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Perspective

Sphingolipids as coenzymes in anion transfer and tumor death

Norman S. Radin*

Mental Health Research Institute, University of Michigan, Ann Arbor, MI, USA

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Abstract—Many kinds of natural sphingolipids and their analogs stimulate or inhibit a wide assortment of biochemical phenomena and enzymes. The puzzle considered here is: how can these lipids control so many different kinds of processes? In almost every study in which a structural comparison was made, an allylic alcohol moiety [–CH=CH–CH(OH)–] was found to be an essential feature of the sphingolipid. Many of those stimulations lead to cell death, emphasizing the importance of allylic sphingolipid structure in the design of chemotherapeutic agents. The proposal offered here is that these lipids function as coenzymes, in which the allylic moiety acts as an anion transferring agent, forming transient phosphate or acyl or peptidyl esters for the synthesis or hydrolysis of phosphoproteins, proteins, and phospholipids. Sphingolipids that *inhibit* these reactions may simply displace the active sphingolipids from their sites in the enzymes' active regions, or bind to the enzymes' allosteric region. This kind of competition could act as a major homeostatic control mechanism. Some of the allylic sphingolipids also generate reactive oxygen, possibly by oxidation of the allylic alcohol group. This explains the need to control redox-controlling metabolites in sphingolipid-controlled processes (e.g., glutathione). Many anticancer drugs that produce apoptosis in tumors possess an allylic alcohol residue, affect protein phosphorylation, and produce reactive oxygen species. They may be therapeutically useful because they control the action of sphingolipids as anion transfer agonists or inhibitors.

1. Introduction

The recent, growing wave of research reports on sphingolipids reveals a strikingly wide range of biological effects of these simple lipids. Ceramide (Cer), the amide of a fatty acid and sphingosine, has received much of this attention because of its ability to kill normal and cancer cells by apoptosis (cell suicide). When Cer was added to cells or changed in cell concentration by metabolites or drugs or radiation, it was found to control cell growth or survival, protein phosphorylation and dephosphorylation, generation of reactive oxygen species (ROS), developmental changes in embryos and young animals, the transport of various substances, and more. Metabolic products of Cer—sphingosine, sphingosine-1-phosphate, Cer-1-phosphate, sphingomyelin, and the glycosphingolipids (particularly the glucosphingolipids, glucosylceramide, lactosylceramide, and the gangliosides)—control some of these properties. In most cases the metabolic products oppose Cer effects, functioning in a dynamic, feedback-controlled system in which hormones, cytokines, and growth factors fluctuate constantly. Some of the Cer metabolites are also involved in metastasis of tumors, the binding and development of microbes and their toxins in their victims, intercellular adhesion, the pathological narrowing of arterial lumens, and higher brain function.

Sphingolipids seem to form rapidly in tumors, and may represent a weak spot that ought to be engaged therapeutically. However, the conflict between the pro- and anti-apoptotic properties of the different sphingolipids makes them difficult to direct. Thus it can be therapeutically frustrating to induce faster Cer synthesis without simultaneously blocking its conversion to metabolites.[1–3] There is a need to understand the mechanisms of all these different effects and to look for an underlying theme in sphingolipid actions. The study of sphingolipid interactions with a variety of proteins has been under active investigation, but no proposal has been offered to describe the nature of the interactions other than a tentative suggestion that Cer exerts allosteric effects or controls the physical properties of membranes and submembrane aggregates (rafts or GEMs).

A clue to the puzzle may lie in dihydroceramide (H_2Cer), the de novo precursor of Cer. It is desaturated enzymatically between carbons 4 and 5 to yield the

* Corresponding author at present address: 10150 Torre Ave., #115, Cupertino, CA 95014-2129, USA. Tel.: +1 408 861 9487; fax: +1 408 446 4913; e-mail: gluconorm@aol.com

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trans-Δ^4 double bond typical of sphingolipids. Cells metabolize H_2Cer like Cer, forming H_2sphingomyelin, H_2glucosylceramide, etc., but the amounts of the saturated analogs are considerably less. Both groups of derivatives undergo hydrolysis as part of their normal turnover, forming H_2Cer and Cer, then free sphingosine and dihydrosphingosine (sphinganine). These amines are recycled to form the more complex sphingolipids or phosphorylated, then degraded. In virtually all the research studies in which Cer and H_2Cer were compared, the latter was inert or even inhibitory. Some researchers have attributed the inertness of H_2Cer to its slightly different physical properties, which may change the physical properties of critical ceramide-rich membranes and thus influence adjacent proteins. Gulbins and Kolesnick[4] have proposed that Cer, derived by the hydrolysis of sphingomyelin (SM), 'provides the driving force that results in the coalescence of microscopic rafts into large-membrane macrodomains. These structures serve as platforms for protein concentration and oligomerization, transmitting signals across the plasma membrane.' Because of the large variety of proteins that enter and function in rafts, the pleiotropic actions of Cer may be explained by this nonchemical mechanism.

Cer differs physically from H_2Cer in its formation of stronger hydrogen bonds between the three dissociable protons at the polar end of the molecule.[5] The protons join with two H_2O molecules to form a stable dihydrate, even in chloroform solution. GalactosylCer was also isolated as a hydrate.[6] Another analysis of the structural difference between the two ceramides concluded that one of the H_2O molecules is bound to the double bond.[7] While these studies were carried out in extremely simplified systems and may thus be physiologically irrelevant, marked changes in the stability of a natural membrane were seen with blood platelets when they were exposed to *N*-acetyl-sphingosine (C_2-Cer), while *N*-acetyl-sphinganine was inert.[8] However, none of these investigations was done with more typical membranes or rafts, and it remains to be seen whether exogenous H_2Cer can even enter into rafts.

The hypothesis that fluidity changes in membranes account for the specificity reported for Cer-induced enzyme activation is questionable. Many kinds of lipids have been added to cells without producing similar activations. Even the use of Cer made from widely different fatty acids (C_2 to C_{18}), or structural variations in the sphingosine moiety, yielded rapid stimulation and apoptosis. Further evidence for the specificity of sphingolipid/protein interactions is given below.

This review proposes a mechanism for the stimulatory action of Cer and other sphingolipids that explains the inertness of sphinganine-based lipids. It is based on the greater chemical reactivity of the allylic group of sphingosine and most sphingolipids. The double bond at Δ^4 brings the hydroxyl at C-3 into the class of allylic alcohols, compounds whose OH group is especially reactive and easily replaced chemically. This reactivity is seen in the ease with which the C-3 OH of Cer is dissociated by strong acid, leaving a cationic carbonium intermediate (carbocation) that readily takes up anionic groups, such as OH^-, MeO^-, or Br^-. The allylic carbocation also readily racemizes or shifts its double bond in the chain (the 'allylic rearrangement') so that the OH^- ion can return to the C-5 position, instead of the C-3 position.

2. The sphingolipid oxidation hypothesis

Allylic alcohols, unlike ordinary alcohols, are relatively easily oxidized by quinones, such as dichlorodicyanobenzoquinone,[9] so it is understandable that Cer interferes with the oxidative activity of mitochondria.[10,11] Oxidation in mitochondria depends on the ubiquinone cycle, in which the lipoidal coenzyme undergoes reduction and reoxidation via the quinone, free radical, and hydroquinone stages. Ubiquinone, like dichlorodicyanobenzoquinone, is a tetrasubstituted *p*-quinone (a double allylic ketone), so one might expect it to oxidize Cer to an allylic ketone, 3-ketoceramide, or to the free radical intermediate like the one formed from ubiquinone. When Cer is added to mitochondria or intact cells, reactive oxygen species (ROS) are produced, conceivably during the oxidation of the C-3 OH, and the complex process of apoptosis begins. Analyses of the steps indicate that Cer starts the apoptogenic process by interfering with ubiquinone function.[10] This capability of Cer is very promising for the therapy of cancer, atherosclerosis, and other diseases of excessive growth.

Allylic ketones undergo Michael condensations with locally available thiols and amines (especially histidine). The major thiol in cells is glutathione (GSH), which can be expected to form a thio ether at C-5 of Cer. GSH does indeed diminish when Cer is added to cells or otherwise forced to accumulate. Many anticancer drugs contain an allylic ketone moiety and also produce a reduction in tumor GSH content.[11] A search for condensation products from labeled Cer might support this hypothesis and lead to improved chemotherapeutic agents. Because of the many roles of ROS and cellular thiols and amines, one might expect Cer oxidation to produce the wide variety of physiological effects that have been reported. Unfortunately, ROS also attack nuclear DNA, so prolonged treatment with drugs that cause Cer elevation in mitochondria, or with drugs that produce ROS, may eventually lead to the appearance of cancer.

GSH is a natural inhibitor of a Mg^{2+}-dependent sphingomyelinase, one of the enzymes that forms Cer by hydrolysis of SM, the major sphingolipid.[12] Thus the destruction of GSH by Cer-induced ROS and condensation leads to the formation of more Cer from the relatively prevalent SM. This sequence of reactions gradually generates an inflationary spiral, leading to increasing concentrations of Cer and ROS, decreasing GSH, and eventual apoptosis. It seems evident that a complete cocktail for cancer chemotherapy should include agents that lower tumor GSH.

It is significant that a surprising number of anticancer drugs in current use or proposed for use contain an allylic alcohol group.[11] Examples of apoptogenic drugs in

this category, some in clinical use: anthracyclines, camptothecin, tetrahydrocannabinol metabolites, mitoxanthrone, irofulven, lapachol, statin metabolites, geranylgeraniol and farnesol, apoptolidin, 3-hydroxymethyl indole, rapamycin, brefeldin A, symbioramide, losartan, paclitaxel and docetaxel, and dihydroxy vitamin D3 among many others. The last of these (D3) rapidly stimulated protein kinase C and may thus act as an agonist, displacing the sphingolipid that normally functions in the kinase.[13] It elevates the level of cell Cer and ROS, causing release of mitochondrial cytochrome *c* and producing tumor cell apoptosis. This suggests that many of these allylic drugs function as agonists for various sphingolipid coenzymes. Many other anticancer drugs are converted to the allylic alcohol form by oxygenases acting near double bonds.

Some anticancer drugs already contain an allylic ketone moiety, resembling the hypothetical keto-Cer. Those drugs may be reduced to allylic alcohols and function as sphingolipid agonists for kinases. Many of the drugs have been shown to generate ROS and lower cell GSH levels. Some have indeed been found to produce Cer.

Other sphingolipids have been shown to generate ROS in vitro too, suggesting that the critical requirement is an allylic alcohol group, and that the other substituents play a secondary role.

3. Phosphoprotein phosphatase activation by sphingolipids

Many of the pleiotropic sphingolipid activities function via control of the phosphorylation state of proteins, now known to control the activity of many enzymes. Cer stimulates several serine/threonine protein phosphatases (the Cer-activated protein phosphatases, CAPP, PPA1. PP2A), while H_2Cer is inactive. This difference, again, can be ascribed to the higher reactivity of the allylic alcohol group and I propose here that the alcohol group acts as a transfer agent. Figure 1 summarizes this thought, in which the Cer is held in a pocket of a phosphatase close to the active site and forced to form a diester with a phosphate group of the substrate phosphoprotein. The diester is then cleaved on the other side of the phosphate group, liberating the dephosphorylated protein. The resultant Cer-3-phosphate ester is then hydrolyzed by the phosphatase, freeing the Cer molecule for a new cycle. This cycle constitutes a coenzyme activity of Cer.

In Figure 1, the OH of Cer is excised by the phosphatase, leaving behind a carbocationic form of Cer, which then combines with an oxygen of the substrate protein's phosphate. However, it is possible that the anionic oxygen of the phosphate ester simply *displaces* the OH of Cer (a reverse form of phosphatase action). Similarly, the release of the phosphate ion from the C-3 position of Cer in the second stage may occur not by hydrolysis but by simple elimination of inorganic phosphate, leaving behind the carbocationic form, which is then ready for the next catalytic cycle. This sort of elimination occurs during the synthesis of farnesyl pyrophosphate (an allylic alcohol phosphate ester) by prenyl synthetase and the reaction of monoterpene cyclases, during which an allylic pyrophosphate ester temporarily forms a carbocationic intermediate.[14]

Numerous researchers have reported finding that Cer (usually one with a short fatty acid moiety, such as

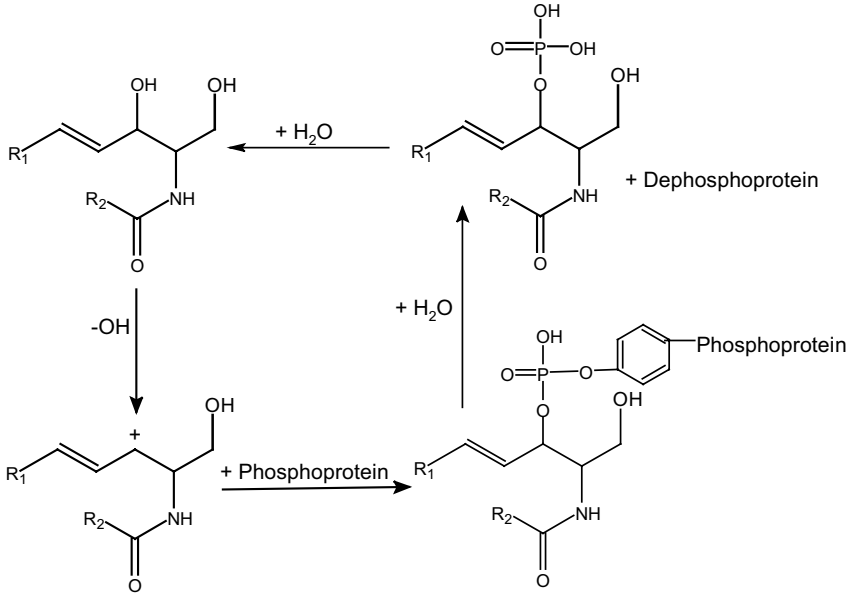

Figure 1. Hypothetical ceramide-catalyzed hydrolysis of a phosphoprotein. A typical Cer ($R_1 = CH_3–(CH_2)_{12}$- and $R_2 = CH_3–(CH_2)_{16}$-) is at the upper left, bound to a phosphoprotein phosphatase. The allylic hydroxyl at C-3 is removed by the enzyme and the resultant carbonium ion condenses with a phosphoprotein, whose hydroxyl-linked phosphate attaches to C-3. This diester is then hydrolyzed at the substrate protein link, forming Cer-3-phosphate, which is then hydrolyzed to regenerate the Cer. In this figure, the phosphoprotein is attached to the phosphate through a Tyr link, but it could also be through a Ser or Thr link. The phosphate linked to Cer is probably also connected to a hydrogen bond with the C-1 hydroxyl.

acetyl or octanoyl) stimulates such phosphatase reactions. Most studies were done with whole cells or cell fractions and only a few were done with purified enzymes. Many of the reports showed that the activation was very fast and dose related. Appreciable phosphatase activity could be demonstrated without adding *any* Cer, probably because some of the phosphatase molecules in the sample already contained bound Cer. Thus the observed activation is due to marshalling of inactive (apo)phosphatase molecules to join the active ones. Yet to be demonstrated, however, is that the phosphatase preparations already contained bound Cer.

Cer-activated protein phosphatases can be blocked very efficiently by nanomolar concentrations of okadaic acid (OA, Merck Index 6891), a natural complex polyether. OA enters a hydrophobic groove of the enzyme, presumably the same groove that normally holds the bound Cer, also very definitely hydrophobic too. Hydrogen bonds form between the enzyme and the OA carboxyl group and two of the OA hydroxyl groups.[15] The hydroxyl at C-24 is allylic, lying very close to an arginine moiety of phosphatase PP1. It seems likely that the strongly cationic guanidine group of arginine can extract this hydroxyl, leaving the allylic carbon of OA (or Cer) in carbocationic form, as postulated in Figure 1. Thus this OH may act like the allylic OH of Cer, forming a phosphate ester with the substrate phosphoprotein, but one that is unproductive (unhydrolyzable). OA is a well-known cancer promoter, able to block the apoptogenic action of Cer. This and other data strongly suggest that protein phosphatase action is required for apoptosis. A good example was seen with protein phosphatase 2A, which with Cer produces dephosphorylation of the anti-apoptotic molecule Bcl2 and thus produces apoptosis.[16] OA blocks this effect. A vital part of the phosphatase, B56 alpha-subunit, binds Bcl2 and apparently also binds Cer. Accordingly, study of connections between compounds containing an allylic alcohol moiety, protein phosphorylation, and cancer cell death should prove fruitful.

A particularly interesting example of Cer function in a protein phosphatase is seen with Akt (protein kinase B). Cer added to glioblastoma cells activated a protein phosphatase to remove two specific phosphate residues from Akt, at Thr308 and Ser473, causing great loss of kinase activity.[17] H_2Cer was inactive and wortmannin increased the effectiveness of Cer. However, the latter stimulated the loss of only the Ser473 phosphate. Wortmannin (Merck Index 10110) is an unusual antifungal antibiotic, generally considered to be an inhibitor of phosphoinositide-3-kinase and DNA-dependent protein kinase. Because wortmannin contains an allylic ketone group attached to conjugated double bonds and a vinylic O atom (apoptosis-promoting structural features), it can be expected to condense with thiol groups, especially in the Cys-rich region near the active site of protein phosphatases. Some of the wortmannin can be expected to undergo reduction to an allylic alcohol, which might behave as a Cer agonist, acting as a coenzyme in Akt dephosphorylation. However, its brief half life (~10 min) might not give the activated phosphatase enough time to cleave *both* phosphate links. The importance of this Cys-rich region has been further indicated by a study with *N*-ethylmaleimide, the reagent that condenses with Cys.[18] As Ruvolo has pointed out,[19] 'Ceramide activation of protein phosphatases has been shown to promote inactivation of a number of pro-growth cellular regulators including the kinases PKC alpha and Akt, Bcl2 and the retinoblastoma protein.' Stated differently: Cer can produce inhibition by stimulating a phosphatase.

4. Ceramide structure and phosphatase activity

A study of the stimulation of protein phosphatases acting on Ser/Thr phosphates[20] showed that the catalytic subunit of PP2A (PP2Ac) was 180% more active in the presence of C_6-Cer. The trimeric (natural) form of PP2A was stimulated 230%, and PP1cα was stimulated 150%. Cer analogs lacking the allylic double bond (C_6-H_2Cer and C_6-phytoCer) were inactive. The *cis*-isomer of C_6-Cer, which retains an allylic structure, could not stimulate the enzymes. This shows that the *trans* form of the allylic double bond is required for proper activation. The D- and L-forms of H_2Cer *inhibited* the enzymes rather effectively (77–91%) at 15 μM, suggesting that they or an appropriate analog may be useful therapeutically.

H_2Cer was found to bind to PP2Ac as strongly as Cer, so its inhibitory activity suggests that the saturated Cer fits into the enzyme's active crevice and displaces Cer. Perhaps other Cer analogs can also enter active enzyme sites and it seems advisable for researchers who synthesize new analogs to test them against Cer-induced phosphate transfer as well as on growth retardation or apoptosis. A particularly interesting one to test would be Cer possessing an extra, conjugated double bond (4,6-diene Cer). This has a more reactive allylic alcohol group and proved much more active than Cer in inducing apoptosis in cancer cells.[21]

An assortment of other Cer analogs either had no effect on the phosphatase or yielded weak inhibition: Cer methylated on the amide N or either OH; 3-ketoCer; Cer with a urea group instead of the amide group; a C≡C bond instead of the normal C=C bond; and ceramides in which the terminal C_{13} chain of sphingosine was replaced by a phenyl group.[20] The hypotheses proposed in this paper predict that most of these analogs are inactive. The triple-bond analog of Cer could conceivably activate the C-3 OH but it does have a somewhat different electron distribution and the shorter bond (compared to C=C) may disturb the fit into the enzymes. Thus the allylic activation hypothesis cannot be fully tested with these data.

5. Protein kinase activation by sphingolipids

Several important kinases were found to be stimulated by Cer and by conditions that lead to Cer accumulation. This coenzyme activity of Cer can be illustrated with Figure 1 by visualizing reversal of the directions of the

arrows and starting with ATP as the phosphate donor instead of a phosphoprotein. The ATP forms a putative transient 3-phosphate ester of Cer in the kinase, which is then joined to a hydroxyl group in an acceptor protein as a diester phosphate. Hydrolysis of the Cer-phosphate bond by the kinase then results in the formation of the phosphoprotein, dissociation of the two proteins, and regeneration of the free allylic O on the Cer. This sequence of steps explains why the addition of a sphingolipid, especially Cer, produces changes in cellular Ca^{2+} distribution.

A member of the protein kinase C family, PKC ζ, was found to rapidly bind Cer (oleoylsphingosine).[22] After contact with ATP and a histone as substrates, in the presence of phosphatidylserine, the amount of phosphorylated histone was found to be several fold higher than the system lacking added Cer. This was demonstrated with enzyme purified by immunoprecipitation. Using diacylglycerol instead of Cer did not stimulate the reaction, but the diglyceride strongly inhibited the effect of Cer. This action is interesting since diglyceride also strongly inhibits the transfer of a phosphocholine moiety from phosphatidylcholine to Cer in the enzymatic synthesis of SM.[23] This may mean that Cer acts as both a substrate and coenzyme for the synthase. This kind of anion transfer coenzyme activity is described below.

Shorter chain Cer (C_6-Cer) also stimulated the purified PKC, while the saturated lipid, C_6-H_2Cer, had no effect on the enzyme.[22] PKC ζ phosphorylated SAPK (stress-activated protein kinase) and C_6-Cer increased the binding between the two proteins. This is the effect to be expected from a coenzyme that forms a link with its substrate protein. The enhancement in enzyme/protein binding may be a general diagnostic test for coenzyme activity by a sphingolipid.

The permissible sphingolipid structures for this kind of activation are much more flexible than the few structures reported for Cer-activated phosphatase action.[20] Cofactor activity is seen even when the fatty acyl chain is quite short (e.g., C_2-Cer) so two long chains are not essential for activity. The oxygen in the amide linkage of Cer is apparently not essential either, since *N*-octyl sphingosine (a 2° amine, not an amide) is also effective in producing apoptosis and protein phosphorylation.[24] *N*-Octyl sphingosine is better than *N*-octanoyl sphingosine in producing apoptosis and equal to C_8-Cer in activating CAPK (ceramide-activated protein kinase). The orientation of the C-3 OH (normally erythro) also was irrelevant.

The hydroxyl at C-1 of sphingosine and Cer is also apparently unessential. Ganglioside GD3 (disialo-lactosylCer) does not have an OH at C-1 but it stimulated purified mitogen-activated protein kinase (MAPK) ~120% within 10 min.[25] It also stimulated formation of ROS, consistent with the idea that the C-3 OH^- was oxidized, as with Cer. The oxidation may yield a free radical in the region undergoing phosphate transfer, adding to the free energy needed for the condensation and cleavage steps.

Exogenous ganglioside GD3 readily penetrated normal human aortic smooth muscle cells but GD3 based on H_2Cer bound only to the cell walls.[25] LactosylCer also greatly stimulated formation of ROS, but ganglioside GM3 (sialyllactosylCer) was inert, perhaps showing the importance of structural differences that may control entry into mitochondria. The concept that glucosphingolipids can act as coenzymes may explain why cells contain so many different glucosphingolipids (hundreds). Presumably the differing oligosaccharide moieties make them specific for different apoenzymes. Over 500 protein kinases are now known. Conversely, it is not surprising that many kinases occur in the sphingolipid-rich rafts (GEMs).[26]

Another example of the unimportance of the C-1 OH is seen with sphingosine-1-phosphate, which induced proliferation of neural progenitor cells and phosphorylation of extracellular signal-regulated kinase (ERK).[27] In a curious feedback arrangement, ERK1/2 or a similar kinase was found to phosphorylate the kinase that makes sphingosine-1-phosphate, acting on its Ser225.[28] Sphingosine phosphate also stimulated phosphorylation of Tyr in Akt by 200% within 4 min in aortic endothelial cells, without any sign of a lag period.[29] This observation, together with the activity of Cer as a *dephosphorylating* agent for Akt,[17] helps explain why Cer and sphingosine phosphate usually produce opposing effects on apoptosis.

Even a sphingolipid lacking the fatty acid and C-1 OH (sphingosylphosphorylcholine = lyso-SM) induces keratin phosphorylation and reorganization of keratin filaments in human epithelial tumor cells.[30] This sphingolipid occurs at particularly high concentrations in patients with ovarian cancer and the authors suggest that the lipid's effect on keratin helps cancer cells metastasize by migrating out through membrane pores. Phosphorylation was observed as early as 5 min and the site of phosphate attachment was quite specific. Sphingosine-1-phosphate, lysophosphatidic acid, *N*-acetyl-lyso-SM (C_2-SM), and a stereomer of lyso-SM could not stimulate the phosphorylation or keratin reorganization. Thus, while proof of allylic sphingolipid-induced phosphorylation was not obtained with a known, purified kinase and with dihydro-lyso-SM, the evidence for similarity to the Cer-induction mechanism is strong.

If Cer acts by forming a transient phosphate ester with the allylic OH, without need for the amide linkage, the C-1 OH, or a long chain fatty acyl group, one could expect that even the simplest sphingolipid should behave similarly. The primary sphingolipid, D-erythro-sphingosine, and its methylated metabolic product (*N*,*N*-dimethylsphingosine), seem to be coenzymes for phosphorylation by various enzymes, including a group called sphingosine-dependent protein kinases (SDKs). An SDK purified from mouse fibroblasts, SDK1, phosphorylated Ser in some members of the 14-3-3 family of modulatory proteins.[31] Cer, sphingosine-1-phosphate, lysoganglioside GM3, and some other lipids, did not stimulate the kinase; unfortunately dihydrosphingosine was apparently not examined.

Dimethylsphingosine was first found in cells when they were incubated with an inhibitor of Cer glucosylation in the presence of interleukin 2.[32] The inhibitor, PDMP (a Cer analog), blocked the synthesis of glucosylCer, causing accumulation of the precursor, Cer. The cells also accumulated three Tyr-phosphorylated proteins, plus monomethyl- and dimethylsphingosine. The latter were evidently due to enhanced hydrolysis of the accumulating Cer and methylation of the free sphingoid base.

An unusual feature of the sphingosine-activated kinase, SDK1, is that its amino acid sequence corresponds very closely to the kinase region of protein kinase Cδ, yet PKCδ was *inhibited* by sphingosine and dimethylsphingosine.[33] When the kinase domain of PKCδ was separated from the enzyme by peptide cleavage with caspase-3, it now responded to the stimulatory action of dimethylsphingosine, with the same substrate specificity as SDK1. Perhaps the *catalytically inactive* segment of PKCδ is able to bind the sphingoid base, causing unproductive distortion of the active region. Thus it is possible to inhibit a sphingolipid-dependent enzyme without actually displacing the active lipid from the active site.

It is significant that some allylic *ketones* are potent inhibitors of kinases that act on protein Tyr residues. Genistein (Merck Index 4402) contains an isoflavone ring (an allylic ketone containing a vinylic ether attached to the allylic double bond) and it counteracts the ability of paclitaxel and vincristine to produce apoptosis via the production of Cer.[34] It down-regulates cyclin B1/CDC2 kinase expression resulting in reduced Bcl2 phosphorylation. This may indicate that the kinase requires Cer to function. Another kinase blocker is herbimycin, an antibiotic containing a benzoquinone ring (double allylic ketone). Quercetin, luteolin, and other examples are allylic ketones that seem to block the action of allylic alcohols (Cer, etc.). Perhaps they can occupy the same site in kinases as the allylic alcohol moiety, thus block sphingolipid coenzyme entrance into the kinase. (See the discussion of wortmannin above.) This thought applies also to many anticancer drugs, as well as normal cell components, that contain an allylic ketone residue. Many of the anticancer drugs also have a vinylic ether or amine or alcohol on the double bond. Thus a sphingolipid, such as Cer, might be especially effective as an apoptosis inducer if such a residue were to be attached to the basic allylic structure.

An intriguing possibility is that Cer participates in the kinase that phosphorylates one of the hydroxyls in reduced ubiquinone. A metabolite of ubiquinone found in urine consists of such a phosphate ester but with a shortened side chain.[35] As mentioned above, Cer interferes with mitochondrial electron transport via the ubiquinone/ubiquinol cycle. It may be that ubiquinone oxidizes the allylic alcohol moiety of Cer (producing ROS) and the reduction product, ubiquinol, is then phosphorylated via a Cer-activated kinase. Another possibility is that some anticancer agents possessing a quinone structure act to oxidize the C-3 OH of sphingolipids, rendering them unable to act as coenzymes.

6. Other hydrolyses/acylations possibly utilizing sphingolipid coenzymes

Cytosolic phospholipase A_2, the enzyme that typically hydrolyzes a carboxylic ester linkage in phospholipids, was found to bind Cer-1-phosphate, which directly activated the enzyme.[36] This finding is consistent with the work cited above, showing that the free C-1 OH of Cer is unnecessary for transfer activity in kinases. It seems likely that the enzyme-bound Cer-1-phosphate acts as a coenzyme, enabling transfer of an anionic fatty acyl moiety from phospholipids to the C-3 carbocation (Fig. 2). The transient 3-O-acyl Cer ester could then be hydrolyzed, yielding the free fatty acid and Cer, completing the coenzyme cycle.

Arachidonic acid is the dominant fatty acid released from phospholipids in this way, and it is interesting that it leads to the prostanoids (some of which contain an allylic alcohol moiety) and also stimulates SM hydrolysis to Cer and phosphocholine. Thus it looks as though the lipase participates in another expanding spiral of gradual increase in Cer that is so typical of Cer-induced apoptosis. But is H_2Cer-1-phosphate inert in this activation process?

C_2-Cer acts as an acceptor for a lysosomal phospholipase A_2 acyltransferase, forming 1-arachidonoyl C_2-Cer.[37] Phosphatidylcholine, phosphatidylethanolamine, and plasmalogen[38] act as donors of the fatty acid. The arachidonate group may attach first to the C-3 carbon of C_2-Cer, then 'swing over' to the C-1 carbon, which is very near. A second enzyme can remove the arachidonoyl moiety by hydrolysis, so a short-chain Cer can act as a coenzyme for the (apparent) hydrolysis of the three major ester-type phospholipids.

If the above mechanism is correct, one could consider the possibility that sphingolipids also transfer other anionic moieties. Compounds containing activated acyl groups (e.g., acyl-CoA, phenolic acetates like aspirin, 1-acyldihydroxyacetone phosphate, enolic acetates, etc.) may transfer acyl groups to the allylic OH of enzyme-bound Cer, then to appropriate receptor amines, such as sphingosine. In the latter case, the product would be Cer, the net effect being a reaction that synthesizes Cer from a phospholipid and free sphingosine without the need for coenzyme A.

A specific Ser moiety in 1-O-acylceramide synthase was found to be essential for activity[37] and was proposed as the intermediate arachidonate binding point, able to transfer the acid of phospholipids to Cer. Perhaps that critical Ser or CySH moieties in sphingolipid-dependent enzymes act in conjunction with the sphingolipid coenzymes.

Peptide hydrolysis is a prominent activity in tumors and cathepsin D, a protease containing an Asp residue at its active site, is found at high levels in many tumors. Cer has been reported to bind to the enzyme and activate it.[39] Perhaps the Asp residue forms an ester with ceramide's allylic OH, which is then activated for exchange

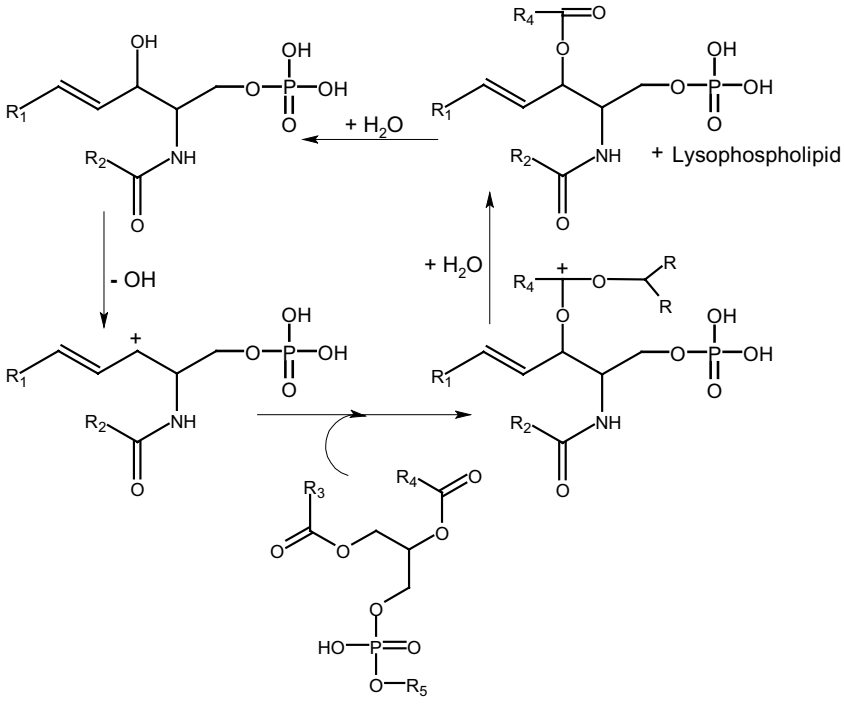

Figure 2. Proposed function of Cer-1-phosphate as coenzyme in hydrolysis of a phospholipid. As in Figure 1, the first step (left side of figure) shows extraction of the allylic OH by the phospholipase. The carbocation next condenses with a phospholipid (R_3 and R_4 are fatty acids), whose carbonyl O at the glyceride C-2 binds to the allylic carbon atom. The product (lower right of figure) now has the plus charge on the R_4 fatty acid's carboxyl carbon. Hydrolysis yields the lysophospholipid and the 3-O-acyl ester of Cer-1-phosphate, which undergoes hydrolysis to regenerate the sphingolipid and release free fatty acid R_4.

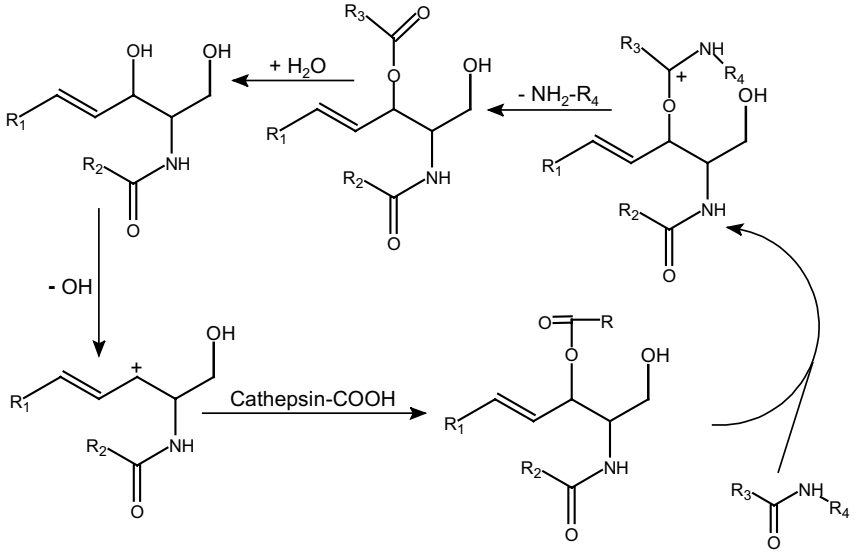

Figure 3. Proposed coenzyme activity of ceramide in protein hydrolysis. The carbocationic derivative of ceramide (bottom left of figure) condenses with the aspartyl COOH of cathepsin (symbolized by R), forming the ceramide's C-3 O-ester. The cathepsin's Asp is displaced by the substrate protein's amide carbonyl (upper right). The cathepsin then cleaves the amide link, leaving part of the substrate still linked to the Cer as a peptide ester (R_3) and the free N-terminal peptide (R_4). Peptide R_3 is then released by hydrolysis, regenerating the coenzyme.

transfer with a peptidyl COOH within the substrate protein (Fig. 3). The purified protease adhered to a Cer-linked affinity column, but not to a H_2Cer column, and was eluted from the column by Triton-solubilized Cer (and not by H_2Cer). An interesting point is that Cer also stimulated the action of pre-pro-cathepsin and its conversion to the final active protease. This paper sets an excellent standard for tests proving that the allylic group is essential for enzyme activation.

Another study appears to show that Cer—but not H_2Cer—activates a protease, prICE.[40] This enzyme cleaves poly(ADP-ribose) polymerase (PARP), yielding an apoptosis-specific fragment. Etoposide, the potent

antineoplastic drug, behaves in vivo like Cer in this respect. It seems significant that the drug undergoes oxidation to form a double allylic ketone, which may be the active factor, thus extending the allylic leitmotif to proteolysis.

A plasma protein, antithrombin III, involved in proteolysis of blood coagulation factors, was found to contain glucosylCer, the precursor of all the glucosphingolipids.[41] Thrombin, the blood coagulant that is blocked by antithrombin, is itself strongly activated by sphingosine-1-phosphate (a major platelet lipid) to generate tissue factor, thus starting the coagulation cascade.[42] Since the enzymes are proteases, these reports may be additional examples of a sphingolipid coenzyme acting as a peptide-transferring agent. It is important that thrombin, like sphingosine phosphate, is also a proliferation stimulator.

7. Comments

Further experimental support for these hypotheses might be obtained by

(1) tracing sphingolipids labeled with radioactive, or fluorescent, or photoactivatable moieties to demonstrate binding to responsive enzymes;
(2) mixing a sphingolipid-activated enzyme with a substrate, looking for enhancement of binding between the enzyme and its substrate;
(3) examining purified anion transfer enzymes for the presence of sphingolipids near the active site;
(4) testing sphingolipid-containing enzymes for release of the bound lipid when treated with a sphingolipid known to inhibit the enzyme;
(5) synthesizing unnatural sphingolipids containing a more reactive allylic alcohol group (double bonds conjugated with the allylic double bond, or extra allylic hydroxyls) to see if they are more active; hydrogenated sphingolipids should be made for comparison;
(6) passing enzyme solutions through a column containing a bound sphingolipid (or mixture of bound sphingolipids as an initial, scouting evaluation) to see if the enzyme binds;
(7) testing drugs containing an allylic alcohol moiety for ability to stimulate anion-transferring enzymes known to require a sphingolipid coenzyme;
(8) synthesizing a sphingolipid that already has a putative anion residue on the allylic OH (such as ceramide-3-phosphate). This would allow one to see if it can insert into an apoenzyme and transfer the linked anion to water or other acceptor substrate. However, the receptor site in the apoenzyme may accept only the uncharged sphingolipid. Other tests will no doubt occur to the reader.

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