De Novo Sphingolipid Biosynthesis in Atherosclerosis

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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

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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

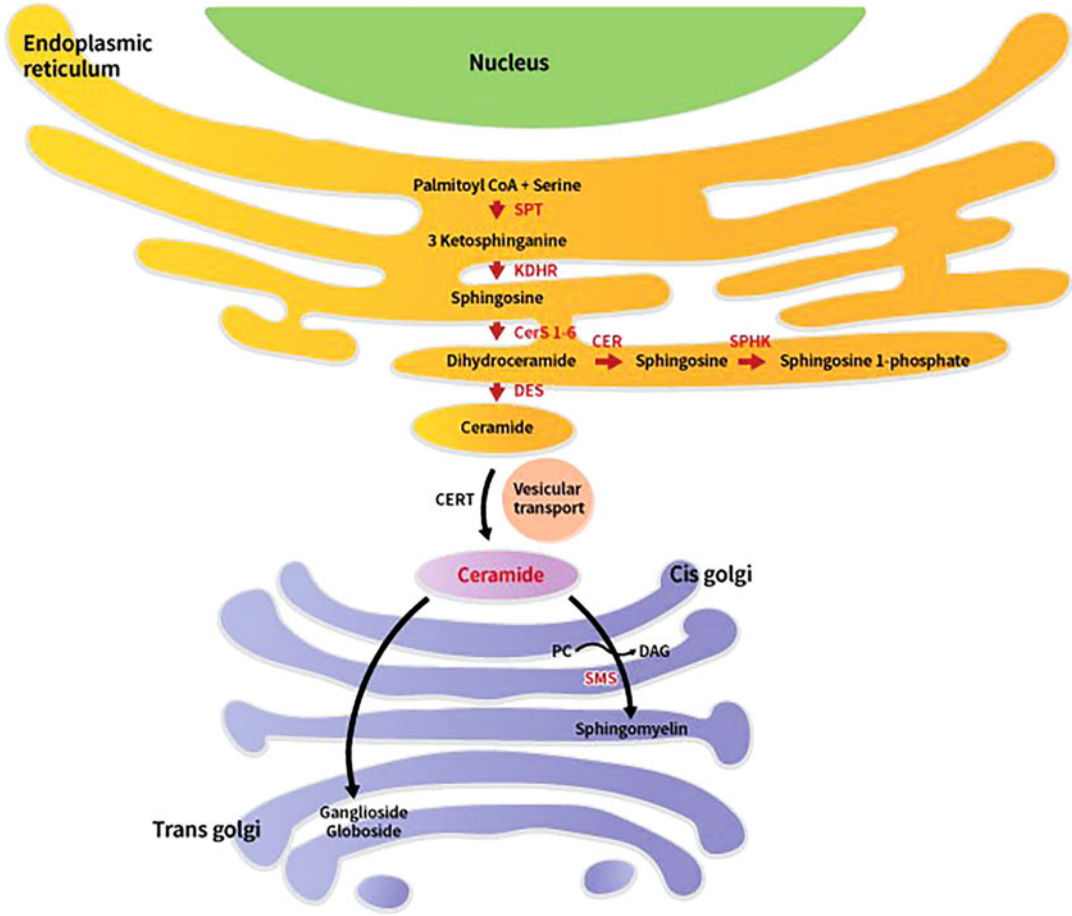

Fig. 3.1 The De novo sphingolipid biosynthesis in mammals. The biosynthetic pathway starts with the condensation reaction of palmitoyl CoA and serine, regulated by a rate-limiting enzyme serine palmitoyltransferase (SPT) to form 3-ketosphinganine followed by a series of enzymatic reactions producing 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.

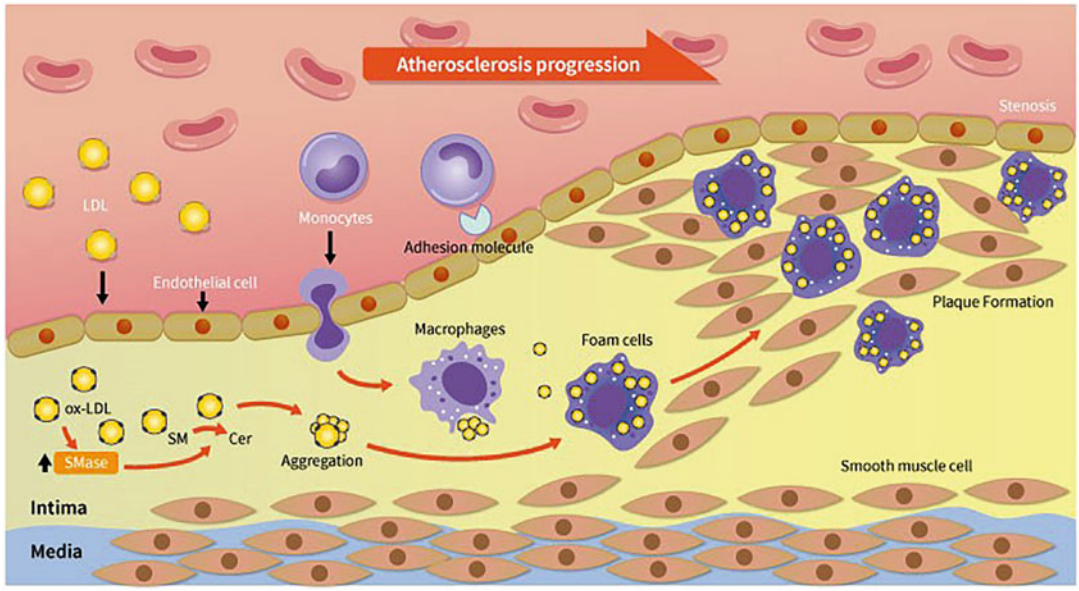

Fig. 3.2 Sphingolipids and atherosclerosis development. LDL accumulation responsible for endothelial dysfunction undergoes oxidative modifications to form oxidized LDL (ox-LDL), inducing an inflammatory reaction causing monocyte infiltration and overexpression of adhesion molecules in endothelial cells. 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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