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Spingolipid *de novo* biosynthesis: a rheostat of cardiovascular homeostasis

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Abstract

Spingolipids are both, fundamental structural components of the eukaryotic membranes and signaling molecules regulating a variety of biological functions. Highly bioactive lipids, ceramide and sphingosine-1-phosphate have emerged as important regulators of cardiovascular functions in health and disease. In this review we discuss recent insights into the role of spingolipids, particularly ceramide and sphingosine-1-phosphate, in the pathophysiology of the cardiovascular system. We also highlight advances into the molecular mechanisms regulating serine palmitoyltransferase, the first and rate-limiting enzyme of *de novo* biosynthesis, with an emphasis on recently discovered inhibitors of serine palmitoyltransferase, ORMDL and NOGO-B proteins. Understanding the molecular mechanisms regulating this biosynthetic pathway may lead to the development of novel therapeutic approaches for the treatment of cardiovascular diseases.

Keywords

Spingolipids; SPT; Nogo-B; cardiovascular diseases

Regulatory mechanisms of SPT

Spingolipid (SL) *de novo* biosynthesis occurs in the cytosolic leaflet of the smooth **endoplasmic reticulum** (ER) (see Glossary) where serine palmitoyltransferase (SPT) catalyzes the first and rate-limiting step, the decarboxylative of palmitoyl-coenzyme A with L-serine to generate 3-ketosphinganine (Figure 1A, B) [1]. The highly conserved SPT enzyme belongs to the oxoamine synthases family and is a membrane-bound heterodimer composed of two subunits, first identified as Lcb1/Lcb2 in yeast [2–4]. In mammals, there is a single Lcb1 homolog, Serine PalmitoylTransferase Long Chain base subunit 1 (SPTLC1), and two homologs of Lcb2, SPTLC2 [5, 6] and SPTLC3 [7]. The SPTLC2 and SPTLC3

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subunits of SPT display distinct expression patterns *in vivo* [7]. Modeling studies using other members of the oxoamine synthases, first AONS [8] and later bacterial SPT [9], suggested that the active site of SPT lies at the heterodimeric interface with each subunit contributing catalytic residues, and this has been confirmed from mutational studies [8, 10, 11]. The topology and the stoichiometry of SPT subunits are still under debate. An initial study by Yasuda et al. proposed Lcb1 as a single transmembrane (TM) protein with an ER luminal N-terminus and a cytosolic C-terminus [12]. Later on, Han et al. demonstrated the presence of three membrane-spanning domains and showed that the first TM domain of both yeast and human LCB1 can be deleted without affecting membrane targeting, formation of the LCB1/LCB2 heterodimer, or enzymatic activity [13].

The stoichiometry of the SPT complex was initially proposed as a heterodimer composed of Lcb1 and Lcb2 in 1:1 ratio [14]. Recently, SPT was suggested to be an octameric complex of four heterodimers resulting from the interaction of SPTLC1 with either SPTLC2 or SPTLC3, depending on the expression pattern of SPT subunits and the cellular sphingolipid requirement [15].

Although SL are recognized as essential membrane components and bioactive signaling molecules [1], little is known about the regulation of their synthesis. Orthologous to the yeast Tsc3 [16], the SPT small subunits a and b (ssSPTa and ssSPTb), are independent activators of mammalian SPT [17]. The interaction of ssSPTa and b with SPT via their single TM domain is sufficient for boosting the activity of the enzyme [18]. Whether or not ssSPTa and b are also regulated at transcriptional or post-translational level is not known.

To date two proteins have been shown to negatively regulate SPT in mammals, the ORosoMucoid-Like (ORMDL) proteins and Neurite OutGrowth inhibitor (NOGO-B), both conserved transmembrane proteins of the ER. Three different genes encode the ORMDL proteins (ORMDL1-3), which display different patterns of expression *in vivo* [19]. The yeast orthologous, Orm1 and Orm2 share ca. 35% of homology with the ORMDLs [19]. The role of the Orm proteins in regulation of SPT was first reported in an elegant study by Breslow and colleagues who showed that the Orms inhibit SPT when the cells have sufficient levels of SL [20], and the lack of Orms significantly increased the activity of SPT of six folds. When sphingolipid synthesis is disrupted by myriocin, an inhibitor of SPT activity [21], the Orms are phosphorylated at multiple sites and the inhibitory brake on SPT is relieved. As predicted, deletion or phosphomimicking mutations in the ORMs cause hyperactivation of SPT [20].

Recent studies have begun to delineate the phosphorylation pathway activated by myriocin as well as by heat stress, leading to Orm phosphorylation. Yeast Protein Kinase 1 (Ypk1) phosphorylates the Orms following myriocin treatment [22] or heat stress [23]. Nutrient depletion also stimulates Orm phosphorylation, but with a different pattern and without affecting SPT activity [24], suggesting that the Orms may have additional biological functions apart from the inhibition of SPT activity.

Furthermore, the morphogenesis checkpoint kinase Swe1 has also been linked to Orm phosphorylation. Yeast lacking Swe1 shows decreased levels of SL and increased sensitivity

to myriocin-induced growth inhibition, suggesting that Swe plays a role in controlling sphingolipid homeostasis [25].

However, whereas the mechanisms responsible for Orm regulation of yeast SPT are being elucidated, regulation of mammalian SPT by the ORMDLs is poorly understood. The ORMDLs lack the N-terminus containing the phosphorylation sites [19], indicating that different regulatory mechanisms control ORMDL-SPT interactions.

Recently, NOGO-B was identified as a negative regulator of SPT activity in mammals [26]. NOGO-B belongs to the large family of Reticulon (RTN) proteins, so called for their localization in the tubular ER through two transmembrane domains separated by a loop of 66-aa named reticulon-homology domain (RHD, Figure 1C) [27]. Of the four Reticulon genes in mammals, Rtn-4 gives rise to three major isoforms, RTN-4A, -4B and -4C (aka NOGO-A, -B, and -C). The topology of NOGO proteins is still under debate. Three possible orientations in the ER membrane have been proposed [28, 29]. While NOGO-A and NOGO-C are expressed in the central nervous system, with low levels of NOGO-C also in the skeletal muscle, NOGO-B is highly, but not exclusively, expressed in the blood vessels [26].

Voeltz et al. elegantly demonstrated that an important biological function of reticulons is to facilitate the formation of the tubular ER network [27, 29]. For other NOGO-related functions, including inhibition of neuronal outgrowth [30] and stimulation of EC migration [31], putative receptors have been suggested as mediators. Given that more than 95% of NOGO proteins are localized in the tubular ER, with the remaining fraction on the cell membrane, the ligand-receptor model proposed remains controversial.

In addition to shaping the tubular ER, a basic intracellular function of NOGO-B is to interact with and inhibit SPT activity. NOGO-B co-localizes with SPT in the tubular ER and co-immunoprecipitates with SPT and the ORMDLs. In the absence of NOGO-B, SPT activity is significantly upregulated by about 40% and the lentiviral-mediated re-expression of NOGO-B restores the activity of SPT to control levels. The inhibitory function of NOGO-B on SPT has been confirmed in murine and human endothelial cells (EC), mouse lung, and heart microsomal preparations, deplete vs. replete of NOGO-B [26]. It is noteworthy to mention that in absence of endothelial NOGO-B not only sphinganine, but also ceramide (C18-, C20- and C22-ceramide), sphingosine and sphingosine-1-phosphate (S1P) are significantly increased. Myriocin treatment markedly reduces S1P production (~50%) suggesting an important contribution of the *de novo* pathway to the endothelial-derived S1P. However, the most abundant ceramides (C16-, C24-, and C26-ceramide) were unchanged. Whether the lack of NOGO-B affects other metabolic steps of the *de novo* pathway, and/or ceramide synthase activity is unknown and further studies are needed to clarify the molecular mechanism/s accountable for that specific changes in SL profile.

Although SPT is fundamental during development [32], the regulatory mechanisms of this enzyme remain poorly understood. Specific molecular interactions of ORMDLs and NOGO-B with SPT complex and their mechanisms of regulation have yet to be identified. Furthermore, how the SPT complex can sense changes in sphingolipid levels and maintain cellular sphingolipid homeostasis is not understood. Are there cell-specific regulatory

mechanisms of SPT in mammals since the ORMDLs and NOGO-B present a specific distribution pattern? Are there other regulatory members of the SPT complex still to be discovered? Lastly, how is SPT regulated in pathological conditions? In exploring strategies for modulating the sphingolipid *de novo* pathway it is imperative to understand regulation, particularly of the rate-limiting step, and the downstream effects of their inhibition, especially considering all the side branches of this pathway.

Locally produced S1P and ceramide in vascular tone regulation

Endothelial S1P-S1P receptor 1 (S1P-S1PR1) signaling: regulator of vascular barrier, tone and flow

Ceramide represents a nodal point of the sphingolipid *de novo* pathway (Figure 1): it can be reversibly converted to higher order SL, including sphingomyelin (SM) and glycosphingolipids; it can be degraded to sphingosine, or a small fraction can be phosphorylated to form ceramide-1-phosphate [1]. Two sphingosine kinases (SPHK1 and SPHK2) phosphorylate sphingosine to generate S1P, which is rapidly secreted outside of the cells, where it can bind to and activate five G-protein coupled receptors, named S1PR1-S1PR5 [33]. In physiological conditions, erythrocytes [34] and EC [35, 36] are major sources of plasma S1P. Reaching concentrations of 0.1–1 nM, plasma S1P is bound to apolipoprotein M (ApoM) of HLD (ca. 60%) and albumin (ca. 35%) [37, 38]. Whether or not these carriers also control the “availability” of S1P to S1PRs is still unclear. In the cardiovascular system, different levels of S1PR1, S1PR2 and S1PR3 are expressed in various cell types. In the EC, S1PR1 is the most abundant receptor [39] (S1PR1 16-fold higher than S1PR3) and regulates vascular development, angiogenesis, endothelial barrier integrity [40] and vascular tone [41].

Low levels of plasma S1P, such as in the absence of SPHK [42] and ApoM [37], and the lack of endothelial S1PR1 [43] cause the disruption of the S1P-S1PR signaling leading to a general increase in vascular permeability. Interestingly, in humans [44] and baboons [45] the severity of sepsis inversely correlates with plasma levels of ApoM. Altogether, these findings suggest the importance of S1P signaling in vascular homeostasis.

The endothelium actively controls vascular tone and blood pressure (BP). Following chemical and mechanical stimuli, including changes in **shear stress**, the endothelium releases different vasoactive factors, including prostacyclin, endothelin, **nitric oxide (NO)** [46], and S1P [35]. Changes in flow induce endothelial synthesis of S1P [35], which in turn is transported outside of the EC through specific spinster-2 transporter [47] to stimulate S1PR1 in an autocrine manner [48] and downstream eNOS [49], of a magnitude comparable to acetylcholine and bradykinin.

Multiple evidences have suggested an important role of S1PR1 in the endothelial response to flow. In absence of S1PR1, changes in flow fail to induce EC alignment and eNOS phosphorylation, *in vivo* as well as *in vitro* [50]. Furthermore, the inhibition of S1PR1 with W146 reduces the vasodilation to flow and the NO production [26], suggesting that S1PR1 is an active player of the mechanotransduction signaling pathway controlling blood flow. A recent study from Galvani and colleagues, by using different genetic mouse models,

demonstrated anti-inflammatory and anti-atherosclerotic functions of the S1PR1 signaling, particularly in vascular points exposed to turbulent flow such as the lesser curvature and branch points *in vivo*, more susceptible to the development of atherosclerotic lesions [51].

A critical role of NOGO-B in controlling endothelial-derived S1P to impact BP through the S1PR1-eNOS autocrine axis was recently demonstrated. Sphingolipid *de novo* biosynthesis is an important, although not exclusive, determinant of endothelial produced S1P and genetic or pharmacological modulation of the *de novo* pathway within the vasculature markedly impacts BP homeostasis. In mice lacking Nogo-B, the endothelium produces higher levels of S1P conferring resistance to endothelial dysfunction and hypertension [26]. Remarkably, the activation of S1PR1 with SEW2871 markedly lowers BP in hypertensive mice. On the contrary, pharmacological inhibition of SPT with myriocin restores BP in mice lacking NOGO to WT levels [26], suggesting that 1) locally produced *de novo* SL, within the vascular wall, are necessary to control vascular functions, despite the high levels of SL in the plasma, and 2) S1PR1 signaling is a novel anti-hypertensive pathway.

The importance of locally produced S1P in vascular tone regulation was further corroborated by other studies. Following carbachol stimulation, a muscarinic M3 receptor agonist, SPHK1 rapidly translocate to the plasma membrane, implicating the contribution of endothelial S1P in the vasodilation induced by muscarinic agonists [52, 53].

Altogether, these findings underline previously underappreciated roles of sphingolipid biosynthesis and signaling in BP homeostasis and provide a framework for investigating whether alterations of NOGO-mediated regulation of sphingolipid metabolism and signals contribute to other cardiovascular diseases, or other pathological conditions linked to the alteration of this pathway.

Vascular produced S1P and myogenic tone

In vascular smooth muscle cells (VSMC), S1P activates S1PR2 and S1PR3 to induce vasoconstriction of the media layer mediated by Ca^{++} /Rho pathway in rodents[41], porcine [54] and human arteries [55, 56].

While endothelial S1P activates the S1PR1-eNOS autocrine signaling, other studies suggested that S1P produced in the vascular wall also participate to the **myogenic tone**. Changes in pressure trigger S1P production in rabbit posterior cerebral arteries [57]. Furthermore, elevated levels of S1P in the vascular wall through Sphk1 overexpression [58] or S1P-phosphohydrolase knockdown [59] increase the myogenic tone of resistance arteries, whereas the overexpression of S1P-phosphohydrolase decreases vascular tone in response to pressure, implicating local produced S1P in myogenic tone regulation. Recently, Hoefer et al. demonstrated that during heart failure the myogenic tone of resistance arteries increases, and the hyperactivation of S1P-S1PR2 autocrine axis is accountable for it [60]. Whether or not the increase in the myogenic tone is due exclusively to an increase of S1P-S1PR2 autocrine loop in the VSMC or also to the disruption of the vasoprotective S1P-S1PR1 signaling in the endothelium, remains an open question.

Clearly S1PRs can be modulated *in vivo* by locally produced S1P. Understanding how S1P autocrine loops in the EC and VSMC change during cardiovascular disease, including hypertension and heart failure, would definitely contribute to our understanding of this pathway in cardiovascular homeostasis but also provide possible pharmacological targets, from the biosynthesis of S1P to its downstream receptors.

Ceramide and vascular tone regulation

The accrual of circulating and local ceramide in certain pathological conditions, including obesity and type-2 diabetes, has been linked to detrimental vascular effects and endothelial dysfunction discussed hereafter. Both glucose and palmitate trigger the *de novo* biosynthesis of ceramide, which interferes with eNOS-derived NO production through the activation of protein phosphatase 2A (PP2A) [61–64] and impact BP [62, 64]. Ceramide also promotes NADPH oxidase activity and ROS formation at the expense of endothelial NO [65]. A recent study from Freed et al. demonstrated that long treatment *ex-vivo* of human resistance arterioles with ceramide triggers the formation of mitochondrial-derived H₂O₂, counteracting the NO which is associated with an inflammatory and dysfunctional state of the endothelium. Interestingly, the inhibition of neutral sphingomyelinase (nSMase) restores the physiological phenotype in arterioles from patients affected by coronary artery disease [66]. In another study, Spijkers L.J. et al. showed an increase of ceramide, but not SM, in the plasma of hypertensive rats and patients. They proposed that endothelial-derived thromboxane A2 was accountable for the hypercontractility of rat carotid arteries induced by ceramide [67]. Altogether these studies support the concept that ceramide accrual is “deleterious” for the endothelial functions.

On the other hand, few studies also reported that acute administration of ceramide induces eNOS-dependent vasorelaxation [68]. It is of noteworthy to mention that C6-ceramide coated catheters limit neointimal hyperplasia while promoting the re-endothelialization of injured arteries [69], suggesting cell-type and pathological state-dependent functions.

Another line of investigation demonstrates a role of ceramide in VSMC contraction. SMase and some ceramide species induce a sustained vasoconstriction of cerebral arteries and venules in rats and dogs [70, 71]. Although preliminary, these studies suggest that ceramide plays an important role in the pathogenesis of hypertension. To date, the paradigms to study ceramide functions in vascular tone regulation mainly involved the administration of exogenous ceramide or nSMase. Genetic mouse models such as *dihydroceramide desaturase 1* heterozygous (*Des1^{+/-}*) has been used to study the role of ceramide in the vasculature in models of diet-induced obesity and type-2-diabetes[62, 64]. The next step would be to manipulate the biosynthetic pathway with potential to result in endogenous changes in ceramide levels, possibly in specific cell types (EC vs. VSMC), to recapitulate more closely pathological conditions such as hypertension.

The role of ceramide in myogenic tone has been poorly investigated. It was recently reported that the lack of Nogo-B in VSMC leads to a selective increase of ceramide species (C18-cer, C20-cer, C22-cer) with specific reduction only in the myogenic tone, leading to a lower BP, suggesting a distinct involvement of ceramide in pressure-induced vasoconstriction [26]. Of course, as central metabolite of an highly dynamic metabolic pathway, we need to keep in

mind that ceramide can give rise to other SL, including sphingosine and S1P that could also account for the biological functions associated to ceramide. Furthermore, whether or not the sources of ceramide (*de novo* vs. SMase) can generate a specific subset of ceramide species, and/or lead to a preferential accumulation in distinct subcellular compartments linked to finite biological functions is unknown. Finally, the identity of the vessels (arteries vs. veins; capacitance vs. resistance arteries), their pathophysiological state, the methods used to evaluate vascular functions, the correlation of vascular function with NO levels, and the amount of ceramides also need to be taken into account when considering the net effect of ceramide on vascular tone.

Sphingolipid *de novo* pathway and S1P in the heart

Based on the current literature, whether changes in plasma levels of S1P may have causative or consequential effects during ischemic heart diseases remain unclear. Clinical studies have demonstrated that following myocardial infarction (MI) plasma levels of S1P decrease in the first days [72], and remain low up to two years [73]. A recent study of Sutter *et al* further confirmed these findings in acute MI patients [74]. Conversely, other studies reported increased [75] or unchanged [76] plasma levels of S1P in patients post-MI. Different reasons could explain these controversies, including when the blood samples were taken (i.e. before, after or during stent implantation), the methodologies employed to quantify S1P, whether or not S1P was normalized to plasma HDL, and lastly the medication given to the patients. Reduced levels of plasma S1P have been also reported in patients affected by coronary artery diseases and they inversely correlate with the severity of the diseases [77, 78]. In spite of discordant changes in plasma S1P following MI in preclinical studies [73, 79, 80], administration of HDL and S1P to the mice protected the heart from ischemia reperfusion injury [81], underlining a cardioprotective function of S1P signaling.

S1P protects cardiomyocyte (CM) from hypoxia-induced damage *in vitro*, as well as from ischemia/reperfusion (I/R) injury inflicted to the heart *ex-vivo* in a **Langendorff perfusion system** (comprehensively reviewed elsewhere [82, 83]). By using systemic knockout mouse models, Means and colleagues elegantly demonstrate an important and redundant cardioprotective role of S1PR2 and S1PR3 from I/R injury [84] *in vivo* by using global knockout mouse models, although a direct role of these receptors on CM biology was not demonstrated. S1PR3 was also shown to mediate HDL-induced protection of the heart from I/R damage [81]. It is still unclear which S1P receptor/s on CM is accountable for the S1P protection from insults.

Pharmacological approaches suggest that S1PR1 mediates the protective actions of S1P on CM *in vitro* [85]. S1PR1 cardiac gene therapy *in vivo* protects the heart from ischemic injury [86]. However, data on the S1PR1 expression in CM are discordant. Although CM seem to express S1PR1 mRNA [85], an elegant study from Chae *et al.* using a β -galactosidase reporter knockin mouse model demonstrated that S1PR1 is mainly expressed in EC and in CM of the atria but undetectable in the ventricle [87], corroborated also by Forrest *et al.* [88]. Interestingly, a very recent study by Keul *et al.* demonstrated that the excision of S1PR1 in CM by using α -myosin heavy chain promoter (α -MHC-Cre) strategy did not affect myocardial infarction compared to WT mice [89], suggesting that S1PR1 does

not play a main role in this pathological scenario. Data on CM *in vitro* showed that S1PR1 plays an important role in intracellular Ca^{++} homeostasis through its inhibitory effect on adenylate cyclase, while the loss of S1PR1 in CM led to age-dependent dilated cardiomyopathy and spontaneous death of the mice [89]. It is noteworthy to mention that α -MHC-Cre mice manifest a Cre- and age-dependent cardiotoxicity [90] warranting caution in the interpretation of these data. Administration of SEW2871, a S1PR1 agonist, preserves the heart from MI damage [91], suggesting that in addition to CM, S1PR1 in the endothelial cells might also contribute to the cardioprotective functions imputable to S1PR1.

Other functions of S1P signaling in the heart, including chronotropic and inotropic effects, have been comprehensively reviewed elsewhere [83, 92]. To date only few studies *in vitro* suggested a pro-hypertrophic function for S1P on CM through S1PR1 [93, 94], while the role of S1P signaling in **pathological cardiac hypertrophy (PCH)** induced by pressure overload is virtually unexplored. A recent study from Zhang *et al.* demonstrated that endothelial S1P protects the heart from inflammation, fibrosis and loss of function during chronic **pressure overload** through the activation of the endothelial S1P-S1PR1-eNOS autocrine signaling [95]. In the heart Nogo-B, identified as inhibitor of SPT (Figure 1), is expressed only in blood vessels and atria but not in ventricular CM. In mice lacking Nogo-B the endothelium produces higher levels of S1P, which activates S1PR1, 3, on the EC surface inducing NO production and endothelial barrier integrity (Figure 2), thus improving vessel compliance to the high pressure and reducing extravasation of plasma proteins and inflammatory cells. Considering the vicinity of capillaries and myocytes, vascular-derived SL, particularly S1P, could also directly impact CM biology. Interestingly, SEW2871 protect the heart from PCH triggered by chronic pressure overload, supporting a cardioprotective role of S1PR1 signaling [95].

In the cardiomyopathies associated to metabolic diseases, the upregulation of the *de novo* pathway and the accumulation of SL, particularly ceramide, have been considered deleterious for the heart (comprehensively reviewed elsewhere [96–98]). However, systemic loss of SPT is genetically lethal [32], while CM-specific deficiency of SPT leads to cardiac fibrosis and dysfunction, despite the reduction in ceramide [99]. In agreement with these findings, the inhibition of SPT by myriocin restores the PCH in Nogo-A/B-deficient mice following pressure overload [95], supporting a cardioprotective role of SPT. Apparently contradictory, these lines of evidence suggest that 1) SL are necessary to maintain cardiovascular homeostasis; 2) SL levels need to be contained in a rather finite physiological window; 3) SL *de novo* biosynthesis has to be tightly regulated because an exaggerated production of SL, such as during metabolic diseases, triggers derangement of cardiovascular functions.

The molecular mechanisms regulating the transition from **physiological cardiac hypertrophy** to PCH remain unclear. Sphingolipid *de novo* biosynthesis and Nogo-B-mediated inhibition of SPT play an important role in the onset of PCH and open new questions. In the heart, Nogo-B is expressed constitutively also in VSMC and transiently in some CM (ca. 20%) following pressure overload. How does Nogo-B-mediated inhibition of SPT in these cell types affect the onset of PCH? Does SPT activity and SL levels change during the pathogenesis of heart failure? And how? Which S1P receptor/s mediate the

cardioprotective functions of S1P? Does the expression of S1P receptors change in PCH? CM-specific knockout mice for the players of this pathway could answer important questions in this field and facilitate possible pharmacological treatment of this condition.

Concluding Remarks and Future Perspectives

A growing body of literature suggests that dysregulation of sphingolipid metabolism and signaling may have a causative role in the pathogenesis of cardiovascular diseases.

ORMDLs and Nogo-B are negative regulators of SPT, the enzyme controlling the rate-limiting step of the SL production. However, the regulatory mechanisms of sphingolipid biosynthesis, and particularly of SPT, in health and diseases are not well understood (see Outstanding Questions).

The upregulation of sphingolipid production, particularly of S1P, and S1P signaling exert protective functions in hypertension [26], PCH [95], atherosclerosis [51] and vascular barrier maintenance [37, 42, 43, 95] mainly through the receptor S1PR1.

Diversely, in others pathological states, such as metabolic diseases, the exaggerated accumulation of SL, particularly of ceramide, has deleterious effects on the cardiovascular system [61–67], suggesting that local SL levels need to be maintained within a limited physiological window. These findings also suggest that relative abundance of SL species (for instance S1P vs. ceramide) may determine the net biological effects, although further studies are needed to confirm the concept of S1P/ceramide rheostat in CV diseases. The concept of S1P/ceramide rheostat in cell fate and cancer has been recently discussed elsewhere [100].

Studies enhancing our understanding of the regulation and functions of SL *de novo* pathway during the pathogenesis of cardiovascular diseases, including hypertension and cardiomyopathy, are strongly warranted to provide a framework for therapeutic modulation of this pathway.

Lastly, in the developments of agents targeting the bioactive SL and its signaling pathways, particularly the SPHKs/S1P/S1PR1, potential cardiovascular effects should be a consideration.

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Glossary

Endoplasmic Reticulum (ER)

It is the largest eukaryotic organelle and spreads in the cytoplasm from the nuclear membrane to the Golgi. It is usually divided in subdomains: the nuclear envelope, the rough ER, the smooth ER and the ER-Golgi intermediate compartment. The rough ER is characterized by a sheet-like morphology, named cisternae, and by the presence of membrane-associated ribosomes responsible for the synthesis of secretory and membrane proteins. The smooth ER, a tubular network devoid of ribosomes, is the site of lipid and

steroid synthesis, carbohydrate metabolism, regulation of calcium levels and drug detoxification. Each subdomain is in communication with the others, forming an interconnected network of tubules, cisternae and vesicles delineated by membranes

Golgi

eukaryotic organelle composed by membranes and vesicles. It is responsible for the post-translational modifications, storage of proteins and other macromolecules, and for their vesicular transport to other intracellular compartments or outside of the cell

Langendorff perfusion system

It is a predominant *ex vivo* model used in cardiac pharmacological and physiological research, in which the isolated heart is perfused in a reverse fashion via the aorta. The backwards pressure of the flow closes the aortic valve and forces the perfusate into the coronary vasculature, supplying nutrients and oxygen to the heart for several hours after the explant

Myogenic tone

is an intrinsic property of vascular smooth muscle cells consisting of contraction of blood vessels in response to a stretch induced by the increase in transmural pressure. This response is independent of neural or hormonal influences

Nitric oxide (NO)

In the vessels NO is synthesized by the endothelial nitric oxide synthase (eNOS). It is a lipophilic molecule easily diffusing through cell membranes and able to act on the vascular smooth muscle cells immediately beneath the endothelial cells to induce vasorelaxation by a cyclic GMP-mediated mechanism

Neurite OutGrowth Inhibitor (NOGO)

so called because Nogo-A, one of three isoforms coded by *Reticulon-4* gene (*Rtn-4*), was reported to repress neurite growth and synaptic plasticity in the central neuron system. To date, these findings remain controversial

ORMDL

ORM1 (Saccharomyces Cerevisiae)-Like protein, where ORM stands for ORosoMucoiD

Pathological cardiac hypertrophy

It consists in the increase of the mass of the heart following pathological stimuli, including hypertension, valve disease, myocardial infarction and genetic mutations. It is characterized by inflammation, fibrosis, alteration of the cardiac structure and functions and represents a risk factor for heart failure

Physiological cardiac hypertrophy

It is a compensatory and reversible response of the heart to physiological stimuli, such as physical activity or pregnancy, characterized by increase in the heart mass with preservation of cardiac structure and function

Pressure overload

it indicates a pathological state of the heart characterized by in excessive intraventricular pressure and deleterious systolic wall stress

Shear stress

is the frictional force that blood flow induces on endothelial cells, the inner layer of the vessel wall

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Box 1: Overview of SLs

SLs are a class of lipids characterized by an eighteen-carbon amino-alcohol backbone, also defined sphingoid base, and are synthesized *de novo* from serine and a fatty acyl-coenzyme A by the enzyme serine palmitoyltransferase. Discovered in brain extracts in 1870s, sphingosine (from “Sphinx”) owes its name to J.L.W. Thudichum “in commemoration of the many enigmas which it presents to the inquirer”. Phosphorylation, acylation and glycosylation of the simplest sphingoid bases, such as sphingosine, phytosphingosine, and dihydrosphingosine, give rise to the variety of complex SLs.

De novo sphingolipid biosynthesis starts in the smooth ER where ceramide is produced (Figure 1), and is completed in the Golgi where the most complex SLs are synthesized, including sphingomyelins (SM) and glycosphingolipids (GSL). The phosphorylation of these sphingoid bases forms the respective –1-phosphate SLs, among which sphingosine-1-phosphate, highly bioactive lipid, is the most studied. The acylation (the addition of a fatty acid chain of length usually between 14 and 26 carbon atoms) of the sphingoid bases sphingosine, dihydrosphingosine and phytosphingosine produces ceramide, dihydroceramide and phytoceramide respectively. Since ceramides are hydrophobic molecules they need a carrier to be moved from the ER to the Golgi, this transport is performed both by vesicles and by the protein CERT. Addition of phosphocholin or, to a small extent, phosphoethanolamine to ceramides forms SM, the major phosphosphingolipids of mammals. Glycosylation of ceramides with one or more sugar residues produces the class of GSL, comprising many members that differ in the number and type of sugar residues attached. Breakdown of complex SLs to ceramide and then to sphingoid bases is called “salvage pathway” and starts in the lysosome. Essential structural membrane component, some of them are also bioactive lipids acting as signaling and regulatory molecules. They are involved in many cellular processes such as cell-stress responses, cell survival, differentiation, cell migration, inflammation, etc. As a consequence, conditions altering this pathway have an impact on many human diseases.

Box 2: Ceramide in Cardiac Hypertrophy

Multiple lines of evidence suggested a deleterious role of ceramide accrual in the cardiomyocytes *in vitro*[1, 2] and *in vivo* in cardiomyopathies associated to systemic metabolic diseases herein and elsewhere discussed[3–6]. Upregulation of the *de novo* SL production was accountable for this metabolic derangement. Genetic manipulations of lipid pathways could recapitulate the myocardial lipotoxicity during metabolic syndromes. Cardiac overexpression of a membrane-anchored lipoprotein lipase increased the uptake of fatty acids (FA) and the accumulation of triglycerides and ceramide in cardiomyocytes, leading to cardiac dysfunction[7]. Interestingly, the inhibition sphingolipid *de novo* biosynthesis with myriocin or the excision of one copy of *Sptlc1* restored the normal function of the heart[7], and lowered ceramide and sphingomyelin, but not diacylglycerol levels, suggesting a causative correlation between the accumulation of ceramide and the cardiomyopathies[8]. Recently, Russo and colleagues reported that in addition to palmitoyl-derived SL, fatty acid overload leads to a conspicuous myocardial accumulation of myristate-derived SL, attributed to the SPTLC3 subunit of SPT and involved in CM apoptosis but not autophagy. Furthermore, the inhibition of SPT with myriocin protected the mice from lipotoxic cardiac hypertrophy[9]. Altogether, these findings are well supported by clinical data showing a direct correlation between plasma ceramide levels and the severity of symptoms and mortality of patients with chronic heart failure[10].

Recently, two independent groups demonstrated that mice challenged with long-term high fat diet accumulate ceramide into the heart and develop to cardiac hypertrophy[11, 12]. Interestingly, the inhibition of the activity[12] or the expression[11] of AMPK re-established a normal metabolic state, and reduced myocardial accumulation of ceramide, suggesting AMPK as an important player in the lipotoxic cardiomyopathy. Nevertheless, other studies have also shown that ceramide levels were not altered in the heart of mice fed with high fat diet[13] as well as in patients with Obesity/type 2 diabetes mellitus[14]. The discordance in these reports might lie in the different approaches employed to measure ceramides, such as immunostaining in the former and mass spectrometry in the latter. However, a recent study from Chokshi et al. elegantly showed that mechanical unloading of the failing heart is sufficient to correct the metabolic derangement in patients[15], suggesting a link between hemodynamic stress and lipid metabolism homeostasis.

Whereas an exaggerated accumulation of SL in the heart is regarded as deleterious, recent lines of investigation have evidenced the requirement of SL to preserve cardiac homeostasis. The genetic deletion of *Sptlc2* gene specifically in cardiomyocytes promotes cardiac fibrosis and dysfunction despite the reduction in ceramide levels[16]. In agreement with these findings, recently Zhang et al. showed that mice lacking Nogo-B, inhibitor of SPT, preserve cardiac function following pressure overload, whereas the inhibition of SPT with myriocin restored pathological cardiac hypertrophy and dysfunction to WT levels. This study also demonstrated that endothelial-derived SL, particularly S1P, play a critical role in protecting the heart from failure when challenged with chronic pressure overload[17]. Apparently contradictory, all these lines of evidence

suggest that SL levels need to be contained in a rather narrow physiological window; hence the regulation of *de novo* biosynthesis of SL is of critical importance to maintain cardiovascular homeostasis. The contribution of this metabolic pathway in cardiovascular diseases is only beginning to be elucidated. More studies need to be done to better understand the role and the regulation of SL metabolism in CV physiology and pathology.

Outstanding Questions Box

- How is SPT regulated in mammalian cells? ORMDLs and Nogo-B have been identified as negative regulator but the molecular mechanisms relieving their inhibitory actions on SPT are unknown. ORMDLs and Nogo-B seem to have a different expression pattern *in vivo*. Are there any cell-specific regulatory mechanisms for SPT? What is the expression pattern of these inhibitory molecules *in vivo* in pathophysiological conditions? Are there other regulatory subunits of the SPT complex still to be discovered?
- Nogo-B inhibition on SPT plays a key role in maintaining cardiovascular homeostasis, mainly through the regulation of the endothelial S1P-S1PR1-eNOS autocrine signaling. However, specific ceramide species are also upregulated in both EC and VSMC. What is the biological function of these SL in the blood vessels? Despite the increase in ceramide levels, the endothelial function was preserved as well as NO production. Is it possible that the rheostat S1P/ceramide dictates the pathophysiological state of the vessels? Are different pools of ceramide (*de novo* vs. SMase) exerting distinct intracellular functions?
- S1PR-mediate signaling exerts cardioprotective functions from MI and PCH, and patients affected by MI have lower plasma levels of S1P. What is the biological role of this change? Is the decrease of plasma S1P due to an impaired S1P production/release or to an increased degradation? And in which cell type/s the synthesis/release of S1P is reduced?
- In absence of Nogo-B, the upregulation of SPT in EC protects the mice from PCH via S1P-S1PR1-eNOS signaling axis. Is Nogo-B expressed in VSMC of the coronary vasculature playing a role during chronic pressure overload? Is Nogo-B expressed in a small fraction of CM following pressure overload playing any role in PCH?
- Excessive accumulation and decrease of ceramide are both deleterious for the heart, suggesting that sphingolipid production must be tightly controlled. What is the role of SPT and how its activity is regulated during the pathogenesis of hypertension or heart failure remain unknown.

TRENDS BOX

NOGO-B and ORMDLs proteins are negative regulators of serine palmitoyltransferase (SPT), the first and rate-limiting step of sphingolipid *de novo* biosynthesis. In blood vessels, Nogo-B-mediated inhibition of SPT plays an important role in the onset of endothelial dysfunction, hypertension and pathological cardiac hypertrophy.

The upregulation of the sphingolipid *de novo* biosynthesis within a physiological range exerts protective functions, particularly through sphingosine-1-phosphate (S1P) signaling, whereas the exaggerated upregulation of this pathway leads to accrual of ceramide in plasma and tissues, leading to cardiovascular dysfunctions.

Understanding the molecular mechanisms regulating the inhibitory actions of NOGO-B and ORMDLs on SPT activity may provide the basis for therapeutic modulation of this pathway.

Figure 1. Sphingolipid biosynthesis and SPT regulation

(A) In the ER membrane, serine palmitoyltransferase (SPT) starts the *de novo* sphingolipid biosynthesis by condensing serine and palmitoyl-CoA to form 3-ketosphinganine, which is further modified to generate ceramide (Cer). Ceramide can also derive from the catabolism of sphingomyelin (SM) in the plasma membrane or in the lysosome. Ceramidase converts ceramide in sphingosine, which in turn is phosphorylated to sphingosine-1-phosphate (S1P) by the sphingosine kinase 1 or 2 (SphK-1/2). S1P is then transported out of the cells through the spinster-2 (Spns2) or other transporters, where it can bind to and activate S1PR1,3 in an

autocrine manner, or it can bind its plasma carriers, albumin and ApoM of HDL. S1P lyase irreversibly degrades S1P in hexadecanal and phosphoethanolamine, representing the exit point of this pathway.

(B) Schematic representation of SPT complex, including SPTLC1/2, its activators ssSPTa/b and negative regulators ORMDL and NOGO-B. **(C)** Upper panel, schematic representation of Reticulon-4 (aka NOGO) isoforms. Reticulon homology domain (RHD) defines the two transmembrane (TM) domains and the loop of 66 aa connecting them. Lower panel, three proposed topologies for NOGO proteins.

Figure 2. S1P, ceramide and their *de novo* biosynthesis in the cardiovascular pathophysiology
Schematic summary of the roles of ceramide, S1P and Nogo-B-dependent inhibition of SPT in the regulation of vascular tone and BP (left panels) and in cardiomyopathies (right panels).