (Jiménez-Rojo et al. 2014; Catapano et al. 2017), such as those containing sphingolipids and cholesterol (Castro et al. 2007; Silva et al. 2007; Staneva et al. 2008; Busto et al. 2010; Busto et al. 2014) (Busto et al. 2010, 2014, Silva et al. 2007), as well as other lipid species, such as free fatty acids (Wang and Klauda 2018; Souza et al. 2011). This type of biophysical studies can be performed both under thermodynamic equilibrium conditions, where the biophysical properties of the model membranes are investigated several hours after the membranes have been prepared and lipid interactions are no longer significantly changing (Sot et al. 2006; Pinto et al. 2008; Varela et al. 2014); or under non-equilibrium conditions, which might include the *in situ* generation of ceramides in the model membranes, through hydrolysis of SM by externally added SMase (Holopainen et al. 1998; Holopainen et al. 2000a; Contreras et al. 2003; Ira and Johnston 2008; Fanani et al. 2009; Silva et al. 2009). In addition to studies in model membranes, molecular dynamics (MD) simulations have also been extensively performed to understand the types of changes that ceramides induce in the lipid bilayer (Guo et al. 2013; Dutagaci et al. 2014; Gupta and Rai 2015; Gupta et al. 2016; Moore et al. 2018; Wang and Klauda 2018, 2019).

Despite using several different ceramides, experimental techniques, types and compositions of model membranes in different studies, most of them are consensual and indicate that ceramides induce strong and complex changes in the biophysical properties of the membranes. Herein, we will focus on the ability of ceramides to form lipid domains with gel phase properties. Additional information regarding the effects of ceramides in the biophysical properties of the membranes can be found in (Castro et al. 2014; Alonso and Goñi 2018).

When mixed with phospholipids, saturated ceramides drive an increase in the packing of the fluid phase, rigidifying the membrane. Ceramides also display a high tendency to self-aggregate into ceramide enriched domains even when present at very low concentrations in the membrane. This is due to their ability to establish a strong and extensive H-bond network, which is conferred by their OH group in the sphingosine backbone, the amide linkage and hydroxyl functional group (Silva et al. 2006; Jiménez-Rojo et al. 2014; Gillams et al. 2015). Nonetheless, the concentration at which ceramide domains start to be formed depends on the structure of the ceramide and the lipids present in the membrane. Palmitoyl-ceramide (C16-ceramide), one of the most studied ceramides, segregates into highly-ordered gel domains with as low as 5 mol% when mixed with the fluid POPC, highlighting the importance of ceramide in a physiological concentration range (Veiga et al. 1999; Hsueh et al. 2002; Silva et al. 2006). Similar results were obtained for stearoyl-ceramide (C18-ceramide) and lignoceroyl-ceramide (C24-ceramide) mixed with POPC (Pinto et al. 2011; Jiménez-Rojo et al. 2014). In contrast, unsaturated C18:1-ceramide and C24:1-ceramide required a much higher concentration, 50 mol% and 30 mol%, respectively, to form gel domains in fluid POPC at room temperature. At physiological temperature C18:1-ceramide is not able to drive gel domain formation in fluid POPC (Pinto et al. 2011).

The gel phase formed by ceramides, particularly by long chain saturated ceramides (e.g., C16- and C18-ceramide) is considered atypical, since it is much more ordered than gel phases formed by other lipids (Ekman et al. 2015), including the sphingolipid SM (de Almeida et al. 2003; Carrer et al. 2009; Pinto et al. 2013). This has been highlighted in studies showing that fluorescent probes that typically report the properties of the gel phase are unable to partition into these rigid domains (Massey 2001; Silva et al. 2006, 2007; Castro et al. 2007; Pinto et al. 2008). Different monolayers studies reveal that ceramides have a solid phase at low temperatures (<20 °C) that transitions to a liquid-condensed state with the increase in temperature. The liquid-condensed state has viscoelastic properties, which means that ceramide domains are resistant to shear deformations. With a further increase in temperature to values above the T_m, ceramide

present a liquid expanded phase that might coexist with microscopic states of liquid-condensed lipid (Catapano et al. 2015, 2017). The coexistence between solid, liquid-condensed and liquid extended phases has already been reported for C16-ceramide (Fanani and Maggio 2011). These studies show that the elastic properties attributed to ceramide domains make them extremely rigid, which result in the impairment in the movement of the molecules (Catapano et al. 2011; Espinosa et al. 2011).

On the other hand, a higher amount of unsaturated ceramides is required to induce gel-fluid phase separation in fluid membranes and, in addition, the gel domains formed by these ceramide species are less ordered (Pinto et al. 2011, 2014; Jiménez-Rojo et al. 2014). Moreover, the gel phase formed by very long acyl chain saturated ceramides is also less ordered compared to those formed by long acyl chain saturated ceramides (Pinto et al. 2011). This is associated to the ability of very long acyl chain ceramides to form interdigitated gel phases, namely partially interdigitated and mixed interdigitated gel phases. These phases, besides being less packed than a non-interdigitated gel phase, are prone to interfere with the membrane structure driving strong morphological alterations and promoting the formation of tubular structures (Pinto et al. 2008; Carrer et al. 2009). These observations suggest that the order of the domains formed by ceramides depends on factors other than the length of the acyl chain structure. Further supporting this hypothesis, is the observation that N-decanoyl-d-erythro-sphingosine (C10-ceramide), a short chain but asymmetric ceramide, also forms highly ordered domains and, in addition, induces transition to an hexagonal phase (Dupuy et al. 2017). These results suggest that the asymmetry between the sphingoid base and the N-acyl chain of ceramides might also be an important factor determining ceramides ability to segregate into ordered domains.

Another important observation is that different ceramides not only form domains with different packing properties but also with different sizes and shapes (Fig. 6.2). Microscopy studies suggest that the size of the gel domains decreases with the increase in the number of carbons in the N-acyl chain of ceramides (Pinto et al. 2011; Varela et al. 2014) (Fig. 6.2). Interestingly, the liquidus boundary in the binary POPC/ceramides phase diagrams, and which defines the concentration at which ceramides start to segregate into gel domains, is very similar for mixtures containing C16-, C18- or C24-ceramides (Silva et al. 2006; Pinto et al. 2008, 2011). Therefore, it would be expected that these saturated ceramides would be able to form gel phase domains to identical extent. However, since the phase diagrams for mixtures containing C18- and C24-ceramides have not been fully determined (Pinto et al. 2011), it is not possible to predict the fraction of gel phase that is formed for each of these mixtures. In addition, even if the different ceramides would form the same amount of gel phase, this could be derived from a high number of small gel domains, or from few very large gel domains. In mixtures presenting phase separation, the size and the shape of lipid domains is determined by a number of factors, such as, line tension at the interface between the two phases, in the case of ceramides/POPC mixtures, the gel and the fluid (Staneva et al. 2009); dipolar repulsion inside the domains, and domain–domain interactions (Perković and McConnell 1997; Holopainen et al. 2001; Vega Mercado et al. 2012). Alteration of any of these factors results in domain shape changes. Moreover, alterations in the hydrogen bonds between ceramides headgroup and the neighbor lipids and/or the surrounding medium, might also influence the shape of the gel domains. We have shown that mixtures of POPC/C16:0-ceramide formed gel domains with a flower-like shape (Fig. 6.2a), while C18:0-ceramide tended to segregate into an elongated network of less irregular and interconnected gel domains (Fig. 6.2b). Interestingly, it was observed that mixtures of POPC with C24:0- (Pinto et al. 2011) or C24:1-ceramides (Varela et al. 2014) (Fig. 6.2c, d) presented sharp interfaces between two phases, which might suggest gel-gel phase separation. This is not unexpected since these very long chain asymmetric ceramides can form interdigitated gel phases that might co-exist in the same mixture (Pinto et al. 2008, 2011). As mentioned

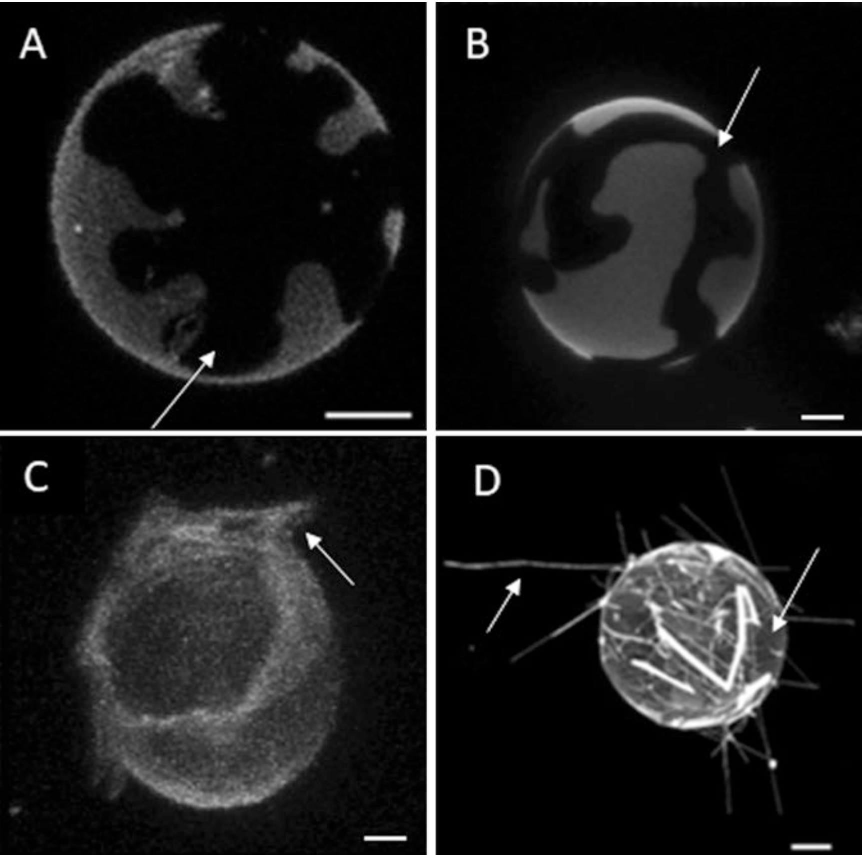

Fig. 6.2 Ceramides-induced gel domain formation and membrane structural alterations. 3D projection images from 0.4 μm confocal slices of giant unilamellar vesicles (GUVs) labeled with Rhodamine-DOPE. GUVs contain POPC with 30 mol% of (**a**) C16-Ceramide, (**b**) C18-Ceramide (**c**) C24-Ceramide and (**d**) C24:1-Ceramide. Gel domains are seen as dark areas that exclude the fluorescent probe. White arrows highlight different alterations induced by ceramides, such as (**a** and **b**) gel domain formation, (**c** and **d**) tubule-like structures, (**c** and **d**) rigid interfaces between domains and (**d**) gel domains with intermediate order properties. Indeed, darker regions as seen in (**a** and **b**) result from total exclusion of the probe highlighting the tightly packed nature of the gel phase; while less dark regions, as seen in (**c** and **d**) indicate partial incorporation of the probe and suggest that these gel domains are less ordered. Scale bar corresponds to 5 μm. Images (**a** and **d**) were adapted from (Varela et al. 2014), (**b** and **c**) were adapted from (Pinto et al. 2011)

above, the presence of interdigitated phases in the bilayer might underlie the formation of the tubular structures observed for these mixtures (Lewis et al. 1994; Pinto et al. 2008; Varela et al. 2014).

Formation of ceramides-enriched gel phase domains has been observed in simple binary mixtures with fluid POPC (Holopainen et al. 2000a, b; Hsueh et al. 2002; Carrer et al. 2006; Silva et al. 2006; Castro et al. 2007; Pinto et al. 2011), but also with fluid DOPC (Ira and Johnston 2008; Slotte et al. 2017; Al Sazzad et al. 2019). In binary mixtures of C16-ceramide/DOPC, ceramide is able to segregate into ceramide-enriched domains at concentrations above 10 mol% (Al Sazzad et al. 2017). The authors further showed that ceramide gel phase was favored in mixtures with POPC when compared with DOPC for the same ceramide concentration (Al Sazzad et al. 2017, 2019). Molecular dynamic (MD) simulation studies have provided additional clues on the effects of ceramides in phospholipid bilayers. For example, MD simulations performed with different concentrations of

C16-ceramide in a DMPC and POPC bilayer further confirmed the ability of ceramides to increase the order of phospholipid bilayers (Dutagaci et al. 2014; Wang and Klauda 2017), as previously shown in experimental studies (Hsueh et al. 2002; Carrer et al. 2006).

Overall, it is consensual that ceramides change the order of fluid phospholipids and drive gel-fluid phase separation in these membranes. However, this effect is not restricted to fluid membranes, and evidence shows that ceramides are also able to affect the properties and lateral organization of gel phase membranes, including in mixtures with DPPC (Massey 2001), DMPC (Holopainen et al. 2000a, b) and DEPE (Jesús et al. 2005). Ceramide and phospholipid gel phase immiscibility was explained by the unfavorable dipolar matching of the lipids (Carrer and Maggio 1999; Carrer et al. 2006). Gel-gel and gel-fluid phase separation was also observed for binary mixtures of ceramides with the sphingolipid SM, depending on the molar ratio between these lipids (Miyaji et al. 2005; Sot et al. 2006; Staneva et al. 2009; Artetxe et al. 2013; Jiménez-Rojo et al. 2014).

Ceramides can be formed by hydrolysis of SM at the PM in response to stress stimuli (Tepper et al. 2000; Kolesnick 2002; Dumitru et al. 2007; Boslem et al. 2012). Therefore, studies that address the interplay between these two lipids are very relevant. Most of the studies so far performed showed that ceramides and SM domains can stabilize each other, and that ceramide's NH group is extremely important in the stabilization of both gel phases (Maula et al. 2011). Leung et al. (2012) confirmed that ceramide and SM mix well in liquid crystalline state as well as in the gel state for high concentrations of C16-ceramide, thus confirming that C16-ceramide stabilizes C16-SM gel. The extent of ceramides/SM miscibility in the gel and the fluid phase is also dependent on the structure of these lipids. Longer chain ceramides (C16 – C24:1) induced heterogeneities in the membrane corresponding to gel domains of both lipids. The heterogeneities caused by C24:1-ceramide are even more complex due to the interdigitation ability of both ceramide and SM (Jiménez-Rojo et al. 2014). It is suggested that the favorable interactions between ceramides and SM might arise from the fact that the small polar head group of ceramide needs the choline headgroup of SM to protect it from interacting with water – "the umbrella effect". The effect of the choline group in the interaction of SM/ceramide was also studied and it was shown that the size of the choline group had no effect on phase separation nor on the properties of both gel phases (Artetxe et al. 2013). In the case of ternary mixtures of POPC/SM/Cer it was shown that ceramide is able to form domains that co-exist with both POPC-rich and SM-rich areas (Castro et al. 2007; Boulgaropoulos et al. 2011). The domains formed by ceramide in these mixtures are extremely ordered with characteristics resembling the gel phase formed by POPC/ceramide mixtures (Silva et al. 2006).

Cholesterol (Chol) is a highly hydrophobic molecule, with a small head group and a planar structure. This sterol is present in most membranes in different percentages and is a key determinant of the fluidity of biological membranes. Indeed, a key biophysical property of Chol, which has been considered as one of its most important features, is its ability to abolish the gel-fluid phase transition of lipids by promoting the formation of the intermediate state – the liquid ordered (l_o) phase (Dufourc 2008). Considering that Chol is highly abundant in the PM, where it is expected to interact with SM to form the widely described lipid raft domains (Goñi et al. 2008; Lingwood and Simons 2010), it is not surprising that several biophysical studies have been performed to understand how Chol and ceramides interact to change the biophysical properties of membranes (Tepper et al. 2000; Scheffer et al. 2005; Castro et al. 2009; Silva et al. 2009; Pinto et al. 2013; García-Arribas et al. 2016). These studies have been made both in the absence (Scheffer et al. 2005; Ali et al. 2006; Scheffer et al. 2006) and presence of SM (Sot et al. 2008; Busto et al. 2010; Pinto et al. 2013; Busto et al. 2014). At low concentrations of Chol, ceramides are still able to form gel domains, a property that is abolished in mixtures with increasing concentration of Chol. It was hypothesized that ceramide

and Chol would compete for the interaction/protection of SM (London and London 2004; Nyholm et al. 2010), however this idea was challenged when it was shown that Chol would solubilize ceramide-enriched gel domains even without the presence of SM (Castro et al. 2009). This evidence has been supported by other studies (Chiantia et al. 2006; Sot et al. 2008; Busto et al. 2014) and critically reviewed in (García-Arribas et al. 2016). For example, Busto and coworkers (Busto et al. 2010; Busto et al. 2014) showed the existence of lamellar gel phases in ternary mixtures of DPPC or SM/Chol/ceramide that were stabilized by the interaction of ceramide and Chol. Moreover, this study suggested that the presence of C16-ceramide and cholesterol in biological systems could drive the formation of two different domains, a Chol-rich and ceramide-rich domain. Furthermore, we showed that ceramides and Chol establish specific interactions in ternary mixtures of these lipids with POPC, driving the formation of a ceramide/Chol-enriched phase with properties in between the gel and the l_o phases (Castro et al. 2009; Silva et al. 2009; Pinto et al. 2013). The most recent results regarding the interaction between these two lipids were performed in red blood cells extracts, a system with a high Chol content (~45%), to which C16-ceramide was added (Garcia-Arribas et al. 2016). The authors showed that ceramide does not exclude Chol, instead it can create a complex gel phase with specific properties that can be important in the regulation of biological processes. Literature evidence regarding Chol and ceramide interactions thus suggest that ceramide and Chol levels might have to be tightly regulated to assure that the properties of different membranes are maintained. Moreover, the presence of Chol/C16-ceramide domains in cell membranes (Goldschmidt-Arzi et al. 2011; Ferreira et al. 2014), the different biophysical properties of ceramide/Chol-enriched domains and the intricate interplay between these two lipids supports the hypothesis that a biophysical mechanism dependent on the interaction between these lipids might be a trigger to initiate biological events.

Although most of the studies to determine the properties of ceramide domains were done in synthetic membranes, Pinto et al. (2014) showed that the results could be transposed to live cells. This study showed for the first time that ceramides with different acyl chains promote different alterations in the fluidity of cell membranes. These alterations were also dependent on the amount of ceramide that was formed and were consistent with the existence of phases with gel properties.

The changes induced by ceramides in the biophysical properties of biological membranes, particularly the formation of ceramide-enriched gel domains, are important at several levels. First, the distinct properties of these domains might determine the specific interactions/partition of integral and peripheral proteins, drugs, pathogens and other molecules with/into the membrane. Some of these entities are likely to be excluded from the tightly packed ceramide-enriched domains but others can be recruited and become temporarily restricted in these ordered regions. While on the one hand this could argue against the dynamics of biological membranes and cellular processes, on the other hand, it suggests that these domains could provide the platform to trigger and enhance signaling events (Geilen et al. 1997; Bollinger et al. 2005; Grassmé et al. 2007; Bieberich 2008; Zhang et al. 2009; Morad and Cabot 2012) and/or could be responsible for trafficking and sorting specific proteins and lipids among cellular membranes. In addition, their different properties also cause changes in membrane curvature that can be sensed by proteins (Jesús et al. 2005; Silva et al. 2012; Doucet et al. 2015; Henne et al. 2015; Prévost et al. 2015) but might also be exploited by pathogens to infect the host cells (Liao et al. 2003; Gulbins et al. 2004; Riethmuller et al. 2006; Schneider-Schaulies and Schneider-Schaulies 2015), or as a mechanism to spread the infection to neighboring cells (Brandstaetter et al. 2012; Menck et al. 2017). Moreover, the negative curvature that is expected to be created by these domains in biological membranes might facilitate the internalization of membrane receptors and be players in other endocytic

events (Chen et al. 1995; Veiga et al. 1999; Jesús et al. 2005; Hernandez et al. 2007; Pepperl et al. 2013). Interestingly, increased levels of ceramides in the PM have been reported upon binding of ligands to several death receptors (Bollinger et al. 2005; Stancevic and Kolesnick 2010; Gajate and Mollinedo 2011), suggesting that the presence of this lipid might be important for receptor-mediated internalization and signaling. In addition to creating regions with different biophysical properties, the presence of distinct domains in the membrane also cause packing defects at the interface between two distinct phases. Such regions are preferential sites for the interaction and partition of small molecules, proteins and pathogens (Bigay and Antonny 2012; Ouberai et al. 2013). These packing defects also alter the permeability of biological membranes and might enhance the exchange of ions and small molecules across the membrane. This interferes with cellular gradients and can impair cellular homeostasis, and ultimately contribute to pathology development, as discussed below.

6.5 Relevance of Ceramide Domains in Health and Disease

Ceramides are involved in an array of cellular processes and several strategies are currently under investigation to take advantage of this lipid and its metabolism (Wymann and Schneiter 2008; Henry et al. 2013). Most of the signaling pathways where ceramides are described to play important roles are related to cell fate (Detre et al. 2006; Van Brocklyn and Williams 2012; Goldkorn et al. 2013; Hartmann et al. 2013; Jiang and Ogretmen 2014; Molino et al. 2017). Nonetheless, ceramides have also been related to different pathologies such as diabetes mellitus type 2 (Aburasayn et al. 2016; Kuzmenko and Klimentyeva 2016), cardiovascular disease (Heneghan et al. 2013), infection by pathogens (Riethmuller et al. 2006; Spindler and Hsu 2012), neurodegeneration (Ben-David and Futerman 2010; Filippov et al. 2012; Czubowicz and Strosznajder 2014; Jazvinšćak Jembrek et al. 2015), among others. Ceramides have also been implicated in wound healing (Tam et al. 2010; Andrews et al. 2014; Castro-Gomes et al. 2016). Despite not being directly associated to ceramide domain formation, evidence shows that different lysosomal enzymes, including aSMase, are released in response to PM injury. It is suggested that aSMase-mediated ceramide formation at the PM is responsible for the invagination of the injured area (Tam et al. 2010; Draeger and Babiychuk 2013). This is one of the few examples where ceramide propensity to induce a negative curvature in membranes might be aiding cell repair.

Most of the studies addressing the pathophysiological implications of ceramides do not provide details on the molecular mechanisms underlying their biological action. Yet, based on the evidence obtained from biophysical studies performed in artificial systems, one of the most accepted hypotheses considers that ceramides modulate cellular pathways through the formation of large ceramide-enriched platforms (Bollinger et al. 2005). There are three main reasons why the formation of these signaling platforms would contribute to the initiation of a signaling cascade, for example. Ceramide-enriched domains would help to spatially organize different receptors and clustering them. Some molecules would be excluded from these ordered areas while others would be recruited. These platforms would help in the stabilization of the whole complex. Moreover, the fact that these domains cluster receptors in a small area might help to amplify the signaling cascade (Grassme et al. 2001a, b; Korzeniowski et al. 2007). There have been several biological studies that suggest that the formation of ceramide-enriched domains is required for certain biological events, such as the clustering of death receptors, including FAS or CD95 (Zhang et al. 2009; Bollinger et al. 2005; Gulbins et al. 2002; Grassme et al. 2001a, b, 2002, 2003), but also for the internalization of pathogens (Riethmuller et al. 2006), and in the development of neurodegenerative diseases (Jana et al. 2009). Ceramide domains might also regulate protein function by direct contact with the proteins in the cytoplasmic leaflet of the membrane. Studies suggest that ceramides could inter-

act directly with phospholipase A2, cathepsin D (Heinrich et al. 1999) and ceramide-activating proteins (Yao et al. 1995), even though no ceramide binding domains have been identified and the molecular mechanisms of these interactions remain unknown. It should however be stressed that in most of the studies, ceramide-domains are identified through the use of anti-ceramide antibodies (Cremesti et al. 2002; Abdel Shakor et al. 2004; Zhang et al. 2009; Gulbins et al. 2016; Burgert et al. 2017), which, might not be completely specific to a single lipid species, particularly one that presents a very small head-group. In addition, the antibodies might induce the formation of larger "domains" than those that would be present in the membrane in the absence of antibodies. In addition, different studies have used different anti-ceramide antibodies. Worth mentioning is a study performed by Burgert and collaborators (Burgert et al. 2017), where they used super-resolution microscopy and an anti-ceramide antibody developed by the group of Bieberich (Burgert et al. 2017) to visualize the distribution of ceramide in two different cells before and after SMase treatment (Krishnamurthy et al. 2007; Burgert et al. 2017). In contrast to other studies, this study suggests that ceramide-enriched domains are in the nanoscale range (approximately 75 nm in cell membranes), and not in the microscopic range (ranging from 900 nm to 2 μm) as suggested in other studies using other types of anti-ceramide antibodies (Grassmé et al. 2002; Krishnamurthy et al. 2007; Zhang et al. 2009). Such differences raise the concern that different antibodies are reporting different cellular features. It would therefore be interesting to compare them in the same experimental settings, particularly in model systems with controlled lipid composition.

Despite being a tool that theoretically enables detecting ceramide domains in cellular membranes, these types of studies do not provide information regarding the biophysical properties of those domains. Indeed, it is not possible to predict whether these domains would display gel phase properties or be in a more fluid phase. Nonetheless, they have been useful to identify whether ceramides are involved in the cell pathophysiology, as it will be discussed below.

6.5.1 Ceramide in Cancer

The clustering of death receptors and the initiation of an apoptotic cascade make ceramide an interesting lipid when it comes to cancer therapy. However, cancer cells seem to have mechanisms preventing the increase in ceramides levels, either by blocking its formation (Kim et al. 2008; Morad and Cabot 2012; Revill et al. 2013) or by increasing the metabolism of ceramides into other lipids species, such as glucosylceramide (GlcCer) (Liu et al. 1999; Bleicher and Cabot 2002; Morad and Cabot 2012). Low levels of ceramides are commonly associated with resistance to apoptosis in different cancer cell lines (Huang et al. 2011; Morad and Cabot 2012; Airola et al. 2015; Chen et al. 2015; Ogretmen 2017). Ceramide-enriched domains are seen as attractive therapeutic targets since these regions are platforms to cluster different death receptors, such as TRAIL2 (White-Gilbertson et al. 2009) and CD95 (Grassme et al. 2001b; Carpinteiro et al. 2008), among others (Pfeiffer et al. 2001; Abdel Shakor et al. 2004; Bionda et al. 2007), and are essential for the formation of death-inducing signaling complexes (DISCs) (Grassme et al. 2001b; Miyaji et al. 2005; Dumitru and Gulbins 2006). Activation of aSMase and formation of ceramide-enriched domains was also reported for cells irridiated with γ-radiation (Garcia-Barros et al. 2003; El Kaffas et al. 2018; Sharma and Czarnota 2019) or UV-light (Sassa et al. 2012; Yao et al. 2013; Dalmau et al. 2018). These studies also suggested that the apoptotic effect of ceramides is related to the activation of different pathways such as the formation of DISC (Zhang et al. 2001; Rotolo et al. 2005) or translocating activated Bax to the mitochondrial membrane (Kashkar et al. 2005).

Most of the biological actions attributed to ceramide-enriched domains in cancer cells are related to their ability to cluster death receptors and initiate different signaling pathways that culminate in apoptosis. Nevertheless, cancer treatment strategies can also benefit from the natural ability of ceramides to self-aggregate into ordered domains to improve the effectiveness of chemotherapy agents such as docetaxel and paclitaxel (van Vlerken et al. 2007; Tekpli et al. 2013; Feng et al. 2014; Hryniewicz-Jankowska et al. 2014;

Zalba and ten Hagen 2017). It would be interesting to investigate if the presence of ceramide-enriched domains would facilitate the interactions of anti-cancer drugs with the plasma membrane and improve their mechanism of cytotoxic action, as suggested for cisplatin (Dimanche-Boitrel et al. 2005; Baritaki et al. 2007). In this respect, biophysical studies in model membranes could be a first approach to obtain molecular details on the mechanisms underlying drugs-membrane interactions.

Another strategy to induce apoptosis in cancer cells is to target the mitochondrial protein Bax. It is suggested that in the mitochondria associated ER membranes (MAMs) ceramide molar ratios are high enough that ceramide molecules organize into domains. These domains would functioning has apoptotic inducers by forming channel like structures, which permeabilize the mitochondria, increasing the release of cytochrome *c* (Colombini 2010; Stiban et al. 2008; Perera et al. 2016; Légiot et al. 2018), stabilizing the insertion of Bax in the mitochondrial membrane, and promoting mitophagy (Garcia-Gonzalez et al. 2018; Légiot et al. 2018). Recent reviews on ceramide channels (Colombini 2017; Abou-Ghali and Stiban 2015) highlight the relation between ceramide and Bcl-2 family proteins. As an example, Bcl-xL inhibits ceramide channel formation by binding to the apolar ceramide tails, in a manner dependent on the length of the ceramide acyl chain (Perera et al. 2012a, b). On the other hand, the interaction of Bax with ceramides is mediated by the polar regions resulting in synergetic permeabilization of the mitochondrial outer membrane (Perera et al. 2012a, b). However, both ceramide and the membrane need to have the correct biophysical properties in order to promote channel formation that will eventually lead to membrane permeabilization (Perera et al. 2016).

Even though many studies place ceramides in the limelight as a key lipid to induce cancer cell death, the role of ceramides in cancer is not straightforward. In fact, different ceramides might have different biological effects. Indeed, it is now recognized that the role of ceramides in cellular fate might be linked to their specific structure, particularly to the length of their N-acyl chains. As an example, C18-ceramide and C16-ceramide were shown to have antagonist effects in cell proliferation: while the endogenous C18-ceramide or an exogenously added C6-ceramide were shown to activate the repression of the hTERT promoter leading to apoptosis, C16-ceramide did not inhibit hTERT promoter expression. This observation supports the idea that endogenous ceramides with different acyl chains might have different physiological roles (Wooten and Ogretmen 2005; Wooten-Blanks et al. 2007). Even in the context of the aforementioned ceramide channel, very long chain ceramides antagonized the channel formation capacity of C16-ceramide and vice versa (Stiban and Perera 2015). Further studies are therefore necessary to elucidate the molecular mechanisms of ceramides biological action and understand why ceramides have been implicated in so many different cellular effects.

6.5.2 Ceramide in Pathogen Infections

There is evidence that a wide range of pathogens use ordered domains present in the PM to infect mammalian cells. *Neissseriae gonorrhoeae* (Grassme et al. 1997; Hauck et al. 2000), *Pseudomonas aeruginosa* (Grassme et al. 2003), *Staphylococcus aureus* (Esen et al. 2001), *Mycobacteria tuberculosis* (Gatfield and Pieters 2000), *Chlamydia trachomatis* (Stuart et al. 2003), HIV (Liao et al. 2003), *Influenza* virus (Scheiffele et al. 1997), Epstein-Barr virus (Dykstra et al. 2001), Rhinovirus (Grassmé et al. 2005), *Plasmodium falciparum* (Samuel et al. 2001), *Toxoplasma gondii* (Persat et al. 2003) and also prions (Naslavsky et al. 1997) are just a few examples. Ceramide domains have been implicated in several steps of infection, such as pathogen internalization (Schmitter et al. 2004), intracellular maturation of phagosomes (Anes et al. 2003), lysis and fusion of phagosomes (Anes et al. 2003), virus budding (Ono and Freed 2001), immune receptor signaling and induction of cell death upon infection and release of cyto-

kines (Inoue et al. 1994; Schultz et al. 2002; Riethmuller et al. 2006). The involvement of ceramide domains in infection by pathogens might be related with the ability of ceramide to have a rapid trans bilayer movement and to induce negative curvature (Ruiz-Arguello et al. 1996; Veiga et al. 1999). The negative curvature promoted by ceramide might also be involved in membrane fusion. There are some pathogens such as *Neissseriae gonorrhoeae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and Rhinovirus that activate aSMase forming ceramide platforms and promote their internalization trough the re-organization of receptors and mediating the internalization signal (Grassme et al. 1997, 2003; Hauck et al. 2000). In some cases the destruction of lipid rafts and/or the inhibition of aSMase prevents the internalization of these pathogens (Grassme et al. 2003). Additionally, the parasite *Plasmodium falciparum* has encoded in its genome a 46-kDa protein similar to a bacterial SMase that is necessary for its internalization (Hanada et al. 2002).

Mycobacteria are intracellular pathogens internalized by macrophages that are able to prevent the maturation of the phagosome and its fusion with the lysosome. Different studies suggest that this bacteria needs ordered domains in the membrane to be able to be internalized (Gatfield and Pieters 2000; Anes et al. 2003; Wu et al. 2018). Once internalized Mycobacteria interferes with the formation of different SLs such as ceramides, to prevent the fusion of the phagosome with the lysosome (Riethmuller et al. 2006). Furthermore, it was shown that the ceramide/nSMase system was important for systemic infection trough the regulation of different signaling cascades (Wu et al. 2018).

Ceramide platforms might have a critical role in the entrance of different virus, eg. HIV. Increasing evidence shows that the interaction between the viral envelope and the CD4+ receptor occurs in ordered regions of the membrane (Gallo et al. 2003; Klasse 2012). It was also shown that ceramide levels are crucial for the internalization of virus. For example, Finnegan et al. showed that in spite of the interaction of HIV protein envelope with CD4+ receptors occur in ordered regions of the membrane, if ceramide levels are too high the membrane becomes extremely rigid and the interaction does not occur (Finnegan et al. 2004). The authors also claim that the increase in the levels of ceramide induces the internalization of the virus through an endocytic pathway that will eventually lead to its degradation.

6.5.3 Ceramide and Neurodegenerative Diseases

The lipid composition of the brain is responsible for mood, perception and emotional behavior (Shamim et al. 2018). Neurodegeneration does not have a unique cause, it results from a multitude of factors that culminate in loss of different brain cells. In numerous neurodegenerative diseases SL metabolism is highly deregulated leading to an altered membrane organization that contributes to the pathogenesis of the disease (Di Pardo and Maglione 2018; Shamim et al. 2018). The PM of neurons is highly enriched in ordered domains and their content is higher in mature neurons compared to immature neurons and astrocytes (Ledesma et al. 1999; Malchiodi-Albedi et al. 2010). Different lysosomal storage diseases (LSDs) cause neurodegeneration related to the accumulation of ceramide and other sphingolipids in the lysosome (Brady et al. 1966; Suzuki and Suzuki 1985; Fuller et al. 2008; Yu et al. 2018). Farber disease (FD) is one of the rarest LSD, and is characterized by the accumulation of ceramide, as it will be described below. There are increasing evidence that elevated levels of ceramide are related to the pathology of different neurodegenerative diseases such as Alzheimer's disease (AD) (Jana et al. 2009; de la Monte 2012; Figuera-Losada et al. 2015), Parkinson's disease (PD) (Xing et al. 2016; Galvagnion 2017; Shen et al. 2017) or Huntington disease (HD) (Pirhaji et al. 2016; Di Pardo et al. 2017). There is a common ground in these diseases namely high levels or ceramides are associated with the induction of the apoptotic signal culminating in neuronal death (Jana et al. 2009;

Czubowicz and Strosznajder 2014). The induction of apoptosis might be related with the formation of ceramide domains at the PM level that will initiate a signaling cascade through the interaction with different proteins such as MAPKs, protein kinase Cζ (PKCζ), ceramide-activated protein kinases (CAPK), ceramide-activated protein phosphatases (CAPP), cathepsin D, and diverse phospholipases (Mencarelli and Martinez-Martinez 2013; Czubowicz and Strosznajder 2014). Moreover, increased ceramide levels stimulate the production of reactive oxygen species (ROS) that will also contribute to the pathology of AD (Huang et al. 2016; Manoharan et al. 2016). Ceramide has also been associated to PD and several studies highlight the importance of this lipid in the pathophysiology of this disease (Mielke et al. 2013; Galvagnion 2017; Lin et al. 2019). The role of ceramide domains has, to our knowledge, not been investigated. It could be interesting to explore if the presence of ordered ceramide domains would affect α-synuclein aggregation and other mechanisms contributing to PD development. Further evidence that ceramide is related to the apoptotic events in neurodegeneration is given by the fact that inhibiting nSMase has a protective effect against cell death and dendritic damage (Figuera-Losada et al. 2015).

6.5.4 Farber Disease

Ceramides have been implicated in different pathologies. However, there is one pathology characterized by the accumulation of ceramides – FD. FD is an autosomal recessive LSD, caused by mutations in the lysosomal acid ceramidase (ASAH1) gene (Sands 2013). These mutations affect the activity of acid ceramidase (ACDase). Consequently, ceramide cannot be efficiently degraded and accumulates within the lysosomes. There are around 50 different diseased-linked mutations associated with the ASAH1 gene (Yu et al. 2018) that are responsible for the wide spectrum of symptoms observed in FD (Levade et al. 2009). Ceramide accumulation is present in most tissues and the classical manifestations include the formation of subcutaneous nodules, joint stiffness and deformation, besides the development of a hoarse voice due to laryngeal involvement. With the progression of the disease, patients can also develop defects in the cardiac, pulmonary and central nervous system (Ehlert et al. 2007). In a relative recent work, Ferreira et al. tried to establish a correlation between the levels of ceramide and the severity of the disease (Ferreira et al. 2014). Taking advantage of an antibody able to recognize specific 2D arrangements of C16-ceramide/chol, i.e., C16-ceramide/chol macrodomains (Scheffer et al. 2006), the authors showed that the accumulation of ceramide in fibroblasts from FD patients occurs not only in late endosomes and lysosomes, but also in mitochondria and PM. The accumulation of ceramide in extra-lysosomal localizations is in accordance to what was described for other lysosomal storage diseases (Futerman and van Meer 2004; Platt et al. 2018). Since there is no evidence that Chol accumulates in FD, this study also suggests that there are specific interactions between ceramides and cholesterol. One hypothesis is that ceramide might recruit Chol to form microdomains with specific biophysical properties. In the absence of Chol, the ceramide domains would be extremely rigid, while the presence of Chol might increase their fluidity conferring properties in between the liquid ordered and the gel phase (Castro et al. 2009; Silva et al. 2009). The high levels of ceramide in FD patients put these cells under constant stress yet the cells do not enter apoptosis. This suggests that ceramides and Chol might interact through a precise mechanism that protects the cells. Another hypothesis is that the formation of structures that shield or sequester ceramides prevent an apoptotic signal, or the transformation into a non-lethal ceramide. Further research aiming at elucidating the role of membrane biophysical properties in FD might contribute to the identification of additional molecular mechanisms underlying this disease.

6.6 Conclusions

The myriad of alterations potentially caused by ceramides in the biophysical properties of membranes, which depend on the ceramide acyl chain structure, sphingoid backbone and interaction with neighboring lipids and aqueous environment, support the hypothesis that specific biophysical mechanisms might underlie the biological and pathological actions attributed to ceramides. Due to their unique biophysical properties, ceramides are expected to cause rapid and significant changes in the fluidity, lateral organization and structure of biological membranes, and provide a fast-cellular response to an insult. Despite the importance of ceramide biophysics in cell function and the extensive number of studies aiming at understanding ceramides biological action, the intersection between these paths is still far from being reached. Progress in the area will depend on close collaboration between researchers from different fields, but also on technological developments that enable better assessment of membrane lipid composition, interactions, trafficking and metabolism in a cellular context. Aside from the experimental difficulties, research focused on understanding ceramide biophysics is promising and will likely contribute to identify molecular mechanisms that regulate cell pathophysiology.

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Sphingolipids as Biomarkers of Disease

Faris Matanes, Waleed O. Twal, and Samar M. Hammad

Abstract

Despite the advancements in modern medicine, there are still difficulties in diagnosing common illnesses. The invasiveness and price of the tests used to follow up certain diseases can be a barrier to proper patient follow-up. Sphingolipids are a diverse category of lipids. They are structural molecules in cell membranes and signaling molecules involved in the regulation of crucial cell functions, including cell growth, differentiation, proliferation and apoptosis. Recent research has shown that abnormal sphingolipid metabolism is associated with genetic and metabolic disease processes. Given their crucial role to maintain homeostasis within the body, sphingolipids have been investigated as potential biomarkers to predict disease in the population. Here we discuss how sphingolipids levels are altered in different diseases, thus illustrating their possible use as diagnostic and prognostic biomarkers for disease.

Keywords

Sphingolipids · Biomarkers · Lipidomics · Ceramide · Sphingosine · Sphingomyelin

Abbreviations

ABCA1	ATP binding cassette family A protein 1
ACS	acute coronary syndrome
AD	Alzheimer disease
ASMase	sphingomyelinase
BMI	body mass index
BNP	B-type natriuretic peptide
C1P	ceramide 1-phosphate
CAD	coronary artery disease
CKD	chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CSF	cerebrospinal fluid
DAG	diacylglycerols
DHC	dihydroceramides
DHSM	dihydrosphingomyelin
FA	fluorescent angioscopy
FFA	free fatty acids
Gb3	globotriaosylceramide

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HbA1c	hemoglobin A1c
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HF	heart failure
HOMA-IR	homeostatic model assessment for assessing β-cell function and insulin resistance from fasting glucose and insulin concentrations
HSAN1	hereditary sensory and autonomic neuropathy type 1
HxCer	hexosylceramide
IDL	intermediate-density lipoprotein
IMT	intima media thickness
LacCer	lactosylceramide
LDL	low-density lipoprotein
LVEF	left ventricular ejection fraction
LysoGb3	globotriaosylsphingosine
MACE	major adverse cardiovascular event
MCI	Mild Cognitive Impairment
MELD	Model for End-Stage Liver Disease
MI	myocardial infarction
MMSE	Mini-Mental State examination
NT-proBNP	N-terminal-prohormone BNP
NYHA	New York Heart Association
PTSD	post-traumatic stress disorder
S1P	sphingosine 1-phosphate
SA1Ps	sphinganine 1-phosphate
SLE	systemic lupus erythematous
SM	Sphingomyelin
SPT	serine palmitoyltransferase
TG	triglycerides
TNF-α	tumor necrosis factor-α
TNM	TNM Classification of Malignant Tumors
VAD	ventricular assist device
VLDL	very low-density lipoprotein

7.1 Introduction

Sphingolipids are a diverse category of lipid molecules, which contain a backbone of sphingoid bases, a set of aliphatic amino alcohols that includes sphingosine, and numerous variations in long-chain hydrocarbon groups, and N-acyl linked fatty acids and head groups. Sphingolipids include sphingomyelin (SM), ceramide, cerebroside, sphingosine 1-phosphate (S1P) and numerous others. Sphingolipids are important constitutions of various membranes in the cell and they can also function as signaling molecules. The most abundant sphingolipids in the circulation are SM, lactosylceramide (LacCer), hexosylceramide (HxCer), and ceramide, (Hammad et al. 2010). The number of carbons in the long-chain hydrocarbon group differentiates the sphingolipid species.

Ceramide serves as the main precursor in sphingolipid biosynthesis and can be synthesized through various pathways (Fig. 7.1). The *de novo* synthesis pathway, takes place in the endoplasmic reticulum, where ceramide can be synthesized from serine and palmitoyl-CoA into more complex sphingolipids (Iqbal et al. 2017). Another common pathway is the salvage/catabolic pathway, in which ceramide can be broken down to serve as the precursor for sphingosine and S1P (Fig. 7.1). These sphingolipids alongside phospholipids, triglycerides (TG) and cholesterols are transported in the blood after being incorporated in lipoproteins: low- and very low-density apoB-containing lipoproteins (LDL, VLDL) and high-density lipoproteins (HDL) (Hammad et al. 2010; Iqbal et al. 2017; Hammad et al. 2012b). S1P can also be transported bound to serum albumin. During the past few decades, sphingolipids have been linked to the pathophysiology of many diseases in the human body. This has led to detailed investigations regarding the metabolism and functions of different sphingolipids. Since different sphingolipids have been linked to cell growth and migration, inflammation, angiogenesis, apoptosis, and senescence (Hannun and Obeid 2018), their role in mediating disease processes is becoming evident.

Sphingolipids were first studied in patients with lysosomal storage diseases, which cause deleterious symptoms in several body systems. Accumulation of sphingolipids can result from deficiencies in enzymes involved in the sphingolipid metabolism pathways (Fig. 7.1). Levels of globotriaosylceramide (Gb3) and

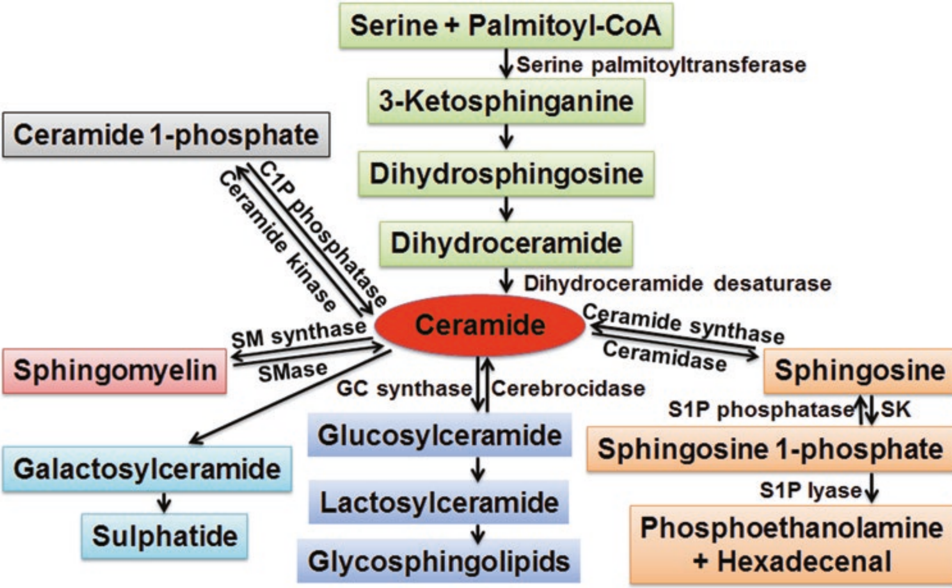

Fig. 7.1 The metabolic pathways for production and degradation of different sphingolipids

globotriaosylsphingosine (LysoGb3) for example were found to be increased in plasma, urine and cellular lysosomes of patient with Fabry disease (Desnick et al. 2003). It was also shown that LysoGb3 serum levels can be used to confirm the diagnosis in individuals with atypical Fabry disease, as well as help in determining the need for treatment (Nowak et al. 2017). This illustrates that measuring sphingolipid levels in the serum or other bodily fluids can predict the effect of the disease and facilitate choosing the correct mode of treatment.

This review discusses the possible use of sphingolipids as biomarkers for certain diseases known to cause high levels of morbidity and mortality to many patients. Relating the level of blood sphingolipids to the presence or progression of these diseases can provide a reliable method of supporting the diagnosis and follow-up treatment, and hence improve outcomes. Since several mass spectroscopy studies have established normal ranges for different sphingolipids in the blood (e.g., Hammad et al. 2010, Scherer et al. 2010), measuring variation in sphingolipid levels from the normal range in patient's fluids is now more attainable.

In this review, we discuss abnormal levels of different sphingolipid species found in different diseases. We also address the use of sphingolipids, mainly in blood, urine and cerebrospinal fluid, as biomarkers of disease due to the routine collection of samples from these sources in clinical settings. Each section summarizes the novel findings reported in a certain body system or disease.

7.2 Cardiovascular Disease

Cardiovascular disease is the number one cause of death in the world. The knowledge of risk factors and pathophysiology of this disease has progressed in the past few decades, but we still lack a simple and accurate method of determining the severity of the stenosis in the coronary arteries, and the probability of developing acute coronary syndrome. The role of ceramides, sphingosine and SM in the diagnosis of coronary artery disease (CAD) and heart failure (HF) are reviewed in this section.

7.2.1 Coronary Artery Disease (CAD)

Ceramides were found to be present in high amount in coronary plaques (Uchida et al. 2017). During percutaneous coronary intervention, fluorescent angioscopy (FA) was used to indicate the presence of ceramide in coronary plaques by giving a characteristic golden fluorescence. Ceramide was detected via fluorescent microscopy in most yellow plaques with a necrotic core but rarely in those without necrotic core. Uchida et al. (2010) reported that this method may imply vulnerability of the plaque to rupture (i.e., thin fibrous cap with necrotic core). This test also provided prognostic factors, as plaques exhibiting golden fluorescence had a thin fibrous cap indicating that they might break off emboli more easily. FA revealed the presence of ceramide in patients with stable angina and also in those with old myocardial infarction (MI). Golden fluorescence in plaques was observed in patients with old MI more often than in those with stable angina, indicating that the former group has higher risk of recurrent acute coronary syndrome (ACS) (Uchida et al. 2017). This illustrates that ceramide detection in a plaque not only indicates the fragility of the plaque, but also can predict the risk of future MI.

Meeusen et al. (2018) examined the prognostic values for ceramides in individuals who have had a major adverse cardiovascular event (MACE) after coronary angiography. They measured plasma ceramide levels in a cohort of 495 individuals before non-urgent coronary angiography, and were then followed for 4 years to document the cardiovascular outcome. Levels of C16:0, C18:0, and C24:1 ceramide were found to be significantly predictive of the combined outcome of MI, stroke, revascularization, and death from any cause after 4 years of follow up. Although C24:0 ceramide was not predictive, using C16:0, C18:0, and C24:1 ceramide as a ratio with C24:0 ceramide strengthened the association with outcome. In both situations, ceramides were still predictive even after adjusting for age, sex, body mass index (BMI), hypertension, smoking, HDL, LDL, TG, glucose, and family history of CAD. The fully adjusted per SD hazard ratios (95% confidence interval) were 1.50 (1.16–1.93) for C16:0, 1.42 (1.11–1.83) for C18:0, 1.43 (1.08–1.89) for C24:1 ceramide, and 1.58 (1.22–2.04) for the ceramide risk score. Furthermore, death of any cause was reassessed at 18 years after the start of the study. Levels of C16:0 and C24:1 ceramide were significantly predictive for all causes of death at 18 years. The ratios of C16:0/C24:0 ceramide, C18:0/C24:0, and C24:1/C24:0 ceramide were significantly predictive and remained significant after adjusting for age, sex, BMI, hypertension, smoking, HDL, LDL, TG, serum glucose, and family history of cardiovascular (Meeusen et al. 2018).

A recent European study investigated the prognostic value of plasma ceramides as cardiovascular death markers in three independent CAD cohorts (Laaksonen et al. 2016). A 0–12-point risk score using the levels of C16:0, C18:0, C24:1 ceramide, and the ratio of those ceramides with C24:0 ceramide was developed. Ceramides, especially when used in ratios, were significantly associated with cardiovascular death in all studies, independent of other lipid markers and C-reactive protein. Another recent European study further examined whether ceramides are associated with MACE among apparently healthy individuals (Havulinna et al. 2016). The results demonstrated that the ceramide-based risk stratification extends beyond the current lipid-based diagnostics and addresses the unmet need for improved identification of high-risk CAD patients. For recurrent MACE, both C16:0 and C24:1 ceramide species showed significant univariate associations, which were calculated as hazard ratios. C18:0 ceramide had a hazard ratio which remained significant at 1.21 (95% confidence interval, 1.11–1.33) after adjusting for the Framingham Risk Score factors (Havulinna et al. 2016). Remarkably, a ceramide-based identification of coronary patients at high cardiovascular risk has recently entered the clinic in the USA, which will allow for a better evaluation of the ceramide utility in clinical diagnosis of cardiovascular disease.

In a different study, Park et al. (2015) used UPLC/Q-TOF MS-based lipidomics approach

and found six plasma lipid metabolites, including SM, ceramide, and glucosylceramide, correlated with an increased risk of MI. Results from their study showed a 1.11–1.17-fold increased risk of MI, but not angina, associated with these sphingolipid markers. In MI patients, strong positive correlation between lipid metabolites related to the sphingolipid pathway, SM, and ceramide and acute inflammatory markers (high-sensitivity C-reactive protein) were found (Park et al. 2015). Thus, the identification of plasma sphingolipid metabolites in CAD patients may uncover the important roles of these metabolites in the development of MI versus angina.

Recently Saleem et al. (2018) addressed the wide variability in response to cardiac rehabilitation in patients with CAD and explored plasma sphingolipid levels as biological correlates of cardiopulmonary fitness in patients with CAD. The results showed higher concentrations of C18:1 SM ($p = 0.01$), C16:0 ($p = 0.02$), C18:0 ($p = 0.002$), C20:0 ($p = 0.02$) and C24:1 ($p = 0.01$) ceramide species, and C18:0 mono-HxCer ($p = 0.02$) were associated with poorer VO_{2peak} at baseline. An improvement in VO_{2peak} was associated with a decrease in C18:1 SM, C16:0, C18:0 and C24:1 ceramide, and C18:0 HxCer (Saleem et al. 2018). Certain long-chain sphingolipids therefore could be useful markers of fitness and response to exercise in CAD.

7.2.2 Heart Failure (HF)

B-type natriuretic peptide (BNP) is a hormone produced by the heart and the N-terminal (NT)-prohormone BNP (NT-proBNP) is a non-active prohormone that is released from the same molecule that produces BNP. Both BNP and NT-proBNP are released in response to changes in pressure inside the heart and can be related to HF and other cardiac problems. Although different assays of detecting BNP and NT-proBNP are available, these assays still have variable sensitivity and specificity (Rawlins et al. 2005). This shows that the discovery of other markers in addition to BNP can be advantageous. Increased total and very long-chain ceramides were found in myocardium and serum of patients with advanced HF (Ji et al. 2017). These values were further increased in the circulation after unloading via a ventricular assist device (VAD); however, ceramide levels decreased in the myocardium following mechanical unloading through VAD placement (Ji et al. 2017). The change in myocardial ceramide levels affected nearly all chain lengths of ceramide species measured (total ceramides and C16:1, C16:0, C20:1, C20:0, C22:1, and C24:1 ceramide species), but the most significant changes were in the levels of C18:1 ceramide (Ji et al. 2017).

Genetic deletion of the serine palmitoyltransferase (SPT) long chain base subunit 2 (*SPTLC2*) gene preserved cardiac function following MI (Ji et al. 2017). This indicates that potential treatments blocking the function of this gene may ameliorate the damage occurring from MI. It was also found that mean serum levels of C16:0 (0.289 vs. 0.229 μM, $p = 0.017$), C18:0 (0.132 vs. 0.089 μM, $p = 0.002$), C20:1 (0.006 vs. 0.003 μM, $p = 0.038$), C20:0 (0.013 vs. 0.009 μM, $p = 0.007$), C22:1 (0.042 vs. 0.022 μM, $p = 0.018$), C24:0 (2.454 vs. 3.09 μM, $p = 0.012$) and C24:1 (1.178 vs. 0.805 μM, $p = 0.0001$) ceramide species were increased in HF patients compared to control subjects (Ji et al. 2017); however, the levels of total ceramides were not different in both groups. In another study, a positive correlation was found between total plasma ceramide levels and the severity of HF and death from HF (Yu et al. 2015).

Polzin et al. (2017) showed that plasma levels of S1P and SM were decreased in individuals with reduced left ventricular ejection fraction (LVEF <40%) compared to patients with normal or mildly decreased LVEF). Regression analysis results showed a negative correlation between plasma S1P and SM and LVEF. In addition, this correlation was applicable in relation to universally used scores; individuals with the New York Heart Association (NYHA) classification class III and IV showed plasma S1P and SM levels lower than those in individuals with NYHA class I and II (Polzin et al. 2017). Youden's index showed an optimal cut-off level of S1P below 843 nM and SM below 77 μM to predict impaired

LVEF. This association is even more evident by the fact that cardiomyocyte-specific depletion of S1P receptor 1 resulted in progressive cardiomyopathy characterized by both systolic and diastolic dysfunction (Keul et al. 2016).

The above reviewed literature demonstrates the potential role of sphingolipids as diagnostic and prognostic biomarkers for HF, and also indicate that drugs targeting sphingolipid metabolism or sphingolipid signaling pathways may have direct effect on the progression of HF and MI.

7.2.3 Hypertension

Hypertension is a leading cause of heart disease, stroke and kidney disease characterized by endothelial dysfunction and changes in vascular tissue architecture (Intengan and Schiffrin 2001). Plasma ceramide levels were compared between 49 hypertensive patients and 18 normotensive controls (Spijkers et al. 2011). In the plasma of individuals with blood pressure over 160/100, mean ceramide levels (±SEM) were significantly higher than normotensive controls (252 ± 23 vs. 185 ± 8 pmole/95 μl sample, $p < 0.05$). In addition, the ceramide levels had a positive correlation with the severity of hypertension. The observed increases were mainly in the concentrations of C24:1 and C24:0 ceramides. Mean plasma levels of S1P (±SEM) were also higher in hypertensive patients than normotensive patients (37.0 ± 1.8 vs. 32.0 ± 1.2 pmol/95 μl sample, $p < 0.05$). However, there were no significant changes in plasma levels of SM and ceramide 1-phosphate (C1P) (Spijkers et al. 2011).

7.3 Cancer

Cancer is the second leading cause of death in the US. Although some screening programs have been successful in reducing the mortality rate in some patient populations, many have failed. Moreover, many cancer types still have no reliable screening program (Buys et al. 2011). There are many risk factors associated with different types of cancers; however, many individuals who suffer from cancer exhibit no risk factors. This indicates the vast impact of finding novel biomarkers on early detection and treatment modalities for the different types of cancer. The levels of sphingosine, ceramides and gangliosides (a ganglioside is a molecule composed of a glycosphingolipid (ceramide and oligosaccharide) with one or more sialic acids linked on the sugar chain) in different types of cancer are discussed.

7.3.1 Ovarian Cancer

Ovarian cancer has the highest mortality rate among all gynecological tumors. Although early stage of this cancer still has a relatively good prognosis, many patients present with late stage due to the vague symptoms or lack of any. Current screening methods might produce some major complications due to their invasive techniques; and importantly they failed to significantly decrease mortality (Buys et al. 2011). Thus, a biomarker, which would be less invasive and would simplify the screening method, is still in need. Studies showed that different sphingolipids were elevated in plasma (ganglioside, lysophospholipids, ceramides, phytosphingosine (Fan et al. 2012)), urine (phytosphingosine (Chen et al. 2012)), and ascitic fluid (ceramides (Shender et al. 2014)) of patients with ovarian cancer.

Whereas ceramide promotes apoptosis, C1P, which is generated from the phosphorylation of ceramide, is mitogenic and has pro-survival properties (Arana et al. 2010). C1P has dual molecular functions: it acts as an intracellular second messenger to promote cell survival, and as an extracellular receptor agonist to stimulate cell migration (Arana et al. 2010). C1P also mediates inflammatory responses through the stimulation of cytosolic phospholipase A2, and the subsequent release of arachidonic acid and prostaglandin formation (Pettus et al. 2003). Plasma levels of C12 C1P, lysophosphatidylcholines, and lysophosphatidylethanolamines were up-regulated in localized epithelial ovarian (early stage) cancer patients compared to benign ovarian tumor/uterine fibroid patients, whereas they were remarkably lower in metastatic epithelial ovarian

cancer patients (Ke et al. 2015). This indicated that the elevation of these metabolites might be associated with ovarian cancer initiation, while their down-regulation in metastatic epithelial ovarian cancer might correlate with cancer metastasis and progression.

7.3.2 Colorectal Cancer

Colorectal cancer is the fourth most common cancer in the US. It is also one of the cancers with the highest mortality rates especially when detected at a late stage. Currently the staging method used is the TNM Classification of Malignant Tumors (TNM). However, many patients with the same stage of colorectal cancer respond differently to treatment; hence, the use of a novel test in addition to TNM staging would be beneficial. In patients with stage IV colorectal cancer the levels of C16:0, C18:0, C18:1, C24:1 ceramide, sphingosine and SM C24:0 had a fold change of 1.68, 1.86, 1.72, 1.51, 1.68 and 1.59, respectively (Separovic et al. 2017). Separovic et al. (2017) concluded that ceramides and SM but not HxCer are implicated in stage IV colorectal cancer (HxCer, a family of sphingolipids comprise both glucosyl- and galactosyl ceramides serve as precursors for more complex sphingolipids and undergo their own metabolic pathways).

7.3.3 Lung Cancer

Most cases of lung cancer are unfortunately diagnosed at a late stage due to lack of symptoms early in the disease course; which emphasizes the necessity of finding novel screening methods. Geometric mean plasma concentrations of S1P and total ceramides were found to be 2.9% ($p = 0.10$) and 5.1% ($p = 0.02$) greater in lung cancer cases compared with controls, respectively (Alberg et al. 2013). Increased S1P levels in plasma were associated with a higher risk of future lung cancers ($p = 0.006$) (Alberg et al. 2013). In addition to the possible use of sphingolipids as markers for cancers, it was proven that the ratio between free oleic acid and ceramide in pleural fluid of lung cancer patients was elevated (Lam and Law 2014). This test was proposed to be useful in suggesting and/or confirming malignant pleural effusions.

7.3.4 Brain Tumors

Brain cancer is one of the most difficult tumors to diagnose since tissue biopsy is typically needed for diagnosis or confirmation of diagnosis. Finding a reliable screening method for brain tumors could be a breakthrough. Higher levels of gangliosides were reported in serum of individuals with brain tumors of neuroectodermal origin (Fish 1996). These tumor cells were shown to shed gangliosides into interstitial spaces and blood. This mechanism was proposed to play a role in increased tumor cell growth and lack of immune cell recognition (Alberg et al. 2013; Valentino et al. 1990).

7.3.5 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a well-known complication of liver cirrhosis. In a clinical study, 249 individuals with cirrhosis were enrolled, 122 had HCC and 127 did not; both age and gender were matched between the two groups (Grammatikos et al. 2016). Serum concentrations of long-chain ceramides (C16:0-C20:0) and very long-chain ceramides (≥C24:0) and dihydroceramides (DHC), the synthetic precursors of ceramides, were measured. There were significant increases in the concentrations of C16:0, C18:0, C20:0, and C24:0 ceramides in HCC patients compared to patients with cirrhosis ($p < 0.001$). C16:0, C18:0 and C24:0 DHC followed the same pattern ($p < 0.001$). The serum concentrations of C24:1 ceramide and C24:1 DHC were not significantly different between the two groups. Sphingosine, S1P and sphinganine 1-phosphate (SA1P) (also known as dihydrosphingosine 1-phosphate) were also reported in higher concentrations in HCC patients compared to cirrhotic patients ($p < 0.001$). Correlation between serum sphingolipid levels and HCC

severity was variable: C16:0 DHC and C16:0 ceramide had a positive correlation with the Model for End-Stage Liver Disease (MELD) score, whereas levels of C20:0 ceramide, C24:0 ceramide, S1P and SA1P were inversely proportional to the MELD score. None of the sphingolipids had an association with the Barcelona Clinic Liver Cancer stages (Grammatikos et al. 2016).

7.4 Respiratory and Lung Disease

Treatment of lung disease is an enormous burden on health care systems worldwide. Most therapies available for lung disease in general are only symptomatic treatment, with only few available methods that are proven to be disease modifying. More effort to find more convenient and feasible methods for early diagnoses and treatment of these illnesses is still needed. The roles of S1P and its receptors, ceramides, SM, and gangliosides are discussed below.

7.4.1 Asthma

Asthma is a disease characterized by airway hyper-responsiveness and inflammation; it affects about 8% of the American population. Sphingolipids were found to be altered in one sub-type of asthma patients, which is exercise-induced asthma (Perzanowski et al. 2017). Although there was no significant difference in serum sphingolipid concentrations among the asthmatic and non-asthmatic school-aged children participated in the study, it was found that in asthmatic children with exercise-induced wheezing, concentrations of C18:0 DHC, ceramides C18:0, C20:0, C24:0, deoxy-C24:1, and SM 24:1 were higher than those in asthmatic children without exercise-induced wheezing. Interestingly, elevated concentrations of C18:0 and C20:0 DHC at age 7–8 years predicted asthma persistence 3 years later. These associations were predominately observed in the non-overweight children that were not altered by seroatopy, suggesting a mechanism independent of allergic sensitization (Perzanowski et al. 2017). In another study, S1P were found in increased amounts in the bronchoalveolar lavage of asthma patients one day after segmental allergen challenge (Ammit et al. 2001). Caution however must be taken in interpreting results from the bronchoalvelolar lavage fluid as it was shown that there are normal levels of certain ceramides in the lavage of healthy individuals (Petrusca et al. 2010).

7.4.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is the third leading cause of death worldwide and smoking is the major risk factor for COPD. Most smokers do not develop COPD; however, those who do have variable clinical phenotypes including airflow obstruction, emphysema, and chronic bronchitis (Friedlander et al. 2007). There is little known about what predisposes certain smokers to develop a particular COPD phenotype. There have been recent reports suggesting that alteration in sphingolipid metabolism may be linked to COPD susceptibility (Ahmed et al. 2014; Telenga et al. 2014). In a study addressing the specific types of sphingolipids associated with COPD in current and former smokers from the COPDGene cohort, the most statistically significant negative association between specific COPD phenotypes and sphingolipid classes was between emphysema and SM, and the most statistically significant positive association was between COPD exacerbations and tri-HxCer (glycosphingolipids) (Bowler et al. 2015). The three plasma sphingolipid species most significantly negatively associated with emphysema were ceramide C16:0 ($p = 0.0006$), ganglioside GM3 C16:0 ($p = 0.0004$), and SM C16:0 ($p = 0.0009$), followed by mono-HxCer C16:0 ($p = 0.002$). Interestingly, these sphingolipids improved the ability to diagnose moderate to severe COPD phenotype beyond just clinical and physiologic covariates.

7.5 Dementia and Mental Diseases

Neurologic and psychiatric disorders are one of the largest burdens on the health care system in the US, and they are among the most difficult illnesses to diagnose and treat. This explains why through the past few decades and even today, many research efforts are being directed towards this field. There is considerable evidence for specific pathology of lipid metabolism in mental diseases, affecting in particular sphingolipids. Such deficits are assumed to interfere with neuronal membrane functioning and the development and maintenance of myelin sheaths (Wattenberg 2019). The possible use of sphingolipids in both diagnosis and treatment of these diseases could be a critical step in improving patient care. In this section, we discuss the novel findings that support the use of sphingolipids as biomarkers for Alzheimer disease (AD) and other neurological disorders.

7.5.1 Alzheimer Disease (AD)

AD and other forms of dementia are currently ranked as the fifth leading cause of death worldwide. Sphingolipids were helpful in screening for AD and interestingly correlated with the pathological manifestations of the disease. This section discusses the findings of multiple reports supporting the use of different sphingolipid species as biomarkers for AD.

In a cross-sectional study comparing 26 individuals with AD and 26 individuals with normal cognition, plasma levels of sphingolipids species were altered in AD compared to control subjects (Han et al. 2011). Eight out of 33 SM species tested, particularly those with long aliphatic chains (C22:0 and C24:0) were significantly decreased in AD ($p < 0.05$) compared to control subjects, whereas ceramide C16:0 and C21:0 were significantly increased ($p < 0.05$). Ratios of SM and ceramide species with identical fatty acyl chains showed more robust discrimination than either metabolite alone. The Mini-Mental State examination (MMSE), a brief screening tool of cognitive status was used to categorize AD patients as mild or moderate (Han et al. 2011). The same findings were seen both in mild and moderate stages of AD with some metabolites also correlated with cognitive performance. For example, MMSE rank was significantly correlated with the rank of the altered mass levels of both SM 20:2 and OH-C25 ceramide (Han et al. 2011).

Cerebrospinal fluid (CSF) metabolite levels usually correlate with those of the blood, because the CSF is reabsorbed into the vascular system and therefore exchanges metabolites with blood. CSF metabolites also correlate well with levels of metabolites in brain cells. A pilot study of phospholipid pattern changes in the CSF showed higher CSF SM levels in patients with probable AD compared to cognitively normal controls (Kosicek et al. 2010). All eight SM species were increased from 20% to 40% in CSF samples from the probable AD group; three of them were statistically significant ($p < 0.05$), probably because of the small sample size. Another study further explored if levels of CSF SM species were correlated with the severity of AD and showed that SM levels were significantly increased ($50.4 \pm 11.2\%$, $p = 0.003$) in the CSF from individuals with prodromal AD compared to cognitively normal controls, but no change in CSF SM levels between mild and moderate AD groups and cognitively normal controls (Kosicek et al. 2012). These results suggested that alterations in the SM metabolism could contribute to early pathological processes leading to AD.

Mielke et al. (2010b) examined baseline serum SM and ceramides as predictors of cognitive impairment from 100 older women enrolled in the longitudinal population-based Women's Health and Aging Study II. Cognitively normal women at baseline were followed up to six times over 9 years. Cross-sectional tests showed that lower levels of serum ceramides and SM were correlated with memory impairment. On the contrary, elevated ceramide levels longitudinally predicted memory impairment (Mielke et al. 2010b). This result was evident by the fact that memory impairment during the 9 years of follow-up was reported in none of the women with blood levels

of ceramide C22:0 in the lowest tertile, and only in one woman with blood levels of ceramides C16:0 and C24:0 in the lowest tertile (Mielke et al. 2010b). In another study, the group investigated in the older women whether serum ceramides can predict all-cause dementia or are specifically associated with AD (Mielke et al. 2012). Twenty-seven percent of the women developed incident dementia; of those 67% were diagnosed with probable AD. Higher baseline serum ceramides, but not SM, were associated with an increased risk of AD; these relationships were stronger than with all-cause dementia. Compared to the lowest tertile, the middle and highest tertiles of ceramide C16:0 were associated with a 10-fold and 7.6-fold increased risk of AD, respectively. The highest tertiles of ceramide C24:0 and LacCer were also associated with risk of AD. Total and HDL cholesterol and TG were not associated with dementia or AD (Mielke et al. 2012).

Mielke et al. (2010a) also examined whether plasma ceramide levels varied by AD severity in a well-characterized clinic sample and were associated with cognitive decline and hippocampal volume loss over one year. The results suggested that very long-chain plasma ceramides C22:0 and C24:0 are altered in Mild Cognitive Impairment (MCI) and can predict memory loss and right hippocampal volume loss among subjects with MCI (Mielke et al. 2010a). The group then examined whether plasma ceramides, DHC, SM, or dihydrosphingomyelin (DHSM) levels and ratios of SM/ceramide or DHSM/DHC were predictive of progression in AD (Mielke et al. 2011). Participants were followed a mean of 4.2 visits and 2.3 years. There were no cross-sectional associations; however, in longitudinal analyses, high levels of DHC and ceramide were associated with greater progression, but findings did not reach significance. In contrast, higher plasma levels of SM, DHSM, SM/ceramide and DHSM/DHC ratios were associated with less progression on the Mini-Mental State Exam, and the Alzheimer's Disease Assessment Scale-Cognitive Subscale; the ratios were the strongest predictors of clinical progression (Mielke et al. 2011).

Recently, Varma et al. (2018) undertook parallel metabolomics analyses in both the brain and blood to identify systemic correlates of neuropathology and their associations with prodromal and preclinical measures of AD progression. They found that C16:0, C18:1, C16:1 SM and C14:1 hydroxy-SM were consistently associated with severity of AD pathology at autopsy and AD progression across prodromal and preclinical stages. Cognitively normal individuals with elevated blood concentrations of all four sphingolipids were significantly associated with increased risk of future conversion to incident AD (Varma et al. 2018). Interestingly, higher baseline blood concentration of SM C18:1 ($p = 0.012$) and C26:1 ($p = 0.050$) were predictive of greater declines in attention and language, respectively. Moreover, the identified sphingolipid species were found to map to several biologically relevant pathways implicated in AD, including tau phosphorylation, amyloid-β (Aβ) metabolism, calcium homeostasis, acetylcholine biosynthesis, and apoptosis. For instance, higher blood concentrations of SM C16:0 and hydroxy-SM C14:1 were associated with greater CSF levels of total and phosphorylated tau in addition to lower CSF levels of Aβ 1-42 (Varma et al. 2018). C16:0 and C18:1 SM, were associated with more AD-like patterns of brain atrophy on magnetic resonance imaging scans measured by the Spatial Patterns of Abnormality for Recognition of Early Alzheimer's disease index (Varma et al. 2018). In brain disorders like AD both the paucity of passage of many molecules through the blood brain barrier, and the difficulty in correlating peripheral markers with brain processes might prevent the serum and CSF molecular level from reflecting the actual levels in the brain.

7.5.2 Psychiatric Disorders

The 2016 National Survey on Drug Use and Health revealed that 18.3% of the American population had a form of mental illness (Ahrnsbrak et al. 2016), and due to this high prevalence, more specific screening methods of these diseases remain needed. In a study measuring the levels of

plasma ceramides in patients with recent, past or no depression, it was found that individuals with recent depression had higher levels of C16:0, C18:0, C20:0, C24:1 and C26:1 ceramides compared to those with no depression or past depression ($p < 0.05$; ceramide measurements were log-transformed and examined as continuous variables in units of counts per second) (Gracia-Garcia et al. 2011). In another family-based study which included 742 individuals, anxiety and depressive symptom scores measured using the Hospital Anxiety Scale showed inverse correlation with plasma SM 23:1 to SM 16:0 ratio (Demirkan et al. 2013).

Sphingolipids were also found to be useful indicators of alcohol toxicity and could be used to distinguish chronic from acute alcohol abuse. In alcohol-dependent patients, Reichel et al. (2010) reported that the activity of acid sphingomyelinase (ASMase), the enzyme that generates ceramide from SM, was increased 3-fold (141 ± 69 vs. 428 ± 220 pmol/ml/h; $p < 0.001$) in peripheral blood cells of acutely intoxicated patients with alcohol dependence, while chronic consumption of alcohol affected the activity of secretory ASMase in blood plasma (Reichel et al. 2011). Further analyses by the same group revealed that the plasma levels of S1P are also increased in alcohol-dependent patients (Mühle et al. 2013). Recently, Mühle et al. (2019) evaluated the potential of the activity of peripheral (secretory) ASMase measurement as a biomarker for depression, its severity, and prospective course. They identified an association of high serum ASMase activity with a stronger improvement of depression assessed independently by self-report and clinician's interview in medicated patients, and concluded that secretory ASMase could be developed as a useful biomarker for predicting the course of depression only after stabilization of medication.

In a study comprised of schizophrenia patients with well-matched healthy control individuals, lipids were extracted from postmortem brain samples (white and gray matter separately) (Schwarz et al. 2008). The membrane phospholipid profile of red blood cell samples from living drug naïve and treated schizophrenia patients was also examined. The data suggested that schizophrenia may involve lipid abnormalities present throughout the body. Ceramide levels were elevated in white mater of the prefrontal cortex of schizophrenia patients, and variations in free fatty acid (FFA) and ceramide concentrations were reported in red blood cell samples from both drug-treated and drug-naive schizophrenic patients (first-onset patients) (Schwarz et al. 2008). Smesny et al. (2013) measured differences in skin lipids (stratum corneum lipids of the epidermal layer) in first-episode schizophrenia patients, as a peripheral model to examine membrane lipid and sphingolipid alterations previously studied in certain brain regions in a population of first-onset schizophrenia patients. Their main finding was a significant difference in the skin composition of ceramides between patients and controls, across several classes of ceramide species.

In a small study of post-traumatic stress disorder (PTSD) in army veterans, we examined plasma pro-inflammatory cytokine levels, ASMase activity, and changes in sphingolipids compared to healthy controls (Hammad et al. 2012a). We found that levels of interleukin 6, interleukin 10, interferon-γ and tumor necrosis factor-α (TNF-α) were higher in PTSD subjects than controls. Plasma ASMase activity (1.6-fold; $p = 0.003$), C18:0 ceramide (1.8-fold; $p < 0.01$), S1P (2-fold; $p < 0.05$), and dihydro-S1P (2.4-fold; $p < 0.05$) were also higher in the PTSD group. Despite the apparent trend of statins in lowering blood cholesterol observed in the PTSD group, pro-inflammatory cytokines and S1P were still higher than in the control group (Hammad et al. 2012a), suggesting other cardiovascular risks such as chronic inflammation remains. We have previously reported that S1P was able to induce increases in released TNF-α, and prostaglandin E2 in macrophages *in vitro* (Hammad et al. 2008). We have also reported that sphingosine kinase, the enzyme that generates S1P through phosphorylation of sphingosine, is released by human monocytic cells in response to lipoprotein-containing immune complexes, generating extracellular S1P that may be involved in sustained activation (Hammad et al. 2006). In a

different PTSD study, it was found that individuals who had been exposed to traumatic episodes during childhood also had elevated interleukin 6, interleukin 10 and TNF-α years after the episode (Hartwell et al. 2013); however, sphingolipid levels were not measured in that study. The data suggest that analysis of both sphingolipids and cytokines could be appropriate indicators of PTSD diagnosis.

7.6 Metabolic Disorders

In this section, we discuss the levels of sphingolipids in Guacher disease and Fabry disease in addition to diabetes mellitus and its complications. Gaucher disease and Fabry disease were some of the earliest diseases to be associated with sphingolipids. Gaucher disease is caused by deficiency of β-glucosidase enzyme, whereas Fabry disease is caused by deficiency of α-galactosidase A enzyme. In both diseases the deficiency of enzymes in the sphingolipid metabolism pathway causes buildup of substrates of these enzymes that leads to disease manifestations. The use of sphingolipids to monitor disease progression and to monitor treatment of these illnesses is discussed. Also, we will focus on the correlation of ceramides, sphingosine and other sphingolipids with diabetes and its complications.

7.6.1 Gaucher Disease and Fabry Disease

Gaucher disease is the most common lysosomal storage disorder. It is caused by a recessively inherited deficiency in lysosomal glucocerebrosidase, which catalyzes the hydrolysis of the glycosphingolipid glucosylceramide to glucose and ceramide in the lysosome. Gaucher disease patients show increased hepatic glucose production and insulin resistance (Langeveld and Aerts 2009). Gangliosides such as GM3 were implicated as negative regulators of insulin sensitivity (Yamashita et al. 2003). Also, abnormalities in GM3 were found to be associated with Gaucher disease (Wennekes et al. 2009). Ghauharali-van der Vlugt et al. (2008) investigated the plasma concentration of GM3 in a large cohort of Gaucher disease patients and reported that plasma GM3 is strikingly increased ($p < 0.0001$) in association with disease manifestation (median = 10.2 μM, range: 4.3–19.1 μM vs. median = 3.6 μM, range 2.7–5.4 μM). Importantly, the increase was comparable to that of glucosylceramide, the primary storage lipid. They concluded that the marked elevations in GM3 may play a role in the insulin resistance of Gaucher patients. Dekker et al. (2011) found that the concentration of plasma glucosylsphingosine, the deacylated form of glucosylceramide, also markedly increased in plasma of symptomatic non-neuronopathic (type 1) Gaucher patients compared to healthy volunteers (median = 230.7 nM, range 15.6–1035.2 nM; vs. median = 1.3 nM, range 0.8–2.7 nM). The ganglioside GM3 correlated with glucosylsphingosine when levels of these two lipids in plasma from individual Gaucher patients were compared ($p = 0.37$) (Dekker et al. 2011). This suggested that plasma glucosylsphingosine can qualify as a biomarker for type 1 Gaucher disease, but its relationship with clinical manifestations of Gaucher disease remain to be determined.

Fabry disease is a rare genetic disorder caused by a defective *GLA* gene, which causes a deficient quantity of the enzyme α-galactosidase. This deficiency leads to elevated plasma levels of lysoGb3, a hallmark of classical Fabry disease. Fabry disease is an X-linked disease that has different manifestations in men and women and more often affects men. In an effort to identify diagnostic biomarkers for Fabry disease, Aerts et al. (2008) measured plasma levels of Gb3 and lysoGb3, a deacylated Gb3 metabolite. They reported that plasma lysoGb3 level is a useful tool to monitor Fabry disease patients. Gold et al. (2013) reported that levels of lysoGb3 were 10 times higher in Fabry disease hemizygotes than levels measured in Fabry disease heterozygote (mean = 94.4 (SD = 25.8) pmol/mL (range 52.7–136.8 pmol/mL), vs. mean = 9.6 (SD = 5.8) pmol/mL (range 4.1–23.5 pmol/mL) and the values for

normal controls were barely detectable (mean = 0.4 (SD = 0.1) pmol/mL (range 0.3–0.5 pmol/mL).

The effect of the enzyme replacement therapy with injections of α-galactosidase A enzyme on plasma levels of Gb3 and lysoGb3 was investigated in Fabry disease patients with classical Fabry disease (van Breemen et al. 2011). As expected, pretreatment levels of Gb3 and lysoGb3 were found to be higher in males compared to females. Plasma levels of Gb3 were increased by 3 folds, whereas lysoGb3 was elevated by 200 folds in the classic Fabry disease males, independent of the age of patients. The enzyme therapy led to prominent reductions of plasma lysoGb3 in Fabry disease males within 3 months ($p = 0.03$), followed by relative stability. In classic Fabry disease females, normal plasma Gb3 levels are commonly found, nonetheless usually abnormally high plasma lysoGb3 levels (15 times higher than healthy individuals) were found (van Breemen et al. 2011). The enzyme therapy led to reduction and stabilization of plasma lysoGb3 in the female patients.

Association of plasma lysoGb3 levels with Fabry disease severity was found to be different between males and females (Rombach et al. 2010). Whereas, plasma lysoGb3 levels correlated well with the severity of the disease in female patients, no correlation was found in male patients (Rombach et al. 2010). Carotid intima media thickness (IMT) showed strong association with plasma lysoGb3 levels in female Fabry disease patients. All classic Fabry disease males had increased IMT, with increased plasma levels of lysoGb3 (Rombach et al. 2012b). LysoGb3 levels were also found to play a role in left ventricular hypertrophy found in Fabry disease patients, with the mass of the left ventricle being correlated with plasma lysoGb3 (Rombach et al. 2012b).

Auray-Blais et al. (2010) found that increases in the urine lysoGb3/creatinine ratio correlated with the concentrations of Gb3, type of mutations, gender, and enzyme replacement therapy status. Urine from healthy controls contained no detectable lysoGb3. Notably, increased urinary excretion of lysoGb3 of Fabry disease patients correlated well with a number of indicators of disease severity (Auray-Blais et al. 2010). Novel isoforms of lysoGb3 were also identified as biomarkers in the urine of Fabry disease patients. These molecules exhibited modifications of the lysoGb3 sphingosine moiety (Auray-Blais et al. 2012; Lavoie et al. 2013). Despite the absence of association between plasma lysoGb3 and renal function in Fabry disease males and females in some studies, LysoGb3 isoform levels correlated well with the enzyme replacement therapy status in males ($p < 0.05$) (Lavoie et al. 2013).

There are certain drawbacks to the use of sphingolipid measurements in Gaucher disease and Fabry disease. Firstly, levels of sphingolipids may depend on disease severity and subtype. Atypical variants of Fabry disease may not follow the trends found in typical Fabry disease patients; plasma lysoGb3 levels in patients with atypical mutations were found to be not significantly different from the normal levels in healthy individuals (Ferraz et al. 2014). Moreover, in a disease process which happens over a long period of time, vascular dysfunction correlates better with lifetime exposure to lysoGb3 rather than with one reading of plasma lysoGb3 level (Ferraz et al. 2014). In addition, male Fabry disease patients were shown to develop antibodies against the infused enzyme treatment, which affected the efficacy of the long-time treatment, while female patients did not develop antibodies against the enzyme (Rombach et al. 2012a).

7.6.2 Diabetes Mellitus and Insulin Resistance

In this section, the association between circulating sphingolipid levels in individuals with diabetes mellitus type 1 and type 2 and in those with complications of diabetes are discussed. Type 2 diabetes is a focal healthcare concern in Western nations, with incidence on the rise in both Europe and the United States. In spite of the fact that diabetes is essentially diagnosed based on high glucose levels in the blood, diabetes has been linked to lipid overload and abdominal obesity, which are well known to promote negative clini-

cal outcomes including cardiovascular disease and kidney failure. Increased organ and plasma fat content (lipotoxicity), plays a central role in the pathogenesis of type 2 diabetes. Changes in sphingolipid synthesis and signaling in tissues such as skeletal muscle, heart, pancreas, liver, and kidney in metabolic disorders including obesity, insulin resistance, nonalcoholic fatty liver disease, and diabetes were previously comprehensively reviewed (Iqbal et al. 2017; Russo et al. 2013).

In addition to their role in the pathogenesis of diabetes, sphingolipids have also emerged as potential biomarkers of diabetes and the metabolic syndrome. It has long been recognized that sphingolipid levels are altered in the blood plasma of diabetic patients (Kremer et al. 1975). Haus et al. (2009) were among the first to quantify the concentration of individual ceramide species in the circulation of patients with type 2 diabetes and healthy control subjects and examine the correlation between plasma levels of ceramide species and insulin sensitivity, and plasma TNF-α concentration as a marker of inflammation. They found that type 2 diabetic subjects had increased (p < 0.05–0.01) concentrations (nmol/ml ± SE) of C18:0 (control: 0.26 ± 0.03 vs. type 2 diabetes: 0.38 ± 0.03), C20:0 (control: 0.09 ± 0.004 vs. type 2 diabetes: 0.11 ± 0.004), C24:1 (control: 0.43 ± 0.03 vs. type 2 diabetes: 0.52 ± 0.04), and total ceramide (control: 2.37 ± 0.19 vs. type 2 diabetes: 3.06 ± 0.26). Insulin sensitivity was inversely correlated with C18:0, C20:0, C24:1, C24:0, and total ceramide concentrations (all $p < 0.01$).

Hanamatsu et al. (2014) investigated the relationship between the molecular species of sphingolipids in serum and the clinical features of metabolic syndrome: obesity, insulin resistance, fatty liver disease and atherogenic dyslipidemia. They found that serum concentrations (μM ± SD) of SM species with distinct saturated acyl chains, C18:0 (15.6 ± 3.0 vs. 12.4 ± 2.0; p = 0.008) and C24:0 (14.4 ± 3.2 vs. 11.4 ± 1.8; $p = 0.012$), were higher in the obese group than in the control group. SM C18:0, C20:0, C22:0 and C24:0 significantly correlated with the parameters for obesity, insulin resistance, liver function and lipid metabolism, respectively; most of the ceramide species were not associated with these parameters. The results suggested that these SM species can serve as novel biomarkers of metabolic syndrome and its associated diseases.

Several groups have profiled sphingolipids in plasma and tissues of various metabolic disease populations, but the results have been confusing. It was reported for example that ceramides and other sphingolipids accumulate in muscle or serum of insulin resistant individuals, and the changes observed were in most cases independent of obesity, physical fitness, FFA, or diacylglycerols (DAG) (reviewed in Chaurasia and Summers 2015). Strong correlations between plasma ceramides and insulin resistance, particularly when considered in concert with levels of inflammatory cytokines were also reported (Chaurasia and Summers 2015). Because others have reported no change in ceramides in individuals susceptible to metabolic disorders, consensus has not been reached (Chaurasia and Summers 2015). The concentration (nM ± SD) of the sphingolipids species, sphingosine (56.62 ± 15.81 vs. 36.64 ± 10.77; $p < 0.013$) and sphinganine (dihydrosphingosine) (16.38 ± 6.21 vs. 11.30 ± 4.7; $p = 0.05$), were found to be elevated in plasma samples of type 2 diabetes patients compared to healthy control subjects (Górska et al. 2005). This may indicate that the rate of ceramide metabolism in the cells of patients with type 2 diabetes is elevated. The impact of obesity on circulating S1P levels and its relationship with markers of glucose metabolism and insulin sensitivity was also investigated (Kowalski et al. 2013). It was found that plasma S1P was elevated in obese humans compared with lean healthy controls (<28%; $p < 0.01$), and was positively correlated with total body fat percentage, BMI, waist circumference, fasting insulin, HOMA-IR (homeostatic model assessment method for assessing β-cell function and insulin resistance from fasting glucose and insulin concentrations), HbA1c (hemoglobin A1c), total and LDL cholesterol.

Information about the mechanisms that regulate the fluctuation of ceramides in response to feeding and other environmental factors are still obscure. In a study assessed the effect of 12-week exercise training on insulin sensitivity and plasma ceramides in obesity and type 2 diabetes, it was found that plasma ceramides in subjects with obesity and normal glucose tolerance were similar to those in subjects with diabetes, despite differences in glucose tolerance (Kasumov et al. 2015). Exercise significantly reduced body weight and adiposity and increased peripheral insulin sensitivity in both groups. Plasma C14:0 (p = 0.007), C16:0 (p = 0.005), and C24:0 (p = 0.0001) ceramide levels were reduced in all subjects after the training period, with decreases in total (r = −0.51, p = 0.02) and C14:0 (r = −0.56, p = 0.009) ceramide being negatively correlated with the increase in insulin sensitivity (Kasumov et al. 2015). Recently, Drazba et al. (2019) examined associations of adiposity and diet quality with circulating ceramides in 96 middle-aged adults and found that BMI is positively associated with C18:0 ceramide ($p < 0.0001$), the ratio between C18:0/C24:0 ceramides ($p < 0.0001$), and the ceramide risk score ($p < 0.009$). Lower C22:0 cermaide values were found to be associated with higher intakes of vegetables (p = 0.02) and whole grains (p = 0.03), and lower intakes of saturated fats (p = 0.04) and added sugar (p = 0.01) (Drazba et al. 2019).

A study in a small, homogenous population by Othman et al. (2012) analyzed the potential of the uncommon class of sphingolipids, deoxysphingolipids, as biomarkers of type 2 diabetes and the metabolic syndrome. They found that the concentrations of the deoxy-sphingoid bases, deoxysphinganine and deoxysphingosine, which incorporate the amino acid alanine rather than serine, were increased in patients with metabolic syndrome, with or without type 2 diabetes. The authors also found that the levels of these compounds had a significant positive predictive value for the metabolic syndrome. They also determined that the concentration of C16-sphingosine, which is derived from myristoyl-CoA rather than palmitoyl-CoA, was decreased in patients with both diabetes and the metabolic syndrome, but not in patients with metabolic syndrome alone. C16-sphingosine was found to have significant predictive value in differentiating patients with type 2 diabetes from prediabetes and control patients (Othman et al. 2012). Thus, it was suggested that levels of alanine-derived sphingoid bases could be a potential biomarker for metabolic syndrome, with or without type 2 diabetes, and levels of C_{16}-sphingosine could be used to detect the development of type 2 diabetes from a compensated insulin resistance status (Othman et al. 2012).

Relatively few studies have directly analyzed the predictive value of sphingolipid levels for diabetes risk. The association between serum metabolites measured by targeted metabolomics and risk of type 2 diabetes was investigated prospectively in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study (Floegel et al. 2013). Serum glycine; SM C16:1; acyl-alkyl-phosphatidylcholines C34:3, C40:6, C42:5, C44:4, and C44:5; and lysophosphatidylcholine C18:2 were independently associated with decreased risk of type 2 diabetes, whereas serum hexose; phenylalanine; and diacyl-phosphatidylcholines C32:1, C36:1, C38:3, and C40:5 with increased risk. The data significantly improved type 2 diabetes prediction compared with established risk factors. The identified metabolites were further linked to insulin sensitivity and secretion in the Tübingen Family study and were partly replicated in the independent KORA (Cooperative Health Research in the Region of Augsburg) cohort (Floegel et al. 2013).

The relationship between lipid metabolism with prediabetes (impaired fasting glucose and impaired glucose tolerance) and type 2 diabetes mellitus was investigated in individuals from the Australian Diabetes, Obesity and Lifestyle Study (AusDiab) and the findings were validated on individuals from the San Antonio Family Heart Study (SAFHS) (Meikle et al. 2013). In addition to the expected associations with DAG, TG and cholesterol esters, type 2 diabetes and prediabetes were positively associated with ceramide (type 2 diabetes: 11.8% difference; p = 0.03, prediabetes: 13.9% difference; p = 0.024), and its precursor DHC (type 2 diabetes: 25.9% differ-

ence; $p = 0.0007$, prediabetes: 24.5% difference; $p = 0.001$). Most of the significant associations in the AusDiab cohort (90%) were subsequently validated in the SAFHS cohort (Meikle et al. 2013). The data showed that the aberration of the plasma lipidome associated with type 2 diabetes is indeed present in prediabetes. Recently, Jensen et al. (2019) measured 15 sphingolipid species and fasting plasma glucose from blood samples collected from 2145 participants without prevalent diabetes in the Strong Heart Family Study at baseline and a mean of 5.5 years after baseline. They found that higher concentrations of most ceramide species and lower LacCer with palmitic acid (C16:0) were associated with higher glucose levels at baseline; no associations between sphingomyelin species or glucosyl-ceramide species with glucose levels were found. Associations of sphingolipid levels with fasting glucose levels at follow-up were similar.

Mounting evidence demonstrates that ectopic fat (defined as storage of TG in tissues other than adipose tissue, such as the liver, skeletal muscle, heart, and pancreas, which normally contain only small amounts of fat) rather than the fat mass drives the risk for type 2 diabetes (Pan et al. 1997). However, there is currently no clinical biomarker other than invasive biopsies or imaging for the presence of ectopic fat. In a cross-sectional study, it was found that among people with obesity serum ganglioside C22:0 and LacCer C14:0 predicted muscle TG; serum DAG C36:1 and FFA C18:4 strongly predicted muscle DAG; and serum TG C58:5, FFA C14:2 and C14:3, phosphotidylcholine C38:1, and cholesterol ester C24:1 predicted muscle ceramide (Perreault et al. 2016). Among endurance-trained athletes, serum FFA C14:1 and sphingosine were significant predictors of muscle TG (Perreault et al. 2016). The combination of serum LacCer C22:0, SM C18:1 and C24:1 predicted insulin resistance in obese and type 2 diabetes subjects, whereas serum TG C50:6 and phosphatidylethanolamine C34:1 together predicted insulin resistance in athletes (Perreault et al. 2016).

Tonks et al. (2016) conducted a comprehensive lipidomic analysis of skeletal muscle and plasma in adiposity-matched insulin-resistant and insulin-sensitive individuals compared to a lean insulin-sensitive group using hyperinsulinemic-euglycemic clamps to further define insulin sensitivity in obesity. They found that in the muscle, insulin resistance was characterized by higher levels of C18:0 sphingolipids (ceramide and GM3), while in the plasma, higher levels of DAG and cholesterol esters, and lower levels of lysophosphatidylcholine and lysoalkylphosphatidylcholine, indicated insulin resistance, irrespective of overweight/obesity. The data suggested that muscle C18:0 sphingolipids may play a role in insulin resistance independent of excess adiposity.

In an effort to develop and validate a plasma lipidomic risk score as a predictive biomarker for future type 2 diabetes and to evaluate cost-effectiveness of the score as a screening tool, Mamtani et al. (2016) have recently proposed a score that is based on plasma concentration of DHC 18:0, lysoalkylphosphatidylcholine 22:1 and triacyglycerol 16:0/18:0/18:1. The score was derived from the San Antonio Family Heart Study (SAFHS), and together with its recalibrated version, were validated in an independent cohort from the Australia-the AusDiab cohort. The participants were type 2 diabetes-free at baseline and were followed-up for maximum of 23.53 years. The score predicted future type 2 diabetes independently of prediabetes with an accuracy of 76%. When combined with risk-stratification methods currently used in clinical practice, modeling studies demonstrated that the score-based risk-stratification combined with metformin supplementation for high-risk individuals was the most cost-effective strategy for type 2 diabetes prevention (Mamtani et al. 2016).

It has been clearly shown that sphingolipids in the circulation can be valuable biomarkers in the prediction of the development of type 2 diabetes. Certain SM species were associated with insulin resistance and the metabolic syndrome, and particular species of ceramides were altered in type 2 diabetes and the prediabetes stage. The detection of changes in the levels of certain plasma sphingolipids could facilitate interventions early in diabe-

tes and may well prevent micro- and macro-vascular diabetic complications later in life.

7.6.2.1 Diabetic Neuropathy

Diabetic neuropathy affects about 30% of individuals with diabetes (Candrilli et al. 2007). Unfortunately, many cases of diabetic neuropathy are diagnosed late in the disease course, which is mainly due to the insidious onset of symptoms in most patients. The application of advanced screening methods to detect early-onset neuropathy would be extremely beneficial to diabetes patients. The role of sphingolipids in diabetic neuropathy was recognized more than 35 years ago (Crepaldi et al. 1983). Ganglioside treatment was evaluated in a multicenter, randomized, double-blind, controlled, cross-over versus placebo trial in 140 insulin-treated diabetic subjects with peripheral neuropathy (Crepaldi et al. 1983). The treatment seemed to have a positive effect on diabetic peripheral neuropathy, improving both symptoms and electrophysiological parameters. Clinically, diabetic neuropathy exhibits a pronounced similarity to the neuropathy of patients with hereditary sensory and autonomic neuropathy type 1 (HSAN1). In HSAN1 patients, sphingolipid metabolism is altered due to gain-of-function mutations to the genes coding for SPT subunits 1 and 2. In these patients, L-alanine and glycine become the preferred amino acid substrates for the mutated enzyme instead of L-serine (Bejaoui et al. 2001; Dawkins et al. 2001; Rotthier et al. 2010), which results in elevated levels of deoxysphingolipids (Penno et al. 2010). These modified sphingolipids cannot be further metabolized to the more complex sphingolipids nor can they be degraded via the normal physiologic sphingolipid catabolic pathways to form 1-phosphate derivatives (Duan and Merrill 2015). Deoxysphingolipids are present at low levels in plasma from normal, healthy individuals, primarily in VLDL and LDL (Bertea et al. 2010).

In a prospective type 2 diabetes cohort who were followed for 8 years plasma levels of the deoxy-sphingoid bases (1-deoxysphinganine, 1-deoxysphingosine) were examined as possible predictive biomarkers (Dohrn et al. 2015). Levels of deoxy-sphingoid bases were elevated not only in patients with metabolic syndrome, impaired fasting glucose and type 2 diabetes and in patients who developed diabetes during the follow-up period, but also were found to be significantly elevated in plasma of patients with distal sensorimotor polyneuropathy (confirmed by electroneurographic examinations). Interestingly, the deoxy-sphingoid bases in that study were detectable in early disease stages, but did not correlate with the clinical course. Because in that study deoxysphingosine was analyzed after total hydrolysis of the samples, measured sphingolipid levels can represent both free deoxysphingosine and deoxyceramides combined.

In an exploratory pilot study, we have recently assessed the associations between multiple sphingolipid species including deoxysphingolipids and free amino acids and the presence of symptomatic neuropathy in a sub-cohort of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions (DCCT/EDIC) type 1 diabetes study (Hammad et al. 2017). Patient-determined neuropathy was based on 15-item self-administered questionnaire (Michigan Neuropathy Screening Instrument) developed to assess distal symmetrical peripheral neuropathy in diabetes. Plasma deoxy-C24:0 ceramide (12.3 ± 3.7 vs. 10.6 ± 4.1 nM, $p = 0.049$), C24:0 ceramide (3184.6 ± 762.7 vs. 2709.5 ± 921.8 nM, $p = 0.039$), and C26:0 ceramide (131.1 ± 39.7 vs. 104.6 ± 35.6 nM, $p = 0.014$) were higher in patients with neuropathy than those without neuropathy. The amino acid cysteine was higher (2.0 ± 1.0 vs. 1.4 ± 0.6 μM, $p = 0.007$) in patients with neuropathy. No differences in other sphingolipids or amino acids were detected. The covariate-adjusted odds ratios of positive patient-reported neuropathy was associated with increased levels of deoxy-C24:0, and deoxy-C24:1 ceramide; C22:0, C24:0, and C26:0 ceramide; and cysteine. Plasma levels of deoxysphingolipids in this cross-sectional type 1 diabetes study correlated negatively with the advanced clinically defined stages of neuropathy. Thus, the data indicated that ceramide species

may have potential diagnostic and prognostic significance in diabetic neuropathy.

7.6.2.2 Diabetic Nephropathy

Diabetic nephropathy is the leading cause of chronic kidney disease, and certainly increases the risk for cardiovascular disease (United States Renal Data System 2014; Chronic Kidney Disease Prognosis Consortium et al. 2010). In a study by Mäkinen et al. (2012a), type 1 diabetic patients from the FinnDiane Study with varying severity of kidney disease (8.3-year follow-up) were investigated to create multimetabolite models of the disease process. In the cross-sectional analyses, patients with no complications had low serum lipids, less inflammation, and better glycemic control, whereas patients with advanced kidney disease had high serum cystatin-C and SM. Prospectively, progressive albuminuria was found to be associated with high unsaturated fatty acids, phospholipids, and IDL and LDL lipids. Whereas earlier progression of albuminuria was associated with poor glycemic control, increased saturated fatty acids, and inflammation, progression of albuminuria was associated with high HDL lipids at longer duration of the disease (Mäkinen et al. 2012b). SM (OR = 2.53, $p = 1.5 \times 10^{-8}$) and large HDL particles (OR ≤ 0.40, $p \leq 3.1 \times 10^{-10}$) were found to be the strongest regressors for kidney disease after the established kidney biomarkers (adjusted by diabetes duration, age and gender) (Mäkinen et al. 2012b). SM was also a significant regressor of urinary albumin excretion rate ($p < 0.0001$) in the multivariate analysis with kidney function, glycemic control, body mass, blood pressure, TG and HDL cholesterol (Mäkinen et al. 2012b).

In the DCCT/EDIC study, we analyzed the plasma concentrations of ceramide species and sphingoid bases and their phosphates to investigate their association with the development of albuminuria in type 1 patients during 14–20 years of follow-up (Klein et al. 2014). Although the majority of patients (59%) exhibited normal levels of albuminuria throughout follow-up, 28% progressed to microalbuminuria and 13% progressed to macroalbuminuria. Generalized logistic regression models were adjusted for DCCT treatment group, baseline retinopathy, gender, baseline HbA1c, age, albumin excretion rate, lipid levels, diabetes duration, and the use of angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs). The results demonstrated that increased plasma levels of very long- (C20:0, C20:1, C22:1, C24:1, C26:0, and C26:1), but not long-chain ceramide species measured at DCCT baseline were associated with decreased odds to develop macroalbuminuria. Although levels of sphingoid bases and their phosphates were not associated with the development of albuminuria in type 1 patients, we noted that there was a striking average 2.5-fold increase in plasma S1P concentration in the cohort of type 1 diabetic patients compared to S1P levels previously observed in plasma from healthy, non-diabetic subjects (Hammad et al. 2010; Klein et al. 2014). It is worthy to note that the observation of increased levels of distinct ceramide species being associated with better outcomes of diabetic nephropathy is opposite to observations in other disease settings. This highlights the potential benefits of the determination of circulating sphingolipid concentrations as predictive, diagnostic, and prognostic biomarkers.

Recently, we examined whether plasma glycosphingolipids (LacCer and HxCer) could predict development of diabetic nephropathy, assessed as macroalbuminuria or chronic kidney disease (Lopes-Virella et al. in press). The data showed that decreases of several long- and very long-chain LacCer were significantly associated with increased risk of progression to macroalbuminuria but not chronic kidney disease. Increases in plasma concentrations of only one HxCer species (C18:1) was significantly associated with increased risk for progression to chronic kidney disease. Our data (Klein et al. 2014; Lopes-Virella et al. in press) in a large cohort of type 1 diabetes supports the possibility that circulating plasma sphingolipids may play an important role in the initial development of diabetic nephropathy.

7.6.3 Liver Cirrhosis

Underlying pathophysiological mechanisms in progression of liver disease to cirrhosis are still obscure. Since S1P-related pathways are associated with inflammation and a number of diseases, such as cancer and cardiovascular diseases, Becker et al. (2017) investigated circulating plasma S1P levels and related molecules such as SA1P and sphingosine in patients suffering from end-stage liver disease in relation of short-term mortality. The authors found that mean plasma concentrations (ng/mL) of S1P and SA1P were significantly lower in patients who deceased within three-months (S1P: 82.43 vs. 141.5; $p < 0.001$, SA1P: 18.69 vs. 34.99; $p = 0.006$) or one-year (S1P: 91.19 vs. 150.14; $p < 0.001$, SA1P: 23.83 vs. 37.32; $p = 0.003$) compared to surviving patients. S1P showed the highest predictive value for one-year survival. It is established that circulating S1P originates from red blood cells and platelets. The authors found that S1P and sphingosine were still significantly correlated with one-year mortality and S1P for three-month mortality after accounting for platelet counts, concentrations of hemoglobin and SA1P concentrations in the multivariate cox regression analysis. The authors also analyzed the patients' results of the MELD score. This scoring system is used for prioritization of patients determined for orthotopic liver transplantation and currently represents the best evaluated mortality score in patients with liver cirrhosis (Kamath and Kim 2007). Plasma levels of S1P and SA1P also showed a negative correlation with the MELD score (S1P: Spearman rank coefficient of correlation: −0.572, $p < 0.001$; SA1P: Spearman rank coefficient of correlation: −0.404, $p < 0.001$); however, sphingosine was not correlated with the MELD score, or could it differentiate between three-month or one-year survivors and non-survivors (Becker et al. 2017). After inclusion of the MELD score in the cox regression analysis for three-month and one-year survival, S1P remained significantly predictive of mortality independently of the MELD score. The results indicated that S1P is a reliable as well as independent risk predictor for mortality in patients suffering from end-stage liver disease.

7.7 Systemic Lupus Erythematous

Systemic Lupus Erythematous (SLE) is an autoimmune disease that affects females more than males with African American females developing more severe manifestation of the disease. SLE can affect many parts of the body, including the joints, skin, kidneys, heart, lungs, blood vessels, and brain. One early symptom that can be indicative of lupus is a photosensitive rash, particularly on the face and upper arms; other early symptoms, including fever, swelling, and stiffness of multiple joints remain undistinguishable. Furthermore, there is not one test that can diagnose lupus; the combination of blood and urine tests, signs and symptoms, and findings from physical examination can lead to the diagnosis. Herein, we describe the findings of recent studies investigating the association of disease activity with circulating sphingolipids in SLE.

Diseases associated with aberrant renal growth including diabetic nephropathy, polycystic kidney disease, and renal cell carcinoma have been associated with changes in glycosphingolipid levels and associated metabolism. Glucosylceramide, LacCer, and ganglioside GM3 are elevated in the kidneys and renal tissues from patients with these diseases and in experimental models of these disorders (Mather and Siskind 2011; Vukovic et al. 2015; Shayman 2016). Importantly, inhibition of glucosylceramide formation with the use of small molecule inhibitors of glucosylceramide synthase reverses or prevents the abnormal renal phenotypes associated with these experimental models; however, the actual basis for this reversal remains unknown (Shayman 2016).

In a lupus mouse model, we showed that total plasma ceramide and SIP levels were higher in the lupus mice compared to controls (Al Gadban et al. 2012). Knowling and colleagues (2015) investigated dysfunctional glycosphingolipid metabolism in lupus nephritis patients and in a

lupus mouse model. They reported that levels glucosylceramide and LacCer were significantly higher in kidneys of nephritic lupus mice compared to non-nephritic lupus mice or healthy controls. In lupus nephritis patients, levels of C16:0 LacCer in the urine were significantly higher compared to lupus patients without nephritis or healthy control subjects ($p < 0.001$). This correlation was still evident even after considering differences in estimated glomerular filtration rate (eGFR).

More recently, Checa et al. (2017) investigated the association of clinical and renal disease activity with circulating sphingolipids in patients with SLE. They found that circulating plasma ceramide and HxCer levels were increased, and sphingoid bases were decreased in SLE patients compared to controls. Specifically, plasma mean concentration (nM) of C16:0 (334 vs. 271), C18:0 (179 vs. 142), C20:0 (118 vs. 95.4) and C24:1 (1380 vs. 1110) ceramide, C16:0 (864 vs. 636), C18:0 (64.8 vs. 54.5), C18:1 (6.3 vs. 5.5) and C24:1 (1430 vs. 1020) HxCer, C24:1 SM (105,000 vs. 93,800) and C16:0 DHC (10.7 vs. 9.0) were elevated in female SLE patients (all at $p < 0.05$). In contrast, levels of sphingosine (9.0 vs. 11.3) and S1P (471 vs. 524) were reduced in the SLE patients ($p < 0.05$). The ratio of C16:0 ceramide to S1P was found to be the best indicator of SLE, and was also associated with active disease indices but not with accumulated damage due to the disease. Levels of HxCer C16:0 and C24:1 discriminated patients with active versus inactive disease. Notably, the enzymes in the sphingolipid metabolism pathway were altered in the SLE patients. Ceramide synthase 5 and S1P lyase were upregulated, while glucosylceramidase and UDP-galactose ceramide galactosyltransferase were downregulated in SLE patients compared to controls. The authors also studied the effect of immunosuppressive treatment in 22 active SLE patients and found that all plasma sphingolipids levels returned to normal after the immunosuppressive treatment.

7.8 Chronic Kidney Disease

It is established that chronic kidney disease (CKD) is associated with an increased incidence of cardiac dysfunction. Early cardiac dysfunction is frequently asymptomatic and under-diagnosed, which often results in a delayed therapeutic intervention. In CKD, the pathogenesis of accelerated cardiomyopathy remains unknown. In animal studies it was determined that elevated ceramide levels can cause dilated lipotoxic cardiomyopathy (Park et al. 2008). Mitsnefes et al. (2014) evaluated the association between serum ceramide levels and markers of cardiac structure and function in children from the Chronic Kidney Disease in Children (CKiD) cohort, which is a prospective observational study of children with mild to moderate CKD. Total serum ceramide levels were found to be significantly higher in CKD children compared to healthy controls ($p < 0.001$), with the proportion of C24:0 metabolites dramatically higher in CKD subjects. LacCer C24:0, and C16:0 were significantly higher in CKD subjects compared to controls ($p < 0.001$) and were identified as an independent predictor of lower systolic function. In CKD children, there was no significant association between ceramides and demographic (age, gender, race, weight, height, BMI), clinical (blood pressure) or laboratory (hemoglobin, serum albumin, serum insulin, HOMA-IR, urine protein/creatinine ratio) parameters. However, LDL-cholesterol levels were significantly correlated with the levels of C16:0 and C24:0 metabolites in univariate analysis. In adjusted multivariate analyses, higher C24:0 and C16:0 metabolites were found to be independent significant predictors of ventricular lower shortening fraction and mid-wall shortening. When those analysis included heart rate from ambulatory blood pressure monitoring data, C24:0 metabolites remained associated with both shortening fraction and mid-wall shortening, while heart rate was not a significant predictor.

7.9 Conclusions and Perspective

In this chapter, we focused on the novel findings demonstrating the advantages of sphingolipids as biomarkers. However, there are still several impediments in the path of using sphingolipid biomarkers in the clinic. Establishing the normal values for the different sphingolipids in the plasma is still a challenge (Burla et al. 2018). The study design, pre-analytical sample handling, precision, accuracy, and reproducibility of lipid quantitation, and patient data sharing are key confounding factors that need to be taken into consideration when assessing the value of sphingolipids as diagnostic and prognostic markers for disease.

Special attention must be paid in choosing plasma versus serum as a sample source for metabolomic studies. Plasma and serum are generated by different separation procedures, where coagulation markedly alters the levels of serum metabolites (Yu et al. 2011; Liu et al. 2018). However, in comparing standard and platelet-free plasma, no differences in metabolite profiles were detected (Liu et al. 2018). One reason for the differences detected in lipid levels between plasma and serum is likely due to the fact that many lipids and lipid-modifying enzymes, are excreted in extracellular vesicles or in soluble forms from platelets, leukocytes, and erythrocytes during the clotting process in serum preparation. It has been proven that this can significantly affect levels of S1P, lyso-phospholipids, prostaglandins, leukotrienes, resolvins and other lipids (Liu et al. 2018; Ono et al. 2013; Dorow et al. 2016; Colas et al. 2014).

Other blood-related pre-analytical conditions that can affect levels of sphingolipids in plasma include type of anti-coagulant, prolonged processing times at different temperatures, hemolysis, and contamination with buffy layer (Hammad et al. 2010; Kamlage et al. 2014). Diseases that increase red blood cell fragility, such as hereditary spherocytosis and possibly other disorders like type 2 diabetes can increase hemolysis during plasma preparation (Da Costa et al. 2013; Lippi et al. 2012). These conditions have to be documented and accounted for. Lipemia is the presence of high levels of suspended lipoproteins particles resulting in blood sample turbidity. Changes in the sample's volume or turbidity because of lipemia can also cause inaccurate results (Nikolac 2014; Kroll 2004).

A position paper by a group of scientists from multiple institutions worldwide recently defined a set of generally accepted guidelines for quantitative MS-based lipidomics of blood plasma or serum, with harmonization of data acquired on different instrumentation platforms (Burla et al. 2018). Since many different demographic characteristics and medications which individuals take may affect sphingolipid levels, the group suggested collecting personal data and medical history for each patient in need of a lipidomics test. These demographics should include age, gender, BMI, ethnicity, fasting status, prescription medications, including drugs directly affecting lipid metabolism (e.g., nonsteroidal anti-inflammatory drugs (NSAIDs), anticoagulants and statins) and also drugs with insufficiently characterized metabolic impact (i.e., hormones, including contraceptives, steroids, diuretics) (Sales et al. 2016; Meikle et al. 2015; Mazaleuskaya et al. 2016). The following medical history parameters could affect the result of the patient's lipidomics test: diabetic/insulin status, HDL/LDL/TG values, blood pressure, full blood count, C-peptide, C-reactive protein, smoking status, alcohol consumption, diet, intake of dietary supplements, type and frequency of exercise, and other information on lifestyle. For example, we showed that fasting and non-fasting samples may have different levels of certain ceramide species (Hammad et al. 2010). Furthermore, age, BMI and systolic blood pressure were all positively and independently correlated with plasma SM levels (Jiang et al. 2000).

The race effect is extremely important as far as sphingolipidomics are concerned; several debilitating diseases, such as SLE, are prominent among African American females, who develop more severe manifestation of the disease compared to Caucasians. The gender of the individual should certainly be taken into account, as plasma and serum levels of DHC, SM and C1P were found to be higher in healthy young

(26.0 ± 4.0 years) non-pregnant females than males (Hammad et al. 2010). In the Baltimore Longitudinal Study of Aging (individuals aged 55 and older) it was found that women had higher plasma levels of all SM species and showed steeper trajectories of age-related increases compared to men (Mielke et al. 2015b). African Americans showed higher circulating SM concentrations compared to Caucasians. Diabetes, smoking, and plasma TG were associated with lower levels of many SM and DHSM, with specificity to SM acyl-chain length and saturation. SM C20:0, C22:0 and C24:0 showed a biphasic relationship in which the age-related increase reached a plateau and then decreased with advanced age. Mielke et al. (2015a) also found that women had higher plasma concentrations of most ceramide and DHC species and showed steeper trajectories of age-related increases compared to men. Ceramides and DHC were more associated with waist-hip ratio than body mass index. Plasma cholesterol and TG, prediabetes, and diabetes were associated with ceramides and DHC, with specificity to the acyl chain length and saturation. Recently, plasma levels of total cermide, C24:0 ceramide and C24:1 ceramide were found to be lower in African Americans with metabolic syndrome compared to African Americans without metabolic syndrome (Buie et al. 2019). Buie et al. (2019) also found a decrease in the plasma concentration of C20:0 ceramide in African Americans with metabolic syndrome compared with whites with metabolic syndrome, and suggested that ceramide metabolism is differentially regulated in African Americans with metabolic syndrome, which may contribute to disparities observed in health outcomes. Hence, in future epidemiological studies it will be extremely important to establish the intra-individual changes in each sphingolipid species in relation to disease onset and progression.

Levels of different lipoproteins in the blood may also affect the levels and thus the actions of sphingolipids; since lipoproteins VLDL, LDL, and HDL particles are the major carriers of sphingolipids in the serum/plasma. However, the main tissue sources of plasma sphingolipids, and the flux rate and half-life of plasma sphingolipids are still obscure. Furthermore, the subcellular location of the crucial pool of sphingolipids that regulate cell function remains unresolved. Clues to the origin of plasma sphingolipids have come from our recent studies that included patients with abetalipoproteinemia and with Tangier disease (Iqbal et al. 2015, 2018). The results identified microsomal triglyceride transfer protein and ATP binding cassette family A protein 1 (ABCA1) as critical determinants of plasma sphingolipid levels.

While it is well known that the majority of the plasma S1P pool is bound to HDL cholesterol (Hammad et al. 2010, 2012b), we and others did not find an association between plasma S1P levels and the total HDL cholesterol fraction, and have shown that S1P transport in lipoproteins was not limited by the concentration of HDL cholesterol in individual subjects due to the high variability of S1P content in HDL particles (Hammad et al. 2012b; Kowalski et al. 2013). Additionally, the relative S1P content per particle was actually highest in the larger VLDL particles (Hammad et al. 2010), possibly explaining the positive correlations between plasma S1P with total and LDL but not HDL cholesterol reported in obesity (Kowalski et al. 2013).

In fact, LDL and HDL particles exhibited potent ability to bind exogenous S1P (Murata et al. 2000; Sattler et al. 2015). Loading with S1P *in vitro* and *in vivo* was shown to fully replenish the absent S1P content of apolipoprotein M (apoM)-deficient HDL particles (apoM on HDL binds S1P) and restore the cardioprotective effect of HDL from CAD patients (Sattler et al. 2015). Thus, it seems possible that lipoproteins interfere with the S1P-induced actions by trapping/scavenging S1P in their particles. It is worth noting that some of the cardio-protective effects of HDL have been attributed to its high S1P content (Karliner, 2013; Keul et al. 2007). The S1P cargo on HDL particles promotes survival of cardiomyocytes and maintains the integrity of vascular endothelium, which enhances blood flow and angiogenesis. As common metabolic disorders have been shown to affect the serum levels of different lipoproteins and therefore sphingolipids, individuals with such conditions need to be first

evaluated for their general health and baseline sphingolipid levels before detecting sphingolipid diagnostic/prognostic biomarkers for other critical medical conditions.

Furthermore, Ståhlman et al. (2013) investigated whether dyslipidemia is required in addition to insulin resistance for the occurrence of an altered HDL lipidome, which may impact HDL functionality. They characterized the HDL lipidome in women from the Diabetes and Impaired glucose tolerance in Women and Atherosclerosis development (DIWA) study. The authors found that small HDL-particles predominated in dyslipidemic subjects compared to the normolipidemic diabetic and control groups, and these HDL particles were distinguished as the primary carrier of ceramides, which is known for promoting inflammation and insulin resistance. In healthy individuals, LDL particles are typically the major carriers of ceramide compared to VLDL and HDL (Hammad et al. 2010; Wiesner et al. 2009). The results from the DIWA study (Ståhlman et al. 2013) indicate that dyslipidemia with and without diabetes can alters the bioactive lipids cargo of HDL, and therefore may affect HDL function. Thus, the analyses of the sphingolipidomics of lipoprotein particles, although more laborious, has the potential for an added value over plasma/serum sphingolipidomics in providing more specific information about not only biomarkers of disease but also mechanistic insights about the pathology and treatment of the disease.

In summary, sphingolipid species were found to be significantly useful markers of disease prediction, diagnosis, prognosis and treatment monitoring. The limitations to their use stated above can still be resolved by carefully detecting comorbidities and adjusting the baseline levels for each individual. It is now speculated that sphingolipids may be the new markers for metabolic and cardiovascular diseases similar to HDL and LDL cholesterol (Hammad 2011; Summers 2018). The fact that ceramides are affected by diet and exercise just like cholesterol strengthens this assumption (Wang et al. 2017; Lankinen et al. 2016; Bergman et al. 2015). All indicators mentioned in this chapter are further illustrated in practice by the fact that Mayo Clinic Laboratories have recently (2016) started performing a diagnostic test that quantifies plasma levels of ceramides to identify patients at higher risk of developing major adverse cardiovascular events.

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8 Inflammatory Ocular Diseases and Sphingolipid Signaling

Richard Grambergs, Koushik Mondal,
and Nawajes Mandal

Abstract

Inflammation is a powerful immune countermeasure to tissue damage and infection. The inflammatory response is complex and requires the involvement of myriad signaling pathways and metabolic processes, all governed by a multitude of regulatory systems. Although inflammation is a vital defense against tissue injury and a necessary step in tissue healing, the mechanisms which modulate the initiation, intensity, and duration of this innate immune response can malfunction and result in inappropriate or out-of-control inflammation, even in the absence of an appropriate stimulus. Though the human eye exists in an immune-privileged microenvironment, it is not spared from this. The eye is neither devoid of immune cells nor is it fully sequestered from systemic immune responses, and is therefore fully capable of ruining itself through localized inflammatory dysfunction and systemic inflammatory disease (Taylor AW, Front Immunol 7:37, 2016; Zhou R, Caspi RR, Biol Rep 2, 2010). In fact, a wide range of ocular inflammatory diseases exist and are major causes of blindness in humans. Advances in the understanding of inflammatory processes have revealed new key pathways and molecular factors involved in the mechanisms of inflammation. Lipids and sphingolipids are increasingly being recognized as having important signaling roles in the pathophysiology of ocular inflammatory diseases. What follows below is a discussion of fundamental inflammatory processes, the place of sphingolipids as mediators of said processes, brief descriptions of major inflammatory ocular diseases, and new findings implicating sphingolipids in their pathogenesis.

Keywords

Sphingolipid signaling · Ocular inflammtion · Innate immunity · Uveitis · Sphingosine 1-phosphate · S1P receptors · Glaucoma · Glucosylceramide

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Abbreviations

AMD	age-related macular degeneration
C1P	ceramide 1-phosphate
Cer	ceramide
CLRs	C-type lectin receptors
COX2	cyclooxygenase 2
DAMPs	Damage-Associated Molecular Patterns
DM	diabetes mellitus
DR	diabetic retinopathy
GlcCer	glucosylceramide
ICAM-1	Intercellular adhesion molecule 1
IOP	intraocular pressure
IRFs	Interferon regulatory factors
LacCer	lactosylceramide
LPS	lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP1	monocyte chemoattractant protein 1
MS	Multiple Sclerosis
NLR	NOD-like receptors
NPDR	Nonproliferative diabetic retinopathy
PAMPs	Pathogen-Associated Molecular Patterns
PDR	proliferative diabetic retinopathy
PRR	Pattern Recognition Receptors
PVD	posterior vitreous detachment
RGC	retinal glial cell
RIP1	Receptor interacting protein-1
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
RPE	retinal pigmented epithelial
S1P	sphingosine 1-phosphate
S1PR2	S1P receptor 2
NF-κB	Nuclear factor-κB
Sph	Sphingosine
TFLL	tear film lipid layer
TLRs	Toll-like receptors
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VLC	very long chain

8.1 Inflammation and Ocular Immunity

The innate immune system is developed to defend against a diverse array of threats. The ability to detect tissue damage and pathogen invasion is provided by 'professional immune cells' such as circulating monocytes and neutrophils, resident and recruited macrophages, dendritic cells, other specialized cells which either reside within tissue or circulate throughout the body, and a variety of 'non-professional' cells (Newton and Dixit 2012). Identification of threats is dependent upon intracellular and surface-bound Pattern Recognition Receptors (PRRs), which detect Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) (Akira 2009; Lampron et al. 2013). PAMPs include nucleic acids, lipoproteins, carbohydrates, and other molecules originating from foreign organisms, while DAMPs are endogenous molecules released by stressed and dying cells. PAMPs and DAMPs associate with and activate PRRs, which then initiate signaling cascades leading to recruitment of leukocytes and the initiation of inflammatory responses. The different families of PRRs are expressed constitutively in macrophages, dendritic cells, and even epithelial and endothelial cells, and are responsible for detecting and initiating responses to different types of PAMPs and DAMPs. These include transmembrane Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). Activation of TLRs, CLRs, RLRs, and certain NLRs initiates signaling cascades involving transcription factors such as Nuclear factor-κB (NF-κB), Interferon regulatory factors (IRFs), and activation of Mitogen-activated protein kinase (MAPK) pathways to upregulate transcription of genes involved in inflammation. The pro-inflammatory genes activated by PRRs code for chemokines, pro-inflammatory cytokines, and proteins which also

modulate PRR signaling (Kawai and Akira 2010). Assembly and activation of inflammasomes, which induce and regulate pro-inflammatory cytokine generation, is a critical role of PRRs. Recent work has shown that activation of inflammasomes and induction of the general inflammatory response are mediated in part by the complement system (Arbore and Kemper 2016). Furthermore, complement has been shown to have a potential role in the resolution phase of inflammation, for example via the actions of C5a and C3a, which stimulate vascular endothelial growth factor (VEGF) expression in post-injury angiogenesis and may be involved in choroidal neovascularization and the development of neovascular age-related macular degeneration (AMD) (Nozaki et al. 2006). The five classic symptoms of inflammation, i.e. erythema, edema, heat, pain, and loss of function, are products of the actions of the pro-inflammatory cytokines produced in response to mechanisms involving PRR signaling. Pro-inflammatory cytokines modify vascular endothelium permeability through the up-regulation of vascular cell adhesion molecule 1 (VCAM-1) and untercellular adhesion molecule 1 (ICAM-1), which stimulate selectin and integrin ligands on endothelial cells, leading to loosening of endothelial tight junctions (Ransohoff et al. 2003). This ultimately has the effect of increasing local blood flow and facilitating the movement of plasma components and immune cells through inflamed tissue, resulting in the redness and swelling characteristic of inflammation. Furthermore, increasing endothelial permeability can also facilitate leukocyte passage across the blood-brain barrier and other obstacles (Takeuchi and Akira 2010).

Ocular tissue exists in an immune-privileged environment which provides a buffer against the free movement of cells and some larger molecules between the eyes and systemic circulation. Immune privilege status in the eye is thought to be conferred by a combination of physical barriers such as the blood-retinal barrier, an immunosuppressive ocular microenvironment created by cell-bound and soluble inhibitory factors within the eye (Caspi et al. 1987; Stein-Streilein 2008), and the lack of direct lymphatic drainage pathways (Stein-Streilein 2008; Streilein 2003; Zhou and Caspi 2010). Certain ocular tissues have been shown to have direct immunosuppressive roles. For instance, retinal pigmented epithelial (RPE) cells have been shown to suppress T cell cytokine production via production of PD-L1 (Sugita et al. 2009b) and stimulate $CD4^+$ T cells to convert to T regulatory cells through constitutive production of the Cathepsin L inhibitor CTLA-2α (Sugita et al. 2008, 2009a). Nevertheless, several inflammatory diseases commonly manifest themselves in ocular tissue and are major causes of blindness and disability worldwide. Increasing emphasis on research regarding the roles of lipids, and specifically sphingolipids, in inflammatory mechanisms has led to their recognition as potential key elements in the pathogenesis of multiple ocular inflammatory diseases. As well as being major structural elements of eukaryotic cells, certain species of sphingolipids, such as ceramide (Cer), ceramide 1-phosphate (C1P), and sphingosine 1-phosphate (S1P) have been shown to act as signaling molecules with regulatory roles in various physiologic and pathogenic processes (Dressler et al. 1992; Hannun and Obeid 2008; Porter et al. 2018; Qi et al. 2017). They have also been tied to wound healing, notably in the cornea (Nicholas et al. 2017). Sphingolipid metabolic diseases are often tied to visual dysfunction. Notorious among these are lysosomal storage diseases, which arise from genetic defects resulting in partial or total loss of lysosomal enzymes that degrade sphingolipids, causing harmful accumulation of precursor molecules. These include GM1 gangliosidosis, Tay Sachs disease, Sandhoff disease, Gaucher disease, etc. These diseases tend to lead to various ocular abnormalities such as the development of "cherry-red macula" (Chen et al. 2014). Other ocular diseases, discussed below, have clear inflammatory components and have been linked to sphingolipid signaling and/or metabolic abnormalities.

8.2 Uveitis

Uveitis is a condition characterized by inflammation of the uvea, which is the pigmented middle layer of the eye containing the anterior uvea (consisting of the iris and ciliary body), and posterior uvea (choroid). Uveitis is responsible for causing blindness in roughly 30,000 people annually in the United States (Acharya et al. 2013). Anterior uveitis is commonly associated with pain, erythema, and photophobia, while intermediate and posterior uveitis can present with "floaters" and visual deficits. Inflammation of the uvea is capable of causing severe damage to the retina, optic nerve, and vitreous, often leading to complications such as macular edema, development of cataracts, and glaucoma (Ness et al. 2017). Uveitis may result from a number of diseases. The etiology of uveitis may be idiopathic, infectious, or noninfectious, with causes ranging from localized viral infection to direct ocular trauma and systemic disease (Rathinam and Babu 2013). Uveitic diseases may be either localized to the eye or may be a manifestation of diseases affecting multiple organ systems, such as systemic sarcoidosis (Caspi 2010), where anterior granulomatous uveitis manifests in up to 70% of cases with ocular involvement (Herbort et al. 2009). Analyses of lipidomics data from aqueous and retinal tissue from an endotoxin-induced uveitis rat model showed significant increases in total sphingolipid levels during the acute inflammatory stage of induced uveitis. Notably, the levels of C_{12} C1P and multiple species of Cer and SM were significantly elevated, suggesting an important role played by sphingolipids in uveitis (Wang et al. 2018). Analyses from Gaucher disease patients have shown increases in glucosylceramide (GlcCer), causing vitreous opacity and subsequent infiltration of macrophages, which may also suggest the involvement of Cer and Cer metabolites in certain forms of uveitis, though uveitis pathogenesis is highly complex and insufficient evidence exists to firmly establish a sphingolipid-mediated mechanism (Astudillo et al. 2016; Fujiwaki et al. 2004). However, the effectiveness of FTY720, an FDA-approved Sphingosine (Sph) analog used to treat Multiple Sclerosis (MS), in treating uveitis and other autoimmune ocular diseases supports a causal relationship of sphingolipid involvement in uveitis and other such diseases (Chen et al. 2014). FTY720 has been shown to suppress experimental autoimmune uveitis in mouse models by substantially reducing peripheral lymphocyte accumulation (Commodaro et al. 2010; Kurose et al. 2000). FTY720 has also been shown to delay retinal degeneration in rat models (Stiles et al. 2016). The discovery that FTY720 is effective in limiting the intensity of uveitis and in delaying retinal degeneration in animal models is strongly indicative of its potential utility in treating inflammatory ocular diseases and highlights the possibility of finding molecular targets within the sphingolipid metabolic pathways to utilize for drug development.

8.3 Diabetic Retinopathy

Diabetic retinopathy (DR) is a common complication of both type 1 and type 2 diabetes mellitus (DM) and involves chronic low-grade inflammation and degenerative neurovascular changes throughout the retina (Abcouwer 2013). It is a leading cause of vision loss and can be expected to develop within 20 years of DM diagnosis (Klein 1987). Left untreated, DR can lead to destruction and detachment of the retina as well as neovascularization of the retina and vitreous through chronic ischemia (Wang et al. 2013). Nonproliferative DR (NPDR) is the early, often asymptomatic form of DR which is characterized by various microvascular abnormalities such as vessel occlusion and microaneurysms. NPDR can exist asymptomatically for years, however, it can cause vision loss through macular edema and is capable of rapid progression to more debilitating forms of the disease, i.e. proliferative diabetic retinopathy (PDR) (Aiello 2003). PDR is an advanced stage of DR, which is characterized by marked proliferation of blood vessels into the retina with increased risk of preretinal and vitreous hemorrhage (Vingolo et al. 2017). VEGF, which promotes vascular permeability and disassembly of endothelial cell junctions, has been

shown to be significantly upregulated in the vitreous of DR patients (Aiello et al. 1994). Elevation of retinal glial cell (RGC) and RPE cell cytokine expression also contributes to retinal vascular inflammation and endothelial damage (Abcouwer 2013; Busik et al. 2008; Mohr 2004). It has also been suggested in animal models that leukostasis of the retinal vasculature is a potentially important contributor to ischemia and endothelial damage, and has a possible role in the mechanisms of ocular inflammation seen in DR (Kim et al. 2005). Retinal neovascularization requires the presence of a collagenous scaffold in the form of the vitreoretinal interface. Disruption of this interface as seen in posterior vitreous detachment (PVD) has been shown to be associated with protection from PDR and has been suggested as a potential surgical treatment for PDR (de Smet et al. 2013). Currently though, no fully effective treatment for DR exists. In humans and animal DR models, SMase increases due to TNF-α and IL-1β were shown to increase Cer generation and subsequently stimulate cytokine-mediated inflammation and regulate retinal microangiopathy (Kim et al. 1991; Mathias et al. 1991; Opreanu et al. 2010, 2011). Cer then activates NF-κB, a ubiquitously expressed proinflammatory transcription factor which stimulates transcription of the cytokines IL-1β, IL-6, and IL-8, monocyte chemoattractant protein 1 (MCP1), and cyclooxygenase 2 (COX2) (Schutze et al. 1992; Xiao et al. 2005). Cer is also capable of COX2 induction in macrophages through stimulation by lipopolysaccharide (LPS) and cEBP activation (Cho et al. 2003). Furthermore, very long chain (VLC) Cer species have been shown to mediate vascular permeability and are decreased in DM animal models (Kady et al. 2018; Tikhonenko et al. 2010). GlcCer may play a role in neuroinflammation and retinal cell death in DR, as abnormal GlcCer accumulation can cause mitochondrial, endoplasmic reticular, and endolysosomal dysfunctions (Astudillo et al. 2016; Fujiwaki et al. 2004). GlcCer has also been shown to increase in retinal neurodegeneration and in hyperglycemic retinal neurons in *in vitro* experiments (Busik et al. 2012; Fox et al. 2006; Opreanu et al. 2011; Sugano et al. 2019). S1P receptor 2 (S1PR2) may influence neovascularization in AMD and DR, as shown by ischemia-induced retinopathy animal models which are S1PR2-deficient and do not exhibit neovascularization (Skoura et al. 2007). Lactosylceramide (LacCer) may also play a role in DR, as evidenced by its apparent involvement in inflammation (Pannu et al. 2005), VEGF-mediated angiogenesis (Kolmakova et al. 2009), and its observed changes in human diabetic retina and vitreous samples (Wilmott et al. 2019). Macrophage influx and choroidal/subretinal neovascularization was also significantly inhibited via blockage of S1P with Sonepcizumab, further demonstrating the extent of sphingolipid involvement in DR (Xie et al. 2009).

8.4 Glaucoma

Glaucoma refers to a family of eye diseases which have been traditionally defined by optic nerve damage from elevated intraocular pressure (IOP). It is one of the world's leading causes of blindness, affecting roughly 80 million people worldwide (Cook and Foster 2012; Quigley and Broman 2006). There are multiple types of glaucoma, all of which may be characterized by progressive optic nerve head degeneration and RGC death, but not all are associated with IOP elevation. Angle-closure glaucoma is characterized by narrowing or complete closure of the anterior chamber angle, and may be due to anatomical predispositions such as defects in the iris or lens (as in primary angle-closure glaucoma) or due to a secondary process such as neovascularization or inflammation (as in secondary angle-closure glaucoma) (Weinreb et al. 2014). Closure of the anterior chamber angle prevents aqueous humor drainage from the anterior chamber, resulting in IOP elevation and optic nerve damage. Open-angle glaucoma patients have increased aqueous outflow resistance due to blockage of the trabecular meshwork, also resulting in gradual IOP elevation and subsequent optic nerve damage (Foris and Gossman 2018). Normal tension glaucoma, however, is characterized by normal or low IOP along with the RGC death, optic nerve degeneration, and visual field defects associated with

other forms of glaucoma involving IOP elevation (Gramer et al. 1986; Quigley 2011). Clearly, there are likely to be other factors at play in the pathogenesis of glaucoma, which are independent of IOP elevation. Reports have strongly suggested the involvement of an inflammatory response in conjunction with glaucomatous neurodegeneration (Luo et al. 2010). Specifically, astrocyte and microglial upregulation of TLR signaling leading to activation of proinflammatory cytokines in the optic nerve head has been identified as a probable contributor (Howell et al. 2011, 2012). Recent findings point to S1PR2 acting as a mediator of trabecular meshwork contractility, likely affecting outflow and potentially having a role in glaucoma pathogenesis (Stamer et al. 2009; Sumida and Stamer 2011). Our own long-term work has shed light on many roles of sphingolipids in the mechanisms of RGC degeneration, which may be applicable to glaucoma. Our lab and others have made novel discoveries pointing to lysosomal accumulation of Cer as being a factor in glaucomatous RGC degeneration (Fan et al. 2016; Hayreh et al. 2009a, b). Furthermore, significant reductions in plasma Sph and S1P have been shown to be associated with primary open angle glaucoma (Burgess et al. 2015). Though data pertaining to the roles of sphingolipids in glaucoma is limited, evidence is growing which suggests an important part played by sphingolipids in the disease's pathogenesis.

8.5 Age-Related Macular Degeneration

Age-related macular degeneration is a degenerative disease of the macula which results in loss of the central visual field. AMD accounts for roughly half of all legal blindness in industrialized countries (Owen et al. 2003). Degeneration of the RPE and subsequent photoreceptor death leading to loss of central vision is the hallmark of both types of AMD. AMD etiology has two forms: dry and wet AMD. Dry AMD involves slow, progressive RPE apoptosis and has a relatively poorly-understood etiology. Dry AMD involves the formation of Drusen between the RPE and the Bruch membrane, leading to RPE and photoreceptor degeneration and progressive geographic atrophy. No effective treatment for dry AMD has yet been developed (Zajac-Pytrus et al. 2015). Wet AMD is characterized by overproduction of VEGF in the RPE, leading to breakdown of the blood-retinal barrier and choroidal/subretinal neovascularization (Nowak 2006). Weak vessel formation may lead to hemorrhage, causing macular scarring and edema, which is the major cause of vision loss in wet AMD (Campochiaro 2013). There are apparent connections between inflammatory mechanisms and AMD pathology. Subretinal Drusen contain a variety of potentially harmful constituents such as lipids, RPE-derived cellular debris, oxidation byproducts, and inflammatory factors including complement components and immunoglobulins (Anderson et al. 2002; Hageman et al. 1999, 2001; Johnson et al. 2000). Factor H (HF1), a major inhibitor of the complement pathway, which is synthesized by RPE, has been shown to accumulate within Drusen and *Y402H* mutation has been identified as a major risk factor for the development of AMD (Hageman et al. 2005). Further associations have been identified between AMD and several complement pathway-associated genes: complement factor H, complement factor H-related 1 and 3, complement factor B, and complement components 2 and 3 (Anderson et al. 2010). Cer-mediated inflammation and apoptotic mechanisms have been linked to RPE cell degeneration in AMD and several ocular degenerative diseases (Zhu et al. 2010). Cer synthesis and oxidative stress are responsible for contributing to mitochondrial permeabilization and caspase-3 activation, followed by apoptotic photoreceptor cell death (Barak et al. 2001; Kannan et al. 2004). Inhibition of Cer synthesis via the SMase inhibitor desipramine has been shown to have a protective effect in oxidative-stressed photoreceptors, preventing apoptotic cell death (Sanvicens and Cotter 2006). We were able to produce a similar photoreceptor protective effect by increasing degradation of Cer to Sph. We achieved this by inducing human ARPE19 cells to overexpress acid ceramidase, an

enzyme which catalyzes the conversion of Cer to Sph, thereby decreasing cellular Cer levels and yielding significant protection from apoptosis (Sugano et al. 2018). The opposite effect has been observed from overproduction of Cer, which accelerated RPE cell death (Zhu et al. 2011). Cer is also implicated in AMD-related RPE degeneration, wherein activation of acid sphingomyelinase results in RPE autophagy dysfunction, complement regulatory protein recycling, endosome biogenesis, and complement activation (Kaur et al. 2018; Natoli et al. 2017; Tan et al. 2016; Toops et al. 2015).

8.6 Dry Eye Syndrome

Dysfunctional tear syndrome, also known as dry eye disease or keratoconjunctivitis sicca, is a multifactorial disorder of the tear film and ocular surface caused by tear deficiency and excessive evaporation (Bron et al. 2014). Dry eye affects roughly 17% of women and 11% of men in the United States (Moss et al. 2000) and is typically seen as a relatively minor condition, though it can cause discomfort, visual disturbance, and ocular surface damage through inflammation (Hessen and Akpek 2014). Dry eye is caused by dysfunction in the lacrimal functional unit, i.e. the synergistic unit composed of the lacrimal glands, eyelids, and ocular surface (Stern et al. 1998). Dysfunction may cause tear film hyperosmolarity and ocular surface inflammation secondary to decreased tear production and/or increased evaporative tear loss. Tear production deficiency dry eye disease can be subclassified into Sjögrens and non-Sjögrens syndrome. Sjögrens syndrome is a chronic autoimmune exocrinopathy involving inflammatory infiltration of the lacrimal glands, causing cell death and tear production deficiency (Fox 2005). Non-Sjögrens dry eye is thought to be age-related and secondary to obstruction of the lacrimal ducts leading to decreased tear output (Damato et al. 1984). Interestingly, diabetes mellitus is significantly associated with non-Sjögrens dry eye (Kaiserman et al. 2005) and growing evidence points to a chronic inflammatory component of dry eye disease, mediated by lymphocytes (Kunert et al. 2000). Conjunctival inflammation is a characteristic clinical symptom of dry eye and in this case may be dependent upon T-cell activation and upregulation of pro-inflammatory cytokines and matrix metalloproteinase (Solomon et al. 2001; Stern et al. 2002). Sphingolipids are involved in maintaining tear film lipid layer (TFLL) integrity, which is essential for proper tear film composition and thereby plays a major role in proper ocular surface lubrication and protection (Lam et al. 2011). Furthermore, SM, short chain GlcCer, and Cer levels have been shown to be elevated in dry eye patient meibomian gland samples, which are the primary source of lipids in the TFLL (Mathers and Lane 1998; Nelson et al. 2011; Nicolaides et al. 1989; Paranjpe et al. 2018; Robciuc et al. 2013; Shine and McCulley 1998). Cer, C1P, and S1P can induce inflammation by arachidonic acid release and prostaglandin formation (Hannun and Obeid 2008; Haversen et al. 2009; Jozefowski et al. 2010), which has been found to be correlated with multiple measures of tear film and meibomian gland dysfunction (Walter et al. 2016). C1P stimulates eicosanoid synthesis (Pettus et al. 2004) and activates cytosolic phospholipase A2 in prostaglandin synthesis (Pettus et al. 2005). However, in addition to inducing inflammation, C1P also has anti-inflammatory roles. For instance, C1P inhibits TNF-converting enzyme (TACE), which is a metalloproteinase that cleaves proTNF to yield its active form, TNFα (Lamour et al. 2011). TNFα activates SphK1 to produce S1P, which then binds ubiquitin E3 ligase TRAF2 and stimulates polyubiquitination of Receptor interacting protein-1 (RIP1), causing phosphorylation of IKK complex and NF-κB activation (Alvarez et al. 2010), which might have roles in various ocular inflammation processes relevant to dry eye disease. Increased Cer levels have also been shown to negatively affect TFLL stability (Arciniega et al. 2013). Altogether, these findings along with our own data strongly support sphingolipid involvement in the pathophysiology of dry eye diseases.

8.7 Conclusion

Recent advances in the understanding of sphingolipid metabolism and signaling as well as inflammatory mechanisms have identified bioactive sphingolipids as mediators in ocular disease processes. Cer, the central molecule in sphingolipid metabolism (Fig. 8.1), has received the most attention for its apparent involvement in apoptosis and stress responses (Pettus et al. 2002). However, it is important to understand that Cer, LacCer, and other sphingolipid metabolites are *classes* of similar lipids with distinct, species-specific effects rather than individual molecules. For example, depending on differences in chain length, degrees of unsaturation, etc. introduced by the >28 distinct enzymes which use Cer as a substrate or product, there are theoretically ~360 different possible variations of Cer. The different variations of sphingolipid species within classes apparently have distinct effects and downstream metabolites (Hannun and Obeid 2011). As if this did not complicate things enough, ocular sphingolipid research is still fairly immature and there is often insufficient foundational knowledge to confidently establish mechanisms explaining the downstream effects of known bioactive sphingolipids, despite association studies strongly sup-

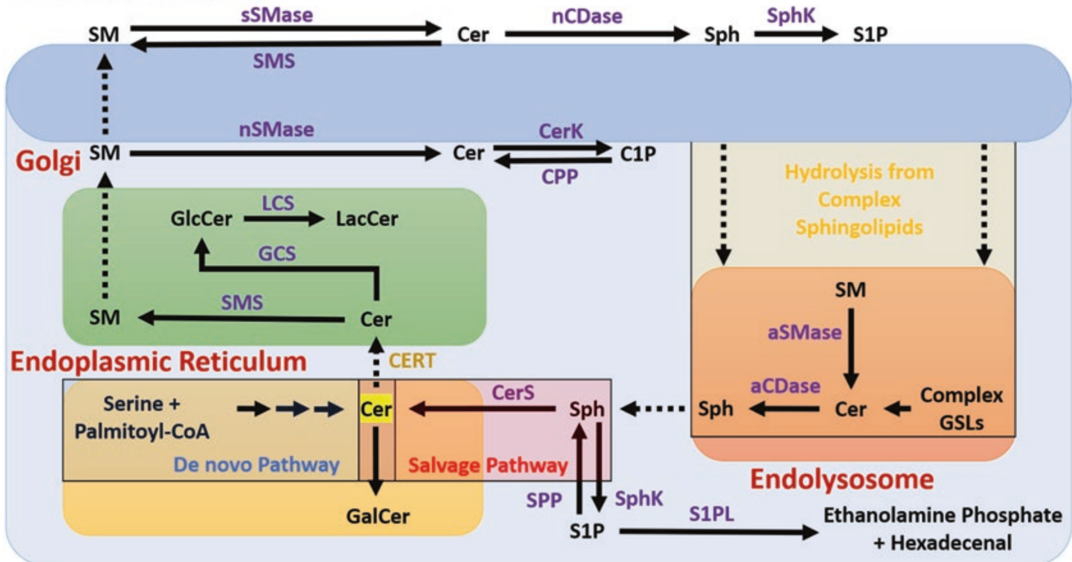

Fig. 8.1 Schematic representation of cellular sphingolipid metabolism. Ceramide (Cer) is produced primarily in the endoplasmic reticulum (ER) from serine and palmitoyl-CoA via a series of reactions in the *de novo* pathway. Cer is then either converted to Galactosylceramide (GalCer) by addition of a galactose or transported from the ER to the *trans*-Golgi, possibly via a trafficking mechanism mediated by Cer Transfer Protein (CERT). In the Golgi, Cer is either converted to Sphingomyelin (SM) by Sphingomyelin Synthase (SMS), or is glycosylated to form Glucosylceramide (GlcCer) by GlcCer Synthase (GCS). GlcCer may be converted to Lactosylceramide (LacCer) by addition of galactose with LacCer Synthase (LCS). SM from the Golgi is transported to the plasma membrane, where it may be converted by cytosolic neutral sphingomyelinase (nSMase) back to Cer, which is phosphorylated by Cer Kinase (CerK) to ceramide 1-phosphate (C1P). Alternatively, SM may be converted to Cer via secretory SMase (sSMase), which is converted to Sphingosine (Sph) by neutral ceramidase (nCDase). Sph is phosphorylated to sphingosine 1-phosphate (S1P) by Sphingosine Kinase (SphK) 1 or 2, which can signal extracellularly via membrane-bound S1P receptors (of which there are 5 known). Complex sphingolipids from the plasma membrane may enter the endolysosomal pathway and be hydrolyzed back to Cer, which is converted to Sph within the lysosome by acid ceramidase (aCDase or ASAH1, N-Acylsphingosine Amidohydrolase 1). From here, Sph is either phosphorylated to S1P or re-acylated back to Cer by Ceramide Synthase (CerS) in the salvage pathway. S1P leaves the sphingolipid metabolic pathway by conversion to ethanolamine phosphate and hexadecenal by Sphingosine 1-Phosphate Lyase (S1PL)

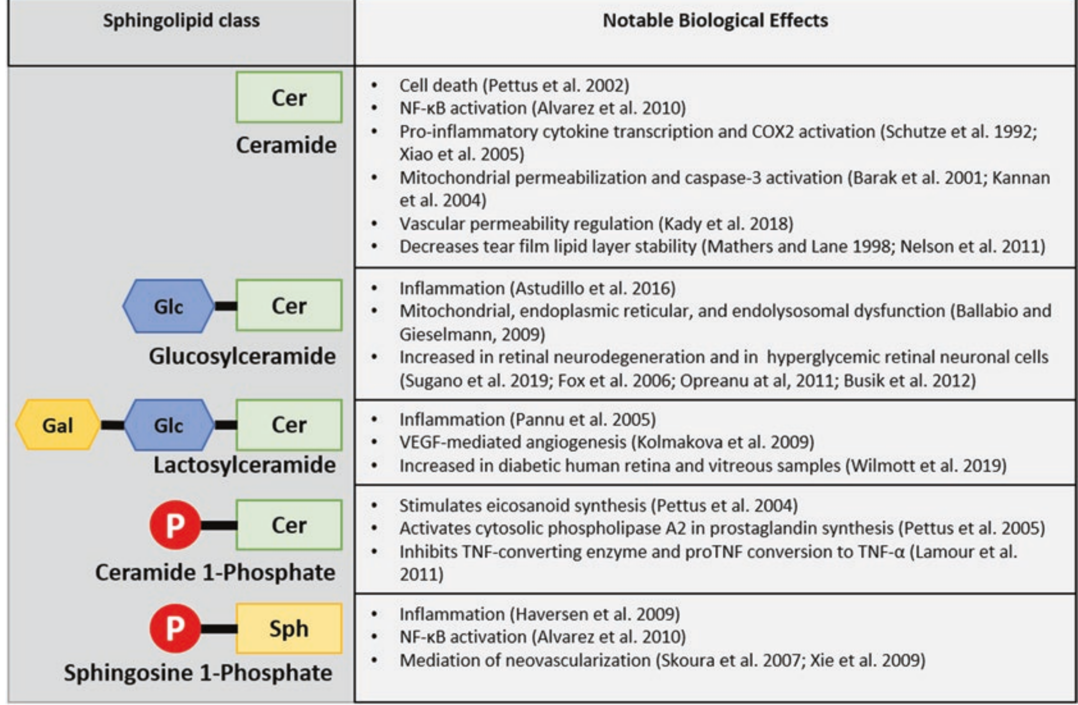

Fig. 8.2 Brief summary of major sphingolipid classes and notable effects relevant to inflammatory ocular diseases. *Cer* ceramide, *Sph* sphingosine, *Glc* glucose, *Gal* galactose, *P* phosphate

porting their involvement in biological processes. Although the exact mechanisms of sphingolipid mediation of ocular inflammatory and degenerative processes are generally incompletely-developed, there is enough evidence to link sphingolipids to several major ocular diseases (Fig. 8.2). Whether these findings implicate them as causative of pathology (in which case they may be targeted for therapeutic treatments) or show that they merely change in response to disease processes (in which case they may be used as diagnostic biomarkers) is, in many cases, also still unclear. That said, the involvement of sphingolipids in inflammation is well-established and the general trend is pointing towards sphingolipids gaining more recognition as important mediators of ocular disease. A number of inflammatory mechanisms are known to rely, at least in part, on sphingolipid signaling and metabolism. Taken into context, these novel discoveries have implications for a wide range of ocular inflammatory diseases. Though the data supporting sphingolipid involvement in the underlying mechanisms of many diseases are limited, efforts to elucidate the mechanisms of these diseases continue to turn up evidence of bioactive sphingolipid signaling and metabolism playing important roles.

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