Minireview

Ceramide-1-phosphate: a novel regulator of cell activation

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Abstract Ceramide-1-phosphate (Cer-1-P) is emerging as a novel bioactive sphingolipid. It is formed by phosphorylation of ceramide catalyzed by ceramide kinase, and has been implicated in different cellular processes. Cer-1-P is mitogenic for fibroblasts, blocks apoptosis in macrophages, controls phagocytosis in neutrophils, and mediates inflammatory responses. Only recently have we started to uncover the signaling pathways that are affected by Cer-1-P. Recent work has demonstrated that cytosolic phospholipase A_2 and acid sphingomyelinase are direct intracellular targets of Cer-1-P, and that it may also induce phosphorylation of extracellular signal-regulated kinase-2 and calcium mobilization. These actions of Cer-1-P seem to be cell type-specific.

Key words: Apoptosis; cell proliferation; Ceramide-1-phosphate; Sphingolipid; Sphingosine-1-phosphate; Sphingomyelinase

1. Introduction

The discovery by Hannun and co-workers [1] of the inhibition of protein kinase C (PKC) by sphingosine in 1986 marked the beginning of a new era in research of lipid signal transduction. In 1987, Kolesnick [2] demonstrated the stimulation of an acid, but not neutral, sphingomyelinase (SMase) activity by exogenous addition of 1,2-dioctanoylglycerol (a permeable analog of diacylglycerol) to GH_3 pituitary cells. This action caused sphingomyelin (SM) hydrolysis and a concomitant increase in ceramide levels that were independent of PKC. It was suggested that sphingolipid-derived metabolites might function as second messengers. This hypothesis was confirmed independently by Hannun's and Kolesnick's groups with the elucidation of the SM pathway and the physiologic functions of ceramide [3–8]. It is now well established that ceramides regulate a variety of physiological or pathophysiological functions including cell proliferation, differentiation, apoptosis, and inflammation [3–10]. To achieve this, ceramides modulate diverse signal transduction pathways by activating or inhibiting key regulatory enzymes including specific serine/threonine kinases [8,11], protein phosphatases [12], PKCζ [13], stress-activated protein kinases also known as Jun nuclear kinases [14,15], mitogen-activated protein kinases (MAPKs) [16,17], phospholipase A_2 (PLA_2) [18], protein kinase B [19,20], and phospholipase D (PLD) [21,22] (Fig. 1). Ceramide formation serves different functions at distinct locations in the cell, and probably the topology of ceramide generation may be a crucial determinant of its impact on cell physiology. However, it is unclear how cells separate signaling pools from metabolic pools. Also, enzymes of ceramide metabolism show distinct subcellular localization and topology (reviewed by Hannun and Obeid [23]). For instance, the plasma membrane of cells contains caveola-associated neutral SMase and a fraction of acid SMase, and the ceramides that are generated by these enzymes may have different functions. For details on SMase activities, enzymology, and compartmentalization, see [24–26]. Ceramide generation at the plasma membrane exerts distinct and specific functions including inhibition of PKC translocation or aggregation of the Fas receptor, but not other effects mediated by endogenous ceramides such as apoptosis or cell cycle arrest [23]. Lysosomal acid SMase-derived ceramides may activate cathepsin D, and the ceramides that are generated in the endoplasmic reticulum, which is the primary site of de novo synthesis of ceramide, have been implicated in mediating apoptosis. The nucleus and mitochondria have also been implicated as sites of ceramide generation, with possible roles in apoptosis [23]. In addition, little is known concerning SM synthase, the enzyme responsible for transferring phosphocholine from phosphatidylcholine to ceramide generating diacylglycerol and SM in cells. This enzyme activity has been implicated in cell regulation and transformation through its ability to modulate the levels of ceramide and diacylglycerol, and it may account, at least in part, for some of the effects previously attributed to phosphatidylcholine-specific phospholipase C [27]. Because natural ceramides are not permeant to cell membranes, many of the studies to determine the role of ceramides in signal transduction are carried out using short-chain cell-permeable analogs, or by exogenous addition of bacterial SMase to cells maintained in culture. However, Ji et al. [28] developed a solvent mixture consisting of ethanol/dodecane (98:2, v/v) that allowed dispersion of natural long-chain ceramide in aqueous solutions, and facilitated its uptake by cells. Using this solvent

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Abbreviations: C_2-ceramide, *N*-acetylsphingosine; C_8-ceramide, *N*-octanoylsphingosine; Cer-1-P, ceramide-1-phosphate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PA, phosphatidate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D; SM, sphingomyelin; Sph-1-P, sphingosine-1-phosphate

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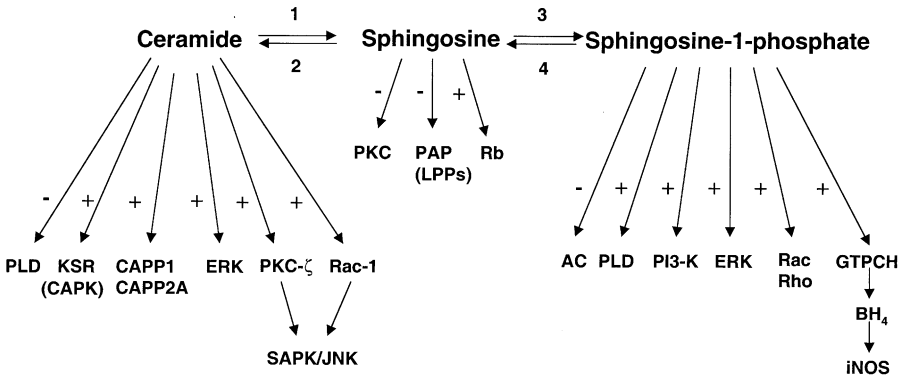

Fig. 1. Some downstream targets of ceramide, sphingosine and sphingosine-1-phosphate. (1) Ceramidase; (2) ceramide synthase; (3) sphingosine kinase; (4) sphingosine-1-phosphate phosphatase. Abbreviations: AC, adenylyl cyclase; BH4, tetrahydrobiopterin; CAPP1, ceramide-activated protein phosphatase 1; CAPP2A, ceramide-activated protein phosphatase 2A; ERK, extracellular signal-regulated kinases; GTPCH, GTP cyclohydrolase; KSR, kinase suppressor of Ras; iNOS, inducible nitric oxide synthase; PAP, phosphatidate phosphohydrolase; LPPs, lipid phosphate phosphatases; Rb, retinoblastoma protein; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLD, phospholipase D; SAPK/JNK, stress-activated protein kinase/Jun N-terminal kinase.

mixture, exogenous ceramide could mimic many of the effects of ceramide-producing agonists such as tumor necrosis factor-α [28]. In addition, ceramide is the precursor of other bioactive sphingolipids that can also regulate cellular functions. Stimulation of ceramidases generates sphingosine (Fig. 2), which can control the activity of key enzymes involved in the regulation of metabolic or cell signaling pathways (i.e. inhibition of the Mg^{2+}-dependent form of phosphatidate (PA) phosphohydrolase [29,30], inhibition of PKC [1], activation of PLD [31], and stimulation of diacylglycerol kinase [32,33]). Many of the effects of sphingosine are known to be mediated through its conversion to sphingosine-1-phosphate (Sph-1-P) [34]. However, this may not always be the case since, for instance in rat fibroblasts, Sph-1-P stimulates PLD rapidly and potently, whereas sphingosine is a slow and weak activator of this enzyme activity [22]. Sph-1-P was first described as a mitogen for cultured fibroblasts [35] and subsequently has been implicated in the regulation of other cellular processes including cell differentiation, migration, angiogenesis and cell survival [36–39]. More recently, our group has demonstrated that Sph-1-P stimulates cortisol secretion in cells of the zona fasciculata of bovine adrenal glands, thereby implicating Sph-1-P in the regulation of steroidogenesis [40]. The observation that Sph-1-P binds to specific cell surface G protein-coupled receptors, together with the discovery that it can be generated intracellularly by the action of growth factors, such as platelet-derived growth factor, points out that Sph-1-P can function as both first and second messenger [38]. These actions of Sph-1-P may differentially affect specific intracellular targets or signaling pathways. In this connection, it has been recently demonstrated that only intracellularly generated Sph-1-P plays a role in the regulation of GTP cyclohydrolase, thereby leading to tetrahydrobiopterin biosynthesis and subsequent stimulation of inducible nitric oxide synthase [41] (Fig. 1).

Another important metabolite that can be generated from ceramide is ceramide-1-phosphate (Cer-1-P), which is formed by the action of ceramide kinase (Fig. 2). There is increasing evidence suggesting that Cer-1-P is implicated in the regulation of vital cellular processes, such as cell proliferation [42,43], apoptosis [44], phagocytosis [45], and inflammation [46,47]. The purpose of this article is to review the role of Cer-1-P as a new bioactive lipid capable of regulating cell activation, and to emphasize the consequences of modifying Cer-1-P levels in cells.

2. Occurrence of Cer-1-P in mammalian cells

Ceramide kinase was first identified in brain synaptic vesicles by Bajjalieh and co-workers [48], and subsequently found in human leukemia (HL-60) cells by Kolesnick and Hemer [11]. Cer-1-P was then found by Dressler and Kolesnick also in HL-60 cells [49]. The latter authors suggested the possibility that a pathway from SM to Cer-1-P via phosphorylation of ceramide by ceramide kinase existed in cells. More recently, Hinkovska-Galcheva et al. [45] observed that endogenous Cer-1-P can be generated during the phagocytosis of antibody-coated erythrocytes in human neutrophils that were primed with formylmethionylleucylphenylalanine. Also, Rile et al. [50] reported that Cer-1-P can be formed in neutrophils

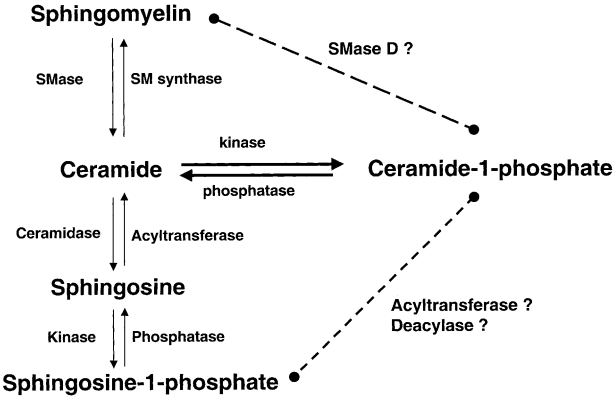

Fig. 2. Generation of bioactive sphingolipids in mammalian cells. Ceramide is produced by the action of SMase on SM. Ceramide can then be converted to ceramide-1-phosphate by ceramide kinase. The reverse reaction is catalyzed by ceramide-1-phosphate phosphatase. Alternatively, ceramide can be acted upon by ceramidases to form sphingosine, which can, in turn, be phosphorylated to sphingosine-1-phosphate. The reverse reaction is catalyzed by sphingosine-1-phosphate phosphatase. SMase D would generate ceramide-1-phosphate directly from SM, but this activity has not been found in mammalian cells. Likewise, interconversion of ceramide-1-phosphate and sphingosine-1-phosphate does not seem to occur in mammals.

upon addition of exogenous cell-permeable [^{3}H]*N*-hexanoyl-sphingosine (C_6-ceramide) to the cells, and Riboni and co-workers [51] have demonstrated that Cer-1-P can be generated from both SM-derived ceramide and the recycling of sphingosine produced by ganglioside catabolism, in cerebellar granule cells. Cer-1-P can also be generated by the action of interleukin 1-β on A549 lung carcinoma (epithelial-derived) cells [46]. More recently, we found that Cer-1-P is present in normal bone marrow-derived macrophages isolated from healthy mice, and that Cer-1-P plays a critical role in cell survival [44].

3. Biological functions of Cer-1-P

Although the existence of Cer-1-P in mammalian cells has been known for some time, no biologic activity had been assigned to this bioactive lipid until we demonstrated that it stimulates DNA synthesis and promotes cell division in rat fibroblasts [42] (Fig. 3). The latter studies were performed using synthetic short-chain Cer-1-Ps (C_2-Cer-1-P and C_8-Cer-1-P) and therefore, these effects were regarded as pharmacological in nature. Progress in understanding the functions of Cer-1-P has been slow probably because the enzymes that regulate its metabolism have not been definitively identified or cloned. Cer-1-P can be considered a sphingoid analog of PA or lyso-PA, two well-known mitogenic agents for fibroblasts [21,52,53]. Of interest, Cer-1-P was more potent than PA or lyso-PA at stimulating DNA synthesis and cell division. In particular, maximal incorporation of thymidine into DNA occurred at concentrations of short-chain Cer-1-Ps that were 5–10-fold lower than those of PA and lyso-PA. In those studies, the phospholipids were presented sonicated in water to cells in culture [21,42]. However, exogenous natural (long-chain) Cer-1-P, which was obtained from bovine brain and contained predominantly stearic and nervonic acids, was not effective at stimulating DNA synthesis unless it was dispersed in a solvent mixture of methanol/dodecane (49:1, v/v) [43]. Like short-chain Cer-1-Ps, natural (long-chain) Cer-1-P stimulated DNA synthesis potently. This action was accompanied by an increase in the levels of proliferating cell nuclear antigen [43], a cell cycle regulator that is present at sites of ongoing DNA synthesis [54]. Another important observation was that

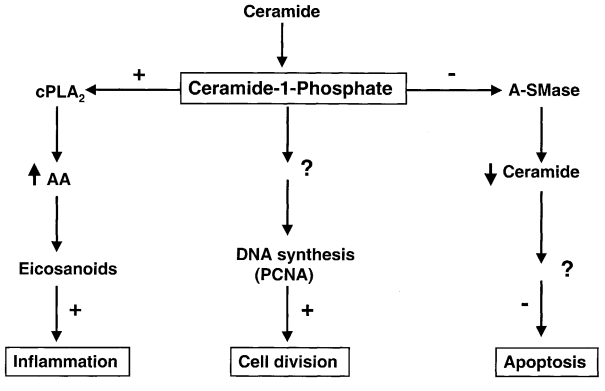

Fig. 3. Signaling cascades regulated by Cer-1-P. Stimulation of ceramide kinase results in Cer-1-P generation. Cer-1-P, by an unknown mechanism, stimulates DNA synthesis, and increases proliferating cell nuclear antigen levels and cell division. Alternatively, Cer-1-P can increase cell numbers by blocking apoptosis through inhibition of acid SMase (A-SMase). In addition, Cer-1-P activates cytosolic (c) PLA_2 initiating signaling through arachidonic acid (AA) release.

Cer-1-P is implicated in phagocytosis. Priming of human neutrophils with formylmethionylleucylphenylalanine followed by addition of IgG-opsonized erythrocytes produced rapid formation of intracellular Cer-1-P, which was associated with phagolysosome formation [45]. To date, little is known about the metabolic pathways that are regulated by Cer-1-P, but unlike other related mitogenic phospholipids, including Sph-1-P, lyso-PA or PA, Cer-1-P does not affect PLD, MAPK (extracellular signal-regulated kinase (ERK)-1/2), adenylyl cyclase, Ca^{2+} mobilization, or the expression of the early genes c-*fos* or c-*myc* in rat or mouse fibroblasts [42,43]. However, induction of ERK-2 phosphorylation, but not ERK-1, by Cer-1-P has been shown in osteoblastic cells [55], and C_2-Cer-1-P-induced Ca^{2+} mobilization has been shown to occur in calf pulmonary artery endothelial cells [56] and thyroid FRTL-5 [57]. Nonetheless, Cer-1-Ps did not induce Ca^{2+} mobilization in fibroblasts [42,43], neutrophils [50], or A549 cells [47], and C_8-Cer-1-P only had a marginal effect on Ca^{2+} mobilization in FRTL-5 cells [57]. Therefore, this effect of Cer-1-P remains controversial. From the above results it can be concluded that the effects of Cer-1-P are cell type-specific.

It was previously reported that C_8-Cer-1-P caused modest increases in the size of cultured chick otic vesicle explants, and that it decreased cell death in serum-starved cultured explants [58]. More recently, our lab has shown that Cer-1-P blocks cell death in bone marrow-derived macrophages incubated in the absence of macrophage-colony stimulating factor [44], a condition known to induce apoptosis in these cells [59,60]. Cer-1-P blocked both DNA fragmentation and the stimulation of the caspase-9/caspase-3 pathway, thereby suggesting that the prosurvival effect of Cer-1-P was due to inhibition of apoptosis [44]. Apoptotic macrophages showed high levels of ceramides and enhanced acid SMase activity compared to healthy cells [39,60]. A key observation was that Cer-1-P completely blocked acid SMase activity in intact macrophages [44], thereby preventing the accumulation of ceramides and promoting cell survival (Fig. 3). Acid SMase was also inhibited by Sph-1-P in intact macrophages [39]. However, unlike Sph-1-P, the inhibitory effect of Cer-1-P involved direct physical interaction with the enzyme, as Cer-1-P also inhibited acid SMase in cell homogenates [44]. This observation supports the hypothesis that the actions of Cer-1-P on intact cells are not mediated through receptor interaction. Also, apoptosis was associated with a substantial decrease in intracellular Cer-1-P, which can account, at least in part, for the activation of acid SMase [44]. This may be relevant, because Cer-1-P could be a physiological inhibitor of acid SMase, necessary for keeping its activity low under normal conditions.

Two recent reports by Pettus and co-workers showed that ceramide kinase mediates cytokine- and Ca^{2+} ionophore-induced arachidonic acid release and prostanoid synthesis, and that Cer-1-P is a direct activator of cytosolic phospholipase A2 in A549 lung carcinoma cells [46,47], a cell line lacking lipoxygenases and cytochrome P450. Specifically, incubation of these cells with interleukin-1β and the Ca^{2+} ionophore A23187 induced an increase in Cer-1-P levels that coincided with activation of Ca^{2+}-dependent ceramide kinase, and was within the relevant time frame of arachidonic acid release [46]. These studies were the first to demonstrate a role for ceramide kinase/Cer-1-P in signal transduction using a molecular approach, and have implicated Cer-1-P in the regulation of inflammatory responses.

The pathological and toxic effects of Cer-1-P have been recognized for some time since a SMase D was identified as the active principle of the venom of the brown recluse spider *Loxosceles reclusa*. SMase D can also be produced by some bacteria including *Corynebacterium pseudotuberculosis* and *Vibrio damsela* [61]. The bite of this spider or infections caused by these bacteria would induce inflammatory responses that are mediated by arachidonic acid release and subsequent generation of eicosanoids, and these actions might be controlled by Cer-1-P.

Cer-1-P can be converted to other bioactive lipids, and therefore one might hypothesize that the effects of Cer-1-P could be mediated through Cer-1-P-derived metabolites. However, ceramides and Cer-1-P are antagonistic signals, and Cer-1-P is unable to mimic the effects of sphingosine or Sph-1-P (i.e. PLD activation or adenylyl cyclase inhibition) [42,43]. In addition, no ceramidases capable of converting Cer-1-P to Sph-1-P have so far been identified in mammalian cells (Fig. 3), and Sph-1-P does not inhibit acid SMase activity in the manner that Cer-1-P does [39,44]. Therefore, Cer-1-P is likely to act on its own to regulate cell activation. However, conversion of ceramide to Cer-1-P might serve as a mechanism to terminate the proapoptotic actions of ceramide, and vice versa, dephosphorylation of Cer-1-P would cause apoptosis. Whether or not these actions have physiological relevance remains to be determined, but it was postulated that at least part of the mechanism whereby ceramides block the mitogenic effect of Cer-1-P may involve stimulation of its metabolism [42]. These observations emphasize the importance for cells to maintain a precise balance in the levels of proapoptotic ceramides and antiapoptotic Cer-1-P, and suggest that the 'rheostat' model proposed by Sarah Spiegel for ceramides and Sph-1-P [62] can also be applied to ceramides and Cer-1-P.

4. Specificity of Cer-1-P actions

A major concern when studying the functions of lipid mediators in metabolism is the specificity of action of the lipid candidates. The detergent-like properties of lipids have been known for a long time. Lipids can affect the integrity of cell membranes, causing loss of cytosolic components and cell lysis. They can also induce non-specific changes in the lipid bilayer of cell membranes thereby leading to alterations in the hydrophobic environment for optimal activity of membrane-associated enzymes. Therefore, lipid specificity is a major requirement when assessing the biological functions of bioactive lipids in cells. Concerning Cer-1-P, Berger et al. [63] reported that *N*-palmitoyl-Sph-1-P, which was complexed to bovine serum albumin, stimulated the incorporation of thymidine into DNA by about 7.9-fold in Swiss 3T3 fibroblasts. By contrast, *N*-palmitoyl-dihydro-Sph-1-P, which lacks the 4–5 *trans* double bond but retains the stereochemical configuration of Cer-1-P, had very little effect on stimulating DNA synthesis. Also, Cer-1-P shows significant specificity when compared to other closely related phospholipids such as Sph-1-P, PA, lyso-PA, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, or phosphatidylinositol. In particular, among all of these phospholipids, only Cer-1-P was able to significantly inhibit acid SMase in a cell-free system [44]. This feature of Cer-1-P makes it suitable for use as a selective inhibitor of acid SMase. In addition, the potency of Cer-1-P to stimulate cell survival was superior for long-chain Cer-1-P compared to short-chain Cer-1-Ps [44]. The effect of Cer-1-P in stimulating arachidonic acid release was also lipid-specific, as Sph-1-P, PA, diacylglycerol and ceramide were ineffective [46]. In addition, treatment of A549 cells with SMase D, which generates Cer-1-P at the plasma membrane of cells, also increased arachidonic acid release [46]. The authors of the latter work concluded that this effect was specific for Cer-1-P, as pretreatment of the cells with SMase C, which hydrolyzes membrane SM to generate ceramides and therefore removes the substrate for SMase D, inhibited SMase D-induced arachidonic acid release [46]. This work demonstrates that SM is required for the action of SMase D and that Cer-1-P is the primary product of SM hydrolysis responsible for stimulating arachidonic acid release. Taken together these studies demonstrate that the cellular activities of Cer-1-P are specific, and support a physiological role for Cer-1-P in controlling cell activation.

5. Metabolism of Cer-1-P

Biosynthesis of Cer-1-P in mammalian cells is catalyzed by ceramide kinase through phosphorylation of ceramides derived from the breakdown of SM, gangliosides, or from de novo synthesis (Fig. 2). The existence of ceramide kinase has been known for over a decade [48], but it has not been cloned until recently [64]. Subsequent identification of Cer-1-P phosphatase in rat brain [65] and hepatocytes [66] suggested that ceramide and Cer-1-P are interconvertible in cells. Cer-1-P can also be converted to ceramide by the action of a PA phosphohydrolase that is specifically located in the plasma membrane of cells [67]. It is now known that this enzyme activity belongs to a family of at least three mammalian lipid phosphate phosphatases [68]. In addition, Cer-1-P could potentially be formed by the action of SMase D, which occurs in a variety of organisms [61]. However, no mammalian SMase D activity has so far been detected.

6. Concluding remarks and future prospects

Cer-1-P has emerged as a new bioactive sphingolipid capable of regulating vital cellular functions. It stimulates cell proliferation, blocks apoptosis, and is involved in phagocytosis and inflammation. By contrast, ceramides are potent inhibitors of cell division and induce apoptosis in a great variety of cell types. In particular, ceramides block cell proliferation induced by mitogenic phospholipids (Cer-1-P, Sph-1-P, PA, or lyso-PA). At least part of this negative regulation of cell growth by ceramides may involve increased degradation of the bioactive phospholipids, possibly by enhancing the activity of specific lipid phosphate phosphatases. Activation of SMases to form ceramides may yield a proapoptotic signal. By contrast, conversion of ceramides to Cer-1-P would stimulate cell division and block cell death. These actions suggest that regulation of the enzymes that are involved in the metabolism of ceramides and Cer-1-P is vital for controlling the overall signal that is finally transmitted in cells. At least one of the mechanisms whereby Cer-1-P blocks apoptosis involves inhibition of acid SMase. Because ceramide and Cer-1-P have opposing effects, these findings add a new dimension to the understanding of the metabolic interrelationship of these metabolites, and suggest that alterations in the balance of the

intracellular levels of ceramides and Cer-1-P can affect cell survival. In addition, Cer-1-P enhances the activity of cytosolic PLA_2 in several cell types including A549 lung adenocarcinoma cells, J774.1 macrophages, and L929 mouse fibroblasts, thereby promoting arachidonic acid release and the subsequent biosynthesis of eicosanoids. The latter observation indicates that Cer-1-P may be an important regulator of inflammatory responses.

Up to now, it is not known whether Cer-1-P is present in plasma, or if it can be released into the extracellular environment upon cell activation. A general feature in cell signaling processes is that a signal, such as ceramide or Cer-1-P, is generated by the action of an enzyme or a group of enzymes acting in concert. The enzyme product will then activate specific downstream effectors leading to a cascade of metabolic reactions that will end up stimulating or inhibiting a biological function. A major challenge for future studies is to establish firmly or to disprove the role of Cer-1-P as a second messenger, and to define the signaling pathways that are regulated by this novel bioactive sphingolipid. The simplest approach to achieve this is to continue to investigate whether Cer-1-P can specifically activate or inhibit known protein kinases and phosphatases, both in vitro and in vivo. Other approaches might include the use of photoaffinity techniques with radioactive Cer-1-P, lipid–protein overlay assays, or large multilamellar vesicle binding assays, so as to detect specific Cer-1-P binding proteins in cells. Also, a better understanding of the interactions between the different metabolites that can be generated from the SMase pathway would be critical for elucidation of the mechanisms that regulate cellular functions, in particular cell proliferation and death, as well as inflammation. Most importantly, the development of inhibitors or activators of the enzymes that affect the intracellular concentrations of Cer-1-P may be crucial for establishing therapeutic strategies for controlling metabolic disorders.

Much effort is now required to elucidate the mechanisms of action of Cer-1-P, and to define the physiological significance of the intracellular formation of this bioactive sphingolipid.

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