The Sphingomyelin Cycle and the Second Messenger Function of Ceramide*  

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It is now well established that glycerophospholipids and their metabolic products (such as diacylglycerol (DAG), inositol trisphosphate, eicosanoids, and platelet-activating factor) function in signal transduction and cell regulation (1-4). On the other hand, a role for metabolic products (such as diacylglycerol (DAG), inositol trisphosphate, eicosanoids, and platelet-activating factor) function in signal transduction has not been well appreciated although sphingolipids exhibit even greater structural diversity and complexity than glycerolipids (5-7). Much of the current understanding of sphingolipid structure and function, however, has focused on the carbohydrate headgroups of the glycosphingolipids (8). The discovery of inhibition of protein kinase C by sphingosine (9) directed attention to the lipid components of sphingolipids and suggested the hypothesis that sphingolipid-derived products may function as second messengers (6). Evidence in support of this hypothesis came with the recent elucidation of the sphingomyelin (SM) cycle and the physiologic functions of ceramide (10, 11). Current results show that the action of a number of extracellular agents results in early activation of a sphingomyelinase that cleaves membrane SM resulting in the formation of ceramide. In turn, ceramide has emerged as a potential mediator of the effects of these agents on cell growth, differentiation, and apoptosis. In cells, ceramide has been shown to modulate protein phosphorylation, the activity of protein kinases, the levels of the c-myec protooncogene, the nuclear factor kB, the activity of phospholipase A2, and prostaglandin release. In vitro, ceramide activates a serine/threonine protein phosphatase with evidence beginning to implicate this phosphatase in the cellular mechanism of action of ceramide. Where examined, the effects of ceramide show structural and stereospecificity commensurate with physiologic roles for the naturally occurring D-eythro-ceramide. Therefore, these studies are beginning to define a novel ceramide-dependent pathway of signal transduction (Fig. 1). This minireview will highlight these developments in our understanding of the SM cycle and the physiologic function of ceramide and its mechanism of action. 

Overview of Ceramide Metabolism 

In analogy with the central role of diacylglycerol in glycerolipid metabolism (12), ceramide plays an equally critical role as a structural and functional component in sphingolipid metabolism (Fig. 2). Because of the emerging role of ceramide as an intracellular effector molecule, enzymes that regulate metabolism of ceramide stand as potential regulators of ceramide levels and consequently ceramide-mediated function. 

The de novo biosynthesis of ceramide (Fig. 2) is initiated by the condensation of serine and palmitoyl-CoA resulting in the formation of 3-ketosphinganine (3-ketodihydrosphingosine), which is subsequently reduced to dihydrosphingosine. Dihydrosphingosine is formed by the amide linkage of fatty acyl groups to dihydrosphingosine. Radiolabeling and pulse-chase studies indicate that ceramide is formed from dihydrosphingosine by the introduction of the trans-4,5-double bond (13, 14). Once formed, ceramide serves as a precursor for all other complex sphingolipids (Fig. 2) (15) such as galactosylceramide, glucosylceramide, acyl ceramides in skin (29), and ceramide phosphate in HL-60 cells (20, 21) but not in fibroblasts (22) through the action of a distinct kinase (19, 20). The biosynthesis of SM involves the addition of a phosphorylcholine headgroup to ceramide primarily through the transfer of choline phosphate from phosphatidylcholine through the action of phosphatidylcholine:ceramide choline phosphotransferase (16). An important feature of this enzymatic activity is that it serves to regulate simultaneously ceramide and diacylglycerol levels (Fig. 2) (17). 

Ceramide also serves as the penultimate sphingolipid in the diverse pathways of sphingolipid catabolism (Fig. 2). Thus, the breakdown of complex glycosphingolipids through the sequential action of acid hydrolases results in the formation of ceramide. Likewise, ceramide is formed from the breakdown of SM through the action of sphingomyelinases of which at least three distinct activities have been described (23-25). This catabolic route is an acidic lysosomal sphingomyelinase, which is deficient in some forms of Niemann-Pick disease. Please refer to Refs. 24 and 25 for recent reviews on sphingomyelinases. 

The catabolism of ceramide has been relatively poorly studied. Three distinct ceramidas have been detected and distinguished by their pH optima (20, 27). These enzymes result in the formation of sphingosine, which in turn may be reincorporated into ceramide or further degraded (28, 29). 

The cellular localization and topology of some of the enzymes involved in ceramide metabolism have been investigated using cell fractionation studies and fluorescent analogs of ceramide, sphingomyelin, and cerebroside (30, 31). The initial enzymes involved in synthesis of dihydrosphingosine appear to reside in the endoplasmic reticulum, but the enzymes that introduce the double bond into ceramide has not been determined. Incorporation of ceramide into more complex sphingolipids occurs in the Golgi apparatus. 

Notwithstanding the major advances in our understanding of intermediary metabolism of ceramide (refer to Refs. 13 and 30 for extensive reviews), major gaps still exist. Mammalian enzymes involved in ceramide biosynthesis remain poorly characterized. 

The yeast Saccharomyces cerevisiae has emerged as an important organism for the dissection of sphingolipid metabolism. S. cereuisiae contains phytosphingosine (4-hydroxyphytosphingosine) as the main long chain amino base and 4-hydroxyceramide as the main ceramide. The predominant complex sphingolipids of S. cereavisiae differ from mammalian sphingolipids in their headgroups. Through the selection of long chain amino base auxotrophs (32), the serine palmitoyltransferase (SPT) of S. cereavisiae was cloned and sequenced (33), thus providing the first molecular insight into an enzyme involved in ceramide biosynthesis. 

The Sphingomyelin Cycle: Discovery, Inducers, and Components 

The hypothesis that membrane sphingolipids could serve in signal transduction pathways suggested that an examination of sphingolipid metabolism in response to the action of extracellular agents may identify regulated pathways of sphingolipid hydrolysis (in analogy with the hydrolysis of inositol phospholipids). In studies conducted in HL-60 human leukemia cells, the addition of 1α,25-dihydroxyvitamin D3, an inducer of differentiation, caused early and reversible hydrolysis of SM and the concomitant generation of ceramide (10). TNF-α and γ-interferon (which induce monocyte differentiation of HL-60 cells similar to 1α,25-dihydroxyvitamin D3) were also found to induce SM hydrolysis (34-36), whereas retinoic acid, dibutyryl cyclic AMP, or dimethyl sulfoxide (which induce granulocytic differentiation) or phorbol esters (which induce macrophage-like differentiation) failed to induce SM hydrolysis (34, 37). Additional inducers of SM hydrolysis have been identified in other cell types including IL-1 (38, 39), dexamethasone (40), and complement components (41) (Table 1).
The studies with 1a,25-dihydroxyvitamin D$_3$ and TNF-α have defined the basic components of the SM cycle (Fig. 1). With TNF-α, this pathway is initiated by interaction with the 55-kDa receptor (42, 43). The action of TNF-α and 1a,25-dihydroxyvitamin D$_3$ then results in activation of a cytosolic neutral sphingomyelinase (44). The mechanisms coupling TNF-α receptors to activation of sphingomyelinase are poorly understood but may involve the intervening activation of phospholipase A$_2$ (45). Completion of the SM cycle occurs with the resynthesis of SM, presumably by the transfer of the choline phosphate headgroup from phosphatidylcholine to ceramide in cells (46). PDMP (Fig. 3), an inhibitor of glycosphingolipid synthesis (47), causes a significant attenuation of its biologic effects may be a consequence of ceramide formation (52).

**Biologic Activities of Ceramide**

The realization that SM hydrolysis was the earliest identified biochemical effect of the action of 1,25-dihydroxyvitamin D$_3$ on HL-60 cells raised the possibility that ceramide, the product of SM hydrolysis, may serve as an important effector. Thus, a major question emerged: is ceramide an intracellular effector mediator? This was initially approached by defining the effects of exogenous ceramides on candidate cellular activities, especially those induced by vitamin D$_3$ and TNF-α. Notably, ceramide emerged as a powerful regulator of cell growth, differentiation, and viability, i.e., major cellular activities of TNF-α.

**Regulation of Cell Growth and Differentiation**—Treatment of HL-60 leukemia cells with C$_2$-ceramide and other water-soluble analogs of ceramide (Fig. 3) was sufficient to induce differentiation of these cells. By morphologic, histochemical, and immunologic criteria, this differentiation was determined to be along the monocytic lineage; thus exogenous ceramides mimicked the action of TNF-α, 1a,25-dihydroxyvitamin D$_3$, and γ-interferon on HL-60 cells (53). In addition, C$_2$-ceramide and analogs exerted specific and potent antiproliferative effects in HL-60 cells observed at concentrations as low as 1–10 μM. Ceramide analogs were also active against other leukemia cells, malignant cells in tissue culture, and normal fibroblasts in logarithmic phase of growth. However, in confluent phase fibroblasts, ceramide enhanced thymidine uptake (22) (this may indicate either mitogenesis or repair following DNA damage).

Ceramide, an Agent of Death—Initial studies with ceramide in different cell systems were hampered by the potent cytotoxicity of ceramide. The realization that this cytotoxicity was specific to the active analogs of ceramide and that it mimicked the cytotoxicity of TNF-α raised the possibility that ceramide functions as an intracellular mediator of cytotoxicity. In fact, TNF-α belongs to a group of extracellular agents that activate endogenous programs of cell death termed apoptosis (54–56). In contrast to tissue necrosis, which occurs in response to severe insults and injury to cells, apoptosis involves an orderly breakdown of cells and cellular macromolecules, which are packaged into apoptotic bodies (57). Although apoptosis is emerging as a major biologic process equal in importance to cell growth and differentiation, it is by far the least understood at a molecular and biochemical level (58, 59).

In myeloid and lymphoid cells, ceramide analogs caused early, potent, and specific internucleosomal DNA fragmentation, a hallmark of apoptosis (60). The effects of C$_2$-ceramide were observed within 2–3 h and occurred at concentrations as low as 1–5 μM, and these effects have been observed in additional cell lines including fibroblasts. Thus, ceramide may mediate the effects of TNF-α on programmed cell death and may participate in other events of apoptosis. For example, ceramide levels are significantly increased in HIV-infected T lymphocytes (61), which are known to undergo apoptosis, suggesting a role for ceramide in the progression of apoptosis.

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2 L. M. Obeid, unpublished observations.
AIDS. Also, ceramide has been shown to increase in ischemia (62) and in denervated muscle (63). The increasingly appreciated roles for apoptosis in the regulation of development, tissue homeostasis, reaction to injury, and oncogenesis suggest important potential functions for ceramide in the regulation of these processes.

Regulation of Protein Secretion by Ceramide—Two recent lines of investigation raise the possibility that ceramide may function as a regulator of protein trafficking. First, it was shown that C₆-ceramide inhibits secretion of vesicular stomatitis virus glycoprotein from infected Chinese hamster ovary cells (64). In another approach, the macrolide brefeldin A, which causes collapse of the Golgi network into the endoplasmic reticulum, was shown to induce SM hydrolysis (65) and to activate the SM cycle in HL-60 cells. The generated ceramide inhibited protein secretion. Thus, ceramide emerges as a potential mediator of the action of brefeldin A.

Role in Inflammation—Studies in human fibroblasts show that ceramide modulates secretion of PG_E₂ in response to the action of IL-1 (38), and ceramide enhances the secretion of IL-2 in lymphocytes (39). Thus, ceramide may participate in IL-1 signal transduction. This, in addition to the role of ceramide in mediating TNF-α action, suggests that ceramide may play a role in modulation of immune function and inflammatory responses.

Specificity of Ceramide Action

The study of lipid mediators has been subject to persistent concerns over the specificity of action of candidate lipid second messenger/effector molecules especially since these molecules tend to be amphipathic and membrane-active. Some lipid effects can result in cell lysis due to detergent-like activity whereas a more valid concern arises from nonspecific effects of lipids in which membrane-associated enzymes and proteins require a hydrophobic environment for optimal activity. The specificity of ceramide was therefore investigated by examining the effects of other lipids, analogs, and isomers of ceramide (see Fig. 3 for structures). A summary of these results indicates that: 1) ceramide shows significant specificity when compared with other closely related lipids such as diacylglycerol; 2) Δ₆-erythro-N-myristoylaminocephylpropanol, a ceramide analog, demonstrates structural and stereospecificity of action (66); 3) Δ₆-erythro-C₆-ceramide is more potent than its enantiomer Δ₆-erythro-C₅-ceramide (67); 4) the closely related Δ₆-erythro-dihydro-C₆-ceramide lacks cellular activity (60, 68). Taken together, these studies demonstrate that the cellular activities of ceramide are specific and support a second messenger function of ceramide in analogy with that of diacylglycerol.

Mechanism of Action of Ceramide

Early Biochemical Effects of Ceramide Action on Cells—The very early effects of ceramide on ceramide levels suggested a number of cellular targets for ceramide (Fig. 1). Thus, ceramide was shown to induce early down-regulation of the c-myc protooncogene in HL-60 cells (34). Also, ceramide was shown to activate the nuclear factor κB in permeabilized (69) but not intact Jurkat T cells (70), raising the possibility that ceramide may be necessary but not sufficient in activating this transactivation factor. In human fibroblasts, ceramide has been implicated as an activator of transcription of the cyclooxygenase gene (38).

Ceramide-activated Protein Kinases—Studies on the effects of sphingosine on the EGFR receptor led to the identification of a sphingosine-induced phosphorylation of the EGFR receptor on threonine 669, which was independent of PKC inhibition (71). In a subsequent investigation of the possible role of ceramide in this phosphorylation, it was shown that C₆-ceramide reproduced this effect, and it was suggested that the effects of sphingosine were mediated by its conversion to ceramide (52) (Fig. 2). This phosphorylation is mediated by a membrane kinase, which is also activated by TNF-α in intact and in cell-free systems (35) and which shares the substrate specificity of MAP kinase (72). Indeed, ceramide has been shown to activate MAP kinase in HL-60 cells (73). At present, it has not been determined whether these kinases are direct or downstream targets for ceramide and what role they may play in mediating the biologic effects of ceramide.

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Ceramide-activated Protein Phosphatase (CAPP) as a Mediator of Ceramide Action—In vitro investigations of the effects of ceramide on protein phosphorylation led to the identification of CAPP as a serine/threonine protein phosphatase activated directly and specifically by ceramide (74). CAPP belongs to the heterotrimeric subfamily of the protein phosphatases 2A group, and it is inhibited potently by okadaic acid (75). Activation of CAPP requires the presence of the B subunit of heterotrimeric protein phosphatases 2A. A role for CAPP as a mediator of ceramide effects is supported by: 1) the specificity of activation of the phosphatase by ceramide (and not by dihydroceramide), which matches the specificity of the biologic activities of ceramide; and 2) inhibition of the effects of ceramide on apoptosis and c-myc down-regulation by okadaic acid.4

Insight From Studies in the Yeast S. cerevisiae—The lcb-1 strain of S. cerevisiae, which lacks the enzyme SPT, requires exogenous long chain bases for viability (32). Such a role in viability is also supported by studies in mammalian cells, since Chinese hamster ovary cells with a thermolabile SPT lose viability (76), primarily due to a loss in SM (77). A second yeast strain, termed SLC, bypasses the requirement for exogenous long chain bases in the absence of the lcb-1 gene. Interestingly, the SLC strain generates a novel glycerolipid that maps to identical headgroups to those found in yeast sphingolipids (78), thus demonstrating that the viability function of sphingolipids in yeast resides in the headgroup. However, the SLC strain shows significant defects in its ability to respond to environmental stresses (79), raising the possibility that the missing sphingolipid backbone (either phytosphingosine or phytoceramide) is important in the stress response. Such a role is further supported by the finding of exogenous long chain bases to restore growth of C₆-ceramide to inhibit growth of S. cerevisiae and to activate CAPP isolated from yeast cytosol (67). Thus, the ceramide pathway of cell regulation appears to be conserved in evolution. Yeast studies should have the added advantage of allowing a molecular genetic approach aimed at examining ceramide-mediated pathways of cell regulation.

Questions and Speculations

As insight into the SM/ceramide pathway develops, a new set of fundamental questions has emerged. 1) What is the extent of extracellular activators of SM hydrolysis and ceramide generation? 2) What are the biochemical and molecular mechanisms involved in coupling receptor activation to sphingomyelinase regulation? 3) What is the cellular localization of the signaling pool of SM? 4) What is the mechanism of action of ceramide, and are there direct targets in addition to CAPP? 5) What are the physiologic substrates for CAPP and how do they impinge on cellular regulation? Thus, although the basic blueprint of this novel signaling pathway can be outlined (see Fig. 1), this should be considered as a tentative outline whose further development requires the next level of biochemical analysis and improved pharmacologic modulators of the SM/ceramide pathway.

Ceramide and Dihydroceramide, the Sphingolipid Yenig and Y'angig of Cell Growth Regulation?—In contrast to ceramide, dihydroceramide is inactive in antiproliferation, differentiation, and apoptosis. The lack of activity of dihydroceramide is not due to decreased uptake or increased metabolism (65) but probably arises from the lack of activity of dihydroceramide on CAPP. Ceramide derives from the long chain base sphingosine (sphing minimine), whereas dihydroceramide derives from dihydrophosphoginisphing (sphingaminine). The only structural difference is the presence of a trans-4,5-double bond in ceramide but not in dihydroceramide (Fig. 3). These considerations raise the intriguing possibility that the sphingolipid double bond serves to impart critical biologic activities to ceramide, thus dissociating the early metabolic steps in sphingolipid biosynthesis from biologic activity.

The SM Cycle as a Prototype for Sphingolipid Signaling and Sphingolipid-derived Second Messengers—Defining the SM cycle and determining the second messenger function of ceramide illustrate the potential role for sphingolipid-derived molecules in signal transduction. The status of other sphingolipid-derived molecules as second messengers is less clear at present. Nonetheless, evidence emerging over the last few years points to important functions for ceramide.

3 C. M. Linnard and Y. A. Hannun, unpublished observations.
Sphingolipid Versus Glycerolipid Signaling: the DAG/Ceramide Matchup—Historically, glycerolipid signaling was defined in the context of mitogenic pathways, and the function of DAG second messengers (and their phorbol ester mimics) has been best elucidated in tumor promotion, mitogenesis, and activation of granule secretory vesicles. In striking contrast, ceramide is emerging as a key regulator of antiproliferative and apoptotic pathways and as an inhibitor of protein trafficking and secretion. Thus, DAG and ceramide may define opposing pathways of cell regulation. Indeed, DAG overcomes some of the actions of ceramide (such as apoptosis). As a simple hypothesis, activation of protein kinase C by DAG may counter the activation of CAPs by ceramide (Fig. 1).

The opposing effects of DAG and ceramide suggest that the relative concentrations of these two neutral lipids may be more critical than the absolute levels of either.

Ceramide as a Tumor Suppressor Lipid—If ceramide is capable of activating antiproliferative and apoptotic pathways, then what can we discern about its normal physiologic function? At a first level, we might elucidate the specific effects of exogenous agents (such as TNF-α). However, ceramide may have an additional function in monitoring the health of cells and tissues and in the response to stress and injury. Thus, increases in ceramide levels may inhibit cell growth as a cellular defense/repair mechanism. More deleterious conditions may result in higher levels of ceramide that go on to initiate apoptosis. Such a role for ceramide is further supported by the identification of a nuclear isoform of CAPP as a known tumor promoter (82). Also, the A subunit of heterotrimeric protein phosphatases 2A (to which CAPP belongs) is a target for the action of tumor antigens (such as SV40 t antigen (83)). Therefore, ceramide may function as a tumor suppressor lipid.

In conclusion, the study of the function of ceramide, its metabolism, and its mechanisms of action presents great rewards in understanding fundamental processes of cell biology including the regulation of proliferation, apoptosis, and protein secretion.

REFERENCES