Sphingolipid Metabolism in Systemic Inflammation

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Introduction

The inflammatory response – induced and regulated by a variety of mediators such as cytokines, prostaglandins, and reactive oxygen species (ROS) – is the localized host's response of the tissue to injury, irritation, or infection. In a very similar and stereotyped sequence, the mediators are thought to induce an acute phase response orchestrated by an array of substances produced locally or near the source or origin of the inflammatory response. Despite its basically protective function, the response can become inappropriate in intensity or duration damaging host tissues or interfering with normal metabolism. Thus, inflammation is the cause and/or consequence of a diversity of diseases and plays a major role in the development of remote organ failure. Better knowledge of the underlying mechanisms of these processes is, therefore, a fundamental pre-requisite fostering the molecular understanding of novel therapeutic targets or diagnostic variables.

Over the past decades, immense attempts have been made to better understand the inflammatory response at the cellular and extracellular level. In the course of these studies, a multitude of lipid mediators of inflammation, such as prostaglandins, leukotrienes, and lipoxins, has been identified and characterized. Much attention has been focused on the function of another major class of lipids, the sphingolipids, which are involved in key regulation processes such as cellular stress response and apoptosis. This chapter highlights the relevance of sphingolipids, their possible role in the regulation of the inflammatory response, and suggests key questions for further research.

Sphingolipids: Structure and Function

Sphingolipids are ubiquitous, inert membrane components of all eukaryotic cells, but are also major constituents of lipoproteins. Most of their functional properties are still being discovered, but there are at least three crucial aspects: structure, recognition, and signal transduction. Originally, sphingolipids were thought to play merely structural roles with rather inert metabolism. However, there has been a fundamental shift in the understanding of their role in modulating various cellular processes such as proliferation, differentiation, induction of apoptosis, and inflammation. The metabolism of sphingolipids comprises a set of highly regulated pathways that serve to control the levels of the individual molecule, their interconversions, and their functions. Most notable of these bioactive molecules are ceramide, ceramide-1-phosphate, sphingosine, sphingosine-1-phosphate, sphingosyl phosphorylcholine, and other derivatives (Fig. 1) [1–3].

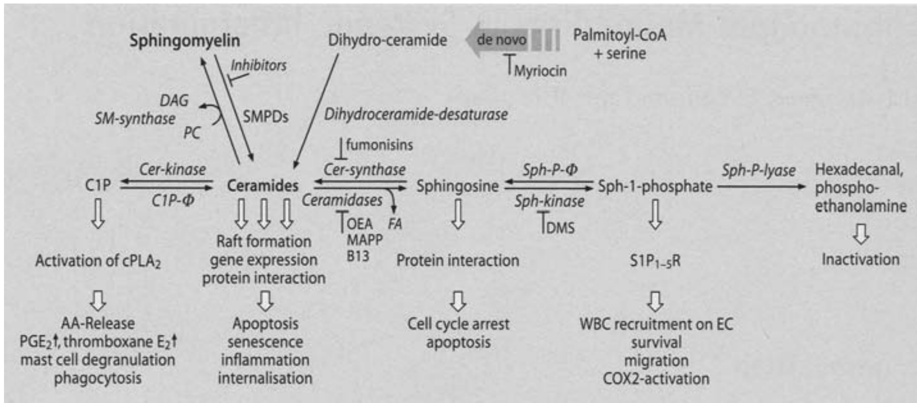

Fig. 1. Sphingolipid metabolism and interconversions. C1P: ceramide-1-phosphate; C1P-Φ: C1P-phosphatase; DAG, diacylglycerol; DMS: N,N-dimethyl-D-erythro-sphingosine; EC: endothelial cell; FA: fatty acid; MAPP: (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol; PC: phosphocholine; PDMP: D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PGE_2: prostaglandin E_2; SMPDs: sphingomyelin phosphocholine diesterases = sphingomyelinases; Sph: sphingosine; Sph-1-P: sphingosine-1-phosphate; Sph-1-P-Φ: Sph-1-P-phosphatase; WBC: white blood cell.

The term 'sphingolipid' generally refers to a number of lipids consisting of a polar head group, which is attached to the primary hydroxy-moiety of ceramides. Ceramides, the central building block of all sphingolipids, consist of a sphingoid base, which is *N*-acylated with fatty acids, differing in length (14–32 carbon atoms) and functionalization (degree of saturation, hydroxylation, etc.). The *de novo* synthesis of ceramides starts with the rate-limiting condensation of palmitoyl-CoA with serine, yielding to dihydroceramide after addition of a long chain fatty acid. The lipid mediator, resulting from desaturation, serves as a precursor for all known sphingolipids, and is further functionalized by addition of a polar head group such as carbohydrates (not referred to in this review) or phosphocholine. In eukaryotic cells, a 4,5-*trans* double bound is essential for bioactivity in lipids with sphingosine backbone. Sphingomyelin (=*N*-acyl sphingosyl phosphocholin) is the other central, but inert storage pool with crucial biophysical functions, which is localized in the outer leaflet of membranes by active flip-flop mechanisms. Removal of the head group – resulting in the generation of ceramide – is catalyzed by the function of several isoforms of sphingomyelinases, which are outlined in detail in Table 1. Accumulation of ceramides in cellular membranes results in the formation of lipid rafts and functional clustering of surface receptors. Another mode of action is performed by direct interaction with proteins in the activation of kinases (e.g., protein kinase C, isoformζ) coupling the action of ceramide to activation of transcription factor, nuclear factor-kappa B (NF-κB) and stress activated protein kinases. In addition, the activation of phosphatases or cathepsin D result in signaling, which enables stimulation of adequate and fine tuned cellular responses. For ceramide clearance, ceramidases hydrolyze the *N*-acyl fatty acid, resulting in the generation of sphingosine, which can be phosphorylated at the primary hydroxy-moiety by isoforms of sphingosine-kinase (SphK) forming sphingosine-1-phosphate (S1P). S1P is cleaved by S1P lyase in an irreversible manner, whereas ceramides or sphingosine can be reversible synthesized by the action of S1P-phosphatase or ceramide synthase, respectively. A

Table 1. Sphingomyelin hydrolyzing enzymes. In human tissues, five RefSeq validated proteins have so far been described, differing in pH-optima, cellular localization and functions. *SMPD1* (sphingomyelin phosphodiesterase 1) codes for the acidic sphingomyelinase, which is either localized in lysosomes by mannose-6-phosphate-receptor/sortilin-dependent trafficking or plasma secreted due to pro-inflammatory stimulation. *SMPD2* codes for a neutral sphingomyelinase-1 (NSM1); however SMPD3 is the *bona fide gene* for NSM2 [83]. A variety of isoform specific inhibitors is described in the right column.

HUGO name (Genbank ID)	pH	cofactor (s)	Localization	Function	Inhibitor(s)
SMPD1 (NM_000534)	~5	none	lysosomes, caveolae, platelets	Metabolic degradation of sphingomyelin; induction of apoptosis, differentiation, inflammation. Congenital deficiency: Nieman-Pick Disease	desipramine, SR33557, NB6, DTT
SMPD1 (NM_000534)	5–7	Zn^{++}	plasma secreted enzyme	Atherogenesis, hemophagocytic lymphohistiocytosis, diabetes, ARDS, sepsis	
SMPD2 (NSMI) (NP_003071)	6.5–7.5	Mg^{2+}	membrane (multi-pass membrane protein)	Differentiation, permeability barrier function	
N.N.	~7	Triton X-100, deoxycholat, phosphatidylserine, (*in-vitro*)	cytoplasma	Differentiation, 1,25-Dihydroxy-vitamin D_3, IFNγ and TNF-α induced signal transduction in HL-60 cells (no RefSeq validation)	Cu^{2+}, Fe^{3+}, Zn^{2+}
SMPD3 (NSMII) (NP_061137)	~7	Mg^{2+}, Mn^{2+} (millimolar); phosphatidylserine, arachidonate	membrane	Hydrolysis of membrane bound SM; differentiation, proliferation, inflammation, apoptosis, lung injury; *bona fide gene*	scyphostatin, glutathione, manumycin, ubichinol, chloro-gentisylchinone, GW4869, C11AG
ENPP7 (NP_848638)	8.0–9.5	bile salts	intestinal lumen; bile, mucosal membrane;	degradation of dietary sphinogomyelin; intestinal PAF-degradation, role in intestinal inflammation or inhibition of colonic tumorigenesis?	

ARDS: acute respiratory distress syndrome; DTT: dithiothreitol; HUGO: Human Genome Organization; IFNγ: interferon gamma; PAF: platelet activating factor; TNF-α: tumor necrosis factor alpha.

major metabolite of ceramide is ceramide-1-phosphate (C1P), which is formed by direct phosphorylation of ceramide by the action of a specific kinase. C1P plays a critical role regulation proliferation, differentiation, apoptosis and generation of eicosanoids *via* direct binding to target proteins, e.g. phospholipase A_2. There is also increasing evidence of a key role in phagocytosis [4], and most importantly, inhibition of SMPD1 by physical interaction has been observed [5].

A major principle in understanding ceramide metabolism (which applies to all other bioactive lipids) is the distinct subcellular localization and topology, which is outlined, e.g., in a review by van Meer and Lisman [6]. In recent years, considerable attention has been given to other types of sphingosine base derivatives, namely 'lyso'-types or *N*-methyl derivatives, which are highly bioactive, however little is known about their origins or function(s).

Beyond the stimulus-dependent activation of sphingolipid metabolism, considerable evidence has been gathered regarding the role of subcellular compartmentalization of the generation and accumulation of individual metabolites. Numerous studies point to a tightly controlled localization of enzyme and lipid pools within the cell, e.g., suggesting a specific, apoptogenic role for endogenously generated ceramide from mitochondrial membranes [7]. Within the different compartments, distinct isoforms of sphingomyelinases perform specific functions *via* generated ceramide, such as inhibition of protein kinase C translocation, inhibition of NF-κB, and aggregation of the Fas receptor [6, 8]. Another mode of action is the endosomal generation of ceramide by acid sphingomyelinase as a direct activator of cathepsin D [9]. Considering that *de novo* synthesis of ceramide is mainly restricted to the endoplasmatic reticulum as well as to membranes associated with mitochondria and the nucleus, the localization and the effects of synthesis inhibitors, such as fumonisins, suggest a pivotal role for ceramide subfractions in mediating apoptosis. Furthermore, recent evidence has indicated the contribution of the mitochondria and the nucleus as major sites in the initiation of apoptosis by ceramide [10].

Due to the dynamic equilibrium of lipid mediators, cells may respond to an extracellular stimulus with perturbation of the balanced 'rheostat' as a consequence of activation of sphingomyelinases followed by accumulation of ceramide and related metabolites. For a better understanding of the sphingolipid flux during the cellular stress response as well as the effects of pharmacological or molecular manipulations of critical enzymes (see below), it is imperative to use reliable measuring systems. Recent molecular advances in the identification of enzymes involved in sphingolipid metabolism and improvements in the analytical equipment, e.g., mass spectroscopy, have facilitated a better understanding of the role and function of sphingolipids [11].

Step by Step: The Hydrolysis of Sphingomyelin

Sphingolipid metabolism is a constitutive process beginning with removal of the head group, phosphocholine, yielding the intermediate product and lipid mediator, ceramide, a reaction catalyzed by a family of enzymes termed sphingomyelinases (or systematically sphingomyelin phosphocholine diesterase, SMPD). The isoforms are distinguished by different pH-optima, localization and cation dependence (Table 1) [12]. Of the five human sphingomyelinases identified so far, the neutral Mg^{2+}-dependent and the lysosomal acid isoform are the most thoroughly studied and, therefore, seem to appear most relevant for generation of ceramide during the stress response [12, 13].

The activity of a neutral pH-optimum, Mg^{2+}-dependent sphingomyelin hydrolyzing enzyme was initially described four decades ago [14]. The maintenance of this isoform's specific activity in both the $SMPD1^{-/-}$ model as well as in cells obtained from patients affected with Niemann-Pick disease Type A, verified that these isoforms are distinct products of different genes [15]. The purified enzyme, termed

neutral sphingomyelinase 1 (NSMI), exhibits an optimal pH at 7.4, an estimated molecular mass around 60 kDa, and a specific activity for hydrolysis of sphingomyelin, but not phosphatidylcholine. The presence of divalent cations such as magnesium or manganese as well as a proper lipid composition containing anionic lipids and unsaturated fatty acids, especially phosphatidylserine and arachidonic acid, are essential for its activity. Recently, it was reported that in caveolae-enriched membrane fractions derived from bovine lung microvascular endothelial cells, a caveolar isoform of neutral sphingomyelinase cross-reacts with specified antibodies against an isoform with a higher molecular weight purified from brain [16].

Two years after the cloning of NSMI, the sequencing and characterization of another mammalian, brain-specific Mg^{2+}-dependent sphingomyelinase, NSMII, was reported [17]. NSMII is also activated by phosphatidyl-serine and other anionic phospholipids, suggesting an enrichment of the protein in the inner leaflet of the plasma membrane, at the mitochondria as well as the endoplasmatic reticulum. Using deletion mutants of the p55 tumor necrosis factor (TNF) receptor, a neutral sphingomyelinase activation domain (NSD) was identified, which is juxtaposed to the death domain of the TNF receptor. A corresponding protein, termed 'factor associated with neutral sphingomyelinase activation' (FAN), was described which binds to NSD, resulting in a functional coupling of the TNF receptor with neutral sphingomyelinase activation [18]. Results demonstrating an interaction of neutral sphingomyelinase with activated C-kinase 1 as well as caveolin-1 further suggest that the formation of multiprotein complexes are involved in the signal transduction from TNF-α to neutral sphingomyelinase, thus regulating its activity [19]. Most recently, it was demonstrated that hydrogen peroxide (H_2O_2)-induced apoptosis of endothelial cells was completely blocked in NSMII-loss of function models [20], highlighting the essential role of NSMII in the induction of apoptosis.

Recent studies have shown that SMPD1 has characteristics of a lysosomal and secretory sphingomyelinase, which both derive from the same gene (*smpd1*) exhibiting differences in the oligosaccharide structure and *N*-terminal proteolytic processing with subsequent differential protein trafficking [21, 22]. As a result the proteins are present in both blood plasma and intracellular lysosomes [12]. Previous studies suggested that the lysosomal mannose-6-phosphate receptor is implicated in SMPD1 trafficking [23]. However, the type I transmembrane glycoprotein, sortilin, is also involved in targeting of the protein: Truncated sortilin partially inhibits lysosomal trafficking and enhances the secretion of SMPD1 [24]. Among the known types of eukaryotic sphingomyelinases, only the secretory variant, SMPD1, has so far been shown to be responsible for extracellular hydrolysis of membrane and lipoprotein bound sphingomyelin [22]. SMPD1 is secreted by macrophages, human skin fibroblasts, and human vascular endothelial cells; the latter are assumed to be the chief source of the enzyme [12, 25]. In endothelial cells, apical as well as basolateral secretion of SMPD1 is stimulated by a variety of pro-inflammatory mediators, including interleukin (IL)-1β, interferon (IFN)γ, IFNβ, TNF-α, platelet activating factor (PAF), and ROS as endogenous mediators, as well as endotoxin as an exogenous mediator [25]. The secreted form of SMPD1 is stimulated by zinc-ions, whereas the lysosomal form is already tightly bound to the cation. Thus, the source of the activity can be distinguished as originating, e.g., from damaged and disintegrating endothelium or from activated cells driven by a pro-inflammatory impetus. An increase to pH 7.4 which is far beyond the optimum for the lysosomal protein as found in plasma, appears to affect only the substrate affinity (i.e., the K_m), but not the activity (V_{max}) of SMPD1 [24], a fact important in estimating the extralysosomal activity of the enzyme [21, 27].

In the following section, cellular mechanisms of the action of sphingolipid mediators are briefly discussed.

Receptor-mediated Effects

The lysosphingolipid, S1P, functions as a ligand for at least five G-protein species coupled to cell surface receptors termed $S1P_{1-5}R$ regulating cell proliferation, apoptosis and motility. Activation by S1P binding results in sequestering of lymphocytes from the circulation to lymph nodes and Peyer's patches. This redistribution effectively reduces T cell numbers at the sites of inflamed tissue or graft sites. TNF-α induced sphingosine kinase 1(SphK1) activity is followed by increased S1P levels and subsequent cyclooxygenase (COX)-2 activation. The inhibition of S1P clearance, e.g. by targeting S1P lyase or S1P phosphatase, augments COX-2 activation and consequently prostaglandin (PG)E2 generation. Exogenous S1P addition dose-dependently reproduced COX-2 induction observed subsequent to TNF-α addition. In neutrophils, the hydrolysis of sphingomyelin increased SphK activity, and S1P generation appears to be a key event in neutrophilic priming by TNF-α and other stimuli [28, 29]. During activation of mast cells, S1P levels increase resulting in the release of inflammatory mediators such as leukotrienes and cytokines. In contrast, sphingosine has an opposing effect on mast cell activation [30].

Cell migration is crucial to the proper functioning of the cells involved in the course of inflammatory processes. The SphK/S1P pathway plays an important role in chemoattractant signaling in myeloid differentiated HL-60 cells [31]. In addition, S1P was found to act as a specific and effective regulator of migration of freshly isolated human neutrophils across endothelial cells [29]. This transmigration, which is essential for the recruitment of white blood cells to the site of inflammation, is enabled by the expression of adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, on endothelial cells.

Interest in sphingolipids as signaling molecules in immune cells increased as it became evident that sphingosine, as well as ceramide, induces apoptosis in T cells, whereas S1P emerged as a counterregulatory principle; in Th2 cells sphingosine (but not ceramide) exerts inhibiting effects on proliferation, implying that individual immunological responses depend on the dynamic balance of sphingolipids [2].

Raft Formation: Ceramide-induced Reorganization of Membrane Receptors

There is currently no defined receptor for ceramide; however, the lipid has been shown to be involved in signal transduction by altering membrane organization and fluidity. Ceramide has the tendency to self-associate and to form ceramide-enriched microdomains that spontaneously fuse to large ceramide-enriched macrodomains, also termed ceramide-enriched platforms. These biophysical properties act to reorganize very small distinct domains in the cell membrane, termed rafts, which serve in the spatial organization of signaling molecules. Thus, ceramide enriched membrane platforms have been shown to mediate clustering (i.e., tighter packing) and recruitment of signaling molecules, while excluding others, and to reorganize the topology of proteins and aggregation of receptors inducing the transduction of signals.

These specialized domains of the cell membrane are central for the spatial organization of receptors and signaling molecules. Upon stimulation, acid sphingomyelinase is translocated to the outer leaflet of the cell membrane, apparently mediated by a fusion of sphingomyelinase containing vesicles with the cell membrane resulting in the cell surface exposure of the enzyme. Generated ceramide then contributes to the formation of both small and large rafts, ceramide-enriched platforms, which in turn may amplify signaling in response to stress, irradiation, ultraviolet light, gamma irradiation, doxorubicin, cisplatin, disruption of integrin-signaling, TNF receptor, CD40, LFA-1, DR5/TRAIL, CD20, FcγRII, CD5, LFA-1, CD28, TNF, IL-1 receptor, PAF-receptor, and CD14 [32].

CD95-dependent apoptosis requires a pre-association of CD95, the formation of the death-inducing signaling complex (DISC), and clustering of CD95 in specific membrane domains. In this context, the acid isoform, activated upon CD95 ligation and initial caspase 8 activation, is translocated and functions upstream of the DISC to mediate CD95 clustering in ceramide-enriched membrane platforms, an event required for DISC formation, yielding full caspase 8 activity and apoptosis [33].

Identical mechanisms seem to be operative in the signaling of apoptosis by other death receptors or stress suggesting a general role of ceramide-enriched platforms in apoptosis and explaining the function of SMPD1 and ceramide in multiple signaling pathways [34, 35].

The metabolic precursor, sphingomyelin, is important for Fas receptor clustering through aggregation of lipid rafts, leading to Fas-mediated apoptosis. Experiments with sphingomyelin synthase (SMS)-defective WR19L cells transfected with the human Fas gene (WR/Fas-SMS$^{-/-}$), and cells that have been functionally restored by transfection with SMS1 (WR/Fas-SMS1), show that expression of membrane sphingomyelin enhances Fas-mediated apoptosis through increasing efficient translocation of Fas into lipid rafts, Fas clustering, DISC formation, and subsequent activation of caspases [34].

Role of Sphingolipids in Regulation of the Immune Response, Susceptibility to Infection, and Triggering of Pathogen-associated Apoptosis

Some pathogens activate SMPD1, which releases ceramide in membrane rafts, structures which enable a host/pathogen interaction by formation of negative membrane curvatures. An abundance of evidence indicates that the formation of ceramide-enriched membrane raft structures facilitates the invasion of various pathogens [37–39]. Often, the final result of ceramide-mediated cellular entry is containment and/or inactivation of the pathogen. The importance of ceramide in pathogen invasion is underscored by studies investigating *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Sindbis virus*, which have been shown to activate SMPD1 resulting in rapid ceramide formation. The strength of these studies was the demonstration that inactivation of SMPD1 nearly completely hindered pathogen internalization. As a result, all pathogen-associated alterations of membrane scaffold have been shown to mediate internalization of bacteria, viruses, and parasites into the host cell, to initiate apoptosis of the host cell upon infection, and to regulate the release of cytokines from infected mammalian cells [37]. On the other hand, ceramide-enriched membrane platforms are also central to the host defense against potentially lethal pathogens like *P. aeruginosa* which, upon infection, trig-

gers activation of SMPD1 and the release of ceramide in sphingolipid-rich rafts within minutes [39]. Failure to generate ceramide-enriched membrane platforms in infected cells results in an unabated inflammatory response, such as massive release of IL-1β and septic death in mice.

In addition, the protozoan, *Leishmania donovani*, was shown to induce ceramide formation by both *de novo* synthesis and SMPD1 activation, resulting in elevated ceramide levels which facilitate the survival of the parasite in the intramacrophageal milieu [41]. Rafts, in addition to playing a crucial role in pathogen entry, have also been shown to serve as platforms for viral assembly or budding and in the intracellular trafficking of phagosomes [42]. It has also been shown that ceramide-enriched membrane platforms are involved in the infection of human cells with pathogenic rhinoviruses [42]. The infection of human epithelial cells with rhinovirus strains triggers a rapid activation of SMPD1, the formation of ceramide in the cell membrane and, finally, the formation of large ceramide-enriched membrane platforms. These events correlate with microtubule- and microfilament-mediated translocation of the enzyme from an intracellular compartment onto the extracellular leaflet of the cell membrane. In agreement with a key role of SMPD1 and ceramide in the infection of human cells with rhinoviruses, genetic deficiency or pharmacological inhibition of the SMPD1 prevented infection of human epithelial cells by rhinoviruses.

The susceptibility to infections of individuals with these diseases, and with elevated plasma levels of SMPD1, which has been observed during the course of sepsis and systemic inflammation (see below), might contribute to an altered immune response rendering individuals susceptible to other, secondary infections, e.g. from colonizing bacteria. All these studies support the notion that rafts and ceramide-enriched membrane platforms function as central structures involved in the infection of mammalian cells by pathogens and as targets for the development of anti-infective drugs.

Role of the Oxidative Balance

The tripeptide, glutathione (GSH), plays a major role in cellular redox homeostasis. Several lines of evidence suggest that ROS, such as superoxide radical ($O_2^{\cdot -}$), H_2O_2, and the hydroxyl radical (·OH), as well as reactive nitrogen species, such as the peroxide radical ($\cdot ONOO^-$) and the cellular redox potential, which is mainly regulated by GSH concentration, are tightly linked to the regulation of sphingolipid hydrolysis.

NSMI activity requires the presence of reducing agents, and recent studies have shown that it is reversibly inhibited by ROS and oxidized glutathione, whereas it is irreversibly inhibited by peroxynitrite [43]. As described above, NSMII activity and trafficking are tightly regulated by the oxidative intracellular status in a complex dynamic process [20]. On the other hand, sphingolipids are also known to play an important role in maintaining cellular redox homeostasis through regulation of plasma membrane oxidants, such as NADPH oxidase, with subsequent disturbance of mitochondrial integrity and induction of apoptosis [44]. Post-translationally, both recombinant and plasma borne SMPD1 are also directly activated in the presence of oxidizing agents by modification either of a 'cysteine switch' or by a copper induced dimerization via disulfide bond formation responsible for an increase in enzymatic hydrolysis [45, 46]. It is, therefore, tempting to speculate that at least some of the above-named stress stimuli stimulate SMPD1 via redox processes. Ceramide itself is

known to trigger the release of ROS, from, for example, endothelial cells [47]. Thus, under oxidative conditions, it might be part of a self-perpetuating and positive feedback mechanism for the post-translational activation of SMPD1. Interestingly, in neutrophils, ceramide generation, CD95 clustering, and apoptosis were dependent on ROS suggesting that an altered redox status initiates ligand-independent death receptor signaling via activation of SMPD1 and clustering of preformed DISC components in lipid rafts [48].

Increased SMPD1 Activity: Cause or Consequence in Organ Failure?

A 2–3-fold increase in plasma sphingolytic activity has been observed in animal models after application of endotoxin or pro-inflammatory cytokines (TNF-α, IL-1β) [49–51]. In a human setting, there is indirect evidence for altered SMPD1 activity during systemic disease. An increased ceramide/sphingomyelin ratio has been reported in septic patients as well an association with a poor clinical outcome [52]. At a cellular level, increased ceramide concentrations have been reported in circulating mononuclear cells of septic patients that were positively correlated with plasma TNF-α levels [53]. In this study, patients with multiple organ failure (MOF) exhibited a more pronounced ceramide accumulation. Furthermore, raised SMPD1 levels have also been reported in children with hypercytokinemia due to hemophagocytic lymphohistiocytosis [54]. In order to achieve a deeper insight into the functional role of SMPD1 during sepsis, we analyzed its presence and activity in patients with various degrees of different concomitant diseases, variable sources of infection, and a broad range of disease severity as reflected by differing levels of inflammatory markers. Circulating SMPD1 was found to be markedly elevated on the first day of sepsis. During the course of the disease, an inverse trend between survivors (decrease) and non-survivors (further increase) as well as a positive association with organ failure scores was observed [45].

The first clinical implications for inhibition of sphingolipid hydrolysis came from studies dealing with the lysosomal activity of SMPD1 in leukocytes obtained from patients with major depression [55]. In addition to an increase in the constitutive activity in peripheral blood mononuclear cells dependent on illness severity, *ex vivo* treatment of the cells with tricyclic antidepressive drugs, such as amitryptiline or imipramine, resulted in a rapid reduction in SMPD1 activity. As reported above, ceramide generation changes the composition of membrane structures thus mediating the formation of platforms, which facilitate receptor clustering and signaling. Thus, alterations in SMPD1 activity may have clinical implications for the regulation of serotonin and dopamine reuptake transporter activity. The pathophysiological significance of altered SMPD1 activity in major depression remains to be further elucidated. The observed increase, however, supports the concept that SMPD1 activity and ceramide generation may function in these molecular phenomena, may contribute to subsequent interference with other enzymes such as phospholipase A_2 and isoforms of protein kinase C, as well as synaptic transmission. It is, therefore, tempting to speculate that SMPD1 activity may be a molecular target for antidepressant drug therapy.

There is also an increasing body of evidence that extracellular hydrolysis of sphingomyelin due to secreted SMPD1 activity may be involved in mediating systemic effects. Induced by the massive release of cytokines and other factors into the circulation, this phenomenon, termed generalization, leads to functional effects

occurring outside the genuine locus of actual infection or treatment, which is often observed in the pathogenesis of MOF or subsequent to radiotherapy for various malignancies. Sathishkumar et al. demonstrated, in patients who underwent high dose spatially fractionation radiation, an elevation of both the level of plasma secreted SMPD1 activity and the concentration of lipoprotein bound ceramide, with a correlation of SMPD1 activity and clinical outcome [56]. Most importantly, a role of SMPD1 and ceramide in the generation of PAF-mediated pulmonary edema was shown by Goggel and colleagues [57]. In an animal study, it was clearly demonstrated that SMPD1 plays a critical role both locally in ceramide generation in the stimulated tissue as well as in the increase of vascular permeability resulting in edema formation. Accumulation of ceramide was paralleled by synthesis and release of aspirin-inhibitable prostaglandin production. SMPD1 deficient mice exhibited 50% less pulmonary edema than wild type animals. Consistent with these findings, agents interfering with ceramide generation as well as anti-ceramide antisera reduced edema formation initially triggered by exogenously administered PAF, bacterial endotoxin, or intratracheal instillation of acid. The latter two models are relevant to the increased permeability during lung edema observed in the clinical condition of sepsis or aspiration-induced pneumonia, which are common and often lethal precipitators of acute lung injury (ALI) in humans [58]. On the other hand, systemic effects were proven by the release of the enzyme into circulation via venous efflux enabling extra-pulmonary ceramide generation at the outer leaflet of remote endothelial membranes [57]. In this context, it is noteworthy that until now plasma secreted SMPD1 is the only enzyme shown to be responsible for extracellular sphingomyelin hydrolysis; activity of NSMII or other sphingomyelinase isoforms has not been observed in plasma [49, 57]. Additionally, SMPD1 translocates to cell surfaces and has activity in the outer leaflet of the cellular membrane; the membrane-associated enzyme may behave differently to recombinant protein administered in solution [34]. However, it is not yet clear whether the enzyme translocated onto the plasma membrane upon CD95 stimulation [33] is identical to the form described by Tabas as the plasma secreted isotype [21].

Signaling by ceramide is also critically involved in molecular mechanisms triggering ischemia/reperfusion injury as well as TNF-α induced organ damage. In the liver, ceramide levels transiently increased in galactosamine/TNF-α-induced liver damage or after the reperfusion phase of ischemia due to early activation of hepatic SMPD1. Inhibition of sphingolytic activity decreased ceramide generation and subsequent increase in surrogates for tissue damage, hepatocellular apoptosis, and mitochondrial targeting of apoptosis-triggering gangliosides, resulting in cytochrome c release [59, 60]. Thus, modulation of sphingolipid signaling may be of therapeutic relevance, e.g., in hepatic tissue injury.

In addition, thrombin activated thrombocytes release SMPD1 [61] and these cells may be a candidate source for SMPD1 because they are critically involved in triggering thrombotic and inflammatory events, e.g., resulting in lung edema [58]. By triggering Weibel-Palade body exocytosis, ceramide activated endothelial cells release von Willebrand factor and P-selectin, which induce leukocyte rolling as well as platelet adhesion and aggregation [62]. This phenomenon, which was also observed after addition of sphingomyelinase, suggests a novel and intriguing mechanism by which ceramide may contribute as an intermediary to vascular inflammation raising a thrombophilic state. A hypothetical schema of the functions of plasma secreted SMPD1 and subsequent ceramide generation during systemic inflammation and infection is outlined in Fig. 2.

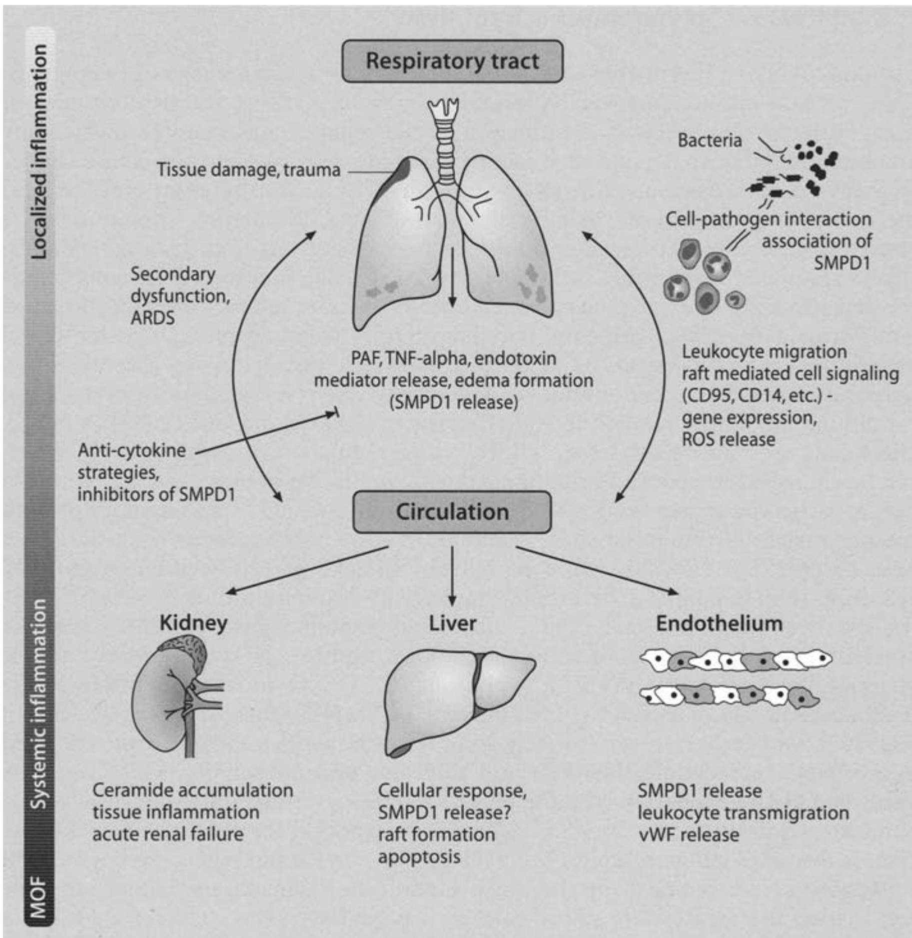

Fig 2. Plasma secreted sphingomyelinase in inflammation. Hypothesized impact of sphingomyelin phosphocholine diesterase (SMPD)1 in the development and exacerbation of systemic inflammation. ARDS: acute respiratory distress syndrome; vWF: von willebrand factor; MOF: multiple organ failure.

It is noteworthy that the observed plasma activities in patients with inflammation of different origins clearly exceed the enzymatic activity reported to induce biological effects in cell culture systems [63, 64]. SMPD1 has been shown to hydrolyze sphingomyelin in low density lipoprotein (LDL) particles [27], which is important considering that most of serum sphingomyelin and nearly half of the ceramides are known to be localized in circulating LDL [49]. Therefore, the accumulation of ceramide in both circulating cells and in lipoproteins may serve as a persistent reference pool reflecting elevated plasma secreted SMPD1 activity. Though not proven, it might, therefore, be possible to discriminate between transient/short-term effects and long lasting/even mild variations by determination of enzyme activity levels in a functional assay or resulting ceramide accumulation. According to our own observations and data from other groups, there is only marginal degradation of plasma ceramide, resulting in the mentioned accumulation of the mediator in lipophilic compartments, such as plasma lipoproteins.

Inhibitors of Sphingomyelin Hydrolysis

The availability of specific pharmacological inhibitors of some enzymes of sphingolipid metabolism and ongoing molecular cloning of some of the key enzymes of these pathways, has allowed examination of the cellular consequences of inducing accumulation of endogenous ceramides or ceramide clearing enzymes. These studies have provided substantial evidence for the role of sphingomyelinases and the lipid mediator, ceramide, in initiating cellular responses. Numerous compounds are known to inhibit NSMI. Structural analogs of the naturally occurring compounds, scyphostatin and manumycin A, have been used [65, 66]; however, these compounds contain a reactive epoxide moiety, which enables them to interact directly and covalently with a variety of proteins, thus hampering the interpretation of biological experiments. For this reason, a panel of compounds was synthesized containing a polyunsaturated fatty acid bound to a chemically less reactive, under physiological conditions, 1,2-amino alcohol derivative with a cyclohexenone moiety [65]. A redox-dependent reversible mechanism is involved in regulation of NSMI activity, which can be abolished by reduced glutathione during induction of programmed cell death due to ischemia in neuronal cells [13]. Structural analogs of sphingomyelin with reactive structural moieties, such as difluoromethylenephosphonic acid, have also been described as effective, non-competitive inhibitors of TNF-α-induced neuronal cell death [67]. In addition, various low molecular weight inhibitors of SMPD1 activity have been identified, including 5'-adenosine monophosphate (5'-AMP); tricyclic antidepressive drugs, such as imipramine, amitryptiline, or desipramine; cationic amphiphilic drugs, such as NB6, L-α-phosphatidyl-D-myo-inositol-3,5-bisphosphate (PtdIns3,5P2), and phosphatidyl-myo-inositol 3,4,5-triphosphate [PtdIns (3,4,5)P(3)]; SR33557; and derivatives of α-mangostin. Tricyclic antidepressants induced rapid intracellular degradation of SMPD1 and abolished enzyme activity, which could be abrogated by preincubation with the protease inhibitor, leupeptin. Interestingly, data obtained using plasmon resonance technology supported the concept of an interaction of the amphiphilic inhibitor with the enzyme and immobilized sphingomyelin containing lipid bilayers, displacing the protein from its membrane bound substrate and rendering it susceptible to proteolytic cleavage [68].

In an experimental model of endotoxic shock, specific inhibition of SMPD1 by the carbazol derivative, NB6, resulted in decreased hepatocellular apoptosis and improved survival rate, providing further evidence for a crucial role of SMPD1 in the pathogenesis of systemic inflammation and subsequent organ failure [45]. Remembering that SMPD1 fulfils crucial functions in host defense, a non-beneficial effect of SMPD1 inhibition during infection of mice with living bacteria in a model of polymicrobial peritoneal contamination and infection with overwhelming mortality after instillation of human feces is feasible (Bunck et al., unpublished data).

FTY720, a substrate for SphK has shown tremendous promise as a regulator of multiple levels of inflammation. FTY720 is chemically derived from myriocin, an ascomycete metabolite, and is metabolized to the phosphor-derivative, FTY-P, by sphingosine kinases to become active as an S1P agonist at four of the five known S1P receptors [69]. FTY720 has been tested as an immunomodulator in renal transplant patients and patients with emphysema. The Sph1P mimetic produces lymphopenia by reducing recirculation of lymphocytes and sequestering them into lymph nodes. FTY-P induces $S1P_1R$ internalization of lymphocytes, which abrogates the interaction with the naturally occurring ligand, S1P, regulating lymphocyte trafficking between lymphoid organs and the sites of inflammatory response. The com-

pound also induces CD31 and β-catenin expression in subcapsular sinus endothelial cells in lymph nodes. The modulation of the inflammatory response by FTY720 may be a useful intervention in a number of inflammatory conditions by targeting bioactive sphingolipid signaling function.

Cracking the Enigma of the Sphingolipids

Distinct analysis of the biological effects mediated by variations in the key sphingolipid enzyme activity or its localization, as well as the complex and structurally diverse composition of the mediators, was hampered for a long time by the lack of an accurate and reliable methodology to measure the sphingolipid factors relevant in, for example, pro-inflammatory signaling. A plethora of papers stress the importance of distinguishing between *de novo* synthesized sphingolipids and degradation products derived from turnover or metabolism from more complex sphingolipids. Over the past decades, sphingolipids, e.g. ceramide, have been discovered to have not only structural but also signaling properties, especially during the stress response. The dynamic balance between sphingolipid metabolites existing in a phosphorylated or dephosphorylated as well as in an acylated or deacylated form, has been recognized as a fundamental factor determining cell fate because of its ambiguous and often opposing properties. On one hand, consistent with their functions as bioactive lipids, ceramide and its metabolites are present in very low levels in the cell's common lipid machinery. On the other hand, the interconvertibility of these mediators and the highly variable kinetics in metabolism present a difficult technical challenge for examining the absolute concentrations at operator-defined time points. This is of particular relevance when various agents for stimulation or analysis of cells consisting of a multitude of cellular subpopulations such as circulating leukocytes are tested. Biological examinations frequently focus on pro-apoptotic or cyto-protective effects alone when mediators are studied. Thus, for a more complete picture it is imperative to analyze all or at least a majority of the lipid mediators potentially responsible for the effects of interest. For this purpose, a number of relatively specific enzymatic methods have been developed based on the use of lipid kinases. These assays are relatively insensitive, time consuming, and require huge amounts of lipid material [70–72]. Derivatization and subsequent separation by high performance liquid chromatography and fluorescence detection have improved detection sensitivity [73, 74]. The problem of co-elution of related, very similar or interfering compounds, however, has to be addressed. In addition, there is an urgent need for the preparation of internal standards for each unique mediator of interest. Methods based on metabolic labeling of the cellular sphingolipid pool with radioactive or fluorescently labeled compounds are also burdened with concerns regarding improper distribution within the cell and the cellular substructures, uneven behavior in the cellular membrane, and questionable metabolic and enzymatic properties in comparison to the naturally occurring structure of interest. Overall this may be an inefficient and inaccurate method for absolute mass level determinations [75–77].

The limitations of these 'one single lipid experiments' can now be addressed by the use of recently developed methodologies, such as liquid chromatography, coupled to subsequent tandem mass spectroscopy resulting in an approach called 'sphingolipidomics' [78, 79]. The use of mass spectroscopy is an intriguing feature for more detailed study of numerous agonists and antagonists and to better address

the effects of previously undetectable variations in concentrations of mediators with a sphingolipid backbone. In fact, the methods of sphingolipidomics also facilitate the determination of isotype specific effects, such as the induction of mitochondrial apoptosis exclusively by C16:0 ceramide [80]. Additional information is also obtained by the analysis of the biological significance of the presence or absence of a double bond in the backbone. This difference cannot be distinguished by conventional methods due to strong structural similarity and the poor discrimination when using enzymatic methods. Saturated analogs, however, are usually biologically inactive [81]. Sphingolipidomics will provide thrilling information on the source and the 'job history' of a unique metabolite by comparative analysis of the levels of its *de novo* precursors versus its degradation products. The convenience of the method can also be applied for the discrimination of cellular effects of extra- or intracellular sphingolytic activity as well as for the determination of the biological effects, e.g., of a selected sphingomyelinase isoform targeted to distinct sub-cellular compartments. Such studies are mandatory for probing the impact of different intracellular sphingomyelin pools [7, 82]. We are convinced that sphingolipidomics will provide useful information on the role, the origin, and the fate of a variety of sphingolipids orchestrating cellular signaling.

Conclusion

There is increasing evidence suggesting a pivotal role of sphingolipids as mediators regulating apoptosis, the cellular stress response, and inflammation. Key regulating enzymes in these processes are sphingomyelinases and ceramidases, generating a fine tuned 'rheostat' between lipid mediators often responsible for opposite cellular effects. The generation of knock-out models and administration of specific low molecular weight inhibitors has enabled the detailed study of the effects at a cellular level. Accumulating evidence emphasizes a critical role of ceramide in systemic inflammation mediated by a plasma secreted isoform of acid sphingomyelinase, SMPD1. An intriguing functional concept for the role of plasma secreted SMPD1 in receptor signaling activation pathways suggests that the enzyme modifies membrane fluidity by the formation of ceramide enriched rafts. Subsequent structural alterations of membrane morphology may then allow rapid and efficient signaling inside the cell, explaining the function of the enzyme in a variety of effects in cellular stress response, but also in the development of MOF during systemic inflammation. Accordingly, plasma secreted SMPD1 is hypothesized not only to function as a signaling molecule *per se*, but also to be involved in the host response and development of remote organ failure. However, for future therapeutic interventions, it is very important to specifically target the enzyme and the ceramide pool in the precise tissue or cell. A therapeutic intervention of ceramide generation might be envisioned to prevent tissue damage during development of organ failure and to prevent infection of mammalian cells with *P. aeruginosa* and other pathogens.

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