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Functions of Sphingolipids and Sphingolipid Breakdown Products in Cellular Regulation

YUSUF A. HANNUN AND ROBERT M. BELL

The discovery that breakdown products of cellular sphingolipids are biologically active has generated interest in the role of these molecules in cell physiology and pathology. Sphingolipid breakdown products, sphingosine and lysosphingolipids, inhibit protein kinase C, a pivotal enzyme in cell regulation and signal transduction. Sphingolipids and lysosphingolipids affect significant cellular responses and exhibit antitumor promoter activities in various mammalian cells. These molecules may function as endogenous modulators of cell function and possibly as second messengers.

UNTIL RECENTLY, INVESTIGATIONS OF THE BEWILDERING array of complex cellular lipids have provided little more than promises of their important cellular functions. Why had these molecules survived eons of evolution when a simple phospholipid would suffice in bilayer formation to delimit cells and to divide the cytoplasm into its organelles and compartments? The answer, while incomplete, has more to do with the breakdown products and metabolites of membrane lipids than with the lipids themselves. Many of these breakdown products and metabolites function predominantly in signal transduction as agonists or as second messengers. They include diacylglycerol (*1*), platelet activating factor (*2*), phosphatidic acid (*3*), arachidonic acid (*4*), prostaglandins (*4*), leukotrienes (*4, 5*), eicosanoids (*4*), thromboxanes (*4*), lipoxins (*5*), inositol phosphates (*6*), and inositol glycans (*7*). Although some of these, such as diacylglycerol and phosphatidic acid, are present constitutively in cells under resting conditions, most of the others are generated when cells are activated.

A recent addition to this growing family of biologically and physiologically active lipids emerged from investigations of sphingolipid breakdown products. When added to cells, sphingolipid-derived molecules, sphingosine and lysosphingolipids, elicit various pharmacologic responses, such as inhibition of platelet and neutrophil activation (*8–10*), inhibition of growth factor action (*11, 12*), modulation of receptor function (*13*), and inhibition of phorbol ester–induced responses (*14–16*).

Studies of the biological roles of sphingosine and lysosphingolipids were spurred by the discovery that these molecules are potent and reversible inhibitors of protein kinase C (*8, 17*). Protein kinase C transduces the action of diacylglycerol second messengers and of phorbol esters (*1*) and is, therefore, a significant target for negative regulation.

The cellular and biochemical effects of sphingosine and lysosphingolipids raise a number of important and as yet unanswered questions. (i) Do lysosphingolipids and sphingosine function physiologically either as long-term modulators of cell activation or as second messengers? (ii) What is the spectrum of pharmacologic activity of these molecules? (iii) Are these molecules causative agents in pathological conditions? (iv) What is the mechanism of action of sphingosine and lysosphingolipids? Are there targets other than protein kinase C? (v) Could these molecules or their analogs be of value as antitumor or chemopreventive agents?

Recognition of the importance of these questions was among several recent developments that generated a renewed interest in sphingolipid metabolism and led to the generation of multiple hypotheses on the role of sphingolipid breakdown products in cellular regulation. In this review, we describe the current state of knowledge about the cellular and biochemical actions of sphingolipids and their breakdown products and evaluate proposed functions for sphingosine and lysosphingolipids.

Although the major emphasis is on sphingosine and lysosphingolipids, an overview of sphingolipid function is warranted because of the integral relation between lysosphingolipids and their parent sphingolipids. Also, the wealth of information on sphingolipids may be useful in future evaluation of the function of their breakdown products, the lysosphingolipids.

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Biological Functions of Sphingolipids

The diversity of sphingolipids has intrigued investigators for many years. In fact, the prefix sphingo derives from the Greek myth of the sphinx and signifies the magnitude of the riddle associated with the function of these molecules (*18*).

At least 300 different sphingolipids are synthesized in various mammalian cell types. Structurally, sphingolipids are composed of a long-chain sphingoid base, an amide-linked fatty acid, and a polar head group at the 1-position (Fig. 1). Except for ceramide, which has a hydroxyl at the 1-position, and for sphingomyelin, which has a phosphorylcholine head group, all other sphingolipids contain carbohydrate head groups and hence are designated glycosphingolipids. These include neutral lipids, which contain from one (cerebrosides) to 20 or more glycose units (*19*), and acidic glycosphingolipids, which contain one or more sialic acid residues (gangliosides) or sulfate monoester groups (sulfatides) (*20*). Most of the gangliosides and complex glycolipids are thought to reside on the outer leaflet of the cell membrane. Recently, however, lactosylaceramide was shown to have a predominantly intracellular location in association with granule-rich fractions of human neutrophils (*21*).

A number of biological and pathological functions are attributed to different sphingolipids (Table 1). The expression of various gangliosides on the cell surface has been correlated with cell transformation and tumor progression. For example, human melanomas express GM_2 ganglioside (*22*), and as melanoma cells progress, the levels of GD_2, another ganglioside, increase. Other gangliosides are expressed in tumor cell lines as well as in oncogene-transfected cells (Table 1). Gangliosides also serve as differentiation markers. Sphingomyelin levels are increased in hairy cell leukemia treated with phorbol esters (*23*) and in 3T3-L1 cells treated with dexamethasone (*24*). The ganglioside GM_3 increases during macrophage-like cell differentiation of HL-60 and U937 promyelocytic and monocytoid leukemia cell lines (*25*). Recently, a GM_3 sialidase activity was observed in growing fibroblasts, with peak activity during the confluent phase of fibroblast growth (*25*). Gangliosides are also thought to participate in various cell regulatory functions. These include cell contact response, contact inhibition, and cell recognition (*26–29*). Additional insight into the functions of gangliosides derives from experiments with specific ligands. For example, the B subunit of cholera toxin, which specifically binds to GM_1 ganglioside molecules, induces a biphasic response in the mitogenesis of 3T3 cells, depending on the cells' state of growth (*30*).

Additional roles for gangliosides in modulating cell proliferation have been examined by the addition of glycolipids and antibodies directed against glycolipids to cultured cells. These studies revealed that added ganglioside reduced the growth rate and saturation density of transformed cells (*31*), normalized the cell cycle, and caused an increase in adhesiveness, differentiation (*32*), and antigenic conversion of cells (*33*). In neuroblastoma cells, GQ_{1b} was found to have nerve growth factor–like activity (*34*). The modulation of gangliosides on the cell surface by the addition of antibodies to glycolipids has also been observed to affect cell growth. The addition of antibody to GM_3, for example, inhibited the growth of hamster NIH and mouse Balb/c fibroblasts (*35*).

The biochemical mechanisms by which gangliosides alter cell behavior are still poorly understood. Certain gangliosides appear to have an effect on protein phosphorylation. Ganglioside-dependent protein kinase activity has been described (*36–38*), whereas other protein kinases are inhibited by gangliosides (*39–41*). These observations raise the question of how gangliosides, which are present on the outer leaflet of the cell membrane, affect the function of intracellular protein kinases and other targets. Transmembrane movement of complex gangliosides from the external cell surface to internal cytoplasmic sites appears unlikely in the absence of specific transporters. Therefore, mechanisms of ganglioside action must involve either specific uptake or transmembrane signalling. In the latter case, gangliosides may be metabolized extracellularly upon specific interaction with ligand to generate the corresponding lysoganglioside or sphingosine (or both), which then crosses the bilayer (see below). In addition, gangliosides, or their metabolites (*25*), may affect receptor-receptor interaction and receptor kinase activity (*11*).

Another hypothesis is that changes in expression of gangliosides on the cell surface during differentiation and cell transformation may be accompanied by changes in the intracellular levels of the corresponding lysosphingolipid or sphingosine. Sphingosine and lysosphingolipids, the putative effectors, would then alter cell function by interacting with specific intracellular targets such as protein kinase C.

Biological Effects of Lysosphingolipids

In contrast to the wealth of information on the diverse biologic functions of sphingolipids, little is known about the biological effects of lysosphingolipids. First, little is known concerning the occurence of this class of lipid molecules. Until recently, only sphingosine, dihydrosphingosine, and psychosine were identified as long-chain amino bases of mammalian cells. The diversity of lysosphingolipids, however, may match that of the parental sphingolipids (Fig. 1), although few lysosphingolipids have been investigated. Second, the cellular levels of sphingosine, psychosine, and other lysosphingolipids are low (*42*), which renders quantitative investigation difficult. Low levels, however, do not preclude the importance of these agents, since low basal cellular levels are found for a number of second messengers, including diacylglycerol and inositol trisphosphate. Third, sphingosine and lysosphingolipids were not suspected of having interesting biological activities.

This situation has changed with the rapid accumulation of evidence that lysosphingolipids and sphingosine alter cell function (Table 2). In addition, sphingosine and psychosine are cytotoxic molecules (*43, 44*); micromolar concentrations can induce lysis and cell death. Psychosine inhibits mitochondrial function and the activity of a cytosolic β-glucosidase (*45*). Sphingosine and related compounds inhibit a number of blood-clotting reactions, especially the activity of tissue factor (*46*), while psychosine hemolyzes red blood cells (*47*).

At low concentrations, sphingosine and other lysosphingolipids inhibit a number of cellular functions (Table 2). In human platelets, sphingosine inhibited secondary aggregation and secretion in response to various platelet activators, but did not affect the initial response or shape change. Inhibition of platelet function was accompanied by inhibition of phorbol dibutyrate binding and by inhibition of protein kinase C activation by endogenous diacylglycerols (*8*). In neutrophils, sphingosine inhibited phorbol ester binding, the induction of superoxide generation, and the secretion of specific but not azurophilic granules (*10*). Sphingosine also inhibited the synthesis of platelet activating factor and leukotriene LTB_4 (*10*). Likewise, sphingosine inhibited the differentiation of HL-60 cells in response to phorbol 12-myristate 13-acetate (*14*), inhibited neurite outgrowth in PC-12 cells in response to nerve growth factor (*12*), and inhibited insulin-stimulated uptake of 2-deoxyglucose in 3T3-L1 fibroblasts (*48*). In A431 cells, sphingosine inhibited the phosphorylation and affinity changes of the epidermal growth factor (EGF) receptor in response to phorbol esters (*13*). In addition, sphingosine led to selective enhancement of phosphorylation of the EGF receptor at sites known not to be direct substrates for protein

kinase C (*13*). Whether the latter effects are independent of protein kinase C or occur through subtle effects on protein kinase C is yet to be determined. In S49 lymphoma cells, sphingosine inhibited the translocation of protein kinase C in response to phorbol myristate acetate (*49*). The lysosphingolipid lyso-GM_1 inhibited the calcium-mediated translocation of protein kinase C from the supernatant to the pellet fraction in homogenates of cerebellar granule cells (*50*). Another lysosphingolipid, lyso-GM_3, inhibited the EGF-dependent

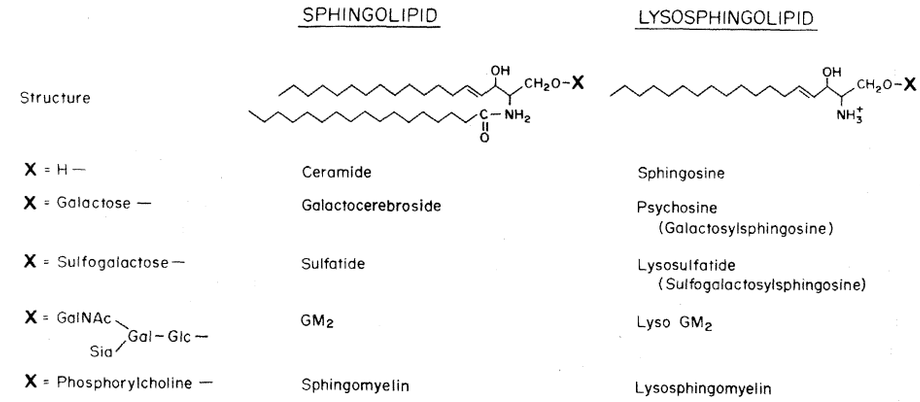

Fig. 1. Structure of sphingolipids and lysosphingolipids. Sphingolipids are derived from ceramide by having different substitutions at the 1-position. Ceramide is composed of a sphingoid base with an amide-linked fatty acyl chain. With the exception of sphingomyelin, which has a phosphorylcholine head group at the 1-position linked through a phosphodiesteric bond, sphingolipids have a glycosidic bond at C-1. These sugar head groups can vary in complexity from a single glucose or galactose, as occurs in cerebrosides, to more complex structures, such as lactosylceramide with two sugars, trihexosides with three, and higher order carbohydrate moieties. Certain subclasses are recognized by additional components, such as sulfatides, which contain sulfate, and gangliosides, which contain sialic acid residues. Lysosphingolipids are based on sphingosine in a manner analogous to the way sphingolipids are based on ceramide. For each parental sphingolipid there is a corresponding lysosphingolipid that has an identical head group at the 1-position but that lacks the amide-linked fatty acyl group at the 2-position. All lysosphingolipids share two important structural features with sphingosine: a charged amine at position 2 and a hydrophobic hydrocarbon tail.

Table 1. Biologic activities of sphingolipids.

Activity	Reference
Tumor antigens	(*73*)
GM_2 expression on human melanoma cells	(*22*)
GD_2 expression upon progression of melanoma	(*74*)
Antibodies to GD_3 have antitumor activity	(*75*)
TerC glycolipid marker for teratocarcinoma	(*76*)
Lactosylceramide marker for colon carcinoma	(*77*)
A sialylated ganglioside as marker for chronic myelogenous leukemia	(*78*)
Glycolipid alteration in *ras*-transfected NIH-3T3 cells	(*79*)
GD_{1a} expressed by rat hepatoma cells	(*80*)
Markers of cell differentiation	(*72*)
Increased sphingomyelin upon differentiation of hairy cell leukemia with phorbol esters	(*23*)
Increased sphingomyelin in 3T3-L1 cells and in polymorphonuclear leukocytes treated with dexamethasone	(*24*)
GM_3 elevation in differentiated HL-60 cells and U937 cells; turnover of GM_3 sialic acid residues during fibroblast growth	(*25*)
SSEA-1 glycolipid in embryo development	
GM_1 and GD_{1a} elevation on differentiation of teratocarcinoma	(*76, 81*)
GM_3 elevation in differentiating intestinal epithelium	(*82*)
I antigen in erythrocyte differentiation	(*83*)
GD_3 elevation in muscle cell differentiation	(*84*)
Ganglioside elevation in neuroblastoma	(*85*)
GM_1 elevation in lymphoid cell differentiation	(*86*)
Role in membrane fluidity	(*87*)
Gangliosides on outer leaflet of bilayer confer rigidity	(*88*)
Correlation of sphingomyelin content with fluidity	(*87*)
Modulation of cell proliferation	
Nerve growth factor–like activity of GQ_{1b}	(*36*)
Neuritogenic and neuronotrophic activities of gangliosides	(*89*)
GM_1 inhibition of Swiss 3T3 cell growth	(*90*)
GM_3 inhibition of growth factor–induced mitogenesis	(*39*)
Inhibition of lymphocyte proliferation	(*91*)
Stimulation of astroglial and neuroblastoma proliferation by exogenous gangliosides	(*34, 92*)
Modulation of protein phosphorylation	
GQ_{1b}-dependent protein kinase activity	(*36*)
Ganglioside-inhibited protein kinase from pig brain	(*40*)
GM_3 inhibition of EGF-dependent tyrosine phosphorylation of EGF receptor	(*39*)
Ganglioside-induced phosphorylation of proteins in myelin	(*34, 92*)
Inhibition of protein kinase C activity by gangliosides	(*41*)
GT_{1b}- and GD_{1a}-activated protein kinase	(*37*)
Ca^{2+}- and ganglioside-dependent protein kinase	(*38*)
GM_3 inhibition of tyrosine phosphorylation of the platelet-derived growth factor receptor	(*90*)
de-*N*-acetyl GM_3 stimulation of tyrosine kinase activity of EGF receptor	(*11*)
Cell contact response	
Inhibition of cell contact	(*26*)
Globoside modulation of neuromuscular junction formation	(*27*)
GM_2 modulation of retinal adhesion	(*28*)
Induction of GD_{1b} and GT_{1b} during neuroglial interaction	(*29*)
Gangliosides as receptors and receptor cofactors	
GM_1 binds B subunit of cholera toxin and mediates its mitogenic effect	(*30*)
G_{1b} gangliosides as receptors for tetanotoxin	(*94*)
Binding of gangliosides to fibronectin	(*95*)
Binding of laminin, thrombospondin, and von Willebrand factor to sulfated glycolipids	(*96*)
Immune recognition	(*97*)
Blood group antigens	(*98*)
Autoimmune antigens	(*99*)
Tumor antigens	(*22, 73–80*)
Differentiation antigens	(*23–25, 81–87*)
Lymphocyte markers	(*19*)
Miscellaneous	
Embryo inversion by a complex glycolipid	(*100*)
Cerebrosides with antiulcerogenic activity	(*101*)
Stimulation of fruiting of *Schizophylium commune* by plant cerebrosides	(*102*)
Modulation of sodium transport by complex gangliosides	(*103*)
"Ganglioside syndrome" in rabbits intensively immunized with GM_1 and GD_{1a}	(*104*)
Glycolipid changes with the cell cycle	(*105*)

tyrosine phosphorylation of the EGF receptor in membrane preparations of A431 cells (*11*).

Mechanism of Action of Sphingosine and Lysosphingolipids

Sphingosine and lysosphingolipids are potent and reversible inhibitors of protein kinase C activity in vitro and in cell systems (*8, 17*). Protein kinase C is an 80-kilodalton protein that has been recently cloned from different cDNA libraries by several groups and found to consist of at least three different isoenzymes; up to six or seven different isoenzymes may constitute a superfamily of protein kinase C's (*51*). The enzyme is physiologically activated by diacylglycerols that are generated by the action of phospholipase C or by phospholipase D and phosphatidic acid phosphatase on membrane phospholipids in response to the interaction of agonists with their cell receptors (*1, 6, 52*). Protein kinase C also serves as the receptor for phorbol esters and other tumor promoters. It is activated by phorbol esters and appears to mediate various biological functions attributed to the action of tumor promoters (*53*). In vitro mechanistic studies of the regulation of protein kinase C suggest that the enzyme requires the presence of a surface (such as a bilayer or a mixed micelle) whereby it interacts with anionic phospholipids (especially phosphatidylserine) in the presence of calcium; in this state the enzyme is surface-bound but inactive. Activation requires the addition of either diacylglycerols or phorbol esters. Once the enzyme is activated, it is able to phosphorylate soluble and membrane-associated protein substrates (*54*). Under these conditions, the enzyme is also capable of autophosphorylation (*55*). Structure-activity relations indicate that protein kinase C is specifically activated by the *sn*-1,2-diacylglycerol stereoisomer. Activation also requires the presence of the hydroxyl at the 3-position, the esters at the 1- and 2-positions, and acyl chains of at least six carbons in length (or a long acyl chain at 1 position and an acetyl group at the 2-position) that will impart sufficient lipid solubility to the diacylglycerol molecule (*56*).

Further studies on the regulation of protein kinase C by lipid molecules showed that enzyme activity was potently inhibited by sphingosine but not by the related ceramide (*8*). Mechanistic studies revealed that sphingosine inhibited diacylglycerol and phorbol ester competitively and calcium noncompetitively. Kinetic studies also indicated that the inhibition was of a mixed type with respect to phosphatidylserine. In the presence of detergent micelles or lipid vesicles, sphingosine did not inhibit the catalytic fragment of the enzyme whose activity is independent of lipid cofactors (*8*). Sphingosine also inhibited phorbol ester binding to the intact protein kinase C and to the amino-terminal lipid binding fragment. Inhibition of phorbol binding or enzyme activity occurred without inhibiting the association of the enzyme with the surface (mixed micelles, in this case). Sphingosine inhibition was subject to surface dilution (inhibition is determined by the surface concentration of the molecule rather than by its molar concentration). Studies in intact cell systems similarly showed sphingosine's effects to be dependent on its concentration in membranes (*8*).

Table 2. Cellular activities of sphingosine and lysosphingolipids.

Activity	Reference
Human platelets: inhibition of secretion and secondary aggregation; inhibition of Na^+/H^+ exchange	(*8, 9*)
Human neutrophils: inhibition of superoxide generation and secretion of specific granules; inhibition of synthesis of platelet-activating factor and leukotriene B_4	(*10*)
HL-60 promyelocytic leukemia cells: inhibition of differentiation	(*14*)
A431 epidermoid carcinoma: increase in affinity of EGF receptor to EGF; inhibition of EGF-dependent phosphorylation of EGF-receptor; stimulation of EGF receptor tyrosine kinase activity	(*13*) (*11*) (*59*)
S49 lymphoma cells: inhibition of protein kinase C translocation	(*49*)
3T3-L1 fibroblasts: inhibition of insulin-stimulated 2-deoxyglucose uptake	(*48*)
PC-12 pheochromocytoma cells: inhibition of nerve growth factor–induced neurite outgrowth	(*12*)
CHO cells: cytotoxicity	(*62*)
Mouse skin: inhibition of induction of phorbol ester–induced inflammation and of induction of ornithine decarboxylase	(*15*)
Lymphocytes: prevention of phorbol ester–induced down-regulation of protein kinase C	(*16*)
Frog skin: inhibition of Na^+ transport	(*106*)
Isolated neurons: inhibition of sustained Ca^{2+} gradients in response to glutamate	(*107*)
Blood clotting: prolonging clotting times and inhibition of tissue factor activity	(*46*)

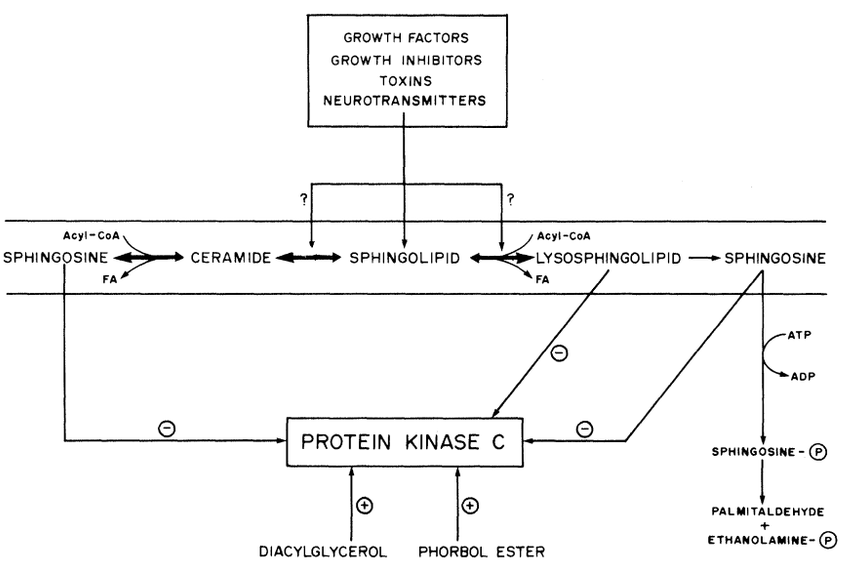

Fig. 2. Sphingolipid turnover and cell regulation. Sphingolipids can be metabolized through successive deglycosylation steps to yield ceramide. Ceramide and possibly other sphingolipids can be *N*-deacylated to yield sphingosine and lysosphingolipids, respectively. Therefore, the breakdown of sphingolipids can generate molecules with cellular activity. At present, protein kinase C is identified as the main target for sphingosine. Protein kinase C is also inhibited by lysosphingolipids, but the range of cellular activity of these molecules has not been fully evaluated (*60*). Sphingosine can be *N*-acylated to yield ceramide or phosphorylated to yield sphingosine-1-phosphate (*108*). Sphingolipid turnover may be stimulated by DAG (*72*), bovine serum albumin (*109*), or dexamethasone (*71*) and may occur in response to the action of neurotransmitters, mitogens, and growth inhibitors. Finally, the interaction of surface sphingolipids with specific ligands such as toxins and cell adhesion molecules may lead to sphingolipid turnover.

In vitro studies of the structure-activity relations of sphingosine indicate that inhibition of protein kinase C requires the hydrophobic character and the positively charged amine (*8*). Sphingosine, *N*-methyl sphingosine, 3-ketosphingosine, and stearylamine all inhibit, whereas ceramide (*8*), *N*-acetyl sphingosine (*8*), and short-chain sphingosines with fewer than 11 carbons (*57*) are inactive as inhibitors (*8, 57*). Similar structure-activity relations were observed in intact cells. Sphingosine, but not *N*-acetyl sphingosine, inhibited protein kinase C activity and phorbol binding in human platelets, HL-60 cells, and neutrophils. Studies with sphingosine analogs having variable chain lengths showed that the 18-carbon molecule had optimal cellular and in vitro activity and that both shorter and longer chain analogs showed progressively less activity (*57*). Sphingosine inhibited the "translocation" of protein kinase C from the cytosol to the membrane fraction upon stimulation with agonists (*49*), and it also inhibited the "downregulation" of protein kinase C (*16*).

The interpretation of the effects of sphingosine on intact cells requires careful investigation of its selectivity and sites of action. Sphingosine has no effect on many of the enzymes involved in signal transduction. At concentrations that inhibited protein kinase C, sphingosine did not affect the activities of phospholipase C, diacylglycerol kinase, cyclic adenosine monophosphate– and cyclic guanosine monophosphate–dependent kinases, and the calcium- and calmodulin-dependent myosin light chain kinase (*8*). However, at higher concentrations, sphingosine inhibited the activities of phospholipase C and myosin light chain kinase (*58*). Inadequate data are available on other enzymes, metabolic pathways, and cellular processes in which sphingosine and lysosphingolipids might exert their physiological or pharmacological effects. Certain effects of sphingosine may be independent of protein kinase C, such as inhibition of the binding of thyrotropin releasing hormone to pituitary cells (*59*), activation of the tyrosine kinase activity of the EGF receptor (*59*), and inhibition of tissue factor activity (*46*). Also, the selectivity of the more polar lysosphingolipids may be different from that of sphingosine (*60*); for example, lyso-GM_3 inhibits tyrosine phosphorylation of the EGF receptor (*11*). Further studies on the selectivity of these molecules and the possible existence of other targets for their action will be of great importance in elucidating their physiological significance as well as their use as pharmacological probes (see below).

Sphingosine and Lysosphingolipids as Pathobiological Molecules

Sphingosine and other long-chain amino bases are cytotoxic to some cell lines (*43, 44, 62*). Sphingosine shows profound cytotoxicity in Chinese hamster ovary cells (*62*). At concentrations greater than required to inhibit protein kinase C, sphingosine caused the death of HL-60 cells, platelets, neutrophils, and macrophages. Psychosine exerts cytotoxity in cell culture systems as well as when it is injected intracerebrally (*43*). Although the mechanism by which lysosphingolipids mediate cytotoxicity and cell dysfunction is not well established, these observations led us to consider the role of lysosphingolipids in the pathogenesis of the sphingolipidoses and other diseases.

The possibility that lysosphingolipids may indeed be the pathobiological molecules in the sphingolipidoses is supported by a number of observations: (i) All lysosphingolipids evaluated were found to be inhibitors of protein kinase C activity and phorbol ester binding (*17*). (ii) Lysosphingolipids accumulate along with the parent sphingolipids in Tay-Sachs, Neiman-Pick, and Krabbe's diseases (*63*). This accumulation occurred to an extent consistent with inhibition of protein kinase C activity (*17*). Lysosphingolipid accumulation in these diseases is consistent with current understanding of sphingolipid metabolism. Cells have the metabolic machinery to synthesize and metabolize lysosphingolipids (*17, 64*), with the same enzymes synthesizing and catabolizing both the parent sphingolipid and the corresponding lysosphingolipid. The deficiency of an enzyme catabolizing a particular sphingolipid results in the accumulation of that sphingolipid in each of the sphingolipidoses. Because the same enzyme catabolizes the corresponding lysosphingolipid, its deficiency is, therefore, expected to result in the accumulation of both the parent sphingolipid and the corresponding lysosphingolipid. (iii) In Krabbe's disease, Miyatake and Suzuki (*65*) hypothesized that psychosine rather than its parent cerebroside was the pathogenic agent. Supporting this hypothesis is the finding that psychosine accumulates to much greater levels in affected tissues than does the parent cerebroside (*63*). Also, when injected intracranially psychosine mimicked the pathophysiology of Krabbe's disease (*43*). The role of psychosine as a pathogenic molecule is also supported in studies with twitcher mice, an animal model of Krabbe's disease (*65, 66*). This "psychosine hypothesis" may be extended to all the sphingolipidoses; in each of these disorders the lysosphingolipid, at low concentrations, inhibits protein kinase C activity, resulting in disturbances in signal transduction, neural transmission, and cell regulation. At higher concentrations, these molecules become cytotoxic (*17*).

Whether the lysosphingolipids have other pathobiologic roles remains unknown at present. Sphingosine levels in HL-60 cells are higher than in other cell lines (*14*), suggesting that developmental trapping of HL-60 cells occurs by elevation of sphingosine and lysosphingolipid levels and the consequent attenuation of protein kinase C activity. Such inhibition would be overcome by the addition of protein kinase C activators, which cause differentiation.

Lysosphingolipids and Structural Analogs as Pharmacological Agents

Sphingosine, lysosphingolipids, and structural analogs are potentially selective inhibitors of protein kinase C and may therefore serve as important pharmacological tools to dissect the role and function of protein kinase C in different cell systems and to develop agents that can selectively prevent the effects of tumor promoters by inhibiting their effects on protein kinase C.

Sphingosine has already been used as a probe to dissect the function of protein kinase C in different cell systems. It has been used successfully to evaluate the role of protein kinase C in signal transduction and short-term cell responses such as occur in human platelets (*8*) and human neutrophils (*9*). In platelets, sphingosine selectively inhibits protein kinase C within a range of concentrations at which it does not inhibit other key elements of signal transduction (see above). The effects of sphingosine on platelets were suggestive of a role for protein kinase C in secondary aggregation that was further confirmed by the combined use of low concentrations of protein kinase C activators (diacylglycerols and phorbol esters) and low concentrations of platelet activators (*8*). Probing with sphingosine in neutrophils has allowed the delineation of a role for protein kinase C in superoxide generation, specific granule secretion, and the synthesis of platelet activating factor and LTB_4 (*9*).

Further use of sphingosine as a pharmacological probe in more complex situations such as cell differentiation and cell transformation is complicated by cytotoxicity, selectivity, and metabolism. (i) Cytotoxicity occurs at concentrations slightly higher than those required to inhibit protein kinase C (*43, 44*). Sphingosine cytotoxicity is affected by many factors, including the vehicle used for

delivery; in neutrophils, the delivery of sphingosine with dimethyl sulfoxide seems to augment its cytotoxicity and decrease its selectivity (*61*), whereas delivery in a 1:1 complex with albumin leads to lower concentrations of free sphingosine and therefore, less cytotoxicity and more selectivity in protein kinase C inhibition (*67*). (ii) The selectivity of sphingosine's effects has not been thoroughly evaluated (see above). Until the full range of targets for sphingosine and lysosphingolipids is determined, care should be exerted in interpreting results when sphingosine is used as a selective inhibitor. (iii) The metabolism of sphingosine may lead to attenuation of its pharmacological effects. In Chinese hamster ovary and HL-60 cells, sphingosine is metabolized to ceramide and other sphingolipids with a half-time of around 6 hours (*14, 62*). Platelets, however, rapidly metabolize sphingosine (*68*) through the action of sphingosine kinase, yielding inactive metabolites (Fig. 2). Other structural analogs of sphingosine, such as *N*-ethyl sphingosine, which appear to be more potent than sphingosine in inhibiting protein kinase C, may offer significant advantages as selective probes. These and other analogs may also be more resistant to metabolism.

The role of protein kinase C in mediating the biological responses of tumor promoters (*53*) suggests the use of protein kinase C inhibitors as chemopreventive agents (antitumor promoters). This is supported by the ability of sphingosine and its active analogs to inhibit phorbol ester–induced responses in various cell systems (Table 2). Therefore, sphingosine and its analogs may have a pharmacological use in the prevention of the action of tumor promoters; this hypothesis is being investigated with experimental models of tumor promotion and carcinogenesis.

Possible Physiological Functions of the Lysosphingolipids

The discoveries that sphingosine and lysosphingolipids inhibit protein kinase C and that sphingosine has cellular activity indicate that the lysosphingolipids may function physiologically. A physiological role for lysosphingolipids is consistent with the current knowledge of cellular machinery for the metabolism of sphingosine, psychosine, and possibly other lysosphingolipids (Fig. 2). Physiologically, these molecules may act in two capacities. According to one hypothesis, low levels of sphingosine or other lysosphingolipids may tonically inhibit protein kinase C and prevent activation by resting levels of diacylglycerol. Protein kinase C would be activated physiologically only upon the generation of diacylglycerol through the breakdown of phosphatidylinositol bisphosphate or other phospholipids. The existence of significant amounts of diacylglycerol under resting conditions has puzzled investigators in the field of signal transduction. Although basal diacylglycerol levels are consistent with their metabolic function in the biosynthesis and catabolism of phospholipids and triacylglycerol, these levels are theoretically sufficient to activate protein kinase C. Therefore, one possibility is that protein kinase C activity is regulated by both positive (diacylglycerol) and negative (sphingosine and lysosphingolipids) effectors (*69*). According to this hypothesis, disturbances in the baseline levels of either diacylglycerols or sphingosine and lysosphingolipids could lead to abnormal regulation of protein kinase C (*70*).

A second hypothesis is that sphingosine and lysosphingolipids function as second messengers. Parental sphingolipids may break down by the action of certain deacylases in response to cellular stimulation to generate the corresponding lyso derivatives. This would be analogous to the generation of diacylglycerol from the breakdown of membrane glycerolipids. Lysosphingolipids may then function to inhibit protein kinase C. Other targets for their action cannot be ruled out at present.

Sphingolipid turnover appears to have a role in signal transduction. Recent observations suggest a role for a "sphingolipid cycle" in the mediation of steroid hormone action. Changes in sphingomyelin levels (*24*) and activation of a neutral sphingomyelinase (*71*) accompany dexamethasone's ability to induce differentiation of 3T3-L1 preadipocyte cells. Also, sphingosine mimics dexamethasone's effects on 2-deoxyglucose uptake (*48*). These observations suggest that changes in sphingolipid metabolism may occur in response to dexamethasone and that the resulting breakdown products such as sphingosine or lysosphingomyelin may mediate some of the actions of steroid hormones.

In other experiments, exogenous diacylglycerol activated a neutral sphingomyelinase in GH_3 pituitary cells, an effect that is not reproduced by phorbol ester and that is apparently independent of protein kinase C activation. The breakdown products of sphingomyelinase action may then lead to the reversal of protein kinase C translocation to the membrane and attenuation of protein kinase C activity (*72*). These data suggest a role for sphingomyelin turnover in the modulation of protein kinase C activity.

Finally, the existence of deacylases that allow sphingolipid turnover could explain the mechanism of action of exogenous sphingolipids as well as ligands that interact with specific sphingolipid molecules. GM_3 and lyso-GM_3 have similar effects on phosphorylation of the EGF receptor (*11*), raising the possibility that endogenously produced lyso-GM_3 may mediate the effects of GM_3. A similar relation may exist between GM_1 and lyso-GM_1. GM_1 inhibits protein kinase C translocation in intact cells but not in vitro (*50*). On the other hand, lyso-GM_1, the deacylation product of GM_1, inhibits protein kinase C translocation in vitro; this suggests that it may mediate the effects of GM_1 in intact cells (*50*). The action of deacylases may also explain the effects of ligands that specifically bind cell surface gangliosides (such as toxins) (Fig. 2). Upon binding to their ganglioside receptors, these ligands may induce the deacylation of gangliosides. The resulting lysogangliosides may then function as second messengers.

Critical testing of these hypotheses requires intensive investigation aimed at answering the following questions.

1) What are the baseline levels of sphingosine and lysosphingolipids and are these levels controlled? Experiments to address this question require the development of sensitive assays that will detect picomolar amounts of sphingosine and the various lysosphingolipids. Such assays will enable investigators to determine whether baseline levels of sphingosine and lysosphingolipids are sufficient to inhibit protein kinase C activity in resting cells. These methods will also allow critical testing of the second messenger hypothesis by monitoring the changes in sphingosine and lysosphingolipid levels in response to various cellular perturbations. Although such assays are being developed for sphingosine, the complexity and variety of lysosphingolipids makes the extension of these assays to measure each of the different lysosphingolipids an enormous task.

2) What are the cell effectors that induce the physiologic turnover of sphingolipids? An obvious set of such effectors includes steroid hormones, tumor necrosis factor, transforming growth factor–β, and other chalones and suppressors of cell growth. As noted above, there is evidence that sphingolipid turnover may have a role in mediating the effects of the corticosteroid dexamethasone on 3T3-L1 cells. Also, the ability of diacylglycerol to induce sphingomyelin breakdown (see above) may have general significance. It suggests that the diverse hormones, growth factors, and neurotransmitters that cause an increase in intracellular diacylglycerol levels may consequently lead to sphingolipid turnover.

3) What are the targets for lysosphingolipid action? Understanding the physiologic significance of sphingolipid turnover requires a careful evaluation of the target (or targets) affected by lysosphingoli-

pids. Although the inhibition of protein kinase C appears to be a selective event at lower concentrations of sphingosine, other targets of action of lysosphingolipids cannot be ruled out at present (*60*).

4) What is the cellular location of sphingosine and lysosphingolipids? Although current thinking places gangliosides and the major sphingolipids predominantly on the outer leaflet of the cell membrane, there are no rigorous studies on the cellular distribution of sphingosine and other lysosphingolipids.

5) What enzymes are involved in lysosphingolipid metabolism, and how are these enzymes regulated? A full evaluation of the physiologic function of lysosphingolipids requires understanding how these molecules are generated and how they are metabolized. Other than the enzymes involved in sphingosine metabolism, little is known about enzymes participating in lysosphingolipid biosynthesis and catabolism. The existence of deacyclases involved in the generation of lysosphingolipids from parental sphingolipids is also unknown.

Conclusion

Sphingosine and lysosphingolipids, the *N*-deacylation products of sphingolipids, elicit a variety of cellular responses, including inhibition of growth factor action, modulation of receptor function, inhibition of platelet and neutrophil function, and antagonism of phorbol ester–induced responses. Many of these effects appear to be a direct consequence of the inhibition of protein kinase C, although the full range of biochemical targets of these molecules has not been evaluated. The diverse cellular activities of sphingolipids and lysosphingolipids suggest that they act as endogenous modulators of cell regulation and possibly as second messengers generated from sphingolipid turnover. Determining the full physiologic significance of sphingolipid metabolism and sphingolipid breakdown products promises to be an exciting area of investigation.

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110. Supported by NIH grant GM38737, and by NCI grant CA46738, and a physicians scientist award (to Y.H.). Y.H. is a Pew Scholar. We thank C. R. Loomis and J. E. Niedel for careful review of this manuscript.

Research Articles

Chromosomal Rearrangement Generating a Composite Gene for a Developmental Transcription Factor

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Differential gene expression in the mother cell chamber of sporulating cells of *Bacillus subtilis* is determined in part by an RNA polymerase sigma factor called σ^K (or σ^{27}). The σ^K factor was assigned as the product of the sporulation gene *spoIVCB* on the basis of the partial amino-terminal amino acid sequence of the purified protein. The *spoIVCB* gene is now shown to be a truncated gene capable of specifying only the amino terminal half of σ^K. The carboxyl terminal half is specified by another sporulation gene, *spoIIIC*, to which *spoIVCB* becomes joined in-frame at an intermediate stage of sporulation by site-specific recombination within a 5–base pair repeated sequence. Juxtaposition of *spoIVCB* and *spoIIIC* need not be reversible in that the mother cell and its chromosome are discarded at the end of the developmental cycle. The rearrangement of chromosomal DNA could account for the presence of σ^K selectively in the mother cell and may be a precedent for the generation of cell type–specific regulatory proteins in other developmental systems where cells undergo terminal differentiation.

THE METAMORPHOSIS OF CELLS OF THE GRAM-POSITIVE bacterium *Bacillus subtilis* into endospores involves the formation of a sporangium consisting of two cell types known as the mother cell and the forespore (*1, 2*). The mother cell and forespore arise by an asymmetric septation, which partitions the sporangium into two unequal compartments. These compartments each receive a chromosome generated by the last round of vegetative DNA replication, but then undergo divergent developmental fates as a consequence of differential gene expression. The forespore can be thought of as a germline cell, because it ultimately becomes the mature spore and gives rise to subsequent progeny. The mother cell, however, is a terminally differentiating cell because it and its chromosome are discarded by lysis when maturation of the spore is complete.

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