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Xian-Cheng Jiang *Editor*

Sphingolipid Metabolism and Metabolic Disease

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Xian-Cheng Jiang
Editor

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Editor
Xian-Cheng Jiang
Department of Cell Biology
SUNY Downstate Health Sciences University
Brooklyn, NY, USA

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Preface

In 2010, Dr. L. Ashley Cowart had edited a book entitled: Sphingolipids and Metabolic Disease for Advances in Experimental Medicine and Biology. Since then, there are a lot new progresses made in the field. It is an urgent task to summary these new discoveries. In last 20 years, metabolic diseases, including obesity, type 2 diabetes, fatty liver, and atherosclerosis, become one of the major concerns of human health. Sphingolipids are closely related with these diseases.

This book discusses the impact of sphingolipid de novo synthesis on metabolic diseases. Major sphingolipids, including ceramide, sphingosine-1-phosphate, sphingomyelin, and glucosylceramide, are the main focus of the discussion. We will also introduce the association of sphingolipids with other bioactive lipids, such cholesterol, phosphatidylcholine, and phosphatidylethanolamine. While highlighting how rare diseases related with abnormal glycosphingolipid metabolism, this publication introduces sphingolipid metabolism related diseases, such as lung diseases and cancers. Since sphingolipid metabolism pathway can potentially serves as drug targets, we will also discuss the new progress on this topic. The potential readers, who will be mainly benefited, will be professional researchers, pharmacophysiologist, and graduate students.

We hope you enjoy the volume.

Brooklyn, NY, USA — Xian-Cheng Jiang

Contents

Sphingolipids and Cholesterol

1

Xian-Cheng Jiang and Zhiqiang Li

Abstract

Sphingolipids and cholesterol are two lipid partners on cellular membranes where they form specific microdomains, named lipid rafts, which mediate specific cell functions. Sphingomyelin (SM) is one of the major sphingolipids. SM and free cholesterol are also two key lipids on the monolayer of plasma lipoproteins, including chylomicron, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL), which participate in lipid transport in the circulation. Thus, sphingolipids and cholesterol play a fundamental role in cell membrane structure and blood lipid transport. In this chapter we will discuss the relationship between both lipids, on the cell membrane and in the circulation, as well as the impact of such relationship in the development of metabolic diseases.

Keywords

Sphingolipids · Sphingomyelin · Cholesterol · Cell membrane lipids · Lipids in the circulation

Abbreviation

SM	Sphingomyelin
SMase	Sphingomyelinase
SPT	Serine palmitoyltransferase
SMS	Sphingomyelin synthase
BLp	Apolipoprotein B-contained lipoprotein
VLDL	Very low-density lipoprotein
LDL	Low-density lipoprotein
HDL	High-density lipoprotein

1.1 Sphingolipids and Cholesterol on Cell Membrane

Sphingolipid biosynthesis occurs via the actions of serine palmitoyltransferase (SPT), 3-ketosphinganine reductase, ceramide synthase, and dihydroceramide desaturase to produce ceramide, which is the central substrate for the production of sphingomyelin through two sphingomyelin synthases (SMS1 and SMS2), sphingosine-1-phospate, and glycosphingolipids [1]. SPT is the first enzyme for sphingolipid de novo biosynthesis and the deficiency of both of its subunits causes lethality [2].

There is a relationship between cell membrane SM and cholesterol. It is known for a long time that SM addition to cells increased cholesterol synthesis [3]. Moreover, SM removal from plasma membrane of cells by treatment with sphingomyelinase, an enzyme that degrades SM,

X.-C. Jiang (✉) · Z. Li
Department of Cell Biology, SUNY Downstate Health Sciences University, Brooklyn, NY, USA
e-mail: xian-cheng.jiang@downstate.edu

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reduced free cholesterol levels on the membrane, through promoting cholesterol translocation from the plasma membrane to the ER [4]. SM and cholesterol interact in biological and model membranes [5]. SMS1 or SMS2 overexpression in hepatocytes significantly increased the levels of intracellular SM and cholesterol and decreases cholesterol secretion in liver cells [6]. In a cell membrane mimicking study, Pathak and London showed that SM/cholesterol/palmitoyl 2-oleoyl phosphatidylcholine, a mixture similar to that in the outer leaflet of plasma membranes, forms nanodomains at physiological temperatures [7]. They also found that Triton X-100 does not induce those domain formations or increase the fraction of the bilayer in the ordered state, although it does increase domain size by coalescing preexisting domains [7]. SM's ceramide moiety interacts hydrophobically with cholesterol sterol-ring systems through hydrogen bonds and van der Waal's forces [8].

Plasma membrane lipid rafts, enriched with cholesterol, SM, glycosphingolipids, have proven to be involved in cell signaling, lipid and protein sorting, and membrane trafficking [9–11]. Studies showed that cellular membranes are not just homogenous mixtures of lipids and proteins, and there are two different phases on cellular membrane: liquid ordered (Lo) and liquid disordered (Ld) phases [12]. In Lo phases, fatty acid chains are stretched, but lipids do not adopt hexagonal lateral arrangement. Lipid rafts are considered Lo, not only enriched in cholesterol and sphingolipids, but also many specific proteins. Non-raft domains of the membrane are considered Ld, which are enriched with non-saturated phospholipids and non-raft proteins [13]. Because of the lack of an appropriate fluorescent probe and the spatiotemporal stability of lipid rafts, the direct observation of lipid rafts has been greatly restricted. However, with the development of new microscopic techniques, more and more evidence of lipid rafts based on the partition and dynamic behavior of SM has been obtained [14]. SM deficiency in lipid rafts is accompanied by reduced cholesterol content [15]. We separated cell membrane SM and cholesterol into two peaks, lipid rafts and non-lipid rafts (based on specific markers), SM is mainly located in lipid rafts [16], while cholesterol is about evenly distributed in lipid rafts and non-rafts [17].

Alterations in lipid rafts could affect cell functions. We reported that SMS2 deficiency significantly attenuated the concentration of toll-like receptor 4 (TLR4)-MD2 complex on the lipid rafts of macrophages after LPS stimulation, and SMS2 depletion reduced TNFα-stimulated lipid raft recruitment of TNF receptor-1 in cells [18]. Further, we found that deficiency in macrophage sphingolipid de novo synthesis significantly lowered SM levels in plasma membrane and lipid rafts. This reduction not only impaired inflammatory responses triggered by TLR4 and its downstream NFκB and MAPK pathways, but also enhanced reverse cholesterol transport [19]. Recently, we showed that hepatocyte plasma membrane SM level is one of the key factors in regulating hepatocyte polarity [20] and sphingolipid de novo biosynthesis is essential for intestinal cell survival and barrier function [21]. Changes in lipid rafts could also affect insulin signaling [22]. Cholesterol extraction with β-cyclodextrin disrupted caveolae in cultured cells and resulted in inhibition of insulin signaling [23–25]. Skeletal muscle membrane cholesterol accumulation is an early, reversible, feature of insulin resistance [26]. We demonstrated that deficiency in SM biosynthesis significantly increased insulin sensitivity and we attributed this primarily to the reduction of SM in plasma membrane SM-rich microdomains and not to plasma membrane ceramide levels [27].

Glycosphingolipids are another important component of lipid rafts on cell membrane [28]. Glucosylceramide (GluCer), one of the simplest glycosphingolipids, plays key roles in physiology and pathophysiology [29, 30]. The deposition or accumulation of GluCer within cells and certain organs results in Gaucher disease [31]. Changes in the level of GluCer are noticed in response to cardiovascular disease and diabetes, as seen in Gaucher disease [32]. GluCer reorganizes cholesterol-containing domains in a fluid phospholipid membrane [33]. Glycosphingolipids in lipid rafts are also important in blocking tyrosine phosphorylation of insulin

receptor and downstream signaling [34–36]. Pharmacological inhibition of glycosphingolipid synthesis had markedly improved insulin sensitivity in rodent models with insulin resistance [37]. Among glycosphingolipids, gangliosides (including GM1, GM2, GM3, and so on) are well-known components in lipid rafts [36, 38]. Changes in ganglioside levels are known to affect the expression of raft-associated proteins on the cell surface and lead to reduced membrane fluidity, resulting in cell dysfunctions, such as impaired signal transduction [38–40]. It was reported that GM1 and GM2 contribute to insulin resistance [41]. GM3 deficiency enhanced insulin receptor activation [42], and GM3 dissociated insulin receptor/caveolin-1 complex, thus resulting in insulin receptor dysfunctionality [43].

In normal situation, ceramide levels in cell membranes are low (0.1–1 mol % of total phospholipid), however, its levels can be higher under cellular stress conditions, such as apoptosis [44]. Previously, researchers have indicated that ceramide level changes in model or cell membranes can induce membrane permeability [45], lipid flip-flop motion [46], and lateral domain segregation [47]. Indeed, ceramide can give rise to highly ordered gel-like domains [48], which could serve as a "membrane platform" clustering receptor molecules and mediating signal transduction processes [49, 50]. It is known that membrane ceramide can be derived from SM by acid sphingomyelinase [51]. Recent two studies indicated the heterogeneity of membrane lipid bilayer, composing cholesterol, SM, and ceramide [52, 53].

Another important impact of SM and cholesterol interaction on plasma membrane is for cholesterol homeostasis, which ensures optimal cholesterol levels in cellular membranes by precise regulation of its synthesis and uptake [54]. About 80% cellular cholesterol is located on plasma membrane, however, cholesterol synthesizing regulator (sterol regulatory element-binding proteins, SREBPs) [55] is located in the ER membrane, which contains only about 1% of total cellular cholesterol [56]. The regulation of cholesterol transport between plasma membrane to ER allows cholesterol sensors in ER to monitor the cholesterol level of plasma membrane [57]. This regulation is determined by plasma membrane lipid rafts, which critically depends on the interaction of cholesterol with SM [58]. Recently, Endapally et al. utilized ostreolysin A (OlyA), a protein that binds to membranes only when they contain both SM and cholesterol and clearly indicated that SM adopts two distinct conformations in membranes when cholesterol is present. OlyA binds only one conformation which contains both SM and cholesterol and it cannot bind to another conformation where SM is free from cholesterol. In cells, levels of SM/cholesterol complexes are held constant over a wide range of plasma membrane cholesterol concentrations, enabling precise regulation of cholesterol levels [59].

The same group of researchers also studied cholesterol homeostasis in the plasma membranes of animal cells using two cholesterol-binding bacterial toxin proteins, perfringolysin O (PFO) and domain 4 of anthrolysin O (ALOD4). They found that cholesterol in the plasma membrane is present in three different pools. The first is an accessible pool that contains mobile cholesterol. Excess cholesterol is transported to ER to terminate SREBP activation and decrease cholesterol synthesis when the cholesterol concentration surpasses a threshold; the second is an SM-sequestered pool. The plasma membrane cholesterol cannot be transported to the ER but can be liberated for transport by SMase treatment; the third is an essential pool. The plasma membrane cholesterol is sequestered by other plasma membrane factors [60]. Hence, SM and the ratio of cholesterol to SM have the potential to markedly alter cholesterol trafficking and homeostasis in cells [61, 62].

Certain SM-binding toxin proteins were also used to evaluate SM/cholesterol interaction. Lysenin is an earthworm toxin which strongly binds to SM [63, 64]. Atomic force microscopy results showed that lysenin assembles into a

hexagonal close packed structure by rapid reorganization of its oligomers on an SM/cholesterol membrane [65]. Lysenin has been used for the study of cell membrane structure. Recently, He et al. utilized nanoscale secondary ion mass spectrometry imaging with ^{15}N-labeled lysenin in combination with ^{15}N-labeled cholesterol-binding proteins to visualize SM-rich and cholesterol-rich domains in the plasma membrane of CHO cells. Their results revealed that SM and cholesterol are not distributed evenly, but are enriched on the surface of microvilli [66]. Sticholysin II is another SM-binding protein. It belongs to the actinoporin family, a group of low molecular weight pore forming toxins that switch from a soluble form to an integral membrane protein without the aid of chaperones and translocons [67]. Sticholysin II is able to discriminate among membrane domains with SM with respect to those enriched with gangliosides [68].

1.2 Sphingomyelin and Cholesterol in the Circulation

Epidemiological studies have shown a positive relationship between total cholesterol concentrations and mortality from coronary heart disease (CHD) [69, 70]. Total cholesterol does not accurately predict the risk of CHD in many patients, because it is the sum of all cholesterol carried not only by atherogenic lipoproteins (i.e., chylomicron, very-low-density lipoprotein [VLDL], low-density lipoprotein [LDL], intermediate-density lipoprotein [IDL]), the so-called bad cholesterol but also by antiatherogenic lipoproteins (i.e., high-density lipoprotein [HDL]) [71], the so-called good cholesterol. SM plays an important role in plasma lipoprotein metabolism including lipoprotein production, cholesterol efflux, cholesterol absorption, reverse cholesterol transport, atherogenic lipoprotein aortic retention, and so on [72]. Metabolic regulation of SM and cholesterol appears to be inter-coordinated [5, 62]. Both SMS1 and SMS2 overexpression in mice increase the atherogenic lipoproteins, which are enriched with SM [73].

1.2.1 Apolipoprotein B (apoB)-Containing Lipoprotein Production

ApoB is the major protein component of chylomicron and VLDL, which are the precursors of chylomicron remnant, IDL, and LDL. Thus, these lipoproteins are also known as apoB-containing lipoproteins (BLp) [74]. Chylomicron and VLDL transport triglyceride (TG) from the intestine and liver, respectively, into the bloodstream [75]. ApoB exists in two forms, apoB100 and apoB48 [76, 77]. Overproduction of BLp is a major cause of accelerated atherosclerosis [78, 79]. The regulation of BLp secretion, which takes place primarily post-transcriptionally [80], is poorly understood. Accumulating evidence suggests that formation of BLp [81–83] is accomplished sequentially. This two-step model postulates that the initial product is a primordial small, dense particle formed during or immediately after apoB translation in the ER. Bulk lipids, most likely TG, phosphatidylcholine, SM, and free cholesterol, are incorporated into the primordial particle to form mature BLp [84]. Multiple factors are involved in BLp maturation. Microsomal TG transfer protein (MTP) is involved in an early phase of lipid addition to apoB [85, 86]. Phospholipid transfer protein (PLTP) may also be involved in the first and second stage of VLDL lipidation [87–89]. Studies in hamsters suggest that de novo synthesized SM is secreted *via* the VLDL/LDL pathway in the liver [23, 24]. Isolated rat hepatocytes secrete SM as a part of BLp [25]. Two major carriers of SM in plasma are VLDL and chylomicrons [20, 90]. Despite these facts, how SM is deposited in nascent BLp remains to be determined. Two candidate proteins could play a role in this process. First, MTP can transfer SM [91] for deposition in nascent BLp. Second, PLTP can transfer SM [92] and promote BLp lipidation [93]. Alternatively, there may be other yet unidentified

proteins that specifically transfer SM. Recently, we found that hepatocyte total SMS blocking can reduce VLDL production. This phenomenon could be related with a reduction of atherogenicity [94].

1.2.2 Cholesterol Absorption

Dietary lipid absorption occurs in the lumen of the small intestine and on the apical surface of enterocytes. Niemann-Pick C1-like 1 (NPC1L1) and the ATP-binding cassette transporters G5/8 (ABCG5/8) are the two major factors mediating net cholesterol uptake. The former mediates cholesterol uptake, and the latter mediates excretion of excess cholesterol into the intestinal lumen [95, 96]. CD36 also participates in cholesterol uptake at the brush border of enterocytes [97, 98]. CD36 [98] and fatty acid transport protein 4 (FATP4) [99] are involved in free fatty acid uptake by enterocytes. It is conceivable that ablation of SMS may reduce the incorporation of SM in enterocyte plasma membrane lipid rafts, a platform for the above-mentioned transporters [97, 100–104], with consequent perturbation of endocytosis and protein recruitment to the plasma membrane, ultimately leading to defective lipid uptake and secretion. It was reported that SM could affect cholesterol absorption in the small intestine [61] and cholesterol uptake by CaCo2 cells [105]. SM in diet affects plasma and tissue levels of cholesterol and reduces cholesterol absorption in rodent small intestines [106–109]. Co-administration of cholesterol and SM leads to inhibition of absorption of both lipids in rats [106].

Reduction of blood total cholesterol by SM consumption in rats and mice makes dietary SM a "functional food" [72]. However, Ohlsson et al. found no significant changes in plasma cholesterol profile after dietary SM feeding in humans [110, 111]. A relatively well-controlled study also indicated that in humans, 1 g/day of dietary SM does not alter the blood lipid profile except for an increased HDL-cholesterol concentration and has no effect on cholesterol absorption, synthesis, and intraluminal solubilization compared to control [112].

1.2.3 Effect of SM on Cholesterol Efflux

Promoting cholesterol efflux to extracellular acceptors is of great importance in the maintenance of cellular cholesterol homeostasis [113, 114]. One of the major functional properties of HDL particles is to mediate cholesterol efflux and consequently promote reverse cholesterol transport (RCT) [115]. SM is the second most abundant phospholipid and major sphingolipid component of HDL [116]. SM content in HDL varies widely [117, 118]. Studies showed that SM 24:1 was the second most abundant species both in HDL_2 and HDL_3, and SM 16:0 was the main species in human HDL and more elevated in HDL_2 than HDL_3 [116, 119]. Therefore, SM levels of HDL could affect HDL metabolism. It was reported that SM accelerated the formation of reconstituted HDL by ApoA1, modified the size and stability of HDL particle. Complete degradation of SM could increase the rate of HDL_3 cholesterol oxidation [120–124]. Moreover, increased SM levels in HDL inhibit the HDL remodeling enzymes lecithin-cholesterol acyltransferase (LCAT) [125] and PLTP activity [126].

ATP-binding cassette (ABC) transporters, including ABCA1 and ABCG1, are involved in HDL-mediated cholesterol efflux [127]. Importantly, plasma membrane SM levels influence cholesterol efflux through regulating ABCA1 and ABCG1 [128, 129]. ABCG1 mediates the efflux of SM and cholesterol from cell membranes depending on their level and distribution in the membrane [130, 131]. We found that SPT subunit 2-haploinsufficient macrophages have significantly lower SM levels in plasma membrane and lipid rafts. This reduction enhanced reverse cholesterol transport mediated by ABC transporters [19]. We also found that SMS1 or SMS2 deficiency promotes cholesterol efflux [128, 132]. However, there is debate about the role of SM-associated cholesterol efflux

[133]. This vague understanding is partially caused by the absence of an approach to determine the interaction of membrane SM and cholesterol in one living cell. Recently, by using of luminol electrochemiluminescence (ECL), Huang et al. revealed the codetermination of SM and cholesterol in cholesterol efflux in one living cell [134].

1.2.4 SM and Atherosclerosis

Atherosclerosis progression is initiated by atherogenic lipoprotein accumulation and oxidation [135, 136], monocyte recruitment, macrophage foam cell formation, and inflammation [137, 138]. Atherosclerosis regression is mediated by lipid lowering, macrophages cholesterol efflux and forms cell effective efferocytosis from the atherosclerotic plaques [138, 139]. Much evidence indicate that SM content in the aortic wall and in plasma is closely related to atherosclerosis. It is well established that SM accumulates in atheroma formed in humans and animal models [140–145]. LDL extracted from human atherosclerotic lesions is much richer in SM than LDL from plasma [146–149]. The ratio of SM to phosphatidylcholine (PC) is increased fivefold in VLDL from hypercholesterolemic rabbits [150, 151]. We found that plasma SM level in *Apoe* KO mice is fourfold higher than in wild-type (WT) animals [152], and this hypersphingomyelinemia together with hypercholesterolemia contributes to increased atherosclerosis [153, 154]. We also found that human plasma SM level is an independent risk factor for coronary heart disease [155, 156]. SM increases from 10% at birth to 48% in patients who had undergone a coronary artery bypass grafting and to 60% in patients who had plaques in their coronary arteries, and SM contributes to atherosclerosis and sudden death [157]. SM is also associated with increased risk of myocardial infarction [158] and human atherosclerotic plaque inflammation [159]. Given that SM is the major sphingolipid in atherogenic BLp, SM biosynthesis should have important impact on BLp production as well as atherogenesis. Our laboratory and others have also discovered that chemical inhibition of sphingolipid biosynthesis significantly decreases plasma SM levels, thus lessening atherosclerotic lesions in *Apoe* KO mice [160, 161].

As suggested by Williams and Tabas, subendothelial retention and aggregation of atherogenic lipoproteins also play a very important role in atherosclerosis [162, 163]. SM-enriched LDL retained in atherosclerotic lesions is acted on by an arterial wall SMase that promotes aggregation and retention, initiating the early phase of atherogenesis [148]. We prepared *Sms2* and *Apoe* double knockout (KO) mice. We found that atherogenic lipoproteins from the double KO mice showed a reduction of their retention in aortas, compared to controls. Importantly, the double KO mice showed a significant reduction in atherosclerotic lesions of the aortic arch and root, compared with controls [164].

1.3 Conclusion

Both cholesterol and sphingolipids are essential lipids in mammalian cellular membranes and in the circulation. It is well known that dysregulation of both cholesterol and sphingolipids is related with metabolic diseases, such as insulin resistance, metabolic syndrome, and atherosclerosis. There are a lot of studies investigating the relationship between cholesterol and metabolic diseases or sphingolipids and metabolic diseases. As we discussed in this chapter, cholesterol prefers to interact with sphingolipids, affecting the structure and function of cell membranes and lipoproteins. Conversely, sphingolipids, especially SM, are involved in cholesterol metabolism, such as cholesterol absorption and RCT. The investigation of the interaction between cholesterol and sphingolipids and the impact of such interactions in the development of metabolic diseases should be the direction in the future.

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Sphingolipids in Adipose: Kin or Foe?

2

Yolander Valentine and L. Ashley Cowart

Abstract

Obesity research has shifted in recent years to address not only the total amount of adipose tissue present in an individual but also to include adipose tissue functions such as endocrine function and thermogenesis. Data suggest that sphingolipids are critical regulators of metabolic homeostasis, and that disruption of their levels is associated with metabolic disease. Abundant data from mouse models has revealed both beneficial and deleterious roles for sphingolipids in adipose function, and numerous human studies have shown that obesity alters circulating sphingolipid profiles. Sphingolipids comprise a large family of interrelated metabolites, and pinpointing specific functions for specific lipids will be required to fully exploit the therapeutic potential of targeting sphingolipids to treat obesity and related disorders.

Keywords

Sphingolipids · Adipose tissues · Lipogenesis · Lipolysis · Inflammation · Metabolic diseases

2.1 Introduction

Though long thought of as an inert energy storage depot, the past decades have revealed adipose tissue to be a highly dynamic organ functioning to regulate whole-body metabolic homeostasis. While a central function of adipose tissue is to store energy during periods of energy excess and to release energy in times of energy paucity, adipose tissue also has endocrine functions and releases numerous factors including hormones, bioactive lipids, and extracellular vesicles bearing diverse cargo to promote inter-organ crosstalk with skeletal muscle, liver, cardiac muscle, hypothalamus, and others. Therefore, adipose tissue serves as a central regulatory hub that senses metabolic status and then relays information to promote adaptive modifications in peripheral organs and tissues.

While obesity/excess adiposity is statistically linked to increased risk of many diseases, it is now becoming increasingly appreciated that functionality of adipose tissue, rather than simply its total amount, is critical for metabolic health. This idea has arisen from the observations that a sub-population of obese individuals is considered healthy with no metabolic dysfunction or

Y. Valentine
C. Kenneth and Dianne Wright Center for Clinical and Translational Research, Virginia Commonwealth University, Richmond, VA, USA

L. A. Cowart (✉)
Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA, USA

Hunter Holmes McGuire Veterans' Affairs Medical Center, Richmond, VA, USA
e-mail: Lauren.cowart@vcuhealth.org

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comorbidities associated with obesity. This phenomenon is referred to as metabolically healthy obesity (MHO). Metabolically healthy obese individuals have BMI over 30 kg/m^2 with no adipose dysfunction even though there is increase in adipose mass. These individuals present with adipocyte hyperplasia, normal adipogenesis, normal insulin sensitivity, normal adipose tissue structure, and adiposity directed toward the legs (and away from abdomen). These observations have provided an impetus for investigating functions of adipose tissue at a more granular level to increase understanding of "healthy" adipose tissue and its role in whole-body homeostasis.

Sphingolipids, including ceramides and S1P, have recently emerged as bioactive mediators of many adipose tissue functions. A plethora of evidence in the literature demonstrates that signaling sphingolipids are elevated in obese mice and human patients. Importantly, while circulating lipids correlate with metabolic pathology, evidence suggests that some of these lipids also serve as drivers for adipose dysregulation in an obesogenic state. Further, these sphingolipids also contribute to inflammation, insulin resistance, and glucose intolerance during adipose dysfunction. Conversely, emerging evidence suggests that in some cases, disruption of adipocyte sphingolipid metabolism in non-obese mice generates a pathological phenotype. These disparate findings suggest that precise regulation of sphingolipid homeostasis is key to adipose function. Therefore, here we will summarize current knowledge regarding specific sphingolipids and their roles in adipose homeostasis and dysfunction.

2.2 Sphingolipid Biosynthesis

De novo sphingolipid biosynthesis starts in the ER with the condensation of palmitoyl CoA with serine to produce 3-ketosphinganine, a reaction which is mediated by the rate limiting enzyme serine palmitoyltransferase (SPT). This reaction is catalyzed by the serine palmitoyltransferase holoenzyme, a heteromeric enzyme with partially distinct subunit composition (for example, in different tissues or disease states) to vary the chain length of the initial product, 3-ketosphinganine. 3-ketosphinganine is reduced to sphinganine (dihydrosphingosine), which is then N-acylated by ceramide synthases (CerS) to form dihydroceramide. Dihydroceramide desaturase (DES1) mediates the removal of the double bond from dihydroceramide to produce ceramide. Ceramide, a bioactive mediator itself, can then undergo conversion into other lipids including sphingomyelin and glucosy/galactosylceramide, precursors for glycolipids. Conversely, catabolism of ceramide by ceramidase enzymes yields sphingosine, which can be phosphorylated by sphingosine kinase 1 or 2 (SphK1/2) to produce the key signaling sphingolipid sphingosine-1-phosphate. SphK1 is generally located in the plasma membrane, while SphK2 is located in the nucleus [1–3]. S1P can act intracellularly or can be exported out of cells by SPNS or ABC transporters. Once outside of the cell S1P can signal by binding any of its five cell-surface G-protein coupled receptors (S1PR1-5) [2]. The major pathways of sphingolipid biosynthesis are summarized in Fig. 2.1.

S1P can be dephosphorylated into sphingosine by S1P phosphatase. This sphingosine can be recycled back to ceramide. Alternatively, S1P can also be degraded by the S1P lyase, which converts S1P to hexadecenal and ethanolamine phosphate. Because sphingosine yielded from the S1P phosphatase or from ceramidase activity can undergo re-acylation to ceramide, which can be readily interconverted between ceramides, glucosylceramides/glycolipids, and/or sphingomyelin, the S1P lyase serves as the only catabolic "exit" from the pathway, and in some cases may be a key regulatory step for total intracellular sphingolipid levels [4].

2.3 Sphingolipids in Adipocyte Differentiation

As noted above, adipose tissue dysfunction may be a key contributor to metabolic disease. For example, experimental evidence suggests that

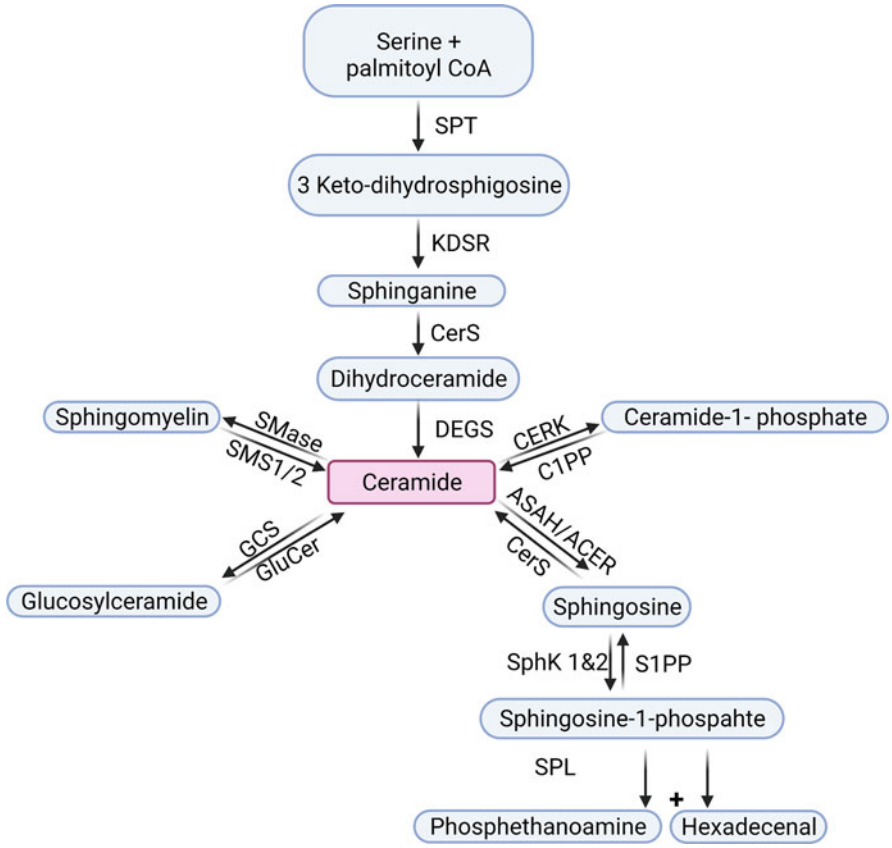

Fig. 2.1 Sphingolipid biosynthesis. *SPT* serine palmitoyltransferase, *KDSR* 3 Ketosphinganine reductase, *CerS* ceramide synthase, *DESG* dihydroceramide desaturase 1, *SM* sphingomyelinase, *SMS* sphingomyelin synthase, *CERK* ceramide kinase, *C1PP* ceramide-1-phosphate phosphatase, *ASAH/ACER* acid ceramidase/alkaline ceramidase, *SphK* sphingosine kinase 1 and 2, *S1PP* spingosine-1-phosphate phosphatase, *SPL* S1P lyase, *GluCer* glucosylceramide, *GCS* glucosylceramide synthase

impaired adipogenesis contributes to adipose metabolic disorders. Adipogenesis is a process that produces mature adipocytes from stem cells [5, 6]. The first phase is the determination phase, wherein mesenchymal stem cells become pre-adipocytes committed to adipocyte lineage and marked by upregulation of pre-adipocyte factor 1 (Pref-1) and C/EBPs. Maturation then completes the adipogenic process. In this step, pre-adipocytes develop a unilocular lipid droplet, along with upregulation of PPARγ and FABP4. These mature adipocytes also start to produce adipokines including adiponectin, a key mediator of metabolic homeostasis via crosstalk with other tissues. When adipogenesis is impaired, existing adipocytes undergo hypertrophy, leading to deleterious outcomes including hypoxia. Because adipose hypertrophy and adipose tissue hypoxia are associated with metabolic disease, adipogenesis via recruitment of stem cells and hyperplasia of adipocytes is key for maintenance of metabolic health.

Sphingolipids play several roles in adipogenesis. For example, ceramide, a sphingolipid associated with differentiation and cell senescence in many contexts, was necessary for pre-adipocyte commitment to terminal differentiation [7]. DEGS1 constitutes the most proximal step in ceramide biosynthesis, desaturating the ceramide precursor dihydroceramide to the

more active lipid ceramide. When DEGS1 was knocked down in the 3T3-L1 pre-adipocyte cell line, cells were arrested at the pre-adipocyte stage and did not differentiate into bonafide adipocytes capable of triglyceride storage [7]. Further, even after "forced" differentiation in vitro, DESG1 depleted 3T3L-1 adipocytes still had elevated levels of PREF1, a pre-adipocyte marker, and lower expression of adipocyte markers of differentiation, including PPARγ and CEBPβ, suggesting impairment of the overall adipogenic program. Functional knockdown of DEGS1 induced cell cycle arrest and subsequently decreased cell proliferation, decreased oxygen consumption, and increased apoptosis. These findings were corroborated by pharmacological approaches demonstrating that treating cells with the DEGS1 inhibitor C8-CCP8 also elevated pre-adipocyte marker PREF1. Additionally, GATA, a critical marker of transition from pre-adipocytes to terminally differentiated adipocytes, was also elevated in vivo in adipose tissue [7]. Together these data show that the conversion from dihydroceramide to ceramide is required for adipogenesis, as inhibiting this step either genetically or pharmacologically attenuated adipogenesis by dampening pre-adipocyte proliferation, and, more importantly, arresting cells at the pre-adipocyte stage.

Another bioactive sphingolipid, sphingosine-1-phospahte (S1P), is also implicated in adipocyte differentiation. S1P's effect on adipocyte processes appears to be quite complex and seems to require a balance in its concentration, rather than S1P being completely present or absent. For example, lower concentrations and short exposure of S1P promoted cell viability in 3T3-L1 pre-adipocytes [8]. However, in the human adipocyte cell line SGBS, concentrations of S1P from 1 mM to 5 mM reduced adipogenesis as indicated by reduced lipid accumulation during the differentiation process. Similarly, S1P upregulated cell cycle/mitotic gene expression in undifferentiated 3T3-L1 pre-adipocytes but had the opposite effect in differentiated 3T3-L1 adipocytes. Interestingly, low concentrations of S1P (100 nM) upregulated genes in the sphingolipid synthesis pathways such as SphK1 and SphK2. S1P receptors 1-3 genes were also upregulated in 3T3-L1 and SGBS differentiated adipocytes. Coordinate upregulation of SphK1/2 and S1P receptors suggests that during the differentiation process the SphK/S1P axis plays an important role, though more work will be required to determine these putative roles and their potential molecular mechanisms.

2.4 Sphingolipid Roles in Obesity

In times of excess influx of energy, adipose tissue can store energy by expanding the size of the adipocyte (hypertrophy) or by differentiating new adipocytes from pre-adipocytes derived from the vascular stroma (hyperplasia). Both hypertrophy and hyperplasia contribute to obesity, though, as mentioned above, hyperplastic adipogenesis is emerging as less deleterious than hypertrophic adipogenesis. Each of these processes alters adipose tissue sphingolipid profiles through several routes (see Fig. 2.2).

Sphingosine kinase 1 and 2 are critical enzymes in sphingolipid biosynthesis and several studies have examined the function on SphK1 and SphK2 on adipose tissue and obesity. In diet induced obese mice, SphK1 and its product S1P were increased in gonadal adipose tissue [9]. Global depletion of SphK1 in mice significantly increased adipose mass, which may have been through hyperplasia, as mice demonstrated reduced adipocyte size but increased adipocyte number. The increase in adipocyte numbers was supported by augmented expression of adipogenesis marker genes PPARγ, FABP4 (AP2), and Pref1 in cultured adipocytes from the gonadal depot [9]. SphK1 deletion also decreased the phosphorylation of PPARγ on Ser 111, which is known to be an inactive form and prevents PPARγ from initiating adipogenesis. Overall, global deletion of SphK1 protected mice from obesity-related aspects metabolic syndrome. These global SphK1 deleted mice were also protected from inflammation with significant reduction with IL6, and TNFα, which is further discussed below.

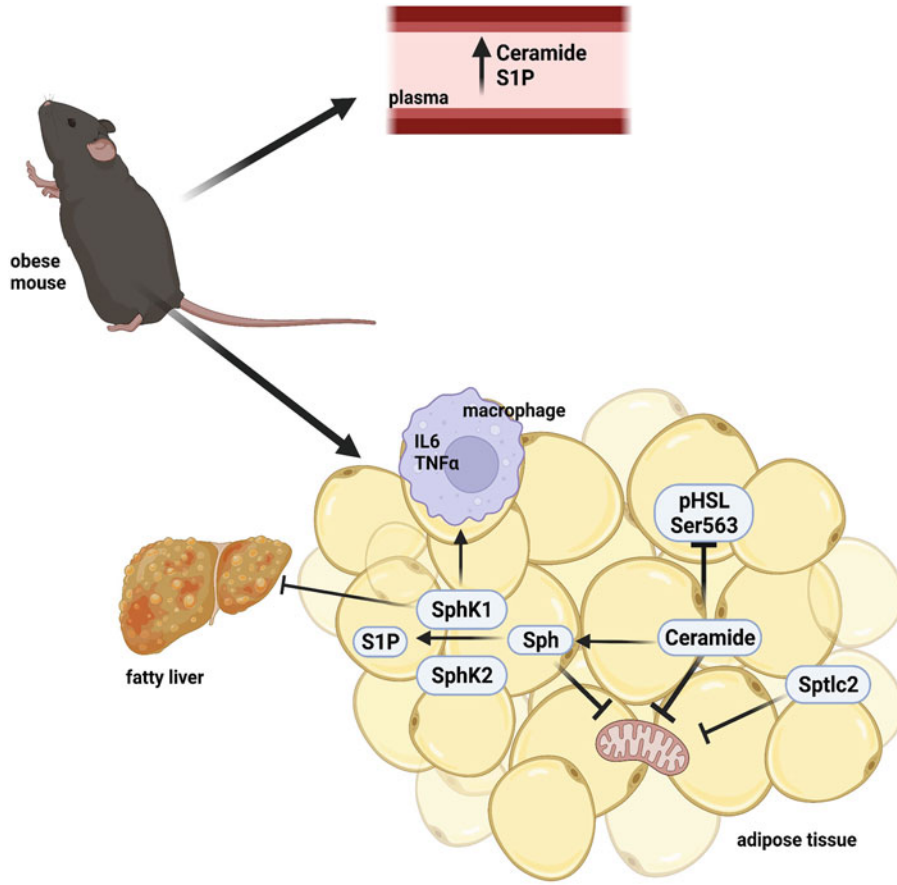

Fig. 2.2 Sphingolipids in obesity

In contrast to the global SphK1 knockout, specific depletion of SphK1 in adipocytes revealed distinct adipocyte-specific functions for SphK1. This selective deletion increased adiposity in mutant mice similar to the global SphK1 deletion, but did not protect from obesity-related metabolic syndrome (observed in SphK1 global deficient mice) [10]. This tissue-specific deletion of SphK1 increased body mass on the control low glycemic isocaloric diet CD, and it also increased the size of adipocytes in the gonadal depot. Mutant female mice had significantly reduced basal levels of ATGL, AbhD5, and FABP4 in the gonadal depots. Similar findings were observed in male mice at basal levels, but this reduction was not significant in male mice. Both male and female mutant mice show reduced AbhD5 and FABP4 expression on a high-fat diet. These results showed that SphK1 in adipose tissue is required for lipolysis basally and in the obese state. As expected, these mutant mice developed glucose intolerance on high-fat diet HFD. Despite these deleterious effects, adipocyte-specific deletion of SphK1 in fact reduced inflammation markers including TNFα and IL6 in the gonadal depot, which is further discussed below.

The deletion of SphK1 in adipose tissue surprisingly led to hepatic steatosis in mice on the HFD. Their liver TAG was also significantly higher than control mice on the obesogenic diet. Liver lipogenic genes FASN and DGAT2 were also elevated in these mutant mice. Proinflammatory markers in the liver, MCP1 and TNFα, were also elevated in obese mutant mice. Similarly, the liver fibrotic marker Col1a1

was significantly elevated in obese male and trending in female mice. This suggests that the dysfunctional adipose tissue led to a NALFD-like phenotype, similar to lipodystrophies, which, due to lack of functional adipose tissue, lead to accumulation of lipids in tissues where lipid storage instigates pathological processes. Together these data show that the crosstalk between adipose and liver is important for metabolic homeostasis, and more importantly that SphK1/S1P axis is vital to optimal adipose-liver crosstalk and maintenance of metabolic health.

The second isoform of SphK, SphK2, has also been studied in relation to obesity and showed similar results to the SphK1. Global deletion of SpK2 provided protection from obesity [11]. SphK2 mutant mice were protected from the normal increase in adipose mass with aging, specifically, 10–20-week-old male mice did not increase their adipose mass. More surprisingly, male SphK2 mutant mice showed a 36% reduction in body fat at 20 weeks and 85% at 52 weeks compared to their WT counterparts. Female SphK2 null mice showed similar outcomes to male mice, where mice showed decreased weight at 22, 50, and 77 weeks old. Together the male and female data confirm that deletion of SphK2 protects mice from age related weight gain. Additionally, these aged mice were also protected from glucose intolerance that is normally associated with weight gain. These mutant SphK2 mice also had higher energy expenditure, but low activity in comparison to WT mice, suggesting that SphK2 inhibits energy expenditure.

Ceramide, a central hub of sphingolipid biosynthesis, is a major contributor to obesity-related metabolic disease [7, 12–14]. Preventing total ceramide synthesis by ablation of DESG1 as previously described in vitro [7] yielded similar results in vivo. Ablation of DEGS1 globally as well as tissue-specific deletion in the liver, adipose tissue, and a double deletion in the liver and adipose, all showed that ceramide is a driver for adiposity. Specifically, these mutant mice showed decreased size of adipose depots and adipocyte size, decreased ceramide species in all adipose depots, liver and muscle, improved glucose tolerance, improved insulin sensitivity, and inhibited HSL activity in adipocytes [13].

Inhibition of de novo sphingolipid synthesis with myriocin, an inhibitor of the serine palmitoyltransferase (SPT), has been shown to prevent obesity across several studies [12, 15, 16]. A thorough obesity study by the Samad group used three obesity models: (1) a genetically obese model with myriocin treatment, (2) WT mice on 60% HFD diet with myriocin treatment at the start of the diet, and (3) WT mice on 60% HFD diet for 8 weeks then myriocin treatment was added to the model and continued for an additional 8 weeks. All three of these models were on 0.3 mg/kg myriocin treatment for 8 weeks and injected every 2 days. Ussher's model used low-fat diet LFD or HFD for 12 weeks then 0.5 mg/kg myriocin treatment for 2 weeks, while Chaurasia's used HFD for 20 weeks and 0.3 mg/kg myriocin (given every 2 days) was added at week 12. Together these three groups showed inhibition of de novo sphingolipid significantly reduced ceramide in the plasma and adipose tissue. Chaurasia showed that myriocin blockade of ceramide synthesis reduced dihydroceramide levels in thermogenic adipose tissue (brown adipose tissue, BAT) and subcutaneous white adipose tissue (sWAT) in mice on HDF, while the Yang group showed that sphingosine and S1P were reduced with ceramide synthesis inhibition. The Chaurasia and Yang groups both found that ceramide reduction decreased adipose weight and body weight in obese mice. Similarly, both Chaurasia and Yang showed that adipocyte size is also reduced with myriocin treatment on HFD. Interestingly, Yang was the only one to show that lean mice treated with myriocin increased sphingosine in their plasma along with elevated level of selected ceramide species (C14, C16, C18, C18:1). These findings coupled with the fact that these lean mice developed slight glucose intolerance show that de novo ceramide synthesis is required in a healthy state to maintain glucose homeostasis. Conversely, data suggest that excessive de novo ceramide synthesis in obesity drives obesity and glucose intolerance. While these studies show exceptional results in mice, they may not be

transferrable to humans because high doses of myriocin elicit adverse reactions in humans including lymphocytopenia, reduced heart rate and headaches [17]. More research is necessary to identify more specific targets (e.g., specific ceramide synthase isoforms).

In agreement with myriocin studies, adipose-specific depletion of Sptlc2, a subunit of SPT, reduced several species of ceramide in gonadal, inguinal, and BAT depots and primary adipocytes isolated from those depots [12]. Dihydroceramides were also significantly attenuated in sWAT and BAT. This trend followed in the epididymal (gonadal) WAT but was not significant. Other species of sphingolipids including monohexosylceramides (MHC), dihexosylceramides (DHC), GM3 gangliosides (GM3), or sphingomyelins (SM) were not affected by Sptlc2 deletion in adipose tissue [12]. The abdominal adipose depot was also reduced in Sptlc2 adipose deficient mice. The absence of Sptlc2 in adipose tissue changed the morphology of adipocytes in inguinal and gonadal depots, reducing adipocyte size, and therefore, these depots were significantly smaller than their controls on NCD and HFD [12], suggesting there was no compensatory hyperplasia.

In addition to alleviating obesity, inhibiting de novo sphingolipid synthesis with myriocin [12, 15, 16] or by Stplc2 deletion [14] increased energy expenditure and oxygen consumption in obese mice. These groups also showed that impediment of de novo ceramide synthesis reduced the respiratory exchange ratio (RER) which is a ratio between CO2 produced and O2 used during respiration [18]. An RER close to 0.7 is indicative of fats being used as a source of energy, while an RER close to 1 indicates that carbohydrates serve as the main energy source. Blocking ceramide synthesis increased metabolic activity by shifting to fats as the main fuel source. Additionally, the obstruction of ceramide synthesis increased metabolic activity by increasing mitochondria uncoupling proteins UCP1 [12, 14] and UCP3 [16]. Even further, deleting Sptlc2 [14] and DEGS [7] in adipocytes showed that obstructing ceramide synthesis elevated the oxygen consumption capacity of mitochondria complex I, II, and IV. This deletion also decreased mitochondria biogenesis and changed the morphology of mitochondria making them larger. Altogether these data suggest that inhibiting de novo ceramide synthesis alleviates obesity by increasing metabolic utilization of fats as the main energy source instead of carbohydrate.

Inhibiting de novo sphingolipid synthesis also affects glucose handling. For example, Sptlc2 mutant mice had lower fed and fasting glucose with slightly improved glucose tolerance [12]. This mutant lowered sphingolipids in adipose tissue, improved insulin signaling, improved adipose glucose homeostasis, and resolved hepatic steatosis [12]. Similarly, impeding de novo ceramide synthesis with myriocin improved insulin sensitivity and glucose tolerance [15, 16, 19]. As discussed above Yang used three obesity models, in all these models myriocin significantly improved glucose tolerance. However, blocking ceramide synthesis with myriocin surprisingly induced slight glucose intolerance in lean mice. This shows that ceramide is needed for normal insulin signaling and glucose metabolism, while excessive ceramide perturbs metabolic homoestasis. Therefore, a moderate amount and/or specific chain-length profiles of ceramide (yet to be determined) are needed for optimal glucose metabolism.

2.5 Sphingolipids and Lipolysis

While lipid storage is the most appreciated function of adipocytes, it is helpful only in as much as stored lipids can be released. This occurs via lipolysis, which is activated to release free fatty acids and glycerol in times of energy demand. Furthermore, the balance between lipogenesis and lipolysis is critical in maintenance of normal adipocyte size. Normal lipolysis can either be enhanced or disrupted by sphingolipids, depending on context and specific lipids involved.

Treatment of adipocytes with dihydroceramide or ceramide (50 μM of C_6 dihydroceramide or C_2

ceramide for 4 h) showed that ceramide and not dihydroceramide reduced isoproterenol-stimulated HSL phosphorylation at Ser 563. This suggests that ceramide reduced activation of PKA, which phosphorylates HSL at Ser563 [20, 21]. This study was done in white adipocytes, and similar results were obtained in brown adipocytes showing that ceramide blocked HSL activation at Ser563 [14]. In contrast, Barbarroja showed that blocking DEGS1 (with C8-CPPC), the enzyme that converts dihydroceramide into ceramide, decreased glycerol release and reduced HSL phosphorylation at Ser600, which is known to induce HSL activity. This study also showed that impeding DEGS1 with C8-CPPC increased phosphorylation of HSL at Ser565 which inactivates HSL. This suggests that DEGS1 and accumulation of dihydroceramide decrease lipolysis mediated by p44/p42MAPKS, which phosphorylates HSL at Ser600, simultaneously facilitating the deactivation of HSL by AMPK that phosphorylates HSL at Ser565 [21]. Together these studies highlight that ceramide's role in lipolysis is complex and greatly understudied. More work is needed to further clarify which HSL phosphorylation sites are regulated by ceramide and the modes of regulation. Additionally, very little emphasis is placed on dihydroceramide which is shown here to reduce lipolysis and prevent deleterious outcomes of obesity in mice [13].

In addition to ceramide, SphK2 also affects lipolysis. Whole-body deletion of SphK2 elevated the expression of adipocyte triglyceride lipase (ATGL), a vital enzyme in the lipolysis, in gonadal WAT under fed and 16 h fasting conditions [11]. ATGL mRNA was also elevated in WAT of 52-week-old mice, suggesting that increased lipolysis may be responsible for the lack of adipose weight increase in these mice. This SphK2 deletion also increased adiponectin and CD36 in older mice. CD36 plays a role in adipocyte recruitment and mediates fatty acid uptake [22]. CD36 deficiency reduced adipocyte recruitment in gonadal depot, and fewer cells from the stromal vascular fraction were committed to the pre-adipocyte stage.

In contrast to the findings of a potential role in suppressing homeostatic, i.e. non-stimulated), lipolysis for SphK2, studies in SphK1 in adipocytes suggested it is required for homeostatic lipolysis. Specifically, depletion of SphK1 in adipose tissue reduced basal lipolysis in the gonadal depot but had no effect on isoproterenol-stimulated lipolysis [10]. This tissue-specific deletion of SphK1 also reduced non-esterified fatty acid (NEFA) in serum and reduced basal glycerol release from tissue explants, consistent with a reduction in basal lipolysis. Additionally, ATGL, FABP4, and ABHG5 (CGI-58) key mediators in the lipolysis pathway were reduced in the mutant mice. Together, this shows that SphK1 is needed for optimal lipolysis in adipocytes.

2.6 Sphingolipid Roles in Adipose Tissue Inflammation

Adipocytes are involved in several other functions that contribute to metabolic disease. Additionally, because adipose tissue is heterogenous and comprised of numerous cell types in addition to adipocytes, including pre-adipocytes, stem cells, macrophages, and endothelial cells, disruption in these adipose-resident cells can contribute to adipose dysfunctions. Further, adipose tissue is involved in crosstalk with other tissues including pancreatic β-cells with insulin signaling and liver with glucose signaling. Adipose crosstalk with β-cells and liver is facilitated by adiponectin [23]. Adiponectin promotes healthy pancreatic β-cell function and insulin sensitivity in the muscle, and both of these organs then produce their respective cytokines to promote normal adipose metabolism [24]. In contrast, leptin, which is elevated in obesity, has negative effects on both muscle and β-cells, impairing insulin signaling and further exacerbating adipose dysfunction. Impairment in insulin and glucose signaling can significantly affect adipocyte functions including lipolysis and lipogenesis. Adiposity also promotes macrophage infiltration which drives inflammation by increasing IL6,

TNFα, and MCP1, all of which further foster adipose dysfunction [24].

Adipose tissue inflammation is a significant component of obesity and contributes to adipocyte dysfunction. Sphingosine kinase 1 is active in IL-6 stimulated adipocyte inflammation. In fact, inflammation induced through lipolysis/β_3 adrenergic pathways requires SphK1 to stimulate IL-6 secretion [25], though the mechanism by which this occurs remains unknown. In contrast, global deletion of SphK1 showed less infiltration of macrophages in the gonadal adipose tissue. S1P is a chemoattractant and recruits macrophages, which may underlie reduced macrophage infiltration in adipose tissue in the SK1 global deletion. Consistent with this, there were significantly less M1 macrophages in obese SphK1 mice compared to WT [9]. A closer analysis of CD11b+/CD11c+ M1 macrophages in adipose tissue showed reduced expression of proinflammatory markers TNFα and IL-6, but increased expression of anti-inflammatory IL-10 cytokine [9]. These SK1 mutant mice also had decreased expression of Suppressor of Cytokine Signaling 3 (SOCS3) which is a contributor to insulin resistance caused by inflammation in adipocytes [9].

Consistent with the SphK1 studies [9, 10, 25], altering sphingolipid synthesis by blocking SPT function with myriocin or deleting Sptlc2 in adipose reduced inflammatory cytokines (IL6, IL4) in adipose tissue and serum [12]. The SphK1 studies discussed above showed that SphK1/S1P axis drives IL6 and TNFα inflammation, which was further supported by work showing that Sphingosine (50 nM, 100 nM) and S1P (50 nM, 100 nM), treatment in adipocytes (cells treated for 3 h) significantly increased IL6 and TNFα [26]. Chaurasia's myriocin and Sptlc2 studies showed elevated sphingosine levels in both studies (S1P levels were not reported), indicating that there may be more sphingolipid recycling or biosynthesis from other adipose-resident cells. However, Sptlc2 deficient mice did not show increased IL6 and TNFα in the sWAT depot (IL1β and IL6 were reduced) but did show IL6 and TNFα augmentation in BAT with elevated sphingosine levels. Instead, sWAT showed increased M2 macrophage anti-inflammatory cytokines such as IL-10 and IL-4. Therefore, more work is needed to determine how Sptlc2 deletion in adipose tissue affects adipose inflammation.

2.7 Brown Adipose Tissue Function

Brown adipose tissue (BAT) is a major thermogenic center in the mouse. BAT is marked by its unique expression of UCP1 and content of brown adipocytes which have high levels of mitochondria and multi-locular lipid droplets. BAT has functions in addition to thermogenesis, and therefore, much emphasis has been made to comprehend how BAT functions, beyond merely thermogenesis. It is established that BAT function decreases with age in mice, and there is some evidence that aging-induced changes in sphingolipids may correlate with loss of BAT function. For example, sphingomyelin and several ceramide species are increased in aging mice [27], along with critical lipolytic proteins [27]. B3 adrenergic receptor (B3AR)-stimulated glycerol production, a measure of lipolysis, was also reduced with aging in BAT. Processes, such as PPAR signaling, protein processing in the ER, and fatty acid degradation and metabolism are impaired in aging mice. Since many of these processes have been linked to sphingolipid signaling, it is likely that modulation of sphingolipid metabolism in aging could maintain BAT function.

In fact, several types of sphingolipids have been shown to affect protein expression in the thermogenic pathway in brown adipocytes (see Fig. 2.3). For example, treating cells with exogenous C16 ceramide attenuated brown adipocyte differentiation by reducing key BAT markers including UCP1, PGC1α, Cidea, Dio2, and PPARγ. Mitochondrial biogenesis genes, TFAM and Cox7, were also reduced with C16 treatment during brown adipocyte differentiation. Consistent with these findings, inhibiting ceramide synthesis with fumonisin B1 (FB1) increased the expression of brown adipogenic markers including UCP1, PGC1, CIDEA, and Dio2 [27]. Analysis of mitochondrial function showed that C16

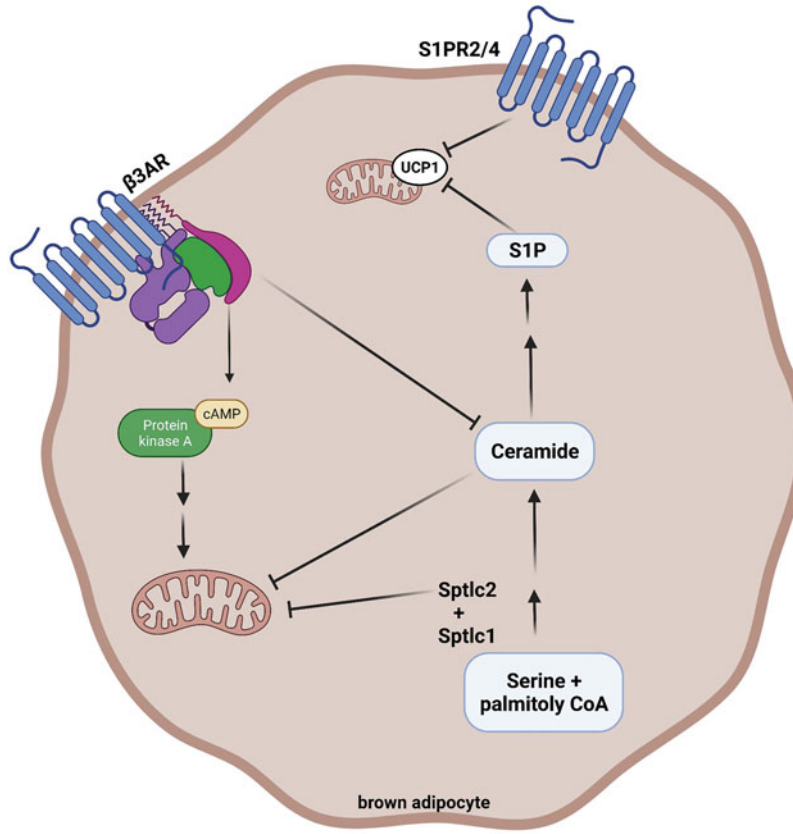

Fig. 2.3 Sphingolipids in brown adipose tissue

ceramide also reduced oxygen consumption, both basally and with UCP1-mediated uncoupling in brown adipocytes. Combined, these data show that ceramides negatively impact expression of brown adipocyte markers and mitochondrial function in aged mice. This concept is further supported by studies indicating that browning stimuli, e.g., B3AR stimulation, negatively regulate C16:0 and Sptlc2 expression in brown adipocytes [14]. Stimulation of B3AR by both cold exposure and the B3AR agonist CL316,243 reduced total ceramide and dihydroceramide in all adipose depots [12]. Sphinganine was significantly reduced in sWAT with CL and trended toward reduction with cold exposure in eWAT and sWAT. This suggested that de novo sphingolipid biosynthesis is reduced with thermogenic activation. SM levels were also reduced with CL 316 243 B3AR stimulation [12], indicating both anabolic and catabolic pathways of ceramide synthesis are decreased by thermogenic stimuli. A functional role for this, however, is supported by the observation that inhibiting sphingolipid biosynthesis augmented the expression of critical brown adipose proteins, including UCP1, PGC1b, PRDM16 [12]. Transcription factors involved in brown adipogenesis including CD137, Tbx1, and TMEM26 were also increased with myriocin [12]. FGF-21 is a key adipokine that enhances PGC1α protein function in adaptive thermogenesis and upregulates UCP1 expression [28]. Ablation of Spltc2 in adipose tissue increased fibroblast growth factor 21 (FGF21) [12]. This suggests that Sptlc2 (and perhaps addition of exogenous ceramides in the above-mentioned studies) dampens thermogenesis through FGF-21 regulation. Altogether, these studies suggest that sphingolipids are downregulated during browning, downregulate brown adipose markers and metabolic function, and thus that their downregulation can promote browning.

It is important to note that at 23 °C, the temperature of most animal facility rooms, mice experience thermal stress [29, 30] and therefore their subcutaneous white depot becomes heterogenous with UCP1 negative cells (white adipocytes) and UCP1 positive cells (beige adipocytes). Depletion of Sptlc2 in UCP1 positive cells resulted in reduced ceramide levels in subcutaneous WAT (results from animals at 23 °C), BAT, and plasma [14]. Thus, reduction of ceramide levels in the subcutaneous white depot is a result of the beige adipocyte population. This depletion of Sptlc2 in UCP1 positive adipocytes also diminished the size of the BAT depot in mutant mice compared to control littermates. Additionally, these mice were partially protected from obesity on an obesogenic diet, displayed less fat mass (reduced BAT and sWAT), and reduced liver weight with protection from steatosis on HFD [14]. Further, these mutant mice with Sptlc2 ablation in UCP1positive adipocytes displayed increased energy expenditure on HFD, increased oxygen consumption, elevated BAT temperature, increased mitochondrial number and size [14]. These results showed that ceramide may impede multiple processes in brown adipose, including mitochondrial morphology and function, thermogenesis, fatty acid uptake, insulin signaling, and lipolysis [12, 14]. The protections gained by deleting Spltc2 in UCP1+ were lost in thermoneutral conditions that turn off BAT activity [14], indicating Sptlc2 and ceramide interference of BAT activity requires B3AR stimulation.

Acid ceramidase (ASAH1) hydrolyzes ceramide into sphingosine and is therefore critical for maintaining the levels of ceramide, sphingosine, and S1P in cells. Deleting acid ceramidase ASAH1 in UCP1 positive cells produced mice with inability to degrade ceramide, resulting in accumulation of ceramide and sphingomyelin in BAT [14]. Surprisingly, sphingosine level was the same in the brown adipose depot between the control and mutant mice. In contrast, sphingosine levels in the subcutaneous and gonadal depots were trending downwards but this was not significant in the mutant mice. Overall, the deficiency of ASAH1 in UCP1 expressing adipocytes showed a phenotype opposite to deletion of Sptlc2 in UCP1 positive adipocytes [14]. This accumulation of ceramide because of ASHA1 deletion in UCP1 positive adipocytes increased the weight of adipose, increased adipocyte size (sWAT and gonadal) basally and on an HFD, decreased energy expenditure, decreased oxygen consumption, and impaired glucose metabolism. In contrast to elevated body temperature with the deletion of Sptlc2 in UCP1 positive adipocytes, deletion of ASAH1 in adipocytes expressing UCP1 significantly reduced the BAT surface temperature of these mice. This was further confirmed with decreased UCP1 expression and reduced mRNA expression of thermogenic markers (CIDEA, PGC1α/β) in the mutant mice. Even further, there was significant reduction in the number of mitochondria present in the brown adipose. These mitochondria also had impaired morphology (increased mitochondria size), consistent with reduced expression in mitochondria transcription factor (Tfam) and mitochondria fission factor (Mff), which is required for mitochondrial fission after biogenesis. Additionally, mitochondrial respiration was also impaired with the deletion of ASAH1 in UCP1 expressing adipocytes by reducing the function of mitochondria complex I and II. This differs from the finding in Sptlc2 deletion in UCP1 positive cells, which showed increased mitochondrial respiration in complexes I, II, and VI, along with increased mitochondrial biogenesis and mitochondria number. Together, in vivo data from the deletion of Sptlc2 or ASAH1 in adipocytes expressing UCP1 support that ceramide may impede UCP1 expression and function and reduce mitochondrial biogenesis, morphology, and function.

Another sphingolipid that affects the brown adipocytes is S1P. S1P presence during adipogenesis reduced both UCP1 and PGC1α expression. S1P signaled through receptors 2 and 4 to reduce UCP1 expression in brown adipocytes [27]. Inhibition of S1PR2 increased glycerol release from brown adipocytes, indicating that S1PR2 also reduced lipolysis in brown adipocytes. Additional studies showed that S1P signals through S1P receptor 1 to reduce

BAT tissue weight and UCP1 expression. Circulating S1P also impeded BAT function [31]. S1P is carried in the blood by apolipoprotein M (ApoM) on high-density lipoprotein (HDL) [32]. Deletion of ApoM decreased gonadal depot weight, increased BAT, and decreased the lipid droplet size in BAT [31], and therefore the S1P content of SpoM may mediate these observations.

2.8 Sphingolipids in Humans

Studies discussed above have largely focused on findings in mouse models. However, these findings do not always translate directly to humans. Nevertheless, studies in humans are largely in agreement with these mouse studies. For example, obese type 2 diabetes mellitus (T2DM) patients demonstrated increased SM and ceramides in subcutaneous WAT [12]. Interestingly, C16 ceramide was significantly elevated in these patients [12], which was also the same species of ceramide that is increased in aging mice and is associated with decreased BAT activity in mice [27]. Selected species of ceramides, namely C14, C16, C24, C24:1, are increased in the white adipose plasma of both obese males and females [33]. Intriguingly, C18 ceramide was increased in the plasma obese males and females; however, in white adipose C16 was significantly reduced in obese females but increased in obese males. This suggests sex-specific metabolism and potentially a function of C18 ceramide in obesity. Increase in total adipocyte ceramide in obese female individuals is associated with reduced adiponectin expression [33]. Similarly, adiponectin expression was reduced in obese mice compared to mice on a normal chow diet [12]. Consistent with these findings, treatment with myriocin, which reduced obesity in mice, restored adiponectin to almost normal levels in mice on an HFD. Elevated adipocyte ceramide in obese female participants also correlated to insulin resistance, but similar results were not observed in male participants. In agreement with mouse studies that inhibiting ceramide synthesis by deleting Sptlc2 [12] and S1P production by deleting SphK1 [10, 25] reduced IL6 driven inflammation, obese individuals with elevated ceramide, sphingosine, and S1P had augmented levels of IL6 in their plasma [33]. While S1P was only elevated in obese non-diabetic patients, sphingosine levels were similar in lean and obese patients [34].

Obese humans (both non-diabetic and diabetic participants) show elevated dihydroceramide levels including dhC16:0, and dhC18:0 compared to lean patients as previously reported in obese mice [12, 34]. Surprisingly, ceramide did not follow this trend but was instead significantly reduced in non-diabetic and diabetic obese patients; sphinganine followed the same trend [34]. A possible explanation for these findings in there was a higher turnover of ceramide in this group since they also showed significant elevation of acid ceramidase level and activity. Additionally, there were increased levels of the SPT subunits SPTLC1 and SPTLC2, elevated SPT activity, and increased acid sphingomyelinase activity in obese non-diabetic and diabetic patients. These results suggest that there may be more de novo sphingolipid synthesis; however, this was not reflected by the levels of ceramide in these patients. Intriguingly, the levels of dihydroceramide were significantly elevated in these individuals suggesting that in humans, obesity upregulates dihydroceramide instead of ceramide. Additional studies in obese humans are needed to confirm this hypothesis. Furthermore, this stands in conceptual contrast to studies in mice where depletion of DEGS1, which essentially "traps" dihydroceramide as such by preventing its desaturation to ceramide, prevented deleterious metabolic outcomes associated with obesity.

In agreement with elevated sphingolipid synthesis in obese patients, SPTLC1 and 2 message and protein were significantly increased in both diabetic and non-diabetic obese patients [34]. These findings also correlated with increased serine palmitoyl transferase activity in both groups of obese patients. Further, there was increase in sphingolipid synthesis through the salvage pathway compared to de novo synthesis pathway in obese patients [34], suggesting

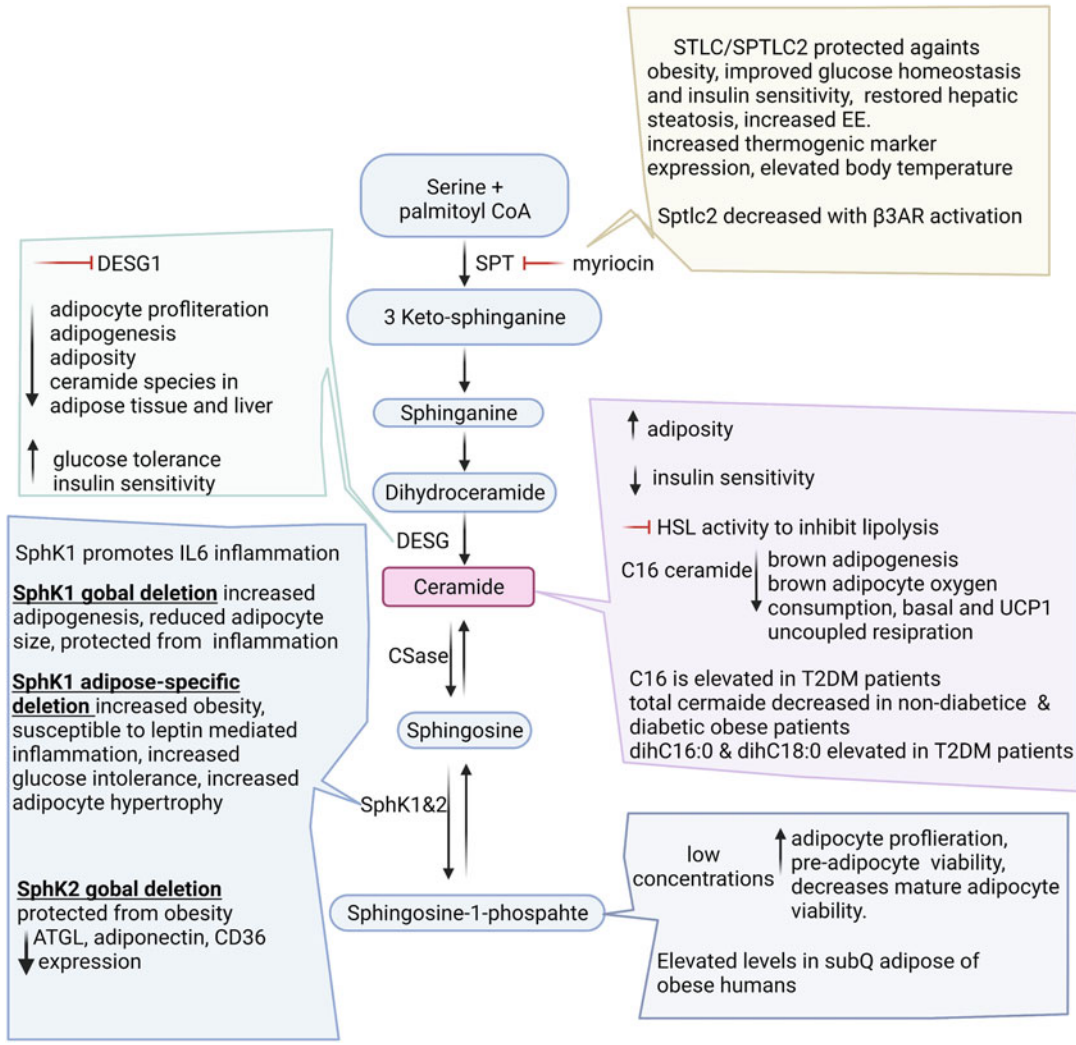

Fig. 2.4 Summary scheme

large-scale ceramide remodeling (e.g., changing chain lengths). These obese individuals had enlarged adipocytes, elevated sphinganine, sphingosine, and S1P in their subcutaneous adipose compared to lean individuals [33].

2.9 Conclusion

We summarized what we discussed in this chapter (see Fig. 2.4). Adipose tissue is a heterogenous and complex tissue and sphingolipid functions in adipose are just the same. Data discussed here supports that sphingolipids have both positive and negative effects on adipose during both obesogenic and healthy states. Sphingolipids, especially ceramides and S1P are elevated in obese mice and may serve as a driver for obesity-related metabolic phenotypes. Therefore, it seems that a balance in the concentration of sphingolipids is needed to prevent obesity-related metabolic phenotypes. Further studies are required to determine the specific amounts and profiles of ceramide that are optimum for adipocyte metabolic homeostasis. Similarly, additional studies are needed to determine S1P's role in

obesity, whether these roles occur via receptor mediated signaling, and, if so, and the specific receptors involved.

Many adipose sphingolipid studies are done in mouse models. While many of the mouse studies have produced promoting results, there has been a bottleneck with transitioning these studies to humans. In fact, there are very few human studies that look at sphingolipids in obese and healthy humans. Further, there have not yet been any major studies addressing sphingolipids in obesity using intervention methods in humans. It is urgent to begin meaningful obesity studies in humans to test whether it is worthwhile to develop sphingolipid-focused treatments to alleviate obesity, adipose dysfunction, and/or the deleterious metabolic outcomes of dysfunctional adipose tissue in obesity.

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De Novo Sphingolipid Biosynthesis in Atherosclerosis

3

Tae-Sik Park, Shivani Devi, Amitesh Sharma, Goon-Tae Kim, and Kyung-Hee Cho

Abstract

Atherosclerosis is the formation of fibrofatty lesions in the arterial wall, and this inflammatory state of the artery is the main cause of advanced pathological processes, including myocardial infarction and stroke. Dyslipidemic conditions with excess cholesterol accumulate within the arterial vessel wall and initiate atherogenic processes. Following vascular reaction and lipid accumulation, the vascular wall gradually thickens. Together with the occurrence of local inflammation, early atherosclerotic lesions lead to advanced pathophysiological events, plaque rupture, and thrombosis. Ceramide and sphingomyelin have emerged as major risk factors for atherosclerosis and coronary artery disease. Currently, the clinical association between de novo sphingolipid biosynthesis and coronary artery disease has been established. Furthermore, therapeutic strategies to modulate this pathway, especially those involving serine palmitoyltransferase and sphingomyelin synthase, against atherosclerosis, cancer, type 2 diabetes, and non-alcoholic fatty liver disease are actively under development. In this chapter, we focus on the relationship between de novo sphingolipid biosynthesis and coronary artery disease.

Keywords

Serine palmitoyltransferase · Sphingomyelin synthase · Ceramide · Sphingomyelin · Atherosclerosis

Abbreviations

CAD	Coronary artery disease
SPT	Serine palmitoyltransferase
SM	Sphingomyelin
SMS	Sphingomyelin synthase
MTP	Microsomal triglyceride transfer protein
SMase	Sphingomyelinase

3.1 Introduction

Coronary artery disease (CAD) is the most prevalent type of cardiovascular disease and accounts for millions of deaths worldwide. Stroke and myocardial infarction, which are responsible for a massive number of deaths, are caused by thromboembolism (blood clots clogged in the arteries) via rupture of atherosclerotic plaques in the carotid artery of various organs, including the

T.-S. Park (✉) · S. Devi · A. Sharma · G.-T. Kim · K.-H. Cho
Department of Life Science, Gachon University, Sungnam, Gyeonggido, South Korea
e-mail: tspark@gachon.ac.kr

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brain and heart [1–5]. Atherosclerosis, a condition of intimal thickening, is the major cause of vascular diseases and is believed to develop from two different pathological mechanisms. First, lipoproteins, especially low-density lipoprotein (LDL), accumulate in the arterial wall and are oxidized to more toxic lipids. Lipid accumulation in the intima and vascular response to lipids thicken the arterial wall. Second, a local inflammatory reaction (known as the primary cause of CAD) to vessel wall injury caused by infections, immune diseases, and diabetes develops. Atherosclerosis progresses with atherosclerotic plaque formation, which interferes with circulation, leading to the progression of coronary heart diseases, including myocardial infarction, heart failure, stable angina pectoris (chest discomfort), stroke, and claudication [6, 7]. Plaque formation, which is responsible for narrowing of the arterial lumen, is initiated by impaired lipid transport, causing endothelial dysfunction. Since the rupture of these plaques is the most common trigger of stroke and acute thrombosis, lipids are the key players in the pathogenesis of atherosclerosis [8, 9].

Impaired lipid metabolism is associated with various metabolic diseases, including type 2 diabetes, non-alcoholic fatty liver disease, insulin resistance, and cardiomyopathy [10]. Overnutrition and obesity elevate the plasma levels of fatty acids (FA), which are taken up by non-adipose tissues, and imported FA is oxidized as an energy fuel or stored as an energy reservoir. When the FA uptake exceeds the levels of oxidation, excess FA is utilized for the nonoxidative synthesis of lipid metabolites. These include triacylglycerol, diacylglycerol, and sphingolipid metabolites. Some of these lipids are inert, while others have anomalous effects on cellular function, called lipotoxicity. One possible pathway to consume the FA surplus is the sphingolipid biosynthetic pathway. Thus, ceramide levels are a biomarker for FA surplus, since FA is a substrate for ceramide biosynthesis [11].

Sphingolipids are a heterogeneous class of lipids, primarily known as the building blocks of the plasma membrane. In addition, they are widely described as bioactive signaling molecules, including ceramides, sphingosine 1-phosphate, sphingosines, sphingomyelin (SM), and other complex sphingolipids [12–14]. Sphingolipids, critically important for inter- and intracellular signaling, comprise a head group of a sphingoid backbone and an FA side chain [15]. When the FA uptake is excessive and lipid accumulation in tissue is followed, metabolic dysfunction occurs due to dysregulation of sphingolipid metabolism. This results in alteration of plasma sphingolipid levels and inappropriate cellular uptake, causing dysfunction. Clinically, the plasma levels of ceramide or SM are correlated with the progression of CAD and are suggested as independent risk factors for CAD [16, 17]. This review describes how sphingolipids and their role in major cardiovascular conditions have changed over the past few years. The findings thus far have consistently proven that sphingolipids are implicated in the progression of atherosclerosis and CAD. Their possible role as prominent biomarkers may lead to more therapeutic approaches as well as diagnostic tools in the future.

3.2 Sphingolipid Biosynthesis

In mammals, sphingolipids are synthesized de novo in the endoplasmic reticulum (ER) and then transported to the Golgi apparatus for further modification and synthesis of more complex sphingolipids [18, 19]. Alternately, they are catabolized from other sphingolipids via the salvage pathway. Sphingolipids consist of a long-chain base backbone as a common structural element that is generated in the rate-limiting step of the sphingolipid biosynthetic pathway. The biosynthesis of sphingolipids may be different among various species; however, the very first step of the pathway, catalyzed by serine palmitoyltransferase (SPT), is conserved across mammals, plants, bacteria, and fungi [20]. The sphingolipid biosynthetic pathway is necessary for the synthesis of ceramide, SM, and glycosphingolipids, which further act as substrates for the synthesis of complex sphingolipids.

The de novo biosynthetic pathway starts from the condensation reaction of an amino acid, serine, and a fatty acyl-CoA catalyzed by the enzyme complex SPT, followed by 3-ketosphinganine reductase, ceramide synthase, dihydroceramide desaturase, and SM synthase (SMS) (Fig. 3.1) [21]. In this review, we focus on the rate-limiting steps in de novo biosynthesis, SPT and SMS, and major effectors ceramide and SM, which affect vessel wall dysfunction.

3.3 Regulation of SPT

SPT is located in the ER membrane and a protein complex consisting of two major subunits, Sptlc1 and Sptlc2, which catalyze the condensation of L-serine and palmitoyl CoA [22]. Sptlc1 and Sptlc2 encode 53- and 63-kDa proteins, respectively, and have homologous sequences with 29% identity [23, 24]. A third major SPT subunit, Sptlc3, has 68% homology with Sptlc2 and 20% homology with Sptlc2. It has been suggested that Sptlc1 is a regulatory subunit, whereas Sptlc2 and Sptlc3 are catalytic subunits with a pyridoxal phosphate-binding domain [25]. The fact that homozygous silencing of Sptlc1 and Sptlc2 in mice is lethal indicates that Sptlc1 and Sptlc2 are essential genes for cell survival [26]. In a previous study, genetic deficiency of Sptlc3 in HepG2 cells resulted in a significant reduction in SPT enzyme activity [27]. The authors proposed that Sptlc3 is an isoform of Sptlc2 and binds to Sptlc1, independently regulating SPT activity to provide sphingolipids for cell requirements. Structural modeling studies using α-oxoamine synthase and bacterial SPT suggested that the active site of SPT is located at the interface of the heteromeric complex, and each subunit contributes to catalytic residues [28, 29]. However, the stoichiometry and topology remain controversial.

Initially, the stoichiometry of the SPT complex was suggested to be a heterodimer composed of Sptlc1 and Sptlc2 in a 1:1 ratio [30]. SPT has been proposed to be an octameric complex of four heterodimers resulting from the binding of Sptlc1 to either Sptlc2 or Sptlc3 [31]. Based on the finding that a third subunit (Tsc3) activates SPT activity markedly and is required for survival at high temperatures in yeast [32], the orthologous SPT small subunits a and b were found to be independent activators of mammalian SPT (ssSPTa and ssSPTb) [33]. However, the mechanism by which this is regulated transcriptionally or post-translationally is not yet known.

SPT enzyme activity is regulated by negative feedback inhibition. Orthologous to the yeast Orm genes, orosomucoid-like (ORMDL) proteins and neurite outgrowth inhibitor (NOGO-B) are found as regulatory transmembrane proteins in the ER. Mammals have ORMDL proteins (ORMDL 1, 2, and 3) that are encoded by separate genes and show different expression patterns in vivo [34, 35]. In this study, Breslow et al. demonstrated that Orm inhibits SPT activity when cellular sphingolipids are sufficient, and silencing of Orm significantly elevates the activity of SPT by sixfold. It differs from the yeast Orm proteins because it does not contain any phosphorylation sites [36]. When cells are treated with myriocin, an SPT inhibitor, to inhibit de novo sphingolipid synthesis, Orm proteins are phosphorylated at multiple sites, and their inhibitory effects on SPT are relieved [37]. Orm deficiency or phosphomimicking mutations drastically enhance SPT activity [34]. Several studies have found that yeast protein kinase 1 phosphorylates Orm proteins when SPT is inhibited by myriocin or when cells have heat stress [38, 39]. The finding that nitrogen starvation in yeast induces phosphorylation of Orm proteins via different mechanisms with no change in SPT activity suggests that Orm has distinct functions in addition to the regulation of de novo sphingolipid biosynthesis [40]. While the mechanism of Orm responsible for SPT activity and de novo sphingolipid synthesis is being elucidated, ORMDL protein-mediated regulation of mammalian SPT has not yet been elucidated. Since ORMDL proteins have no phosphorylation sites, mammalian SPT is regulated differently by these proteins [35]. The mechanism of SPT regulation by ORMDL proteins requires further study.

Additionally, NOGO-B has been identified as a negative SPT regulator in mammals. It is a

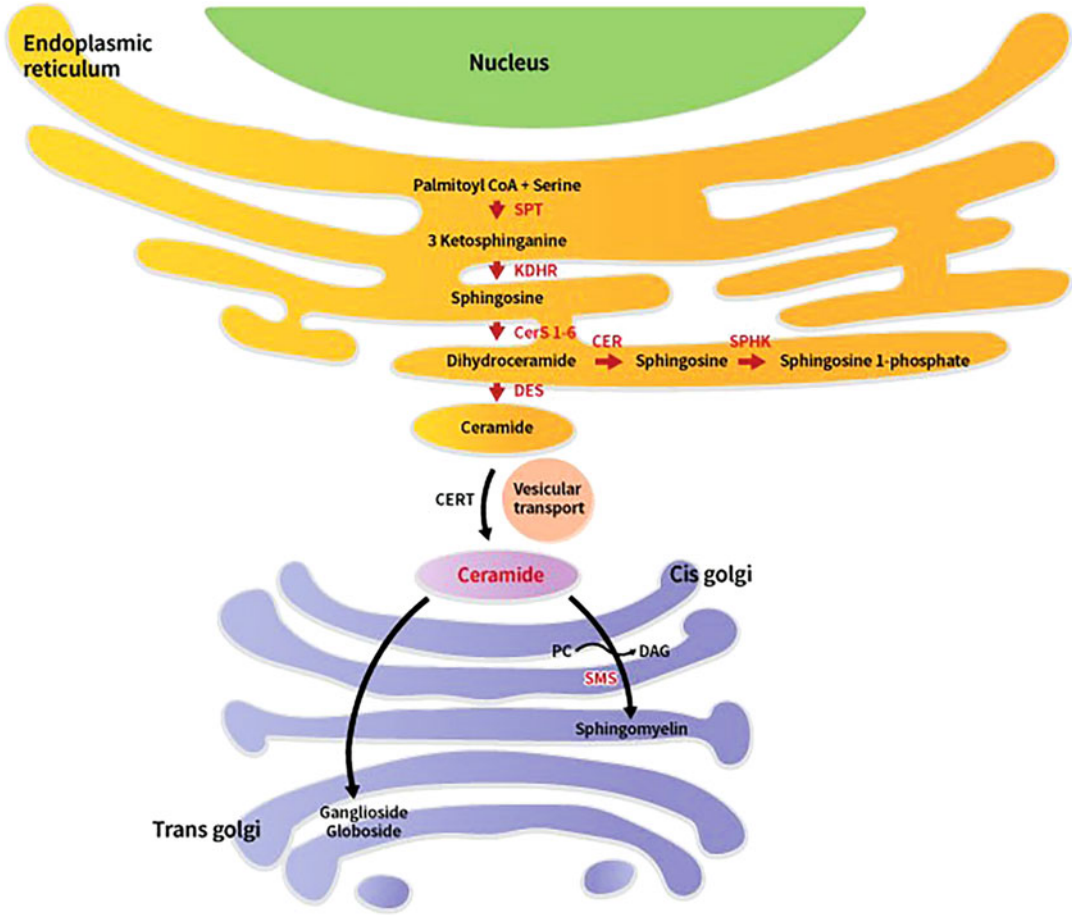

Fig. 3.1 The De novo sphingolipid biosynthesis in mammals. The biosynthetic pathway starts with the condensation reaction of palmitoyl CoA and serine, regulated by a rate-limiting enzyme serine palmitoyltransferase (SPT) to form 3-ketosphinganine followed by a series of enzymatic reactions producing ceramide and other complex sphingoid bases. Ceramides are generated in endoplasmic reticulum and transferred to Golgi via vesicular transport or ceramide transfer protein (CERT). Sphingomyelin synthase (SMS) synthesizes sphingomyelin and diacylglycerol (DAG) by transferring phosphocholine from phosphatidylcholine (PC) to ceramide. *SPT* serine palmitoyltransferase, *KDHR* 3-ketodehydrosphingosine reductase, *CerS* ceramide synthase, *SPHK* sphingosine kinase, *DES* dihydroceramide desaturase, *CERT* ceramide transfer protein, *SMS* sphingomyelin synthase, *PC* phosphatidylcholine, *DAG* diacylglycerol

reticulon (RTN) family protein, which is localized in the tubular ER through two transmembrane domains separated by a loop of the RTN-homology domain with 66 amino acids [41]. Rtn-4, one of the four RTN genes in mammals, produces three major isoforms, NOGO-A, NOGO-B, and NOGO-C, which are expressed in the central nervous system; NOGO-C is expressed in the skeletal muscle at low levels, and NOGO-B is highly expressed in the blood vessels [42]. The biological role of RTNs is to facilitate the formation of tubular ER networks [43]. NOGO proteins function as mediators to inhibit axonal extension of neurons [44] and stimulate migration of endothelial cells by binding to putative receptors [45].

A principal function of NOGO-B is to shape the tubular ER and inhibit SPT activity. Deficiency of NOGO-B elevates SPT activity, and lentiviral re-expression of NOGO-B restores

SPT activity to normal levels in murine and human endothelial cells [42]. Co-immunoprecipitation studies confirmed the interaction of SPT with NOGO-B and ORMDL proteins and the close interaction of these proteins. Since SPT is the first enzyme involved in sphingolipid biosynthesis, the absence of endothelial NOGO-B elevates overall sphingolipid metabolites, including sphinganine, ceramide, sphingosine, and sphingosine 1-phosphate. Among the ceramide species, C18-, C20-, and C22-ceramides increased, while C16-, C24-, and C26-ceramides were not altered. This finding suggests the involvement of NOGO-B in SPT as well as ceramide synthases; however, further studies are needed to elucidate the mechanism of sphingolipid metabolism.

Although the importance of SPT has been proposed in cell survival, neuron development, and cardiovascular function, the exact regulatory mechanism has not yet been elucidated. Regulation of SPT by interaction with ORMDMs and NOGO-B should be studied further. In particular, how SPT can sense sphingolipid levels and adjust to the level changes is not yet understood in terms of ORMDL proteins and NOGO-B. Understanding how pathological conditions affect de novo sphingolipid synthesis will contribute to the application of sphingolipid modulation in the development of diagnostic and therapeutic methods.

3.4 Role of Ceramide in Lipoprotein Metabolism and Vascular Regulation

Elevation of circulating and local ceramide levels in atherosclerosis and cardiac dysfunction has been associated with deleterious effects on the vascular wall and cardiomyocytes. Ceramides are present in very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) and approximately 3% of total plasma sphingolipids [46]. They are evenly distributed in apoB-containing lipoproteins and HDL. Hussain et al. showed that mice deficient in hepatic and intestinal microsomal triglyceride transfer protein (MTP) (L-I-Mttp$^{-/-}$) have reduced plasma ceramide levels by 90%, while patients with abetalipoproteinemia who lack apoB-containing lipoproteins have reduced ceramide plasma levels by approximately 80% [47]. These findings suggest that MTP plays an important role in ceramide transport to apoB lipoproteins and apoB lipoprotein assembly. However, it is unclear whether other proteins are involved in the supply of ceramide to lipoproteins.

The total ceramide in plasma is a combined outcome because all cells synthesize ceramides. However, since the apoB lipoproteins in charge of most plasma ceramides are only synthesized in hepatocytes and enterocytes, synthesized ceramides in other cells may mostly be used as components of the cells and not for secretion. In addition, HDL contains a significant amount of plasma ceramides. Phospholipid transfer protein and cholesterol ester transfer protein may transfer ceramide from apoB lipoproteins to HDL during HDL synthesis or reverse cholesterol transport; however, it is not clear how ceramide is transported to HDL.

Pathological conditions with dysregulation of glucose or FA metabolism cause elevation of plasma ceramide levels via the activation of de novo sphingolipid biosynthesis. The accrual of ceramide interferes with endothelial nitric oxide (NO) synthase-derived NO production through the activation of protein phosphatase 2A [48]. Treatment of ex vivo human resistance arterioles with ceramide induces the formation of mitochondrial H_2O_2, which is associated with an inflammatory response in the endothelium, leading to endothelial dysfunction [49]. This is attributed to ceramide-mediated activation of NADP oxidase and reactive oxygen species (ROS) formation by depleting endothelial NO [50]. Conversely, inhibition of neutral sphingomyelinase (SMase) restores the physiological dysfunction of the arterioles in patients with CAD [49]. Collectively, these findings suggest that ceramide causes endothelial dysfunction.

The contraction of vascular smooth muscle cells (VSMCs) is also regulated by ceramides.

SMase and some ceramide species induce sustained vasoconstriction of the cerebral arteries and venules in rats and dogs [51, 52]. Heterozygous dihydroceramide desaturase 1 mice ($Des1^{+/-}$) have been studied to elucidate the role of ceramide in the vasculature of obese and type 2 diabetes mouse models [48, 53]. The mechanism of action of ceramide in vascular contraction is poorly understood. In part, NOGO-B deficiency in VSMCs causes a selective increase in ceramide species (C18-, C20-, and C22-ceramides) and lower blood pressure [42]. Notably, SPT regulation leads to alteration of various sphingolipid metabolites, including ceramide, and it is difficult to propose that this outcome is derived from altered ceramide levels only. Additionally, ceramide generation from the de novo (SPT) or salvage pathway (SMase) may have distinct effects, such as accumulation in a specific subcellular organelle, and is associated with separate disease generation.

3.5 Role of SM in Atherosclerosis

SM is the most abundant sphingolipid in lipoproteins and cellular membranes and constitutes approximately 87% of total sphingolipids and 20% of total phospholipids in the plasma [46, 54]. ApoB lipoproteins are major sources of plasma SM, as evidenced by L-I-$Mttp^{-/-}$ mice, which have lower plasma SM levels by 73% [47].

Therefore, the involvement of plasma SM levels in atherosclerosis has been studied. Various processes have been implicated in the early development of atherosclerosis, including lipoprotein oxidation, lipoprotein aggregation, endothelial dysfunction, monocyte recruitment, macrophage chemotaxis, foam cell formation, and smooth muscle cell migration and alteration (Fig. 3.2) [55]. There is evidence indicating that SM levels are positively correlated with the development of atherosclerosis. SM accumulates in atheromas from human patients and animal models [56, 57]. Retained atherogenic lipoproteins in the vessel wall are excellent substrates for secretory SMase, making this enzyme a leading candidate for the arterial wall SMase that hydrolyzes LDL-derived SM and causes subendothelial LDL aggregation [57]. Ceramide, a product of SMase, contributes to LDL aggregation in the intima, which is an early step in atherogenesis. The ratio of SM to phosphatidylcholine (PC) is increased five-fold in VLDL from hypercholesterolemic rabbits [58]. ApoE knockout mice, a well-known atherogenic animal model, demonstrated four-fold increased levels compared to WT mice [59]. Moreover, a diet enriched with 1% SM significantly elevated plasma SM levels, LDL aggregation, and atherosclerotic lesions in LDL receptor knockout mice [60]. Based on these findings and clinical information, Jiang et al. proposed that human plasma SM levels and the SM/PC ratio are independent risk factors contributing to CAD [16].

The following clinical results (Table 3.1) also confirm the correlation between plasma SM levels and CAD. Nelson et al. reported an association between plasma SM levels and three measures of *subclinical* cardiovascular disease (carotid intimal-medial wall thickness, ankle-arm blood pressure index, and Agatston coronary artery calcium score) among 6,814 middle-aged, asymptomatic adults in the Multi-Ethnic Study of Atherosclerosis [61]. These observations are consistent with the hypothesis that the plasma SM level is a standard cardiovascular disease risk factor that predicts the risk of subclinical disease. In contrast, another study showed that a high plasma SM level was not associated with an increased risk of adjudicated *incident* CAD in population-based adults free of clinical cardiovascular disease at baseline [63]. Recently, association studies between sphingolipid species and type 2 diabetes depending on pregnancy state, gestational state, and various ethnic groups have been performed [77–79]. Indeed, these clinical lipidomic studies demonstrated an association between type 2 diabetes and the level of a certain class of ceramide or SM. These results suggest that the SM level can be a diagnostic risk factor for CAD and type 2 diabetes, depending on the degree of progression and disease status.

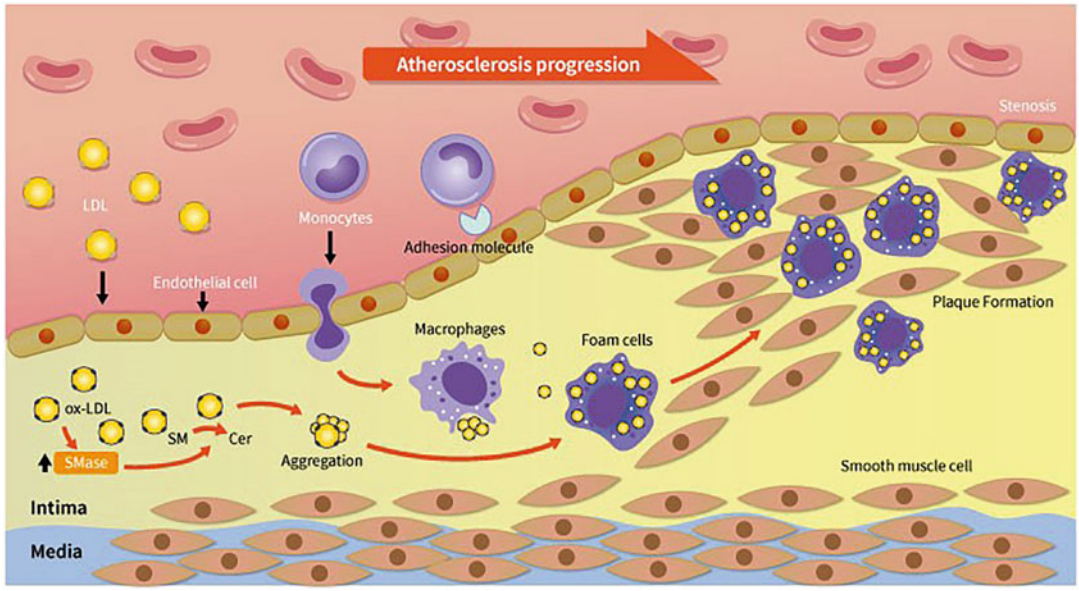

Fig. 3.2 Sphingolipids and atherosclerosis development. LDL accumulation responsible for endothelial dysfunction undergoes oxidative modifications to form oxidized LDL (ox-LDL), inducing an inflammatory reaction causing monocyte infiltration and overexpression of adhesion molecules in endothelial cells. Monocytes circulating in the blood enter into the intima, mature into macrophages, and form foam cells by accumulating ox-LDL aggregates. *LDL* low-density lipoprotein, *ox-LDL* oxidized low-density lipoprotein, *Cer* ceramide, *SM* sphingomyelin, *SMase* sphingomyelinase

There are two possible modulation methods for the sphingolipid pathway to prevent atherosclerosis. The first was to reduce the expression of secretory SMase activity. Previously, SMase-deficient ApoE KO mice showed decreased development of early atherosclerotic lesions and reduced retention of atherogenic lipoproteins compared to ApoE KO mice matched for similar lipoprotein levels [80]. The second was inhibition of de novo SM synthesis by reducing the SM levels in atherogenic lipoproteins synthesized in the liver or intestines and ultimately sub-intimal retention and aggregation. However, heterozygous and total liver-specific ablation of Sptlc2, a major SPT subunit, reduces all major plasma SM and apoB lipoprotein levels but induces jaundice by impairing the development of adherens junctions and causing tumorigenesis [81]. Therefore, a subtle modulation of sphingolipid metabolism in the liver or intestine will be necessary to induce therapeutic effects on lipoprotein metabolism and atherosclerosis.

3.6 SMS and Atherosclerosis

SMS is the last enzyme involved in SM biosynthesis. It catalyzes the formation of SM and DAG by transferring phosphocholine from PC to ceramide. Thus, SMS is a pivotal enzyme for regulating the levels of important lipid signaling mediators and associated pathological conditions.

Among the three members of the SMS gene family found in the cell, SMS1 and SMS2 are selectively distributed in the trans-Golgi apparatus and have catalytic activity [82, 83]. An association study demonstrated that downregulation of SMS2 protects against clinical conditions, including atherosclerosis [84, 85] and hepatosteatosis [86]. Jiang et al. elegantly demonstrated that adenoviral overexpression of SMS1 and SMS2 elevates SM proportions of apoB lipoproteins and decreases SM levels in HDL. ApoB lipoproteins from both SMS1 and SMS2 adenovirus-treated mice were significantly

Table 3.1 Clinical studies investigating the role of diverse ceramides and SM as cardiometabolic biomarkers

Year	Population	Correlating sphingolipids (↑, higher; ↓, lower)	Clinical endpoint	References
2000	CAD patients ($n = 279$)	↑ Total SM	CAD	[16]
2006	Multi-Ethnic Study of Atherosclerosis ($n = 6814$)	↑ Total SM	Subclinical CAD	[61]
2009	CAD patients ($n = 33$)	↑ Total ceramides	Incident CAD	[62]
2010	Multi-Ethnic Study of Atherosclerosis ($n = 6814$)	No correlation between CAD and plasma SM levels	CAD	[63]
2013	CAD patients ($n = 211$)	↑ SM 38:2	CAD	[64]
2014	CAD patients ($n = 304$)	↑ Total ceramides ↑ SM	Acute coronary syndrome	[65]
2014	CAD patients ($n = 258$)	↑ C16:0, C18:0 ceramides	Cardiovascular death	[66]
2015	CAD patients ($n = 581$)	↑ C16:0, C18:0, C24:0, and C16:0/C24:0 ceramide ratio	MACE	[67]
2015	CHF patients ($n = 423$)	↑Total ceramides	Mortality	[68]
2016	Three CAD cohorts ($n =$ 80, 51, and 81)	↑ C16:0, C18:0, C24:1, and C16:0/C24:0 ceramide ratio	Cardiovascular death	[69]
2016	Healthy individuals ($n =$ 8101)	↑ C16:0, C18:0, C24:1, and ratios with C24:0 ceramides	Cardiovascular death	[70]
2017	CAD patients ($n = 111$)	↑ C16:0 and C18:0 ceramides ↑ SM 18:1	CAD and depression	[71]
2017	PREDIMED trial ($n = 980$)	↑ C16:0, C22:0, C24:0, and C24:1 ceramides	Non-fatal acute myocardial infarction, non-fatal stroke, cardiovascular death	[72]
2018	Strong Heart Family Study (SHFS) ($n = 2086$)	↑16:0 ceramide ↑ 18:0, 20:0, 22:0, and 24:0 SM	SHFS cohort	[73]
2018	Participants before non-urgent coronary angiography ($n = 265$)	↑ C16:0, C18:0, C24:1, and ratios with C24:0 ceramide	MACE	[74]
2018	Two cohorts ($n = 2462$ and $n = 3134$)	↓ C24:0/C16:0, C22:0/C16:0 ceramide ratios	Incident CAD and total mortality	[75]
2019	Cardiovascular Health Study ($n = 1179$)	↑ 16:0 ceramide ↑ 18:0 SM	Incident CAD	[76]

CAD coronary artery disease, *CHF* chronic heart failure, *MACE* major adverse cardiac events, *PREDIMED* the Prevencion con Dieta Mediterranea

aggregated after treatment with a mammalian SMase, whereas the lipoproteins from control animals did not aggregate [87]. In a subsequent study, SMS2 KO and SMS2 liver-specific transgenic (LTg) mice were constructed and characterized [88]. SMS2 KO mice on a high-fat diet (HFD) had significantly reduced plasma SM levels, while SMS2 LTg mice had increased SM levels, but with no change found in other lipids. ApoB lipoproteins from SMS2 LTg mice displayed a stronger tendency to aggregate after SMase treatment, as shown in the reports of adenoviral overexpression. While SMS2 deficiency increased plasma apoE levels by twofold, SMS2 LTg decreased these levels by 1.8-fold. Moreover, SMS2 KO mice had an activated cholesterol efflux from macrophages, whereas SMS LTg mice had no efflux (efflux prevented) [88]. These results suggest that SMS2 is a key

player in the regulation of plasma and liver SM levels in mice.

Disordered apoptosis is important in atherogenesis because the death of lipid-rich foam cells promotes lipid core formation in the vessel wall [89]. Genetic manipulation of SMS activity alters cellular DAG and ceramide, which may contribute to apoptosis. Pharmacological inhibition of SMS reduces cellular DAG levels and activity of protein kinase C (PKC), which is activated by DAG [90]. While ablation of SMS1 or SMS2 significantly reduces DAG levels, overexpression of SMS1 or SMS2 elevates DAG levels in THP-1 macrophages [91]. Cellular DAG is an activator of the conventional novel PKC, a family of serine/threonine kinases that regulate a line of cellular processes, including pro-apoptotic and pro-survival activities. In both CHO cells and THP-1 macrophages, siRNA-mediated knockdown of SMS1 or SMS2 reduced intracellular SM and DAG levels, respectively, and lipopolysaccharide-mediated apoptosis was reduced [91]. In this study, overexpression of SMS1 or SMS2 elevated cellular ceramide levels, as well as SM levels. This mismatch may be attributed to the bidirectional activity of SMS enzymes [83]. In addition, the ratio of ceramide to SM increased in cells overexpressing SMS. This may represent another contributing factor for the increased apoptotic potential of the cells, given that ceramide is a bioactive lipid for pro-apoptotic events. In a recent report, pharmacological inhibition or genetic knockout of SMS2 decreased the generation of M2-type macrophages in vitro and reduced tumor weight and lung metastatic niche formation in a 4T1-triple negative cancer mouse model [92]. These results indicate that modulation of SMS may result in different outcomes depending on the cell type and disease status.

3.7 SMS2 in Macrophages and Endothelial Cells

Macrophages play an important role in the formation of atherosclerotic lesions in the vessel walls. Accumulated LDL in the intima initiates atherogenesis, followed by infiltration of circulating monocytes into the vessel wall, maturation into macrophages, and lipid-laden foam cell formation. Liu et al. transplanted SMS2 knockout bone marrow into LDL receptor knockout ($Ldlr^{-/-}$) mice, creating a mouse model with macrophage-specific SMS2 deficiency. SMS2 ablation in macrophages reduced the aortic arch, root, and entire aorta compared with transplantation with wild-type macrophages [84]. Further plaque morphology analysis confirmed that SMS2 deficiency in macrophages reduced the necrotic core area and collagen content in atherosclerotic lesions. Downregulation of SMS1 and SMS2 in macrophages resulted in reduced TLR4 on the cell surface [91]. Recently, Prymas et al. demonstrated that silencing of SMS1 and SMS2 led to a depletion of SM in cells, and the TRIF-dependent signaling pathways of TLR4 were inhibited. These results indicate that LPS-induced pro-inflammatory response in macrophages is regulated by SMS via downregulation of SMS and regulates atherogenesis in the vessel wall [93].

Dysfunction of endothelial cells is the pathological basis of atherosclerosis. Oxidative stress and mitochondrial dysfunction are the major causes of endothelial dysfunction [94]. Oxidative stress leads to a depletion of intracellular antioxidants and elevation of ROS levels, causing lipid peroxidation and degeneration of biological macromolecules to develop vascular endothelial dysfunction [95]. The expression of SMS2 in human umbilical vein endothelial cells (EC) was upregulated when cells were treated with H_2O_2. In addition, SMS2 overexpression promoted apoptosis and macrophage adhesion of H_2O_2-induced ECs and upregulated the expression of β-catenin. In contrast, the SMS inhibitor Dy105 decreased the levels of endothelial cells and β-catenin. These findings indicate that SMS2 activity is closely associated with endothelial dysfunction via the Wnt/β-catenin signaling pathway, and SMS2 inhibition may inhibit this event.

3.8 Pharmacological Modulation of SPT and SMS2 in Atherogenesis

Since ceramide and SM in the plasma play an important role in the development of atherosclerosis and coronary artery events, pharmacological confirmation of the therapeutic effects and development of the inhibitors of de novo sphingolipid biosynthesis have been reported. For therapeutic purposes, sphingolipid metabolism correcting pathophysiological disease conditions is the most critical. Modulation of SPT and SMS has been actively studied for pharmacological modulation of atherosclerosis and CAD.

3.8.1 SPT

Identification of SPT inhibitors initiated with the characterization of naturally occurring compounds, including myriocin, sphingofungins, and lipoxamycin. These compounds are potent and highly selective for SPT, inhibiting fungal and mammalian SPT in cell-free preparations [96–98]. Structurally, these inhibitors resemble the transient intermediate postulated to form during the condensation of serine and palmitoyl CoA. Since these inhibitors act on the first step in the de novo sphingolipid pathway, major sphingolipid metabolites, such as ceramide and SM, are reduced in both cultured cells and in vivo [96–98]. Earlier, these compounds have drawn attention as antifungal or immunosuppressive agents.

The association between SPT inhibition and atherosclerosis has been reported with the use of myriocin. Park et al. and Hojjati et al. have reported that oral or intraperitoneal administration of myriocin reduces plasma ceramide and SM levels and atherosclerosis in ApoE KO mice [99, 100]. Although intraperitoneal administration of myriocin did not alter plasma lipid levels, oral administration reduced plasma cholesterol and triglyceride levels. This result suggests that myriocin reduces the absorption of cholesterol in the small intestine. Additionally, myriocin increases insulin sensitivity and reduces non-alcoholic hepatosteatosis [101, 102]. Thus, SPT inhibition by myriocin has direct effects on anti-atherogenic vascular effects and acts as a lipid-lowering agent.

Genetically modified heterozygous Sptlc1 and Sptlc2 mice are healthy and viable despite the embryonic lethality of homozygous ablation. Heterozygous Sptlc1 knockout mice absorbed less cholesterol owing to decreased Niemann-Pick C1-like1 (NPC1L1) and ABCA1 levels and increased ABCG5 levels in the SPT-deficient small intestine. SM levels in the apical membrane of enterocytes also decreased [103]. Together, these results suggest that SPT deficiency reduces cholesterol absorption by downregulating NPC1L1 and ABCA1 proteins in the apical membranes of enterocytes via reduced SM levels in the apical membrane. Therefore, SPT could be an alternative therapeutic target for hypercholesterolemia and atherosclerosis.

In addition to atherosclerosis, cancer is another disease condition that can be modulated by SPT inhibition. Sano et al. synthesized 137 pyrazolopyridine derivatives and validated the relationship between in vitro SPT inhibition and lung cancer cell growth [104]. In a subsequent study, high-throughput screening and medicinal chemistry efforts led to the identification of structurally diverse SPT inhibitors. Their anti-tumor activity was observed in a PL-21 xenograft mouse model [105]. Genin et al. identified novel potent SPT inhibitors for type 2 diabetes and dyslipidemia. These imidazopyridine and pyrazolopiperidine derivatives reduce plasma ceramides, enhance insulin sensitization in diet-induced obese mice, and improve lipid profiles, such as elevation of HDL levels and reduction of triglyceride levels in cholesterol/cholic acid-fed rats. Unfortunately, these compounds cause gastric enteropathy after chronic dosing in rats [106]. Various efforts to regulate SPT pharmacologically for the development of novel therapies are ongoing. The precautionary point is that SPT is the first step in de novo sphingolipid synthesis and alters the cellular levels of various bioactive sphingolipid metabolites. Therefore, fine-tuning of the manipulation of a specific sphingolipid metabolite is

important for the future development of therapies for various chronic diseases to improve disease-specific efficacy.

3.8.2 SMS2

SMS-mediated SM synthesis is implicated in atherogenesis, endothelial dysfunction, and macrophage polarization. Recently, a line of compound development has been reported for therapeutic purposes. Adachi et al. identified a 2-quinolone derivative as an SMS2 selective inhibitor with an IC_{50} of 950 nM and more than 100-fold selectivity for SMS2 over SMS1 using a high-throughput enzymatic assay [85]. Additionally, SMS2-deficient mice are protected from diet-induced obesity, fatty liver, and type 2 diabetes [86, 107, 108]. Mo et al. discovered 4-benzyloxybenzo[d] isoxazole-3-amine derivatives as potent and highly selective SMS2 inhibitors [109]. Among them, the 15w compound had good pharmacokinetic properties in vivo and attenuated chronic inflammation in db/db mice for further development of a therapy against inflammation-associated metabolic dysfunction. Further, 15w showed anti-tumor efficacy against triple-negative breast cancer [92]. Based on these study findings, further medicinal chemistry efforts identified a representative 2-(benzyloxy)-N-arylbenzamide derivative, Ly93, with a high selectivity over SMS1 and a nanomolar range of IC_{50} [110]. Ly93 dose-dependently reduced apoB secretion from Huh7 cells but also significantly reduced SMS activity and increased cholesterol efflux from macrophages in cell studies. Additionally, it dose-dependently attenuated atherosclerotic lesions in the aortic root and the entire aorta, as well as the macrophage content in lesions in apoE gene knockout mice. In a subsequent study, Huang et al. demonstrated that HFD-induced insulin-resistant C57BL/6 mice treated with Ly93 were more sensitive to insulin than were untreated mice and showed improved insulin tolerance [111]. In particular, phosphorylation of IRS-1, Akt, and GSK-3β increased and sensitized insulin signaling. Yukawa et al. synthesized 1,8-naphthyridin-2-one derivative 37 as a potent and selective SMS2 inhibitor with a nanomolar range of IC_{50} [112]. This compound reduced hepatic SM levels in mice and exhibited a good pharmacokinetic profile. Collectively, SMS2 is a novel therapeutic target for chronic diseases, including atherosclerosis, inflammation, and insulin resistance; the development of SMS2 inhibitors to find a potent drug candidate in industrial areas is actively ongoing.

3.9 Conclusion

Atherosclerosis is a CAD that is a major cause of mortality worldwide, especially in developed countries. While the current risk factors are predictive parameters for CAD, the broad variability in the development of the disease is difficult to elucidate. Modulation of ceramide and SM in the de novo sphingolipid biosynthetic pathway has been clinically proven to be a new approach for understanding and treating the disease. The development of pharmacological agents to manipulate the levels of ceramide and SM for the treatment of CAD is currently ongoing. Additionally, sphingolipid biosynthesis is tightly associated with the development of cancer, type 2 diabetes, obesity, and non-alcoholic fatty liver disease. SPT and SM are new therapeutic targets for these chronic diseases, and the strategic goal is to manage ceramide and SM in the plasma and target cells. Although fine-tuning is necessary to manage the essential components (sphingolipid metabolites) in the cell, this pathway has a high therapeutic value for the treatment of chronic diseases. Thus, pharmacological modulation of sphingolipid biosynthesis can provide a therapeutic strategy to treat patients with atherosclerosis and CAD.

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Serine Palmitoyltransferase Subunit 3 and Metabolic Diseases

4

Museer A. Lone, Florence Bourquin, and Thorsten Hornemann

Abstract

Sphingolipids (SL) are a class of chemically diverse lipids that have important structural and physiological functions in eukaryotic cells. SL entail a long chain base (LCB) as the common structural element, which is typically formed by the condensation of L-serine and long chain acyl-CoA. This condensation is the first and the rate-limiting step in the de novo SL synthesis and catalyzed by the enzyme serine palmitoyltransferase (SPT). Although palmitoyl-CoA is the preferred substrate, SPT can also metabolize other acyl-CoAs, thereby forming a variety of LCBs, which differ in structures and functions. The mammalian SPT enzyme is composed of three core subunits: SPTLC1, SPTLC2, and SPTLC3. Whereas SPTLC1 and SPTLC2 are ubiquitously expressed, SPTLC3 expression is restricted to a few specific tissues. The SPTLC1 subunit is essential and can associate with either SPTLC2 or SPTLC3 to form an active enzyme. Depending on the stoichiometry of the SPTLC2 and SPTLC3 subunits, the spectrum of SPT products varies. While SPTLC1 and SPTLC2 primarily form C_{18} and C_{20} LCBs, the combination of SPTLC1 and SPTLC3 produces a broader spectrum of LCBs. Genetic and population based studies have shown that SPTLC3 expression and function are associated with an altered plasma SL profile and an increased risk for cardiometabolic diseases. Animal and in vitro studies showed that SPTLC3 might be involved in hepatic and cardiac pathology and could be a therapeutic target for these conditions.

Here we present an overview of the current data on the role of SPTLC3 in normal and pathological conditions.

Keywords

Serine palmitoyltransferase · Serine palmitoyltransferase subunit 3 (Sptlc3) · Sphingolipid biosynthesis · Sphingolipids · Metabolic diseases

4.1 The Serine Palmitoyltransferase and Long Chain Base Synthesis

Sphingolipids (SL) are an essential class of lipids and found in some prokaryotes (*Bacteroides*, *Sphingomonads*) and all eukaryotes [1]. Structurally, SL are defined by the presence of long chain amino-hydroxy alkanes also called long chain bases (LCB). The LCB formation is the first and

M. A. Lone · T. Hornemann (✉)
Institute for Clinical Chemistry, University Hospital and University of Zürich, Zürich, Switzerland
e-mail: thorsten.hornemann@usz.ch

F. Bourquin
Institute for Biochemistry, University of Zürich, Zürich, Switzerland

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rate-limiting step in the de novo SL synthesis and catalyzed by the serine palmitoyltransferase (SPT). Typically, SPT condenses an activated fatty acid with L-serine in a PLP dependent reaction. LCBs can vary in the number of carbons, hydroxylation as well as in their saturation state [2]. Some LCB structures are specific to certain species [3]. Most variations in the LCB spectrum arise due to the broad and variable substrate specificity of the SPT enzyme although some LCB modifications are also introduced downstream of SPT. SPT metabolizes acyl-CoA's of variable structure and length, and the formation of odd and even numbered LCBs as well as methyl-branched structures was reported across species [3]. The 18-carbon dihydroxy amino alkene, sphingosine (SO, d18:1) is the most abundant LCB in mammals including humans. However, plants and yeast generally contain the saturated trihydroxy derivate, phytosphingosine (PhytoSO, t18:0), whereas shorter LCBs (C_{14} and C_{16}) are commonly found in insects (*Drosophila*) while *C. elegans* forms iso-branched C_{17} LCBs [4, 5]. Under conditions of serine deficiency, SPT can also metabolize L-alanine and glycine as alternative substrates, which results in the formation of a non-canonical and neurotoxic class of 1-deoxysphingolipids [6].

SPT belongs to the family of pyridoxal 5-phosphate (PLP)-dependent a-oxoamine synthases (POAS) similar to the 5-amino levulinic acid synthase that is involved in heme metabolism. In contrast to other members of this family, which are mostly soluble cytosolic proteins, SPT is an integral membrane protein and located at the outer membrane of the endoplasmic reticulum (ER) [7, 8]. In mammals, the SPT core subunits are encoded by the three genes *SPTLC1*, *SPTLC*2, and *SPTLC3*. A minimally functional SPT enzyme consists of the SPTLC1 subunit in conjunction with either SPTLC2 or SPTLC3 [9]. Accessory subunits (ORMDL3, ssSPTa, ssSPTb) that modulate activity and substrate specificity have been identified in yeasts and eukaryotes (Fig. 4.1). In response to cellular SL levels, a conserved family of integral ER membrane proteins (ORMDL1-3) controls SPT activity and SL de novo synthesis [10]. Budding yeast encodes for two isoforms (Orm1 and Orm2), while mammals express three ORM orthologues (ORMDL1-3). The two accessory small proteins, ssSPTa and ssSPTb are specific to mammals and reported to regulate the synthesis of C_{18} and C_{20} LCBs [9, 11]. Here, ssSTPa promotes the use of palmitoyl-CoA forming C_{18} LCBs, while the presence of ssSPTb enhances the activity with stearoyl-CoA forming a C_{20} LCB. The expression of ssSPTb appears to be specific to neuronal tissue and a pathologically increased synthesis of C_{20} LCBs due to a gain-of-function mutation in the *ssSPTb* gene causes neurodegeneration and macular defects in mouse models [12].

In contrast, SPTLC3 appears to form a rather large spectrum of non-canonical LCBs in mammals especially in humans. SPTLC3 was cloned more than a decade ago [13] but despite a significant association with different metabolic traits in several genome-wide studies, its physiological function remains largely unknown. However, recent reports shed new light on its function and relevance.

4.2 Evolution of SPT and Structure

As an essential metabolic enzyme, SPT has evolved from simpler to more complex forms of life. This relates to gene copy numbers, structural heterogeneity of the enzyme as well as function. The bacterial SPT from *Sphingomonas paucimobilis* is a soluble homodimer and transcribed from a single gene [14], whereas in yeast (*S. cerevisiae*), SPT is a heteromeric and membrane bound enzyme that is encoded by the two genes *LCB1* and *LCB2* [1]. *Arabidopsis* encodes for a homologue of SPTLC1 and for two homologues of SPTLC2 (AtLCB2a and AtLCB2b) [15]. AtLCB2a/b are 405 amino acid long proteins that share 95% identity and appear to be ubiquitously expressed and functionally redundant in *Arabidopsis* [16]. A third, SPTLC2 related but truncated coding sequence (At3g48790) is present in tandem with AtLCB2a. This truncated form is likely the result of a gene duplication event and lacks 140 residues of the

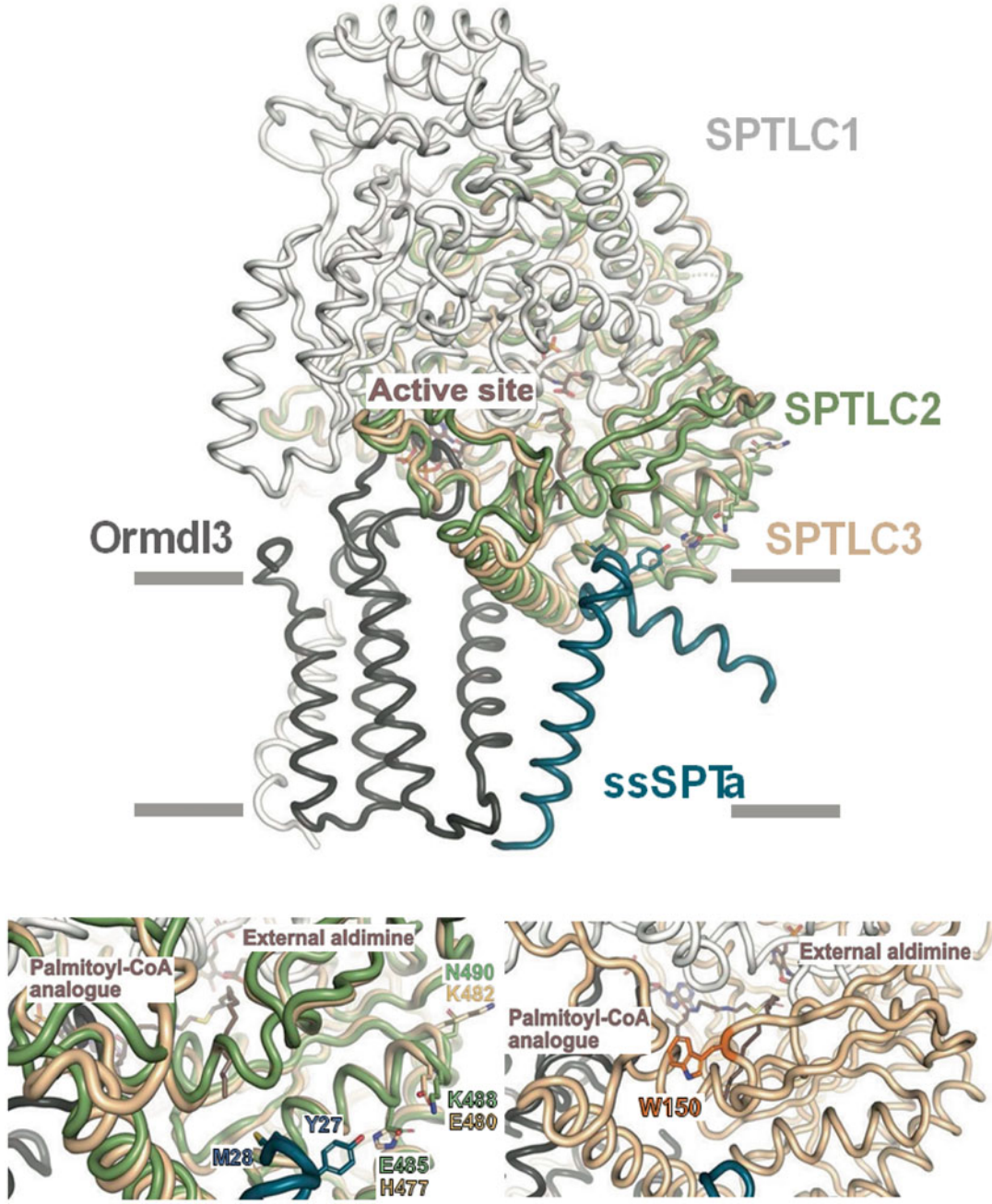

Fig. 4.1 SPTLC1-SPTLC2 and SPTLC1-SPTLC3 protein structure: Overlay of SPTLC1-SPTLC2 and an SPTLC1-SPTLC3 based dimer. The SPTLC1-SPTLC3 structure was modeled in silico using the recently published cryo EM structure of the SPT complex [7, 8]. Closely located to the residues Met-28 and Tyr-27 is a helical domain. The charge distribution of this helix differs considerably between SPTLC2 and SPTLC3. Residues Tyr-27 and Glu-485 in SPTLC2 and His-477 in SPTLC3 (*upper right*) can be assumed to form a gate to the long chain base binding site. At physiological pH, Glu-48 in SPTLC2 is negatively charged and the gate is expected to be open due to a repulsion between the hydrophobic Tyr-27 and Glu-485. In contrast, Tyr-27 and the neutral His-477 of SPTLC3 build hydrogen bonds and form a closer gate. At acidic conditions, the repulsion of the then positively charged His-477 and Tyr-27 is expected to bring SPTLC3 into a more open conformation compared to physiological pH. Further differences are to be found in the charge distribution over the entire helix

N-terminus. However, the gene product was not yet detected at protein level.

Human SPTLC3 is also slightly shorter in length than SPTLC2 (552 vs. 562 amino acids, respectively) and shares about 68% overall identity with SPTLC2. The two subunits are rather conserved in their central regions and than at their N- and C-terminal ends [13].

Both SPTLC2 and SPTLC3 bear a conserved PLP binding motif with a central Lysine residue that is absent in SPTLC1. This lysine is essential for the transferase reaction, as PLP forms a Schiff's base with the amino acid substrate, creating an external aldimine (Fig. 4.1). Therefore, SPTLC2 and 3 are considered to be the catalytically active subunits, whereas SPTLC1 appears to be important for protein stability, membrane insertion, and serves as a focal point to control enzyme activity.

The cryo EM structure of the human SPT complex (SPTLC1, SPTLC2, ORMDL3, and ssSPTa) was published recently in two independent studies [7, 8]. The structures provide a good insight into the mechanisms of substrate recognition, catalytic activity, and enzyme regulation. They show that SPTLC1 and 2 are both involved in the recognition of the acyl-CoA head group, whereas the acyl-tail is mostly stabilized by residues from SPTLC2. The amino acid environment around the acyl-CoA binding pocket is important for activity, as mutations of these SPTLC2 residues either decrease or completely block enzyme activity [8]. In addition, a single amino acid residue from ssSPTa (Met28) appears to participate in the acyl chain coordination [8]. The large hydrophobic methionine protrudes into the acyl binding pocket of SPTLC2, acting as a plug that defines the possible length of the acyl chain that can be accommodated within the pocket [8]. The corresponding amino acid in the human subunit ssSPTb is Val25 which leaves more room in the acyl binding pocket. Consequently, it was demonstrated that the exchange of Met28 to valine increases the affinity of SPT enzyme towards the bigger stearoyl-CoA [8, 17, 18].

Based on the published structure data we generated an in silico model of an SPTLC1-SPTLC3 dimer (Fig. 4.1). In comparison, the SPTLC1-SPTLC3 dimer showed a different configuration of the substrate binding pocket that likely allows more variability in the biding of different acyl-CoA structures. The biggest differences was seen at the interface to ssSPTa, in close vicinity to the residues ssSPTa Met28 and Tyr27 (Fig. 4.1 lower left). The structural changes around Met28 can be expected to influence the substrate binding properties of the enzyme. A not yet solved aspect is the role of the flexible protein termini that could not be resolved in the recently published structures [7, 8]. They may interact with lipids or other proteins influencing substrate binding and product spectrum.

4.3 Role of SPTLC3 in Sphingolipid Metabolism

The two subunits SPTLC1 and SPTLC2 are expressed ubiquitously and the relative mRNA levels vary little between tissues. In contrast, SPTLC3 is specifically expressed in certain tissues and mRNA levels between these tissues vary significantly. SPTLC3 shows moderate expression in liver, significant expression in skin, and very high expression levels in placenta and trophoblasts [13]. The cellular and physiological reasons for this tissue specificity are not yet understood, but it is reasonable that SPTLC3 expression is highest in those tissues that benefit most from a broad LCB spectrum. This might explain the significantly high expression in skin,

Fig. 4.1 (continued) (*upper right*). The residue Lys-488 of SPTLC2 is replaced by Glu-480 in SPTLC3, reversing the charge in this area. The residue Asn-490 of SPTLC2 is replaced by Lys-482 in SPTLC3, introducing a positive charge at the very beginning of the helix. Models were generated by the SWISS-MODEL server using the automated mode and selecting the model with 100% coverage and the best global model quality estimate (GMQE) and qualitative model energy analysis (QMEAN) [48–52]

which has a highly complex arrangement of SL and derivatives that contribute to the hydrophobic barrier that prevents the body from transdermal water loss. However, SPTLC3 expression in human placenta and trophoblast cells are many-fold higher than in skin [13] but it is currently not clear whether such a broad spectrum of SLs is of funtional importance for the placenta. Unfortunately, SPTLC3 knockout (KO) models that could address these questions are not yet available.

In *Arabidopsis,* the deletion of either AtLCB2a or AtLCB2b is well tolerated with no observable consequences on plant growth [16]. Both forms appear to generate similar LCB profiles and can complement LCB auxotrophy in SPT deficient yeast cells [16]. However, the mammalian SPTLC2 and SPTLC3 subunits appear to diverge functionally. The earliest indications to functional divergence between the two enzymes came from the fact that in vitro activity of SPTLC3 but not of SPTLC2 is inhibited in the presence of Triton-X-100 (0.2%) [12]. This detergent sensitivity is unlikely a result of the disruption of SPTLC1-SPTLC3 interaction, which is efficiently maintained even at higher detergent concentrations (0.5%) [9]. The effect is likely related to non-covalent structural modification of the enzyme as the activity of the soluble SPT from *S. paucimobilis* is also inhibited by the detergent [13], perhaps through interference with the acyl-CoA binding site.

Unlike human SPTLC2 and *Arabidopsis* AtLCB2a/b, SPTLC3 only partially rescues LCB auxotrophy in LCB1/LCB2 deficient yeast cells [11]. In animals, systemic SPTLC2 deficiency is lethal, whereas SPTLC3 deficient models have not yet been reported. A conditional KO of SPTLC2 in cardiomyocytes causes cardiac dysfunction, myopathy, and fibrosis in mice [19]. In response, SPTLC3 expression is induced in KO cells but this does not compensate for the SPTLC2 deficiency [19]. These differences between the two subunits could be attributed to different activities. Whereas the combination of SPTLC1/SPTLC2 and ssSPTa preferentially metabolizes palmitoyl-CoA (Fig. 4.2a, c), the combinations of SPTLC1/SPTLC3 showed a broader affinity for fatty acids of variable length (Fig. 4.2b, c). The length of the metabolized acyl-CoA's ranges from myristic (C_{14}) to stearic acid (C_{18}), including acyl-CoA's with even and odd carbon chains. The human SPTLC3 is effectively metabolizing penta (C_{15})- and heptadecanoic (C_{17}) acid to generate odd chain 17- and 19-carbon LCBs but has poor activity with nonadecanoic (C_{19}) acid. The presence of the ssSPTb subunit shifts this pattern and increases activity of both SPTLC2 and SPTLC3 towards stearoyl-CoA forming C_{20} LCBs although the capacity to form these LCBs is still higher for SPTLC3 [9]. In addition, human SPTLC3 also forms an *anteiso* branched omega-3-methyl LCB ($meC_{18}SO$) which uses *anteiso*-methyl-palmitate as a substrate. Branched chain fatty acids (BCFAs) are formed from branched chain amino acids (BCAA) such as Ile, Leu, and Val. In fact, stable isotope tracing showed that primarily Ile contributes to the formation of both, odd chain $C_{17}SO$ and branched $meC_{18}SO$. *Anteiso*-methyl-palmitate is generated from the intermediate precursor molecule, 2-methyl-butyryl-CoA, whereas pentadecanoic (C_{15}) acid is formed by the elongation of propionyl-CoA, an end-product of the BCAA catabolic pathway. Interestingly, BCAA were shown to be significantly elevated in metabolic diseases such as T2DM [20] suggesting that also $C_{17}SO$ and $meC_{18}SO$ levels might be increased in these conditions.

Interestingly, the formation of $meC_{18}SO$ seems to be specific to humans as $meC_{18}SO$ is present in human plasma and in purified human lipoproteins but was absent in the plasma of mice [9]. However, it is not clear whether this is because of intrinsic differences in the activity of human and mouse SPTLC3 or differences in the substrate availability [21]. The availability of relevant fatty acid substrates such as pentadecanoic acid and *anteiso*-methyl-palmitate is a significant factor determining the overall LCB spectrum in SPTLC3 expressing cells.

The physiological function of SPTLC3 derived SLs is currently not known. SLs with different LCB and N-acyl profiles might affect the biophysical properties of membranes. It has been shown that the length of the LCB in

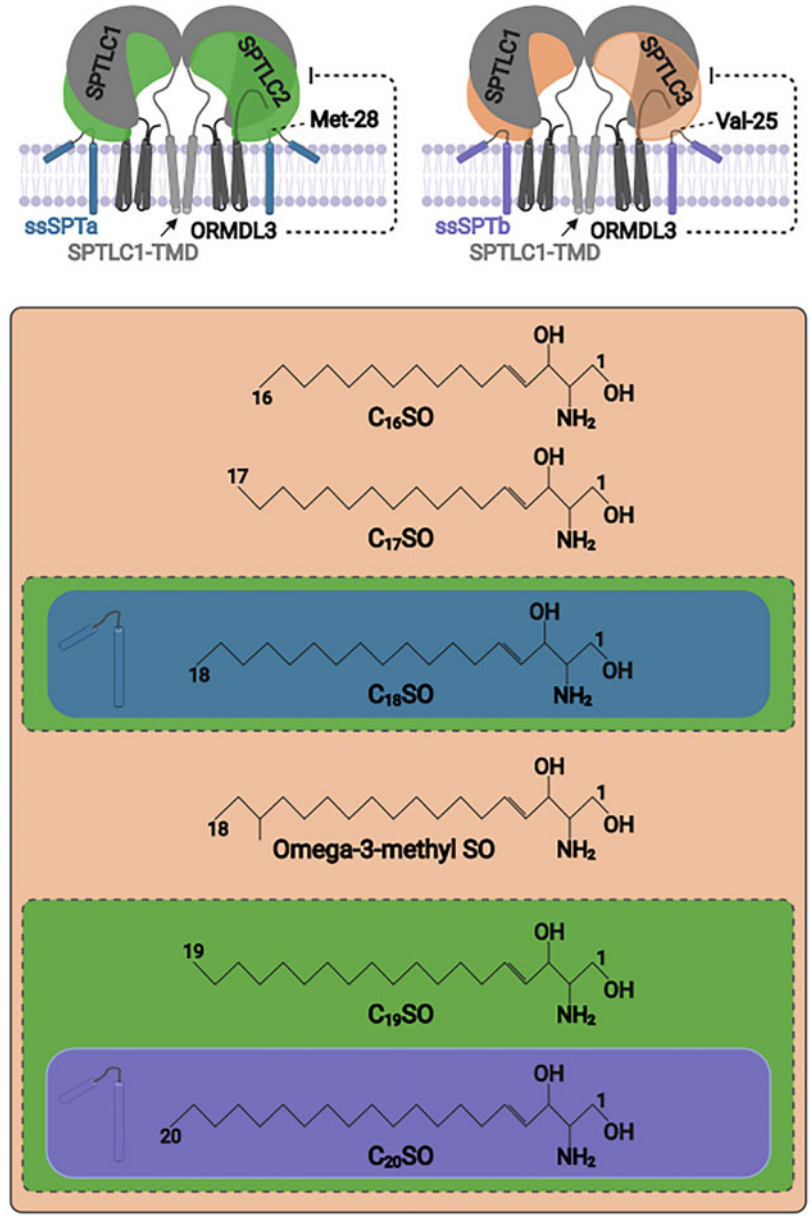

Fig. 4.2 SPT subunit composition and the resulting LCB product spectrum: (**a**) SPTLC1 + 2 and (**b**) SPTLC1 + 3 based SPT enzyme. The specificity of each subunit combination is shown through the inset-overlap (**c**). *Colors* in the *insert* correspond to the protein subunit in the structures above. The SPTLC1 + SPTLC2 based enzyme generates primarily C_{18} (d18:0) and to a lesser extend C_{20} (d20:0) based LCBs. In contrast, the SPTLC3 subunit (*top right* and *large-brown inset* below) forms a broader range of LCBs in the range of C_{16}–C_{20}. The synthesis of C_{16}, C_{17}, and the omega-3-methyl sphingosine is exclusive to the SPTLC3 subunit, whereas the activity of the SPTLC2 and 3 overlaps concerning the formation of C_{18} and C_{20} LCBs. In combination with SPTLC2, the small subunit ssSPTa (*blue* protein *top left/blue square inset*) increases C_{18} LCB synthesis, whereas ssSPTb increases the formation of C_{20} LCBs (*purple top right* for both subunits). ORMDL3 interacts with SPTLC1 and thereby controls enzyme activity

ceramides is inversely associated with the amount needed to induce lateral segregation in vitro [22]. In these experiments, the LCB chain length had a stronger effect on segregation than the N-linked acyl chain. In addition, phosphorylated atypical LCBs might alter sphingosine-1-phoshphate (S1P) and S1P-receptor (S1PR) mediated signaling pathways. For example, it was reported that S1PR activation is S1P-alkyl chain length dependent [23]. Additionally, C_{20}S1P was recently shown to diminish signaling from SP1R2 [24].

4.4 SPTLC3 in Metabolic Diseases

SNPs in *SPTLC3* have been significantly associated with several lipid traits in genome-

wide association studies (GWAS) and repeatedly reported as significant influencers of circulating SL levels [25–31]. Sphingolipids, ceramides, and sphingomyelins are independent markers for cardiovascular disease [32] and plasma ceramides were shown to be relevant biomarkers to assess the severity of cardiovascular dysfunction [33, 34]. Tabassum et al. showed that SNP rs364585 in the SPTLC3 locus was associated with reduced plasma ceramides and a decreased risk for intracerebral hemorrhage [29]. Animal studies have shown that the SPTLC3 dependent ceramides formation is increased in the heart of mice on high fat diet (HFD). The addition of C_{16}SA (d16:0) but not of C_{18}SA (d18:0) was toxic to cultured cardiomyocytes in vitro and linked to a non-apoptotic pathway [35].

SNPs in the SPTLC3 locus also appear to be associated with changes in other lipids classes in liver and plasma. Mirkow et al. showed that the SNP rs168622 was associated with an elevated SPTLC3 expression and increased hepatic lipid content [36], whereas other studies linked SNPs in SPTLC3 with altered plasma LDL-C levels [25, 28, 31]. This association with plasma cholesterol could emanate from an intimate intertwining of the SL and cholesterol metabolism, co-transport in plasma, and their alliance in establishing functional eukaryotic membrane microdomains. Sphingomyelin and ceramide levels regulate cholesterol synthesis by modulating SREBP activation and HMG-CoA reductase activity [37]. Similarly, sphingomyelin sequestration of cholesterol within cellular membranes could set off futile cycles of synthesis and unloading.

However, given the fact that SPTLC3 generated SL account for a relatively small fraction of total SL in human plasma, these strong associations are rather surprising.

Metabolically, it was shown that SPTLC3 expression is influenced by a variety of factors. Shah et al. [38] reported a significant increase in SPTLC3 expression in epididymal adipose tissues of mice on HFD with a modest decrease in SPTLC2 expression. Others reported induction of hepatic SPTLC3 expression in mice on HFD [39–41]. SPTLC3 expression was also associated with insulin resistance in adipose [38] hepatic tissues [39, 41] and upregulated in steatotic livers of mice supplemented with homocysteine [42]. This points to the fact that steatosis, irrespective of its nature, induces the expression of SPTLC3. In addition, SPTLC3 expression appears to be related to plasminogen activator inhibitor 1 (PAI1) as PAI1 deficiency decreased SPTLC3 expression in mice on HFD [38]. Similarly, cannabinoid receptor 1 (CB_1R) antagonists were reported to decrease hepatic SPTLC3 expression and improve insulin sensitivity in mice on HFD [39]. Polyunsaturated fatty acids and sulforaphane also reduced SPTLC3 expression, prevented from steatosis, and improved insulin sensitivity [41, 42]. Inhibition of SL synthesis by myriocin or siRNA mediated downregulation of SPTLC3 levels appears to induce similar response in vitro indicating that SPTLC3 could be a therapeutic target in metabolic conditions.

The association of SPTLC3 with cardiometabolic conditions in animal models is further supported by a recent clinical study including 2′302 ethnically Chinese Singaporean participants and a long-term follow-up of 11 years. Lipidomics analysis showed a significant correlation of C_{16}SO based sphingolipids with obesity and diabetes [43]. Unfortunately, the authors did not include C_{17}SO and meC_{18}SO based SL in their analysis but speculate on altered SPTLC3 function in response to metabolic conditions. It will be important to see whether obesity and insulin resistance correlate with increased hepatic SPTLC3 expression in humans as it was shown for mice.

The evidence that SPTLC3 related SNPs are determining tissue or plasma SL levels and also involvement in cardio-metabolic conditions is strong, but information on how these associations translate to enzymatic function and pathology is missing.

4.5 SPTLC3 in Other Diseases

Several mutations in SPTLC1 and SPTLC2 cause the rare peripheral neuropathy HSAN1 [6]. The

HSAN1 mutations shift the substrate preference of SPT towards L-alanine and glycine, leading to the formation of an atypical class of neurotoxic 1-deoxySL [6]. Plasma 1-deoxySLs are also increased under conditions of serine deficiency and in this context involved in the course of the rare retinopathy (macular telangiectasia type 2) and cancer [44, 45]. However, this alternative activity with L-alanine and glycine seems to be specific for SPTLC2 and was not yet reported for SPTLC3.

Recently, another group of mutations in SPTLC1 was associated with early onset of amyotrophic lateral sclerosis (ALS) [46]. In contrast to the SPT-HSAN1 mutations, the SPT-ALS mutations cluster in exon 2 of *SPTLC1* and result in a pathologically increased SL formation.

So far, no mutations in SPTLC3 have been associated with either HSAN1 or ALS. However, Gonzaga-Jauregui et al. reported a male patient (30 years) with a SPTLC3p.W150R variant, which was predicted to be loss of function mutation. This SPTLC3 variant was associated with a sensory neuropathy, deformation, and atrophy of the extremities and some bulbar involvement [47]. The authors showed that suppressing SPTLC3 expression in zebrafish causes defects in axons of motor neurons. Expression of human wild type SPTLC3 but not of the SPTLC3 p.W150R variant suppressed these defects [47]. Structurally, the mutation is replacing a hydrophobic residue (W150) with a positively charged arginine that might impair the binding of the hydrophobic substrate (Fig. 4.1, lower right). This indicates that SPTLC3 activity is also relevant to neuronal developmental and might contribute to C_{20} LCB formation in neuronal tissue, in particular as C_{20} LCB synthesis by SPTLC3 is greatly enhanced in presence of ssSPTb [9].

Taken together, it appears that SPTLC3 expression is involved in the generation of cell/tissue specific SL profiles. SPTLC3 activity might be modulated genetically but also by substrate availability and the presence of other components of the SPT complex. However, the mechanistic details of how SPTLC3 generated SLs affect metabolism, membrane integrity, and cell signaling need to be addressed in future work.

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Molecular Mechanisms of Sphingolipid Transport on Plasma Lipoproteins

5

Jahangir Iqbal, Ali Al Qarni, and Ahmed Bakillah

Abstract

Sphingolipids are biomolecules with diverse physiological functions in signaling as well as plasma membrane structure. They are associated with either cellular membranes or plasma lipoproteins and any changes in their levels may contribute to certain metabolic diseases. Sphingolipids are evenly distributed in lipoproteins and may be used as prognostic and diagnostic markers. Mechanisms involved in the transport of sphingolipids have been recently explored and here we discuss the most recent advances in the molecular mechanisms of sphingolipids transport by lipoproteins. It has been shown that microsomal triglyceride transfer protein (MTP) and ATP-binding cassette transporter family A protein 1 (ABCA1) play an important role in plasma sphingolipid homeostasis. However, the exact mechanisms are not well known. Though much research has already been done to emphasize the impact of sphingolipids changes in many pathological disorders, understanding mechanisms by which circulating lipoproteins assist in transporting sphingolipids may provide novel information that may help in devising strategies to therapeutically target these pathways to treat various metabolic disorders.

Keywords

ABCA1 · Abetalipoproteinemia · Ceramides · Hexosylceramides · Lipoproteins · MTP · Sphingolipids · Sphingomyelin · Sphingosine-1-phosphate · Tangier disease

Abbreviations

ABCA1	ATP-binding cassette transporter A1
apoA1	Apolipoprotein A1
apoB	Apolipoprotein B
apoM	Apolipoprotein M
Cer	Ceramides
CERT	Ceramide transfer protein
CETP	Cholesteryl ester transfer protein
C1P	Ceramide 1-phosphate
ER	Endoplasmic reticulum
GluCer	Glucosylceramides
HDL	High density lipoproteins
HexCer	Hexosylceramides
LactCer	Lactosylceramides
LCAT	Lecithin–cholesterol acyltransferase
LDL	Low density lipoproteins
MTP	Microsomal triglyceride transfer protein

J. Iqbal (✉) · A. Al Qarni · A. Bakillah
King Abdullah International Medical Research Center (KAIMRC)-Eastern Region, King Saud Bin Abdulaziz University for Health Sciences (KSAU-HS), Ministry of National Guard-Health Affairs (MNG-HA), Al Ahsa, Saudi Arabia
e-mail: iqbalja@ngha.med.sa

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PLTP	Phospholipid transfer protein
SR-B1	Scavenger receptor B1
SM	Sphingomyelin
SMS1	Sphingomyelin synthase 1
S1P	Sphingosine-1-phosphate
VLDL	Very low density lipoproteins

5.1 Introduction

Sphingolipids are not only involved in membrane organization as structural lipids but also act as essential signaling biomolecules [1]. The homeostasis of sphingolipids is maintained by regulating their synthesis and degradation in the cells. Ceramides (Cer) act as key precursors in the de novo synthesis of other sphingolipids such as glycosphingolipids, sphingomyelin (SM), sphingosine-1-phosphate (S1P), etc. [1]. Sphingolipids can also be synthesized through salvage pathway by the re-acylation of sphingosine in the lysosomes to form Cer or its derivatives [2]. Newly synthesized sphingolipids are secreted and transported by lipoproteins or remain associated within cells as structural lipids. In recent years new knowledge has been gained in understanding intracellular transport and secretion of different sphingolipids. Here, we summarize an overview of the most recent advances that have been made to understand pathways involved in the intracellular transport of sphingolipids by lipoproteins and highlight deficiencies that need to be resolved in future research.

5.2 Biosynthesis of Sphingolipids

Biosynthesis of sphingolipids involves either de novo synthesis in the endoplasmic reticulum (ER) or catabolism of other sphingolipids via the salvage pathway [1, 2]. Cer acts as central precursor molecules in the synthesis of other sphingolipids. They are transported from the ER to Golgi via either transport vesicles or ceramide transfer protein (CERT) [3]. The mode of transport from the ER to Golgi may determine the fate of Cer as a precursor molecule for biosynthesis of different sphingolipids [3]. CERT dependent transport of Cer from the ER to Golgi appears to be necessary for the synthesis of SM [4, 5] as dysfunctional CERT in cells reduces the levels of SM but not glucosylceramides (GluCer) [5]. Sphingomyelin synthase 1 (SMS1) inside the Golgi lumen helps in the synthesis of SM from Cer by transferring a phosphorylcholine group from phosphatidylcholine onto Cer [6, 7]. The synthesized SM is then transported to the plasma membrane to form lipid rafts with cholesterol [7]. Synthesis of GluCer from Cer that are transported via transport vesicles from ER to Golgi occurs on the cytoplasmic side of the *cis* Golgi. Translocation of Cer from the lumenal side to cytoplasmic side for the biosynthesis of GluCer involves possibly unidentified transporters. These glycosylated Cer can be translocated back to the Golgi lumen and transferred to the *trans* Golgi network by both vesicular and non-vesicular transport for the biosynthesis of lactosylceramides (LactCer) which are precursors for more complex glycosphingolipids [3]. Besides SM and GluCer, Cer can also be de-acylated in the ER to form sphingosine, which is further phosphorylated to form S1P. They can also be phosphorylated in the Golgi to form ceramide 1-phosphate (C1P) [8].

5.3 Lipoproteins as Lipid Transporters

Since lipids are insoluble in water, they need to associate with proteins in order to transport in the circulation [9, 10]. Lipoproteins are complex particles containing both polar and non-polar lipids along with apolipoproteins. Classification of lipoproteins is based on their size and flotation densities [9]. They are mainly classified into four major entities from low density to high density as chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDLs) [9]. Liver and intestine are the main two organs that produce these lipoproteins. Chylomicrons are the largest size triglyceride rich particles which share a common structural apolipoprotein, apolipoprotein B

(apoB), with VLDL and LDL. Conversely, HDL lacks this protein and contains apolipoprotein A1 (apoA1) as the core structural protein. Assembly of chylomicrons and VLDL takes place in the intestine and liver, respectively, and essentially requires the presence of MTP for the lipidation of apoB [11, 12]. Formation of LDL occurs after the hydrolysis of triglycerides on VLDL by the lipases. These LDL are then taken up by the peripheral tissues as a source of fatty acids through LDL receptors.

Unlike chylomicrons and VLDL, HDL is synthesized extracellularly after the secretion of apoA1 by the enterocytes and hepatocytes. ApoA1 from intestine and liver interact with the membrane bound ABCA1 and accept phospholipids and unesterified cholesterol to form discoidal pre-β HDL which is then converted to HDL in the plasma after the esterification of cholesterol by lecithin–cholesterol acyltransferase (LCAT) [13, 14]. HDL accepts more cholesterol from these peripheral tissues through ABCA1 and ABCG1 cholesterol efflux which is then delivered to the liver and steroidogenic tissues after the interaction with the scavenger receptor B1 (SR-B1) [15].

Lipoproteins play an important role in the absorption and transport of dietary lipids in the circulation to peripheral tissues. Any abnormality in the functioning of MTP or ABCA1 leads to conditions that affect the lipoprotein levels in the circulation. Abetalipoproteinemia is one of the rare diseases characterized by a defect in the assembly or secretion of plasma VLDL and chylomicrons due to mutations in MTP [16]. Similarly, mutation in ABCA1 leads to a condition called Tangier disease which is characterized by low HDL levels in the plasma [17].

5.4 Secretion and Transport of Sphingolipids

Plasma sphingolipids may be used as potential biomarkers for several metabolic disorders [6, 18–25]. However, there is not much information known about the mechanisms that regulate plasma sphingolipids. It was recently shown that the pathways involved in the transport of sphingolipids from the intestine and liver are more complex than those involved in other lipids [26, 27]. Like other lipids, sphingolipids also need apolipoproteins for their transport in the circulation. There are around 200 species of sphingolipids that are distributed on chylomicrons, VLDL, LDL, HDL, and albumin in the circulation [28–30]. These sphingolipids may be secreted from the cells and associated intracellularly with apoA1 or apoB-containing lipoproteins or effluxed to extracellular acceptors such as HDL or albumin from the plasma membrane. Here, we summarize the recent advances that have been made to understand the molecular mechanisms that are involved in the transport of sphingolipids.

5.4.1 Transport of Ceramides

Ceramides which constitute around 3% of plasma sphingolipids are equally distributed in apoB- (~49%) and apoA1-containing (~41%) lipoproteins (Table 5.1) [30]. However, similar to triglycerides, transport of Cer to the plasma is critically dependent on the presence of MTP in the liver and intestine. We have previously shown that mutation in *MTTP* gene in abetalipoproteinemia subjects or mice lacking hepatic and intestinal MTP (*L,I-Mttp*$^{-/-}$) resulted in ~82–95% decrease in plasma Cer compared to controls (Tables 5.2 and 5.3). Furthermore, tissue-specific ablation of MTP suggests that liver and intestine are both important in regulating the levels of Cer in the plasma [27]. This observation points to significant role MTP plays in Cer transfer to apoB-containing lipoproteins for their transport to plasma [26]. It is possible that other unknown proteins and mechanisms may exist to enrich lipoproteins with Cer. We have also shown that deficiency of ABCA1 in the intestine and liver tends to associate with lower plasma Cer levels (Table 5.4) [27], but the decrease was not as significant as that shown in MTP deficiency (Table 5.3) [26]. Similar findings were observed in Tangier subjects where the decrease in the plasma Cer level was only 35% as opposed to

Table 5.1 Distribution of sphingolipids on human plasma lipoproteins

	VLDL	LDL	HDL	HDL2	HDL3
Cer	8.8	39.9	—	28.7	22.6
SM	3.2	32.9	—	34.1	29.8
HexCer	8.0	49.1	42.0	—	—
LactCer	8.2	46.4	44.4	—	—
S1P	1.3	3.7	—	16.4	78.6

Data were partially adapted and modified from references [30, 34] and are presented as percent distribution among different lipoproteins

an 82% decrease in abetalipoproteinemia subjects compared to controls (Table 5.2). Interestingly, only hepatic ABCA1 and not intestinal ABCA1 play a major role in regulating the plasma Cer level (Table 5.4). Although ABCA1 may be involved in determining the plasma levels of Cer to some extent, but the data points towards MTP as being the major determinant of plasma Cer.

It is intriguing to see that transport and plasma levels of Cer are completely dependent on the presence of MTP and apoB-containing lipoproteins since Cer are synthesized by other cells also, apart from enterocytes and hepatocytes, that do not synthesize and assemble apoB-lipoproteins. Whereas hepatocytes and enterocytes may synthesize Cer for delivery to other tissues, it is possible that the non-apoB synthesizing cells may use Cer not for secretion but to synthesize other sphingolipids. Although the presence of MTP may be critical in determining the levels of plasma Cer, significant amounts of Cer are also found on HDL particles [30]. Not much is known about the mechanisms of incorporation of Cer onto HDL particles. It is likely that other plasma lipid transfer proteins such as phospholipid transfer protein (PLTP) which transfers phospholipids between HDL particles and cholesteryl ester transfer protein (CETP) which transfers cholesteryl ester from HDL to apoB-containing lipoproteins in exchange for triglycerides may transfer Cer from apoB-lipoproteins to HDL. Additionally, the degradation of SM by sphingomyelinases or direct efflux of Cer from plasma membranes may also contribute to HDL Cer. Further studies are needed to investigate all potential possibilities.

5.4.2 Transport of Sphingomyelin

Sphingomyelin constitutes around 20% of total phospholipids [31] and 87% of total sphingolipids [30] in the plasma. It is the most abundant sphingolipid in lipoproteins and is distributed around ~36% in VLDL/LDL and ~64% in HDL (Table 5.1) [30]. SM uses both apoB-dependent and apoB-independent pathways to reach circulation. Absence of MTP in abetalipoproteinemia subjects and liver and intestine (*L,I-Mttp*$^{-/-}$) mice was associated with a decrease of ~41% and ~59% (Tables 5.2 and 5.3), respectively, in plasma SM levels compared to controls [26]. Conversely, absence of ABCA1 in Tangier subjects and mice was associated with a reduction of about 40% and 86% (Tables 5.2 and 5.4), respectively, in plasma SM levels [27]. These data suggest that MTP and ABCA1 could play a major role in modulating plasma SM levels in both humans and mice. Interestingly, liver specific ablation of MTP or ABCA1 in

Table 5.2 Plasma levels of sphingolipids in Abetalipoproteinemia and Tangier disease subjects

	Cer (μM)	SM (μM)	HexCer (μM)	LactCer (μM)	S1P (μM)
Normal subjects	5.87	280	3.72	2.06	0.67
Abetalipoproteinemia	1.06 (−82%)	164 (−41%)	5.21 (+40%)	1.66 (−19%)	1.64 (+145%)
Tangier disease	3.84 (−35%)	167 (−40%)	1.15 (−69%)	3.64 (+77%)	1.19 (+78)

Data were partially adapted and modified from references [26, 27]
Values in parentheses represent percentage change from normal subjects

Table 5.3 Plasma levels of sphingolipids in MTP knockout mice

	Cer (μM)	SM (μM)	HexCer (μM)	LactCer (μM)	S1P (μM)
Wildtype	1.48	63.8	3.54	0.08	1.22
L-Mttp$^{-/-}$	0.44 (−70%)	19.6 (−69%)	ND	ND	0.93 (−24%)
I-Mttp$^{-/-}$	0.57 (−61%)	48.8 (−24%)	ND	ND	1.01 (−17%)
L,I-Mttp$^{-/-}$	0.07 (−95%)	26.4 (−59%)	2.85 (−20%)	0.11 (+38%)	0.69 (−43%)

Data were partially adapted and modified from references [26, 27]
Values in parentheses represent percentage change from wildtype mice
ND not determined, *L-Mttp*$^{-/-}$ liver specific MTP knockout, *I-Mttp*$^{-/-}$ intestine specific MTP knockout, *L,I-Mttp*$^{-/-}$ liver and intestine specific MTP knockout

mice decreased the plasma SM levels by ~69% and ~88% (Tables 5.3 and 5.4), respectively [27], suggesting that liver may be the major contributor of SM in the plasma.

Neither MTP nor ABCA1 is involved in the synthesis of Cer or SM. However, MTP has been shown to transfer both Cer and SM between vesicles in vitro, suggesting that it can add Cer and SM during apoB-lipoprotein synthesis [26]. Similar to its role in cholesterol efflux, we predicted that ABCA1 might also be involved in SM efflux. However, ablation, downregulation, or overexpression of *ABCA1* gene did not affect SM efflux to HDL suggesting that it may not directly participate in SM efflux [27]. These studies suggest that ABCA1 is indirectly involved in bringing SM to plasma compartment. It is possible that reduction of HDL due to absence of ABCA1 in Tangier subjects or ABCA1 ablated mice may indirectly diminish SM efflux to plasma HDL via an unknown membrane-embedded protein. Reduction in plasma SM in SMS2 knockout mice suggest that synthesis of SM by SMS2 on the plasma membrane may also contribute to HDL SM [32]. Furthermore, ability of PLTP to transfer SM between vesicles in vitro suggests that it may be involved in the transfer of SM from VLDL to HDL [33]. Further studies are needed to evaluate the possibility of other transporters in SM efflux.

5.4.3 Transport of Glycosphingolipids

Out of more than 50 species of complex glycosphingolipids that make up about 9–10% of plasma sphingolipids, the most abundant are GluCer and LactCer. They are mostly distributed on VLDL/LDL (~54–57%) and HDL (~42–44%) (Table 5.1) [30, 34]. Although reduction in plasma glycosphingolipid levels ameliorates atherosclerosis [35], very little is known about their origin in plasma lipoproteins. Unlike SM and Cer, MTP neither transfers glycosphingolipids between vesicles in vitro nor does its deficiency in humans and mice have any effect on plasma glycosphingolipids concentrations [26]. It is possible that glycosphingolipids may be effluxed to HDL in the plasma through yet unidentified protein(s) or mechanisms. ABCC1 and ABCA12 have been shown to transport GluCer in vitro [36] and in keratinocytes [37]. However, their role to efflux glycosphingolipids to apoA1 or

Table 5.4 Plasma levels of sphingolipids in ABCA1 knockout mice

	Cer (μM)	SM (μM)	HexCer (μM)	LactCer (μM)	S1P (μM)
Wildtype	1.55	98.4	5.62	0.10	1.18
L-Abca1$^{-/-}$	0.63 (−59%)	12.0 (−88%)	0.84 (−85%)	0.04 (−60%)	0.49 (−58%)
I-Abca1$^{-/-}$	1.36 (−12%)	114.0 (+16%)	6.48 (+15%)	0.11 (+10%)	1.55 (+31%)
L,I-Abca1$^{-/-}$	0.61 (−61%)	13.3 (−86%)	0.64 (−89%)	0.02 (−80%)	0.53 (−55%)

Data were partially adapted and modified from references [26, 27]
Values in parentheses represent percent change from wildtype mice
L-Abca1$^{-/-}$ liver specific ABCA1 knockout, *I-Abca1*$^{-/-}$ intestine specific ABCA1 knockout, *L,I-Abca1*$^{-/-}$ liver and intestine specific ABCA1 knockout

HDL in plasma is still unknown. We have recently shown for the first time that ABCA1 is a critical determinant of plasma glycosphingolipids [27]. Deficiency of ABCA1 in humans and mice was associated with reduced plasma hexosylceramides (HexCer) levels. A reduction of ~69–89% in the levels of HexCer in Tangier subjects and liver and intestine ABCA1 knockout (*L,I-Abca1*$^{-/-}$) mice (Tables 5.2 and 5.4) possibly suggest the existence of additional mechanisms in their transport from tissues to the plasma compartment. Hepatic ABCA1 plays a crucial role in determining plasma HexCer level since tissue-specific ABCA1 ablation in mice had predominant effect on their plasma levels (Table 5.4) [27]. In contrast, ablation or chemical modification of ABCA1 in Huh7 cells had no effect on GluCer efflux to HDL suggesting that ABCA1 may not be directly involved in their efflux [27]. These data may implicate that the reductions in plasma HexCer in ABCA1 deficient humans or mice may be secondary to low levels of HDL which may be needed to interact with different transporter proteins on plasma membranes for the efflux of HexCer. Further studies are needed to identify possible transporters that are involved in this process.

We have previously shown that plasma concentrations of LactCer in humans and mice differ significantly with humans containing higher concentrations of LactCer than mice [26, 27]. Furthermore, deficiency of ABCA1 reduces LactCer only in mice but not in humans (Tables 5.2 and 5.4) [27] suggesting that their levels may be regulated by different mechanisms. Conversely, studies have shown that deficiency of MTP in mice and humans has no effect on plasma LactCer (Tables 5.2 and 5.3) [26]. One plausible explanation is that ABCA1 and MTP probably do not play a significant role in regulating plasma LactCer level. Further studies are needed to determine potential proteins that are involved in this process.

5.4.4 Transport of Sphingosine 1-Phosphate

Sphingosine 1-phosphate is a bioactive signaling molecule abundant in plasma [38] and found mainly on HDL and albumin [29, 39]. Among all the lipoproteins, about 95% of S1P is present on HDL (Table 5.1). Majority of plasma S1P comes from erythrocytes, platelets, and endothelial cells [39, 40] with some contribution from liver [41]. In our recent studies, we have shown that abetalipoproteinemia or Tangier subjects have no significant difference in the levels of S1P compared with controls suggesting that neither MTP nor ABCA1 may be involved in the transport of S1P in humans (Table 5.2) [26, 27]. Even though we observed a decrease of around 43–55% in the plasma SIP levels in MTP and ABCA1 deficient mice (Tables 5.3 and 5.4), this difference was not statistically significant [26, 27]. These data suggest that MTP and ABCA1 may not play a significant role in S1P transport from the cells to the circulation. Several proteins have been implicated in regulating the levels of plasma S1P. Cell surface membrane protein, spinster2, exports S1P to plasma [42]. Apolipoprotein M (apoM) plays an important role in the transport of S1P to plasma [43, 44]. Besides apoM, PLTP has also been shown to play a role in regulating plasma S1P levels [45].

5.5 Conclusion

In conclusion, we have summarized here that dependence of sphingolipids on lipoproteins as transporters is different among sphingolipids family. Sphingolipids utilize different mechanisms and carriers for their transport from cell membrane to plasma. Although MTP and ABCA1 seem to play an important role in regulating the levels of plasma sphingolipids (Fig. 5.1), their transport involve more complex and diverse mechanisms than are known for glycerolipids and sterol transport through apoB-lipoproteins or HDL. ABCA1- and MTP-dependent pathways may not be universal for all sphingolipids. Some of the sphingolipids such as LactCer, sphingosine, S1P, dihydrosphingosine, dihdyrosphigosine-1P, and dihydroceramide may reach circulation independently of these pathways [26, 27].

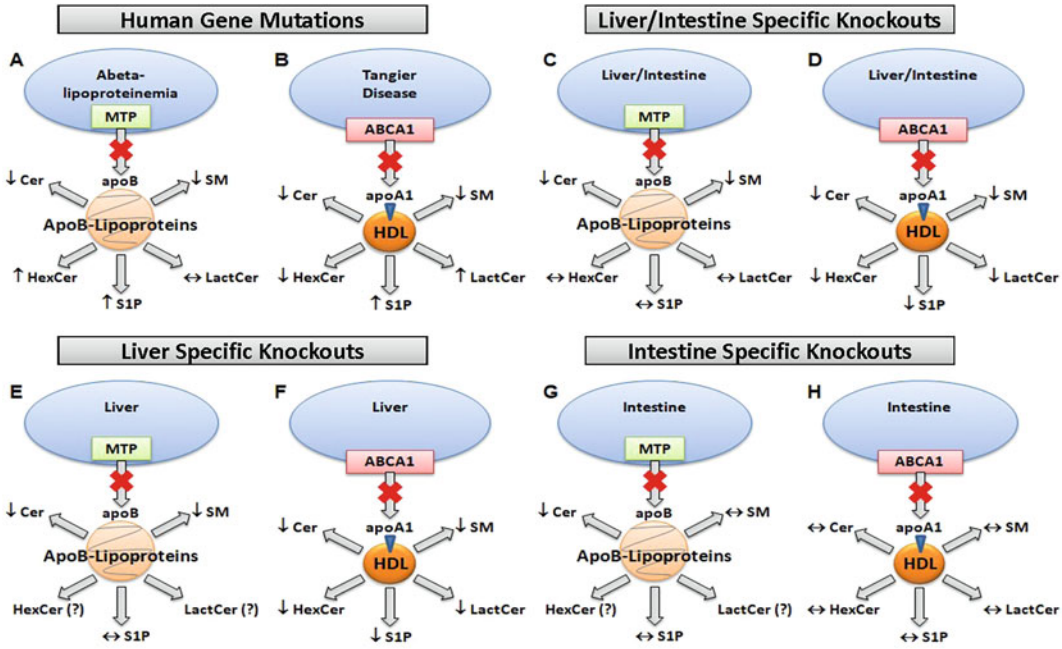

Fig. 5.1 Impact of MTP and ABCA1 deficiencies on plasma sphingolipids transport. MTP and ABCA1 play an important role in the regulation of sphingolipids in the plasma mainly through their transport to apoB-lipoproteins or HDL. Presence of MTP and ABCA1 is critical for the transport of Cer and SM to plasma in humans and mice (**a**–**d**). Levels of Cer in the plasma are changed by both hepatic (**e**) and intestinal (**g**) MTP activity. Conversely, only hepatic (**f**) and not intestinal (**h**) ABCA1 regulates Cer level in the plasma. Furthermore, levels of SM in the plasma are modulated by the presence of MTP and ABCA1 in the liver only (**e**, **f**) and not in the intestine (**g**, **h**). In contrast to SM which is regulated by both hepatic MTP and ABCA1, only hepatic and not intestinal ABCA1 regulates the levels of HexCer, LactCer, and S1P in the plasma (**f**)

Our recent findings suggest that transport of sphingolipids apparently involves at least three different mechanisms (Fig. 5.1). First, MTP may contribute to plasma levels of sphingolipids by directly transferring Cer and SM to apoB-containing lipoproteins [26]. Second, HDL may act as an acceptor for Cer, HexCer, and SM from cells [27]. Third, some of the sphingolipids such as LactCer and S1P may reach circulation via different pathways independently of apoB-containing lipoproteins and HDL [26, 27]. The reason why sphingolipids use different mechanisms for their transport might be related to their diverse functions. These new findings highlight the importance of plasma lipoproteins in transporting sphingolipids and provide novel insights into mechanisms of sphingolipids secretion and transport. Further studies are warranted to unravel the existence of multiple mechanisms involved in the sphingolipids transport pathways.

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Sphingosine 1-Phosphate Metabolism and Signaling

6

Yan Hu and Kezhi Dai

Abstract

Sphingosine 1-phosphate (S1P) is a well-defined bioactive lipid molecule derived from membrane sphingolipid metabolism. In the past decades, a series of key enzymes involved in generation of S1P have been identified and characterized in detail, as well as enzymes degrading S1P. S1P requires transporter to cross the plasma membrane and carrier to deliver to its cognate receptors and therefore transduces signaling in autocrine, paracrine, or endocrine fashions. The essential roles in regulation of development, metabolism, inflammation, and many other aspects of life are mainly executed when S1P binds to receptors provoking the downstream signaling cascades in distinct cells. This chapter will review the synthesis, degradation, transportation, and signaling of S1P and try to provide a comprehensive view of the biology of S1P, evoking new enthusiasms and ideas into the field of the fascinating S1P.

Keywords

Sphingosine 1-phosphate · Sphingolipid metabolism · Receptor signaling

Y. Hu · K. Dai (✉)
Department of Psychiatry, School of Mental Health, Wenzhou Medical University, Wenzhou, Zhejiang, PR China
e-mail: daikezhi@wmu.edu.cn

6.1 Synthesis of S1P

The synthesis of S1P is illustrated in Fig. 6.1. Although it has been documented that the synthesis of S1P could occur intra- and extracellularly [1], recent reports favor the notion that intracellular synthesis is the major contribution of S1P production under physiological condition [2]. The instant reaction to produce S1P from sphingosine involves two characterized kinases, namely sphingosine kinase (SK or SphK) 1 and SK2 [3–6].

6.2 S1P Kinases

Although two human genes encoding SK1 and SK2 are located on different chromosomes with *SK1* on the chromosome 17 and *SK2* on the chromosome 19, they are evolutionarily conserved lipid kinases with five highly conserved motifs (named C1–5) [7, 8]. Among them, a C4 motif is considered as unique to the SKs superfamily [8] and confines substrate recognition [9]. Analysis of crystal structure of SK1 determined a two-domain architecture model with the C-terminal domain accommodating a substrate-binding pocket formed by C4 and C5 motifs [10], providing a prototype for understanding the molecular basis of the catalytic sites and substrate specificity of SKs. Both SK1 and SK2 phosphorylate dihydro- and sphigosine, although phosphorylation catalyzed by SK1 is much more

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efficient than by SK2 [11]. SK2 has broader substrate spectrum, including phytosphingosine [6] and the spingoid base analog FTY720 or its derivative [12–15].

Bulk majority of SK1 is present in the cytosol; however, a small portion of SK1 is translocated to the plasma membrane, cytoskeleton, and intracellular organelles, such as endoplasmic reticulum (ER) and phagosome upon stimulation [16–18]. The compartmentalization and translocation of SK1 not only make it closer to its substrate, sphingosine, which is embedded in the lipid bilayer, but encounter with its regulators [8]. For example, extracellular signal-regulated kinase (ERK) 1/2-mediated phosphorylation of SK1 at Ser225 promotes conformational change of SK1, which leads to plasma membrane translocation and an increase of catalytic activity [19]. Moreover, interacting with the calcium- and integrin-binding protein 1 is required for induction of membrane translocation of SK1 in a calcium-dependent manner [20]. In contrast, SK2 was found shuttling between the cytoplasm and the nucleus [19]. SK2 has 234 more amino acids (AAs) than that of SK1 (384 AAs), in which additional nuclear localization and exportation signal motifs are located [21, 22]. The S1P generated in the nucleus was reported to involve in epigenetic regulation of gene transcription [23]. SK2 is also found in intracellular organelles, including mitochondria [24] and ER [25]. Interestingly, apoptotic cells release SK2, which in return increases local S1P concentration in regulation of immune responses [26]. These in vitro studies indicate that SK1 and SK2 may regulate different pools of S1P despite many shared properties.

Indeed, studies from knockout (KO) mouse models showed that Sk1 and Sk2 contributed differently to tissue and serum levels of S1P, but share some redundant functions, which allowed them to compensate each other for the single KO mice to survive since none of the double KO mice survived beyond embryonic day 13.5 due to intracranial bleeding [27, 28]. The single KO mice have normal or slightly reduced S1P levels in most tissues, while the serum S1P in Sk1-KO mice was reduced by 50% while the Sk2-KO mice has an increased serum S1P level [29, 30]. However, the lymphocyte cellularity and distribution in the lymphatic organs, which is controlled by S1P, was not affected in the Sk1-KO mice, indicating that reduced serum pool of S1P was sufficient for the lymphocyte trafficking [27]. Further studies found that Sk1-KO mice exhibited normal immune responses in mouse model of thioglycollate-induced peritonitis or collage-induced arthritis [31]. In other words, the S1P gradient (will discuss in detail below) regulating lymphocyte traffic was not disrupted by Sk1 deletion. Instead, the reduced serum S1P level by Sk1 deletion resulted in more severe neuroinflammation in a LPS-induced brain injury model [32], whereas the increased serum S1P by Sk2 deficiency reduced atherosclerotic lesions in a mouse model of atherosclerosis [30], suggesting the serum level of S1P is rather likely affecting vascuar functions via endothelail $S1P_1$ [33].

6.3 S1P Degradation

S1P level is determined by the balance of synthesis and degradation (Fig. 6.1). As discussed above, the synthesis of S1P is mainly catalyzed by two specific kinases; the degradation of S1P also requires several designated enzymes. On the one hand, S1P is dephosphorylated to sphingosine by three lipid phosphate phosphatases (LPPs, encoded by *Lgpps* in mouse genome) and two S1P-specific phosphatases (SPPs, encoded by *Sgpps* in mouse genome) identified up to date [34]. On the other hand, S1P is irreversibly hydrolyzed by the S1P lyase (SPL, encoded by *Sgpl1* in mouse genome) [35].

6.4 S1P Phosphatases

Both SPPs and LPPs belong to the type 2 lipid phosphate phosphohydrolases family with three conserved domains at the predicted enzyme active site, despite LPPs have broader substrate specificity than that of SPPs [34, 36]. LPPs are located on the plasma membrane with its active site on the outer leaflet and thus dephosphorylate

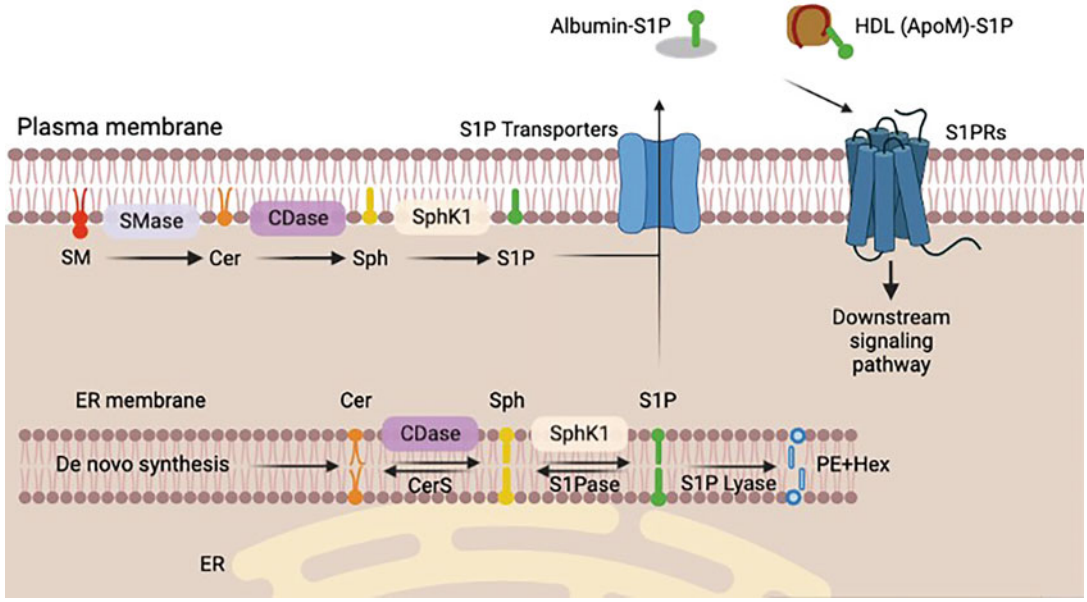

Fig. 6.1 Schematic presentation of intracellular metabolism of sphingolipid and extracellular transportation of S1P. In the ER, ceramide (Cer) generated from the de novo synthesis pathway is hydrolyzed by ceramidase (CDase) to yield sphingosine (Sph) which in return is phosphorylated by Sph kinase 1 (SphK1) to produce S1P. S1P is constantly dephosphorylated by S1P phosphatase (S1Pase) to recycle Sph back to synthesis of sphingolipid. Conversely, S1P is degraded by S1P lyase to yield phosphoethanolamine (PE) and hexadecenal (Hex). The two end products of S1P breakdown are consumed by a salvage pathway to enter the cycle of phospholipid metabolism. In the plasma membrane, sphingomyelin (SM) is catalyzed by sphingomyelinase (SMase) and subsequentially CDase to yield Sph for SphK1 to generate S1P. The intracellular synthesized S1P is actively transported across the plasma membrane by designated S1P transporters and transfer onto carriers to produce albumin-S1P and ApoM-S1P for signaling transduction when they bind to S1P receptors (S1PRs)

the extracellular pool of S1P [36]. Other substrates of LPPs include lysophosphatidic acid (LPA), phosphatidate, ceramide 1-P, and FTY720-P. And mouse genetic studies suggest that LPA is likely the favorable substrate of LPPs [36]. In contrast, SPPs are more substrate-specific phosphatases that dephosphorylate S1P, dihydro-S1P, and phyto-S1P, mediating the recycling of sphingoid bases into different metabolic pathways [37–39]. Mammalian SPPs locate in the ER and consist of two isoforms, SPP1 and SPP2 [34]. SPP1 regulates the intra- and extracellular levels of S1P and is essential for cell viability in vitro [40]. Gene-deletion studies revealed that *Sgpp1*-KO mice developed an ichthyosis-like phenotype a few days after birth; *Sgpp1*-KO keratinocytes had increased intracellular level of S1P and accelerated cellular differentiation [41]. *Sgpp2*-KO mice exhibited normal pancreatic islet size but defective β-cell proliferation when challenged with either high-fat diet or a specific β-cell toxin; mechanistic studies showed that SPP2 plays pivotal role in ER stress and proliferation of the β-cell [42]. These findings indicate that LPPs and SPPs are dispensable for the S1P levels in tissues or the circulation.

6.5 S1P Lyase

Unlike LPPs and SPPs that recycle sphingosine, SPL cleaves S1P to yield ethanolamine phosphate and hexadecanol taking the sphingoid base out of recycling [43]. SPL is a single-pass ER membrane protein exerting its enzymatic activity in homodimers [44] and is highly expressed in

tissues with rapid cell turnover, including small intestines, colon, thymus, and spleen [45]. High level of SPL expression is associated with low level of S1P in the tissues whereas its absence in the red blood cells (RBCs) and platelets maintains high S1P level in the plasma. Therefore, SPL is the key enzyme guarding the chemotactic S1P gradient between tissue and blood, enabling egress of lymphocytes [46].

The pivotal role of SPL in regulation of development, metabolism, and inflammation has been illustrated in *Sgpl1*-KO mouse models [47]. Besides lymphopenia, the *Sgpl1*-KO mice were reported to manifest developmental retardation, thymic atrophy, neutrophilia, hyperostosis, and nephrosis [48–52]. Lack of SPL leads to intra- and extracellular accumulation of S1P which has toxic effects [53], as well as dysregulation of global lipid metabolism, such as increased ceramides, sphingomyelins, phospholipids, triacyglycerols, diacylglycerols, and cholesterol esters [52]. In a mouse model of chemically induced colitis, the intestinal epithelial cell-specific deletion of *Sgpl1* led to more severe disease conditions and promoted colon carcinogenesis [54]. Gene polymorphisms associated with *SGPL* have been shown to be related to the susceptibility of late-onset Alzheimer's disease [55]. Recent discoveries about a new inborn error of sphingolipid metabolism caused by loss-of-function mutations of *SGPL* closely translated the previous findings obtained from cultured cells and mouse models, indicating that SPL is essential for sphingolipid homeostasis.

6.6 Transportation of S1P and Transporters

S1P contains a hydrophilic head group and a hydrophobic tail, which renders itself water incompatible. S1P requires designated binding partners, like transporters and carriers (Fig. 6.1), to move around along the circulation.

As a lysophospholipid molecule, intracellular S1P requires transporters to export across the cytoplasmic membrane. Initially, in vitro studies suggested that some of the ATP-binding cassette transporter family members were candidates as S1P transporters and might be responsible for exporting S1P to plasma. However, results of S1P measurement in plasma from gene-deletion mouse model ruled out this possibility (reviewed by Hla et al. [56]). The protein spinster homolog 2 (Spns2) is the first identified mammalian S1P-specific transporter [57–60]. Spns2 is enriched in vascular and lymphatic endothelial cells (LECs), regulating S1P concentration mainly in the lymph [60]. Spns2 in LECs is essential to maintain S1P gradient between lymphatic tissue and lymph and governs lymphocyte migration and survival [60–62], suggesting Spns2 is functioning on the apical membrane of LECs. A recent study, interestingly, provided evidence that Spns2 partners with the major facilitator superfamily domain-containing protein 2a (Mfsd2a) to release S1P into basolateral space of mouse brain ECs and to preserve the blood brain barrier function [63]. Mechanism underlying membrane localization of Spns2 in different ECs merits further investigation. Mfsd2b is the second definitively verified S1P transporter which is required for exporting S1P out of RBCs, sustaining static plasma S1P concentration [64]. Under thrombosis condition, Mfsd2b is required for the activated platelets to release S1P [65]. Mouse genetic studies on Mfsd2b and Spns2 have fortified the notion that RBC-S1P is the most important source for the plasma S1P, whereas the LEC-S1P is the major contributor of the lymphatic S1P. Therefore, targeting specific S1P transporters might be a promising therapeutic strategy to differentially inhibit S1P signaling on different cell types [65, 66].

Both transporters are multi-pass transmembrane proteins and mainly expressed on the cell surface [64, 67, 68]. Two recent reporters on 3D structure of Mfsd2a orthologs revealed the tropisms and critical amino acids of the lysolipid-binding cavity of MFD family proteins [69, 70], providing analogous blueprint for the substrate-binding cavity of Spns2 and Mfsd2b. Based on the resolved crystal structure of Mfsd2a [70] and computational predictions by AlphaFold [71], one may postulate that the

S1P-binding pockets of Spns2 and Mfsd2b are composed of 12 transmembrane helices divided into two pseudo-symmetric six-helices bundles. Several pocket-lining amino acids of human MFSD2B derived from homologous analysis with MFSD2A are embedded in the transmembrane helixes and critical for substrate export [69, 70], suggesting that S1P gains access into the binding pocket is likely from within the membrane bilayer [64]. However, further investigation is obligated to justify the amino acids which define the S1P-binding specificity.

6.7 S1P Carriers

As an amphipathic lipid in nature, there is no free S1P in the aqueous phase. It has been estimated that ~54% of plasma S1P is associated with HDL, ~35% is bound to albumin, and the rest might associate with other lipoproteins [72, 73]. The HDL-associated apolipoprotein M (apoM) is the solo high affinity S1P-binidng protein identified to date with a well-defined binding pocket inside [74]. ApoM increased not only S1P in the plasma by increasing its solubility and protecting it from degradation, but also modulate the homeostasis of plasma S1P levels [74, 75]. ApoM regulates S1P function by facilitating its interaction with its receptors, thus enhances its biological functions in maintaining endothelial barrier and limiting endothelial inflammation [33, 76, 77]. Recently, an engineered S1P chaperone apoM-Fc was reported to efficiently activate S1P receptors in vitro and in vivo, providing potential therapeutic strategy for hypertension and ischemia-reperfusion injury of vital organs [78]. However, how and when S1P gets into apoM remain mysterious.

In contrast to the specific binding of apoM and S1P, albumin-associated S1P can be more considered as its free form [79]. The presence of albumin may only increase the solubility of S1P instead of actual physical binding. Further studies showed that albumin-associated S1P plays a controversial role in the pathogenesis of atherosclerosis, comparing with the protective role of apoM/HDL-associated S1P [80]. Nevertheless, fair amount of serum S1P is still present in the double KO of *apoM* and *albumin* mice [73] rendered further investigation for precise regulation of serum-S1P.

6.8 S1P Signaling Receptors

The extracellular S1P evokes intracellular signaling transduction via its five high affinity cognate receptors, named as $S1P_1$-$S1P_5$ [81]. And these receptors belong to the class A G-protein coupled receptor (GPCR) family [82]. The cell-type specific and dynamic expression of these five receptors differs vastly in various stages of life. Numerous publications have reported very broad expression and functionalities of $S1P_1$, $S1P_2$, and $S1P_3$ [83]. The confirmed cell types include, but not limited to, endothelial cells (ECs), immune cells, neural cells, myocytes, and fibroblasts. Interestingly, the same receptor residing on different cell types exerts opposite or similar regulatory consequences, while different receptors expressed on the same cell type may act opposingly or coordinatingly [83]. For instance, activation of $S1P_1$ in EC and macrophage is anti-inflammatory, whereas activation of astrocyte-$S1P_1$ increases release of pro-inflammatory cytokines; $S1P_1$ promotes vascular barrier function, while $S1P_2$ and $S1P_3$ damage the barrier in ECs; $S1P_2$ and $S1P_3$ function cooperatively to induce fibrosis in various tissues [84–87]. Recent studies using $S1P_1$ reporter mouse models presented exciting observations of spatial-temporal activation of $S1P_1$ related to endothelial transcriptional regulation [88] and immune cell maturation [89], which opens a new avenue for studying the heterogeneity of S1P/$S1P_1$ signaling.

$S1P_1$ is one of the most abundant and studied GPCRs. Many earlier effects have been focused on the binding partners (CD44, CD69, and β-arrestin), downstream signaling effectors ($G\alpha_i$, ERK, PI3K, Akt, phospholipase C, and Rac), and enzymatic modifiers of the intracellular domains of $S1P_1$ (Akt, GPCR kinase 2, and DHHC5). With more advanced genetic and proteomic screening technologies, LPA receptor 1 [90] and endoglin [91] were recently identified as the

crosstalk receptors of S1P/$S1P_1$ signaling. The former is also a GPCR acting upstream of $S1P_1$ to suppress S1P/$S1P_1$ signaling in lymphatic ECs [90]. The latter is an auxiliary receptor of TGF-β superfamily members acting downstream of $S1P_1$ to regulate EC barrier function and angiogenesis [92]. Although the crystal structure of $S1P_1$ was resolved together with a fusion protein tag and an antagonist, it at least provided a prototype for understanding how S1P gains access to its receptors and the substrate-binding pocket [93]. The authors suggested that S1P might slide into the amphipathic pocket, lined with charged and polar amino acid residues for the head of phospho-sphingolipid, from within the membrane bilayer. However, how the exogenous S1P maneuvers its way into the pocket demands more experimental innovations and logical inductions.

In contrast, expression of $S1P_4$ and $S1P_5$ is restricted to certain cell types, for instance, $S1P_4$ in myeloid cells, including T cell, B cell, macrophage or monocyte, neutrophil, eosinophil, mast cell, and dendritic cell [81, 83]. Functions of $S1P_4$ have been implicated in regulation of cell trafficking and differentiation [51, 94]. Expression of $S1P_5$ is limited to NKT cell [95] and oligodendrocyte [96, 97] though its mRNA was found in other myeloid cell types. However, the proposed function of $S1P_5$ has encountered contradictory findings [83]. Further experiments are required to sort out the role of $S1P_5$ signaling.

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Sphingomyelin Synthase Family and Phospholipase Cs

7

Xian-Cheng Jiang and Yeun-po Chiang

Abstract

The sphingomyelin synthase (SMS) gene family has three members: SMS1 and SMS2 have SM synthase activity, while SMS-related protein (SMSr) has no SM synthase activity but has ceramide phosphorylethanolamine (CPE) synthase activity in vitro. Recently, we found that SMS family members are a group of phospholipase Cs (PLC). SMS1 and SMS2 are two phosphatidylcholine (PC)-PLCs and SMSr is a phosphatidylethanolamine (PE)-PLC. SMS family members not only influence SM levels but also influence the levels of diacylglycerol (DAG), PC, PE, and glycosphingolipids, thus influencing cell functions. In this chapter, we will discuss the recent progress in the research field of SMS family and will focus on its impact on metabolic diseases.

Keyword

Sphingomyelin synthase · Phospholipase C · Metabolic diseases

Abbreviation

PC Phosphatidylcholine
PE Phosphatidylethanolamine
PLC Phospholipase C
SMS Sphingomyelin synthase

7.1 Sphingomyelin Synthase 1

Sphingomyelin (SM) biosynthesis occurs via the actions of serine palmitoyltransferase (SPT), 3 ketosphinganine reductase, ceramide synthase, and dihydroceramide desaturase to produce ceramide, which is a substrate for the production of sphingomyelin (through sphingomyelin synthase, SMS) (Fig. 7.1) [1, 2]. SM synthase has two isoforms, SMS1 and SMS2 [2, 3]. SMS1 is encoded by the *SGMS1* gene which is located on human chromosome 11 (mouse chromosome 19), with 11 exons [4]. Human SMS1 consists of 413 amino acids [2, 3]. Various alternative SMS1 mRNA transcripts, with different exon combination and length of 5′- or 3′-UTR, were found [5]. Truncated SMS1 mRNA transcripts which do not yield full-length SMS1 protein were also discovered [6, 7]. It has been reported that certain circular noncoding RNAs (circRNAs), which contain sequences of 5′-UTR and/or exonic portions of the *SGMS1* gene, were found in human, rat, and mouse tissues [8]. These SMS1 circRNAs might play a role in the regulation of SMS1 expression, through binding certain microRNAs [8]. Human SMS1 translation could be regulated through its mRNA 5′-untranslated region [4].

X.-C. Jiang (✉) · Y.-p. Chiang
Department of Cell Biology, SUNY Downstate Health Sciences University, Brooklyn, NY, USA
e-mail: xian-cheng.jiang@downstate.edu

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The three-dimensional structure of SMS remains unresolved so far. Thus, little is known about its overall folding and structure of catalytic site. However, there are two computer models that have been reported. Using two proteins (with known structures) as templates and combined with homology and molecular dynamics, a computer model of human SMS1 was proposed by us 12 years ago [9]. The conserved Histidine 285, Histidine 328, and Aspartate 332 along with the respective distances between residues are depicted [9]. A recent study also proposed another human SMS1 computer model which showed that (1) the residence time of PC is shorter than SM; (2) SMS1 binding site is extremely conserved and three amino acids, Aspartate 101, Arginine 220, and Asparagine 358, are involved in SM synthesis; and (3) the hydroxylation of PC increases the rate of conversion from PC into SM [10].

SMS1-generated SM plays an important role in transferrin trafficking and cell proliferation [11] as well as involved in attachment and infection with Japanese encephalitis virus [12]. SMS1 deficiency reduces bone formation due to impaired osteoblast differentiation [13]. A very recent report indicated that SMS1 mediates hepatocyte pyroptosis to trigger nonalcoholic steatohepatitis [14]. SMS1 is essential for male fertility in mice [15]. We and another research group reported that global SMS1 deficiency exhibited moderate neonatal lethality [16, 17], i.e., 25% of homozygotes die during the first 3 weeks (the remainders can grow to adulthood). *Sms1* knockout (KO) mouse homozygous crosses did not yield viable progeny [17]. It has been reported that *Sms1* KO mice exhibited reduced body weight, β cell mitochondrial dysfunction, insulin secretion inhibition, and insulin resistance [16]. *Sms1* KO mice have lipodystrophy which is related with an induction of oxidative stress in adipose tissues [18]. SMS1 is the major isoform in macrophages, while SMS2 is the major one in the liver [17]. The expression levels of both in the rest of tissues are comparable [17]. To evaluate atherogenicity, we transplanted *Sms1* KO mouse bone marrow into LDL receptor KO mice ($Sms1^{-/-} \rightarrow Ldlr^{-/-}$). After 3 months with a Western type diet, these animals showed a significant decrease of atherosclerotic lesions in the root and the entire aorta, compared with WT→$Ldlr^{-/-}$ mice [17].

Based on hydrophobicity analysis, SMS1 contains six transmembrane α-helices connected by extramembrane loop domains with a sterile alpha motif (SAM) at its N-terminal [2]. The SAM domain plays an important role in cell functions, such as development, signal transduction, and transcriptional regulation, through protein/lipid and protein/protein interactions [19]. However, the proteins interacting with SAM domain of SMS1 have not been identified until recently. It is known that both SMS1 and glucosylceramide synthase (GCS) are involved in a sphingolipid metabolic branch point (Fig. 7.1). SMS1 catalyzes the transfer of phosphocholine from phosphatidylcholine to ceramide to form SM, whereas GCS catalyzes the transfer of glucose from UDP-glucose to ceramide to form glucosylceramide (GluCer) [20]. Similar to SMS1, GCS is located in the Golgi apparatus [21]. There is an interaction between the SMS1

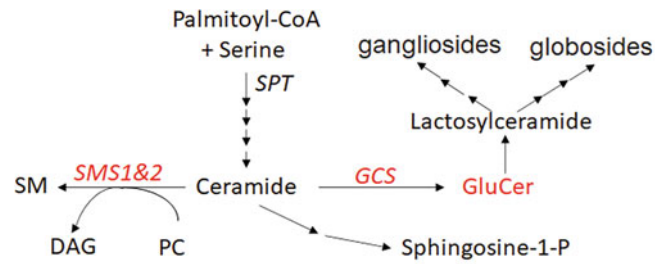

Fig. 7.1 Scheme for ceramide, SM, and GluCer formation

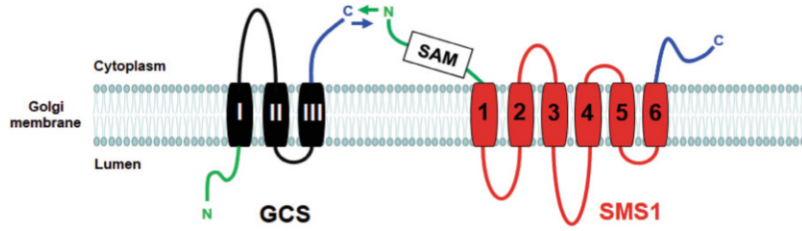

Fig. 7.2 SMS1 and GCS complex formation on Golgi membrane

N-terminal SAM domain and GCS C-terminal domain to form a SMS1-GCS complex in the Golgi (Fig. 7.2), controlling the metabolic fate of ceramide in the organelle [22]. The formation of the SMS1-GCS complex increases SM synthesis and decreases GCS synthesis [22]. In normal tissue, the formation of the SMS1-GCS complex can serve as a switch that controls the steady state levels of GluCer. However, the complex in the situation of SMS1 deficiency is depleted, thereby greatly releasing GCS activity and promoting GluCer biosynthesis, which was observed in our study [17].

Very recently, we studied the metabolic consequences of SMS1 deficiency-mediated GluCer accumulation. Liver-specific *Sms1*/global *Sms2* double-knockout (dKO) exhibited severe liver steatosis under a high-fat diet. Further, old (more than 6-month-old) dKO mice had liver damage, inflammation, and fibrosis, compared with *Sms2* KO and wild type mice. RNA sequencing analysis indicated the induction of various genes which are involved in lipogenesis, inflammation, and fibrosis. Furthermore, we found that GluCer administration promoted hepatocyte to secrete more activated TGFβ1, which could stimulate more collagen 1α1 production in hepatic stellate cells, thus promoting fibrosis. Additionally, GluCer accelerated more β-catenin translocation into the nucleus, thus promoting tumorigenesis. Significantly, human nonalcoholic steatohepatitis patients had higher levels of liver GCS and higher levels of serum GluCer. Our observation implicated that GluCer accumulation is one of the triggers stimulating the development of nonalcoholic fatty liver disease into nonalcoholic steatohepatitis, then, fibrosis and tumorigenesis [23].

7.2 Sphingomyelin Synthase 2

SMS2 is encoded by the *SGMS2* gene which is located on human chromosome 4 (mouse chromosome 3) with consist of 365 amino acids [2, 3]. SMS2 shares highly identical in protein sequence with SMS1 but has no SAM domain in its N-terminal. Same as SMS1, SMS2 is predicted as a six-pass transmembrane protein but predominantly localized on plasma membrane, with a minority portion found in Golgi [9]. Thus, SMS2 contributes mainly to plasma membrane SMS activity [2, 3]. While SMS1 plays a role of SM producer, SMS2 was shown mostly a regulator of the SM in plasma membrane [2, 3]. We and other researchers have shown that SMS2 expression positively correlates with SM levels in the lipid rafts of plasma membrane [24–26]. SMS2 is required for the maintenance of plasma membrane microdomain fluidity [27] and is directly linked to cell membrane lipid messengers that play a role in cell survival and apoptosis [24, 28]. In contrast to the neonatal lethality found in *Sms1* KO mice, *Sms2* KO mice are health and viable [29, 30].

SMS2 is one of the major determinants for plasma and liver SM levels in mice [29]. We and other researchers have found that SMS2 deficiency prevented high-fat diet-induced obesity and insulin resistance [30, 31]. Moreover, we found that liver SMS2 overexpression promoted fatty acid uptake and liver steatosis, while SMS2 deficiency had an opposite effect, in comparison with their respective controls. Importantly, the exogenous ceramide supplementation to Huh7 cells (a human hepatoma cell line) reduced the expression of PPARγ2 and its target genes, CD36 and FSP27 [32]. Thus, SMS2 deficiency-

mediated ceramide induction suppressed lipogenesis in hepatocytes [32]. We prepared *Sms2* and *Apoe* double-knockout (KO) mice. They showed a significant decrease in plasma lipoprotein SM levels. There was a reduction of atherogenic lipoprotein aorta retention in the double KO mice. Importantly, the double KO mice showed a significant reduction in atherosclerosis, compared to controls [33].

G-protein coupled receptor family C group 5 member B (GPRC5B) recruitment of Src family kinases has been implicated in diet-induced insulin resistance [34]. GPRC5B-mediated phosphorylation of sphingomyelin synthase 2 (SMS2) by Fyn is a crucial step in the development of insulin resistance [35]. SMS2 deficiency inhibits osteoclastogenesis by decreasing RANKL expression in mouse primary osteoblasts [36]. A recent paper reported that SMS2 deficiency suppresses steatosis but worsens fibrosis in the liver in a specific condition with choline-deficient, L-amino acid-defined, high-fat diet [37]. In a very recent study, we indicated that SMS2 deficiency ameliorated cerebral ischemic reperfusion injury through reducing the recruitment of toll-like receptor 4 to lipid Rafts [38]. We also prepared liver-specific *Sms1*/global *Sms2* double KO mice to evaluate the effect of hepatocyte SM biosynthesis in lipoprotein metabolism. We found that 2-month-old double KO mice significantly reduced their hepatocyte SM levels and reduced very low-density lipoprotein production [39]. This phenomenon could be related with a reduction of atherogenicity.

In general, SMS2 deficiency decreases dietary induced obesity, insulin resistance [31], and atherosclerosis [33, 40]. We and others have suggested that SMS2 could be a therapeutic target for metabolic diseases, including NAFLD, type 2 diabetes, and atherosclerosis [30–33, 41–43]. In fact, there are some progress in the development of SMS2 specific inhibitors. A selective SMS2 inhibitor ameliorates diet-induced insulin resistance via the IRS-1/Akt/GSK-3β signaling pathway [44]. The *db/db* mouse is an animal model for diabetic dyslipidemia [45]. A specific SMS2 inhibitor reduced chronic inflammation in *db/db* mice [46]. We have a specific chapter to discuss this aspect.

It has been reported that the potent anticancer property of 2-hydroxy-oleic acid (2OHOA) is mediated by its activation of SMS which induced SM accumulation [47]. Further, the same group researchers reported that 2OHOA had opposite effect towards SMS1 and SMS2 [48]. However, we found that 2OHOA inhibited rather than activated purified rSMS1 and rSMS2 in a dose-dependent fashion [49]. Thus, 2OHOA is not a SMS activator and that its anticancer property should be related to other mechanisms.

7.3 Sphingomyelin Synthase-Related Protein

Sphingomyelin synthase-related protein (SMSr) is the third member of the SMS family. SMSr is encoded by the *SAMD8* gene which is located on human chromosome 10 (mouse chromosome 14), consisting of 414 amino acids [2]. SMSr is conserved throughout the animal kingdom [2, 50] and ubiquitously expressed in all tested tissues [51]. SMSr is located on endoplasmic reticulum (ER) [52]. Like SMS1 and SMS2, SMSr also has six transmembrane domains and cytoplasmic N and C termini [2, 50] as well as a conserved triad of two histidine and one aspartate residue [2, 9, 50]. However, unlike SMS1 and SMS2, SMSr does not have SM synthase activity but instead catalyzes synthesis of an SM analog, ceramide phosphoethanolamine (CPE), in test tubes [52]. Interestingly, SMS2 [53] and SMS1 [54] are also capable to synthesize CPE in vitro.

It has been reported [52] that *Smsr* gene knockdown (by siRNA) in cultured HeLa and Drosophila S2 cells could lead to a significant increase in ER ceramide levels and a collapse of the early secretory pathway. Thus, the researchers hypothesized that SMSr regulates ceramide synthesis in ER. The same group of researchers also reported that abnormal ceramide accumulation can lead to mislocalization of ceramide to mitochondria, triggering the mitochondrial apoptosis pathway, suggesting that SMSr might be a

suppressor of apoptosis [55]. However, another group researchers reported that *Smsr* knockdown in multiple cell lines did not alter sphingolipid biosynthesis, including ceramide [56].

To examine SMSr function in vivo, we generated *Smsr* KO mice which were fertile without obvious phenotypic alterations. Interestingly, quantitative MS analyses of plasma, liver, and macrophages from the KO mice revealed only marginal changes in CPE levels which were extremely low [54]. Further, ceramide levels in the plasma, liver, and macrophages from the KO mice were not significantly different from those of WT controls [54]. Similar phenomenon was also observed independently by another research group [57]. Furthermore, since both SMS2 and SMSr have CPE synthase activity in test tubes [52, 53], *Smsr/Sms2* double KO mice were used to evaluate CPE-related biology. Unexpectedly, the double KO mice had no obvious impact on mouse development or fertility [57]. While SMSr is widely expressed in all tested tissues, including the brain, blocking its catalytic activity did not affect ceramide levels or secretory pathway integrity in the brain or any other tissues [57]. Then, two fundamental questions are asked: (1) is SMSr responsible for the production of the trace amount of CPE in tissues? and (2) is CPE synthesis a real biological function of SMSr? It could be possible that the trace amount of CPE in the circulation could come from microbiota which contains high levels of CPE [58] and is located in the lumen of intestine. Based on these unexpected results, we even raised a concern 6-year ago: "given the fact that tissue CPE levels are extremely low, it is not clear why SMSr is needed and so highly conserved in the animal kingdom" [54]. Thus, SMSr's real biological function was a big puzzle for more than 15 years.

Like SMS1, SMSr has a SAM domain [2]. SMSr's ER residency relies on homotypic oligomerization mediated by its SAM domain [59]. SMSr functionally interacts with diacylglycerol kinase δ through its SAM domains and such an interaction represents a new pathway independent of phosphatidylinositol turnover [60]. These observations were linking the function of SMSr with lipid metabolism, other than sphingolipid metabolism. This will be the focus of the rest of this chapter.

7.4 SMS Family Is a Group of Phospholipase Cs

Phospholipase C cleaves the phospholipid into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) or phosphorylcholine (P-choline) or phosphorylethanolamine (P-ethanolamine). Phosphatidylinositol-specific phospholipase C (PI-PLC) converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG and IP_3 each control diverse cellular processes and are also substrates for synthesis of other important signaling molecules. Mammals express six families of PI-PLCs each with both unique and overlapping controls overexpression and subcellular distribution [61].

Phosphatidylcholine-specific phospholipase C (PC-PLC) or phosphatidylethanolamine-specific phospholipase C (PE-PLC) cleaves either PC or PE moiety, thereby generating DAG and P-choline [62] or P-ethanolamine. Considering the large abundance of PC and PE, it is possible that PC-PLC hydrolysis of PC or PE-PLC hydrolysis of PE can produce a more sustained DAG elevation than PI-PLC cleavage of phosphatidylinositol does [63]. Moreover, PC and PE are the major lipid components on cell membrane [64], it is conceivable that PC-PLC and PE-PLC activities are important in maintaining cell membrane integrity and function.

Although bacterial PC-PLC was cloned [65], mammalian PC-PLC has not yet been cloned. Mechanistic PC-PLC studies in mammalian cells and in vivo have to rely on small molecule inhibitors of PC-PLC, such as tricyclodecan-9-yl-potassium xanthate (D609) [66]. Similarly, bacterial PE-PLC has also been cloned [67]; however, mammalian PE-PLC is still totally unknown. Interestingly, D609 actions are widely attributed to inhibiting PC-PLC and it also inhibits sphingomyelin synthase (SMS) [68].

As known from SM synthase reaction, SM formation can be separated into two steps:

(1) PC-PLC step, where PC is hydrolyzed into P-choline and DAG and (2) SM formation step, where P-choline is added onto ceramide to form SM. Similarly, CPE formation can also be separated into two steps: (1) PE-PLC step, where PE is hydrolyzed into P-ethanolamine and DAG and (2) CPE formation step, where P-ethanolamine is added onto ceramide to form CPE (Fig. 7.3). Besides six membrane-spanning domains, SMS1, SMS2, and SMSr contain four highly conserved sequence motifs, designated D1, D2, D3, and D4 [2]. Motifs D3 (C-G-D-X_3-S-G-H-T) and D4 (H-Y-X_2-D-V-X_2A-X-Y-I-T-T-R-L-F-X_2-Y-H), containing conserved amino acids His-His-Asp, are similar to the C2 and C3 motifs in lipid phosphate phosphatase (LPPs) which form a catalytic triad mediating the nucleophilic attack on the lipid phosphate ester bond [69]. In fact, the cloning of mammalian SMS gene family was based on the knowledge of LPP sequence [2]. Thus, potentially, SMS family is a functional PLC family.

Very recently, Murakami and Sakane [70] reported that SMSr, as a multi-glycerophospholipid PLC (or pan-PLC), generates diacylglycerol via the hydrolysis of glycerophospholipids in the absence of ceramide. Although we observed similar phenomenon that SMSr is a PLC, importantly, there is a significant difference between our results and theirs. Murakami and Sakane showed that rSMSr has an PC-PLC activity [70] and we found that rSMSr has no such activity [71]. Our observation is consistent with the knowledge that SMSr has no SM synthase activity, which consume PC and ceramide [2]. Moreover, Murakami and Sakane [70] showed that SMSr can also hydrolyze phosphatidic acid (PA) to form DAG, but we did not find such an activity too [71]. PA does not contain phosphodiester bone as PE and PC, thus, the rationale that SMSr has phosphatidic acid phosphatase (PAP) activity (which is different from PLC activity) is not that obvious. Given the fact that mammals express six families of PI-PLCs, with at least 13 members [61], and SMSr has no PS-PLC, PG-PLC, PC-PLC, and PAP activities [71], it is not likely that it has PI-PLC activity. Thus, we believe that SMSr is a specific PE-PLC but not a pan-PLC and its specificity is an important property of SMSr.

Interestingly, SMSr-mediated PE-PLC activity is not calcium dependent and can be inhibited by D609 in a dose-dependent fashion [71]. Thus, our study together with the recent study [70] clearly solved a long-time puzzle, i.e., what is the real activity of SMSr in vivo? SMSr is a PE-PLC but not a CPE synthase in vivo. Importantly, SMSr can regulate steady state levels of PE in vivo [71], and it should be a new tool for PE-related biological study.

A question was asked more than 20-year ago: does SMS account for the putative PC-PLC? [72] Since SMS family was not cloned until 2004 [2], we had no clue for PC-PLC at that time. Given the fact that D609 can inhibit both PC-PLC and SM synthase activities [72], we hypothesize that both SMS1 and SMS2 have PC-PLC activity, i.e., producing DAG through hydrolysis of PC in the

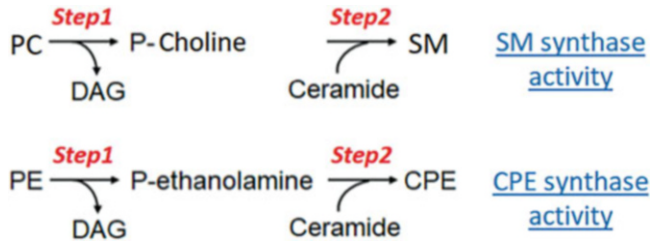

Fig. 7.3 SMS1/SMS2 mediated SM formation and SMSr-mediated CPE formation. The first step of the reaction is hydrolysis of PC or PE (like PC-PLC or PE-PLC activity) to form P-choline or P-ethanolamine and diacylglycerol (DAG). The second step of the reaction is adding P-choline or P-ethanolamine onto ceramide to form sphingomyelin (SM) or ceramide phosphrylethanolamine (CPE)

absence of ceramide. Indeed, so far, we have good evidence to show that both SMS1 and SMS2 have PC-PLC activity [73]. First, purified recombinant SMS1 (rSMS1) and rSMS2 have PC-PLC but not PE-PLC activity. Second, we prepared liver-specific *Sms1*/global *Sms2* double-knockout (dKO) mice. We found that liver PC-PLC activity was significantly reduced and steady state levels of DAG and PC in the liver were regulated by the deficiency, in comparison with wild type mice. Third, we, respectively, expressed *Sms*1 and *Sms*2 genes (using adenovirus) in the liver of the dKO mice and found that SMS1 and SMS2 expression can hydrolyze PC to produce phosphocholine and DAG. Thus, SMS1 and SMS2 exhibit PC-PLC activity in vitro and in vivo [73]. Although DAG is known as an activator of certain protein kinases (PKCs) [74], DAG generated from different sources may have variable effects on PKCs. For example, DAG derived from phosphatidylinositol 4,5 biphosphate hydrolysis can activate PKCs whereas DAG produced from cannot activate PKCs [75]. SMS/PC-PLC generated DAG pool may be different from PI-PLC generated DAG pool.

We concluded that SMS family is a PLC family. SMS1 and SMS2 are two specific PC-PLCs, while SMSr is a specific PE-PLC. The biological and pathological functions of SMS-mediated PLC activity deserve further investigation.

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Sphingolipid Metabolism and Signaling in Endothelial Cell Functions

8

Linda Sasset and Annarita Di Lorenzo

Abstract

The endothelium, inner layer of blood vessels, constitutes a metabolically active paracrine, endocrine, and autocrine organ, able to sense the neighboring environment and exert a variety of biological functions important to preserve the health of vasculature, tissues, and organs. Sphingolipids are both fundamental structural components of the eukaryotic membranes and signaling molecules regulating a variety of biological functions. Ceramide and sphingosine-1-phosphate (S1P), bioactive sphingolipids, have emerged as important regulators of cardiovascular functions in health and disease. In this review we discuss recent insights into the role of ceramide and S1P biosynthesis and signaling in regulating endothelial cell functions, in health and diseases. We also highlight advances into the mechanisms regulating serine palmitoyltransferase, the first and rate-limiting enzyme of de novo sphingolipid biosynthesis, with an emphasis on its inhibitors, ORMDL and NOGO-B. Understanding the molecular mechanisms regulating the sphingolipid de novo biosynthesis may provide the foundation for therapeutic modulation of this pathway in a variety of conditions, including cardiovascular diseases, associated with derangement of this pathway.

Keywords

Sphingolipid de novo biosynthesis · Serine palmitoyltransferase (SPT) · Ceramide · Sphingosine-1-phosphate (S1P) · Endothelial cells · Nogo-B

8.1 Endothelium: Structure and Functions

In 1628 William Harvey first described a network of vessels separating the blood from tissues. In 1661 Marcello Malpighi reported the existence of capillaries and red blood cells (RBC) by using the microscope. Only in 1800s Wilhelm His observed and coined the word "endothelium" to describe the inner layer lining of blood vessels and cavities. The endothelium has been initially considered as an "inert" physical barrier between blood and tissues. Over the years, the concept of endothelium gradually evolved from that of semi-permeable barrier to a metabolically active paracrine, endocrine, and autocrine organ, able to sense the neighboring environment and exert a variety of biological functions important to maintain physiological homeostasis of the vasculature, tissues, and organs (Fig. 8.1).

L. Sasset · A. Di Lorenzo (✉)
Department of Pathology and Laboratory Medicine, Cardiovascular Research Institute, Feil Brain and Mind Research Institute, Weill Cornell Medicine, New York, NY, USA
e-mail: and2039@med.cornell.edu

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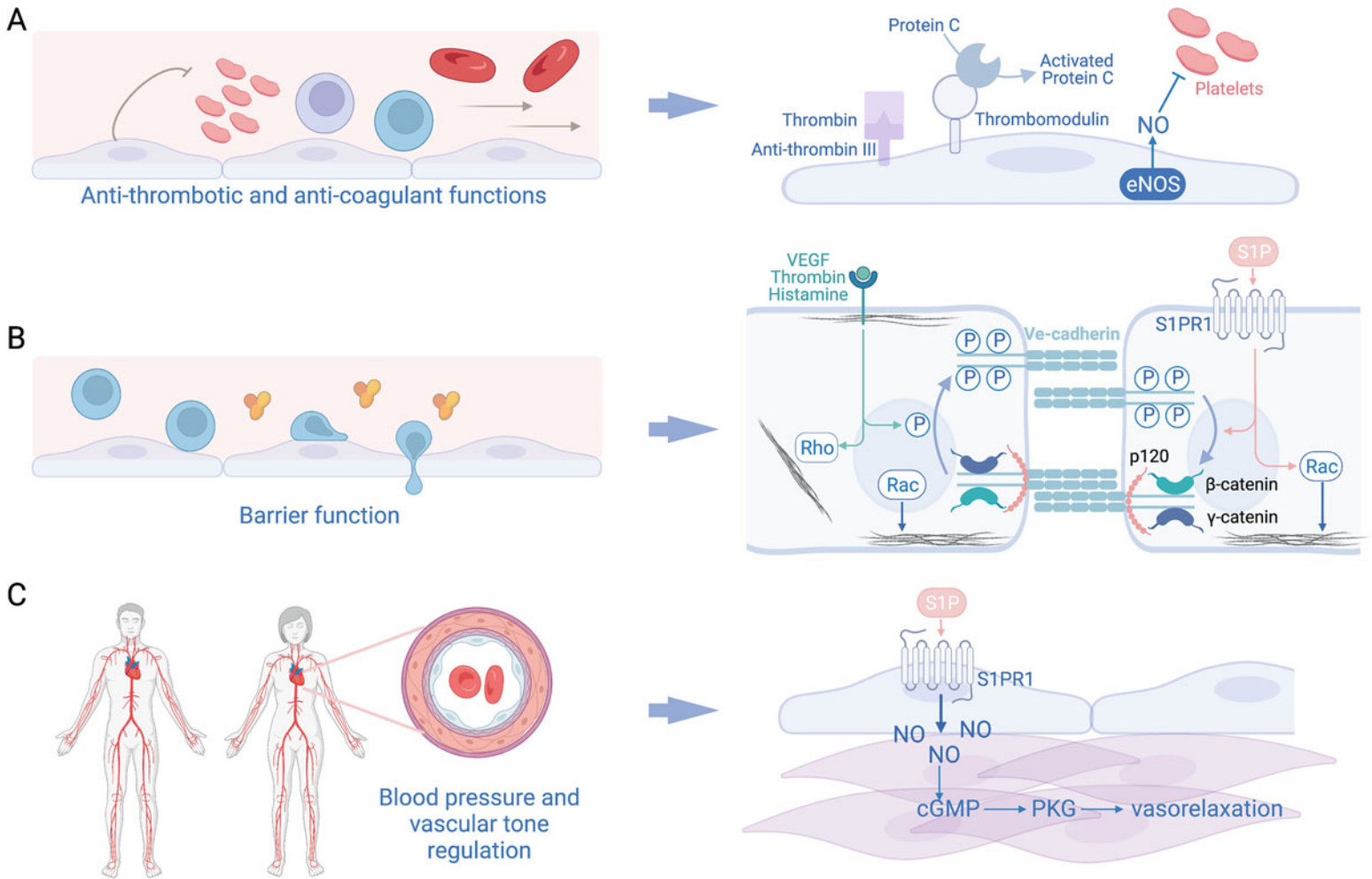

Fig. 8.1 Endothelial functions. (**a**) The endothelium preserves blood fluidity by providing an anti-thrombotic and anti-coagulant surface and factors, such as anti-thrombin III, thrombomodulin, and NO. (**b**) Adherens junctions provide a functional barrier regulating paracellular permeability. They are composed by the transmembrane

Fig. 8.1 (continued) proteins VE-cadherin, linked cytoplasmatically to a different intracellular binding partner, including p120-catenin, β-catenin, and γ-catenin, favoring the interaction of VE-cadherin complex with the cytoskeleton. Pro-permeability signals such as VEGF, thrombin, and histamine trigger VE-cadherin phosphorylation and Rho-dependent stress fibers formation, leading to barrier disruption. Signals that reinforce the endothelial barrier integrity, such as S1P, trigger VE-cadherin dephosphorylation and clustering and Rac-dependent cortical actin formation. (**c**) The endothelium regulates vascular tone and blood pressure, through the production of vasoactive molecules. NO is a potent endogenous vasodilator via cGMP-PKG pathway. S1P is a potent activator of eNOS-derived NO. *NO* nitric oxide, *eNOS* endothelial nitric oxide synthase, *VEGF* vascular endothelial growth factor, *S1P* sphingosine-1-phosphate, *S1PR1* S1P receptor 1

Endothelial cell-to-cell adhesion is mainly maintained by tight and adheres junctions (AJ), which are transmembrane adhesive protein complex that interacts with intracellular cytoskeleton [1], providing a functional barrier regulating paracellular permeability and cellular polarity. As ultrastructure studies began to unfold in the 1960s, the endothelium appeared having a heterogeneous structure throughout the vascular tree, mirroring its diversified functions. For instance, the endothelium of the arteries and arterioles in the heart, brain, and muscle is non-fenestrated and continuous, with organized and tighter junctions than post-capillary venules. The latter are the primary site of plasma proteins and leukocytes extravasation during inflammation; therefore, the endothelial junctions are more loose and disorganized to better support their functions [2]. Discontinuous endothelium is characteristic of the liver sinusoids, containing large fenestrae (open pores across the cell lacking diaphragm), poorly formed basement membrane, and high endocytic capacity, supporting the metabolic clearance of the liver [3]. In tissue with high filtration rates such as glands and glomeruli, the endothelium of the capillaries is continuous and presents fenestrae, the majority of which is provided by diaphragms, subcellular structure [2, 4].

In resting state, the endothelium regulates the exchange of oxygen and nutrients between the bloodstream and tissues, controls blood flow, and refrains plasma proteins and leukocytes from extravasating [2, 5]. All endothelial cells (EC) of the vasculature preserve blood fluidity by providing an anti-thrombotic and anti-coagulant surface and factors [6]. For instance, the activation of thrombin and the formation of fibrin clot is inhibited by the tissue factor pathway inhibitors (TFPIs), anti-thrombin III bound to heparan sulfate proteoglycans, and by thrombomodulin, which converts the functions of thrombin from pro-coagulant to anti-coagulant (Fig. 8.1). The endothelium also contributes to the activation of the fibrinolytic system. For instance, EC can produce tissue-type and urokinase-type plasminogen activators (tPA and uPA), converting the inactive plasminogen to active plasmin, an enzyme able to degrade fibrin [7], forming the clot. EC also prevent platelet activation by sequestrating von Willebrand factor in Weibel–Palade bodies (intracellular granules), degrading adenosine-5′-triphosphate (ATP) via the membrane-bound ecto-adenosine diphosphate (ADP)-ases CD39 and CD73, and releasing factors such as endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) and prostacyclin (PGI_2), which are able to increase intracellular cGMP and cAMP in platelets and inhibit their activation [7].

One of the first functions recognized to the endothelium is the capacity to produce a variety of vasoactive molecules able to control vascular tone, therefore dictating the equilibrium between tissue metabolic demand and blood supply (Fig. 8.1). The initial observation from Furchgott and Zawadzki that the removal of the endothelium abolished the vasodilating effects of acetylcholine (Ach) [8] led to the identification of NO as the main endogenous regulator of vascular tone [9, 10], via cGMP [11, 12]. The association of endothelial eNOS with caveolin-1 keeps the enzyme in a low active state localized to the caveolae, which are invaginations of plasma membrane microdomains of lipid rafts composition [13]. The activity of eNOS is regulated by a complex and multilayered process. Following the activation of specific receptors on EC by agonists (i.e., Ach, bradykinin, vascular endothelial growth factor, or insulin), intracellular Ca^{2+} increases, and the complex Ca^{2+}/calmodulin can interact with eNOS and displace caveolin-1, thereby enhancing its activity. Other proteins, such as heat shock protein 90 (HSP90), can interact with eNOS and enhance its activity [14]. Different phosphorylation sites have been identified in eNOS, which can modulate its activation. The Ser1176 of eNOS (Ser1177 in human and bovine) has been the most studied and when phosphorylated by phosphoinositide 3-kinase (PI3K)-Akt heightens eNOS activity [15, 16]. Other kinases can phosphorylate eNOS at Ser1176, including PKA (protein kinase activated by cAMP), AMPK (activated by AMP), and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II). By using L-arginine as substrate, eNOS forms NO and the co-product

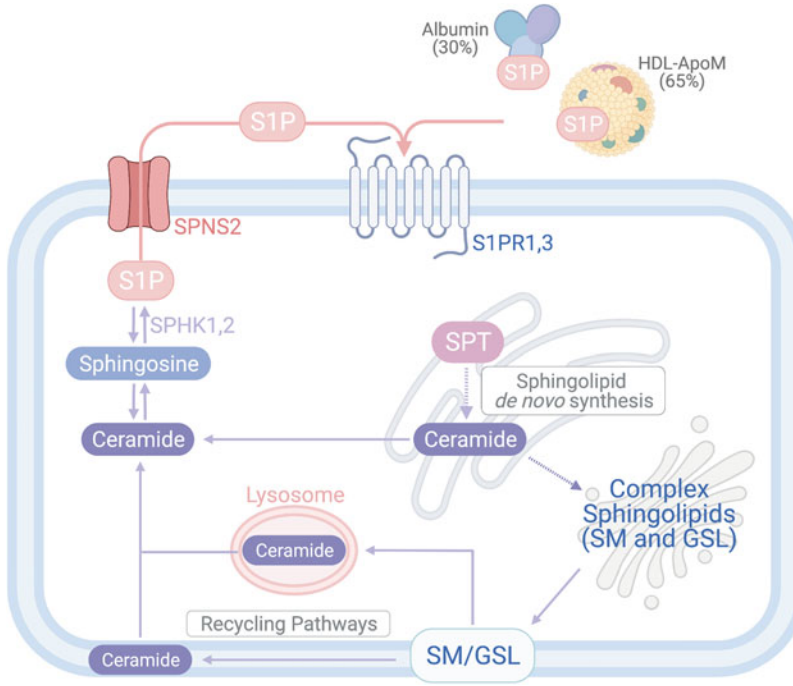

Fig. 8.2 Sphingolipid biosynthetic pathways. SPT initiates de novo SL biosynthesis in the ER, where multiple enzymatic reactions generate ceramide. Ceramide can also derive from the catabolism (recycling pathway) of sphingomyelin and glycosphingolipid in the plasma membrane or in the lysosome. Ceramide can be degraded to sphingosine, which in turn is phosphorylated to S1P. S1P is then transported outside EC by SPNS2, where it can activate endothelial S1PRs or bind to its carrier, HDL-ApoM and albumin, to be transported into the circulation. *SPT* serine palmitoyltransferase, *SL* sphingolipids, *S1P* sphingosine-1-phosphate, *S1PR* S1P receptor, *SM* sphingomyelin, *GSL* glycosphingolipid, *SPHK1,2* sphingosine kinase, *SPNS2* spinster-2

citrulline [14]. NO is a free radical gas that can rapidly diffuse across biological membranes to the subjacent vascular smooth muscle cells of the media, where it induces vasorelaxation via cGMP-mediated mechanisms, including the decrease of intracellular Ca^{2+}, and the activation of myosin light chain phosphatase, dephosphorylating myosin light chain [17].

The loss of the endothelium to accomplish any of these functions is defined "*endothelial dysfunction*" and represents an early event in the onset of cardiovascular diseases [18, 19]. For instance, pre-clinical and clinical studies have demonstrated that endothelial dysfunction precedes the onset of atherosclerosis and hypertension [20–22] and contributes not only to development of the lesions but also to later clinical complications.

8.2 Sphingolipid Pathway

Sphingolipids (SL) are a class of structurally and functionally diverse lipids, discovered in brain extracts in the 1870s. Sphingosine, from "Sphinx," owes its name to J. L. W. Thudichum "in commemoration of the many enigmas which it presents to the inquirer." Recent advances in mass spectrometry and lipidomics have enabled a sensitive and accurate characterization of the sphingolipidome, and to date the LIPID MAPS Lipidomics Gateway (www.lipidmaps.org) lists almost 5000 distinct chemical entities within this lipid class.

Structurally, SL are characterized by an 18-carbon amino-alcohol backbone called sphingoid base, N-acylated with fatty acids, that can be saturated or mono-saturated, with chain

lengths ranging from 14 to 26 carbon atoms. Sphingolipid de novo biosynthesis starts in the cytosolic membrane of the endoplasmic reticulum (ER) with the condensation of L-serine and a saturated acyl-coenzyme A, typically palmitoyl-CoA, by serine palmitoyltransferase (SPT), first and rate-limiting enzyme of the pathway [23] (Fig. 8.2). In the ER ceramide synthases 1–6 N-acylates the sphingoid base with fatty acids to give rise to dihydroceramides [24], and dihydroceramide desaturase converts dihydroceramides in ceramides [25], central metabolites of the pathway. Ceramides can be modified in their head group to form complex SL. Sphingomyelins (SM), major SL in plasma membranes, are formed by the addition of phosphocholine or phosphoethanolamine to ceramides by sphingomyelin synthase. Addition of sugars by galactosyltransferase and glucosyl-ceramide synthase forms glycosphingolipids (GLS) [26]. SM and GLS biosynthesis occurs in the Golgi. Because of their hydrophobicity, ceramides are transported to the cis-Golgi for SM synthesis mainly by CERT (ceramide transfer protein) [27] and by vesicular transport to the trans-Golgi for GSL synthesis [28]. All complex SL can be degraded to ceramide by the activity of specific hydrolases removing the head groups [26]. SM and GLS can be both catabolized to ceramide in the plasma membrane or internalized through the endosomal pathway and degraded by sphingomyelinases (SMase) and glucosidases to ceramide in the lysosomal compartment [26].

Although ceramide is primarily converted into complex SL, it can also be phosphorylated by ceramide kinase in ceramide-1-phospate [29] or hydrolyzed to sphingosine and fatty acid by ceramidases [30]. Ceramide hydrolysis occurs in the lysosome or in the ER by acid or alkaline ceramidase, respectively. Sphingosine can be "salvaged" into the sphingolipid pathways, or phosphorylated to form sphingosine-1-phosphate (S1P), a potent bioactive lipid, by the two sphingosine kinases 1 and 2 (SPHK1 and 2) [31]. SPHK1 is mainly located in the cytoplasm, SPHK2 in the ER and in the nucleus [31]. S1P can be dephosphorylated by S1P phosphatase to sphingosine or degraded by S1P lyase to hexadecenal and phosphoethanolamine. The latter occurs in the cytosolic membrane of the ER and is the only enzymatic reaction in which a SL is converted into a non-SL molecule [32], thus constitutes the only exit of the pathway.

Functionally, SL are important building blocks of cell membranes [33]. Together with cholesterol, ceramides and SM are enriched in lipid rafts, small plasma membrane domains of nano- and microscale [34–36]. Lipid rafts are hypothesized to form by self-association of SL, favored by their long and mostly saturated hydrocarbon chain, that allow a tightly packing in the bilayer. Cholesterol molecules are believed to fill the voids between the associating SL, producing domains more ordered than the surrounding membranes [37, 38]. Lipid rafts are enriched in proteins post-translationally modified by glycosylphosphatidylinositol (GPI) anchor [35, 38], and protein–protein interactions are proposed to stabilize the small and dynamic rafts, leading to the formation of larger structures [39]. Ceramide-rich domains are another kind of SL-enriched domain in the plasma membrane, larger than the lipid rafts and devoid of cholesterol. Produced by the hydrolysis of SM to ceramide by SMase in response to external stimuli (i.e., TNFα), they are also postulated to exhibit higher order than the surrounding bilayer and to be enriched in GPI-anchored proteins [40]. Both lipid rafts and ceramide-rich domains are also platforms where tyrosine kinase receptors and G-protein couple receptors (GPCR) cluster [41, 42]. By modulating the membrane properties, SL can influence the activity of transmembrane proteins and their associated cellular processes, such as signal transduction, endocytosis, intracellular trafficking [34, 43].

SL function also as potent signaling molecules, and to date, the best characterized bioactive SL are S1P and ceramides [44]. More recently additional signaling functions have been ascribed to dihydroceramide [45], sphingosine [44], and ceramide-1-phosphate [46]. While S1P controls different cellular functions by activating five different GPCR, namely S1PR1–5 [44, 47], ceramides function mainly intracellularly as second messenger by modulating the activity of

target proteins, such as protein phosphatases PP2A and PP1 [48, 49] and protein kinase Cζ [50]. Biological roles of S1P and ceramide in EC will be discussed later in this chapter.

8.3 S1P Biosynthesis, Secretion, Carriers

Sphingosine-1-phosphate (S1P), one of the most studied bioactive lipids, is generated by a two-step process: the hydrolysis of ceramide by ceramidases and the subsequential phosphorylation of sphingosine by SPHK1,2 [47]. In healthy state, erythrocytes [51, 52] and EC [53–55] are major sources of plasma S1P, while platelets represent an additional source in pathological conditions [56]. Once formed, endothelial S1P is rapidly transported outside by the transporter Spinster homolog 2 (SPNS2) [57, 58].

In mammals, S1P is spatially compartmentalized in blood (~1 μM) and lymph (~0.1 μM) circulation, while it is almost undetectable in interstitial tissue, including lymphoid organs (<1 nM) where S1P lyase activity is high, creating an S1P gradient across the endothelial barrier [59, 60]. This gradient is particularly important for immune cell trafficking. Lymphocytes egress into circulation by an S1PR1-dependent sensing of S1P gradients at the tissue–circulatory interface [61, 62]. Once in circulation, lymphocyte S1PR1 is mostly internalized and desensitized due to the high S1P concentration, allowing lymphocytes to overcome the S1P-dependent attraction to blood and return to lymphoid tissues [63].

Because of its lipophilic nature, plasmatic S1P is mainly carried by high density lipoprotein (HDL, ~65%) and albumin (~35%) [64]. In HDL, S1P is carried by apolipoprotein M (ApoM) in a lipid-binding pocket formed by eight antiparallel β-sheets [65, 66]. Interestingly, ligand-binding studies have demonstrated that S1P binds to ApoM with an IC_{50} of 0.9 μM, corresponding to plasma concentration of S1P [65, 66]. In ApoM knockout mice ($ApoM^{-/-}$), plasma HDL-bound S1P is virtually absent, resulting in increased vascular permeability due to a disruption of the endothelial barrier function [66], and increased blood pressure (BP) due to diminished endothelial S1P-NO signaling activation [54]. Human studies reported an inverse correlation between the severity of sepsis with the plasma levels of ApoM and S1P [67, 68]. Similarly, in an *E. coli*-induced model of sepsis in baboons, ApoM and S1P plasma levels progressively dropped over time, reaching half the concentration at the 24 h time point [68]. Patients with stable coronary artery disease (CAD) and myocardial infarction (MI) have lower levels of circulating HDL-S1P than healthy individuals [69, 70], with an impaired capacity to stimulate eNOS-derived NO production [71]. While not associated with the extent of lesion stenosis or restenosis, HDL-bound S1P inversely correlates with the severity of CAD [72]. Interestingly, genetic variants of ApoM have been associated with CAD [73, 74].

While these findings suggest a role for ApoM-bound S1P signaling in maintaining cardiovascular health, further studies are needed to understand the pathological significance of altered levels and/or functions of HDL-bound S1P in disease settings such as atherosclerosis.

8.4 S1P and Ceramides in Endothelial Barrier Functions

Vascular permeability is governed by the tightness of the endothelial barrier (cell-to-cell contact), which ensures a proper compartmentalization between the blood and interstitial space, allows the exchange of small molecules (i.e., gases O_2, CO_2, sugar, salts), and refrains macromolecules (i.e., immunoglobulins, albumin) and immune cells from extravasating. The failure to maintain endothelial barrier integrity results in excessive increase of permeability, a hallmark of different inflammatory conditions [75, 76], including rheumatoid arthritis, healing wounds, tumors.

The core component of endothelial AJ is the transmembrane protein vascular endothelial cadherin (VE-cadherin, Fig. 8.1b). Its extracellular domains form trans-cadherin interactions between neighbor EC, while the intracellular domain mediates interactions with a complex of intracellular binding partners, including β-catenin, p120-catenin, and γ-catenin, essential for junctional stability, signaling, and cytoskeleton anchorage [77, 78]. Lee and colleagues demonstrated that S1P reinforces the assembly of AJ by increasing VE-cadherin and β-catenin localization at the cell-to-cell contact sites in HUVEC (human umbilical vein EC), through S1PR1 and S1PR3 activation [79]. The enhancement of AJ was coupled to stress fibers and cortical actin formation, mediated by Rac and Rho GTPases activation [79]. Rac activity is necessary to heighten the endothelial barrier, and S1PR1 is mainly accountable for Rac activation via $G_{\alpha i}$-dependent PI3K signaling, although S1PR3 can also signal, in part, to Rac. A follow-up study by Garcia et al. showed that in different types of EC, S1P increases the trans-endothelial electrical resistance (TEER), a readout of the barrier strength. The increase in TEER was induced by the activation of PTX-sensitive S1PRs and Rho/Rac GTPases. They also showed that S1P enhances the re-establishment of the endothelial barrier following thrombin-induced disruption [80]. Other studies supported the role of S1P as endothelial barrier-enhancing factor and identified additional mechanisms by which S1P controls cytoskeleton remodeling [81]. Consistent with these findings, mice endothelial knockout for *S1pr1* (*S1pr1*^{ECKO}) subjected to reverse Arthus reaction, an established model of acute immunocomplex-mediated vascular injury and inflammation, showed increased pulmonary extravasation of albumin-bound Evans blue dye, increased lung weight, as well as a greater number of white and RBC in the bronchoalveolar lavage fluid compared to *S1pr1*$^{f/f}$, index of an impaired endothelial barrier [82]. Exaggerated permeability and inflammation were also reported in the lung of *S1pr1*^{ECKO} treated with LPS [83]. Furthermore, the increase of plasma S1P following LPS treatment was shown to enhance the expression of S1PR1, S1P metabolizing enzymes, and transporters (i.e., SPHK1 and SPNS2), hence favoring the re-establishment of endothelial barrier [83].

In vivo manipulation of S1P levels with different genetic approaches underlined the importance of circulating S1P in preserving the endothelial barrier functions and in refraining plasma proteins and leukocytes from extravasating. Mice global knockout for sphingosine kinase 1 (*Sphk1*$^{-/-}$) showed an enhanced pulmonary edema in response to LPS and thrombin [84]. Moreover, SPHK1 is activated following thrombin stimulation and contributes to the recovery of the barrier following the breakdown. Camerer and colleagues demonstrated that mutant mice lacking erythrocyte-derived S1P (gene specific *Sphk1/2*$^{-/-}$ mouse model), lowering circulating S1P levels [56], presented vascular hyperpermeability associated with the formation of EC gaps, and an exaggerated leakage in response to platelet-activating factor (PAF) and histamine. Both the transfusion of mice with wild type (WT) RBC, restoring plasma S1P to control levels, and the acute administration of S1PR1 agonist, reduced vascular leakage and EC gaps in venules [85]. It has been also demonstrated that platelet-derived S1P enhances the endothelial barrier integrity in human and mice [86, 87] and contributes to reinforce the high endothelial venule cell barrier in lymph nodes during the immune response [88], underlying that both RBC and platelet-derived S1P exerts barrier-enhancing functions.

Endothelial-derived S1P is also important to maintain the integrity of the barrier. Murine lung EC isolated from *Spns2*$^{-/-}$ mice showed reduced TEER compared to control and downregulated SPNS2 expression following LPS or pro-inflammatory cytokines, suggesting that a reduction of endothelial-derived S1P during inflammation may contribute to barrier disruption. In support of the in vitro data, albumin leakage was significantly enhanced in bronchoalveolar lavage of *Spns2*$^{-/-}$ mice [89]. A recent study from Simmons et al., by specific deletion of *Spns2* in lymphatic EC using Lyve1-Cre, demonstrated that endothelial-

derived S1P is critical to preserve high endothelial venule barrier integrity, survival, and functions related to immune cell trafficking [90].

Christofferesen et al. showed the importance of ApoM of HDL-bound S1P in preserving the endothelial integrity [66, 91]. ApoM$^{-/-}$ mice, with about 50% reduction in plasma S1P, showed ca. 40% increase in lung vascular permeability in basal conditions compared to controls [66, 91]. Plasma reconstitution with ApoM-bound S1P, or treatment with an S1PR1 receptor agonist, restored the endothelial integrity and lowered lung permeability [91]. The importance of ApoM-bound S1P was also corroborated in a model of acute inflammation. Following a subplantar injection of carrageenan, a pro-inflammatory agent, ApoM$^{-/-}$ mice showed an exaggerated vascular leakage and leukocytes extravasation compared to WT. This phenotype was reversed by adenoviral-mediated ApoM overexpression [91].

Whereas much effort investigated the impact of S1P on endothelial barrier integrity, and its underlying mechanisms, the role of ceramide in healthy and disease states remains poorly defined. Several cellular stressors (i.e., pro-inflammatory cytokines, oxidative stress, ionizing radiations) activate neutral and/or acid SMase at the cell membrane and lysosome, respectively, resulting in an increase of ceramides [92, 93], linked to hyperpermeability. An early study by Goggel et al. showed that the activation of acid SMase and ceramide production contributes to the PAF-induced lung edema. Both SMase inhibitor ARC39 and genetic deletion of acid SMase in mice attenuated pulmonary edema formation [94], supporting a role for SM-derived ceramide in endothelial barrier disruption. Interestingly, acid SMase activity is elevated in a variety of pathological conditions associated with vascular hyperpermeability, such as acute lung injury [94] and sepsis [95] in animals, and in septic human patients [96].

By using bovine and human pulmonary EC in vitro, Lindner et al. showed that exogenous C6:0-ceramide (30 μM) significantly decreased TEER after 90 min [97]. Another study showed that in primary rat and human microvascular EC, cigarette smoke induced endothelial barrier disruption in a dose- and time-dependent manner, by a mechanism involving ROS-dependent activation of neutral SMase and ceramide elevation. The morphological changes included stress fiber formation, downregulation of zonula occludens-1 (ZO-1, forming TJ), and intercellular gap formation, which were ameliorated by neutral SMase inhibition. The addition of C6:0-ceramide (20 μM, 2 h) was able to recapitulate the disruption of endothelial barrier by cigarette smoke [98].

While these studies suggest an involvement of SMase-derived ceramide in the endothelial barrier disruption by a stressor, additional studies are necessary to underpin the mechanisms underlying these effects. For instance, ceramides can alter membrane biophysical properties and impact junctional strength, dynamics, and/or intracellular binding partners of the VE-cadherin complex, resulting in the breakdown of cell-to-cell contacts. Ceramides can also interact with intracellular targets, such as PP1, PP2A, PKCζ [48–50] which in turn signal to EC junctions and alter their stability. Lastly, ceramides can also impact oxidative stress [99] and induce cytoskeleton modification [100] to impact the barrier integrity.

8.5 S1P Signaling in Endothelial Control of Vascular Tone

The endothelium lines the interior walls of blood vessels, therefore it is in constant direct contact with circulating signals and can sense hemodynamic changes, such as shear stress of the flowing blood, in response to which it releases vasoactive factors including NO, PGI2, endothelin, and thromboxane [101]. S1P has been described as a potent activator of AKT-eNOS pathway [102, 103], comparable to other NO agonists (i.e., VEGF).

Several studies showed that low concentrations of exogenous S1P (0.1-100 nM) were able to vasodilate pre-constricted rodent resistance arteries ex vivo by activating

endothelial NO production [104], mainly via S1PR1 [105]. It has been shown that HDL-bound S1P induces vasorelaxation of thoracic aorta rings ex vivo mainly via S1PR3 [106]. On the contrary, a recent study from our group revealed S1PR1 and not S1PR3 as main mediator of albumin-bound S1P-induced vasorelaxation in resistance arteries, which are relevant to BP regulation. In this study, second-order mesenteric arteries (MA) were mounted in the pressure myograph system using Krebs solution (containing salts and glucose), allowing to recapitulate hemodynamic conditions of blood flow and pressure as in vivo. In this setting, vasorelaxation in response to S1P was blunted in MA of $S1pr1^{ECKO}$, but not $S1pr3^{-/-}$ mice, suggesting that in resistance arteries S1P vasorelaxation is mainly mediated by S1PR1 [105]. Considering the heterogeneity of EC, it is possible that S1PR distribution differs throughout the vascular tree (i.e., aorta vs. resistance arteries vs. capillaries), thus the role of S1PR1 and S1PR3 in mediating S1P vasodilation. Another consideration is that the carrier to which S1P is bound (albumin vs. HDL) might differentially activates S1P receptor subtypes [107]. FTY720 (Fingolimod, Gilenya) is an FDA-approved immunomodulator for the treatment of relapsing-remitting multiple sclerosis [108]. It blocks the egress of lymphocytes from lymphoid tissues by downregulating S1PR1 expression [109]. FTY720 is an analogue of S1P and is phosphorylated in vivo by SphK2 [110]. P-FTY720 targets 4 out of 5 S1P receptors, S1PR1 and S1PR3–5 [111], with highest affinity for S1PR1 [112]. Like S1P, P-FTY720 (1 nM-10 μM) induces eNOS-dependent vasodilation of murine thoracic aorta rings ex vivo [113]. However, when given chronically, FTY720 is a functional antagonist and induces the internalization and proteasomal degradation of S1PR1 [114]. In MA from mice treated chronically with FTY720 (0.3 mg/kg), vasodilation in response to S1P is suppressed, both in normotensive and hypertensive conditions [105]. Altogether, these findings underline that an important function of S1P is to control vascular tone mainly via endothelial S1PR1 activation, and in part via S1PR3, of eNOS-NO signaling.

8.6 Endothelial Ceramide in Vascular Tone Regulation

The elevation of circulating and local ceramide in certain pathological conditions, including obesity, type 2 diabetes (T2D), and atherosclerosis, has been linked to adverse vascular effects and endothelial dysfunction. Different eNOS-activating agonists, such as vascular endothelial growth factor, bradykinin, ATP, S1P, insulin, or fluid shear stress can induce the phosphorylation of eNOS at Ser1176, leading to NO production and vasodilation, which is impaired when endothelial dysfunction sets off.

Ceramides can directly interact with different intracellular targets and modify their functions and/or localization, including the repressive factor Inhibitor 2 (I2PP2A), disrupting its inhibitory interaction with protein phosphatase 2A (PP2A) [115]. Studies from the Symons's lab demonstrated that in bovine aortic EC in vitro exposure to palmitate leads to ceramide accumulation and its binding to I2PP2A, resulting in PP2A activation, which in turn dephosphorylates Ser1177-eNOS [116, 117]. As consequence of PP2A-eNOS interaction, eNOS also dissociates from AKT and HSP90 complex, resulting in an overall reduced NO production [116, 117]. In vivo approaches using high fat diet (HFD) model of obesity-induced T2D showed that ceramides are increased in mouse aorta compared to standard chow diet-fed mice, and this correlated with worsen Ach-mediated vasodilation of aortic rings, eNOS phosphorylation, and hypertension [116, 117]. Both the inhibition of ceramide biosynthesis with the SPT inhibitor myriocin and the heterozygous deletion of the dihydroceramide desaturase (DES1) [116], or the inhibition of PP2A [117], ameliorated Ach-mediated vasodilation, eNOS phosphorylation, and improved hypertension in HFD-fed mice, suggesting that decreasing vascular ceramide content improves the functions. It is

noteworthy to mention that in these studies ceramides have been measured in aorta and not in resistance arteries, accountable for BP regulation [26, 116, 117]. Furthermore, even if palmitate-induced ceramide accrual in EC in vitro has been demonstrated by different studies [116–118]; however, whether these changes occur also in EC in vivo in pathological settings is yet to be demonstrated, although inferred. In vivo, EC are exposed to a variety of plasma components, shear stress and circumferential stress due to the blood flow and pressure, which are not present in vitro. Further studies are needed to understand how ceramides change and impact on endothelial dysfunction and its sequela in vivo.

While different studies characterized how excessive ceramide contributes to endothelial dysfunction, the role of ceramide in the healthy endothelium received less attention. EC are bathed in plasma rich in SL. Cells can use sphingosine via recycling pathway to build ceramide and more complex SL [26]. Thus, it is conceivable to assume that endothelial de novo biosynthesis is dispensable to preserve sphingolipid homeostasis.

Recently, we showed that mice defective of endothelial de novo sphingolipid production, by specific *Sptlc2* deletion ($Sptlc2^{ECKO}$), have decreased plasma ceramide and S1P (ca. 30% and 50%, respectively), while SM were not affected, suggesting that the endothelium actively contributes to maintain circulating levels of ceramide an S1P. In liver EC deleted in vitro of *Sptlc2*, ceramide and hexosyl-ceramide were also significantly reduced, but not SM, at the time points assessed (48 h post-4-hydroxytamoxifen treatment). SM are the most abundant SL in the EC, and longer time points might be required to see an impact on their levels in vitro. Indeed, measurements of SL in MA demonstrated a significant decrease of SM, suggesting that endothelial de novo biosynthesis is required to maintain sphingolipid homeostasis. BP is significantly lower in $Sptlc2^{ECKO}$ compared to controls ($Sptlc2^{f/f}$). S1P and ceramides function as rheostat of eNOS-derived NO production. While S1P is a potent activator of eNOS, ceramide levels inversely correlate with eNOS activity. In the absence of SPTLC2, ceramides levels are low, and I2PP2A remains bound to and inhibits PP2A, resulting in increased eNOS activity. Indeed, P-VASP, downstream target of NO-cGMP signaling, is significantly increased in the thoracic aorta of $Sptlc2^{ECKO}$ mice, supporting the lower BP. Although apparently beneficial, the decrease of ceramide below homeostatic levels impairs the endothelial control of vascular tone leading to dysfunction [119]. Specifically, endothelial-mediated vasorelaxation to VEGF, insulin, S1P, thrombin, PGI_2 and flow was markedly impaired, except for Ach. These data clearly demonstrate that the activation of AKT-eNOS-cGMP axis was preserved in $Sptlc2^{ECKO}$ MA (i.e., vasorelaxation to Ach) and suggest that the decrease of endothelial ceramides, although heightened basal activation of eNOS, compromised the signal transduction. Indeed, VEGF-mediated phosphorylation of VEGFR2 and downstream AKT-eNOS signaling were impaired in EC lacking *Sptlc2*. The addition of C16:0-ceramide restored ceramide levels, as well as VEGF signaling in EC in vitro, suggesting that ceramides are necessary to preserve endothelial membrane signal transduction. It is likely that ceramides can modulate membrane biophysical properties, and/or the partition of receptors in specific membrane microdomains, and/or directly affect membrane receptor functions.

It is noteworthy to mention that multiple studies have used exogenous short chain ceramides (C2:0-, C6:0-ceramides), which are not present in mammals, to study how ceramide elevation impact vascular tone. Although it has been shown that short chain ceramides are deacylated and recycled into long-chain ceramides once administered [120], a process estimated to require ca. 1 h in lung epithelial cells (A549), how exogenous short chain ceramides change ceramide profile in vascular cells, and whether exogenous short chain or endogenous ceramides mediate the observed effects is often unknown. Thus, the results from studies using this approach need to be carefully interpreted.

The formation of reactive oxygen species (ROS) underlines endothelial dysfunction in

diabetes and CV conditions [121]. In addition to a direct role in inhibiting eNOS activity, ceramides also promote ROS production by increasing NADPH oxidase activity [99, 122]. Li et al. have shown that treatment of HUVEC with C6:0-ceramide triggers ROS production in a concentration-dependent manner. These effects led to decreased bioactive NO, restored by the addition of tetrahydrobiopterin, a cofactor used by eNOS in the conversion of L-arginine to NO [99]. In a follow-up study, C2:0-ceramide was shown to increase superoxide production in bovine coronary arterial EC via activation of NADPH oxidase, followed by peroxynitrite accrual, findings that correlated with impaired bradykinin-mediated vasodilation of small bovine coronary arteries ex vivo. Mechanistically, ceramides induced the translocation of $p47^{phox}$, a NADPH oxidase subunit, to the membrane, enhancing the enzyme activity [122]. It has been also proposed that ceramide-induced lipid rafts formation favors the aggregation of NADPH oxidase subunits, $p47^{phox}$ and $gp91^{phox}$, to form the active enzyme producing ROS [123]. Similarly, carotid arteries from mice overexpressing CuZn superoxide dismutase, a ROS scavenger, were resistant to ceramide-reduced vasodilation to Ach compared to controls [124]. Growing evidence implicate altered mitochondrial dynamics and mitophagy in endothelial dysfunction, particularly in diabetes [125]. Although how ceramides contribute to impaired mitochondrial dynamics and mitophagy is an ongoing area of research, ceramide impact on mitochondria biology in EC remains poorly investigated.

Interestingly, it has been shown that in patients with CAD the endothelial mediator of flow-induced dilation shifts from NO to mitochondrial-derived hydrogen peroxide (H_2O_2) [126, 127]. Despite both being vasorelaxant factors, whereas NO elicits anti-inflammatory and anti-thrombotic pathways, H_2O_2 has pro-inflammatory and prothrombotic effects [128]. Recently, Freed et al. demonstrated that C2:0-ceramide treatment of healthy adipose arterioles triggered mitochondrial H_2O_2 production, which mediated the vasodilation in response to flow. Inhibition of neutral SMase with GW4869 restored endothelial NO as flow-mediated dilator in arterioles from adipose tissue and atria of patients with CAD [129]. A complementary study corroborated the role of ceramide in triggering H_2O_2-mediated vasodilation to flow in arterioles isolated from human adipose tissue and treated with neutral ceramidase or SPHKs inhibitors, both increasing ceramides [130]. These data revealed another mechanism of action of ceramide accrual in the onset of endothelial dysfunction.

Altogether, these studies support the concept that physiological ceramide levels need to be tightly regulated within a narrow window to preserve endothelial functions, and that both, ceramides decrease and accrual, are "deleterious" and lead to dysfunction.

8.7 Endothelial-Derived S1P: A Mechanotransduction Signaling in Blood Flow Regulation

The half-life of S1P in the plasma is about 15 min [55], suggesting the presence of high-capacity cellular sources to preserve plasma S1P levels in the low micromolar range. In addition to RBC [56] the endothelium is an important source of plasma S1P [54, 55, 119]. Changes in shears stress due to the flowing blood stimulate the release of S1P from the endothelium [55]. However, the biological significance of endothelial-derived S1P is not limited to sustain the circulating carrier-bound S1P, but is critical in regulating blood flow. EC can sense and respond to changes in fluid stress, which is the frictional force per unit area from flowing blood acting on the endothelium. Blood flow regulation is important not only to match the metabolic requirements of a tissue by providing nutrients, oxygen, and removing waste, but also for vascular morphogenesis and physiology.

A recent study from our group provided direct evidence of this S1P function. The increase of flow led to vasorelaxation of WT MA mounted in a pressure myograph system via activation of eNOS and NO production. However, genetic

deletion of endothelial *Spns2* markedly suppressed the vasorelaxation to flow [54], directly implicating the endothelial-derived S1P in the activation of S1PR1-eNOS signaling in blood flow regulation. This phenotype was supported by a significant reduction of phosphorylated-VASP (vasodilator-stimulated phosphoprotein), a downstream target of the NO-cGMP pathway, in thoracic aorta [131]. These results suggest that endothelial-derived S1P and autocrine S1PR1-signaling is necessary to preserve vascular functions and blood flow regulation [54].

Both S1PR1 and S1PR3, expressed by the healthy endothelium, can activate eNOS via PI3K-AKT pathway [102]. However, vasorelaxation in response to flow was dramatically suppressed in $S1pr1^{ECKO}$, but not $S1pr3^{-/-}$ MA, and this phenotype correlated with reduced plasma NO levels [105]. Similar results were obtained in MA lacking *Sptlc2*. Flow-induced vasorelaxation was significantly reduced in $Sptlc2^{ECKO}$ MA [119]. Furthermore, ex vivo treatment of MA with W146, a competitive antagonist of S1PR1, suppressed flow-mediated vasodilation and eNOS activation in concentration-dependent manner [53]. These findings clearly identified endothelial-derived S1P and the autocrine S1PR1-eNOS-signaling as critical mechanotransduction pathway regulating blood flow.

An elegant study of Jung et al. demonstrated that the genetic deletion of endothelial *S1pr1* resulted in misalignment of the aortic endothelium with blood flow, compared to control morphological alignment of EC lacking *S1pr1* [132]. In vivo, the deletion of endothelial S1PR1 led to an abnormal hypersprouting and poorly perfused vasculature in the retina [132].

Altogether these studies propose S1PR1 as a critical mechanotransduction signaling. Flow induces endothelial S1P production, which is transported outside of the cell via SPNS2, and can induce vasodilation by activating S1PR1-eNOS-NO signaling (Fig. 8.3). These findings also suggest that a deranged endothelial S1P production/degradation, and/or transport by SPNS2, may impair vascular functions and contribute to the pathogenesis of atherosclerosis, hypertension, and metabolic-related cardiovascular disorders.

8.8 S1P and Ceramide Rheostat in Blood Pressure Homeostasis

BP is determined by cardiac output, defined as the volume of blood ejected by the heart per minute, and total vascular resistance, which is the

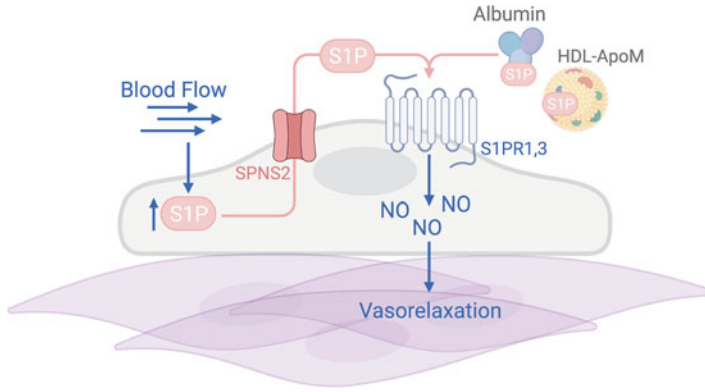

Fig. 8.3 Endothelial-derived S1P: a mechanotransduction signaling in blood flow regulation. Changes in blood flow stimulate the synthesis of S1P by EC. Once produced, S1P is transported outside of the endothelial cells by SPNS2. Endothelial-derived S1P can act in an autocrine or paracrine manner on S1PRs. Endothelial S1PR1 activation is critical in blood flow-mediated vasodilation via eNOS-derived NO production. *S1P* sphingosine-1-phosphate, *S1PR* S1P receptor, *SPNS2* spinster-2, *eNOS* endothelial nitric oxide synthase, *NO* nitric oxide

resistance exerted by all the vasculature on the flowing blood. BP is very dynamic and regulated by the interplay of heart (i.e., morphology and function); kidney, controlling extracellular fluid and blood volume via the pressure-natriuresis mechanism; neuroendocrine system (i.e., the sympathetic nervous system, renin-angiotensin-aldosterone system); and peripheral vascular resistance. In this regard, generalized vasodilation usually leads to a decreased systemic BP, but may also occur in specific tissues causing a local augmentation of blood flow. Endothelial dysfunction, mainly defined as the loss of the endothelium to induce a NO-mediated vasodilation in response to changes in flow, Ach, or other NO-stimulating agents, comprises the impairment of all the endothelial properties, including barrier integrity, antithrombotic and anti-inflammatory functions. In the past three decades endothelial dysfunction emerged as a pivotal and initial event in the onset of hypertension, atherosclerosis, and other CV diseases [18–20]. Dysfunctional endothelium is no longer able to meet an organ's needs and to preserve its functions, and over time contributes to end-organ damage, such as kidney and heart failure, cognitive impairments, stroke, etc. [133].

In in vivo anesthetized rats, acute administration of S1P (0.2 mg/kg) evoked a rapid decline of heart rate and mean arterial BP, followed by a transient hypertension concomitant to the increase in heart rate [134]. While the acute activation of endothelial S1PR1 and S1PR3 and downstream NO signaling is most likely responsible for the initial drop in BP, the following elevation in BP might be due, in part, to the functional antagonism of S1PR1 downregulating eNOS activation [102, 103] and NO bioavailability, and by activation of smooth muscle S1PR2/3 leading to vasoconstriction via Rho-kinase activation [135, 136]. However, mice systemically lacking S1PR3 [105] and S1PR2 [137] did not show any difference in BP at baseline. Consistently, when challenged with chronic infusion of AngII, *S1pr3*$^{-/-}$ mice developed hypertension to the same extent of control mice [105], arguing against the role of S1PR3 in hypertension. It is possible that in absence of S1PR3, smooth muscle S1PR2 may play a compensatory role in systemic BP regulation. Nonetheless, myogenic tone, which is the vasoconstriction of resistance arteries in response to pressure increase, was significantly reduced in *S1pr3*$^{-/-}$ MA [105]. Thus, although dispensable in systemic BP regulation, it is possible that S1PR3 controls blood flow in specific vascular beds, such as MA [105] and cerebral arteries [135, 136]. On the contrary, S1PR1 signaling is critical for BP homeostasis. In a recent study we reported that mice *S1pr1*^{ECKO} have a higher BP compared to mice *S1pr1*$^{f/f}$, both in physiological and hypertensive conditions. Vasodilation of *S1pr1*^{ECKO} MA in response to S1P and flow was significantly reduced, as the downstream eNOS-derived NO signaling [105]. Interestingly, chronic administration of FTY720, which dramatically downregulates endothelial S1PR1 in blood vessels, significantly elevated BP in healthy and in AngII-induced hypertensive mice [105]. This hypertensive phenotype correlated with impaired endothelial-dependent vasodilation of MA to Ach, S1P and flow, supporting vasculo-protective functions of endothelial S1PR1 signaling. Additional studies underlined the role of S1PR1 in BP regulation. A single dose of FTY720 increased the BP of spontaneously hypertensive rats after 24h [138], while chronic administration of FTY720 significantly raised BP in normotensive rats in a dose-dependent manner [139]. Clinical studies using FTY720 corroborated the role of S1PR1 in BP regulation. In patients, a sustained elevation of BP (≈4–5 mmHg) was observed after 2 months of FTY720 treatment [140]. Altogether, animal and human studies underline the importance of the S1P-S1PR1 pathway in maintaining vascular and BP homeostasis [105, 140].

Endothelial S1PR1 is activated by both circulating carrier-bound S1P and endothelial-derived S1P in autocrine manner. Our group demonstrated that endothelial deletion of *Spns2* (*Spns2*^{ECKO}) leads to a significant elevation in BP and impairment of BP dipping [54], which is the difference between the diurnal and nocturnal mean BP. Altered BP dipping has been associated with a higher risk of CV events [141, 142].

Because plasma S1P increases in hypertension [54, 143, 144], it was postulated a pro-hypertensive role. When challenged with chronic infusion of AngII, *Spns2*^{ECKO} mice developed an exaggerated hypertension, although plasma S1P remained ca. 40% reduced in *Spns2*^{ECKO} mice compared to control (*Spns2*$^{f/f}$), arguing against a pro-hypertensive role for plasma S1P [54]. Interestingly, cardiac hypertrophy was also worsened in hypertensive *Spns2*^{ECKO}. Both the increase in systemic BP, which causes a hemodynamic stress on the heart, and the decrease of local endothelial-derived S1P and NO, exerting cardioprotective functions [145, 146], might contribute to aggravate the cardiac hypertrophy in *Spns2*^{ECKO} mice. Interestingly, *Spns2*^{ECKO} mice showed a decrease not only in S1P, but also in ceramides in both EC and circulation, and lowered systemic BP compared to controls [119]. In these conditions, the impact of ceramide prevails on S1P. Specifically, the decrease of ceramide-dependent PP2A activation is accountable for the increased eNOS phosphorylation and NO production at baseline, hence the lower BP [119].

ApoM-bound S1P also contributes for BP homeostasis. Mice *ApoM*$^{-/-}$ showed higher systolic BP at baseline and following chronic infusion of AngII, correlating with worsen endothelial dysfunction and cardiac hypertrophy compared to control [54]. These data underline the importance of endogenous ApoM-bound S1P in preserving endothelial functions and BP homeostasis and, suggest that ApoM-bound S1P could be exploited therapeutically to treat CV conditions such as hypertension. In a follow-up study with Hla's group, we reported that administration of an engineered ApoM, named ApoM-Fc, which does not need to bind HDL and has longer half-life than native ApoM, leads to a sustained anti-hypertensive effect in mice lasting ca. 7 days. ApoM-Fc-bound S1P can activate S1PR1-NO signaling and lower BP, as demonstrated by the treatment with W146, an S1PR1 antagonist, abolishing its BP lowering effects [147].

To understand the role of S1P in BP homeostasis, initial studies used mice *Sphk1* or *Sphk2* knockout, of a combined deletion of their alleles, since the deletion of both genes is embryonically lethal [148]. However, these studies led to confusing outcomes for different reasons. First, the degradation of S1P by S1P lyase is the only catabolic exit of sphingolipid pathway; therefore, the deletion of *Sphks* results in the loss of S1P formation and a backlog of ceramides, adding a confounding factor to the interpretation of the phenotypes. Second, whereas circulating levels of S1P are reduced in *Sphk1*$^{-/-}$ [143, 149] and *Sphk1*$^{+/-}$ *Sphk2*$^{-/-}$ [56, 85] mice, they are increased in *Sphk2*$^{-/-}$ [150, 151] mice, probably for an overcompensation by SPHK1 [151]. At baseline, BP was not affected in mice *Sphk1*$^{-/-}$ [143, 152–156] and *Sphk2*$^{-/-}$ [152, 154, 155], nor in mice overexpressing SPHK1 [157]. Several groups studied the role of SPHKs in regulating BP in response to acute AngII administration in vivo. Furuya et al. showed that AngII administration (i.p.) induced higher BP elevation in *Sphk2*$^{-/-}$ but not in *Sphk1*$^{-/-}$ mice compared to controls. However, it is difficult to ascribe this protective effect to SPHK2-derived S1P or to the excessive elevation of plasma S1P when *Sphk2* is deleted [152]. On the contrary, Wilson et al. showed that BP elevation following AngII was reduced in mice *Sphk1*$^{-/-}$, and considering that *Sphk1*$^{-/-}$ mice have lower BP at baseline than WT, the BP increase between the two groups is of similar magnitude [156].

More consistent results have been obtained with chronic infusion of AngII. Siedlinski et al. reported that *SphK1* mRNA and protein expression was upregulated in MA and aorta of AngII-treated WT mice [143], and AngII-induced BP elevation was significantly lower in *Sphk1*$^{-/-}$ mice WT mice [143, 156]. Meissner et al. showed that both *Sphk1*$^{-/-}$ and *Sphk2*$^{-/-}$ mice are protected from AngII-induced hypertension, with a more profound effect in the *Sphk2*$^{-/-}$ [154]. Furthermore, administration of the SPHK2 antagonists ABC294640 or K-145, but not the SPHK1 antagonist N, N-dimethylsphingosine, significantly reduced BP in hypertensive mice, suggesting that SPHK2 contributes to the onset of hypertension [154]. Altogether these data suggest a role for SPHK2, and in part SPHK1, in the pathogenesis

of hypertension. Data in *Sphk2*$^{-/-}$ support an anti-hypertensive rather than pro-hypertensive role of plasma S1P, considering that is higher in *Sphk2*$^{-/-}$ mice [150, 151]. Furthermore, the backlog of ceramide can also contribute to some of the observed phenotypes. However, all the studies have been performed in mice global knockout for *Sphks*, and to better dissect the specific roles of SPHK1 and SPHK2 in BP homeostasis (i.e., vasculature, immune system) further studies will be required in tissue-specific knockout mice.

Although additional studies are required to elucidate how S1P signaling is impaired in hypertensive conditions, collectively these studies provide significant evidence of its role in the regulation of BP homeostasis.

8.9 Endothelial Fine-Tuning of Sphingolipid De Novo Biosynthesis: Role of Nogo-B and ORMDLs

Localized in the cytosolic leaflet of the smooth ER, SPT is formed by two major subunits, SPTLC1 and SPTLC2, ubiquitously expressed [158–160]. A third subunit, SPTLC3, with tissue-specific distribution, was recently identified and can form an active complex with SPTLC1 [160]. The active site is located at the interface of the two SPT subunits [161–163]. Importantly, SPTLC3 has a higher activity with lauryl- and myristoyl-CoA, generating C14- and C16-sphingoid bases [164], whereas SPTLC2 forms mainly C18- and C20-sphingoid bases.

Altered sphingolipid homeostasis has been implicated in different pathological conditions, including CV [165, 166], metabolic [167] and inflammatory [93] diseases, and cancer [168]. Yet, under normal physiology, sphingolipid levels are tightly regulated. Mechanisms governing cellular sensing of SL and accordingly sphingolipid biosynthesis remain poorly understood. SPT small subunits a and b (ssSPTa and ssSPTb) have been identified by the Dunn's group as enhancer of SPT activity through their single transmembrane domain [169, 170]. In 2010, Weissman's group reported ORMDL1–3 proteins (Orm proteins in yeast) as negative regulator of SPT activity [171]. ORMDLs are a conserved family of ER membrane proteins, ubiquitously expressed in fetal and adult tissues. ORMDL1, 2, and 3 share more than 80% of identity in the same species, and 96–99% of homology between human and mouse [172]. Orthologues of the ORMDLs have been reported in yeast, plants, microsporidia, urochordates, and invertebrates. Identity between mammal and yeast ORMDLs/Orms is limited to 30–40% [172]. Structurally, ORMDLs are anchored to the ER membrane by 4 transmembrane domains [173–175], with both N- and C-terminus facing the cytosol [176]. Breslow et al. demonstrated that lowering SL with myriocin triggers the phosphorylation of Orms, which releases its interaction with SPT, hence heightening sphingolipid de novo biosynthesis [171]. However, this regulatory mechanism is not shared by mammalian ORMDLs as they lack the N-terminus hosting the phosphosites in yeast [172]. Nonetheless, in eukaryotic cell lines, the absence of ORMDLs increases the activity of SPT [171, 177], corroborating their inhibitory function on SPT. How ORMDLs interaction with SPT is regulated in mammals has attracted much attention. ORMDLs expression can be induced both at transcriptional level by allergenic and inflammatory stimuli (i.e., OVA, LPS, IL-4, IL-13) [178], and at translational level by increased expression of catalytically active SPT [179], although the underlying molecular mechanisms remain unknown. Studies from Wattenberg's group using cell-free isolated membranes and permeabilized cells showed that non-physiological ceramides (C6:0- and C8:0-ceramide), and endogenously produced ceramides, can acutely downregulate the activity of SPT, probably by interacting with SPT-ORMDLs complex [173, 177]. Although this proposed mechanism has been demonstrated in HeLa cells, whether it is physiologically relevant is yet to be demonstrated. Two recent crystallographic studies have elegantly showed that ORMDL3 directly inhibits SPT activity by

inserting its N-terminus in the opening between SPTLC1 and SPTLC2, therefore preventing substrates access to the catalytic domain [174, 175]. How ORMDL3 inhibition of SPT is dynamically regulated by SL levels remains a standing question. In this regard, our group recently discovered that ORMDLs are constitutively hydroxylated at Pro137, which is an obligatory step for their ubiquitination and proteasomal degradation. This mechanism preserves SPT activity at steady state by refraining ORMDL levels. Moreover, we identified S1P as the key sphingolipid sensed by EC via S1PRs. S1P-activation of S1PR1,3 signaling stabilizes ORMDLs to negatively regulate SPT and maintain sphingolipid homeostasis [180]. Interestingly, genetic disruption of S1P-S1PRs signaling pathway at different levels, by the deletion of *SphK1,2*, or *S1pr1,3* or *Spns2*, relieved the inhibition on SPT with consequent increase of ceramide levels. In particular, the deletion of *Spns2* highlighted the importance of endothelial-derived autocrine S1P signaling to maintain not only the vessels response to blood flow, but also sphingolipid homeostasis. *Spns2* deletion caused ceramide accrual, mitochondrial dysfunction, and impaired signal transduction, all leading to endothelial dysfunction, an early event in the onset of cardio- and cerebrovascular diseases [180] (Fig. 8.4). It is noteworthy to mention that, while S1P is protective for many of the endothelial functions discussed in this chapter, an excessive increase may lead to a deleterious inhibition of sphingolipid biosynthesis via S1PRs-ORMDLs. It is likely that the disruption of this newly described S1P-ORMDL-SPT signaling in EC might be implicated in the pathogenesis of several conditions characterized by deranged SL metabolism, such as diabetes, cardiomyopathies, and neurodegeneration.

ORMDL3 has been the most studied among the ORMDL isoforms, especially considering that single nucleotide polymorphisms (SNP) increasing its expression have been associated with asthma (rs8076131 and rs4065275) [181, 182]. Consistently, global overexpression of human ORMDL3 in mice led to airway remodeling, inflammation, and hyperresponsiveness to methacholine versus control mice [183]. Vice versa, deletion of *Ormdl3* specifically in lung epithelial cells protected the mice from airway hyperresponsiveness, inflammation, mucus secretion, and collagen deposition [184]. Finally, mice *Ormdl1/3*$^{-/-}$ manifested SL accrual in the sciatic nerves and reduced level of myelinization [185], underlying the importance of ORMDL proteins in preserving sphingolipid homeostasis in higher eukaryotes.

Later in 2015, our group discovered Reticulon-4B (RTN4B, aka NOGO-B) as a negative regulator of SPT activity in mammalian cells (Fig. 8.4) [53]. NOGO-B belongs to the family of reticulon proteins, localized in the membrane of the tubular ER (aka smooth ER), contributing to the tubulogenesis of the ER network [186]. RTN proteins are characterized by two transmembrane domains separated by a loop of 66-aa, named reticulon-homology domain (RHD) [186, 187]. Rtn4 gene gives rise to three major splicing isoforms, namely NOGO-A, NOGO-B, and NOGO-C. While NOGO-A and -C are mainly expressed in the central nervous system [187], and low levels of NOGO-C are found the skeletal muscle [188], NOGO-B is highly expressed, but not exclusively, in the blood vessels [53]. In physiological conditions, NOGO-B is also expressed in bone marrow cells [189] and in cardiomyocytes of the atria but not ventricles [190]. In this regard, growing evidence demonstrated an upregulation of myocardial RTN4 expression in cardiomyopathy in mice and humans [191–193], implicating a role for NOGO in heart diseases. In EC the most abundant isoform is NOGO-B, while NOGO-A is undetectable [190].

8.9.1 NOGO-B Regulates BP and Vascular Tone Via SPT

NOGO-B binds to and downregulates SPT activity of ca. 40% in both murine and human EC in vitro and in murine lung microsomes. In the absence of NOGO-B, SPT activity and

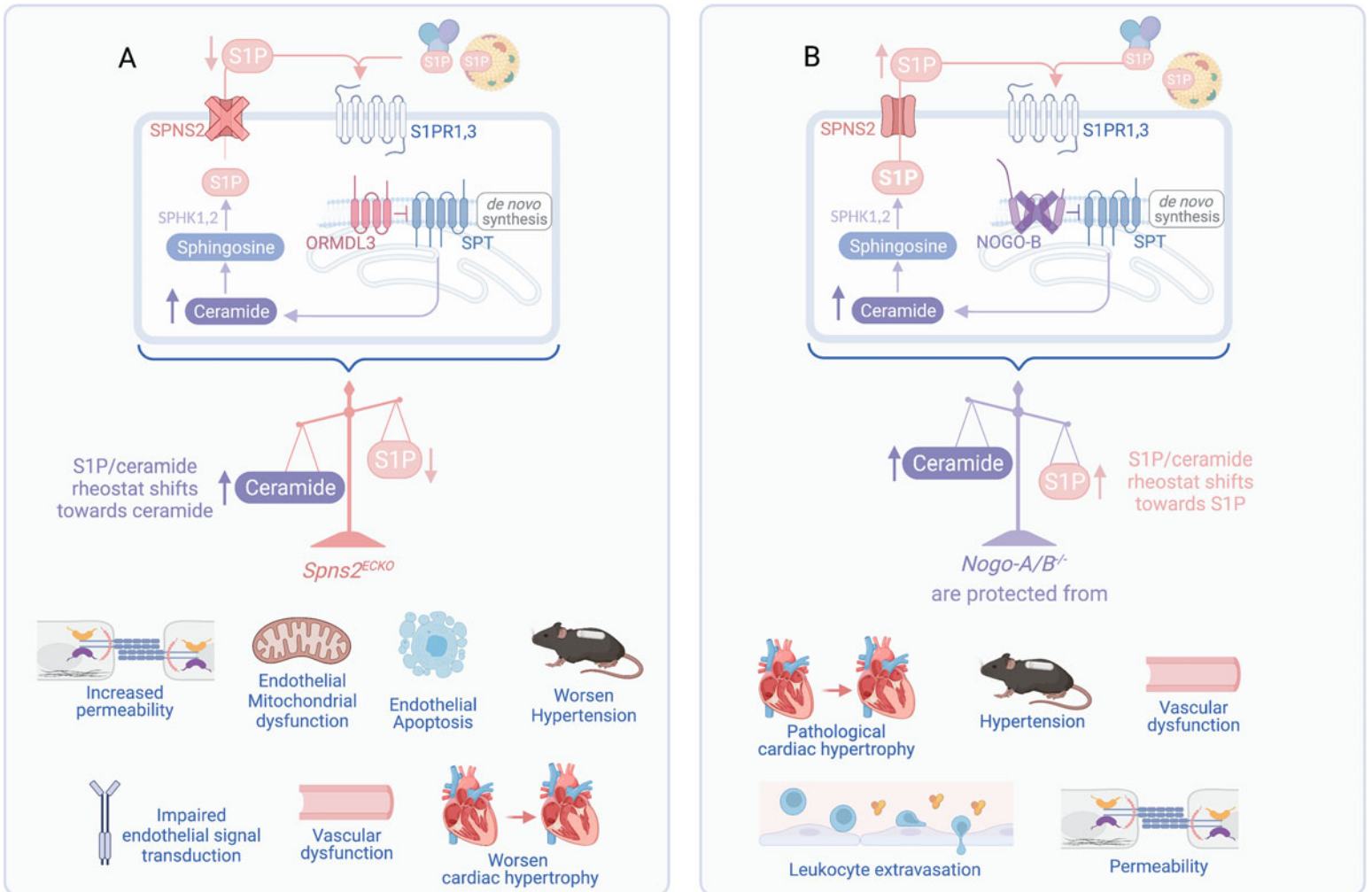

Fig. 8.4 Endothelial fine-tuning of sphingolipid de novo biosynthesis: role of Nogo-B and ORMDLs. (**a**) In absence of Spns2 (*Spns2*^{ECKO}), S1P-S1PRs dependent stabilization of ORMDL3 is disrupted, leading to an upregulation of SPT activity. Ceramide build up whereas S1P is no longer transported outside of the cells, thus

Fig. 8.4 (continued) autocrine/paracrine endothelial S1P signaling is lost. In these conditions, the S1P/ceramide rheostat shifts towards ceramides, resulting in generalized endothelial dysfunction, including barrier impairment, mitochondrial dysfunction, apoptosis, impaired signal transduction, dysregulation of vascular tone, hypertension, and severe cardiac hypertrophy. (**b**) In absence of Nogo-B (*Nogo-A/B*$^{-/-}$), the upregulation of SPT activity leads to the beneficial increase of both ceramide and S1P levels, within a window of opportunity. The rheostat S1P/ceramide shifts towards S1P beneficial effects, resulting in reduced pathological cardiac hypertrophy, vascular dysfunction, hypertension, leucocyte extravasation, and permeability. *S1P* sphingosine-1-phosphate, *S1PR* S1P receptor, *SPT* serine palmitoyltransferase, *SM* sphingomyelin, *GSL* glycosphingolipid, *SPHK1,2* sphingosine kinase, *SPNS2* spinster-2

sphingolipid levels, particularly S1P, are increased [53]. However, the molecular basis governing the dynamic interaction of NOGO-B/SPT, for instance in response to changes in sphingolipid levels, remains to be elucidated. Mice lacking Nogo-A/B globally (*Nogo-A/B*$^{-/-}$) and specifically in EC (*Nogo-A/B*^{ECKO}) are born at Mendelian ratios. In physiological conditions, they only present a significant lower systolic BP than controls (ca. 90 vs. 115 mmHg, respectively). Strikingly, both *Nogo-A/B*$^{-/-}$ and *Nogo-A/B*^{ECKO} mice were protected from developing high BP following chronic infusion of AngII, suggesting a pro-hypertensive role of NOGO-B. These in vivo findings were supported by vascular reactivity studies ex vivo. The loss of NOGO-B preserved vasorelaxation of MA in response to Ach and flow, which correlated with higher cGMP levels in thoracic aorta, index of endothelial NO production [53]. Mechanistically, in the absence of NOGO-B endothelial-derived S1P was markedly upregulated, hence the S1PR1-NO signaling. Interestingly, treatment of *Nogo-A/B*$^{-/-}$ mice with myriocin restored BP to pathological levels (from ca. 110 to 140 mmHg in AngII-treated mice), due to the downregulation of S1P-S1PR1-NO signaling. Although in the absence of NOGO-B, SPT activity increases and results in the elevation of both ceramide and S1P, this upregulation is within a "window of opportunity" that upregulates endothelial S1P and S1PR1-NO signaling without activating PP2A via ceramide. Overall, these findings indicate the Nogo-B downregulation of SPT activity is relevant to vascular diseases and endothelial dysfunction.

8.9.2 NOGO-B and Barrier Function

In addition to eNOS activation, S1P can enhance the endothelial barrier function via Rac-mediated cytoskeleton remodeling [80] and VE-cadherin dephosphorylation, as discussed in Sect. 8.4 [79]. The loss of NOGO-B enhances endothelial S1P production in vitro, which correlates with higher TEER enhanced barrier function [193]. Plasma S1P is also significantly elevated in *Nogo-A/B*$^{-/-}$ mice. Interestingly *Nogo-A/B*$^{-/-}$ mice were protected from myocardial permeability and inflammation following pressure overload (induced by transverse aortic constriction), whereas myriocin treatment abolished these beneficial effects. On the contrary, SEW2871, an agonist of S1PR1, was able to reduce inflammation following pressure overload [193], suggesting that endothelial S1P-S1PR1-mediated barrier enhancement in absence of NOGO-B refrains plasma proteins and leukocytes from extravasating and damaging the heart. It is noteworthy that while the loss of NOGO-B did not affect EC adhesion molecules expression (ICAM-1 and CD31) [194], exogenous addition of S1P was able to induce a significant and dose-dependent increase in VCAM-1 and E-selectin [195]. This difference may be due to the fact that in EC lacking NOGO-B the increase in S1P is into physiological levels, whereas exogenous S1P was added to EC in the μM range (1–5 μM), significantly higher than concentrations used for in vitro studies (100-300 nM), thus might overcome beneficial effects of S1P. In models of acute inflammation, such as carrageenan-induced paw edema and zymosan air-pouch, the enhanced barrier function in *Nogo-A/B*$^{-/-}$ resulted in decreased vascular leakage and extravasation of inflammatory cells, particularly neutrophils and monocytes [194]. Studies in vitro of endothelial–leukocyte interactions under flow demonstrated that paracellular trans-endothelial migration of neutrophils and monocytes was markedly suppressed in *Nogo-A/B*$^{-/-}$ EC, supporting the enhanced barrier [194]. Mechanistically, in the absence of NOGO-B the upregulation of local S1P production counterbalances VE-cadherin phosphorylation and stress fiber formation induced by ICAM-1 cross-linking [194], thus restraining the opening of the endothelial junctions during inflammation, in line with similar S1P antagonizing effects reported for VEGF and thrombin-mediated barrier disruption [80]. Accordingly, mice overexpressing S1PR1 specifically in the endothelium showed a decreased expression of ICAM-1, confirming that S1P-S1PR1 signaling suppresses the expression of inflammatory genes.

8.9.3 Endothelial NOGO-B and Pathological Cardiac Hypertrophy

In absence of endothelial NOGO-B, SPT activity is upregulated. *Nogo-A/B*$^{-/-}$ mice are protected from cardiac hypertrophy, fibrosis, and failure following chronic pressure overload [193]. Myriocin treatment inhibits SPT and reinstates the pathological phenotype in *Nogo-A/B*$^{-/-}$, most luckily by suppressing local S1P-S1PR1 signaling. Interestingly, *Nogo-A/B*^{ECKO} mice are protected from developing pathological cardiac hypertrophy, recapitulating the phenotype observed in *Nogo-A/B*$^{-/-}$. Mechanistically, the upregulation of S1P/eNOS/NO pathway in the absence of NOGO-B exerts a protective effect on the pressure overloaded heart by different means, including the reduction of permeability and inflammation by enhancing the EC barrier and cardioprotective effects of endothelial S1P [146], as well as of NO [145, 196, 197], on cardiomyocytes.

Altered sphingolipid metabolism has been implicated in cancer, and CV, immune and neurodegenerative diseases [198]. Thus, it is not surprising that more than one regulatory mechanism is in place regulating the de novo biosynthesis to preserve sphingolipid homeostasis in response to metabolic and environmental stimuli. This pathway is dynamical regulated in response to the changes of the microenvironment (i.e., inflammatory stimuli, radiations, blood flow, hemodynamic stress in the heart, etc.). We are just beginning to understand how sphingolipid levels are sensed by the cells and the multilayered regulatory mechanisms maintaining sphingolipid homeostasis. The stabilization of ORMDL by S1P signaling identified in EC might be also implemented in other cell types. Unraveling the regulatory mechanisms of sphingolipid biosynthesis may provide the framework for therapeutics to restore homeostasis in disease settings.

8.10 Plasma Ceramide as Biomarker for Major Cardiovascular Events and Other Conditions

Traditional serum lipid biomarkers of CV diseases are triglycerides and cholesterol. In the recent years, multiple studies demonstrated a strong relationship between the levels of circulating ceramides and the incidence of adverse CV events, such as CAD, MI, hypertension, and stroke, both with non-fatal and fatal outcomes [144, 199–207].

Most of these studies identified three major ceramide species, C16:0-, C18:0-, and C24:1-ceramide, positively associated with CV disease incidence [199], secondary CV events [201, 205], and CV mortality [200, 202, 203]; whereas C24:0-ceramide negatively correlated with CV mortality [200, 202, 203]. C24:0- and C24:1-ceramide positively correlated with the severity of hypertension [144]. Levels of C16:0-, C22:0-, and C24:0-ceramide associated with increased risk and severity of ischemic stroke [206]. Another study positively correlated C20:0- and negatively correlated C24:1-ceramide with acute ischemic stroke. Moreover, the ratios S1P/C24:1-ceramide and C24:0/C24:1-ceramide were particularly increased in acute stroke patients, suggesting a potential use as biomarkers [207]. CERT1 (cardiac event risk test 1) was developed for clinical practice and is currently in use at Mayo Clinic. Based on the concentrations of C16:0-, C18:0-, and C24:1-ceramide, alone or in their ratio to C24:0-ceramide, CERT1 stratifies patients into four CV risk categories [201]. A second risk score, CERT2, was developed taking into consideration that also phosphatidylcholine has shown prognostic values for CV events, but is not currently used in clinical practice [201, 208]. However, the inclusion of these scores in clinical practice would allow a more aggressive interventions in patients at higher risk. A recent study of Poss and colleagues evaluated a more comprehensive panel

of 32 SL species in the plasma of patients with CAD, including dihydroceramides, ceramides, glucosylceramides, dihydroSM, SM, sphingosine, and sphinganine. Thirty out of 32 SL measured were elevated in patients affected by familial CAD versus healthy controls and correlated with the severity of the disease [204]. They proposed a new risk score, named SIC (sphingolipid-inclusive CAD), including C18:0-dihydroceramide, C18:0-, C22:0-, and C24:0-ceramide, C24:1-dihydroSM, C18:0- and C24:0-SM, and sphingosine, expected to outperform CERT1 in the stratification of patients with CAD [204].

In addition to CV diseases, plasma C18:0-, C20:0-, and C24:1-ceramide are also significantly elevated in obese patients with T2D compared to lean healthy individuals and correlated with the severity of insulin resistance [209]. Interestingly, the ratio C18:0/C16:0-ceramide was predictive of T2D 10 years before the diagnosis [210], and C16:0-, C22:0-, C24:0-, C24:1-dihydroceramides were increased and predictive of T2D 9 years before the diagnosis [211]. While it is known that EC and RBC are major sources of plasma S1P [53, 54, 212], little is known on the cellular sources of ceramides in circulation. We have recently demonstrated that endothelial deletion of SPTLC2 leads to ca. 50% and 30% reduction in plasma S1P and ceramides, respectively, but not in SM [119]. A significant reduction in plasma S1P (ca. 40%) was also achieved by genetic deletion of endothelial SPNS2, supporting the role of the endothelium as source of both plasma S1P and ceramide [54, 58]. Based on clinical data, it is conceivable that the endothelium contributes, at least in part, to remodel plasma ceramide landscape in patients affected by CV and metabolic diseases, including CAD, heart failure, and diabetes. Thus, in addition to predict major CV events, plasma ceramides might also function as biomarkers of the diseases state of blood vessel. It is noteworthy to mention that how SL change in the diseased versus healthy endothelium, and their impact on the vascular pathology, remains poorly understood. The increase of endothelial ceramide in CV diseases and diabetes has been inferred by in vitro findings but never measured in vivo. Thus, further studies are needed to understand how sphingolipid metabolism changes and contributes to endothelial dysfunction, culprit event in the pathogenesis of cardiovascular and cerebrovascular diseases.

8.11 Gene Variants of Sphingolipid Pathway and Cardiovascular Diseases

Several rare monogenic disorders have been associated with mutations impairing the activity of proteins of sphingolipid metabolism, and most of them manifest neurological disorders, like in hereditary sensory and autonomic neuropathy type 1, and Niemann–Pick, Gaucher, Krabbe, Fabry, and Farber diseases [213]. Growing literature reported the association between single nucleotide polymorphism (SNP) of sphingolipid metabolism genes and CV diseases. In 2009, Hicks et al. conducted a Genome-Wide Association Study (GWAS) between 318,237 SNPs and the levels of circulating SL species in five different European populations. They found SNPs in SPTLC3, CERS4, SGPP1 (S1P phosphatase), and FADS1–3 (fatty acid desaturase) associated with circulating sphingolipid levels. Moreover, SNP in SPTLC3 correlated with the incidence of MI [214]. A recent study identified 28 SNPs close to the SPTLC3 locus that significantly associated with reduction in plasma C22:0- and C24:0-ceramide [215], and increased levels of these ceramide species have been linked to CAD and heart failure. A genetic variant in dihydroceramide desaturase (DEGS1), leading to L175Q mutation (rs191144864), causes a partial loss-of-function [216]. Patients with this mutation have increased dihydroceramides and a modest decrease in three established ceramide ratios (C16:0-, C20:0-, and C24:1- in ratio with C24:0-ceramide) linked to CV diseases, suggesting that this SNP in DEGS1 may have protective functions [216]. Moreover, egs *Degs1*$^{-/-}$ mice on leptin-deficient background or fed an obesogenic diet have reduced hepatic steatosis and insulin resistance. Chaurasia et al. [217], suggesting that DEGS1 L175Q may protect from obesity and diabetes. A SNP in

CERS2 (rs267738) leading to a partial loss-of-function [218], and three in CERS6 (rs3845724, rs4668102, and rs4668089) mapping in an intronic region [219], have been associated with metabolic disease and T2D, respectively. Hla's group characterized the functional consequences of several SNPs in S1PR1 [220]. R120P mutation (rs149198314) abrogates the ability of S1PR1 to bind S1P, and therefore its endocytosis and downstream signaling. I45T (rs148977042) and G305C (rs146890331) mutations impair S1PR1 desensitization process. Thus, the efficacy of FTY720 treatment might be affected in individuals carrying these S1PR1 mutations. Finally, R13G mutation in S1PR1 was found enriched in a high CV risk population, but it appeared to protect from CAD [220], although in vitro data of S1PR1 R13G mutant showed no functional differences with the native receptor. SNPs SphK1 [221] associated with increased CV events; SNPs in NOGO-B (rs17046570) and ORMDL3 (rs7216389 and rs9303277) positively correlated with CAD [212, 222].

Circulating concentrations of several key components of sphingolipid metabolism are under genetic control, and it is conceivable to think that other variants in their loci can be accountable for the disruption in the SL homeostasis seen in the cardiometabolic diseases.

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9 Cholesterol Metabolism in Chronic Kidney Disease: Physiology, Pathologic Mechanisms, and Treatment

Xiaoyue Pan

Abstract

High plasma levels of lipids and/or lipoproteins are risk factors for atherosclerosis, nonalcoholic fatty liver disease (NAFLD), obesity, and diabetes. These four conditions have also been identified as risk factors leading to the development of chronic kidney disease (CKD). Although many pathways that generate high plasma levels of these factors have been identified, most clinical and physiologic dysfunction results from aberrant assembly and secretion of lipoproteins. The results of several published studies suggest that elevated levels of low-density lipoprotein (LDL)-cholesterol are a risk factor for atherosclerosis, myocardial infarction, coronary artery calcification associated with type 2 diabetes, and NAFLD. Cholesterol metabolism has also been identified as an important pathway contributing to the development of CKD; clinical treatments designed to alter various steps of the cholesterol synthesis and metabolism pathway are currently under study. Cholesterol synthesis and catabolism contribute to a multistep process with pathways that are regulated at the cellular level in renal tissue. Cholesterol metabolism may also be regulated by the balance between the influx and efflux of cholesterol molecules that are capable of crossing the membrane of renal proximal tubular epithelial cells and podocytes. Cellular accumulation of cholesterol can result in lipotoxicity and ultimately kidney dysfunction and failure. Thus, further research focused on cholesterol metabolism pathways will be necessary to improve our understanding of the impact of cholesterol restriction, which is currently a primary intervention recommended for patients with dyslipidemia.

Keywords

Cholesterol metabolism · Chronic kidney disease · Apolipoprotein · Lipotoxicity · Cholesterol accumulation

Abbreviations

ABCA1	ATP-binding cassette transporter (ABC) A1
ABCG1	ATP-binding cassette subfamily G member 1
ACAT1	Acyl CoA: cholesterol acyltransferase 1 sterol-*O*-acyltransferase 1
Apo	Apolipoprotein
ApoA1	Apolipoprotein A1
ATP	Adenosine triphosphate

X. Pan (✉)
Present Address: Department of Foundations of Medicine, New York University Long Island School of Medicine, Mineola, NY, USA
e-mail: Xiaoyue.Pan@nyulangone.org

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Bmal1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
BMI	Body mass index
Cd36	Scavenger receptor class B
CKD	Chronic kidney disease
$Clk^{\Delta 19/\Delta 19}$	*Clock* mutant mice
CVD	Cardiovascular disease
ESKD	End-stage kidney disease
FASN	Fatty acid synthase
FXRs	Farnesoid X receptors
GFRs	Glomerular filtration rates
HDAC1	Histone deacetylase 1
HDL	High-density lipoprotein
HK2 cells	Human proximal tubule cells
HIF	Hypoxia-inducible factor
HMGCR	3-Hydroxy-3-methylglutaryl-CoA reductase
KO	Knock out
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptor
IncRNAs	Long noncoding RNAs
LPL	Lipoprotein lipase
LOX-1	Lectin-like oxLDL receptor-1
LXRs	Liver X receptors
miRNAs	MicroRNAs
MTP	Microsomal triglyceride transfer protein
NAFLD	Nonalcoholic fatty liver disease
NCEH1	Neutral cholesterol ester hydrolase 1
NPC1	Niemann-Pick C1
NPC1L1	NPC1-like intracellular cholesterol transporter 1
NRs	Nuclear receptors
Nrf2	Nuclear factor-erythroid factor 2-related factor 2
NS	Nephrotic syndrome
PCSK9	Proprotein convertase subtilisin/kexin type 9
PHD2	Hypoxia-inducible factor prolyl hydroxylase 2
PKD	Polycystic kidney disease
PLTP	Phospholipid transfer protein
PPARs	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
RPTECs	Renal proximal tubule epithelial cells
Shp	Small heterodimer partner
SPRING	SREBF pathway regulator in Golgi 1
SR-A1	Scavenger receptor class A
SR-B1	Scavenger receptor class B type 1
SREBPs	Sterol regulatory element-binding proteins
USP20	Ubiquitin C-terminal hydrolase 20
VLDLs	Very light density lipoproteins

9.1 Introduction

Human kidneys are critical organs that perform essential functions, most notably the removal of waste products, excess fluids, and acid from the body via the formation and excretion of urine. Healthy kidneys filter approximately 140 L of blood and generate 2 L of urine per day. Lipids in the kidney are mainly in the form of sphingolipids, cholesterol, and fatty acids which are detected in nearly equal ratios to one another. Membrane lipids function as channel regulators (e.g., K^+, Na^+, Ca^{2+}, Kir2.1, and Cl^- channels) and ion transporters in kidney [1]. Lipids also provide significant contributions to signaling, cellular bioenergetics, and the formation of cell membranes that are critical for normal kidney function [1–3]. Studies focused on genetic and non-genetic lipid dysfunction in the kidney have revealed links between local fat deposits and the pathogenesis of kidney disease [4–6].

Thirty-seven million people in the USA are currently living with chronic kidney disease (CKD) [7]. The common causes of CKD include both type 1 and type 2 diabetes, hypertension, heart disease, aging, sleep disorders, and COVID-19 [8–18]. A family history of kidney dysfunction is also a prominent risk factor for CKD. Kidney failure can result in numerous signs and symptoms, including itchiness, muscle cramps, nausea and vomiting, anorexia, dependent edema, polyuria, oliguria, dyspnea, and sleep difficulties. Abnormalities in lipid metabolism have been described in patients at all stages of CKD [19–22].

Many factors can result in acute and chronic kidney injury [23] including dysfunction of mitochondrial [24, 25] and lysosomal pathways in association with oxidative stress and dysregulated autophagy [26]. One or more of these dysfunctional responses can promote, modulate, and accelerate kidney injury and CKD.

Both transcriptional and post-transcriptional mechanisms that have an impact on gene regulatory pathways can contribute to the pathogenesis of acute kidney injury and CKD. For example, noncoding RNAs, including microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) have recently been identified as versatile regulators of gene expression. Several characterized miRNAs modulate post-transcriptional regulation of low-density lipoprotein receptor (LDLr) activity in human cells; genetic mutations within the RNA binding motif protein 25 have been associated with alterations in both LDL and cholesterol levels [27]. Several studies have demonstrated that the circadian clock gene promotes transcriptional mechanisms that contribute to the pathogenesis of CKD [28–31].

Epigenetic regulation involves several specific biochemical processes including DNA methylation, histone modifications, histone variants, isoform incorporation, nucleosome remodeling, and RNA interference [32, 33]. Common histone modifications include N-terminal acetylation, methylation, sumoylation, ubiquitination, citrullination, and poly ADP-ribosylation. These modifications can have a profound impact on pathophysiological processes and the risk of developing renal cancer [33]. For example, Inoue et al. [34] reported that inhibition of histone deacetylase 1 (HDAC1) and HDAC2 activities may limit the progression of human proteinuric kidney diseases via the regulation of the transcription factor, early growth response 1. These results suggest that HDAC1 and HDAC2 may be attractive targets for therapeutic intervention.

Numerous pathways play significant roles in regulating kidney injury. Pathways involving nuclear receptors [35, 36], glycogen synthase kinase 3 [37], and de novo nicotinamide adenine dinucleotide biosynthesis [38] have been implicated in disorders including metabolic syndrome, acute kidney injury, and CKD. Xenobiotic transporters [39], as well as glucose-associated metabolic pathways [40–42], glucose transporters [43, 44], and glucose reabsorption transporters [45, 46] also play critical roles in promoting these disorders.

While dyslipidemia can develop as a result of defects in cholesterol, fatty acid, and sphingolipid metabolism, the detailed mechanisms leading to dysfunction of glomerular podocytes and proximal tubular epithelial cell function remain unclear [47, 48]. Likewise, we have little understanding of how metabolic dysregulation of lipoproteins, fatty acids, and cholesterol contributes to acute kidney injury and CKD [21, 49, 50].

There are several methods available that can be used to study lipid metabolism in the kidney. Several recent studies featured lipidomic approaches to characterize dysregulated lipid metabolism in kidney disease [51]. Under physiologic conditions, high-density lipoprotein (HDL) regulates reverse cholesterol transport and thus controls circulating lipid levels. Abnormalities in HDL synthesis and/or regulation can result in cardiovascular disease and may contribute to the development of nephrotic syndrome (NS) and advanced CKD [48]. Recent work by Rao et al. [52] featured a Sequential Window Acquisition of all Theoretical Spectra-Mass Spectrometry (SWATH-MS) approach to measure early changes in the lipid content associated with acute kidney injury. This technique was combined with Matrix-Assisted Laser Desorption/Ionization (MALDI) and tissue imaging. A recent study by Subramanian et al. [53] featured transcriptomic analysis of single cells from human kidney organoids; the findings were highly reproducible and facilitated reductions in the number of off-target cells used for mouse transplantation or human fetal kidney studies. This group reported a positive relationship between expression of LY6/PLAUR domain containing 1, serine protease 23, and cadherin 6 with human glomerular disease based on the evaluation of single cells isolated from organoids or human kidney tissue [53]. Organoid single-cell profiling approaches have been used to identify

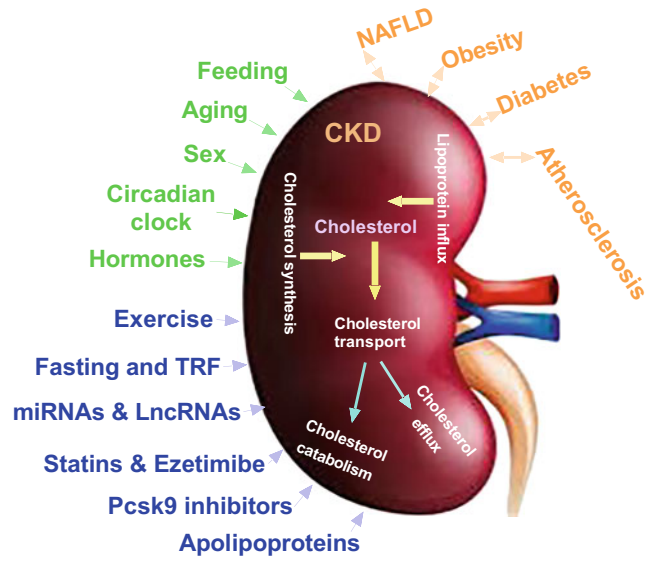

Fig. 9.1 Several behavioral, clinical disease and genetic factors contributing to cholesterol-related CKD and nephrotic syndrome according to the published data from several groups

transcriptional signatures of injured podocytes and glomerular epithelial cells that are associated with the development of glomerular disease [54].

Variation in metabolism can result in altered lipid structure both intracellularly and within the membrane of kidney cells [49, 50, 55]. Functional disorders and injuries sustained by podocytes have been linked to dysfunctional lipid metabolism, including pathways involving cholesterol, triglycerides, and fatty acids. Dysfunctional lipid metabolism contributes to fatty kidney disease, acute kidney injury, diabetic kidney disease, type 1 and type 2 diabetes, cardiovascular disease (CVD), and CKD, which are all disorders that feature progressive deterioration of podocytes and tubular epithelial cell function [21, 56–62]. Results of ongoing basic and clinical research have provided us with the understanding that high levels of plasma cholesterol are among the most significant risk factors associated with the development of atherosclerosis. It is also clear that lipotoxicity and lipid accumulation can promote renal cell dysfunction and apoptosis [63]. Thus, high levels of plasma cholesterol also represent a risk factor for kidney disease. An understanding of physiology and pathophysiological activities of cholesterol metabolism and cholesterol-associated gene expression in the kidney will be essential for the development of future management strategies and targeted therapeutics for the treatment of CKD, nephrotic syndrome (NS), metabolic syndrome, and CVD.

This review highlights several recent studies aimed at elucidating the genetic mechanisms of cholesterol transport, de novo synthesis, and catabolism in the kidney (Fig. 9.1). The molecular biology of renal sterol transporters and cholesterol-associated gene expression patterns at homeostasis and under pathophysiologic conditions in the podocytes and renal proximal tubule epithelial cells (RPTECs) will also be considered.

9.2 Cholesterol Metabolism in Kidney

Cholesterol and cholesterol metabolic pathways play critical roles both in the development of the kidney during gestation and in controlling its normal functioning during adult life [11, 58]. Cellular cholesterol homeostasis represents a balance of de novo synthesis and catabolism as well as cholesterol influx and efflux in podocytes, tubular epithelial, and other cells [2, 6, 58]. De novo synthesis of cholesterol occurs mainly in the brain, liver, and intestine, while the kidney has been identified as limited synthesis of cholesterol

[61]. High levels of plasma cholesterol have been identified as independent risk factors associated with the progression of renal disease, and cholesterol accumulates in the human kidney in association with hyperlipidemic states and CVD [14, 64]. Accumulation of cholesterol in the kidney can lead to CKD. Increasing amounts of cholesterol taken up from the plasma into the tubular epithelial cells can lead to a decline in renal function in association with decreased expression of the lipoprotein receptor, megalin, and suppression of cell proliferation in renal proximal tubule [65]. Therefore, cholesterol is a critical regulator of megalin expression in RPTECs. Cholesterol synthesis, catabolism, influx, and efflux are all critical steps involved in cholesterol metabolism. These pathways include de novo synthesis of cholesterol, the influx of lipoprotein from the circulation, lipid catabolism and cholesterol efflux, lipoprotein binding to cholesterol in the circulation, and cholesterol excretion by reverse transport mechanisms [66]. Because each of these steps takes place within the kidney, any disorder affecting one or more of these pathways will have a direct impact on this organ. Cholesterol metabolism is a multistep process that is regulated by several genes expressed in RPTECs.

9.2.1 Lipoprotein Influx

Low-density lipoproteins (LDLs) are derived principally via de novo synthesis and absorption from dietary sources. High plasma levels of cholesterol-LDL in plasma can result in uptake into cells within the kidney via one of the several distinct receptors. Unmodified LDL particles bind to LDL receptors. By contrast, modified cholesterol-LDL, including oxidized, nitrated, and carbamylated forms, as well as advanced glycation end product LDLs inhibit the functioning of this receptor. Modified cholesterol-LDLs interact with and are taken up via cellular scavenger receptors, including the scavenger receptor class A (SR-A1), class B (Cd36), and the lectin-like oxLDL receptor-1 (LOX-1, also known as OLR-1). The process of cholesterol influx into renal cells is similar to that characterized in other tissues as well as macrophages. While only low levels of LOX-1 expression can be detected at homeostasis, this receptor is upregulated in response to increasing levels of plasma cholesterol resulting from a high cholesterol diet, diabetic kidney disease, and atherosclerosis [67, 68]. LDL receptor, CD36, and SR-A1 expression positively correlated with the level of cholesterol detected in podocytes and RPTECs [69, 70].

9.2.2 Cholesterol Synthesis

Cholesterol homeostasis is largely regulated by cholesterol synthesis in the endoplasmic reticulum. This process relies directly on the actions of sterol regulatory element-binding proteins (SREBPs), which are transcription factors that regulate the expression of genes involved in cholesterol and fatty acid synthesis during physiological and pathophysiological processes [71, 72]. For example, SREBP1α promotes global lipid synthesis and growth, SREBP1c and SREBP2 are involved in fatty acid synthesis, energy storage, and cholesterol regulation, respectively. Activation of Srebp1c results in lipid-mediated cellular stress (lipotoxicity) that contributes to metabolic diseases [71, 72] and engages pathways that provide feedback mechanisms to prevent the over-accumulation of cholesterol within cells. Wang et al. [64] found that mice with type 2 diabetes (i.e., the FVBdb/db loss-of-function mutation of the leptin receptor model) display increased levels of SREBP1 and SRBP2 proteins in the renal nuclear extracts compared with control mice. In addition, increased levels of mRNA encoding acetyl-CoA carboxylase, fatty acid synthase (FASN), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) were detected in FVBdb/db mice, which resulted in an increase in renal cholesterol and triglyceride content. High plasma glucose levels also result in increased expression of mRNAs encoding Srebp1α, Srebp1c, and FASN, as well as increases in SREBP1 protein, which ultimately result in triglyceride accumulation in the kidney

[73]. Collectively, these studies suggested that increased expression of SREBPs promotes renal lipid accumulation, glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria in a mouse model of type 2 diabetes [58] and that Srebps play central roles in regulating cholesterol synthesis in the kidney.

Results from several human and animal studies (with some notable exceptions) suggest that cholesterol synthesis in the kidney acts acutely to augment lipotoxic dysfunction [74]. For example, Herman-Edelstein et al. [58] examined a series of diagnostic kidney biopsies of patients diagnosed with diabetic nephropathy and found that expression of mRNAs encoding HMG-CoA reductase, HMG-CoA synthase, and Srebp2 was not substantially different from that detected in normal kidney tissue. The results of this study suggest that cholesterol influx and efflux be the more important regulators of cholesterol accumulation in the diabetic kidney. Recently, Loregger et al. [75] identified SPRING (SREBF pathway regulator in Golgi 1) as a glycosylated Golgi-resident membrane protein that is required for SREBP-mediated regulation of cholesterol metabolism. This result suggests that SPRING also plays an essential role in controlling cholesterol synthesis in these cells.

Several nuclear receptors (NRs), including liver X receptors (LXRs), peroxisome proliferator-activated receptors (PPARs), and farnesoid X receptors (FXRs), among others, play vital roles in regulating cholesterol transport [36] in association with CKD. Of note, FXRs are involved in several distinct pathways associated with lipid metabolism, suggesting that these receptors are not only important for controlling cholesterol synthesis, they may also regulate CKD via pathways that promote oxidative stress, inflammation, endoplasmic reticulum stress, and fibrosis [36].

9.2.3 Cholesterol Transport

Circulating levels of cholesterol are determined by rates of de novo synthesis in the gastrointestinal tract and its interactions with lipoproteins to form cholesterol-ester-rich lipoprotein particles. Cholesterol is transported in circulation by two major lipoproteins. The first of these is high-density lipoprotein (HDL) which is often referred to as "good cholesterol"; the second, LDL, is frequently considered to be "bad cholesterol." Cholesterol-LDL particles are the major source of cholesterol that can be taken up into cells by LDL receptor [76]. Cholesterol-containing LDL particles bind to the LDL receptor followed by endocytosis and fusion of the cholesterol-LDL-containing vesicle with an intracellular lysosome [77]. Lysosomal enzymes break down the particles and convert LDL to its constituent amino acid, thereby releasing free cholesterol. Next, the un-esterified cholesterol is transported by Niemann-Pick C1 and C2 proteins (NPC1 and NPC2) from the endosome/lysosome complex to the endoplasmic reticulum and ultimately to the plasma membrane [77, 78]. NPC1 is a multi-spanning protein found in the outer membrane of late endosome [79, 80]. NPC2 is found within the lumen of the late endosome [80, 81]. The increase in cholesterol within the cell results in the formation of cholesteryl esters via activation of Acyl CoA: cholesterol acyltransferase 1 sterol-O-acyltransferase 1 (ACAT1) and signals a reduction in both LDL and cholesterol synthesis, the latter via the inhibition of HMGCR and squalene epoxidase [82–87].

Accumulation of free cholesterol may result in cytotoxicity, suggesting the importance of cholesterol esterification for maintaining free cholesterol at levels that are optimal for cell membrane function. The enzyme, ACAT1 localized to the endoplasmic reticulum converts free cholesterol to cholesterol esters; the newly formed cholesterol esters are then stored in lipid droplets [88, 89]. Diminished levels of ACAT1 result in reductions in cholesterol ester and lipid droplet formation in human podocytes [90]. Similarly, neutral cholesterol ester hydrolase 1 (NCEH1) is a single membrane-spanning type II membrane protein with three functional domains (i.e., N-terminal, catalytic, and lipid-binding) [91] that contributes to the initial step of reverse cholesterol transport [92, 93]. While these enzymes are likely to play important roles in cholesterol

metabolism, their involvement specifically in renal metabolism remains to be elucidated.

9.2.4 Cholesterol Efflux

Reverse cholesterol transport, or efflux, involves several steps in the kidney that are similar to those that have been characterized in other tissues [94–96]. As a first step, cholesterol is exported from somatic cells and linked with HDL. The ATP-binding cassette transporter (ABC) A1 (ABCA1) and ATP-binding cassette subfamily G member 1 (ABCG1) play essential roles in this process [97]. Next, the cholesterol linked to HDL is esterified by the actions of the enzyme lecithin-cholesterol acyltransferase (LCAT) and is carried in the core of the HDL particle to the liver. Cholesterol esters are delivered to hepatocytes via interactions with Scavenger receptor class B type 1 (SR-B1). Finally, cholesterol is excreted from the liver into the bile either directly or after conversion to bile acids. It is then secreted into the intestines and excreted in the feces. The process of cholesterol efflux from macrophage foam cells is perhaps the most relevant with respect to atherosclerosis. Our group has demonstrated that reverse cholesterol transport plays an essential role in the induction of atherosclerosis in Clock mutant mice ($Clk^{\Delta 19/\Delta 19}$) mice as well as aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1)-gene-deleted mice [98, 99]. Dysfunction of cellular cholesterol efflux pathways is a risk factor associated with the development of renal dysfunction, atherosclerosis, and CKD.

Interestingly, Merscher-Gomez et al. [100] found that diminished levels of ABCA1 resulted in a reduction in cholesterol efflux and accumulation of cholesterol in renal podocytes of patients diagnosed with diabetic nephropathy. Interestingly, the phenotype resulting from downregulation of ABCA1 included diminished cholesterol efflux and increases in renal cholesterol levels that were similar in type 2 diabetic mice and human diabetic nephropathy [58, 100]. Downregulated cholesterol efflux and decreased expression of Abca1 that result in dysfunctional lipid metabolism and lipid accumulation can also induce the synthesis of reactive oxygen species (ROS) which will contribute to the progression of animal diabetic nephropathy [21, 57]. Another recent study revealed that Oxysterol binding protein-like 7 regulated both ABCA1 and cholesterol efflux in cultured human podocytes from patients with kidney disease [61]. Similarly, Tsun et al. [36] found that reductions in ABCA1, ABCG1, and SR-B1 were associated with diminished levels of apolipoprotein A1 (ApoA1) and HDL-cholesterol in RPTECs from mice with type 2 diabetes as well as in human kidneys. This phenotype was preceded by the development of nephropathy; mice with nephropathy secondary to type 2 diabetes expressed the lowest levels of Abca1, Abcg1, and Sr-b1 in this study [101]. Likewise, NRs, including LXRs, PPARs, and FXRs, among others have a significant role in regulating cholesterol transport. LXRα and PPARα are major transcription factors that control Abca1 expression and promote cholesterol efflux [102–104]. Renal function is also influenced by LXRα and PPARα; loss of renal LXRα and PPARα resulted in fatty acid and cholesterol accumulation in podocytes and RPTECs in the kidney [36, 100, 105]. Collectively, these data suggest that decreased expression of cholesterol transporters in RPTECs will result in cholesterol accumulation and might contribute to the development of diabetic nephropathy in mice.

9.2.5 Cholesterol Catabolism

Cholesterol metabolism plays an essential physiological role in supporting normal renal function and, together with cholesterol catabolism, contributes to the pathophysiology associated with CKD. Cholesterol can be broken down into numerous important catabolites, including acetyl-CoA, propionyl-CoA, succinyl-CoA, and pyruvate, which are compounds used by different carbon assimilating pathways [106]. As part of this process, acetyl-CoA and pyruvate are substrates for the citric acid cycle that are used to generate ATP [106]. Cholesterol can also be

converted into adrenocortical and sex hormones or degraded into bile acid. Griffin et al. [106–109] reported that chronic infection of bone marrow-derived macrophages with the bacterial pathogen, *Mycobacterium tuberculosis*, results in the activation of the cholesterol catabolic methylcitrate cycle and the transcriptional regulator, Rv1129c. Although there are few to no observations describing cholesterol catabolism and its role in renal cellular function, future studies might explore the role of these pathways in CKD and other renal diseases to discover new and effective therapies.

Circulating LDL binds to glycosaminoglycans in the glomerular basement membrane and increases its permeability. Accumulation of filtered lipoprotein in mesangial cells induces their proliferation to the point at which they interfere with the basolateral membrane [110]. RPTECs can metabolize some of the filtered lipoproteins; the fraction that remains is altered during its passage down the nephron. If the intraluminal pH is close to the isoelectric point of apolipoprotein B (ApoB) or ApoE, the luminal lipoprotein will precipitate, thereby initiating or aggravating tubule-interstitial disease [110–112]. Ongoing abnormal lipid processing may lead to chronic progressive kidney disease.

9.3 Pathological Pathways Associated with Obesity, Diabetes, Atherosclerosis, and NAFLD That Contribute to the Development of CKD

It is critical to understand that the term "kidney disease" is not a single entity but rather a set of disorders that range from simple lipid accumulation and can extend to include inflammation, necrosis, and fibrosis. The distinct histopathologic conditions are likely to have different effects on the risk of developing CKD, as they involve the kidney alone or result in alterations to the hormonal and inflammatory environment of the entire body. As a result, it is difficult to differentiate between pathophysiologic pathways that are directly associated with CKD, rather than those that promote, for example, overall atherosclerosis. Thus far, we know that hyperlipidemia is a risk factor that can lead to kidney damage and ultimately chronic kidney disease. Simple lipid accumulation in the kidney can progress to acute renal injury and dysfunction. Lipid droplets are typically detected in proximal tubule cells in patients with NS; likewise, filtered lipoproteins such as HDL may be chemically altered on passage through the nephron [113]. One can anticipate that high lipid levels in tissues other than liver or adipose will cause cellular injury and dysfunction. Unfortunately, there are currently no non-invasive diagnostic tools available for clinical use that will permit differentiation between simple cholesterol accumulation in the kidney and CKD. This can only be determined by examination of a renal biopsy sample. However, this invasive procedure is used only in the most severe cases of CKD in which patients exhibit chronically elevated serum levels of renal enzymes and lipids markers.

Several recent studies evaluated the relationship between CKD and clinical conditions that include obesity, diabetes, atherosclerosis, and aberrant cholesterol metabolism. However, these studies were limited because they used only indirect measures to evaluate CKD (e.g., serum levels of kidney enzymes, lipids, and apolipoproteins). While clinical studies based on ultrasonography are more reliable than those based on renal enzyme measurements alone, several of these remained uninterpretable because they did not include a case-control, prospective, or retrospective cohort, and made no adjustments for potential confounding clinical factors. Furthermore, many of these clinical studies were limited to select patient cohorts; as a result, these studies enrolled comparatively few patients and had overall low statistical power. Magnetic resonance imaging is another non-invasive method that has been used to evaluate CKD. Magnetic resonance imaging-based phenotyping has been used to measure the number of glomeruli and changes in volume distribution change with or without a reduction nephron number [114, 115]. It is of course easier to evaluate the impact of lipid accumulation and its role in promoting the

progression of glomerular and tubulointerstitial lesions in experimental animal models of chronic glomerulopathies, obesity, diabetes, and atherosclerosis.

In the sections to follow, we summarize the current data on several diseases including obesity, diabetes, atherosclerosis, and NAFLD-related CVD and kidney injury.

9.3.1 Obesity

Kidney disease is frequently associated with obesity. Kidney disease is diagnosed more commonly in patients who are obese than in their normal-weight counterparts, and obesity is observed more commonly in patients diagnosed with CKD than in others [20]. Consistent with this finding, clinical reports have revealed that a body mass index (BMI) $\geq$30 is a strong risk factor for kidney disease [113]; this phenomenon has also been reported in animal studies and supported by results from in vitro studies. For example, recently, Sun et al. [116] reported that a high-fat diet can induce oxidative stress, resulting in renal glomerular and tubular injuries. Oxidative stress can also activate the expression of HMGCR and induce renal cholesterol accumulation in cultured proximal tubule (HK-2) cells [117].

9.3.2 Diabetes

Similar to obesity, results from several studies have shown that in animal models and patients diagnosed with diabetic nephropathy, renal cholesterol accumulation has been associated with glomerulosclerosis and renal fibrosis due to accumulation of cholesterol in the podocytes induced by oxidative stress, mitochondrial dysfunction, inflammation, and cellular apoptosis [66, 118–121]. Sun et al. [122] reported that activation of SREBP cleavage-activating protein, SREBP-2, and HMGCR resulted in cholesterol accumulation in the kidney of streptozotocin-induced type 2 diabetic rats maintained on a high fat/sucrose diet. In this model of type 2 diabetes, the HMGCR inhibitor, atorvastatin, inhibited cholesterol synthesis and reduced renal lipid accumulation.

9.3.3 Atherosclerosis

Several clinical and animal studies have characterized the relationship between CKD and atherosclerosis [9, 123–125]. For example, in the ApoE knockout (KO) mouse model of atherosclerosis, mice respond with increased plasma and renal cholesterol when maintained either on routine chow or a high cholesterol diet [126]. Mice maintained on a high cholesterol diet developed more extensive kidney injury compared to mice on the routine chow diet [126]. The accompanying mechanistic studies revealed that the high cholesterol diet induces renal dysfunction via the reduction of Klotho expression [127]. In addition, RPTECs from ApoE KO mice exhibit enhanced uptake of oxidized-LDL when compared to controls. Likewise, the high-fat diet enhanced total and LDL-cholesterol in ApoE KO mice over levels observed in wild-type controls [128]. Notably, several studies have reported a strong positive association between the dysregulated expression of markers of kidney function and the severity of tissue histology. For example, ApoE KO mice exhibited reduced creatinine clearance, higher serum levels of urea and creatinine, and higher levels of collagen deposition compared with wild-type control mice. Overall, the ApoE KO mouse model can be used to explore the underlying pathophysiological mechanisms linking hypercholesterolemia and CKD that are similar to those identified in humans [129, 130].

9.3.4 NAFLD

The pathophysiology of NAFLD is linked to that of CKD through multiple pathways, including atherogenic dyslipidemia, metabolic syndrome, low-grade inflammation, type 2 diabetes mellitus, arterial hypertension, a prothrombotic state, increased serum uric acid levels, and unbalanced

levels of hormones and vitamins [131]. We recently found that Clock mutant mice develop NAFLD; by contrast, mice treated with cobalt chloride respond with activation of hypoxia-inducible factor (HIF), induction of tissue injury, and activation of Hif1α protein. Further mechanistic studies revealed that mutant Clock mice exhibit downregulated expression of mRNAs encoding Hypoxia-inducible Factor Prolyl Hydroxylase 2 (PHD2) and PHD3 and thus upregulate Hif1α protein to promote CD36-induced fatty acid uptake in the liver [132]. Recently studies indicated that the hypoxia-inducible factor Hif1β may also play a significant role as an antifibrotic agent in the pathogenesis of CKD [133, 134].

Together, the associations lining CKD with preclinical and clinical obesity, diabetes, and atherosclerosis have strength, conformity, timeliness, and biological consistency. These relationships are at least in part due to the coexistence of obesity, diabetes, atherosclerosis, NAFLD, cholesterol accumulation, dysglycemia, insulin resistance, hypertension, inflammation, and dyslipidemia in this patient cohort. However, accumulating evidence indicates that these associations might be partially independent of these pathologic conditions, most notably in cases in which CKD is characterized by systemic and as well as site-specific renal inflammation, as is the case in NS. At this time, we can conclude that numerous factors might have a pathogenic role that links CKD and/or NS to the development and progression of CKD.

9.4 Clinical Factors Contributing to Cholesterol-Related CKD

Strong associations link CKD and NS to clinical conditions that include obesity, diabetes, NAFLD, and atherosclerosis. Given the role played by cholesterol metabolism in each of the aforementioned conditions, one might hypothesize that CKD may also be associated with derangements in cholesterol metabolism. CKD is also associated with numerous additional factors, including age, sex, and circadian rhythm. As noted earlier in the text, these factors, together with inflammation, fibrosis, glucose metabolism, and insulin resistance, as well as a variety of related metabolic factors also have an impact on lipid metabolism [29].

9.4.1 Sex

Several clinical and animal model studies have shown that sex is a critical factor that influences the development and progression of CKD [135, 136]. One clinical study that enrolled type 2 diabetic patients reported differences between males and females in the assessment of the associations between HDL-cholesterol and the progression of diabetic kidney disease [137]. Specifically, males were at greater risk of developing CKD and death compared with females [136]. Animal model studies have also suggested that sex differences can influence the progression to renal injury in response to a high cholesterol diet [138]. Mechanistic studies revealed that estrogen receptors play a major role in the differential responses to renal injury exhibited by males and females [35].

9.4.2 Aging

Both males and females are at greater risk of developing CKD in response to aging [139]. CKD is diagnosed more frequently in aged patients than in their younger counterparts; likewise, the aging and elderly are more likely to be diagnosed with CKD than are younger patients [140]. The results of several animal model studies provide examples of aging processes that lead to renal dysfunction. For example, aged mice exhibit increased mesangial expansion and decreased glomerular filtration rates (GFRs) [126, 141]. Hypercholesterolemia leads to early renal injury in mice; aging promotes the clinical progression of this condition [121]. Recently, Chu et al. [142] reported that enhanced expression of glutathione peroxidase-1 resulted in diminished oxidative stress via the actions of nuclear factor-erythroid factor 2-related factor

2 (Nrf2) to limit pathology and the extent of proteome remodeling in the aged mice. Nrf2 is also a regulator of lipid metabolism [143] and directly associates with LXRα to regulate cholesterol and bile acid metabolism [144]. The role of Nrf2 in renal cholesterol metabolism remains unclear.

9.4.3 Hormones

As mentioned earlier in the text, kidney injury and dysfunction can be attributed to the actions of several hormonal factors. Among these, hormones modulation angiotensin II-mediated decreased expression of ABCA1 and increased expression of LDL receptors, Srebp2 and HMGCR [145]. The net effect of these alterations includes decreased cholesterol efflux and increased cholesterol synthesis, resulting in cholesterol accumulation and injury in renal podocytes [146].

9.4.4 Circadian Rhythm

Disruptions in the circadian rhythm are well-known risk factors contributing to the development of metabolic diseases. Recently, we reported that the circadian clock regulates plasma cholesterol, and apolipoprotein (Apo)AIV levels, and influences the development of atherosclerosis in *Clock* mutant and *Bmal1*-deficient mice [98, 99, 147]. Other unpublished studies have revealed that mutations in the *Clock* gene result in increased expression of cholesterol metabolism-related genes and their encoded proteins in the kidney. Several groups have reported that the circadian clock plays an important role in the regulation of renal function. In this regard, a recent study also revealed the influence of circadian rhythmicity and the role of circadian clock-associated genes on the GFR in podocytes [148]. Renal excretion of electrolytes is also controlled by circadian clock genes [30, 149, 150]**.** Likewise, several population-based studies have documented the relationship between CKD and sleep disorders, with reference to systemic inflammation, oxidative stress, fibrosis, the high disease incidence of obesity, diabetes, atherosclerosis, NAFLD, CVD, and overall morbidity and mortality. Our studies have shown that aged *Clock* mutant mice develop NAFLD via activation of Hif1α to promote CD36-mediated fatty acid uptake in the liver [132]. Additional studies will be needed to determine whether *Clock* mutant mice use this pathway for the development of NS or CKD. It will also be useful to target other outcomes, including atherosclerosis, NAFLD, as well as CKD in these mice.

9.4.5 Feeding

Cholesterol synthesis is affected by fasting and feeding. Fasting can limit cholesterol synthesis via the downregulation of Srebp2, while feeding enhances this pathway by increasing the expression of this gene [71, 151]. Recent studies have revealed that feeding also induces cholesterol synthesis by Srebp2-mediated upregulation of HMGCR expression specifically in the liver [152]. Mechanistic studies have revealed that feeding can activate phosphorylation of the mechanistic target of rapamycin complex 1 as well as the actions of ubiquitin C-terminal hydrolase 20 (USP20) [153]. USP20 prevents HMGCR degradation [153]. There are several studies that have found that feeding induced fatty acid synthesis in kidney [74]. However, the effect of feeding on renal cholesterol has not been clearly demonstrated. While several findings in liver provide evidence suggesting that fasting and feeding may be associated with a means to prevent CKD, the underlying mechanisms remain controversial in kidney.

9.5 Therapeutic Targets and Interventions

An improved understanding of the regulation of various disorders, disease states, and factors contributing to the regulation of cholesterol metabolism in the kidney that are impaired in CKD will provide us with insight into the

potential for novel therapies. These therapies may target cholesterol metabolism-related genes and lipoproteins and may include fasting, exercise, cholesterol metabolism-related protein inhibitors, apolipoprotein peptide mimetics, and microRNA, as well as other factors designed to control cholesterol metabolism in the kidney.

9.5.1 Fasting and Time-Restricted Feeding (TRF)

While results of recent animal model studies have revealed that fasting can slow the development of polycystic kidney disease (PKD) [154–157], there is currently no information on the impact of intermittent fasting in patients diagnosed with CKD. Additional studies will be needed to determine whether fasting serves to limit or increase the risk of poor health outcomes. Likewise, time-restricted feeding (TRF) has been examined in both animal models and human clinical studies [158–161]. Time-restricted food intake has a proven positive impact on patients diagnosed with metabolic syndrome [159, 161, 162]. We have shown that TRF is effective at controlling plasma triglyceride and cholesterol levels in wild-type as well as *Clock* mutant mice and that the *Clock* gene mediates intestinal absorption of both triglycerides and cholesterol [163, 164]. However, the impact of TRF on the development and progression of kidney disease remains unclear. More studies will be needed to understand how TRF regulates cholesterol metabolism in the kidney as well as its use as a potential therapeutic strategy in kidney diseases [165].

9.5.2 Exercise

Many studies have suggested that physical exercise may be an effective way to prevent CKD and progression to end-stage kidney disease (ESKD). For example, exercise prevented the kidney damage resulting from hyperlipidemia in mice that undergo exercise [48, 128]. Similarly, participation in exercise therapy correlated with improved renal function in patients diagnosed with atherosclerosis and CKD; exercise resulted in reductions in triglyceride levels, increases in HDL-cholesterol levels, and improved estimated GFR in these patients [123, 166].

9.5.3 Statins and Ezetimibe

Extensive clinical testing has revealed that HMGCR inhibitors (e.g., statins), cholesterol absorption inhibitors (e.g., ezetimibe), cholesterol ester transfer protein inhibitors, and cholesterol acyltransferase inhibitors are effective at reducing LDL-cholesterol to very low levels as well as improving levels of HDL. Of note, statins can limit the rate of progression of disease in patients with mild-to-moderate CKD, but not patients diagnosed with ESKD [167]. Interestingly, several studies have revealed that statins have no impact on oxidative stress, inflammation, HDL deficiency, or mortality secondary to atherosclerosis in patients with ESKD [167]. Similarly, antioxidant vitamins were also shown to be ineffective at targeting oxidative stress, inflammation, or overall mortality associated with ESKD. Many of these therapeutic agents are limited by high rates of adverse events and side effects. Thus, it seems unlikely that these therapies will be used extensively in patients diagnosed with CKD. Of note, while bile acid sequestrants and the adenosine triphosphate citrate lyase inhibitor, bempedoic acid, can both promote decreased levels of LDL [168], they were overall less effective than statins. However, several groups have reported that fibrates, a lipid-modifying agent, increase lipoprotein lipase activity and decrease plasma triglyceride, cholesterol, and LDL levels to control cholesterol accumulation in the kidney [66, 118, 169–171].

Emerging experimental evidence suggests that ezetimibe, which is an inhibitor of NPC1-like intracellular cholesterol transporter 1 (NPC1L1) can reduce total and LDL-cholesterol, triglyceride, and ApoB levels and increase circulating levels of HDL-cholesterol. Ezetimibe can be used to promote reductions in cholesterol levels in patients diagnosed with CKD or NS [172]. In addition, ezetimibe administered together with

simvastatin is effective at controlling cholesterol influx in patients diagnosed with CKD [173]. Collectively, these studies suggest the potential benefits of combination cholesterol-lowering therapy in patients diagnosed with CKD.

9.5.4 Apolipoproteins

Several recent studies have revealed that elevated serum levels of ApoA1 were significantly associated with the development of new cardiovascular outcomes as well as with cardiovascular-related mortality in patients diagnosed with ESKD [174]. The ApoA1 target is a component of a distinct pathway that regulates cholesterol metabolism. ApoA1 can activate LCAT and can interact with ABCA1 to regulate levels of HDL-cholesterol and with SR-BI to promote selective lipid uptake and cholesterol efflux. While this protein is pro-inflammatory in the absence of ApoE, ApoA1 can also inhibit the expression of endothelial adhesion molecules.

Similar to ApoA1, ApoAIV has also been identified as a predictor of cardiovascular risk in patients with CKD. ApoAIV can enhance LCAT activity and regulate lipoprotein lipase (LPL) to promote cholesterol efflux from cells. ApoAIV also has characterized antioxidant and anti-atherogenic properties [175, 176]. Several clinical studies have documented high serum levels of ApoAIV in early-stage CKD patients. Similarly, patients diagnosed with ESKD patients have on average more than two times higher serum levels of ApoAIV compared with control groups [177–179]. High concentrations of ApoAIV were also detected in the urine; this result suggests that ApoAIV undergoes glomerular filtration followed by reabsorption in the RPTECs under homeostatic conditions [177–179].

ApoB can promote the formation of chylomicrons in the intestines, stimulate the formation of nascent very light density lipoproteins (VLDLs) in the liver, and serve as a ligand for plasma LDL [180, 181]. Microsomal triglyceride transfer protein (MTP), ApoB, and ApoAIV [182] assemble and secrete ApoB-containing lipoprotein within chylomicrons and as VLDL [29, 180, 182]. Two inhibitors that target ApoB-containing lipoproteins have recently been introduced for the treatment of patients diagnosed with homozygous familial hypercholesterolemia [183]. Lomitapide is an inhibitor of MTP activity, while the antisense oligonucleotide, mipomersen, prevents the translation of ApoB mRNA, thereby preventing its synthesis [184]. Recent studies clearly document that both lomitapide and mipomersen can decrease LDL-cholesterol levels by 30–50% [184, 185].

Wolfrum et al. [186] found that ApoM is required for the formation of preβ-HDL lipoprotein and cholesterol efflux to form HDL and decrease atherosclerosis in a mouse model study. These results suggest that ApoM may be an important apolipoprotein that might be targeted to regulate kidney function. Beckerman et al. [187] explored the expression of risk-associated alleles of APOL1in an animal model and identified those essential for podocyte function as well as those contributing to glomerular disease. ApoL1 has been identified as critically associated with the pathogenesis of CKD [188, 189].

Taken together, these studies have revealed mechanisms underlying apolipoprotein-mediated regulation of cholesterol metabolism in the kidney and have documented their influence on renal function and the pathogenesis of CKD. It will be important to develop a larger understanding of apolipoprotein biology and function specifically in the kidney and to translate these findings into therapeutic agents that are effective at decreasing the risk of developing CVD and CKD.

9.5.5 Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Inhibitors

Several studies have explored findings linking the enzyme, PCSK9 to the development of hypercholesterolemia. PCSK9 inhibitors regulate Srebp2 and LDL receptor activity and can be used clinically to manage hypercholesterolemia and reduce the risk of developing atherosclerosis. Specifically, PCSK9 controls hepatic degradation of the

LDL receptor, thereby regulating postprandial lipoprotein metabolism. PCSK9 also modulates cholesterol efflux in the intestines and thus the extent of its excretion in the feces [174]. PCSK9 also decreases glucose-stimulated insulin production, promotes central nervous system development, and limits β-cell apoptosis [190, 191]. However, serum PCSK9 levels were not associated with the development of CVD in patients diagnosed with CKD or ESKD [174]. Future treatment of dyslipidemia and high levels of LDL-cholesterol in patients with CKD or ESKD will rely on our capacity to target functional biomarkers such as PCSK9 and may include new therapeutic strategies, notably monoclonal antibodies (i.e., evolocumab and alirocumab) [192].

9.5.6 miRNAs and lncRNAs

Long noncoding RNAs (lncRNAs) are attractive both as diagnostic biomarkers and therapeutic targets in diseases associated with lipid metabolism [193]. Likewise, several miRNA biomarkers are currently in use as clinical therapeutics and may ultimately play a principal role in the treatment of patients diagnosed with CKD [194]. Many miRNAs have been explored in clinical research [195, 196]. For example, Fujii et al. [197, 198] identified three circulating CVD-associated miRNAs in aged CKD patients who survived the Fukushima earthquake in 2016. Rayner et al. [199, 200] found that miR-33 could regulate both HDL biogenesis in the liver and cellular cholesterol efflux via its actions on Abca1, Abcg1, and Npc1. miR-33 also regulates the activation of SREBF2, a factor that controls cholesterol synthesis in both animal and human cells [199, 200]. Specific miRNAs also regulate apoB-containing lipoprotein production [201], including miR-122, miR-34a, and miR-30c [201–206]. The efficacy of miR-34 and miR-122 has been examined in both preclinical and clinical treatment studies [207]. Administration of miR-30c results in decreased levels of LDL, while treatment with miR-33 decreases HDL, plasma cholesterol, and triglyceride in animal models. Soh et al. [204] found that administration of miR-30c resulted in diminished expression of MTP, a transport protein that modulates secretion of VLDL from the liver. Of importance, mi30c can function as an MTP inhibitor that reduces plasma cholesterol levels in Apoe KO mice with no significant side effects [204, 208]. Similarly, miR-613 regulates cholesterol metabolism via its impact on LXRα in experiments performed using human monocytic cell macrophages. miR-206 also controls cholesterol efflux via regulation of LXRα expression in macrophages. miR-128-2 promotes cholesterol homeostasis by targeting RXRα in human liver cells, and miR-142-3p regulates cholestasis through the orphan nuclear receptor, small heterodimer partner (SHP) in mice maintained on a cholic acid-rich diet. Long noncoding RNA maternally expressed 3 induces cholestatic liver injury via its association with polypyrimidine tract binding protein 1 which accelerates SHP mRNA decay [209]. Likewise, miR-205 downregulates cholesterol synthesis via SQLE-mediated inhibition in prostate cancer cells [210]. Among the LNCs, Inc-MGC was found to regulate the development of diabetic nephropathy in animal model studies [211]. Likewise, the results of several studies have revealed that administration of lincRNA-DYNLRB2-2 increases cholesterol efflux via a distinct pathway [212–215]. Specifically, lincRNA-DYNLRB2-2 enhances Abeca1 and reduces toll-like receptor 2 expression in macrophages [213]. lncRNA CHROME also plays a significant role in promoting cholesterol efflux and HDL biogenesis, thereby controlling cholesterol homeostasis in primate model [193]. Sallam et al. [216] demonstrated that the lipid-responsive noncoding RNA LeXis modulates lipid metabolism; expression of LeXis results in decreases in serum cholesterol and reductions in sterol synthesis via the SREBP pathway.

To determine whether these miRNAs and lncRNAs are effective specifically in the kidney, we will need to develop simple and effective methods to evaluate cholesterol accumulation at this locale. Intestinally, lipoproteins not only transport lipid carriers, but also small molecules including vitamins, hormones, solutes, and

miRNAs [217]. Therefore, agents that interact with lipoproteins and interfere with their physiologic activities might have a direct impact on pathophysiological processes and cardiovascular risk in patients diagnosed with CKD. For example, Yan et al. [215] reported that systemic delivery of cholesterol-tagged siRNAs designed to target 12/15-lipoxygenase yielded clinical improvements in a mouse model of type 1 diabetes. These results suggest that cholesterol-tagged siRNAs may be a novel and important therapeutic approach for the prevention and treatment of CVD and diabetic nephropathy, it will be useful to decrease CKD and NS [216, 218].

9.6 Cross-Talk Between Cholesterol and Sphingolipid Metabolism

Analogous to cholesterol, sphingolipids also play an important role in kidney biology and renal disease. Sphingolipids constitute the fourth largest class of membrane lipids. While sphingomyelin levels are highest in the brain and nervous tissue, they are mainly detected in the intestine, liver, and kidney. Sphingolipids are formed from components that include choline, fatty acids, phosphoric acids, and sphingosine.

Sphingolipid metabolism engages in cross-talk with pathways associated with cholesterol metabolism. For example, Puri et al. [219] have shown that sphingolipid storage promotes the accumulation of intracellular cholesterol via cleavage of Srebp1. The results of this study suggest that sphingolipids may play a wider role than anticipated in controlling cholesterol metabolism. Plasma phospholipid transfer protein (PLTP) activity can control the levels of circulating HDL-cholesterol [220–222]. Diminished levels of PLTP activity have been associated with changes in the reverse cholesterol transport and HDL-cholesterol levels [223]. Recently, Zhang et al. [224] showed that inducible PLTP KO mice exhibit decreased levels of total plasma cholesterol and triglycerides and can thus inhibit the development of atherosclerosis. A dysfunction-inducing defect in PLTP might elucidate the therapeutic utility of this target for the treatment of atherosclerosis [224–226]. Increased levels of PLTP have also been associated with obesity and insulin resistance in both human and animal studies [222].

9.7 Conclusions and Future Directions

Collectively, the results reviewed in this manuscript provide a clear demonstration that changes in lipid distribution, as well as the responsiveness of kidney cells to lipid-mediated signaling, may both be factors contributing to changes taking place in the normal kidney and the development of renal disease.

Many of the familiar risk factors associated with obesity, diabetes, atherosclerosis, and NAFLD also contribute to the likelihood of developing CKD. However, recent work has revealed that age, sex, circadian rhythm, inflammation, dietary considerations, and tissue-cross talk also contribute to the development of CKD. Other factors that may contribute to CKD, for example, the nature of the intestinal microbiota, bile acids, glucose, insulin, and neuropeptides, will be the subject of a future discussion.

Plasma lipids are attuned to environmental change. However, the contributions of cholesterol-containing plasma lipoproteins and their role in promoting homeostatic, as well as dysfunctional renal function, require further elucidation. In addition, we need to develop a larger understanding of how factors that include nutrient intake, sleep deprivation, and stress has an impact on lipid, apolipoprotein, and lipoprotein levels in mesangial cells, endothelial cells, podocytes, and RPTECs. Findings from experiments designed to address these questions may provide us with an opportunity to develop lifestyle-based strategies to prevent or at very least limit the rate of progression of CKD and renal damage secondary to CVD, diabetes, obesity, and NAFLD. Future studies will be needed to develop these critical research directions focused on lipids, apolipoproteins, and lipoproteins and their roles in the pathogenesis, progression, and mortality associated with acute kidney injury and CKD.

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Sphingolipids and Asthma

10

Tilla S. Worgall

Abstract

Asthma is the most prevalent chronic respiratory disease worldwide and the leading serious chronic illness in children. Clinical characteristics are wheezing, reversible airway obstruction, airway inflammation, and airway hyperreactivity. Asthma susceptibility is influenced by genes and environment. 17q12–21 is the most significant genetic asthma susceptibility locus and single nucleotide polymorphisms (SNPs) within that high-risk locus are linked to increased expression of the Ormdl sphingolipid biosynthesis regulator (ORMDL) 3. ORMDL3 is an endoplasmic reticulum protein that stabilizes the serine palmitoyl transferase (SPT) complex that regulates sphingolipid de novo synthesis. Sphingolipids essential for formation and integrity of cellular membranes and bioactive molecules that regulate key cellular processes can be synthesized de novo and through recycling pathways. Their metabolism is tightly regulated through feedback regulation. ORMDL3 inhibits de novo synthesis when it engages subunit 1 of the SPT complex. This chapter focuses on the effect of decreased sphingolipid synthesis on asthma features and summarizes studies in mouse models and in children with and without asthma.

Keywords

Sphingolipids · Childhood asthma · Airway hyperreactivity · ORMDL3

Abbreviations

FEV1/FVC	Forced expiration in the first second/forced vital capacity
GWAS	Genome Wide Association Study
ORMDL	Ormdl sphingolipid biosynthesis regulator
PBMC	Peripheral blood monocytes
PCLS	Precision cut lung slices
SNP	Single nucleotide polymorphism
SPT	Serine palmitoyl transferase
SPTLC1	Serine palmitoyl transferase long-chain base subunit 1
SPTLC2	Serine palmitoyl transferase long-chain base subunit 2
ssSPTa	Small subunit SPT A

10.1 Asthma Is a Common Disease

Asthma affects more than 300 million people worldwide and is the most common chronic respiratory medical condition in childhood, affecting

T. S. Worgall (✉)
Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, USA
e-mail: tpw7@cumc.columbia.edu

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six million children in the United States [1, 2]. Asthma is a complex disease and childhood and adult asthma differ with respect to severity and comorbidity [3]. In order to improve consistency in genetic and environmental correlations, the observable characteristics of the asthma phenotype have been further classified into endotypes that are defined by specific biological mechanisms [4]. Current thought is that childhood asthma is a developmental disorder in which interactions between common genetic variants and environmental exposure to viruses, allergens, pollution at critical times during the development of the immune system and the airways play a critical role [5]. And, that genetic risk factors of adult-onset asthma are largely a subset of the genetic risk for childhood asthma suggesting a greater role for non-genetic factors in adult-onset asthma [6]. Typical asthma phenotypic features are airway hyperreactivity, inflammation, mucus overproduction, and smooth muscle hypertrophy [7, 8]. The majority of patients have allergic asthma that presents with eosinophilic airway inflammation, increased total and antigen-specific IgE levels, blood eosinophilia, and sensitization to various allergens of which house dust mites and Alternaria alternata are very prevalent [9, 10]. Treatment options consist of β-2 agonists to decrease airway smooth muscle contraction, corticosteroids to reduce inflammation, and monoclonal antibodies to antagonize inflammatory mediators.

10.2 17q12–21 Is a High-Risk Asthma Locus

A GWAS study in 2007 identified 17q12–21 as a high-risk asthma locus and showed that single nucleotide polymorphisms (SNPs) that increase ORMDL3 expression contribute to the risk of childhood asthma [11]. Consecutive studies confirmed the significance of 17q21, extended the association to additional gene candidates, specifically to the adjacent gasdermin-B (GSDMB) and replicated the results in children and adults in different ethnicities and localities [12–17]. SNPs associated with childhood asthma were consistently associated with increased transcript levels of ORMDL3 [11, 18–22] and with wheezing illness, a characteristic of very early onset asthma [22, 23].

10.2.1 ORMDL Proteins Are Evolutionary Conserved Endoplasmic Reticulum Proteins

There are three human ORMDL proteins (ORMDL1, ORMDL2, and ORMDL3) that are encoded on chromosomes 2q32.2 (ORMDL1), 12q13.2 (ORMDL2), 17q21.1 (ORMDL3). These proteins have the same size (153 aa acids), similar molecular weight (ORMDL1 17,371 Da, ORMDL2 17,363 Da, ORMDL3 17,495 Da), and a high homology with orthologues in yeast, plants, invertebrates and vertebrates, and with each other. Human ORMDL1 shares 83% identity with ORMDL2 and 84% identity with ORMDL3 [24]. ORMDL 2 and ORMDL 3 expression is highest in myocytes, immune and epithelial cells, while ORMDL1 is more widely expressed (heart, brain, lung, liver, skeletal muscle and kidney) [25].

10.2.2 ORMDLs Mediate Sphingolipid Homeostasis by Regulating De Novo Synthesis

Serine palmitoyl transferase (SPT) is the first and rate limiting step of de novo synthesis. The SPT complex converts a fatty acid, mostly the C16:0 palmitoyl-CoA with an amino acid (mostly serine) to generate 3-keto sphinganine that in turn is rapidly degraded to sphinganine, the substrate for sphingosine kinases that generate sphinganine-1-phosphate, and ceramide synthases that generate dihydroceramides. Introduction of a 4,5-bond in the sphingosine backbone of dihydroceramides generates ceramides that are metabolized to more complex sphingolipids such as glucosylceramides and sphingomyelins [26].

Yeast studies showed that Orm1 and Orm2 that physically interact with long-chain base subunit 1 (Lcb1) regulate sphingolipid homeostasis and protein quality control [27, 28].

Deletion of Orm proteins increases sphingolipid de novo synthesis and induces a constitutive unfolded protein response that is reversed when sphingolipid synthesis is inhibited with myriocin [28]. Endoplasmic reticulum stress in turn induces Orm2 transcription through activation of calcium and calcineurin dependent pathways [29]. Regulation occurs post-transcriptional through Orm phosphorylation in response to sphingolipid depletion. Phosphorylated Orm1 and Orm2 can no longer inhibit sphingolipid de novo synthesis [27].

Human ORMDLs function similarly, although they are not regulated by phosphorylation. With adequate SPT subunit concentrations ORMDL proteins become regulatory [30, 31]. Overexpression of each ORMDL inhibits sphingolipid synthesis and deletion of all ORMDLs increases sphingolipid synthesis [32–34]. Deletion of ORMDL3, increases SPT activity and synthesis of C16 ceramides and sphingomyelin in A549 cells more than deletion of ORMDL1 and ORMDL2 [33].

10.2.3 ORMDLs Stabilize the Multi-Dimeric SPT Homocomplex

Structure and assembly of the SPT complex, elucidated by two independent groups in 2021, reveal that SPT is a double dimer complex composed of two SPTLC1 subunits, two SPTLC2 (or SPTLC3) subunits, and two ssSPTa units (SPT small subunit A). ORMDLs bind the N-terminus of subunit SPTLC1 and thus stabilize the complex while the ssSPTa units anchor SPTLC2 to the endoplasmic reticulum membrane and form a substrate binding tunnel [35, 36]. De novo synthesis proceeds when the ssSPT subunit engages a long-chain substrate in the catalytic tunnel. De novo synthesis is inhibited when the catalytic site within the tunnel is occupied by myriocin or by the N-terminal of ORMDL that blocks accessibility to the tunnel. The experiments suggest however that an additional, currently unknown factor is likely required for ORMDL to mediate inhibition. Because levels of ORMDL and SPT are the same in high and low sphingolipid conditions, SPT regulation could be mediated by conformational changes [30]. The structural data are consistent with the prior observation that ORMDL regulates SPT activity through post-transcriptional mechanisms that are independent of ORMDL mRNA expression [32, 37]. These studies also showed that inhibition occurs in response to increased ceramide levels and that it depends on functional SPT. Interestingly, free cholesterol promotes degradation of ORMDL1 in macrophages, but it is not known whether this mechanism extends to different cell types and the other ORMDLs [38]. It is thus still unclear what regulates ORMDL to function as an inhibitor, i.e., occupy the substrate binding tunnel, and what regulates de-repression.

10.3 ORMDL3 and Asthma

Genetic variations in 17q21 that increase ORMDL3 have prompted multiple studies in mice and humans with the obvious goal to understand whether or not ORMDL3 is causally related to asthma pathology. Early ORMDL3 overexpression and knockout studies suggested that inflammation, mainly through activation of the unfolded protein response, could be the functional link to asthma [39]. The identification of ORMDL3 as a regulator of mammalian sphingolipid de novo synthesis 3 years after the initial GWAS study [27] suggested that this large lipid class of membrane constituents and bioactive signaling molecules may also be involved in asthma pathogenesis. Sphingolipid synthesis is decreased in mouse models that overexpress human or murine ORMDL3, but phenotypes differ starkly [40, 41]. Overexpression of human ORMDL3 induces airway remodeling and airway responsiveness characteristic of asthma, while overexpression of murine ORMDL3 does not alter or induce key asthma features in mice

[40]. By the same token however, ORMDL3 knockout protects mice from developing allergic airways disease triggered by Alternaria alternans [42]. The model shows a marked decrease in pathophysiology, including airway hyperreactivity and airway eosinophilia induced by Alternaria without activation of the unfolded protein response. The protection was lost when ORMDL3 was reconstituted in bronchial epithelial cells of these ORMDL3 knockout mice.

A complicating factor of transgenic models is that unphysiological and unregulated (over)-expression could affect the stoichiometry of ORMDL3 and SPTLC1, and thus feedback regulation [30]. In-vitro experiments in lung epithelial cells and macrophages demonstrate that small increases in ORMDL3 expression decrease ceramide levels, while higher expression increases ceramide production through recycling pathways resulting in sphingolipid overload that might also characterize ORMDL knockout models [32, 43, 44].

10.4 Decreased Sphingolipid Synthesis and Asthma

The following highlights experimental and clinical asthma cohort studies that link decreased sphingolipid synthesis to the 17q21 genotypes and asthma and how sphingolipid synthesis might be a target for asthma therapies.

SPT Inhibition Increases Airway Reactivity To understand the relevance of decreased sphingolipids, asthma features were assessed in wild-type mice treated intranasally with SPT inhibitor myriocin and in haploinsufficient SPTLC2$^{+/-}$ mice in which sphingolipid synthesis and mass are decreased by 40–50%. In contrast to complete SPTLC knockout that is embryonically lethal, haploinsufficient mice are healthy [45]. We evaluated the effects of acute and chronic decrease of sphingolipid synthesis on inflammation, mucus production, and airway remodeling and assessed methacholine-mediated airway constriction in mice and explanted murine and human bronchial airways. All experiments were carried out without prior sensitization to allergens (i.e., house dust mites, ovalbumin, Alternaria alternata).

Myriocin inhibits sphingolipid synthesis by physically blocking the substrate channel of SPT that is formed by SPTLC2 and ssSPTa [46, 47]. Of note is that myriocin is also a potent immunosuppressive that alters S1P receptor-mediated lymphocyte egress and inhibits lymphocyte proliferation and generation of allo-reactive T lymphocytes [36, 46, 48, 49].

In first experiments, myriocin was directly administered to the respiratory tract of wild-type mice or added to incubation media of murine and human bronchial rings. Within 3 h after installation, myriocin decreased lung sphinganine and total ceramides and increased airway reactivity that was determined by changes in central airway resistance in response to nebulized methacholine. Baseline lung mechanical properties such as static compliance were not altered. Application of myriocin for 90 min to human or murine bronchial rings equally showed a dose-dependent increase in bronchoconstriction [50].

Decreased SPTLC2 Activity Increases Airway Reactivity To ensure that the effects are not mediated by mechanisms related to the immune suppressive function of myriocin and to further distinguish acute inhibition of sphingolipid de novo synthesis from a habitually decreased sphingolipid de novo synthesis and decreased sphingolipid mass, we used SPTLC2$^{+/-}$ mice. Airway resistance was equally increased in response to methacholine in Sptlc2$^{+/-}$ compared to Sptlc2$^{+/+}$ controls and no differences were detected in the baseline mechanical lung parameters, including static compliance. Contractile response to methacholine was also increased in bronchial rings isolated from Sptlc2$^{+/-}$ mice compared to bronchial rings isolated from Sptlc2$^{+/+}$ controls. Notably, very similar results were obtained in an allergic asthma model in which co-administration of myriocin with house dust mites during a 2-week sensitization period

dramatically increased methacholine stimulated airway constriction compared to house dust mites alone [51].

In the $SPTLC^{+/-}$ and $SPTLC^{+/+}$ controls or myriocin-treated wild-type and wild-type controls, there were no differences with regard to lung histology, cell composition of bronchioalveolar lavage, or expression of tumor necrosis factor-α (TNF-α), inflammatory cytokines interleukin-6 (IL-6) and IL-1β that are found in allergic and chronic asthma. These characteristics are consistent with the association of ORMDL3 polymorphisms with non-allergic asthma.

10.5 Blood Sphingolipids Are Decreased in Children with Non-Allergic Asthma

Plasma and serum sphingolipids have been investigated in different asthma pheno- and endotypes in patients of different ages using different methodological approaches. In a study of adult patients with allergic asthma, serum ceramides (C16, C18, C18:2, C24, C24:1) were higher in allergic asthma patients than in controls, and higher ceramide C16 and C24:0 distinguished uncontrolled from controlled patients [52]. In a study of house dust mite allergized adult asthma patients, ceramide levels were not different compared to controls, but asthma patients failed to mount an increase in sphinganine in response to allergic stimulation [53]. In another adult cohort, no effect of annotated ORMDL3 asthma SNPs was found on total plasma long-chain bases albeit by a method that cannot distinguish whether analytes originate from the de novo or the recyling pathway [54].

In a study of children with clinically mild disease and releative normal lung function but lower FEV1/FVC and atopy increased serum dihydroceramide C18 and ceramide C20 at ages 7 and 8 years predicted asthma persistence at ages 10 to 11 years [55]. In another study that measured ceramides and sphingomyelin in children with different asthma endotypes, decreased sphingomyelin distinguished non-allergic childhood asthma from allergic childhood asthma [56]. Moreover, plasma sphingolipid analysis of 500 children enrolled in the 'Copenhagen prospective studies on asthma in childhood' (COPSAC) birth cohort found that lower concentrations of ceramides and sphingomyelins at the age of 6 months were associated with an increased risk of developing asthma before age 3. At the age of 6 years, lower concentrations of sphinganine-1-phosphate were associated with increased airway resistance [57].

Our group investigated the effect of asthma-associated SNPs on sphingolipid synthesis and mass in children with non-allergic and allergic asthma and controls. To this end we enrolled 61 children with physician diagnosed asthma and 59 children without asthma and measured sphingolipids in whole blood and plasma and determined sphingolipid de novo synthesis in peripheral blood monocytes (PBMC). Eosinophilia was used to stratify patients with allergic asthma (>300 eosinophils/μL). Patients with non-allergic asthma (<300 eosinophils/μL) had lower whole blood dihydroceramides (C18, C18:1, C24:1), ceramides (C18, C20, C22, C24, C24:1), and sphingomyelins (C18, C18:1, C24:1) compared to controls. When comparing patients with allergic asthma to patients with non-allergic asthma, we found significantly higher dihydroceramides (C18, C18:1), ceramides (C18, C24), and sphingomyelins (C18, C18:1, C24:1) in the allergic asthma patients but no difference when compared to controls with eosinophilia. Similar results were obtained when the asthma group was stratified by IgE as a marker for atopy. Strikingly, these results were seen only in whole blood but not in plasma that is devoid of erythrocytes that have a high membrane content and PBMC in which ORMDL3 expression is high. In plasma there was a trend for increased dihydroceramide C24:1 for asthma patients (non-allergic and allergic) that did not reach significance, and significantly increased C20 ceramide in patients with allergic asthma compared to non-asthma controls with high eosinophilia, confirming earlier studies that showed high plasma ceramide C20 in exercise-induced wheezing [55].

Together the strong association of non-allergic asthma with decreased sphingolipids is consistent with the original report that associated non-allergic asthma with increased ORMDL3, and with results obtained in the COPSAC study that indicate a sphingolipid-associated childhood asthma endotype with an early onset of symptoms and increased airway resistance by the age of 6 years that is already present in infancy and is associated with 17q21 genetic variants and expression of SPT enzymes [57].

10.5.1 Plasma Sphingolipids Are Higher Than Controls in Non-Allergic and Allergic Asthma

Notably, in our study, plasma sphingolipids in the same population showed significantly increased ceramide C20 in asthma patients with high eosinophils, and a trend for increased dihydroceramides C24:1 in asthma patients with both low and high eosinophils ($p < 0.035$ by *t*-test but not significant when correcting for a false discovery rate of 0.05). The strikingly different results obtained in whole blood and plasma are not understood well. They alert on the one hand to the difficulty in comparing studies in different matrixes and cohorts but also to the possibility that decreased whole blood and increased plasma sphingolipids in the same individual reflect different sphingolipid pools originating possibly through compensatory mechanisms.

10.5.2 Risk Alleles Correlate with Whole Blood Sphingolipids

To further investigate the relationship between genetic variations at 17q21 and sphingolipids, we determined five asthma-associated 17q21 SNPs (rs7216389, rs8067378, rs4065275, rs8076131) [11, 14, 20, 25, 58–61]. SNPs for rs8067378, rs4065275, and rs12603332 did not correlate with whole blood sphingolipids, but risk alleles in the originally identified rs7216389 and rs8076131 that are expression quantitative trait loci (eQTLs) for ORMDL3 correlated with decreased dihydroceramides (C16, C18, C24) and three ceramides (C16, C18,20) in a genotype phenotype specific manner.

10.5.3 Sphingolipid De Novo Synthesis Is Decreased in Asthma and Associated with an Asthma-Risk Genotype

Given the high expression of ORMDL3 in T and B cells, PMBCs were used to assess de novo sphingolipid synthesis by measuring incorporation of stable isotope labeled serine ($C^{13}N^{15}$) into sphinganine [59]. PBMC from all children with asthma compared to all controls generated significantly less stable labeled sphinganine and sphinganine-1-phosphate ($p < 0.05$). When these data were stratified by the rs7218369 genotype, de novo synthesized sphinganine and sphinganine-1-phosphate were lowest with the asthma-risk TT genotype (CC + CT > TT, $p < 0.05$), and risk allele A (GG + AG > AA) for rs8076131 and consistent with decreased sphinganine-1-phosphate found in the COPSAC study by Rago et al. [57].

10.5.4 Increasing Sphingolipid De Novo Synthesis Decreases Airway Reactivity

We also evaluated if increasing sphingolipid synthesis can normalize excessive airway reactivity. These experiments were carried out in $Sptlc2^{+/-}$ mice using the FlexiVent system and in precision cut lung slices (PCLS) from $Sptlc2^{+/-}$ mice. Sphingolipid de novo synthesis was increased with chloride channel inhibitor GlyH101 and with fenretinide, an inhibitor of ceramide desaturase. These substances were chosen based on our previous observation that expression of defective CFTR as well as inhibition of CFTR increase sphingolipid de novo synthesis [62]. Whether the mechanisms behind increased de novo sphingolipid synthesis are related to an

increase in intracellular volume induced by inhibition of CFTR is not known [63, 64]. Fenretinide, on the other hand is a drug that inhibits ceramide desaturase, leading primarily to decreased synthesis of ceramides in the de-novo pathway. This inhibition triggers an increase of de novo sphingolipid synthesis and affects recycling pathways [65]. GLYH101 and fenretinide increased sphinganine and dihydroceramides in human bronchoepithelial and smooth muscle cells, and fenretinide also increased ceramides and sphingomyelins.

Experiments were carried out in PCLS from $SPTLC^{+/-}$ mice to specifically assess constriction of small airways in addition to global airway constriction that was evaluated with the FlexiVent system. Incubation with GlyH101 for 15 h decreased methacholine-induced airway contraction in PCLS. Incubation for 15 min, sufficient to inhibit the chloride channel, but insufficient to significantly increase sphingolipid synthesis, had no effect. By the same token, incubation with increasing concentrations of fenretinide for 15 h decreased methacholine-induced contraction in a dose-dependent manner in PCLS and bronchial rings isolated from $SPTLC^{+/-}$ mice.

The results strongly suggest that targeting the imbalance of sphingolipids in asthma to oppose airway hyperresponsiveness can serve as a therapeutic target for asthma [66].

10.6 Conclusion

GWAS studies alerted to a potential role of sphingolipid metabolism in asthma when genetic variation in the asthma locus 17q21 was associated with increased expression of ORMDL3 with childhood asthma. Early ORMDL3 overexpression and knockout studies suggested that inflammation, mainly through activation of the unfolded protein response, could be the functional link to asthma [39]. The identification of ORMDL3 as a regulator of mammalian sphingolipid de novo synthesis 3 years after the initial GWAS study suggested that this large lipid class of membrane constituents and bioactive signaling molecules may also be involved in asthma pathogenesis [27]. Mice that are haploinsufficient for SPTLC2 show that decreased sphingolipid synthesis is sufficient to increase airway reactivity without affecting airway inflammation, mucus production, eosinophilia that characterize allergic asthma. Sphingolipid de novo synthesis is decreased in children with asthma, and whole blood sphingolipds are decreased in children with non-allergic asthma compared to controls. Children with allergic asthma have higher whole blood sphingolipids than children with non-allergic asthma, suggesting an additional effect of allergy on sphingolipid mass. These differences were seen in whole blood but not in plasma. Results obtained in mice indicate that increasing sphingolipid synthesis can normalize airway reactivity. Collectively, the data link increased airway reactivity to decreased sphingolipid synthesis, and genetic variations in 17q21 asthma locus to decreased sphingolipid synthesis and decreased whole blood sphingolipids in non-allergic childhood asthma. The studies establish a role of sphingolipids in asthma and serve as a basis to explore whether increasing sphingolipid de novo synthesis and exogenously increasing metabolites of the de novo pathway affect airway reactivity [67].

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Manifold Roles of Ceramide Metabolism in Non-Alcoholic Fatty Liver Disease and Liver Cancer

11

Kai Wang, Yiran Wei, Ruijuan Xu, Yiyi Li, and Cungui Mao

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disorder manifested in hepatic fat accumulation (hepatic steatosis) in the absence of heavy alcohol use. NAFLD consists of four major stages ranging from simple steatosis or non-alcoholic fatty liver (NAFL) to more advanced stages, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis. NFLAD may further advance to hepatocellular carcinoma (HCC). Primary causes of NAFLD are obesity and obesity-associated insulin resistance (IR). As a result of the obesity pandemic, NAFLD has become one of the most common liver disorders worldwide and both the incidence and mortality rate of HCC that develops from NAFLD are increasing steadily. As treatment options are not available for advanced NAFLD, a better understanding of the molecular mechanisms for NAFLD development and progression is urgently needed. Emerging evidence suggests that dysregulation of the metabolism of sphingolipids contributes to development and progression of NAFLD and NAFLD-associated HCC. The present chapter summarizes roles of bioactive sphingolipids, ceramides, sphingosine, and sphingosine-1-phosphate (S1P) and their metabolizing enzymes in NAFLD and HCC.

Keywords

Ceramide · Non-alcoholic fatty liver disease (NAFLD) · Non-alcoholic steatohepatitis (NASH) · Hepatocellular carcinoma (HCC) · Insulin resistance

11.1 Overview of NAFLD and HCC

Non-alcoholic fatty liver disease (NAFLD) is a metabolic disorder manifested in hepatic fat accumulation (hepatic steatosis) in the absence of heavy alcohol consumption in past medical history. NAFLD exhibits a spectrum of conditions ranging from simple steatosis or non-alcoholic fatty liver (NAFLD) to non-alcoholic steatohepatitis (NASH) or cirrhosis, which may further advance to hepatocellular carcinoma (HCC) [1]. Primary causes of NAFLD are obesity and obesity-associated insulin resistance (IR). As a result of the obesity pandemic, NAFLD has become one of the most common liver disorders worldwide and currently affects around 25% of

K. Wang · R. Xu · Y. Li · C. Mao (✉)
Division of Hepatobiliopancreatic Surgery, Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

Department of Medicine and Cancer Center, The State University of New York at Stony Brook, Stony Brook, NY, USA
e-mail: Cungui.Mao@stonybrook.edu

Y. Wei
Division of Hepatobiliopancreatic Surgery, Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China

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the general population of Western countries [2, 3]. NASH is expected to become the leading cause of liver transplant [3]. However, NASH currently has no effective treatment apart from lifestyle interventions [4]. Therefore, there are unmet medical needs for understanding the molecular and cellular mechanisms for the progression of NAFLD to NASH and cirrhosis and developing novel approaches to treating advanced NAFLD based on an understanding of the pathogenesis of this disease.

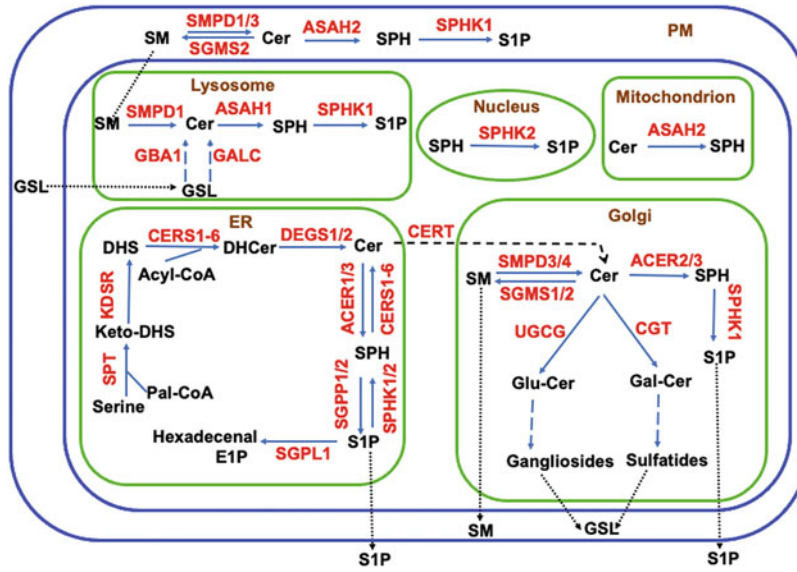
Obesity [2] and IR [5] have been suggested to be major risk factors for NAFLD. Obesity and IR result in accumulation of free fatty acids (FFAs) in the liver [2]. Hepatic FFAs are metabolized into many lipotoxic species that cause various forms of hepatocellular stress, including oxidative stress, endoplasmic reticulum (ER) stress, and cell death [1], thus leading to liver damage, inflammation, and fibrosis, which are the hallmark comorbidities of NASH [6]. The ER plays central roles in calcium ion storage, lipid biosynthesis, and protein sorting and processing [7]. Accumulation of unfolded proteins in the ER lumen or impairment of the ER membrane integrity results in ER stress which in turn activates a series of signaling processes called the unfolded protein response (UPR) [8]. Transient activation of the UPR restores the homeostasis of the ER while chronic UPR activation due to persistence of ER stress results in cell death and cellular injury. Chronic UPR activation has been observed in liver and/or adipose tissue of dietary and genetic murine models of obesity, and in human obesity and NAFLD [8]. The chronic responses to cell death and cellular injury lead to chronic hepatocyte turnover, the recruitment of immune cells, and activation of hepatic stellate cells (HSCs), thus contributing to the development of liver fibrosis and cirrhosis [9–11]. It has become evident that, besides apoptosis, necroptosis is a highly relevant form of programmed cell death (PCD) in the liver [9, 12, 13]. In addition to PCD, increasing studies have implicated autophagy in the pathogenesis of NAFLD [14]. Autophagy is a lysosomal degradative pathway that promotes cell survival by supplying energy under the stress of energy crisis or by removing damaged organelles and proteins after cellular injury [15]. An initial study suggests that autophagy mediates the breakdown of lipids in hepatocytes [16]. Subsequent studies have implicated autophagy in regulating several pathological effects of NAFLD, such as insulin sensitivity, hepatocellular injury, innate immunity, fibrosis, and liver carcinogenesis [14].

However, lipotoxic species that mediate ER stress, cell death, and autophagy in the context of NAFLD have not been completely identified. Increasing studies have implicated bioactive sphingolipids, such as ceramides, sphingosine, and sphingosine-1-phosphate (S1P), in both initiation of NAFLD and its progression to HCC.

11.2 Metabolism of Ceramides in NAFLD

11.2.1 Dysregulation of Metabolism of Sphingolipids in the Liver of Patients with NAFLD

Increasing studies have demonstrated that ceramides, a subclass of sphingolipids, are potential lipotoxic mediators of saturated FFAs in driving NAFLD onset and progression [17, 18] . Palmitic acid, the most abundant hepatic FFA in NAFLD patients, is an essential precursor of ceramides and other sphingolipids [19, 20] (Scheme 11.1). Palmitic acid is activated to form palmitoyl-CoA, which is condensed with serine into keto-dihydrosphingosine by the action of serine palmitoyltransferase (SPT) [19]. Keto-dihydrosphingosine is reduced to form dihydrosphingosine [20], which is acylated by various fatty acids to form dihydroceramides with various acyl-chains by the action of 6 (dihydro)ceramides synthases (CERS1–6) [21, 22]. Dihydroceramides are then converted to ceramides by the action of dihydroceramide desaturase. These enzymatic steps occur in the ER and belong to so-called the de novo pathway for ceramide formation. Once generated in the ER, ceramides are transported from the ER to the Golgi complex where they are then incorporated into more complex sphingolipids



Scheme 11.1 Metabolism of sphingolipids. *Cer* ceramide, *CGT* galactosylceramide synthase, *Dhcer* dihydroceramide, *DHS* dihydrosphingosine, *E1P* phosphoethanolamine, *Gal-Cer* galactosylceramide, *GLS* glycosphingolipid, *Glu-cer* glucosylceramide, *KDSR* ketodihydrosphingosine reductase, *Pal-CoA* palmitoyl-CoA, *SPH* sphingosine, *S1P* sphingosine-1-phosphate, *SM* sphingomyelin

such as sphingomyelins and glycosphingolipids. Sphingomyelins on the plasma membrane can be hydrolyzed to form ceramides by the action of neutral sphingomyelinase. This metabolic pathway is termed the sphingomyelin-hydrolysis pathway for ceramide formation. Complex sphingolipids can also be transported back to the lysosomes where they are converted back to ceramides, which are hydrolyzed into sphingosine by the action of ceramidases [23, 24]. Sphingosine can be converted back to ceramides by the action of CERS, which is termed the salvage pathway for ceramide formation. Therefore, there are three major metabolic pathways that lead to the formation of ceramides in cells, including the de novo, sphingomyelin-hydrolysis, and salvage pathways. In vitro cellular studies demonstrated that oversupply of palmitate markedly increases the levels of ceramides in hepatocytes [25]. Several clinical studies demonstrated that the hepatic content of ceramides is elevated in patients with NAFLD. Apostolopoulou et al. [26] found that the hepatic levels of ceramides were increased in NASH patients compared to NAFL patients or healthy individuals. Luukkonen et al. [27] demonstrated that the hepatic levels of ceramides and dihydroceramides were elevated in patients with steatosis plus IR compared to patients with steatosis. However, Vvedenskaya et al. [28] showed that the hepatic levels of ceramides were similar between healthy individuals and patients with NAFL or NASH. Several other studies demonstrated that the hepatic levels of ceramides were also increased in murine models of NASH [29–32]. Accumulated ceramides are derived from both de novo pathway and sphingomyelin-hydrolysis pathway. Enzymes in both ceramide-generating pathways have been shown to be upregulated in the liver of mice with NAFLD, including SPT, CERS1, CERS2, CERS4, CERS6, and SMPD1 [33–37]. These results indicate that NAFLD is indeed associated with increased hepatic levels of ceramides.

As mentioned earlier, ceramides can be hydrolyzed into sphingosine by the action of 5 different ceramidases including acid ceramidase (ASAH1) [38], neutral ceramidase (ASAH2) [39], alkaline ceramidase 1 (ACER1) [40], alkaline ceramidase 2 (ACER2) [41], and alkaline ceramidase 3 (ACER3) [42]. As such, the hepatic content of sphingosine may be increased with increasing FFAs and ceramides in NAFLD patients and animal models of NAFLD. Indeed, the content of sphingosine was also increased in human hepatocytes treated with palmitate, a cellular model of steatosis [43, 44]. Hepatic sphingosine has been shown to be elevated in several

mouse models of NAFLD [26, 29, 30, 45] although it remains unclear whether this is also the case in patients with NAFLD.

As sphingosine can be phosphorylated to form S1P by the action of SPHK1 and/or SPHK2 in liver cells, several studies have shown that hepatic levels of S1P are increased in patients with NAFLD [30] and murine models of NAFLD [46–48].

11.2.2 Role of Ceramide and Sphingosine in Hepatocyte Injury

In addition to structural components of membranes of mammalian cells, ceramides are bioactive lipids implicated in regulating oxidative stress [49], ER stress [50], and PCD [51, 52], which are key drivers of hepatic injury in NASH. This indicates that increased hepatic ceramides may contribute to the pathogenesis of NAFLD. In line with this notion, several animal studies demonstrated that inhibiting the generation of ceramides protected mice from diet-induced NASH. Treatment with myriocin, a specific and potent SPT inhibitor that inhibits the biosynthesis of ceramides, reduced liver damage and fibrosis, and expression of the inflammation markers, IL-1β, MCP-1, and TNFα [53]. Myriocin has also been found to be effective in suppressing high-fat diet (HFD)-induced steatosis, inflammation, fibrosis, and apoptosis in *LDLr* $^{-/-}$ mice, a genetic mouse model of NASH [54]. Moreover, myriocin was also shown to reduce the severity of NAFLD in rats fed diet enriched in fat and cholesterol [55]. In contrast to myriocin, treatment of ceramide per se was found to induce hepatic steatosis by enhancing hepatic lipogenesis [56]. These results suggest that ceramides or their metabolites accumulated in the liver may drive NASH onset and progression.

Indeed, several recent studies revealed the distinct roles of specific ceramide species in the pathogenesis of NAFLD. We showed that knocking down ACER3 increased the levels of $C_{18:1}$-ceramide and alleviated oxidative stress and cell death in human hepatocytes oversupplied with palmitic acid [57]. We also demonstrated that knocking out the mouse *Acer3* protected mice from hepatic oxidative stress and death of hepatocytes in a mouse model of NAFLD likely by increasing hepatic levels of $C_{18:1}$-ceramide [57]. These results suggest a protective role of the unsaturated long-chain ceramide in NAFLD. *CerS2* haploinsufficiency decreased very-long-chain ceramides while inducing a compensatory increase of C_{16}-ceramide in mice, resulting in inhibition of beta-oxidation and advanced steatohepatitis [58], suggesting either a protective role of very-long-chain ceramides or a pathological role of C_{16}-ceramide in NAFLD. A series of studies demonstrated that increased C_{16}-ceramide due to *Cers6* deficiency promoted lipogenesis and impaired mitochondrial respiration in NAFLD with obesity background [58–60]. Additionally, C_{16}-ceramide treatment per se induced steatosis in mice by upregulating lipogenesis [56]. Interestingly, *Cers5* knockout decreased the levels of C_{16}-ceramide in various tissues including the liver in a feeding-independent manner and protected mice from weight gain, white adipose tissue inflammation, and hyperglycemia after HFD challenge [61]. These results suggest that C_{16}-ceramide produced by the action of different CERS isozymes has distinct roles in regulating NAFLD.

Similar to ceramides, previous studies indicate that sphingosine also functions as a bioactive lipid to mediate PCD in human cells in response to different stressful insults, such as the pro-death cytokine TNF-α [62], glucocorticoid [63], serum deprivation [64], or oxidative stress [65]. However, which ceramidase is responsible for generating the pro-death sphingosine remained unclear until our recent studies have firmly established that ACER2 is a major ceramidase responsible for production of sphingosine that mediates cell death in response to a variety of stress stimuli. We demonstrated that genotoxic cancer chemotherapeutic agents [66] or ionizing radiation increased the levels of sphingosine in human tumor cells in a p53-dependent manner by upregulating ACER2 [67]. Knocking out ACER2 inhibited not only the generation of sphingosine but also PCD in response to DNA-damaging

agents, whereas overexpression of ACER2 was sufficient to induce both sphingosine generation and PCD in cells [66, 67]. Furthermore, we identified ACER2 as a novel transcriptional target of p53, explaining why DNA-damaging agents and ionizing radiation upregulate both ACER2 and sphingosine in cells in a p53-dependent manner [67]. Many previous studies suggest that ceramides act as bioactive lipids to mediate PCD in tumor cells in response to different forms of stress, including DNA damage [68–70]. However, we demonstrated that an increase in the levels of ceramides due to ACER2 knockout or knockdown in cells failed to induce cell death [66], suggesting that ceramides that serve as endogenous substrates of ACER2 do not directly mediate PCD. These results suggest that sphingosine converted from ceramides by the action of ACER2 may also mediate the pathogenesis of NAFLD by inducing PCD of hepatocytes. Gulibositan et al. recently reported that hepatocyte-specific knockout of *Sphk2*, which phosphorylates sphingosine into S1P, significantly impaired insulin sensitivity and glucose tolerance in HFD-fed mice [71]. Intriguingly, the mechanistic study by the same group found that hepatocyte-specific ablation of *Sphk2* increased the hepatic levels of sphingosine without affecting those of S1P, suggesting that sphingosine may be a bona fide mediator of HFD-induced insulin resistance and glucose intolerance [71].

11.2.3 Role of S1P in Steatosis and Inflammation in NAFLD

Emerging evidence suggests that S1P plays roles in inflammation and fibrosis in the context of NAFLD. In vitro study reported that SPHK1 expression protected hepatocytes from lipotoxicity by inhibiting IRE1α activation and JNK phosphorylation and thereby ER-stress-associated apoptosis of hepatocytes [72]. However, in vivo studies found that knocking out *Sphk1* alleviated steatosis and hepatic inflammation in a mouse model of NAFLD, suggesting a role of S1P in NAFLD progression [48, 73]. In contrast, *Sphk2* knockout mice were found to be more susceptible to NAFLD induction likely due to the compensatory upregulation of *Sphk1* [47], further confirming the pathological role of S1P in NAFLD.

11.3 Sphingolipid Metabolism in HCC

11.3.1 Dysregulation of Ceramide Metabolism in HCC

Several studies demonstrated that ceramide metabolism is dysregulated in HCC in humans and animals. Krautbauer et al. found that most ceramide species (C_{16}-$C_{24:1}$) were decreased in human HCC tissues compared to their matched nontumor hepatic tissues [74]. In line with this study, Ismail et al. [75] and Li et al. [76] showed that the hepatic levels of ceramides were significantly decreased in human HCC tumors compared to paired nontumor hepatic tissues. In contrast to the above studies, Miura et al. found that the hepatic levels of several subclass sphingolipids, including ceramides, were significantly increased in human HCC tumors compared with their matched nontumor hepatic tissues [77]. The discrepancies among these studies might be attributed to variations in the etiology of HCC patients. Preclinical studies also indicate that hepatic levels of ceramides are altered in chemically induced liver tumors compared to nontumor liver tissues in mouse models of HCC. Haberl et al. [78] demonstrated that the levels of ceramides were higher in liver tumors than in nontumor hepatic tissues in mice treated with the carcinogen N-nitrosodiethylamine (DEN).

11.3.2 Role of Ceramides in HCC

Ceramides derived from either the de novo or sphingomyelin-hydrolysis pathway have been implicated in regulating HCC development and progression. Targeting specific CERS with a genetical approach has revealed that ceramides

with different acyl-chains may have distinct roles in regulating HCC in mice. It has been shown that *Cers2* deficiency decreased the hepatic levels of very-long-chain ($C_{22–24}$) ceramides while increasing those of C_{16}-ceramide and promoted sporadic liver tumor formation and chemical-induced hepatocarcinogenesis in mice [79, 80]. These results suggest that very-long-chain ceramides have a tumor suppressor role in HCC or that long-chain ceramide has an oncogenic role in HCC. Silencing CERS6, which synthesizes C_{16}-ceramide, was found to mediate the cytotoxicity of antifolate methotrexate in HCC cell, suggesting that C_{16}-ceramide might be a mediator of chemotherapeutic agents in HCC [81]. CERS4 was found to be upregulated in HCC tissues and its upregulation promoted the proliferation and survival of HCC cells [82]. These data suggest that ceramides with different chain lengths and saturation degrees, which are produced by specific CERSs, may function distinctly in HCC.

The sphingomyelin-hydrolysis pathway is also involved in the pathogenesis of HCC. Neutral sphingomyelinase 1 encoded by the gene *SMPD2* was found to be downregulated in HCC tissues and its downregulation negatively correlates with poor long-term survival of patients with HCC [83]. Neutral sphingomyelinase 2 encoded by the gene *SMPD3* was identified as a tumor suppressor-like gene that negatively correlates with early recurrence of human HCC after curative surgery [84]. *Smpd3* deficiency in mice promoted survival and proliferation of cancer stem-like cells, resulting in spontaneous HCC. Unexpectedly, *Smpd3* deficiency increased both sphingomyelin and C_{16}-ceramide levels in mouse HCC tissues, especially in cancer stem-like cells. The increased sphingomyelin and C_{16}-ceramide were attributed to the compensatory upregulation of *Cers5* [85]. These results suggest that sphingomyelinases play an anti-cancer role in HCC.

On the other hand, inhibition of glucosylceramide synthesis was also found to suppress HCC. Jennemann et al. found that glucosylceramide synthase encoded by the *UGCG* gene was significantly overexpressed in human HCC tissues as compared to nontumor liver tissues and that knockout of the mouse *Ucgc* specifically in the hepatocyte inhibited HCC initiation and progression in a chemically induced HCC model [86]. Su et al. found that ganglioside synthesis was increased in the livers of an animal model of activation and expansion of liver cancer cells, and pharmacological inhibition of ganglioside synthesis suppressed proliferation and sphere growth of liver cancer cells [87]. Guri et al. [88] demonstrated that activating the mTOR signaling pathway specifically in the liver through the liver-specific knockout of both *Tsc1* and *Pten* increased synthesis of fatty acids and sphingolipids including glucosylceramides in the liver, resulting in hepatosteatosis and HCC, which occurred sequentially. Inhibiting sphingolipid biosynthesis with myriocin, which specifically inhibits SPT, or knocking down hepatic Ugcg by RNA interference markedly reduced liver tumor numbers in *Tsc1* and *Pten* double knockout mice. This study provides compelling evidence that increasing glucosylceramides or more complex glycosphingolipids may promote NAFLD development and progression to HCC.

The breakdown of ceramide catalyzed by ceramidases plays important role in regulating cancer-related pathobiology [24]. Pharmacological and siRNA inhibition of acid ceramidase was found to inhibit growth of liver tumor xenografts of HepG2 cells and enhanced the cytotoxicity of daunorubicin on HCC cells by upregulating oxidative stress and apoptosis [89]. The expression of ACER3 was found to be upregulated in different liver cancer cell lines compared to normal liver cells and its increased expression inversely correlates with the overall and disease-free survival of HCC patients [90]. Knockdown of ACER3 inhibited cell growth and promoted apoptosis in HCC cells but had no influence on growth or apoptosis in normal hepatocyte cells. Similar to ACER3, Liu et al. found that ACER2 was also upregulated in HCC and its upregulation promoted HCC cell survival and migration [91]. These results suggest that increased alkaline ceramidases promotes liver tumorigenesis likely by increasing hydrolysis of ceramides.

11.3.3 Role of S1P in HCC

SPHK-derived S1P has been shown to function as an oncogenic lipid of HCC by promoting survival, migration, and proliferation in HCC cells. SPHK1 was found to be upregulated in human HCC tissues compared to adjacent non-tumorous liver tissues, and the overexpression of SPHK1 was associated with advanced malignancy and poor prognosis of HCC [92–95]. Bao et al. found that the SphK1-induced migration and invasion of HCC cells was mediated by the S1P receptor S1PR1 [92]. Mu et al. demonstrated that SPHK1 could mediate the migration of hepatoma cells induced by hepatocyte growth factor (HGF) [96]. SPHK1-mediated invasion and metastasis were also attributed to induction of the mesenchymal transition (EMT) in HCC cells as increased SPHK1 accelerates lysosomal degradation of the cell-cell adhesion molecule E-cadherin (CDH1) [97]. Notably, this study found that SPHK1-produced S1P bound to TRAF2 and stimulated lysine 63-linked ubiquitination and beclin1 activation, resulting in autophagic degradation of E-cadherin and thereby EMT [97]. SPHK1 has been shown to mediate HCC proliferation by activating the Ras/ERK, MEK1/2, FAK/MLC-2, Wnt5A/b-catenin, Akt/GSK3β, and Akt/NF-kb signaling pathways [98–100]. In addition to regulating the malignancy of HCC, *Sphk1* deletion in mice was found to suppress carcinogenesis in a chemically induced HCC model, supporting a role of SPHK1 in promoting hepatocarcinogenesis [101]. SPHK2 has a similar role in HCC. SPHK2 mRNA levels were found to be increased in HCC tissues and positively correlated with intra- and extra-hepatic recurrence [95]. Shi et al. reported that SPHK2 overexpression was associated with regorafenib resistance in HCC by activating NF-κB and STAT3 [102]. Beljanski et al. also reported that inhibition of SPHK2 in combination with sorafenib suppressed cell growth through the MAPK pathway in HCC [103]. Interestingly, S1P lyase was also increased in HCC tissues, and higher S1P lyase mRNA levels in HCC were associated with increased proliferation and poorer differentiation of HCC [95]. These results suggest that S1P may have an important role in liver tumorigenesis and that targeting the S1P pathway may improve the therapy of HCC.

11.3.4 Therapeutic Role of Exogenous Ceramide in HCC

Exogenous treatment of ceramide and sphingosine have been demonstrated to exert cytotoxic effects in HCC. Short-chain ceramide, including C_2- and C_6-ceramide, are well-studied antitumor lipids that induce growth arrest and cell death in various types of cancer [104]. Since Obeid et al. demonstrated for the first time that C_2-ceramide induces PCD [105], short-chain ceramides have come into the spotlight of cancer research. C_2-ceramide was later shown to induce cell death in HCC cell, including apoptosis and necrosis, possibly by downregulating Bcl-2 and inhibiting respiratory chain to produce reactive oxygen species (ROS), exhausting ATP, and impairing mitochondria function [106, 107]. C_6-ceramide was shown to induce apoptosis in HCC cells concurrent with release of cytochrome c and activation of caspase-3 without affecting mitochondrial respiratory chain [107]. Co-administration of C_6-ceramide was also found to enhance cytotoxic and pro-apoptotic effects of mTOR complex 1/2 (mTORC1/2) dual inhibitor AZD-8055 in a panel of HCC cell lines and primary cultured human HCC cells, with no adverse effect on growth and survival of normal human hepatocytes [108]. Nanoliposomal C_6-ceramide, which can be administered intravenously and has improved bioavailability and solubility [109], was then applied in several preclinical studies to evaluate its therapeutic efficiency in HCC. Tagaram et al. reported that nanoliposomal C_6-ceramide administration suppressed HCC growth in mice engrafted with HCC cells by reducing tumor vascularization and proliferation, inducing tumor cell apoptosis, and inhibiting phosphorylation of AKT [110]. More recently, in addition to tumor growth suppression, nanoliposomal C_6-ceramide was found to improve antitumor immune response [111]. Nanoliposomal C_6-ceramide injection

could reduce numbers of tumor-associated macrophages and their production of ROS while inducing differentiation of tumor-associated macrophages into M1 phenotype of macrophages, which reduce immune suppression and increase activity of anti-cancer $CD8^+$ T cell [111]. Similar to liposomal C_6-ceramide, liposomal C_8-ceramide was also found to induce apoptosis in HCC by activating caspase pathways and apoptosis signal-regulating kinase 1 (ASK1)-Jun N-terminal protein kinase (JNK) signaling, and injection of liposomal C_8-ceramide inhibited HepG2 xenograft growth in severe combined immuno-deficient mice and improved their survival [112]. The liposomal short-chain ceramides were also found to enhance the effect of several chemotherapeutic drugs. Co-treatment with nanoliposomal C_6-ceramide and vinblastine synergistically inhibited growth in HCC cells, probably by inhibiting autophagy flux and increasing apoptosis [113]. Similarly, liposomal C_6-ceramide augmented the growth inhibitory effects of mTOR complex 1/2 inhibitor AZD-8055 in HCC mice xenografts with HCC cells [108]. Notably, co-loading C_6–ceramide with sorafenib into liposomes synergistically increased antitumor activity in HCC cells with reduced systemic toxicity of sorafenib [114]. Similar to ceramides, sphingosine treatment was found to induce apoptosis by suppressing the activation of AKT kinase and upregulating caspase pathways in HCC cells [115–117]. All the data highlight the anti-cancer activities of ceramides and sphingosine mainly by inducing cell death in HCC, which warrants further clinical studies to evaluate the potential of ceramide and sphingosine treatment for HCC.

11.4 Conclusions

Recent findings strongly suggest that specific ceramide species, sphingosine, sphingosine-1-phosphate, and other sphingolipids play prominent roles in NAFLD and liver tumorigenesis. Targeting the metabolism of these bioactive sphingolipids may represent a novel approach to halting NAFLD development and/or its progression to HCC. Therefore, an important area of future study in the sphingolipid field is to develop novel drugs targeting ceramide metabolism to enhance therapeutic response and improve survival outcome in HCC patients.

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Drug Development in the Field of Sphingolipid Metabolism

12

Zhibei Qu and Lu Zhou

Abstract

Sphingolipids are the major lipid components on cellular membranes especially on lipid raft regions, intermediating various important biological functions for eukaryotic cells. Sphingolipid metabolism pathways can utilize sugar, protein, nucleic acid, and other metabolites participating lipid transport in the circulation, play an essential role in maintaining cell homeostasis and are related to a variety of different diseases including lysosomal storage disorders (LSDs), Gaucher disease, etc. The dynamic balance of sphingolipid levels in organisms is regulated by a series of sphingolipid synthases, hydrolases, and metabolic enzymes, such as sphingomyelinase (SMase), sphingomyelin synthase (SMS), serine palmitoyltransferase (SPT), ceramide synthase (CerS), glucosylceramide synthase (GCS), etc. Thus, sphingolipids and its related enzymes are potential targets for drug discoveries and receive great research interests by medicinal chemist. In this chapter, we will discuss the relationship between sphingolipids and the regulating enzymes involved in sphingolipid metabolisms, and systematically summarize the advances in the development of new drugs in the field.

Z. Qu · L. Zhou (✉)
School of Pharmacy, Fudan University, Shanghai, China
e-mail: zhoulu@fudan.edu.cn

Keywords

Sphingolipid metabolism · Ceramide synthase · Sphingomyelin synthase · Serine palmitoyl transferase · Sphingomyelinase · Drug development

Abbreviations

AAL	Alternaria alternate lycopersici
CerS	Ceramide synthase
FB1	Fumonisin B1
GCS	Glucosylceramide synthase
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
LSD	Lysosomal storage disorders
SM	Sphingomyelin
SMase	Sphingomyelinase
SMS	Sphingomyelin synthase
SPT	Serine palmitoyltransferase

12.1 Background

Sphingolipids are essential lipids involved in regulating cell functions and maintaining metabolic homeostasis in organisms [1]. These lipids share a sphingoid base backbone which is *N*-acylated with various fatty acid chains. Sphingolipids can be divided into three structural classes [2], including sphingoid bases and derivates (i.e., sphingosine, sphingosine-1-

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phosphate), ceramides, and complex sphingolipids. Complex sphingolipids can be further divided into phosphosphingolipids (i.e., sphingomyelin, C1P), neural glycosphingolipids (Glc-Cer, Gal-Cer, Lac-Cer, etc.), and acidic glucosphingolipids (i.e., gangliosides).

From the perspective of macro-molecular metabolic pathways, the sphingolipid metabolism pathways can utilize sugar, protein, nucleic acid, and some metabolites in other lipid metabolism pathways (L-serine, Acyl-CoA, fatty acid, etc.) as raw materials for de novo synthesis of sphingolipids. In addition, some key active molecules (such as phosphoethanolamide, phosphor-choline, phosphor-inositol, DAG, etc.) generated by sphingolipid catabolism can participate in the anabolism of other substances, maintaining metabolic homeostasis of organisms [3].

The dynamic balance of sphingolipid levels in organisms is regulated by a variety of sphingolipid synthases, hydrolases, and metabolic enzymes. Functional deficiency or loss of some essential enzymes would directly break the balance, further leading to the occurrence of various diseases including lysosomal storage disorders (LSDs). LSDs are a class of inherited metabolic diseases. Typical LSDs are caused by mutations in genes that encode certain hydrolases and/or activators, preventing cells from producing these functional proteins abnormally, and then resulting in a large accumulation of related substrates in the cells. For example, Gaucher disease is a functional deficiency of glucocerebrosidase caused by mutation of GBA1 gene, resulting in abnormal accumulation of glucosylceramide. And glucosylceramide synthase inhibitors (e.g., Genz-112,638) were proved to alleviate the Gaucher disease.

Furthermore, abnormal sphingolipid metabolism is prevalent in many common diseases. Ceramide is a well-recognized signaling molecule mediating cell death. Although recent studies have shown that different types of ceramides could regulate cell growth and death differently [4], the lipotoxicity caused by ceramide in obesity and inflammation disease could not be ignored [5]. Therefore, studying the in vivo synthesis pathway of ceramide could lead new strategies for the treatment of related diseases. Ceramide can block Akt signaling pathway by activating PP2A and PKCζ, further regulating cell growth and other signals [6]. By inhibiting insulin stimulation of Akt [7] and activating the expression of some inflammatory factors (STAT3, etc.) [8], ceramide contributes to a variety of metabolic diseases.

In a high-fat diet, excessive fatty acid intake activates the intracellular palmitoylation metabolic pathway, in turn regulates the transcriptional activation of the de novo synthases of ceramide, Sptlc2 and CerS, further promotes the synthesis of ceramide and leads to obesity and its syndromes [9]. Interestingly, the sphingolipid metabolism of certain intestinal flora can also stimulate the synthesis of ceramide in the host, thereby promoting the development of inflammation and metabolic diseases [10]. Therefore, inhibition of ceramide synthase (such as CerS) is a potential therapeutical strategy.

Serine palmitoyl transferase (SPT) complex is the first enzyme in de novo biosynthesis of ceramide, which locates at the upstream of the entire sphingolipid synthesis pathways. In mammals, the SPT complex is composed of two large subunits, SPTLC1 and SPTLC2/3, and two small subunits, SSSPTA and SSSPTB [11]. SPT is very important in maintaining the balance of sphingolipid metabolism in eukaryotes. Homozygous SPTLC1- or SPTLC2-deficient mice are embryonic lethal, while heterozygous SPTLC1/2-deficient mice remain healthy [12, 13]. Compared with normal mice, the levels of sphingolipids (such as sphingosine, ceramide, and S1P) in tissues and plasma of SPTLC1/2-deficient mice are significantly reduced. Inhibiting SPT in cells using SPT inhibitors enables growth inhibition of some fungi and tumor cells [14–16], indicating that SPT activity is indispensable for the eukaryotic cell. The missense mutation of SPTLC1 gene is the main cause of the congenital disease hereditary sensory neuropathy type I (HSN1). High expression of SPTLC2 can promote the synthesis of ceramide in liver, activate the JNK signaling pathway, and then lead to insulin resistance [17, 18]. Inhibition

of SPT also has a certain effect on alleviating atherosclerosis and obesity metabolic syndrome [19, 20]. In addition, SPT can also affect the assembly of lipid rafts on biological membranes by regulating the synthesis of sphingolipids, thereby promoting the localization of the NS protein of HBV and the replication of viral nucleic acids [21]. Certain inflammatory factors (neutrophil elastase, etc.) can upregulate SPT activity to promote the synthesis of ceramide, which in turn lead to inflammation [22]. In recent years, the crystal structure of human SPT complex has been reported, laying an important structural foundation for the study of the mechanism of SPT and the development of inhibitors targeting SPT [23].

Sphingomyelin (SM) is an important direct metabolite of ceramide, which participates in the formation of cell (organelle) membranes and the conduction of various signals. The level of sphingomyelin in organisms is mainly regulated by sphingomyelin synthase (SMS) and sphingomyelinase (SMase). SMase catalyzes the decomposition of SM into ceramide, which plays a key role in maintaining the balance of ceramide. Based on the optimal working pH of enzyme, SMase can be divided into three types, including aSMase, nSMase, and alk-SMase. aSMase is expressed in almost all types of cells and has intracellular lysosomal form and extra-cellular secreted form depending on localization. The aSMase is normally located in the endosome/lysosome compartments. During cell stress and disease, aSMase can be preferentially transported to the outer lobes of the cell membrane and secreted into the extra-cellular space [24]. The activation of aSMase promotes the accumulation of a large amount of ceramide in the cell membrane, causing metabolic disorders, inflammatory reactions, or cell apoptosis, and ultimately leading to disease [25]. For example, in the pathological model of cystic fibrosis (CF) with Cftr gene defect, the defect or inhibition of aSMase activity can alleviate the CF caused by excessive accumulation of ceramide in respiratory cell [26]. The aSMase activation induces the accumulation of ceramide, which will promote the rapid death of tumor cells. Thus aMase could be a potential therapeutic target for tumors [27]. The nSMase located in the cell membrane has also been shown to regulate the release of inflammatory factors and the accumulation of Aβ by regulating the levels of SM and ceramide, thereby participating in the regulation of inflammation and neurodegenerative diseases [28]. Alk-SMase is expressed only in mammalian intestinal mucosa (also in human liver), and functions through the secretion of intestinal mucosal epithelial cells into the intestinal lumen, involved in the regulation of the metabolism and absorption of intestinal sphingolipids and then affecting the progression of diseases such as inflammation [29].

Interestingly, while SMase can improve the level of ceramide and perform certain physiological functions like ceramide, a SM synthase with the opposite function to SMase, SMS, also has physiological functions such as promoting the development of inflammation, cardiovascular disease, and metabolic syndrome. This may be related to the diversity of the SMS family's catalytic function and the difference in subcellular distribution. The SM generated by SMS1 and SMS2 can be transported to cell membrane to participate in the assembly of lipid rafts on cell membrane, promoting the function of lipid raft-related proteins. For example, SMS2 can stabilize the localization of CD36 to lipid rafts on cell membrane, thus promoting the cellular absorption of fatty acids [30]. SMS-produced DAG is considered to be a second messenger that activates the PKC-JNK axis, impairing insulin action and inducing insulin resistance [31]. SMS can maintain the CD14-TLR4 complex localization in cell membrane and promotes its function, thereby activating LPS-induced inflammatory signaling pathway downstream of TLR4, promoting the release of the inflammatory factor TNF-α, and aggravating the progression of associated inflammation [32]. In addition, SMS employing ceramide as a substrate, to somewhat alleviates the effect of ceramide overaccumulation in promoting apoptosis, and it can promote tumor growth and drug resistance by activation of certain signaling pathways (e.g., TGF-β/Smad) and promoting cytokines (e.g., BCL-2) expression [33].

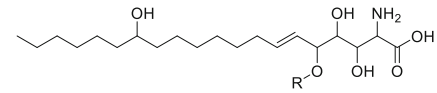
In summary, sphingolipid metabolism plays a vital role in maintaining homeostasis. Sphingolipid metabolism has its unique and complicated operating mechanism, involved in not only the formation of cells, but also the transformation or transmission of intra- and extra-cellular substances and signal transduction. The balance of sphingolipid metabolism plays a key role in modulating the normal development and growth of the body. The imbalance of sphingolipid metabolism caused by a variety of factors can directly or indirectly lead to the occurrence of diseases, suggesting that pharmaceutical intervention of sphingolipid metabolism may be a new way to treat certain diseases. However, in the complicated sphingolipid metabolism pathway, the effects of certain metabolites on diseases are subtle and sometimes even two-sided, suggesting the need for more comprehensive consideration and scrutiny when intervening in metabolic regulation.

12.1.1 Serine Palmitoyl Transferase Inhibitors

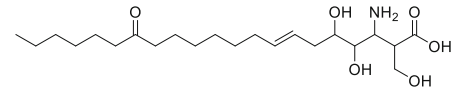
Serine palmitoyl transferase (SPT) complex is the first enzyme in de novo biosynthesis of ceramide, which locates at the upstream of the entire sphingolipid synthesis pathways [34]. SPT is very important in maintaining the balance of sphingolipid metabolism and inhibiting SPT in cells shows significant growth inhibition for eukaryotic cells. Inhibition of SPT also has a certain effect on alleviating atherosclerosis and obesity metabolic syndrome. Thus, SPT inhibitors are potential drugs for sphingolipid-related metabolic disorders.

SPT inhibitors can be divided into two subclasses. The first type was the substrate-mimics of SPT complexes. SPT has two natural substrates, palmitate and serine. Both mimics can be developed as potential SPT inhibitors.

In 1992, Marcia M. Zweerink and coworkers proved two known compounds, sphingofungin B and C, separated from *Aspergillus fumigatus*, showed the anti-fungi activity by inhibition of the sphingolipid synthetic pathways via SPT complexes [14]. In 1995, Yurika Miyake et al. proved myriocin as a highly efficient and selective SPT inhibitor using CTLL-2 cells as enzyme source, with a remarkably low IC50 of 0.3 nM [35].

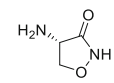


R = H sphingofungin B
R = Ac sphingofungin C

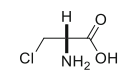


ISP-1/myriocin

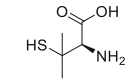
Moreover, a series of serine analogs such as L-Cycloserine [36], β-chloro-L-alanine [37], and L-penicillamine [38] were reported to show SPT inhibition activities but none was as good as myriocin and its derivates.



L-Cycloserine

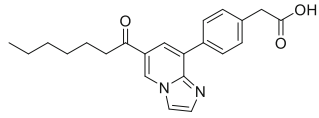


β-Chloro-L-alanine

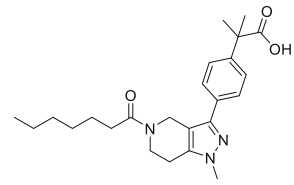


L-penicillamine

Medicinal chemists discovered a series of SPT inhibiting compounds which are non-analogs of SPT substrates. For example, Michael J. Genin screened two new imidazopyridine and pyrazolo-piperidine compounds (compound 1 and 2) [19], and found them showed good SPT inhibition properties in vitro (1: IC50 ~ 5 nM; 2: IC50 ~ 64 nM). In vivo tests showed that both compounds significantly reduced ceramide levels in DIO mice and promoted HDL levels.

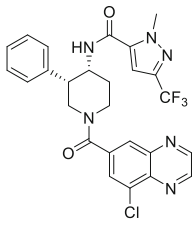


compound 1

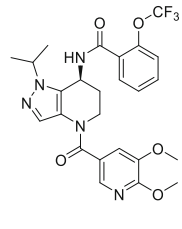


compound 2

Masahiro Yaguchi et al. discovered a new type of SPT inhibitor—compound 3, which has a significant effect on the disease of pl-21 acute myeloid leukemia PDX mice at an oral dose of 3 mg/kg [15]. And Ryutaro Adachi et al. obtained a new class of compounds with inhibitory SPT activity through enzyme-level activity screening, such as compound A (IC50 ~ 0.76 nM), which is effective for the growth of non-small cell lung cancer HCC4006 cells, with good in vitro activity (EC50 ~ 3.9 nM) [16].

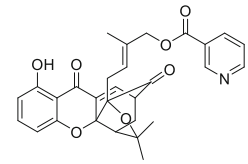


compound 3



compound A

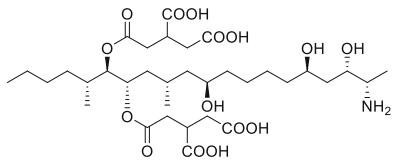
Dominic G. Hoch et al. obtained a class of gambogic acid and its structurally related xanthone derivatives (compound 18) by combining proteomics and metabolomics as a class of first-in-class mammalian SPT covalent inhibitor, and proved that its main mechanism is covalently bound to SPT small subunit B (SPTSSB) to destroy the formation of SPT complex [39].



compound 18

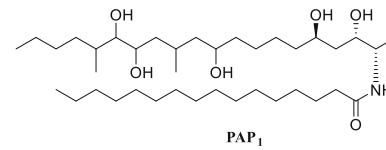
12.1.2 Ceramide Synthase Inhibitors

Ceramide is an intermediate in the biosynthetic pathway of lipids and a cell signaling molecule, catalytically produced by Ceramide synthase (CerS) [40]. CerS consists of six subtypes (CerS1–6) that distribute in different tissues and catalyze the synthesis of different ceramides. Elevated ceramide levels cause many disorders such as inflammation and obesity-related syndromes, and the development of CerS inhibitors is essential for potential therapeutical methods to metabolic diseases.



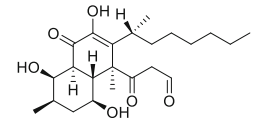
Fumonisin B_1

IC_{50} (CerS) = 0.1 μM



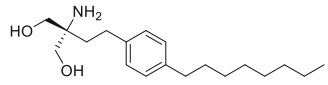
PAP_1

Fumonisin B_1 (FB_1), a mycotoxin isolated from *Fusarium moniliforme* by Gelderblom and coworkers, can inhibit CerS through substrate structural similarity (IC_{50}: 0.1 μM) [41], but is toxic that caused esophageal cancer, birth disability, and growth disorders [42]. Its derivatives, such as hydrolyzed esterification product PAP_1, had a better inhibitory effect but showed higher cytotoxicity [43].

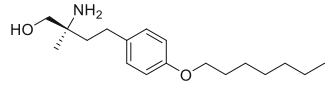


Australifungin
IC_{50} (CerS) = 0.093 μM

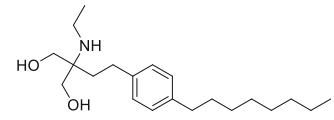
Australifungin, a toxin isolated from *Sporormiella australis* by Mandala and coworkers, had broad-spectrum antifungal activity against human pathogenic fungi [45], but the high chemical reactivity of the α-diketone and β-ketoaldehyde functional groups limited its use [46].



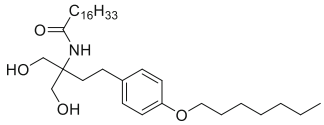
FTY720
IC_{50} (CerS) = 6.4 μM



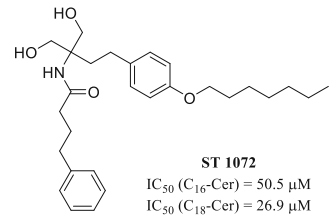
AAL(S)



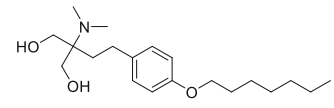
ST 1058
IC_{50} (C_{16}-Cer) = 25.7 μM
IC_{50} (C_{18}-Cer) = 50.7 μM
IC_{50} (C_{24}-Cer) = 60.6 μM



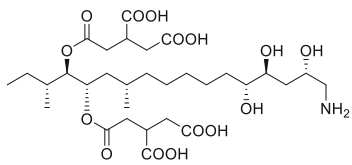
ST 1060
IC_{50} ($C_{24:1}$-Cer) = 61.3 μM
IC_{50} (C_{24}-Cer) = 63.6 μM



ST 1072
IC_{50} (C_{16}-Cer) = 50.5 μM
IC_{50} (C_{18}-Cer) = 26.9 μM
IC_{50} (C_{24}-Cer) = 81.3 μM



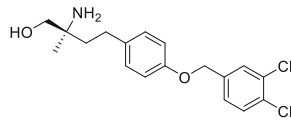
ST 1074
IC_{50} (C_{18}-Cer) = 21.4 μM
IC_{50} ($C_{24:1}$-Cer) = 28.7 μM



AAL-Toxin
IC_{50} (CerS) = 0.035-0.04 μM

Alternaria alternate lycopersici toxin (AAL-toxin), a fungal toxin isolated from *Alternaria alternata f.* sp. *lycopersici* by Bottini and coworkers, competitively inhibited ceramide synthase (IC_{50}: 0.04 μM) [44].

FTY720 was a synthetic analog of sphingosine and inhibited in a similar manner to FB_1 (IC_{50}: 6.4 μM), but the two had different inhibition efficiencies for long and short chain ceramide synthesis [47]. AAL(S) was an unphosphorylated FTY720 analog that could be used to study diseases associated with CerS1 [48]. The ST series of compounds were derivatives of FTY720 main chain modification with selective inhibitory effects [49]. P053, a small-molecule compound synthesized by Nigel Turner and coworkers, selectively inhibited CerS1 (IC_{50}: 0.5 μM), might be used to treat obesity [50].

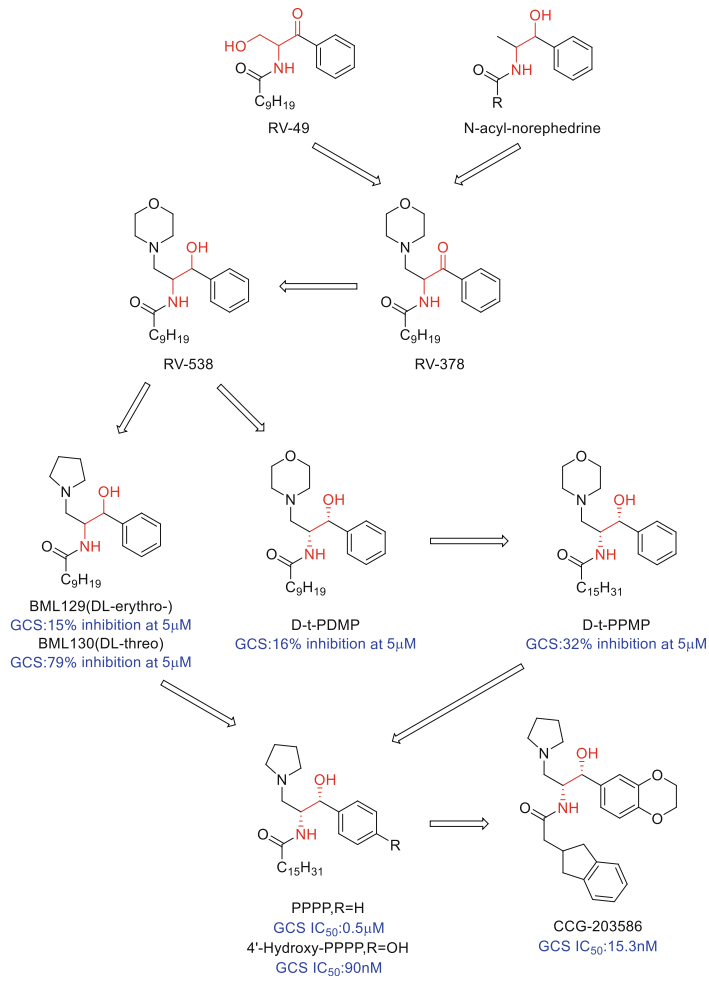


P053
IC_{50} (CerS1) = 0.5 μM

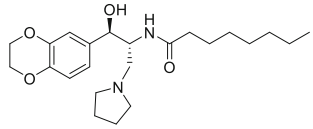
12.1.3 Glucosylceramide Synthase Inhibitors

Glucosylceramide synthase (GCS, also known as UCGC), catalyzes the conversion of ceramide to glucosyl ceramide [51]. GCS small-molecule inhibitors have been reported primarily for the treatment of lysosomal storage disorders—Gaucher's Disease and Fabry disease and emerged as promising studies related to type II diabetes and tumor resistance in recent years. They are generally divided into two categories by chemical structure.

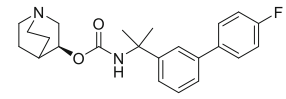
One class is ceramide analogs. The precursors of the PDMP family are RV-49 (70% inhibition at 0.3 mM) and *N*-acyl-norephedrine (82% inhibition of the most active compound at 0.3 mM). A non-competitive GCS inhibitor, RV-378 (72% inhibition at 150 mM), was synthesized by introducing a morpholine group at 1-position. The 3-position ketocarbonyl group of RV-378 is reduced to form a more potent analog RV-583 (84% inhibition at 37.5 mM) that is a competitive GCS inhibitor [52, 53]. RV-583 was originally a mixture of four stereoisomers. And only the D-threo-PDMP (1S,2R) is active against GCS [54]. By replacing the morpholinyl group of RV-538 with a pyrrolidinyl group, a pair of enantiomers BML-129 and BML-130 that showed growth inhibition of several kinds of cancer cells were produced [55]. PPMP, P4, and 4′-Hydroxy-P4 were prepared on the basis of structural modifications of D-threo-PDMP and BML-129/130 [56–58]. The most recent compound of the PDMP family is CCG-20358, which turns the alkyl chain linked to the *N*-acyl group into a benzocyclopentane to increase the rigidity of the entire molecule. It can cross blood–brain barrier [59].



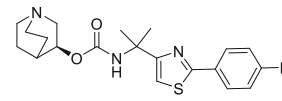
An effective GCS inhibitor eliglustat (Genz-112,638) is also a ceramide analog [60]. Further development on it revealed two brain-penetrant heterocyclic compounds Genz-667,161 and Genz-682,452 (venglustat) with reduced chiral centers [61–63]. Exelixis identified a more active GCS inhibitor (EXEL-0346) through high-throughput screening and hit optimization [64]. Recently, a novel CNS-permeable GCS inhibitor, T-036, was discovered [65].



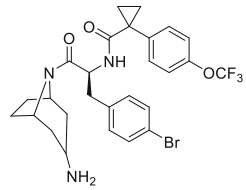
eliglustat(Genz-112638)
GCS IC_{50}=16nM
Cell IC_{50}=2nM



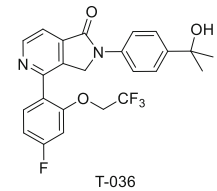
Genz-667161



venglustat(Genz-682452)

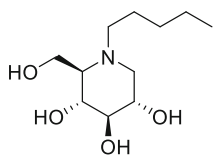


EXEL-0346
GCS IC_{50}=2nM

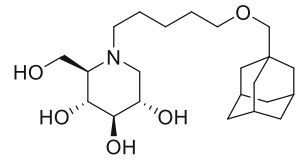


T-036
GCS IC_{50}=31nM

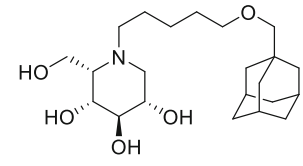
The other class is *N*-alkyl-deoxynojirimycins (DNMs). Miglustat (Zavesca), an alkyl iminosugar that mimics the transition state of the cationic intermediate in glycosylation reactions, is a competitive inhibitor of GCS. It is approved for the treatment of type I Gaucher's disease and Niemann–Pick disease. An optimized iminosugar (AMP-DNM) is a more effective GCS inhibitor [66]. Subsequently, it was found that ido-AMP-DNM, the C5-epimer of AMP-DNM, had a slightly stronger inhibitory effect on GCS [67]. Various iminosugar-based GCS inhibitors have been identified, which differ not only in the nature of the *N* substituent but also in the configuration of the piperidinyl iminosugar [68, 69]. However, DNMs are less selective for GCS and generally active against GBA1 and GBA2 as well. Some investigators designed a hybrid structure of two classes of GCS inhibitors, but with greatly reduced activity against GCS and active against GBA1 and GBA2 [70].



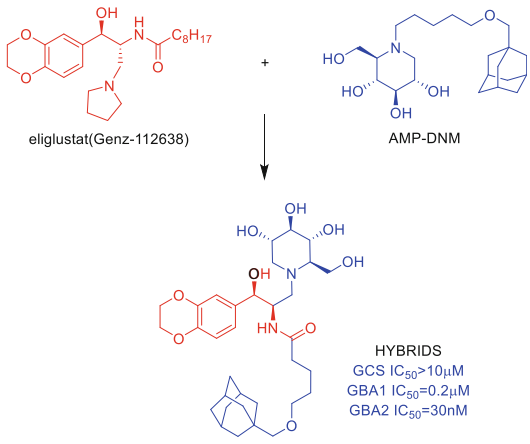
miglustat
GCS IC_{50}=50μM
GBA1 IC_{50}=400μM
GBA2 IC_{50}=230nM



AMP-DNM
GCS IC_{50}=150-220nM
GBA1 IC_{50}=0.2μM
GBA2 IC_{50}=1nM



L-ido-AMP-DNM
GCS IC_{50}=150nM
GBA1 IC_{50}=2μM
GBA2 IC_{50}<1nM

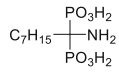


12.1.4 Sphingomyelinase Inhibitors

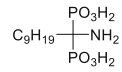
Sphingomyelinase (SMase) is an enzyme that hydrolyzes sphingomyelin to produce phosphocholine and ceramide. So far, at least 6 subtypes of SMase have been identified, mainly based on their optimal pH value and cofactors [71]. Among them, acid sphingomyelinase (A-SMase) is the most important subtype, and its biological activity accounts for 90% of the total SMase activity [72].

There are now growing evidence that the activation of SMase and the accumulation of ceramide play an essential role in the development of various human diseases. For example, the inhibitions of SMase activity prevent ischemic-stress-induced neuronal death [73], improve acute lung injury caused by repeated airway lavage [74], and reduce apoptosis in hepatic ischemia-reperfusion injury [75]. The A-SMase activity is also associated with major depression [76]. Therefore, the discovery of potent SMase inhibitors is of great significance for the development of drugs for the prevention and treatment of related diseases.

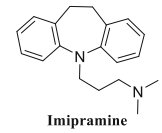
To date, a variety of inhibitors against A-SMase and N-SMase have been reported. The inhibitors of A-SMase are divided into two types, the direct inhibitors and the functional inhibitors. Direct inhibitors are characterized by not requiring high lysosomal drug concentrations as a prerequisite for inhibiting A-SMase, and there are few known examples of direct inhibitors [77]. For instance, several bisphosphonates have strong selective inhibition on A-SMase, among which the compound 7b and 7C have better inhibitory activity [78], imipramine can inhibit A-SMase4 [79]. Functional inhibitors are characterized as cationic amphiphilic substances, inducing the dissociation of A-SMase proteins from the endolysosomal membrane to inactivate A-SMase, including Astemizole and Amlodipine, etc. [80].



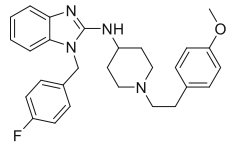
7b
IC_{50}(A-SMase)=0.04±0.01μM
(2009)



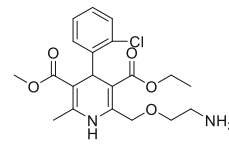
7c
IC_{50}(A-SMase)=0.02±0.00μM
(2009)



Imipramine
80-90% inhibition at 50μM
(2009)

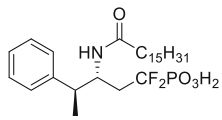


Astemizole

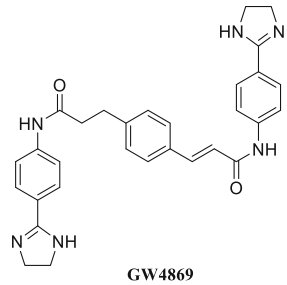


Amlodipine

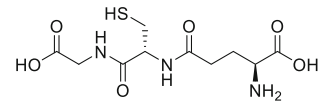
There are synthetic molecules and natural products as N-SMase inhibitors. Luberto and coworkers synthesized a series of compounds, among which GW4869 exhibited significant inhibitory activity against N-SMase both in vitro and cellular models. This compound is a non-competitive and selective inhibitor, which means GW6948 did not inhibit A-SMase [81]. Soeda synthesized a series of difluoromethyl analogs (SMAs) of sphingolipids, among which SMA-7 has better inhibitory activity. Liu found that N-SMase was inhibited in vitro by physiologically relevant concentrations of glutathione (GSH) and was activated in GSH-depleted cells [82], whereas glutathione acts as a natural inhibitor of N-SMase in the reduced (GSH) and oxidized (GSSG) forms [83].



SMA-7
IC_{50}(N-SMase):3.3 μM

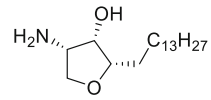


GW4869
IC_{50}(N-SMase):1 μM

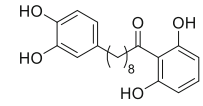


Glutathione
95% inhibition at 5mM

Microbial-derived natural products were screened as SMase inhibitors. Ryuji isolated a known compound, Alutenusin, from cultures of *Penicillium* sp. as a selective N-SMase inhibitor [84]. Tanaka isolated Schyphostatin from the mycelial extract of *Dasyscyphus mollissimus*, which was found to be a competitive inhibitor of N-SMase [85]. Schyphostatin was the most potent natural product against N-SMase [86], but unable to inhibit A-SMase activity [87]. Arenz discovered some analogs of Manumycin A are also the irreversible inhibitors of N-SMase, whose inhibitory ability is strongly influenced by their hydrophobic side chains [88].

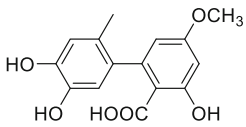


Jaspine B
EC_{50} (SMS) = 5 μM
(2009)

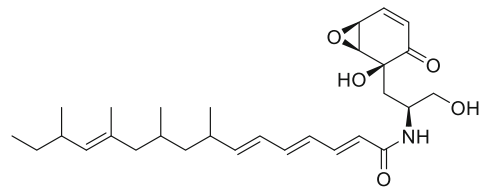


Malabaricone C
IC_{50} (SMS2) = 1.5 μM
selectivity (SMS2 vs SMS1) = 2
(2019)

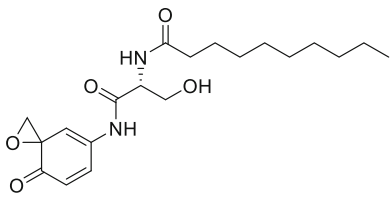
The first reported SMS inhibitor was D609, which had been known as a selective PC-PLC inhibitor before. Meng and coworkers found that



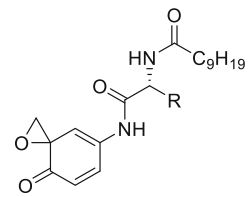
Alutenusin
IC_{50}(N-SMase)=28μM
(1999)



Schyphostatin
IC_{50}(N-SMase)=49.3μM
(1997)



2
88% inhibition at 100μM
(2001)



a:R=CH_3
b:R=$CH_2C_6H_5$

3a,b
3a:33% inhibition at 100μM
3b:23% inhibition at 100μM
(2001)

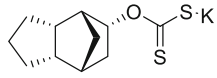
12.1.5 Sphingomyelin Synthase Inhibitors

With the increasing understanding of sphingomyelin synthase (SMS) family proteins, SMSs were found associated with the occurrence and development of various diseases [89]. A variety of SMSs inhibitors have been developed, and some of them have been evaluated in disease models.

D609 was capable of inducing U937 cell death by apoptosis, which was associated with the inhibition of SMS activity [90]. A significant increase in the intracellular level of ceramide and a decrease in that of sphingomyelin (SM) and diacylglycerol were observed, suggesting that SMS is a potential target of D609 and inhibition

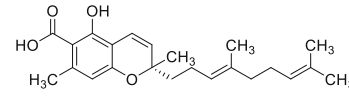
of SMS may contribute to D609-induced tumor cell death. However, D609 is very unstable under aqueous conditions due to the carbonodithioate structure, which restricted its further application in in vivo study.

New types of SMS inhibitors including natural products and synthetic molecules have been developed in the past 10 years. Jaspine B, an anhydrophytosphingosine derivative isolated from the marine sponge *Jaspis* sp. by Salma and coworkers, inhibited the activity of sphingomyelin synthase (IC_{50}: 5 μM) and induced cell death in SMS1-depleted cells but not SMS1-overexpressed cells [91].



D609
IC_{50} (SMS) = 177 ~ 600 μM
(2004)

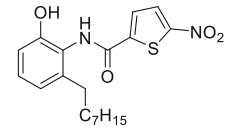
Malabaricone C, isolated from the fruits of *Myristica cinnamomea* King by Othman and coworkers, was reported as an SMS inhibitor [92]. It exhibited multiple efficacies, including reduction of weight gain, glucose tolerance improvement, and reduction of hepatic steatosis in high-fat diet-induced obesity mice models. However, Malabaricone C was also reported to inhibit α-Glucosidase [93] and cholinesterase [94] as well, suggesting that it was a multi-target natural product.



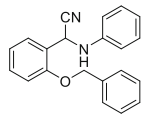
Daurichromenic acid
IC_{50} (SMS2) = 4 μM
selectivity (SMS2 vs SMS1) = 1.8
(2020)

Another natural product Daurichromenic acid (DCA), isolated from *Rhododendron dauricum* by Deepak and coworkers, was found as a sphingomyelin synthase inhibitor [95]. In addition, DCA was proved to inhibit amyloid β aggregation. Although these natural products showed moderate inhibition against SMS, they all acted on two or more targets. Thus, these compounds were not ideal chemical tools to study the potential roles of SMS in disease models.

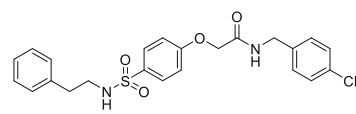
Medicinal chemists have put efforts into synthetic SMS inhibitors and made great progress in recent years. Swamy and coworkers designed a series of ceramide mimics based on ginkgolic acid which is a natural product and an SMS inhibitor. Among them, compound **5** showed moderate activity [96].



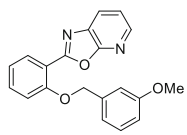
Compound 5
IC_{50} (SMS2) = 3 μM
selectivity (SMS2 vs SMS1) = 1.7
(2018)



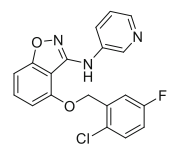
D2
IC_{50} (SMS2) = 14 μM
(2014)



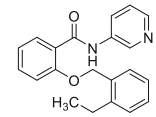
SAPA 1a
IC_{50} (SMS1) = 5.2 μM
(2015)



QY16
IC_{50} (SMS2) = 3 μM
selectivity (SMS2 vs SMS1) > 30
(2017)

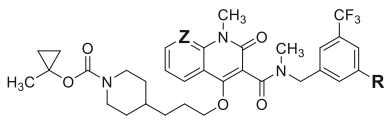


Compound 15w
IC_{50} (SMS2) = 100 nM
selectivity (SMS2 vs SMS1) = 560
(2018)



Ly93
IC_{50} (SMS2) = 91 nM
selectivity (SMS2 vs SMS1) = 1400
(2019)

A research group from Fudan University, China contributed to the development of SMS inhibitors. Deng and coworkers performed structure-based virtual screening in combination with chemical synthesis and bioassay [97]. They found a hit compound D2, which was the first small-molecule SMS inhibitor with potency close to the micromolar range. Based on the structure of lead compound D2, Qi and coworkers designed a series of oxazolopyridine derivatives with good selectivity [98]. Through a similar approach, Li from the same research group developed a new series of SAPA compounds, among which SAPA1a showed the best in vitro activity [99]. Progress of SMS inhibitors was made by Mo and coworkers in 2018 [100]. They developed 4-benzyloxybenzo[d]isoxazole-3-amine derivatives as potent and highly selective SMS2 inhibitors. Among them, compound 15w demonstrated good pharmacokinetics and attenuated chronic inflammation significantly in db/db mice. This was the first reported oral available selective SMS2 inhibitor. In the coming year, Li developed another oral available SMS2 inhibitor Ly93, with reported highest selectivity till then (1400-fold over SMS1) [101]. The 2-benzyloxybenzamide derivative Ly93 significantly decreased the plasma SM levels of C57BL/6 J mice and was capable of dose-dependently attenuating the atherosclerotic lesions in the root and the entire aorta in apolipoprotein E gene knockout mice. These preliminary molecular mechanism-of-action studies revealed SMS2 function in lipid homeostasis and inflammation process, which indicated that the selective inhibition of SMS2 would be a promising treatment for inflammation.



	Cmpd.	Z=	R=	IC_{50} (SMS2, nM)	selectivity (SMS2 vs SMS1)
(2017)	**3**	CH	CF3	6.5	150
(2020)	**37**	N	OMe	45	53

Another research group from Japan also made progress in this field. Adachi and coworkers developed a human SMS2 enzyme assay with a high-throughput mass spectrometry-based screening system and found a hit compound with the 2-quinolone scaffold. Further modification of the hit compound led to a potent and selective SMS2 inhibitor (compound 3, IC_{50}: 6.5 nM) [102]. Recently, the research group developed a new compound **37** as an effective in vivo tool for the study of the SMS2 enzyme. Compound **37** showed promising efficacy in reducing hepatic sphingomyelin levels in a mouse model [103].

12.2 Conclusion

Sphingolipid metabolism pathways play an essential role in maintaining cell homeostasis and are related to a variety of metabolic diseases, such as insulin resistance, metabolic syndrome, etc. The dynamic balance of sphingolipid levels in organisms is regulated by a variety of sphingolipid synthases, hydrolases and metabolic enzymes, such as sphingomyelinase (SMase), sphingomyelin synthase (SMS), serine palmitoyltransferase (SPT), ceramide synthase (CerS), glucosylceramide synthase (GCS), etc. The above-mentioned enzymes are potential targets for drug discoveries in metabolic disorders and receive a lot of research interests.

Medicinal chemists have discovered various natural products and synthetic small molecules targeting sphinogolipid metabolism-related enzymes. From the primary discovery of myriocin as an SPT inhibitor in 1990s, Fumonisin B1 as CerS inhibitor in early twenty-first century, to the very recent advances in SMS inhibitor recognition of Ly93 by Fudan University, hundreds of compounds were reported to be potential drugs to the perturbation of sphinogolipid metabolism and several of them come to preclinical stages. However, there are remaining challenges in drug development towards sphinogolipid metabolism. (1) The affinity of small-molecule inhibitors to target protein need great improvement. Except very few compounds mentioned in the chapter reaches a low IC50 to sub-100 nM level, the main inhibitors

to SMS, SMase and other related enzymes showed a weak affinity hindering their movements to clinical drugs. (2) Inhibitors with better selectivity are needed. Sphinogolipid metabolism is fundamental to the survival of cells and mammals, the lack of selectivity of sphinogolipid metabolism-related inhibitors will cause severe side effects or even death. (3) Distinguishing of clear indications of sphinogolipid metabolism-related drugs are favored. Due to the lack of understanding of sphinogolipid metabolism in the development of diseases, we do not have a lot of knowledge of the indications of sphinogolipid metabolism-related inhibitors. More clinical trials need to be done to clarify their therapeutic effects for human.

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Rare Diseases in Glycosphingolipid Metabolism

13

Hongwen Zhou, Zhoulu Wu, Yiwen Wang, Qinyi Wu, Moran Hu, Shuai Ma, Min Zhou, Yan Sun, Baowen Yu, Jingya Ye, Wanzi Jiang, Zhenzhen Fu, and Yingyun Gong

Abstract

Sphingolipidoses is a cluster of genetic rare disorders regarding glycosphingolipid metabolism, classified as lysosomal storage disorders (LSD). Here, we focus on eight inheritable diseases, including GM1 gangliosidosis, GM2 gangliosidosis, Fabry disease, Gaucher's disease, metachromatic leukodystrophy, Krabbe disease, Niemann–Pick disease A and B, and Farber disease. Mostly, pathogenic mutations in the key enzyme are loss-function, resulting in accumulation of substrates and deficiency of products. Thus, cellular overload of substrates causes lipotoxicity, which is deleterious to cellular and organ function. In the terms of clinical manifestations in sphingolipidoses, multiple systems and organs, especially central nervous system (CNS) are usually affected. As for diagnosis strategy, enzymatic activity assay and genetic sequencing are helpful. Up till now, limited treatment approaches have approved for treating sphingolipidoses, with some potential strategies for further evaluation. In general, enzyme replacement therapy (ERT), substrate reduction therapy (SRT), and molecular chaperones are feasible choices for enzyme deficiency disorders, but these therapies are limited to relieve CNS lesions and symptoms due to prevention from blood–brain barrier. Other possible treatments such as gene therapy, bone marrow transplantation (BMT), and hematopoietic stem cell transplantation (HSCT) need further evaluation.

Keywords

Glycosphingolipid · Sphingolipidoses · Lysosomal storage disorder · GM1 gangliosidosis · GM2 gangliosidosis · Fabry disease · Gaucher's disease · Metachromatic leukodystrophy · Krabbe disease · Niemann–Pick disease A and B · Farber disease

Abbreviations

AAV	Adeno-associated virus
ACDase	Acid ceramidase enzyme
ASA	Arylsulfatase A
AST	Aspartate aminotransferase
BMT	Bone marrow transplantation
CNS	Central nervous system
CSF	Cerebrospinal fluid
DBS	Dried blood spots
ERT	Enzyme replacement therapy
FD	Fabry disease

H. Zhou (✉) · Z. Wu · Y. Wang · Q. Wu · M. Hu · S. Ma · M. Zhou · Y. Sun · B. Yu · J. Ye · W. Jiang · Z. Fu · Y. Gong
Department of Endocrinology and Metabolism, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China
e-mail: drhongwenzhou@njmu.edu.cn

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FDA	Food and Drug Administration
GALC	Galactocerebrosidase
Gb3	Globotriaosylceramide
GCase	Glucocerebrosidase
GD	Gaucher's disease
GM2-AP	GM2 activator protein
Hex	β-hexosaminidase
HSCT	Hematopoietic stem cell transplantation
KD	Krabbe disease
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
LSD	Lysosomal storage disorders
MCP-1	Monocyte chemoattractant protein 1
MLD	Metachromatic leukodystrophy
MRI	Magnetic resonance imaging
mRNA	messenger RNA
MSD	Multiple sulfatase deficiency
NB-DGJ	N-butyldeoxygalactonojirimycin
NPD	Niemann–Pick disease
Sap-B	Saposin B
SD	Sandhoff disease
SMA-PME	Spinal muscular atrophy with progressive myoclonic epilepsy
SRT	Substrate reduction therapy
TSD	Tay–Sachs disease
α-Gal A	α-galactosidase A
β-Gal	β-galactosidase

13.1 GM1 Gangliosidosis

13.1.1 Condition and Genetic Defects

GM1 storage disorder termed GM1 gangliosidosis is a rare neurodegenerative lysosomal storage disease caused by β-galactosidase (β-Gal) deficiency due to mutation in the GLB1 gene [1, 2]. The GLB1 gene (MIM *611458) is located on the short arm of the chromosome 3 (3p22.3), comprising 16 exons. Up to now, about 293 different genetic variants associated with GM1 gangliosidosis have been reported and most of them are categorized as missense mutations [3].

The overall incidence of GM1 gangliosidosis has been estimated to be 1 in 100,000–200,000 live births wordwide [4], with elevated incidence in some certain populations, which may be biased by the founder's effect [4–7]. For instance, increased prevalence was previously reported in Porto Alegre city at south Brazil (1:1700) [5, 6], in Malta (1:3700) [7], which may be closely related with a higher frequency of disease carriers, around 8.3% at the village of Pelendri in Cyprus [8] and 1:30 in Malta [7].

β-Gal is the first enzyme in the catabolism of complex glycosphingolipids, responsible for the degradation of glycoproteins, glycolipids, and keratan sulfate, all of which perform essential roles in cellular signaling and structure [9]. This deficiency results in abnormal deposition of GM1 gangliosides in neuronal organs and glycosaminoglycans or glycopeptides in visceral tissues [10, 11].

13.1.2 Lab Findings

Definitive diagnosis of GM1 gangliosidosis mainly relies on assaying the enzyme activity of β-Gal and genetic testing of GLB1 gene. Residual β-Gal activity in fibroblasts isolated from patients, measured using the artificial 4-methylumbelliferyl β-galactopyranoside substrate, varied in different disease subtypes, namely 0.07% to 1.3% of control values in infantile patients, 0.3% to 4.8% in the juvenile form, and up to 9% in adults [12].

Several objective biomarkers can reflect disease diversity of GM1 patients and would be useful for evaluating disease status and therapeutic response to clinical trials, including GM1 ganglioside concentration, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), neuron-specific enolase, myelin basic protein and brain proton magnetic spectroscopy indexes [13, 14].

Targeted lipidomic is used to measure the storage of GM1 gangliosides in cerebrospinal fluid (CSF) samples. Although valuable, lipidomics analysis requires a liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay only available in specialized laboratories. By contrast, AST and LDH are more commonly applied

to monitor disease severity and treatment responses. It has been demonstrated that AST and LDH levels were obviously increased in the CSF samples collected from late-infantile patients, but not in most juvenile patients. Thus, AST and LDH could be ideal candidates for indicating disease severity [15, 16]. Furthermore, in peripheral blood samples from GM1 gangliosidosis patients, AST levels were correlated with disease severity, but LDH remained unchanged [13]. As a result, AST was emphasized to be a potential biomarker for clinical application [13]. Furthermore, recent studies have shown that patients with the infantile form showed a tenfold higher plasma chitotriosidase activity than those with the late-infantile form, indicating a correlation between chitotriosidase activity and clinical phenotype [17].

Except for the biochemical indicators in CSF and peripheral blood, brain proton magnetic spectroscopy indexes, such as *N*-acetylaspartate and myoinositol, have been proposed to monitor investigational approaches for GM1 gangliosidosis [18, 19]. Electroencephalography, 7T magnetic resonance imaging (MRI), and magnetic resonance spectroscopy were used to evaluate lesions in the brain of GM1 patients. Besides, pathologists have demonstrated that the brain is sometimes slightly infiltrated with inflammatory cells in GM1 gangliosidosis patients, which considered as pathophysiological responses to the lipid overloading in neurons and the abundance of antigenically acting gangliosides [20]. It is necessary to examine the expression of a panel of inflammatory markers in the CNS.

Overall, tracking of disease progression through clinical assessment will remain critically important, sensitive biomarkers are important tools for monitoring disease progression and evaluating the efficacy of treatment strategies.

13.1.3 Clinical Features and Diagnosis

The clinical manifestations of GM1 gangliosides results from the massive storage of GM1 gangliosides and related glycoconjugates in various tissues, especially in the CNS [21]. GM1 gangliosides in the gray matter of GM1 gangliosides patients could accumulate as much as threefolds more than that of healthy individuals [22]. Besides, GM1 gangliosides also accumulates in the viscera, producing complications such as hepatomegaly, cardiomegaly, skeletal deformities, joint stiffness, and muscle weakness.

Cognitive decline, muscular hypotonia, and seizures were the most frequent neurologic symptoms. Based on the age of onset and severity of symptoms, GM1 gangliosidosis has been classified into three major phenotypes: type I (infantile, MIM #230500), type II (late infantile/juvenile, MIM #230600), and type III (adult, MIM #230650). (1) The type I infantile form is the most severe, with life expectancy less than 3 years [21, 23], characterized by early onset between birth and 6 months with psychomotor regression, cherry-red spot, visceromegaly, and facial and skeletal abnormalities. (2) The type II form usually starts between 7 months and 3 years of age with slowly progressive neurological signs, such as early locomotor problems, seizures, strabismus, lethargy, muscle weakness, and terminal bronchopneumonia. Comparing to type I, dysmorphisms and skeletal changes are less severe [24]. (3) The adult form, also known as type III, the mildest phenotype of the disease, with onset between 3 and 30 years of age, is featured by cerebellar dysfunction, slurred speech, dystonia, mild vertebral deformities, and short stature [21]. Overall, the diagnosis of GM1 gangliosidosis is often delayed, particularly in the patients with milder forms or unclassical characteristics.

The residual activity of the mutant β-Gal directly linked with disease severity, as the enzymatic activity nearly absent in type I-associated mutations, but a small amount of residual activity in type II and type III patients [21, 25].

In rare cases, patients present with peripheral manifestations of β-Gal deficiency but without CNS involvement, for instance, one type of such disease termed as Morquio B disease, also called mucopolysaccharidosis IVB. The deficiency of β-Gal enzymatic activity, in combination with neuraminidase deficiency, is also present in a

different disorder named galactosialidosis [26], due to a defect in the protective protein/cathepsin A [27], which stabilizes the β-galactosidase/neuraminidase complex [28]. Meanwhile, it should be pointed out that the predominant clinical symptoms of galactosialidosis are mainly resulted from severe neuraminidase deficiency, instead of the partial β-Gal enzyme deficiency [21].

Diagnosis of all subtypes of GM1 gangliosidosis is often achieved by β-Gal enzyme activity assay [1] and genetic testing through whole-genome sequencing or -exome sequencing of the GLB1 gene [29]. Furthermore, prenatal diagnosis is possible via amniocentesis especially for those candidates with GM1 gangliosidosis family history, or for those from both parents are carriers of a pathogenic allele [30].

At the same time, the detection of biochemical markers and neuroimaging changes are playing important roles during diagnosis and treatment.

13.1.4 Treatment and Advances

At present, there is no current effective FDA-approved treatment for GM1 gangliosidosis yet, only symptomatic and supportive therapy is available for these patients. Several therapeutic approaches have been explored by reducing substrate accumulation and promoting GM1 ganglioside catabolism. Targeted restoration of GM1 ganglioside catabolism occurs through gene therapy [31], stem cell transplantation [32], enzyme replacement therapy [33], and pharmacological chaperones [34] to increase the β-Gal activity, reduce neuronal lysosomal storage lesions, prevent the onset of neurological signs gait abnormalities, and extend the lifespan.

To date, the gene therapy based on adeno-associated virus (AAV) serotype 9 has been developed for GM1 gangliosidosis [35]. Related studies exhibited extraordinary efficacy in animal models, which demonstrated that AAV-mediated gene transferred to the brain can restore β-Gal expression in CNS and prevent the onset of neurological signs. Moreover, a phase I/II clinical trials is ongoing to evaluate the safety and efficacy of AAV-mediated GLB1 delivery by intravenous injection [30].

SRT is another alternative possible approach, which relies on residual β-Gal activity to remove ganglioside that has already accumulated. Therefore, it would not be effective enough for those symptomatic infantile GM1 patients with scarce or no residual β-Gal enzyme activity.

Hopefully, it has recently been reported that *N*-butyldeoxygalactonojirimycin (NB-DGJ), which functions as pharmacological chaperone that binds directly to misfold protein, improving its folding to increase its stability and promote its proper trafficking, showed favorable outcomes in infantile GM1 patients. NB-DGJ enhanced the activity of β-Gal up to ~4.5% of control activity within the first 24 h, and further significantly increased to ~10% within 6 days [36].

Besides, previous research has demonstrated that microglia/Mϕ activation plays a central role in the pathological process of GM1 gangliosides [20]. In this case, anti-inflammatory drugs might be an effective choice for the GM1 gangliosides, along with other proven therapies such as SRT [37] and bone marrow transplantation [38].

In the perspective of future available therapies, we are expecting more effective therapeutic methods that can be applied to clinical treatment for GM1 ganglioside patients.

13.2 GM2 Gangliosidosis

13.2.1 Condition

GM2 gangliosidosis is rare autosomal recessive LSD resulted from the deficiency of β-hexosaminidase (Hex) or GM2 activator protein (GM2-AP) activity, leading to the accumulation of GM2 ganglioside in the lysosome in neurons [39, 40]. GM2 gangliosides can be catabolized by the lysosomal hydrolases Hex through the hydrolysis of the N-acetylgalactosamine residues in normal conditions [41]. Hex are formed by the dimerization of α (*HEXA, MIM *606869*) and β (*HEXB, MIM *6068732*) subunits. Additionally, GM2-AP plays an important role in GM2

gangliosides degradation by presenting the gangliosides to α subunit of Hex [42].

GM2 gangliosidosis is caused by inherited enzyme deficiency, so that the accumulation of GM2 gangliosides deposits in the brain and surrounding organs, resulting in a series of clinical manifestations.

13.2.2 Genetic Defects

The GM2 gangliosidosis can be divided into Tay–Sachs disease (TSD, MIM #272800), Sandhoff disease (SD, MIM #268800), or GM2-AP deficiency (AB variant, MIM #27750), caused by the mutations in the genes of *HEXA, GM2A, and double mutations of HEXA and HEXB* (MIM *613109) [41]. Very few GM2A mutations have been reported in GM2 gangliosidosis [40].

13.2.3 Clinical Features

GM2 gangliosidosis are autosomal recessive, progressive neurodegenerative disorders.

TSD can be divided into three subtypes: classic infantile, juvenile, and adult late onset according to when symptoms appear. The incidence is approximately 1 in 320,000 newborns. Classic infantile is the most common and severe phenotype, losing the ability to turn over, sit, or crawl in 6 months old [43]. Seizures, axial hypotonia, cherry-red spot, regression in developmental milestones, and exaggerated startle response are the clinical features of TSD. Neuroimaging may implicate bilateral thalamic involvement, brain atrophy, and hypomyelination [40].

SD can also be divided into three subtypes and is clinically almost indistinguishable from TSD in neurodegenerative disorders, with infantile cases presenting as the most severe and rapidly progressing condition [22]. Ataxia, myoclonus, motor regression, psychotic episodes, intellectual disability, and progressive clumsiness are the clinical features of SD. Cerebellar atrophy can be discovered by neuroimaging [40].

GM2-AP deficiency is featured with dysphagia, muscle atrophy, cerebellar ataxia, dysarthric speech, manic depression, muscle weakness, psychotic episodes, and severe cerebellar atrophy, which are similar to those of TSD and SD [22].

The classical infantile form, with severely defective Hex enzyme activity, develops in infancy with developmental retardation, paralysis, dementia, blindness, and death usually by 2 or 3 years of age [40, 44]. Juvenile and adult types of late onset have a late onset with milder symptoms [45].

13.2.4 Diagnosis

In addition to the clinical characteristics, diagnosis is also supported by neuroimaging characterized by hyperdensity of basal ganglia, which can be accompanied by other changes in white matter and sometimes prominent, but non-specific, cerebellar atrophy [44].

Detection of Hex enzyme activity in peripheral blood can confirm the diagnosis [41]. At least, genetic testing of the *HEXA, HEXB,* and *GM2A* genes allows confirmation of all GM2 gangliosidosis subtypes [40].

13.2.5 Treatment

There is no proven treatment for TSD/SD and only supportive care is suggested for patients [22]. Several approaches have been explored for new treatments for GM2 gangliosidosis, including ERT, HSCT and BMT, SRT, pharmacological chaperone therapy, gene therapy, and gene editing approaches with AAV or CRISPR system [46].

ERT is a therapeutic alternative conceived in 1964 in which the lysosomal enzymes can be uptake through endocytosis and delivered to the lysosomes [47]. However, IDEAMLD phase I/II clinical trial (NCT01510028) showed that ERT may not be a definitive treatment for GM2 gangliosidosis with limited enzyme spread to the neuraxis [41]. Maegawa et al. found that pyrimethamine was the most prospective

pharmacological chaperone for HexA because it can induce a threefold increase in enzyme activity on TSD fibroblasts [48, 49]. However, clinical trials with pyrimethamine showed an increase in enzyme activity but with little effect on the CNS manifestations [41, 50, 51]. What's more, the intraperitoneal administration of EtDO-PIP2 can reduce the content of brain and liver gangliosides in SD mice, suggesting a potential treatment for ganglioside storage diseases with CNS manifestations [52]. HSCT can offer a potential alternative approach by delivering WT HexA and HexB with a patient's own blood cells [46]. Additionally, gene therapy with AAV or CRISPR has been proved a 56% extension of the lifespan, a significant improvement motor function, and a slight therapeutic effect on the CNS in SD mice [53, 54]. It is possible that the co-administration of different therapies for GM2 gangliosidosis patients may be required.

13.3 Fabry Disease

13.3.1 Condition and Genetic Defects

Fabry disease (FD, MIM #301500) is a rare X-linked lysosomal storage disease caused by mutations in the GLA gene (Xq22; MIM *300644) resulting from deficient or absent activity of the lysosomal enzyme α-galactosidase A (α-Gal A) and glycolipid accumulation in many tissues [55], mainly in the form of globotriaosylceramide (Gb3). In 1898, Johann Fabry [56] and William Anderson [57] reported the skin lesion of Fabry disease, termed as angiokeratoma corporis diffusum. Nowadays, more than 1000 mutations in patients with Fabry disease have been identified [58]. According to individual genotype, the range of α-Gal A mutation phenotypes can vary widely [59]. In fact, the impacts of most reported α-Gal A mutations remain unclear [60]. Furthermore, an accurate estimation of the prevalence is complicated by the great phenotypic heterogeneity. The estimated incidence of FD is 1 in 117,000 live male births [61]. Because the atypic variant of the disease is underdiagnosed, the incidence might be underestimated in previous newborn screening surveys, and it may be much higher. The actual incidence of the disease may be approximately 1 in 8800 individuals in European [62–64]. In Asia, even higher frequencies have been noted, approximately 1 in 1600 males [65].

13.3.2 Clinical Features

The early-onset Type 1 "Classic" and Type 2 "Later-Onset" phenotypes are two major subtypes. Type 1 "Classic" is a classic variant subtype with systemic symptoms that begin in childhood and Type 2 "Later-Onset" is a late-onset variant subtype that develop in adulthood [66]. As FD is an X-linked disease, clinical manifestations are generally more severe and its progress is more rapid in males than in females. Some representative manifestations include periodic crisis of severe pain in the distal extremities, chronic neuropathic pain, sweating abnormalities (anhidrosis and hypohidrosis), angiokeratomas distributed in groin, hip and periumbilical areas, corneal (cornea verticillate) and lenticular opacities [67]. While in females, these symptoms present 5–10 years later and some even may be asymptomatic [68]. Besides multiple organs can be suffered, renal failure, left ventricular hypertrophy, gastrointestinal symptoms, strokes, and myocardial fibrosis often appear after 30 years old. In some cases, left ventricular hypertrophy might be the only manifestation [69].

13.3.3 Diagnosis

Within a detailed family history and physical examination, α-Gal A activity should be measured. α-Gal A activity $<1\%$ is highly suggestive for the diagnosis of classic FD [70]. Biopsy of the affected organ and "zebra bodies" are the distinctive characteristics [71, 72]. Elevated lyso-Gb3 in plasma and in urine is helpful for both initial diagnosis and long-term monitoring. Obviously, genetic testing of GLA is the golden criteria. Recently, some new methods have been explored to further elevate the

accuracy, such as sensitive LC-MS method [73], dried blood spots (DBS), peripheral blood mononuclear cells measurement [74], and the proteomics approach [75]. Besides, calnexin, g-enolase, galectin-1 and relative miRNAs are new markers that have shown promising potential.

The chronic pain of FD should be discerned from fibromyalgia or rheumatologic pain diseases [76]. Systematic abnormities such as unexplained left ventricular hypertrophy, stroke, and renal failure should be screened and other diseases that also have the similar symptoms should be excluded [77].

13.3.4 Treatment

Current two treatment methods are intravenous administered ERT with agalsidase-α or agalsidase-β, and oral pharmacological chaperone therapy (Migalastat) or enzyme stabilizers along with other symptomatic remedies. Bone marrow transplantations may preferably be performed on young FD patients. Despite the above methods have achieved definite clinical effects, drawbacks like infusion-associated reactions, limited tissue penetration, and the formation of neutralizing antidrug antibodies cannot be negligible [78, 79]. Fortunately, there have been relative remedies to mitigate these effects [80, 81]. Nowadays, ERT, SRT, messenger RNA (mRNA), and gene therapy are novel methods [82]. Pegunigalsidase-alfa (PRX-102, Protalix Biotherapeutics) and Moss-αGal (Greenovation biopharmaceuticals) are two new forms of ERT [83]. Novel developed SRT molecules, like venglustat/ibiglustat [84] and lucerastat [85], aim to reduce production of Gb3 by inhibiting glucosylceramide synthase [86]. The mRNA therapy is a new class of therapy, and the administration of α-Gal mRNA can help stimulate the production of α-Gal [82]. For gene therapy, several different vectors (retroviral, lentiviral, adenoviral, adeno-associated viral and non-viral vectors) have been explored and may be an effective treatment option [87]. Additionally, Judit [88] explored α-Gal-loaded nanovesicles to treat FD and showed improved efficacy and good safety profile.

13.4 Gaucher's Disease

13.4.1 Condition

Gaucher's disease (GD) is a rare autosomal recessive disease characterized by a variety of clinical phenotypes, and yet it is the most common type in LSD. In 1882, Philippe Gaucher observed unusual cells in a patient's spleen with splenomegaly and reported the disease for the first time. The prevalence of GD is about 1/40,000 to 1/50,000 worldwide, while it rises to approximately 1/600 in Jews of Ashkenazi population [89].

13.4.2 Genetic Defects

The GBA1 gene (MIM *606463) is located on chromosome 1 (1q22) and encodes glucocerebrosidase (GCase), one of the lysosomal enzymes. Homozygous or compound heterozygous pathogenic mutations in GBA1 gene damage the function of glucocerebrosidase in hydrolyzing its substrate, glucosylceramide, into β-glucose and ceremide, thus leading to the abnormal accumulations of glucosylceramide in macrophages [90]. More than 400 mutations in GBA1 gene have been discovered until recently, of which N370S, L444P, 84GG, and IVS2(+1) are most prevalent [91, 92]. The dominant mutations in Jews of Ashkenazi population are N370S, 84GG, IVS2(+1), and R496H [93, 94].

13.4.3 Lab Findings

Detection of GBA1 gene mutations based on genetic sequencing is necessary to establish the diagnosis of GD, which becomes a common diagnostic method due to the development in molecular science [95].

The measurement of GCase activity in patients is a requisite for diagnosing GD. Usually, the

residual activity of GCase in GD patients is approximately between 10% and 15% compared to the normal standard [96].

The accumulation of glucosylceramide transforms macrophages into the typical "Gaucher cells" characterized with a "crumpled tissue paper" appearance. Those pathological cells deposit in several organs (such as liver, bone marrow, and spleen) and are the cause of most symptoms in GD. The presence of Gaucher cells in bone marrow aspiration helps to confirm the diagnosis of GD, but it's not regarded as a routine examination [92].

About 90% of GD patients have thrombocytopenia with a different extent, meanwhile anemia and leukopenia are comparatively rare [97].

Chitotriosidase, CCL18, glucosylsphingosine, and ferritin are biomarkers of GD. Gaucher cells can produce a considerable amount of chitotriosidase and CCL18, resulting in high levels of these types of substances in plasma [98, 99]. Glucosylsphingosine, a newly found biomarker, is more sensitive than chitotriosidase and CCL18 regarding condition monitoring and is elevated in GD patient's plasma as well [100]. The concentration of ferritin often elevates in GD, and excess iron in plasma is likely to accumulate in the liver and bone marrow, which may indicate the appearance of bone complications [101].

In imaging examinations, magnetic resonance imaging is recommended to evaluate the lesion in the liver, the spleen, and bone marrow in GD, because it is not radioactive and is suitable for monitoring the disease in repeated examinations [102].

13.4.4 Clinical Features and Diagnosis

According to the presence of neurological manifestations, GD is divided into 3 types, including non-neuronopathic GD (GD1, MIM #230800), acute neuronopathic GD (GD2, MIM #230900), and chronic neuronopathic GD (GD3, MIM #231000). The common phenotypes include splenomegaly, hepatomegaly, abnormal hemogram and, in some cases, impairments in bones. In neuronopathic GD, clinical features related to the central nervous system are important to identify patients in the early onset of disease. However, the correlation of phenotype and genotype remains uncertain as GD patients' clinical manifestations are of high heterogeneity [103].

GD1, the most common type of GD, accounts for 90% to 95% of GD patients in Europe and North America [92]. Since the appearance and extent of clinical symptoms vary in different individuals, GD1 can be diagnosed at any age with a median age from 10 to 20 years old [104]. Most GD1 patients have enlarged or even massive spleen and sometimes have enlarged liver as well. Hematological disorders can be observed in part of patients in the forms of mucocutaneous bleeding and postoperative hemorrhage resulting from thrombocytopenia mainly [105]. Bone manifestation, like acute or chronic bone pain, is another typical symptom in GD1 patients, especially in children, and has a relationship with bone deformities, lesions and mass decline [106, 107]. With the progression of the disease, the respiratory system can be affected by the infiltration of Gaucher cells in lung presented with the interstitial disease [108]. Furthermore, about 35% of children diagnosed with GD1 had growth retardation and delayed puberty in a past cohort study [109].

GD2 is often characterized by early and severe clinical symptoms within the first year of life. Although patients also exhibit splenomegaly, thrombocytopenia, and growth retardation, the diagnosis of GD2 relies on distinguishing the feature of neurological impairment from non-neurological GD. Typical signs include rigidity of neck and trunk, difficulty in swallowing, laryngeal stridor, trismus, and oculomotor paralysis [110]. Central apnea accounts for about 50% of death in GD2, as Gaucher cells infiltrate lung leading to dysfunction of the respiratory system [111]. Of note, perinatal lethal form of GD2, also known as fetal GD, is the rarest, while at the same time, the most severe type exhibits hydrops fetalis, ichthyosis, facial dysmorphia, and central nervous system degeneration, resulting in fetal death in utero or soon after

birth [111, 112]. This emphasizes the importance of carrying out newborn screening, next-generation sequencing, and carrier testing among high-risk populations to achieve early diagnosis of GD and minimize negative consequences.

Apart from observing visceral involvements which is similar to GD1, the identification of oculomotor nerve impairment, a slowed or absent horizontal saccadic eye movement, is necessary in diagnosing GD3 patients [113]. This can be the only neurological sign in some cases. GD3 patients may develop other severe neurological manifestations such as myoclonic seizures, cerebellar ataxia or spasticity and dementia [114, 115]. GD3a, GD3b, and GD3c are classical subtypes of GD3. Progressive myoclonic epilepsy constitutes the clinical feature in GD3a patients, and the symptom is less severe in type 3b comparatively [95, 114]. GD3c, the cardiac phenotype of GD3, is described with cardiac manifestations, of which calcification of the aortic and mitral valves and calcification of the aorta are the most typical characteristics [116].

Taken together, diagnosis of GD is mainly based on GBA1 gene sequencing mutations and the measurement of remaining GCase activity as mentioned above. The identification of Gaucher cells in biopsy of bone marrow and enlarged spleen can help confirm the diagnosis when GD patient has no obvious symptom, shows unexplained worsening blood count or displays no response to enzyme treatment [117].

13.4.5 Treatment

Two well-established treatments of GD are ERT and SRT.

ERT applies recombinant GCase via intravenous injection to compensate for the lack of GCase in cells. ERT for GD first appeared in the 1990s [118], and three types of ERT drugs, including imiglucerase, velaglucerase, and taliglucerase, have been applied in clinical use under marketing authorizations. These drugs can alleviate visceral and hematological involvements effectively. However, none of them improves neurological symptoms without the ability to cross the blood–brain barrier [95].

In SRT, the partial inhibitor restrains the function of the enzyme that synthesizes glucosylceramide to reduce the substrate of GCase. Miglustat and Eliglustat are two oral SRT drugs that have been approved officially to treat GD. Miglustat can be a replacement drug therapy when patients are intolerant of ERT [117]. Even though Miglustat can cross the blood–brain barrier, it exerts a subtle influence on neurological manifestations [119]. Given that Eliglustat, a ceramide analog, is a more potent drug and has fewer side effects than Miglustat and ERT drugs, it is now recommended as first-line therapy in the treatment of adult GD1 patients [120]. Patients are required to have CYP2D6 genotyping before using Miglustat, which is decomposed by cytochrome P450, to prevent certain drug–drug interactions [92]. Still, Eliglustat is unable to get into the central nervous system to ease neurological symptoms [121].

Splenectomy, orthopedic surgery, liver transplantation, and other symptomatic treatments are not regarded as routine therapies after the appliance of ERT and SRT, unless severe visceral complications exist, such as splenic rupture and pathological bone fractures.

As new emerging therapies, both gene therapies and the usage of molecular chaperones need further investigation and clinical trials [122, 123].

13.5 Metachromatic Leukodystrophy

13.5.1 Condition

Metachromatic leukodystrophy (MLD, MIM #250100) is an extremely rare autosomal recessive lysosomal storage disease and the estimated birth prevalence rate is 1.4–1.8 per 100,000 [124]. Most patients were of Caucasian ethnicity, followed by Asian [125]. Based on the age of onset, MLD is divided into three subtypes, namely late-infantile form (before 30 months), juvenile form (between 2.5–16 years), and adult

form (after 16 years) [126]. Late-infantile patients account for the largest proportion of all clinical types of MLD patients [125].

13.5.2 Genetic Defects

MLD is an auto-recessive hereditary disorder characterized by arylsulfatase A (ASA) deficiency or, more rarely, defective sphingolipid activator protein B, saposin B (Sap-B) [125]. ASA plays an important role in the degradation of sulfatides which are mainly found in the myelin membranes and Sap-B assists this process by solubilizing the hydrophobic lipid [127]. Patients with MLD often show increased levels of 3-O-sulfogalactosylceramide (sulfatide) and 1-(3-O-sulfo-beta-D-galactosyl) sphingosine (lyso-sulfatide) [128, 129]. Sulfatides accumulate in several tissues, such as kidney, gallbladder, or bile ducts [130], but the progressive accumulation especially affects oligodendrocytes and Schwann cells, and leads to demyelination and axon loss, causing neurological symptoms [128, 130].

ASA is encoded by ARSA gene (22q13.33, MIM *607574), with 8 exons [125, 131]. Now more than 250 mutations of the ARSA gene have been reported [132], while the most common disease-causing variants are splice donor site variant c.465 + 1G > A, missense variants c.1283C > T (p.Pro428Leu) and c.542 T > G (p.Ile181Ser) [125, 133]. The splice donor site variant c.465 + 1G > A is regularly identified in the most severe late-infantile form [125, 133, 134]. However, missense mutations are the most common mutations observed in the late-infantile form, which leads to misfolding of ASA in the endoplasmic reticulum and subsequent proteasomal degradation, thus no functional enzyme can reach lysosome, subsequently causing sulfatides accumulation [134]. The other two missense variants mentioned above are usually detected in adult or juvenile patients [125, 133].

Sap-B is encoded by PSAP gene (MIM #176801), which is mapped to chromosome 10q22.1 and with 15 exons [125]. ASA activity is usually normal in patients with MLD due to Sap-B deficiency.

13.5.3 Clinical Features and Diagnosis

The clinical features of MLD are heterogeneous according to the subtypes. In patients with late-infantile form, impaired gross motor function is usually the first manifestation, such as abnormal movement patterns, walking difficulties, affected gait, and motor regression [134–136]. Motor function completely lost in late-infantile patients occurs no later than 40 months [135]. In late-infantile form, peripheral neuropathy symptoms usually appear before the central neuropathy symptoms and the symptoms usually manifest as clumsiness, muscle weakness, sensory deficits, and areflexia [126]. As the disease progresses, patients can develop other symptoms, such as mental regression, spastic tetraparesis, visual and auditory impairment, bulbar palsy, and seizures [126].

In juvenile patients, MLD is usually characterized by cognitive impairment and behavioral changes, which usually manifests as academic performance deterioration, attention difficulties, and disruptive behavior, followed by motor regression, such as slowness of movements, gait disturbances and balance impairment, and epilepsy [136, 137].

Adult patients often have symptoms of mental deterioration and behavioral abnormalities firstly [138]. Adult patients can also have neurological symptoms including pyramidal and cerebellar symptoms, usually accompanied by modification of speech [138]. The mean survival of adult patients is longer than the two other types [139]. In addition to nervous system, gallbladder is usually involved, such as polyp, gallstone, and increased thickness, and MLD patients is prone to neoplastic gallbladder abnormalities [140].

Whole-exome sequencing or whole-genome sequencing can be used to detect mutations to help genetic diagnosis [141–144]. To measure ARSA enzyme activity, samples from leukocyte lysate, or rarely, fibroblast lysate, can be incubated with synthetic chromogenic substrate *p*-nitrocatecholsulfate [145], and the assay can be conducted at 0 °C to reduce the interference of arylsulfatase B (ARSB) which can also hydrolyze the substrate [146]. To further improve

accuracy, $AgNO_3$ can be added to inhibit ARSB [146]. This assay can be read out photometrically [145]. A novel detection method using deuterated natural sulfatide substrate to measure ARSA enzyme activity in leukocytes or DBfS by LC-MS/MS is developed, which has high specificity and sensitivity [147]. Measuring blood sulfatides or sulfatides excretion in urine by LC-MS/MS is helpful for diagnosis in MLD [148, 149].

MRI is an important tool in the diagnosis of MLD. On proton density and T2-weighted images, diffuse and symmetric hyperintensities within the white matter can be seen, which is consistent with demyelination [150]. The corpus callosum is usually the initial area to be involved and abnormalities then spread to the periventricular white matter, while the subcortical fibers are spared at the early stage [137]. Within the abnormal white matter, there are hypointense signal abnormalities which can be divided into two appearances on T2I: tigroid stripes extending radially [134, 150] and dots like leopard-skin appearance [150, 151]. Proton magnetic resonance spectroscopy can evaluate motor and cognitive function by measuring the neuronal metabolite N-acetylaspartate levels which is a biomarker for neuronal and axonal loss [152]. Nerve conduction studies can detect decline of the peripheral nerve conduction velocity which is an early symptom to help diagnosis [138, 153].

Adult patients with MLD might be misdiagnosed with Alzheimer disease for early-onset progressive dementia [154]. Besides, the similar psychiatric symptoms of the adult MLD might lead to wrong diagnosis, such as manic depression, schizophrenia, organic disorders, or alcoholism [155].

Some ARSA gene alterations known as polymorphisms lead to an 10%–15% of normal ASA enzymatic activity, which is enough to prevent symptoms of disease, and these alleles are called pseudodeficiency alleles [156]. Sulfatide excretion in a 24-h urine sample helps distinguish ASA pseudodeficiency from MLD, in which MLD patients exhibit a large amount of sulfatide excretion [134].

Multiple sulfatase deficiency (MSD) is a rare disorder characterized by the deficiency of multiple sulfatases and combines the clinical features of diseases caused by deficiencies of individual sulfatases [157]. Some MSD patients with similar neuropathy symptoms, high protein in cerebrospinal fluid, and high sulfatide excretion in urine might be misdiagnosed as MLD, which can be distinguished by detecting other or total sulfatases, and specific manifestations including facial dysmorphia, skeletal deformities, and ichthyosis [134].

13.5.4 Treatment and Advances

HSCT can improve ASA enzyme activity and limit disease progression by halting further demyelination and immunomodulation [158–160]. Because of slow replacement of ASA deficient resident tissue, HSCT is only suitable for asymptomatic juvenile and adult patients, or patients with mild nervous system involvement, while the beneficial effects may be temporary and restricted to CNS symptoms [126, 137, 161, 162].

Gene therapy of MLD can be divided into bone marrow stem cell gene therapy and in vivo gene therapy [163]. Genetically modified autologous HSC by retrovirus and lentivirus transplantation can provide more functional enzyme for effected tissues and reduce the risk of graft-versus host disease, while it is only suitable for those who are identified at pre-symptomatic stage, which limits the application of this strategy [156, 164, 165]. In vivo gene therapy has developed three vectors: optimized adeno virus, adeno-associated virus, and lentivirus vectors [163]. AAV-based gene therapy is believed to be a promising way to transmit the gene message for ARSA gene directly to the CNS for sustained metabolic correction within the body [164, 166].

ERT has showed success in the treatment of many other LSDs, while the effectiveness of ERT in treating MLD is challenged by blood–brain barrier which is considered a powerful barrier preventing high molecular weight substances from reaching brain tissue [156, 167]. To solve

this problem, intracerebroventricular and intrathecal ERT agent delivery are developed [156, 167].

13.6 Krabbe Disease

13.6.1 Condition

Krabbe Disease (KD, MIM #245200) was first described in 1916 by the Danish neurologist, Knud Krabbe [168]. It is a rare autosomal recessive lysosomal disorder due to deficiency of β-galactocerebrosidase, affecting primarily cerebral white matter and peripheral nerves [169]. The incidence of KD is about 1:100,000–1:250,000, and more than 90% of cases are proved as severe forms which often start during the infant time. Meanwhile, the adult-onset form of KD probably leads to the disability [170]. KD is a long-term disease that can be life-threatening especially in the early-onset form which can lead to death in early infancy [171].

13.6.2 Genetic Defects

KD is an autosomal recessive neurodegenerative disorder typically caused by a deficiency of the lysosomal enzyme galactocerebrosidase (GALC), encoded by gene GALC (14q31, MIM *606890). Up to date, more than 140 disease-causing mutations and polymorphisms have been reported, including missense, deletion nonsense, insertion, and deletion insertion variants. The effects of mutations are complicated due to the concomitant presence of polymorphisms [172]. Besides, a child at 3.5-month-old was reported as atypical KD with mutation in the prosaposin gene (PSAP, 10q22.1, MIM *176801), leading to saposin A deficiency, which functions as a kind of sphingolipid activator protein [173]. Another pathogenic mutation in PSAP was reported in a 7-month-old girl strongly suggested KD, but without enzymatic evidence nor genetic findings in GALC [174].

13.6.3 Clinical Features

Galactosylceramide, direct substrate of GALC, is the major lipid species in myelin, kidney, and epithelial cells of the small intestine and colon. Owing to this biology property, GALC loss-of-function mutations mainly lead to defects in nervous system, urinary system, and digestive system. The classifications for KD can be various. The classic one divides KD into five forms which is based on the age at onset of neurological symptoms: early-infantile (0 to 6 months), late-infantile (7 to 12 months), later onset (13 months to 10 years), adolescent (11 to 20 years) and adult (from 21 years on) [169]. The first two forms account for most of cases while the adult form is very rare [175]. In the terms of central neuropathy, Krabbe's leukodystrophy is the outcome of demyelination in the brain, also known as globoid cell leukodystrophy.

The early-onset infantile KD is a severe rapidly progressing, demyelinating disease, and has typical clinical features including hyperirritability, feeding difficulties, stiffness, elevated temperature, psychomotor regression, convulsive seizures, and vision loss. The other form of KD is late onset, the symptoms of which can be very various. Blindness, muscle weakness, and arrest of intellectual development are always evident. Other symptoms include behavioral problems, ataxia, and spastic paraparesis [176, 177].

13.6.4 Diagnosis

GALC activity measurement, genetic analysis, and psychosine measurement are the main diagnostic criteria which are performed only when the symptoms occur. Some early clinical evidence should be discovered to distinguish the KD in an early time [178].

Magnetic resonance imaging is one of the important diagnostic features and biomarkers for the description of the CNS pathology of KD [169]. The features differ according to the age of disease onset. The infantile onset form often

presented with white matter hyperintensities which involve the dentate nuclei and cerebellar white matter. For the juvenile form, the parieto-occipital white matter is affected while the cerebellum is always normal. But the data for adult-onset patients is quite limited [179].

Shoko Komatsuzaki's study showed the earlier onset of disease was strongly associated with a higher cerebrospinal fluid protein concentration which means it may be considered as a diagnostic biomarker in the appropriate clinical background of KD. However, since other diseases such as CNS infections and demyelinating diseases also have the similar laboratory findings, cerebrospinal fluid protein concentration can be very limited to specifically distinguish KD from other differential conditions [177].

The first-tier measurement of GALC enzymatic activity in DBS of the newborn alone can be unreliable due to common pseudodeficiency GALC gene variants. The psychosine has emerged as a valuable biomarker for diagnosis and prognosis of KD [180]. As for atypical KD with PSAP mutations, genetic sequencing supplemented with functional validation may be the proper practice at current status.

13.6.5 Treatment and Advances

KD is a long-term debilitating disease that can be life-threatening. Treatment options for KD include HSCT, gene therapy, bone marrow cell transplant, substrate reduction therapy, ERT, and the use of small molecules "chemical chaperones," but some of which haven't been conducted on human [181].

Currently, there is no FDA or EMA approved treatment for KD. Two investigational compounds, recombinant human GALC and ibudilast were granted orphan drug designation by the FDA. The former one produced by a method known as "recombinant DNA technology," was also assigned orphan drug designation by the EMA in 2011 [171].

Some experimental therapeutic approaches are also focused on hematopoietic stem cells which can replace the missing enzyme. However, it is risky, expensive, and of uncertain efficacy [175].

Recent information reported by a group of public health and medical professionals showed that the diagnosis and prompt initiation of HSCT conducted before 30 days of life can improve the future symptoms considerably. Several states of America have begun considering about the necessity of KD newborn screening [182]. Inversely, some people question the efficacy of newborn screening for KD due to the false positives of the tests, and the efficacy of early HSCT [183].

However, prenatal diagnosis should be performed with measurement of GALC enzyme activity or molecular genetic testing if the family member was diagnosed of KD. Carriers testing of the family members is useful to prevent the risk for future pregnancies with potential disease [172].

13.7 Niemann–Pick Disease A and B

13.7.1 Condition

Niemann–Pick disease (NPD) is a genetic lipid storage disease, inherited in autosomal recessive mode, and characterized by developing hepatosplenomegaly, neuropathic symptoms, and recurrent lung infections close to birth. The first NPD patient reported by German pediatrician Albert Niemann in 1914 was an Ashkenazi Jewish infant. In 1961, Allen C. Crocker described four subtypes of NPD, type A to type D. Later, two more types, type E and F were delineated. Nevertheless, NPD-A (MIM #257200) and NPD-B (MIM #607616) are two classic forms with the majority, with frequency suggested to be 1 in 250,000 individuals [184]. From a worldwide perspective, approximately 1200 cases have been diagnosed as NPD-A or NPD-B. NPD-A is also known as the classic infantile form or the intermediate protracted neurovisceral form, while NPD-B is recognized as the visceral form [185], mainly based on clinical manifestations. The signs and symptoms of NPD-B are similar with NPD-A, but not as severe as NPD-A, thus NPD-B patients usually survive into adulthood.

13.7.2 Genetic Defects

NPD-A and NPD-B are caused by homozygous or compound heterozygous mutation of SMPD1 gene (MIM *607608), which is located on human chromosome 11 (11p15.4), encoding a lysosomal enzymatic protein termed acid sphingomyelinase (ASM). This 631-amino acid protein mainly functions in the hydrolysis of sphingomyelin to ceramide and phosphorylcholine [186, 187]. Respectively, ASM activity is completely absent in NPD-A, but is residual remaining in NPD-B. Pathogenic mutations in SMPD1 result in deficiency of acid sphingomyelinase, which leads to reduced degradation of sphingomyelin. Over time, the buildup of sphingomyelin causes lipotoxicity, further arouses cellular dysfunction or even apoptosis. Cell death further non-selectively impairs fundamental function of organs including the brain, lungs, the spleen, and the liver.

13.7.3 Clinical Features and Diagnosis

Symptoms of NPD are closely related with the tissues and organs where sphingomyelin abnormally accumulates, including peripheral symptoms (hepatosplenomegaly) and central symptoms (dementia, dysarthria, dysphagia, and ataxia). NPD-A is distinguished from NPD-B with impaired neurological function [188]. Most NPD-A patients show severe neuropathic symptoms and shorter lifespan compared to NPD-B subjects [185]. Moreover, typical cherry-red spots of the retina macula are common in NPD-A affected infants, and often combined with fatal interstitial lung diseases [189]. An early-stage diagnosis of NPD is established in observing the deficiency of acid sphingomyelinase activity in white blood cells or cultured skin fibroblasts. Prenatal diagnosis is anticipated via measuring sphingomyelinase enzymatic activity, or the neonatal screening panel based on the gene sequencing technique on uncultured or cultured chorionic villus sampling, or cultured amniocytes [190].

13.7.4 Treatment and Advances

Liver transplantation might be efficient for NPD individuals with severe liver and pulmonary dysfunction [191]. On the one hand, ERT, especially in NPD-B patients, has been proven worked in previous clinical trials [192]. On the other hand, no curable treatment is available for NPD-A so far. Meanwhile, recent study [193] has revealed that cerebellomedullary cistern injection of AAV serotype 9 encoding human ASM can effectively restore ASM activity in ASM knockout mice, which indicates the possibility of genetic therapy in clinical trials in NPD-A as well as other lysosomal storage brain disorders. Enzyme replacement therapy or gene therapy for treating NPD and other human sphingolipid disorders might be promising in the foreseeable future.

13.8 Farber Disease

13.8.1 Condition

Farber disease, also known as Farber's lipogranulomatosis (MIM #228000), is an extremely rare autosomal recessive lysosomal storage disorder caused by mutations in the ASAH1 gene (MIM *613468). The first case was referred as "disseminated lipogranulomatosis" by Sidney Farber in 1952 [194]. Meanwhile, the mutated ASAH1 gene contributes to another disorder known as spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) [195]. It was originally believed that Farber disease and SMA-PME were two independent diseases. However, there is an overlap in their clinical phenotypes with the appearance of more case reports. To date, less than 200 reported cases of Farber disease or SMA-PME have been identified

worldwide based on literature search and the true incidence of this disease remains unknown.

13.8.2 Genetic Defects

The ASAH1 gene is mapped to chromosome 8p21.3-p22 and encodes human acid ceramidase enzyme (ACDase), which is a lysosomal hydrolase that degrades bioactive lipid ceramide into sphingosine and fatty acid [196, 197]. Mutations in ASAH1 lead to deficiency in ACDase and abnormal accumulation of ceramides in various tissues of the body, such as joints, bones, brain, liver, and spleen, and ultimately cause various clinical features.

13.8.3 Lab Findings

The ACDase activity assay measured in cultured skin fibroblasts and leukocytes, which is generally reduced to less than 10% of control cells, could provide valuable evidence for the diagnosis of Farber disease [198, 199]. Moreover, the significantly decreased enzyme activity in amniocytes and fetal fibroblasts is also employed in prenatal diagnosis [200].

In addition, excess ceramide concentrations could be detected in cultured cells as mentioned above or in biopsy specimens and body fluid samples from Farber disease patients [201–203]. In the recent decades, the fundamentally development of liquid chromatography and mass spectrometry allowed for detailed detection and quantification of ceramide species and their derivatives, which has been applied to demonstrating increased amount of ceramide species in tissues and cells and provided novel insights into the pathologic mechanism of Farber disease [204]. Based on this technique, C26:0 ceramide has been reported as a potential sensitive biomarker candidate for early detection and intervention [205].

The morphological change of subcutaneous nodules or other tissues from Farber disease patients mainly includes granulomas and lipid-laden macrophages (foam cells) infiltration. Ultrastructural analysis revealed the presence of characteristic comma-shaped curvilinear tubular Farber bodies, spindle-shaped bodies, and zebra-like bodies among different cell types [206–208].

Recent studies also found that monocyte chemoattractant protein 1 (MCP-1) was increased in plasma of Farber disease patients and animal models, which is consistent with the phenotype of inflammatory infiltration in tissues [204, 209]. It was suggested that MCP-1 could be a sensitive cytokine biomarker for differential diagnosis of Farber disease.

13.8.4 Clinical Features and Diagnosis

The clinical symptoms of Farber disease patients are varied and complicated. The most classic three phenotypes consist of subcutaneous nodules near joints, progressive immobilization and deformation of joints and hoarseness due to nodules formation within the larynx. Moreover, Farber disease is characterized as seven types via different clinical manifestations, age of onset, and the severity of this disorder. The following Table 13.1 summarized the main phenotypes of Farber disease and SMA-PME.

The primary clinical diagnosis of Farber disease is based on the classic triad as mentioned above. Generally, Farber disease might be initially misdiagnosed as juvenile idiopathic arthritis, rheumatoid arthritis, and juvenile hyaline fibromatosis due to its rarity and similar manifestations in joints and subcutaneous nodules [197, 213]. Therefore, further biochemical and genetic analyses are required. Enzyme activity assay and quantitation of excess ceramides described above are the two most methods assisting in definite diagnosis of Farber disease. Gene testing provides a conclusive diagnosis, especially for those with mild or non-classical symptoms, and SMA-PME patients.

13.8.5 Treatment and Advances

As for the management of this disease, there is no effective therapeutic method to date. The basic treatment are anti-inflammatory medications and physical therapy to relieve pain and related

Table 13.1 Clinical phenotypes of Farber disease

Classification	Age of onset	Main clinical features	Involved organs	Severity/ survival period
Type 1 (classic form)	Infancy	Classic triad[a], dyspnea, pneumonia, progressive neurological impairment (developmental delay, hypotonia, muscle atrophy, and seizures)	Skin, joints, larynx, lungs, and nervous system	Severe/ infancy
Type 2	Infancy	Classic triad and slight neurological problems	Skin, joints, larynx, and nervous system	Moderate/ early-mid childhood
Type 3	Infancy–toddler stage	Classic triad and intellectual disability	Skin, joints, larynx, and nervous system	Mild/young adulthood
Type 4 (neonatal-visceral subtype)	Neonatal stage	Hepatosplenomegaly and visceral histiocytosis	Liver, spleen, lungs, thymus, and lymphocytes	Severest/ early infancy
Type 5	Infancy–toddler stage	Progressive neurological deterioration: Seizures, quadriplegia, loss of speech, myoclonus and intellectual disability; nodules and joint involvement	Nervous system, skin, and joints	Moderate–severe/early childhood
Type 6 (combined Farber and Sandhoff disease variant-1 case [210])	Infancy (6 months)	Classic triad, hypotrophy, and cherry-red macular spots	Skin, joints, and larynx	Moderate/ early childhood (2.5 years)
Type 7 [211, 212] (Prosaposin deficiency with intact ASAH1)	Infancy	Hepatosplenomegaly, seizures, myoclonus, and respiratory failure	Liver, spleen, nervous system, and lungs	Severe/ infancy
SMA-PME	Childhood	Walking difficulties, sporadic falls, muscle weakness, myoclonic epilepsy, and tremors	Nervous system	Moderate/ adolescent

[a]Classic triad: subcutaneous nodules, joint contractures, and voice hoarseness

symptoms. HSCT was reported for the first time to treat a classic Farber disease case in 1989 [214]. Recently, a follow-up study evaluated the effects on 10 Farber disease patients who underwent HSCT within 15 years [215]. All the data revealed that abnormal inflammatory response could be corrected after HSCT, while neurological symptoms and signs could not be relieved. Therefore, for Farber disease patients without nervous system involvement, HSCT might be effective.

ERT has been commonly applied to clinical treatment of lysosomal storage disease such as Gaucher disease [216]. A recent study demonstrated that human recombinant acid ceramidase (rhACDase) derived from Chinese hamster ovary cells overexpressing ACDase significantly reduced ceramide in fibroblast from a Farber disease patient [217]. Meanwhile, the researchers administered rhACDase to the asah1$^{P361R/P361R}$ mutant mice (Farber disease mice) and found that the treatment improved their survival rate, normalized spleen size, decreased plasma MCP-1 levels, reduced macrophage infiltration in liver and spleen, and ceramide accumulation in tissues [217]. It was suggested that ERT might be promising treatment for Farber disease and further investigations are urgently required for clinical translation.

13.9 Summary

In this chapter, we give a brief review about these eight diseases belonging to sphingolipidoses. To further compare similarities and differences between them, Table 13.2 including genetic

Table 13.2 Summary of diseases of sphingolipidoses

Gene	Disease	Inheritance	Enzyme/protein	Major accumulated substrate	Affected organs	Treatments	Potential therapy
GLB1	GM1 gangliosidosis	AR	β-Galactosidase	GM1 gangliosides	CNS, liver, heart, bones, joints, muscle	Not proved yet	Gene therapy, HSCT, ERT, molecular chaperones, SRT, BMT
HEXA HEXB GM2A	GM2 gangliosidosis Tay–Sachs disease Sandhoff disease GM2-AP deficiency	AR	β-Hexosaminidase GM2 activator protein	GM2 gangliosides	Brain, muscle	Not proved yet	ERT, HSCT, BMT, SRT, molecular chaperones, gene therapy
GLA	Fabry disease	XL	α-Galactosidase A	Globotriaosylceramide	Kidney, heart, gastrointestinal system, cardiovascular	ERT, enzyme stabilizers, bone marrow transplantation	SRT, mRNA and gene therapy
GBA1	Gaucher's disease	AR	Glucocerebrosidase	Glucosylceramide	Liver, bone marrow, spleen, CNS	ERT, SRT	Gene therapy, molecular chaperones
ARSA PSAP*	Metachromatic leukodystrophy	AR	Arylsulfatase A * Saposin B	Sulfatide, lyso-sulfatide	Peripheral nerve, CNS, kidney, gallbladder, bile ducts	HSCT	Gene therapy, BMT, ERT
GALC PSAP*	Krabbe disease *atypical form	AR *AR	Galactocerebrosidase *Saposin A	Galactosylceramide	Brain, kidney, intestine, colon *Neurological dysfunction	Not proved yet	HSCT, gene therapy, BMT, ERT, SRT, molecular chaperones
SMPD1	Niemann–Pick disease A and B	AR	Acid sphingomyelinase	Sphingomyelin	Brain, lung, spleen, liver	ERT for NPD-B Liver transplantation	Gene therapy
ASAH1	Farber disease	AR	Acid ceramidase	Ceramide	Skin, joints, bones, larynx, brain, lung, liver, spleen	Not proved yet	HSCT, ERT

AR autosomal recessive, *BMT* bone marrow transplantation, *CNS* central nervous system, *ERT* enzyme replacement therapy, *HSCT* hematopoietic stem cell transplantation, *mRNA* messenger RNA, *NPD-B* Niemann–Pick disease B, *SRT* substrate reduction therapy, *XL* X-linked

characteristics, molecular changes, clinical manifestations, and treatments is attached as follows. Overall, limited effective choices have been approved for treating sphingolipidoses, especially for those patients affected with CNS. Thus, further investigations are indeed warranted for better understanding and managing these life-threatening disorders.

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