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Enhanced ceramide-induced apoptosis in ceramide kinase overexpressing cells

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Abstract

We evaluated how increased levels of ceramide kinase (CerK) would impact the growth of COS-1 fibroblasts and RBL-2H3 basophils. The low CerK activity in these cells was strongly up-regulated upon recombinant expression of CerK. CerK-overexpressing COS-1 cells depended on higher concentrations of serum for their growth and displayed many filipodia. The two CerK-overexpressing cell lines were more sensitive to C2-ceramide-mediated apoptosis, and this correlated with the production of C2-ceramide-1-phosphate by CerK. This study indicates that ceramide kinase may participate in the control of cell growth, and establishes a novel assay that will be valuable for testing ceramide kinase inhibitors.

Keywords: Ceramide; Ceramide-1-phosphate; Apoptosis; Differentiation; Proliferation

Ceramide kinase (CerK) was cloned in 2002 [1] and is so far the only established enzyme that uses ceramide as a substrate to produce ceramide-1-phosphate (C1P). We recently characterized a CerK-like protein and variants thereof, all of which, however, did not display CerK activity [2]. A multi-lipid substrate kinase, alternatively named acylglycerol kinase, was also identified as another close homolog, but its activity towards ceramide remains controversial [3–5]. C1P was first described in brain synaptic vesicles, HL60 cells and neutrophils [6–8]. There is evidence that C1P stimulates cytosolic phospholipase A_2 mediated arachidonic acid production and eicosanoid synthesis [9], where it synergizes with sphingosine-1-phosphate (S1P) [10]. Other studies have pointed to a role of ceramide kinase and/or C1P in: cell proliferation (reviewed in [11]), mast cell degranulation [12], regulation of acidic sphingomyelinase, and control of apoptosis and survival in bone marrow derived macrophages [13,14], calcium mobilization in GH4C1 pituitary and Jurkat T-cells [15,16], and dopamine release in PC12 cells [17].

The importance of ceramide kinase (CerK) in cell signaling has largely relied on exogenous addition of its reaction product, ceramide-1-phosphate (C1P) to cultured cells. The procedure used for C1P delivery, as well as the extent of C1P dephosphorylation arising upon treatment, both may impact the outcome [18]. It is also still unknown how exogenously added C1P signals to produce intracellular effects. We chose to evaluate the consequence of CerK overexpression, an approach that allows for modulation of C1P levels directly inside the cell. The results reported here establish a novel assay to test putative ceramide kinase inhibitors, and suggest that the levels of CerK activity/C1P might need to be regulated to determine cell fate.

Materials and methods

Materials. C2 and C8 ceramides were from Cayman. C16-ceramide was from Sigma. [γ-^{32}P]ATP (10 mCi/ml, 3000 Ci/mmol), [^{32}P]orthophosphate (10 mCi/ml) were from Amersham Biosciences. Complete™

Abbreviations: CerK, ceramide kinase; C1P, ceramide-1-phosphate; Cer, ceramide; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; GlcCer, glucosylceramide; GCS, glucosylceramide synthase; NBD, nitrobenzo-2-oxa-1,3-diazole; SM, sphingomyelin; SMS, sphingomyelin synthase; TLC, thin layer chromatography.

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protease inhibitors tablets were from Roche Molecular Biochemicals. OneShot® TOP10 competent *Escherichia coli* cells and TrypLE Select were from Invitrogen.

Stable cell lines generation. CerK cDNA, corresponding to GenBank™ Accession No. AB079066, was obtained and subcloned in Gateway™ compatible entry vectors as described previously [19]. A *Sac*II/*Asc*I fragment, blunt ended with T4 DNA Polymerase, was ligated into the pMono-Blasti vector (Invivogen), previously digested with *Mlu*I and *Eco*RV, using Fast Link ligation reagents (Epicentre). COS-1 (cultured in DMEM/10% FCS at 37 °C/5% CO_2 in a humidified atmosphere) were seeded at 10^5 cells/well in 6-well plates. After 24 h, cells were transfected with 3 μg DNA, using FuGENE 6 (Roche Molecular Biochemicals). Selection was started 24 h after transfection by addition of 10 μg/ml blasticidin. After clonal dilution, the obtained clones were screened for expression of CerK in a CerK kinase assay (see below). Stable RBL-2H3 cells (cultured in MEM-E 1×, 20% RPMI 1640/10% FCS at 37 °C/5% CO_2) were generated following the same procedure.

Fluorescence microscopy. COS-1 cells were seeded into 4-chambered coverslips (LabTEK™ II—Nalge Nunc) at 2.2×10^4 cells/chamber. Twenty-four hours later, cells were transfected with 0.6 μg plasmid (encoding CerK, N-terminally flagged with GFP) per chamber using FuGENE 6. Live-cell fluorescence microscopy was done 24 h after transfection as already described [20].

Ceramide kinase assays. For in vitro kinase assays, cells were scraped into lysis buffer (10 mM MOPS, pH 7.2; 2 mM EGTA, 150 mM KCl, 2% Triton X-100, 1 mM DTT, and protease inhibitors). The suspension was homogenized by 20 strokes in a Potter–Elvejhem homogenizer and used immediately. Kinase activity assays were performed as described previously [20].

For cell-based ceramide kinase assays, stably transfected cells were seeded in a 6-well plate at a density of 2×10^5 cells/well. Cells were incubated for 2 h at 37 °C, 5% CO_2, with C2-, C8-, or C16-ceramide at a concentration of 10 μM. After 2 h, [^{32}P]orthophosphate (10 mCi/ml) was added to a final concentration of 300 μCi/ml and cells were incubated for another 2 h. Cells were then scraped and spun down, the supernatants discarded, and the pellets washed with 500 μl HBSS supplemented with 10 mM EDTA. After spinning again and discarding supernatants, pellets were resuspended in 100 μl of HBSS/EDTA. Lipid extraction, saponification, and analysis on TLC plates were performed as already described [20]. Plates were dried and directly exposed to Kodak X-Omat Blue XB-1 films, and processed for autoradiography using an Agfa Curix 60 developer. For experiments using fluorescent ceramide, cells were incubated with NBD-labeled C6-ceramide (Molecular Probes) at a concentration of 5 μM for 2 h at 37 °C, 5% CO_2. Afterwards, cells were washed once with HBSS/EDTA. Lipid extraction and analysis was performed as in [20].

Viability and apoptosis. For viability assays, we used resazurin, a fluorescent redox indicator (Alamar Blue, Serotec). Cells were incubated with 10% (v/v) Alamar Blue at 37 °C for ~2 h. Fluorescence was recorded (excitation, 530 nm; emission, 600 nm; and cutoff, 590 nm) in a Spectramax Gemini XS Fluorescence Microplate reader (Molecular Devices).

For apoptosis measurement the serum content of the medium was reduced to 1%, 24 h after seeding. Cells were then incubated with vehicle (0.1% EtOH) or 10 μM C2-ceramide for 9 h. After a wash in PBS, cells were detached, washed again twice with cold PBS, resuspended in 1× binding buffer, and analyzed by FACS using the Annexin V PE Apoptosis Detection Kit I (BD #559767), according to the manufacturer's instructions.

Results

Overexpression of ceramide kinase in COS-1 cells reduces proliferation

Ceramide kinase (CerK) activity is barely detectable in COS-1 cells when measured in crude cell lysates (Fig. 1A). Consistently, addition of NBD-C6-ceramide to COS-1 cells in culture leads to glucosylceramide (GlcCer) and sphingomyelin (SM) formation, as a result of GlcCer synthase (GCS) and SM synthase (SMS) activities; however, C1P is not detected (Fig. 1B). Transfection of COS-1 cells with WT-CerK results in a strong increase in CerK activity as measured in vitro (Fig. 1A) and in a switch of the metabolism of added NBD-C6-Cer towards C1P, indicative of effective competition for access to ceramide between GCS, SMS, and CerK (Fig. 1B). This observation supports earlier findings that recombinant CerK mainly localizes at the Golgi complex [19,22], where GCS and SMS1 are also present [21,22].

CerK overexpressing COS-1 cells proliferate normally in the presence of 10% FCS. However, reducing the FCS concentration has more impact on these cells as compared to control COS-1 cells (transfected with the empty vector). Typically, a twofold decrease in proliferation rate was

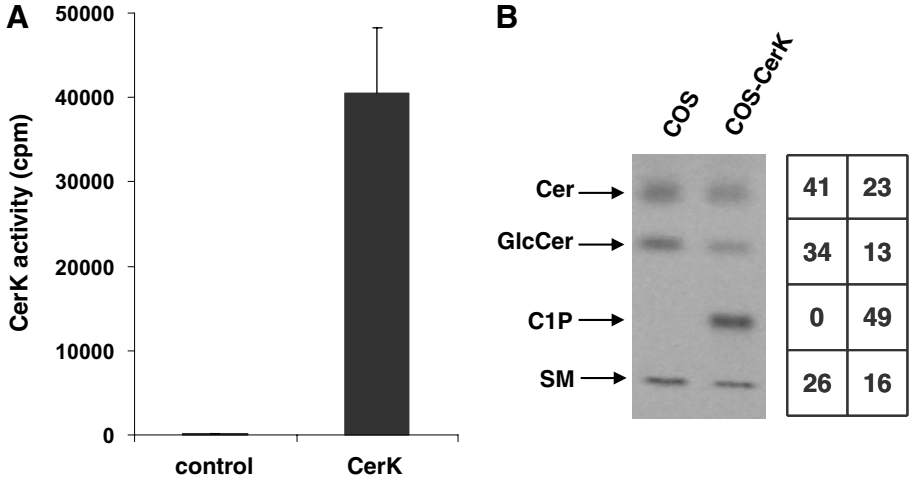

Fig. 1. Overexpression of CerK in COS-1 cells. (A) CerK-overexpressing COS-1 cells and empty vector-transfected controls were lyzed and analyzed in vitro using [^{32}P]ATP, for their ability to phosphorylate C8-ceramide. (B) A cell based assay with NBD-ceramide was used to analyze ceramide metabolites: overexpression of CerK increases C1P formation at the expense of the other anabolites (GlcCer and SM). Left, TLC plate; right, scanned intensities of the various metabolites.

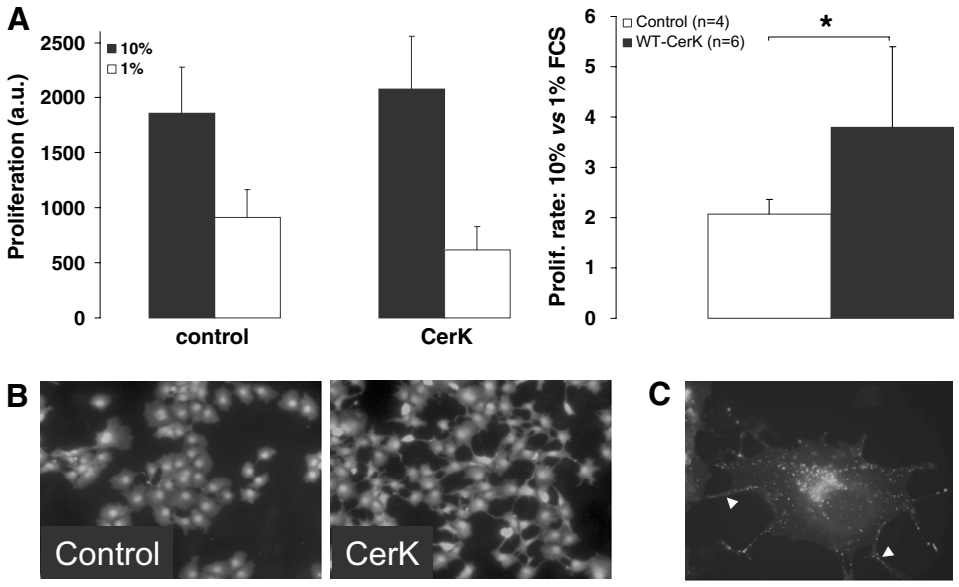

Fig. 2. Reduced growth of CerK-transfected COS-1 cells in low FCS-containing medium. (A) Proliferation of control and CerK-overexpressing stable cell lines was monitored over 5 days and measured with Alamar Blue as described in Materials and methods. Left, proliferation values in 10% or 1% FCS; right, growth rate in 10% versus 1% FCS. n represents the number of individual clones used in this experiment; * indicates statistical significance at $P < 0.05$ using the Student t-test. (B) Phase contrast microscopic analysis of stable cell lines. (C) Magnified picture of a COS-1 cell-overexpressing GFP-CerK, analyzed by fluorescent microscopy performed as described in [20]. Filipodia, where GFP-CerK is localized, are indicated with arrowheads.

observed for control cells when FCS concentration was lowered to 1%, whereas the decrease approached fourfold in CerK-overexpressing cells (Fig. 2A). The morphology of CerK-overexpressing cells was more elongated, displaying plasma membrane extensions and a reduced ability to reach confluence (Fig. 2B). Upon recombinant expression of a GFP-tagged CerK, we noticed that the recombinant protein is present in cell membrane extensions, such as shown in Fig. 2C. We recently showed that CerK dynamically traffics between the Golgi apparatus and the plasma membrane, relying on its PH domain, and using microtubules [20]. It is, therefore, possible that CerK, trafficking to the plasma membrane, enables formation of these cellular extensions at the expense of cell proliferation.

CerK-overexpressing COS-1 cells are more susceptible to C2-cer-induced apoptosis

The use of short chain ceramides, that are cell permeable, has been shown to induce apoptosis in many cell systems (reviewed in [23]). COS-1 cells do respond to C2-cer, but with only limited decrease in viability, even at 10 μM (Fig. 3A). Remarkably, CerK overexpression enhanced the sensitivity of COS-1 cells to C2-cer-mediated cell death. A treatment with 10 μM C2-cer typically reduced the viability of CerK-overexpressing cells by more than 60% after 24 h, whereas that of control COS-1 cells was reduced by 30% only (Fig. 3A). Furthermore, after 9 h of treatment CerK-overexpressing cells had initiated apoptosis, as indicated by Annexin V detection, and 15% had already reached the late apoptotic/necrotic stage, as shown from the double detection of Annexin V and 7-AAD (Fig. 3B). Therefore, increased cell death in CerK-overexpressing cells occurs as a result from enhanced apoptosis.

Ceramide-induced apoptosis in CerK-overexpressing COS-1 cells correlates with its phosphorylation by CerK

We next examined the effects of ceramide on cell viability, as a function of acyl chain length. Treatment of COS-1 cells with C2- or C16-cer decreased cell viability in a concentration-dependent manner (not shown), reaching about 30% of vehicle-treated controls at 10 μM (Fig. 3C). C8-cer, in contrast, had virtually no effect on viability up to 10 μM, in line with already published observations [18]. Overexpression of CerK sensitized COS-1 cells to C2-cer-mediated cell death (Fig. 3C). CerK overexpression, however, did neither sensitize cells to C16-cer-mediated cell death, nor did it promote sensitivity of cells to C8-cer (Fig. 3C). These observations prompted us to look at the ability of these exogenously added ceramide species to serve as substrates for CerK in CerK-overexpressing COS-1 cells. Only C2-cer could be significantly phosphorylated; C8-C1P was barely detectable, and C16-C1P was not formed at all (Fig. 3D). Therefore, enhanced apoptosis of CerK-overexpressing cells in response to ceramide correlates with the ability of the ceramide species to be phosphorylated by CerK. In vitro studies have shown that C2-, C8-, and C16-ceramides can be readily phosphorylated by CerK, provided the right conditions are met [4]. That only C2-cer could efficiently serve as a substrate when added exogenously to cells, therefore, suggests that only C2-cer may cross the plasma membrane and reach the Golgi complex, where CerK is mainly localized.

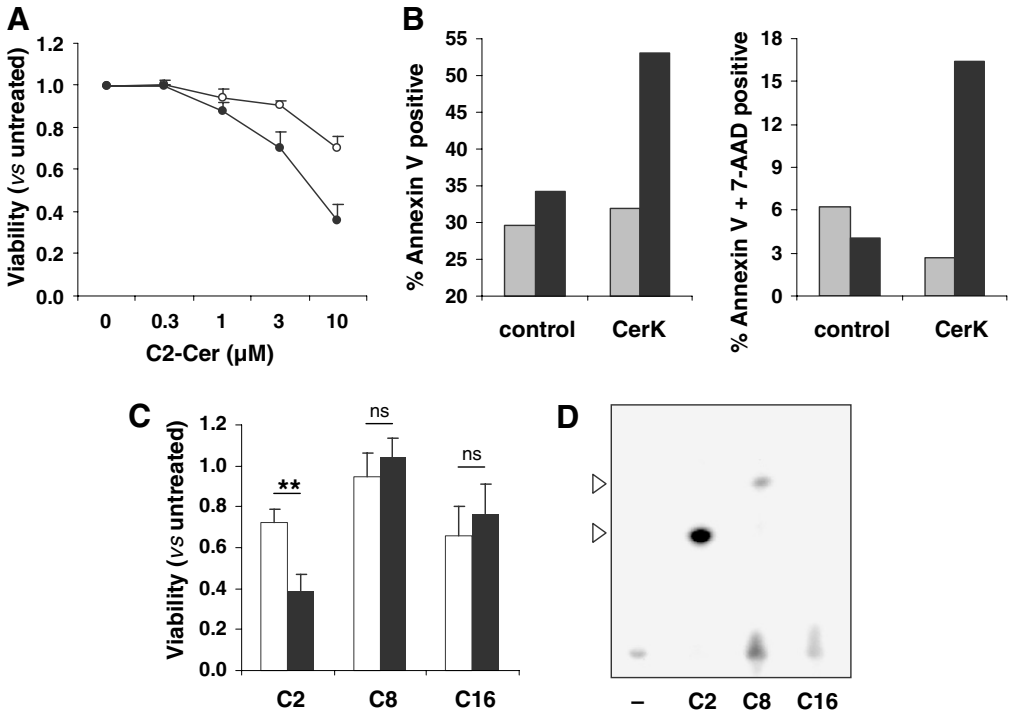

Fig. 3. CerK overexpression sensitizes COS-1 cells to C2-cer-mediated apoptosis. (A) Control (○) and CerK-overexpressing (●) stable cell lines, at a density of 0.4×10^4 cells/well in a 96-well plate, were incubated for 24 h with 1% FCS in the presence of various concentrations of C2-ceramide. Alamar Blue (10% final concentration) was added subsequently to measure the residual viability. (B) Control and CerK-overexpressing stable cell lines were incubated for 9 h in the absence (grey bars) or presence (black bars) of 10 μM C2-ceramide before assessment of apoptosis. Left, cells in early apoptosis (Annexin V-positive); right, cells in late apoptosis/necrosis (Annexin V and 7-AAD positive). This is one of three independent measurements with similar results. The high basal Annexin V signal was always observed, and therefore probably represents background due to the handling of adherent cells. (C) Control (empty bars) and CerK-overexpressing (full bars) stable cell lines were incubated for 24 h in the presence of 10 μM of C2-, C8- or C16-ceramide. Alamar Blue (10% final concentration) was added subsequently to measure the residual viability. ** indicates statistical significance at $P < 0.01$ using the Student *t*-test. (D) CerK-overexpressing stable cell lines were incubated with [^{32}P]orthophosphate and 10 μM of C2-, C8- or C16-ceramide as described in Materials and methods. Lipid were extracted, saponified, and run on TLC. The lower (higher) arrowhead indicates C2-C1P (C8-C1P).

High susceptibility of CerK-overexpressing RBL-2H3 cells to C2-cer-mediated cell death

To investigate whether the above mentioned effects would be limited to COS-1 cells, we evaluated the basophilic derived RBL-2H3 cells stably expressing CerK. RBL-2H3 cells have been used as a model system to study mast cell biology; a recent study in particular indicates that CerK may contribute to mast cell activation following IgE/Ag stimulation [12]. As for COS-1 cells, overexpression of CerK in RBL-2H3 promoted a switch in ceramide metabolism following exogenous addition of fluorescent ceramide: GlcCer and SM were the predominant metabolites formed in control RBL-2H3 cells, which formed very

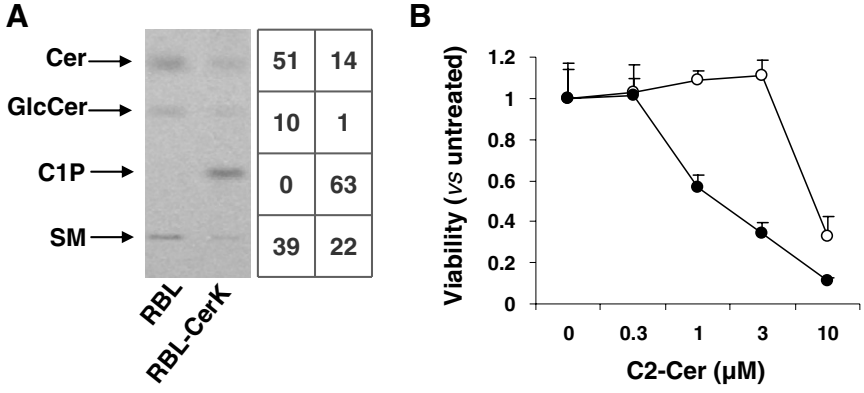

Fig. 4. CerK overexpression sensitizes RBL-2H3 cells to C2-cer-mediated apoptosis. (A) Cell-based assay with NBD-ceramide: overexpression of CerK increases C1P formation at the expense of the other anabolites (GlcCer and SM). Left, TLC plate; right, scanned intensities of the various metabolites. (B) Control (○) and CerK-overexpressing (●) stable cell lines, at a density of 0.4×10^4 cells/well in a 96-well plate, were incubated for 24 h with 1% FCS in the presence of various concentrations of C2-ceramide. Alamar Blue (10% final concentration) was added subsequently to measure the residual viability.

low C1P, whereas CerK overexpression led to a strong increase of C1P made at the expense of GlcCer and SM (Fig. 4A). Further, in line with the observations made using COS-1 cells, CerK-overexpressing RBL-2H3 cells were strongly sensitized to C2-cer-mediated cell death (Fig. 4B). Therefore, the sensitization by CerK to ceramide-induced cell death is not restricted to a particular cell type.

Discussion

The experiments reported here show that overexpression of CerK in two unrelated cell lines is sufficient to switch the metabolism of exogenously added ceramide towards ceramide-1-phosphate (C1P), at the expense of other metabolites such as glucosyl ceramide (GlcCer) and sphingomyelin (SM). This indicates that the enzymes responsible for synthesis of these metabolites (CerK, GCS, and SMS) can compete with each other for substrate availability. We show that cells overexpressing CerK are sensitized to ceramide-mediated apoptosis. How death occurs in CerK-overexpressing cells exposed to ceramide may result from different inputs. First, it is possible that reduction in the amount of GlcCer and SM, which are usual markers of immortalized cell lines, is a priming factor. Second, cell death may occur as a consequence of increased C1P levels, i.e. phosphorylation of the added cell permeable ceramide species in addition to already elevated endogenous C1P levels. Third, death may have occurred as a result of these additive effects, combined with toxicity of the short chain ceramide itself. Regarding the latter, there has been debate whether short chain ceramides are physiologically relevant or not. In fact, LC/MS measurements have recently revealed that these short chain species might have been overlooked [24] and, therefore, there is a need to investigate this further. Although it would clearly be important to evaluate the effect of pro-apoptotic stimuli that generate long-chain ceramides, the fact that C2-ceramide induces apoptosis in CerK-overexpressing cells already represents a novel and valuable paradigm for the testing of putative CerK inhibitors.

The findings here contrast with current understanding of C1P biology. Indeed, C1P was reported to promote cell growth or survival in fibroblasts and macrophages, respectively (reviewed in [11]). In these studies, high concentrations (up to 50 μM) of C1P were added exogenously. It is at present unknown how C1P signals into the cell and exerts its proliferative and pro-survival effects. Whether through GPCR signaling, transport across the cell, or simply accumulation at the plasma membrane, has not been clarified yet. Furthermore, we recently showed that C1P is efficiently dephosphorylated when added to cells [18] which complicates experimental settings because not only C1P but metabolites thereof are likely present. Here, we addressed the consequence of increased CerK/C1P levels on cell growth and apoptosis, based on C1P that is generated inside the cell. Whereas, treatment with exogenous ceramide is transient, the use of an overexpression system allows for sustained production of C1P. The observation that CerK-overexpressing cells display an elongated shape, with increased filipodia where CerK itself is localized (Fig. 2), suggests that CerK may contribute to cell growth in a broad sense, i.e. not restricted to proliferation. One could actually speculate that C1P signals differently, being proliferative when added to the cell, or alternatively, favoring differentiation or even being pro-apoptotic when formed endogenously. As preliminary evidence indicates that C1P is present in plasma (R.A. Sabbadini, personal communication), the possibility that C1P biology, akin to S1P, has both extra- and intra-cellular signaling arms will be very interesting to explore. Of note regarding the present work, recent data also indicate that S1P accumulation, in some instances, can lead to apoptosis, and that this is receptor independent [25].

Acknowledgments

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