Apoptosis (2007) 12:923–939
DOI 10.1007/s10495-007-0721-0

Sphingolipids and cell death

Albert Morales · Hyunmi Lee · Felix M. Goñi · Richard Kolesnick · José C. Fernandez-Checa

Published online: 6 February 2007

Abstract Sphingolipids (SLs) have been considered for many years as predominant building blocks of biological membranes with key structural functions and little relevance in cellular signaling. However, this view has changed dramatically in recent years with the recognition that certain SLs such as ceramide, sphingosine 1-phosphate and gangliosides, participate actively in signal transduction pathways, regulating many different cell functions such as proliferation, differentiation, adhesion and cell death. In particular, ceramide has attracted considerable attention in cell biology and biophysics due to its key role in the modulation of membrane physical properties, signaling and cell death regulation. This latter function is largely exerted by the ability of ceramide to activate the major pathways governing cell death such as the endoplasmic reticulum and mitochondria. Overall, the evidence so far indicates a key function of SLs in disease pathogenesis and hence their regulation may be of potential therapeutic relevance in different pathologies including liver diseases, neurodegeneration and cancer biology and therapy.

Keywords Ceramide · Gangliosides · Membranes · Transbilayer trafficking · Mitochondria · Endoplasmic reticulum

A. Morales · J. C. Fernandez-Checa (✉)
Liver Unit and Centro de Investigaciones Biomédicas Esther Koplowitz, IMDiM, Hospital, Clínic i Provincial, Instituto Investigaciones Biomédicas August Pi i Sunyer (IDIBAPS), CIBER-HEPAD and Department of Cell Death and Proliferation, Instituto Investigaciones Biomédicas Barcelona, Consejo Superior de Investigaciones Científicas,
08036 Barcelona, Spain
e-mail: checa229@yahoo.com

H. Lee · R. Kolesnick
Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center,
1275 York Avenue, New York, NY 10021, USA

F. M. Goñi
Unidad de Biofisica (Centro Mixto CSIC-UPV/EHU), and Departamento de Bioquimica, Universidad del Pais Vasco,
48080 Bilbao, Spain

Introduction

Evidence has been provided during recent years that sphingolipids (SLs) are more than just mere structural components of biological membranes. Many different stimuli generate or stimulate the upregulation of SLs, particularly, ceramide which, upon interaction with downstream targets, mediate specific cell responses that range from proliferation to growth arrest, apoptosis, differentiation or recognition. Intriguingly, some SLs such as ceramide and sphingosine 1-phosphate (S1P) exert opposing functions in the regulation of cell death and survival, and hence the relative balance between ceramide/S1P determines the fate of cells in response to specific stimuli [1, 2]. The role of ceramide in cell death has been best characterized and has attracted much attention, despite the fact that its relevance was called into question due to technical issues related to its determination and the suggestion that ceramide generation might be a consequence rather than a cause of cell death [3, 4]. However, the validation of the methods for ceramide measurement and the availability of clear-cut evidence indicating that ceramide generation occurs prior to the commitment of the effector phase of apoptosis [5], have reinforced a causal role for ceramide as an early mediator of cell death in response to many inducing stimuli [1, 2, 5]. Moreover, the activation of sphingomyelinases (SMases) which hydrolyze sphingomyelin pools in membranes provide a strategy for the fast generation of ceramide

(within minutes to hours) in response to numerous inducers, consistent with the notion that ceramide generation serves as a second messenger that actively participates in the initiation of cell death pathways [6, 7]. In addition to the ability of ceramide to act as a transducing signal and as a cell death promoter, ceramide serves also as a precursor for complex glycosphingolipids (GSLs) and gangliosides synthesized in the Golgi, which then distribute to different membrane compartments [8]. This subfamily of SLs shares with ceramide the carbon backbone to which different sugars and sialic acid residues are added, implying that certain biological functions are common for both ceramide and GSLs, while others are exclusive for the latter. The purpose of this review is to bring up to date recent data on the role of ceramide and SLs in cell death, the mechanisms involved and the relevance in disease pathogenesis.

Metabolism and regulation of sphingolipids

Ceramide is the prototype SL that has been most intensively studied in relation to cell death induction and in stress response. Ceramide can be generated by different pathways, and since its metabolism into other GSLs or transformation into survival derivatives, such as S1P, modulate its participation in cell death mechanisms, we will briefly review its generation and biotransformation (Fig. 1).

Ceramide generation

The capacity for the *de novo* ceramide biosynthesis is widespread among cell types and tissues. Ceramide *de novo* synthesis occurs on the endoplasmic reticulum (ER) with the condensation of L-serine and palmitoyl-CoA to form 3-ketosphinganine, catalyzed by the pyridoxal phosphate-dependent enzyme serine palmitoyl transferase (SPT), the rate limiting enzyme in ceramide biosynthesis [9]. 3-ketosphinganine is then reduced to the sphingoid base sphinganine and acylated to generate dihydroceramide by the (dihydro)ceramide synthase. Dihydroceramide is then oxidized to ceramide catalyzed by dihydroceramide desaturase, which introduces a *trans*-4,5 double bond. Mammals and other species including plants have multiple ceramide synthase genes, whereas most of the other enzymes in the SLs biosynthetic pathway exist in only one or two isoforms [10]. Although the reason for this diversity of genes expressing ceramide synthase is uncertain, it implies an important role for ceramides containing specific fatty acids in cell physiology and death regulation [11, 12]. The *de novo* ceramide synthesis is necessary for many cellular functions as inferred from the detrimental consequences of its inhibition. Fumonisins, mycotoxin contaminants of maize, inhibit (dihydro)ceramide synthase and are known to cause cancer, leukoencephalomalacia, pulmonary edema, and liver and kidney toxicity [9, 13, 14]. By inhibiting (dihydro)ceramide synthase, fumonisins cause the accumulation of sphinganine in tissues, serum, and urine, which is widely used as a biomarker of fumonisin exposure. The accumulation of sphinganine appears to be responsible for many of the deleterious effects of these mycotoxins, although depletion of complex SLs needed for optimal membrane functions may contribute to the manifestations of toxicity.

In addition to the *de novo* synthesis, ceramide can arise from hydrolysis of sphingomyelin(SM)-engaging SMases,

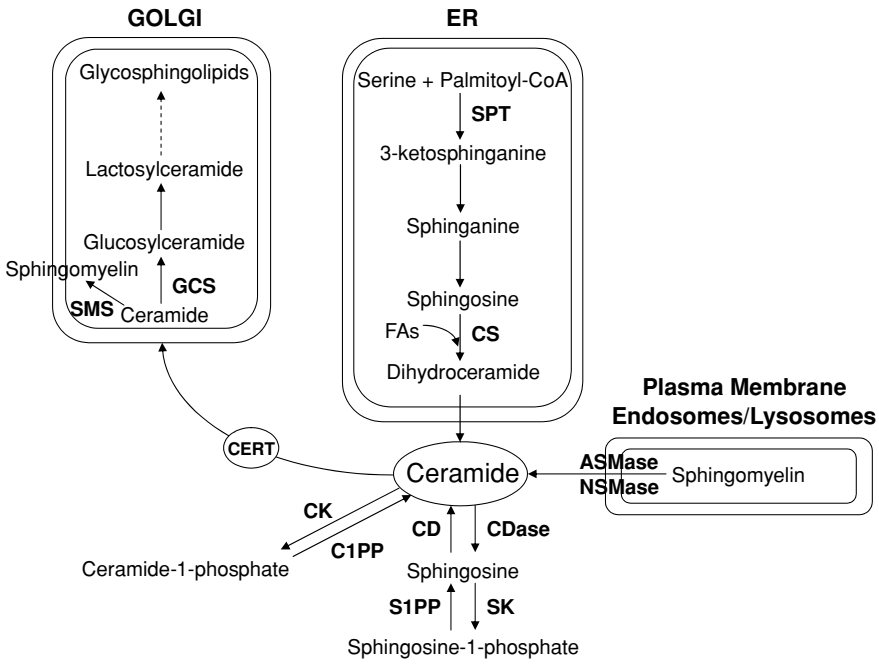

Fig. 1 Ceramide generation and biotransformation. Ceramide can arise from the ER where the molecular machinery for its *de novo* synthesis resides as well as from sphingomyelin hydrolysis at the plasma membrane and intracellular acidic compartments (endosomes/lysosomes) via NSMase and ASMase activation. The trafficking of ceramide to the Golgi fuels the synthesis of complex GSLs including gangliosides. The metabolism of ceramide involves its conversion into S1P via the concerted action of CDases and SK and into C1P via CK that antagonize the role of ceramide in cell death pathways

contributing to increased ceramide levels over a period of minutes to hours. Several SMases have been characterized of which two classes are of relevance in signaling [6, 7]. The membrane-bound neutral SMases (NSMase) exhibit an optimum pH of 7.4 and are being characterized at the molecular level [15–18]. Three mammalian NSMases have been cloned. While the function of NSMase1 is uncertain as its biochemical characterization revealed a lyso-platelet activating factor-phospholypase C activity, NSMase2 exhibits a requirement for Mg^{2+} and a pH optimum of 7.4 and it is membrane bound. However, its specific expression in brain questions the relevance of this enzyme in SLs metabolism and in cell death. Recently, Krut et al. reported a third NSMase3 which, as NSMase2, exhibited Mg^{2+} dependency and pH optimum of 7.4 but, unlike NSMase2, is ubiquitously expressed [19]. NSMase3 is distributed mainly in the ER and Golgi and becomes activated very quickly upon stimulation with TNF. However, since TNF binding to its plasma membrane receptors initiates a complex signaling device, the relevance and mechanisms of this ER/Golgi-based NSMase in TNF signaling and cell death remains to be further clarified. Furthermore, acidic SMases (ASMases) exhibit a pH optimum of around 4.8 of which several isoforms have been described [20, 21]. Three different pools or ASMase isoforms have been described. An acidic lysosomal/endosomal ASMase consistent with its strict acidic pH requirement, a secretory ASMase associated with inflammation and stress responses, and a receptor-activated ASMase, which undergoes a translocation to the outer cell membrane in specific microdomains, where serves as signaling platforms by cell surface receptors such as Fas [20–22]. In addition, the ceramide pool released upon ASMase activation has been shown to contribute to TNF-mediated death by downstream mechanisms that ultimately target and recruit mitochondria [23, 24]. A key feature in this function was the observation that lysosomotropic agents that abolish intracellular vesicle acidification prevented ASMase-induced ceramide generation by TNF or exogenous ASMase and subsequent hepatocellular death [25, 26]. In addition, preventing the endocytosis of exogenous ASMase via mannose 6-phosphate receptor blocked its activation, ceramide generation and hepatocellular death, suggesting that the trafficking of ASMase into acidic vesicles is necessary for TNF-mediated death. Thus, although both NSMase/ASMase release ceramide in cells, their relative contribution to cell death signaling may depend on several conditions including the kind of apoptotic stimuli used and the cell type studied.

Finally, besides these pathways, recent observations have provided evidence for a novel path in which ceramide is generated from the processing of GLSs at the plasma membrane. Using conditions in which sugar units of GM3 were detached, Valaperta et al. showed the formation of ceramide at the plasma membrane in human fibroblasts [27]. The production of ceramide from GM3 occurred under conditions that blocked endocytosis or lysosomal activity, and the overexpression of the plasma membrane ganglioside sialidase Neu3 correlated with a higher production of ceramide in the plasma membrane. Moreover, recent evidence has disclosed the presence of ceramides in mitochondria with the detection of ceramide synthase and reverse ceramidase in these organelles [28, 29]. One caveat to these observations, however, was the contamination with the ER (endoplasmic reticulum)-related compartment mitochondria-associated membrane (MAM). However, highly purified mitochondria and MAM indicated the presence of both ceramide synthase and reverse ceramidase, although the latter enzyme activity was barely detectable in microsomes [29]. Overall these studies demonstrate the involvement of mitochondria in the metabolism of ceramides through different pathways, thereby supporting the hypothesis that the topology of ceramide formation could determine its function.

Ceramide metabolism and trafficking

Once generated, ceramide may be converted into a variety of metabolites (Fig. 1). Deacylation by ceramidases (CDases) yields sphingosine, which may be phosphorylated by sphingosine kinase (SK) to S1P, another sphingolipid with an important role in cell signaling [1, 2]. Two distinct SKs, SK1 and SK2, have been cloned and a SK1 knockout mice have been generated [30, 31]. Interestingly, the S1P levels are only significantly reduced in serum but not in tissues from SK1 knockout mice, suggesting that other mechanisms of S1P generation exist.

In addition, ceramide may be phosphorylated by ceramide kinase (CK) generating ceramide 1-phosphate (C1P), which in turn, may be dephosphorylated back to ceramide by the C1P phosphatase. The existence of C1P was first reported in human leukemia (HL-60) cells [32, 33], and it was found that C1P was synthesized from ceramide derived from SM but not from GSLs, suggesting that a specific pathway extended from SM to C1P [32]. CK was first identified in brain synaptic vesicles [34], and human CK has been recently cloned by Sugiura and co-workers [35]. Similar to SIP, C1P has mitogenic properties and promotes cell survival [33].

Although the *de novo* ceramide synthesis occurs in the ER, its transport to other cellular sites regulates its conversion into other derivatives. Ceramide can be transferred to the Golgi through different mechanisms including vesicle-dependent and independent processes [11, 36]. A novel cytoplasmic protein named CERT that mediates the ATP-dependent transport of ceramide from the ER to the Golgi in a vesicle-independent manner has been identified recently [37]. CERT contains several lipid binding domains,

including a phosphatidylinositol-4-monophosphate-binding (PtdIns4P) domain and a putative domain for lipid transfer START, also present in cholesterol-binding proteins such as StAR and MLN64. The C-terminal START domain of CERT is responsible for its ability to specifically target ceramide extracted from phospholipid bilayers to the Golgi [38]. CERT-mediated transport of ceramide from the ER to the Golgi mediates SM synthesis, in a process regulated by oxysterol binding protein· OSBP and vesicle-associated membrane protein–associated protein, VAP [39]. Moreover, the S1P phosphohydrolase, which regulates sphingolipid metabolism and apoptosis, has been shown recently to be involved in the anterograde membrane transport of ceramide from the ER to the Golgi [40].

Ceramide in the Golgi may be converted to SM by transfer of phosphorylcholine from phosphatidylcholine via SM synthase. Alternatively, specialized glycosyltransferases transfer a glucose or galactose residue in a α-glycosidic linkage to the C1-hydroxyl of ceramide to produce glucosylceramide (GluCer) or galactosylceramide [41, 42]. Most of the GSLs arise from the glucosylation rather than galactosylation of ceramide, a step catalyzed on the cytosolic surface of the Golgi by the rate-limiting enzyme glucosylceramide synthase (GCS) [43]. GluCer is then transferred to the lumenal leaflet of the Golgi, where it is modified by the addition of a galactose moiety to produce lactosylceramide from which most of gangliosides derive by the action of specific glycolipid-glycosyltransferases [44] (Fig. 1). For instance, sequential addition of one, two, or three sialic acids to lactosylceramide results in the formation of GM3, GD3, and GT3, respectively. Since ceramide provides the carbon backbone of GSLs, their synthesis is dependent on the availability of ceramide generation. Indeed, ganglioside generation, which is involved in cell death after exposure to inflammatory cytokines o chemotherapeutic drugs [8], is dependent on the activation of specific SMases to provide the ceramide needed for ganglioside synthesis (and consequent ceramide formation by these compounds). In this regard, although fenretinide-induced apoptosis involves several intermediates, this synthetic retinoid illustrates the stage in which SMases-regulated ceramide generation is linked to its biotransformation into ganglioside GD3 to ultimately cause the demise of cancer cells [45, 46].

Biophysics and effects on membranes

The recognition of SLs as signaling lipids regulating a variety of cellular processes has stimulated the investigation of the biophysical properties of these lipids, particularly sphingosine and ceramide, and their effect on biological membranes [47, 48].

Sphingosine

Sphingosine is the basic building block of SLs and can be dispersed in water behaving as a surface-active amphiphile. Due to its apparent pK_a of 9.1 sphingosine exhibits a positive charge under physiological conditions, underlying its interaction with anionic and zwitterionic phospholipids in membranes. In mixtures with most phospholipids, sphingosine rigidifies the membranes as evidenced by measurements of surface pressures in lipid monolayers at the air-water interface of egg phosphatidic acid monolayers [49]. Due to its positive charge at physiogical pH, sphingosine modulates the interaction of cations such as Ca^{2+} to membranes and has been exploited in the preparation of positively charged liposomes to study the membrane binding of a number of macromolecules including DNA and enzymes. Indeed, using a combination of fluorescence spectroscopy, fluorescence microscopy, and differential scanning calorimetry, Kinnunen et al observed that the binding of DNA to a bilayer composed primarily of egg phosphatidylcholine depended critically on the presence of sphingosine in the liposomes, and the attachment of DNA to sphingosine-containing membranes could be reversed by the addition of negatively-charged phosphatidic acid in the bilayers [50].

Moreover, sphingosine exhibits the ability to permeabilize membranes to small solutes. The ability of sphingosine to form channels in planar lipid bilayers was described in red blood cell membranes and resealed ghosts membranes characterized by short open lifetimes and diameters smaller than 2 nm [51, 52]. Sphingosine-increased permeability caused the stabilization of gel domains in membranes, raising their melting temperatures and increasing the transition cooperativity. The consequences of these features were the formation of structural holes in the membrane as a result of the lateral phase separation from the "more rigid" to "less rigid" domains, which provides the molecular framework for the leakage of aqueous solutes to the extravesicular medium.

Ceramides

Ceramides are found in nature with *N*-fatty acyl chains containing from 2 to 28 carbon atoms. While those with C16 to C24 fatty acids are most common, these species exhibit a negligible solubility in water. In contrast, ceramides containing C2 to C6 fatty acids are more frequently used in experimentation due to their enhanced solubility. Indeed, free ceramides (e.g., with a fatty acid C12 or longer) do not exist in solution in biological fluids or in cytosol and they belong to the category of "non-swelling amphiphiles", implying that they cannot even give rise to micelles or other aggregates in aqueous suspension. When mixed with phospholipid monolayers or bilayers, ceramides induce

major effects on the behavior of phospholipids including an increased molecular order, lateral phase separation and domain formation, induction of transbilayer (flip-flop) lipid movements, and membrane permeabilization that may contribute to many of the biological effects of ceramide.

Increased lipid order

The degree of lipid chain order (in particular the "dynamic order" that is usually related to bilayer fluidity) is often estimated using fluorescence probes such as diphenylhexatriene (DPH), whose fluorescence polarization increases with increasing molecular order (decreasing fluidity). Ceramides have been shown to increase acyl chain order in a dose-dependent fashion in phospholipid bilayers in the fluid lamellar phase [53, 54]. Moreover, *in situ* generation of ceramide through the action of SMase on bilayers containing phosphatidylcholine and sphingomyelin also lead to an increased DPH fluorescence polarization, an effect confirmed by the determination of the polarization of transparinaric acid fluorescence in POPC fluid bilayers [54]. Thus, the rapid formation of ceramide within membranes results in the formation of microdomains enriched in this lipid called rafts, thought to be of relevance in the downstream signal transduction by this lipid, due to the existence of specialized proteins in these structures.

Lateral phase separation

Ceramide has been shown to induce the separation of ceramide-rich and ceramide-poor domains in phospholipid/ceramide bilayers, with ceramide partitioning largely into the gel phase of phospholipids [55]. The use of a pyrene-labeled phospholipid, a fluorescent probe that is sensitive to lateral mobility and to the local concentration of fluorophore in the membrane, allowed the detection of ceramide-enriched microdomains in fluid phosphatidylcholine membranes [53, 54]. Measurements of surface potentials in monolayers reveal that molecular dipole potentials may play an important role in lateral phase separation, with favorable ceramide-PC dipolar matching as a key determinant of close molecular interactions [56]. Epifluorescence studies of sphingomyelin monolayers degraded by the addition of SMase, indicated that the generation of ceramide *in situ* altered surface topography by inducing phase separation into condensed (ceramide-enriched) and expanded (sphingomyelin-enriched) domains [57, 58]. Ceramide-enriched domains in sphingomyelin/ceramide vesicle bilayers, rather than monolayers, have been described recently by Sot et al. [59]. While pure egg sphingomyelin bilayers exhibited a narrow transition centered at 39°C, egg ceramide, even at low proportions (5 mol%), had the effect of widening the phase transition shifting it to higher temperatures. Indeed, the endotherms of ceramide-containing samples had a clearly asymmetric shape, indicating formation of high-T melting ceramide/sphingomyelin domains. In addition, observations on giant unilamellar vesicles doped with the fluorescent probe DiI_{C18}, that partitions preferentially into the more fluid membrane regions, indicated that pure sphingomyelin vesicles appeared uniformly stained, whereas those containing egg ceramide displayed dark areas, corresponding to rigid, ceramide-rich domains. Increasing ceramide concentrations caused a parallel increase in dark areas that changed their shape from circular to elongated or worm-like [59].

Transmembrane (flip-flop) lipid motion

Using fluorescent ceramide analogues, Bai and Pagano determined that the half-time of flip-flop transbilayer diffusion of ceramide was much faster than the corresponding fluorescent analogs of phosphatydilcholine or sphingomyelin but slower than that of diacylglyerol [60]. Additional observations in egg phosphatidylcholine giant unilamellar vesicles to which a small percentage of ceramides (about 0.1% total lipid) were added revealed a change in shape from prolate to pear-shaped vesicle [61], implying a transmembrane diffusion of ceramide, that was confirmed by flip-flop experiments with a spin-labeled ceramide analog incorporated into large unilamellar vesicles.

Related studies using different procedures demonstrated that ceramides induce flip-flop motion in model and cell membranes [62, 63]. Using a liposome containing a ganglioside only in the outer monolayer and entrapped neuraminidase, the *in situ* generation of ceramide from sphingomyelin hydrolysis by a SMase demonstrated the hydrolysis of ganglioside under conditions in which no neuraminidase was released from the vesicles. Moreover, sphingomyelin hydrolysis was accompanied by fluorescence energy transfer to an impermeable acceptor in the outer aqueous medium in liposomes or erythrocytes ghosts that have been labeled with a fluorescent energy donor in the inner leaflet [60, 61]. Similar observations were made when egg ceramide was added externally to pre-formed vesicles. Interestingly dihydroceramides were unable to promote flip-flop movements [62]. These observations may provide a plausible mechanism linking the sphingomyelin hydrolysis that occurs in the outer monolayer of the plasma membrane to the intracellular sites where ceramide acts.

Membrane permeability

In addition to the above effects early observations indicated that ceramide exerted a direct permeabilizing effect in model membranes due to the presence of the amide group of the ceramide, which serves as a link between the hydrocarbon chains, giving rise to lateral hydrogen bonds within

the membrane lipids that influence membrane stability and permeability [64]. Using large unilamellar vesicles (LUVs) composed of sphingomyelin, phosphatidylethanolamine and cholesterol at a 2:1:1 mol ratio, Ruiz-Argüello et al showed that upon treatment with SMase, the resulting ceramide generation caused the leakage of entrapped fluorescent dye to the exogenous media [65], an effect independent of the specific lipid composition of LUVs or the type of dye entrapped. Moreover, the generation of ceramide in LUVs by the action of *Bacillus* cereus SMase led to the release of entrapped fluorescein-derivatized dextrans of molecular mass ≈20 kDa [66]. In addition, using planar lipid bilayers and electrophysiological detection methods, ceramide has been shown to cause the formation of channels in isolated mitochondria [67, 68]. Even though the permeabilizing effect of ceramide has been observed at physiological relevant concentrations, the contribution of this membrane permeabilizing effect of ceramide on mitochondria activation and subsequent release of cytochrome c in death pathways remains to be further clarified, in particular as to how it relates to the key role of Bcl-2 family members.

Although not every biophysical property of SLs has been correlated with a given physiological event, some of these properties are likely to play important roles, particularly in cell death. Specifically, the lateral phase separation and the membrane flip-flop lipid motion induced by ceramide may contribute to the formation of signaling platforms in specific domains of the membrane bilayer and to the movement of ceramide within membrane leaflets to gain access to specific targets (see below). In addition, ceramide-induced flip-flop motion can be related to the loss of membrane asymmetry and presence of phosphatidylserine in the plasma membrane outer leaflet that occur during apoptosis. Finally, despite the fact that ceramide may disrupt the biophysical features of different membranes within the cell (e.g. plasma membrane or mitochondria), given the limited capacity for spontaneous intracellular diffusion of natural ceramide due to its physicochemical properties, the topology and membrane sidedness of ceramide generation may be critical determinants of its impact on cell death.

Role as second messenger in cell death

Lipid second messengers in signal transduction are usually produced rapidly and transiently and have well defined physiological target proteins. Ceramide has been established over the past decade as a second messenger due to its rapid and transient generation that modulates a variety of physiologic and stress responses [1, 2, 6, 69]. As expected for a second messenger, a variety of stress stimuli and agents generate cellular ceramide levels either by *de novo* synthesis or sphingomyelin hydrolysis (Fig. 2). While cellular ceramide levels generated via *de novo* synthesis in response to different agents represents a late and more sustained phase of ceramide stimulation, the activation of SMases constitutes a rapid means for ceramide formation. Several possible intracellular target enzymes for ceramide have been proposed, including the protein kinases PKCζ [70, 71], kinase suppressor of Ras (KSR) [72], Raf-1 [73], protein phosphatases

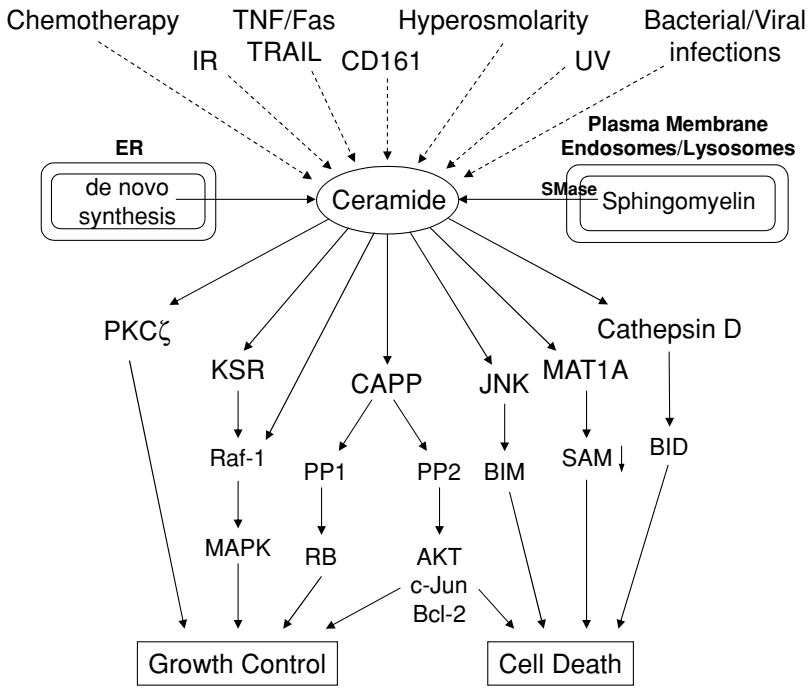

Fig. 2 Second messenger action of ceramide. Cellular ceramide levels increase in response to a variety of stimuli and agents from chemotherapy to death ligands, radiation or viral/bacterial infections either by *de novo* synthesis or sphingomyelin hydrolysis via SMases. The variety of cellular responses resulting from the increase in ceramide levels, related to growth control (proliferation, differentiation, arrest) or cell death, is accounted by a signaling cascade determined by a primary line of ceramide targets which in turn act on several other targets that ultimately contribute to the modulation of growth control and cell death

PP1 and PP2A [2, 74, 75], and JNK [76], which contribute to the control of cell growth and cell death through various downstream players including members of the Bcl-2 family (Fig. 2). Based on the structural similarity between ceramide and diacylglycerol, one might anticipate that ceramide would bind to defined consensus motifs in the primary sequence of its target proteins. Evidence indicates that in some of these proteins, ceramide specifically binds a C1B lipid-binding domain to activate target proteins, although other less well-defined binding sites have also been identified in other proteins such as the catalytic (C) subunit of protein phosphatase PP2A. As shown recently, PP2A activation by ceramide has been suggested to function as a physiological Bax regulatory phosphatase that through Bax dephosphorylation activates its proapoptotic function [77].

Another ceramide target is cathepsin D, a protease that co-localizes with ASMase in endosomes [78] (Fig. 2). Cathepsin D has been shown to bind ceramide directly resulting in its activation that mediates TNF-induced cell death signaling [78]. Recent studies have indicated that TNF-induced cathepsin D activation is dependent on functional ASMase expression, which in turn, targets Bid cleavage, leading to activation of caspase9/3 [79]. Interestingly, cathepsin D and Bid colocalized with Rab 5, suggesting that endosomal compartments represent a scenario where acid proteases and BH3-only Bcl-2 members meet in a stage orchestrated by ASMase-induced ceramide generation. More intriguingly, ceramide derived by ASMase target cytolosic proteins such as methionine adenosyl transferase 1A (MAT1A) and γ-glutamylcysteine synthase in parenchymal cells [24, 25], thus linking the stress response to ceramide generation and the regulation of GSH synthesis and oxidative stress. Interestingly, the effect on MAT1A was specific for ASMase, while the upregulation of γ-GCS was observed by either NSMase or ASMase [24], although the net increase in hepatic GSH levels was only observed with NSMase. Consistent with its acidic requirement, the blockade of vesicular acidification prevented the effect of exogenous ASMase or TNF on MAT1A downregulation [25]. Whether these effects of ceramide on these enzymes are direct or not are currently unknown.

Another mode of ceramide function in cell death signaling involves its function in the re-organizing plasma membrane infrastructure. When generated within sphingolipid- and cholesterol-enriched plasma membrane rafts [80] via the action of a secretory form of ASMase [81], ceramide spontaneously self-associates, likely via hydrogen bonds and van Der Waal forces, inducing raft coalescence into large transmembrane signaling platforms [22, 82]. These platforms serve as sites for protein oligomerization and complex formation, a near universal requirement for transmembrane signaling, perhaps in part by serving as targeting sites for ceramide-binding proteins. Ceramide-rich raft platforms transmit signals for diverse cellular stresses including physical stress, (UV-light, γ-irradiation), bacterial (P. aeruginosa, S. aureus, N. gonorrhea) and viral (Sindbis-virus, Rhinovirus) infection, chemotherapy (doxorubicin, cisplatin), and inflammatory mediators (CD95-ligand, TNF, TRAIL, CD40-ligand, B7), and for FcγRII capping and integrin receptor inactivation [22, 83]. The demonstration that ceramide is required for generation of specialized cell membrane structures, which mediate stress-induced cell death, defines a mechanism by which ceramide can serve pro-apoptotic function in diverse cellular systems. In line with this, recent data indicate that ceramide generated by CS activation may play a similar role in mitochondrial membranes (Kolesnick and Fuks, unpublished data). Furthermore, recent studies indicate that ceramide functions as a molecular device to detect and sense environmental stress that operate in certain cells [84–87]. In this context, distinct ceramide-operating sensor cells may control cell fate in select organs by engaging distinct topologically ceramide pools, such as the ASMase plasma membrane raft axis or the ceramide synthase mitochondrial pathway. For instance, the ASMase/ceramide transducer is utilized by germ cells in ovaries [84], hepatocytes in liver [23, 24, 26], microvascular endothelium in the lung [88], GI tract and CNS, while irradiated GI crypt stem cells sense stress by utilizing the CS/ceramide transducer [89]. Further, ceramide transduction is not fixed for a particular stress type as the same stress may engage ceramide signaling in one organ and a different sensor cell-transduction pathway in another organ, and consistent with this, ASMase has been shown to contribute to Fas-mediated cell death in hepatocytes but not in T lymphocytes [90]. In addition, ceramide generation through NSMase stimulation has been reported to contribute to cell death caused by various stimuli including TNF in breast cancer cells or ethanol in HepG2 hepatoma cells [91, 92]. Thus, it appears that depending on the cell type or stimuli used, ASMase or NSMase may differentially contribute to cell death.

In addition to these described targets that mediate ceramide action in cell death and stress response, ceramide has been recently shown to modulate autophagy, an evolutionary conserved lysosomal pathway involved in the turnover of long lived proteins and organelles, that starts with the formation of a multilayer membrane-bound autophagosome that sequesters fractions of the cytoplasm [93–97]. Although the visualization of autophagosomes in dying cells has led to the belief that autophagy is a nonapoptotic form of programmed cell death, evidence indicates that in certain conditions autophagy may function as a pro-survival rather than a pro-death mechanism, and the inhibition of macroautophagy triggers apoptosis [97]. The role of ceramide in the modulation of autophagy involves two non-mutually exclusive mechanisms, such as the interference with the inhibitory class I PI3K signaling pathway and the stimulation of the expression of the autophagy gene beclin 1. In addition, ceramide

has been shown to activate BNIP3, a member of the BH3-only subfamily that interacts with the mitochondrial outer membrane [94]. Hence as the roles of ceramide in cell phyisiology and biology increase, it is likely that novel putative molecular targets will be uncovered.

Mechanisms involved in SLs mediated cell death

The role that SLs play in the stress response and cell death has sparked considerable interest in deciphering the mechanisms involved. Cell death can occur through diverse biochemical and morphologic features that shape distinct forms of cell death from apoptosis to necrosis and from caspase-dependent to caspase-independent apoptosis [98, 99]. Mitochondria have been shown to play a fundamental role in the integration of cell death pathways, although the final phenotype of cell death depends on several factors including the degree of mitochondrial dysfunction, mechanism of mitochondrial membrane permeabilization, caspase activation or ATP depletion. In addition to this key role of mitochondria, activation of death pathways occurs in the ER as well, and there seems to be a cross-talk between mitochondria and ER in cell death pathways [99–104]. Here we will describe the evidence indicating the participation of ER and mitochondria in ceramide/SLs-mediated cell death (Fig. 3).

ER stress

The ER fulfills multiple cellular functions most notably in protein and lipid management, and disturbances in the normal functions of the ER lead to an evolutionarily conserved cell stress response, the unfolded protein response (UPR) [105, 106]. The lumen of the ER is unique in several aspects as it contains the highest concentration of Ca^{2+} within the cell because of active transport of calcium ions by Ca^{2+} ATPases. The ER lumen is embedded in an oxidative environment, in part due to the predominant presence of GSSG rather than GSH, which is critical for formation of disulfide bonds and proper folding of proteins destined for secretion

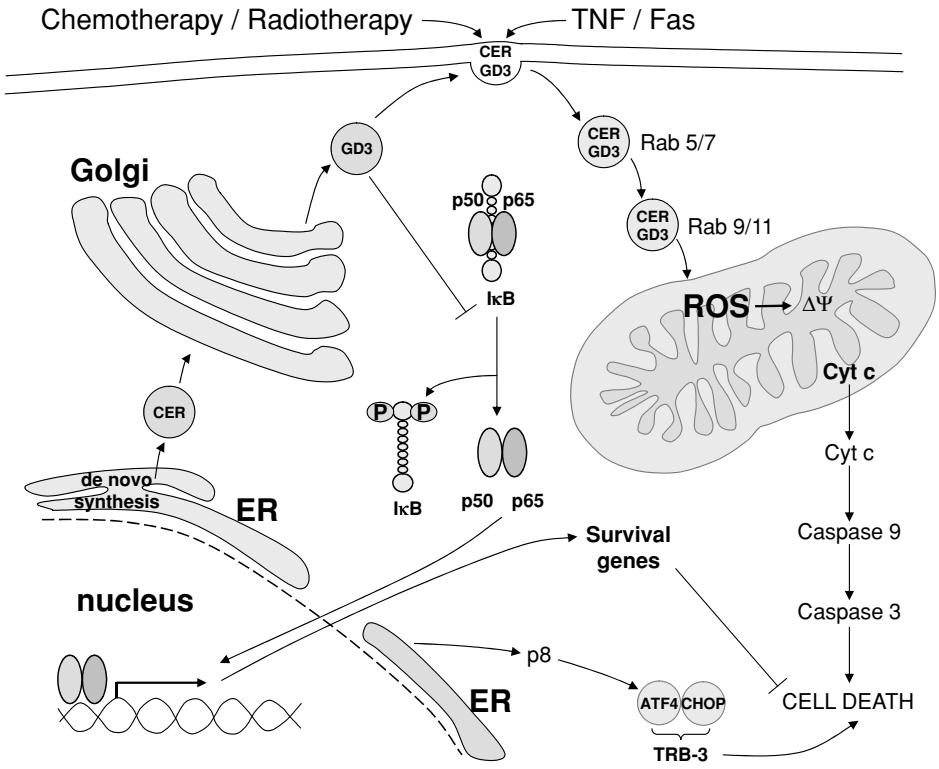

Fig. 3 Trafficking and organelle-specific intiation of cell death pathways by SLs. The increase and distribution of ceramide and GD3 to various organelles promote cell death using different strategies. The mitochondrial targeting of GD3 and mitochondrial ceramide increase in response to stress or death ligands stimulate the mitochondrial permeabilization and apoptosome assembly that culminates in caspase activation. In addition, the *de novo* synthesis of ceramide in the ER induces an ER stress response that induces an ER-specific death mechanism involving an intricate cascade of intermediates not shown here for simplification that include Ca^{2+} release from the ER, sustained JNK activation, and caspase 12 activation among other (see text). In the particular case of THC, the accumulation of ceramide via *de novo* synthesis in the ER induces the stress protein p8, which contributes to cell death through an ATF4-CHOP-TRB-3 cascade. In addition, GD3 has been shown to block the nuclear translocation of DNA-competent κB complexes resulting in the inactivation of survival genes that can be exploited to potentiate or synergize with cancer therapies

or display on the cell surface [107]. Because of its role in protein folding and transport, the ER is also rich in Ca^{2+}-dependent molecular chaperones, such as Bip/Grp78, Grp94, and calreticulin, which stabilize protein folding intermediates. Early during UPR unfolded proteins causes dissociation of chaperones Bip/Grp78 from ER resident kinases such as type-I ER transmembrane protein kinase (IRE1), the PKR like ER kinase (PERK) and the activating transcription factor6 (ATF6). Glucose deprivation, aberrant Ca^{2+} ER regulation, viral infection or proteasome impairment has been described to lead to ER stress mediating the UPR [105, 106, 108]. The initial goal of the UPR is to adapt to the changing environment, and reestablish normal ER function with two major programs involved. The translational arm of the UPR constitutes a short-term adaptation that reduces the load of newly synthesized polypeptides entering the ER lumen, while the transcriptional arm constitutes a long-term adaptation that increases the capacity of the organelle to handle unfolded proteins. In addition, a recent third effector has been described consisting in the degradation of mRNAs encoding secreted and membrane proteins [109, 110]. Moreover, secondary adaptation signals include the activation of transcription factors ATF4 that regulate genes involved in aminoacid transport, glutathione (GSH) synthesis and resistance to oxidative stress [111]. However, excessive and prolonged ER stress triggers cell death, usually in the form of apoptosis caused by the activation of a number of intermediates including transcription factor CHOP and caspase 12 [108, 112].

The presence of gangliosides at the ER membrane implies that these molecules regulate membrane dynamics and structure in this organelle; hence, changes in ganglioside composition, sugar modifications, and local concentration can alter not only their behavior but also that of other membrane components. It has been shown that gangliosides at the ER act as key modulators of Ca^{2+} flux [113, 114]. Efflux of Ca^{2+} from the ER occurs via two types of channels that reside in the ER membrane, the Ca^{2+}-gated Ca^{2+}-release channel or ryanodine receptor (RyaR) and the inositol 1,4,5-triphosphate-gated Ca^{2+}-release channel, the IP3 receptor (IP3R). Ca^{2+} influx from the cytosol to the ER lumen is mediated by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). For instance, accumulation of GluCer, which is the main storage product in Gaucher disease, increases Ca^{2+} mobilization from intracellular stores in cultured neurons, likely via amplification of the response of the RyaR to agonists, which leads to ER Ca^{2+} release [115, 116]. Isolated microsomes from the brain of a Sandhoff disease mouse model that accumulate GM2 show substantially reduced rates of Ca^{2+} uptake because the V_{max} of sarco/endoplasmic reticulum Ca^{2+}-ATPase SERCA is reduced in those organelles, and this effect of GM2 is prevented if the mice are treated with a specific inhibitor of glycolipid synthesis that reduces GM2 storage, directly implicating this ganglioside in the function of SERCA [116]. In addition to alterations in Ca^{2+} homeostasis, GM1 accumulation due to deficiency of lysosomal beta-galactosidase leads to upregulation of GRP78 and CHOP and activation of JNK2 and caspase-12 that contribute to neuronal cell death in a mouse model of GM1-gangiosidosis [117].

In addition to complex SLs and gangliosides, the *de novo* synthesized ceramide has been shown to elicit an ER stress-dependent death mechanism in tumor cells in response to tetra-dydrocannabinol (THC) [118]. THC induced apoptosis in C6 glioma cells and U87MG cells that is prevented by ISP-1, a selective inhibitor of SPT. The accumulation of ceramide synthesized *de novo* upregulated the levels of p8 (also known as candidate of metastasis 1), a protein that belong to the family of HMG-I/Y transcription factors implicated in the control of cell fate (Fig. 3). Consistent with its function in cell death regulation, upregulation of p8 by THC led to the activation of ATF-4 and stress-regulated protein tribble homolog 3 (TRB-3) also known as cell death-inducible kinase, NIPK, and ISP-1 preincubation prevented these events. Moreover, the addition of cell-permeable C2-ceramide recapitulated the effects seen with THC, including the upregulation of p8 and subsequent ER stress signs such as induction of ATF4, CHOP and TRB3 expression. An interesting feature of these observations is the fact that pharmacological blockade of the mitochondrial respiratory chain prevented THC-induced cell death but not p8 upregulation, supporting an ER-mitochondria cross-talk in ceramide-induced cell death [118]. Thus, in addition to the accumulation of complex GSLs, which had been implicated in activation of the ER stress pathway, ceramide itself activates the ER stress due to upregulation of the stress protein p8. A key feature of the latter observations is that the ceramide-ER stress axis seems to occur only in transformed or tumor cells, which undoubtedly makes this very attractive for cancer therapy. However, the molecular mechanism for the specificity of this pathway in cancer cells but not in healthy cells remains to be understood.

Mitochondrial targeting

The role of mitochondria in cell death regulation is a relative recent landmark in cell physiology/biology with respect to its long held view as the cells energy power plants, and the subject of intensive investigation [119, 120]. The key to this function is the discovery that a number of specialized proteins that play a primary role in the activation of cell death pathways are released from the space between the inner and outer mitochondrial membranes into the cytosol, in a process that is preceded by the permeabilization of mitochondrial membranes by mechanisms that are not completely understood [119–122]. Indeed, many different cell

death-inducing stimuli (e.g. radiation, chemotherapy, death ligands, Ca^{2+}, reactive oxygen species, ROS) act on mitochondria stimulating the release of several intermembrane proteins of which cytochrome c has been most extensively characterized. Consistent with its role as a second messenger, whose levels increase in response to many cell death-inducing agents that operate through mitochondrial targeting, ceramide has been shown to interact with components of the mitochondrial electron transport chain accounting for the stimulation of ROS, mitochondrial depolarization and overall mitochondrial dysfunction [123–126]. These events in turn induce the mitochondrial membrane permeabilization and release of cytochrome c into the cytosol that assist in the apoptosome assembly and activation. In cell-free assays using purified rat liver mitochondria it was first shown that the addition of ceramide C2 directly induced a burst of ROS generation predominantly from the complex III of the mitochondrial electron carriers and blockade of the mitochondrial electron transfer [123, 127, 128], thus contributing to the mitochondrial depolarization and dysfunction observed during cell death. Moreover, the relevance of these observations with isolated mitochondria was of further significance due to the findings of increased ceramide levels in purified mitochondria from cells exposed to different agents such as TNF, Fas, reoxygenation after hypoxia or UV irradiation [24, 123, 124]. These results imply either that the stimulated ceramide levels traffic to mitochondria or that ceramide is generated *in situ* in these organelles. Consistent with the latter, Birbes et al. observed that the enforced mitochondrial targeting of bacterial sphingomyelinase in MCF7 resulted in mitochondrial ceramide increase that caused cytochrome c release and apoptotic cell death [129]. Moreover, ceramide can be generated locally in these organelles through several mechanisms including the presence of a reverse ceramidase activity [28], or a ceramide synthase [29] implying that mitochondria have the ability to generate ceramide *de novo*. These observations suggest the existence of an independent and highly regulated sphingolipid metabolism in mitochondria that contribute to the accumulation of ceramide in these organelles modulating cell death pathways, although more details about this pathway remain to be uncovered.

Due to the negligible solubility of ceramide that limits its intracellular diffusion, synthetic positively charged ceramide analogues have been designed to model the interaction of ceramide with mitochondria. For instance, laboratory design cationic long-chain ceramide [pyridinium bromide D-*erythro*-C_{16}-ceramide, LCL-30] has been shown to target negatively charged mitochondria. LCL-30 is highly cytotoxic to human colon carcinoma SW403 cells and other cancer cell lines due to its preferential accumulation in mitochondria, resulting in the loss of mitochondrial membrane potential, release of mitochondrial cytochrome c and caspase 3 and 9 activation [126]. Intriguingly, LCL-30 seems to metabolize ceramide into S1P as the decrease in mitochondrial C16-ceramide levels correlated with the appearance of S1P, while this was not observed in the cytosolic and extramitochondrial fractions, suggesting the presence of a mitochondrial SK. Moreover, positively charged ceramides has been shown to increase the inner mitochondrial membrane permeability due to the activation of specific ion transporters sensitive to cyclosporine A and 1, 3-dicyclohexylcarbodiimide, suggesting the presence of specific ceramide targets in the mitochondria that mediate the permeability of the inner and outer mitochondrial membranes [130].

In addition to the possibility that ceramide-mediated mitochondrial dysfunction may occur through interaction with specific components, recent evidence has shown the ability of ceramide to form channels in mitochondrial outer membrane at physiologically relevant concentrations [68]. Indeed, the permeability of the mitochondrial outer membrane correlates directly with the level of ceramide in the membrane, and the concentration of ceramide at which significant channel formation occurs is consistent with the level of mitochondrial ceramide that occurs during the induction phase of apoptosis. A similar mechanism of action has been shown for sphingosine in model membranes and isolated mitochondria, forming channels with short open lifetimes and diameters less than 2 nm [51, 52]. However, the contribution of this putative function of sphingosine to the release of cytochrome c and apoptosis is uncertain due to the smaller size of the channel formed to allow the passage of cytochrome c and to the metabolic conversion of sphingosine to ceramide by a ceramidase-dependent mechanism.

The underlying mechanisms for the trafficking of the generated ceramide to mitochondria in response to apoptotic stimuli (e.g. TNF/Fas, UV radiation) have not been elucidated. Whether this movement occurs by vesicle-dependent or independent processes remains unknown. In contrast, the mitochondrial trafficking of ganglioside GD3 has been better characterized [131–135]. As ceramide, cell GD3 levels increase in response to apoptosis stimuli and consequently the down-regulation of GD3 synthase, the enzyme responsible for GD3 synthesis from its precursor ganglioside GM3, prevents Fas-, TNF- or β-amyloid-induced cell death [135, 136]. While most of the evidence for the apoptotic role of GD3 has been derived from *in vitro* studies with isolated mitochondria, recent studies in CEM cells and cultured hepatocytes indicated the trafficking and physical interaction of GD3 with mitochondria in response to apoptotic stimuli [131, 133]. Most of GD3 was present at the plasma membrane in resting hepatocytes, however, in response to TNF exogenous ASMase or ionizing radiation, GD3 underwent a dramatic redistribution that involved first its disappearance from the plasma membrane followed by its trafficking to mitochondria [133]. The colocalization of GD3 with mitochondria was preceded by its location in early to late endosomes via coordinated

secretory/endocytic vesicular trafficking, and the disruption of this pathway prevented the interaction of GD3 with mitochondria sparing sensitized hepatocytes to TNF exposure. These findings suggest that endosomal vesicles trafficking through actin cytoskeleton may be part of the TNF/Fas multicomponent signaling complex delivering death signals, e.g., GD3, to mitochondria.

Finally, recent observations have indicated the existence of lipid microdomains in mitochondria that contribute to the apoptosis of lymphoblastoid CEM cells induced by Fas [137]. The presence of GD3, the voltage-dependent anion channel-1 (VDAC-1) and the fission protein hFis1 are structural components of a multimolecular signaling complex, in which Bcl-2 family proteins (t-Bid and Bax) are recruited, suggesting a new scenario in which mitochondria-associated lipid microdomains can act as regulators and catalysts of cell fate.

Inactivation of survival pathways

The transcription factor NF-κB is known to induce a plethora of target genes that modulate a great variety of cellular responses such as proliferation, inflammation, and cell survival, hence playing a pivotal role in carcinogenesis and tumor progression [138]. While ceramide does not interfere with this surveillance system, ganglioside GD3 has been shown to abort the activation of NF-κB by preventing the nuclear translocation of active DNA-binding competent κB members to the nuclei in response to TNF or ionizing radiation, thus preventing the induction of antiapoptotic genes [139, 140]. Using GSL derivatives, it was shown that while the N-fatty acyl sphingosine moiety common to both ceramide and GD3 is necessary for its ROS stimulating effect, the presence of sugar residues in the backbone of ceramide is required in blocking the nuclear translocation of NF-κB by a mechanism that remains to be characterized and that may possibly involve the nuclear localization signal of the κB complex members. The exploitation of this dual role of GD3 in apoptosis, as mitochondrial ROS stimulator and NF-κB-inactivating agent, has been recently shown in human hepatoma (HepG2) cells that are highly resistant to current cancer therapy [140]. However, this proapoptotic role of GD3 is not general for all cancer cells, and its ability to disable survival programs is modulated by the acetylation status of GD3, since acetylated GD3 has been reported to have antiapoptotic activity [141]. Indeed, cells resistant to the overexpression of the GD3 synthase actively convert de novo synthesized GD3 to 9-O-acetyl-GD3, while prevention of 9-O-acetyl-GD3 accumulation reconstitutes GD3 responsiveness and apoptosis [142]. Thus, the enforced expression of GD3 synthase in tumor cells may have a potential therapeutic relevance in cancer therapy as the resultant GD3 expression may exhibit a dual mechanism in cell death including the mitochondrial targeting and the inactivation of NF-κB-mediated survival genes.

Thus, although GD3 exhibits a dual mode of action in apoptosis through its ability to cause ROS generation from mitochondria and disabling NF-κB-mediated survival pathways, the generation of ceramide at the very sites that govern cell death pathways such as the ER and mitochondria defines the central role of this SL in cell death regulation and hence in pathophysiology.

Sphingolipids and disease pathogenesis

Given the role of SLs as intermediates of many different apoptotic stimuli and that the relative balance between proapoptotic and antiapoptotic SLs determines cell fate, the modulation of SLs may be of potential therapeutic relevance in disease pathogenesis.

Liver diseases

Since TNF overproduction is a key player in liver pathobiology the mechanisms underlying the hepatocellular susceptibility to TNF are of relevance in the progression of several liver diseases including ischemia/reperfusion (I/R) damage, steatohepatitis and fibrogenesis. In examining the role of SMases in hepatocellular apoptosis by TNF, we observed that hepatocytes lacking ASMase were resistant to TNF-mediated apoptosis/necrosis and fulminant liver failure *in vivo* caused by D-galactosamyne/LPS challenge [23, 25, 143]. Detailed analyses of transducing intermediates indicated that the signaling events upstream of mitochondria including NF-κB activation and mitochondrial Bax translocation were preserved in ASMase-deficient hepatocytes [23, 25], yet the generation of mitochondrial ROS was defective in ASMase$^{-/-}$ hepatocytes sensitized to TNF/Fas through mitochondrial GSH depletion [143], suggesting that the onset of local oxidative changes within mitochondria are necessary for TNF induced hepatocellular death. These results therefore indicate that the translocation of BH1-3 multidomain Bcl-2 family members, for example Bax, appears to be independent of ASMase, yet downstream steps of mitochondria, including cytochrome c release and caspase activation, seem to require ASMase. Although our observations do not support a role for NSMase in TNF-mediated hepatocellular death, previous studies have shown that FAN, an adapter factor associated with NSMase activation, is involved in TNF–induced apoptosis in fibroblasts and in LPS-induced lethality in D-galactosamine sensitized mice [144, 145]. However, whether the lack of FAN, in addition to the modulation of NSMase, regulates the production of other cytokines such as IL-8 or granulocyte/macrophage colony-stimulating factor secreted in response to TNF that may affect the course of liver damage remains to be established.

Consistent with the role of ASMase in TNF-mediated hepatocellular death, ASMase ablation by siRNAs *in vivo* has been shown to improve the survival during total hepatic I/R in mice by preservation of mitochondrial function [24]. These findings revealed that ceramide generated by ASMase targets JNK which in turn contributes to BimL phosphorylation and translocation to mitochondria. Furthermore, consistent with this notion, the potent activation of JNK by exogenous ASMase has been reported in primary hepatocytes, mediating bile acid induced hepatocellular death [146]. Moreover, the connexion JNK activation and BimL has been observed in Fas-mediated liver damage and free fatty acids dependent hepatocyte lipoapoptosis [147, 148]. Thus, the modulation of ceramide generation through ASMase mediating TNF/Fas-induced hepatocellular death may be a promising therapeutic approach to steatohepatitis [143], hepatic I/R damage [24] or alcohol-induced liver injury, in part, through activation of GCS and subsequent ganglioside generation that may target mitochondria weaken by depletion of mGSH [see 8 and references therein]. Finally, S1P has been shown to protect liver sinusoidal endothelial cells from ethanol-induced apoptosis, while the antiapoptotic role of SK through S1P formation has been involved in the protection of stellate cells in bile duct ligation-induced hepatic fibrosis [149, 150]. Hence not only the generation of ceramide but its metabolism into anti-apoptotic S1P derivative through concerted action of CDases and SK may have an important role in nonparenchymal cell fate and hence fibrogenesis.

Neurodegeneration

The brain is particularly enriched in SLs and disregulation of SLs metabolism contributes to neurological disorders. SLs, including ceramide and gangliosides, along with cholesterol are vital components of specialized membrane domains that may influence a wide range of metabolic activities and responses to changing environmental conditions through protein-lipid interactions. Enhanced long-chain ceramide and galactosylceramide levels as well as free cholesterol have been found in cell membranes from vulnerable brain regions of Alzheimer disease (AD) patients compared with the same brain region of normal subjects and to a less vulnerable region of the AD patients [151]. The progressive impairment of short-term memory and emotional disturbances that characterize AD results from the dysfunction and death of neurons in the hippocampus and associated regions of the cerebral cortex and limbic system. Although the pathogenesis of AD is not completely understood, these abnormalities are believed to derive, in part, from oxidative stress and excessive production and accumulation of the amyloid β-peptide (Aβ), particularly the 42 amino acid peptide, both inside and outside of neurons due to the processing of the amyloid precursor protein (APP). While cultured hippocampal neurons are susceptible to Aβ-1-42 peptide-induced death accompanied by enhanced cholesterol and ceramide levels, pre-treatment of cells with ISP-1, a SPT blocker (Fig. 1), reduces ceramide levels and protects cells against Aβ-1-42 apoptosis [151]. In addition, Aβ-1-40/Aβ-25-35 and fibrillar Aβ-1-42 peptides have been reported to activate the NSMase-ceramide pathway resulting in the killing of oligodendrocytes and human primary neurons [152, 153], thus involving a pathogenic role for ceramide in the white matter dysfunction and neuronal loss that typify AD. Furthermore, although perturbations in lipid metabolism (cholesterol and SLs) are known to modulate APP processing, recent evidence has shown that altered APP processing can itself directly affect cholesterol and SLs metabolism, suggesting a positive feedback loop in AD in which lipid alterations promote Aβ-1-42 formation and Aβ-1-42, in turn, exacerbates the lipid abnormalities in neuronal membranes [154]. In addition to the Aβ-1-42-NSMase pathogenic link, the activation of ASMase has been involved in cerebral ischemia and stroke [155]. Transient focal cerebral ischemia activates ASMase, increases ceramide levels, and production of inflammatory cytokines in wild-type mice, but not in mice lacking ASMase. Neurons lacking ASMase exhibit decreased vulnerability to excitotoxicity and hypoxia, which is associated with decreased levels of intracellular Ca^{2+} and oxyradicals, suggesting that drugs that inhibit ASMase may prove beneficial in stroke patients. Finally, altered GD3 metabolism has been observed in multiple neurological disorders. LPS or bacterial stimulation of cultured microglia have been reported to stimulate the synthesis and secretion of GD3 resulting in toxicity to primary oligodendrocytes via mitochondrial targeting and dysfunction and caspase activation [156]. Moreover, Aβ-induced GD3 synthesis has been shown to mediate neuronal cell death [136]. Thus, these findings define a role for enhanced ceramide/GD3 levels in the white matter degeneration associated with neurological disorders including AD, HIV-induced neurodegeneration or medulloblastoma, and hence drugs targeting these proapoptotic SLs may be of clinical relevance.

Cancer biology and therapy

Given the critical role of SLs in cell death/survival regulation, the alteration in SLs metabolism has a significant impact on cancer biology and therapy. In particular the balance of ceramide to S1P generation is known to modulate cell fate in response to cancer therapy and alterations in SLs metabolism may thus contribute to multidrug resistance in cancer cells [1, 2]. Since CDases modulate the ceramide/S1P ratio through phosphorylation of sphingosine by SKs, these ceramide metabolizing enzymes promote carcinogenesis and

determine the efficacy of cancer therapy. For instance, acid CDase inhibition by a newly developed ceramide analogue, B13, induces apoptosis in cultured human colon cancer cells, and prevents liver metastases *in vivo* [157]. Furthermore, anthracycline therapy (e.g. daunorubicin, DNR) has been shown to activate acid CDase but not neutral CDase by a post-transcriptional-dependent mechanism in established human (HepG2 cells) or mouse (Hepa1c1c7) hepatoma cell lines as well as in primary cells from murine liver tumors, but not in cultured mouse hepatocytes [158]. Consequently, acid CDase silencing by small interfering RNA or pharmacological inhibition with N-oleoylethanolamine (NOE) enhanced the ceramide to S1P balance compared to DNR alone, sensitizing hepatoma cells (HepG2, Hep-3B, SK-Hep and Hepa1c1c7) to DNR-induced cell death, through a mechanism involving mitochondrial targeting. Importantly, *in vivo* siRNA treatment targeting acid CDase reduced tumor growth in liver tumor xenografts of HepG2 cells and enhanced DNR therapy [158]. Thus, CDases promote carcinogenesis through the enhanced conversion of the proapoptotic SL ceramide to its antiapoptotic derivative S1P, and hence their antagonism constitutes an attractive target for enhanced cancer therapy. Indeed, anti S1P antibody has recently been shown to be an effective therapy in the reduction of growth, invasion and angiogenesis in multiple tumor lineages [159]. These findings expand the role of S1P beyond its antiapoptotic function, and suggest that S1P not only modulates the survival of tumor cells themselves, but this lipid promotes the actions of angiogenic factors that favors tumor growth and expansion.

In conclusion due to the instrumental action of SLs in cell death/survival pathways, the modulation of their metabolism may have profound implications in disease pathogenesis. While in healthy tissues the aim of this intervention is to diminish or prevent the generation of proapoptotic SLs, such as ceramide or GD3, to reduce cellular loss that underlie tissue dysfunction, in cancer therapy the goal will be to implement strategies that either enhance the accumulation of ceramide/GD3 or that block the generation of S1P to sensitize tumor cells to current cancer therapies. As the multiple biological facets of SLs are uncovered and more detailed knowledge of their mechanisms of actions are revealed it is likely that these discoveries may pave the way to exploit the therapeutic potential of the modulation of SLs metabolism in diseases.

Acknowledgments The work presented was supported in part by the Research Center for Liver and Pancreatic Diseases, P50 AA 11999; and grant 1R21 AA014135, funded by the US National Institute on Alcohol Abuse and Alcoholism; Plan Nacional de I+D grants SAF (2003-04974; 2006-06780); and Red Temática de Investigación Cooperativa G03/015, Red de Centros C03/02, PI 05/1285 and FIS (06/0395) supported by Instituto de Salud Carlos III, Spain, and the European Lipidomics Initiative (Elife). A.M. is a Ramon y Cajal investigator.

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