Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages

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Abstract It was reported previously that ceramide-1-phosphate (Cer-1-P) is mitogenic for fibroblasts (Gómez-Muñoz, A., P. A. Duffy, A. Martin, L. O’Brien, H-S. Byun, R. Bittman, and D. N. Brindley. 1995. Mol. Pharmacol. 47: 883–889; Gómez-Muñoz, A., L. M. Frago, L. Alvarez, and I. Varela-Nieto. 1997. Biochem. J. 325: 435–440). We now show that Cer-1-P prevents cell death in bone-marrow-derived macrophages (BMDMs) after withdrawal of macrophage colony-stimulating factor (M-CSF). Removal of M-CSF is known to induce apoptosis in these cells. Cer-1-P blocked activation of the caspase-9/caspase-3 pathway and prevented DNA fragmentation, indicating that the enhancement of cell survival was due to inhibition of apoptosis. M-CSF deprivation resulted in activation of acid sphingomyelinase (A-SMase). Exogenously added Cer-1-P inhibited A-SMase in intact BMDMs at concentrations that also prevented apoptosis. Cer-1-P also inhibited A-SMase in cell homogenates, suggesting a possible direct physical interaction of Cer-1-P with the enzyme. In conclusion, these data demonstrate that Cer-1-P blocks apoptosis in BMDMs through inhibition of A-SMase, thereby reducing ceramide generation. This adds a new dimension to the understanding of the metabolic interrelationship of ceramides and Cer-1-P, and shows how altering the balance of intracellular levels of these mediators can affect cell survival.—Gómez-Muñoz, A., J. Y. Kong, B. Salh, and U. P. Steinbrecher. Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. J. Lipid Res. 2004. 45: 99–105.

Supplementary key words sphingosine-1-phosphate • caspases • cell survival

The breakdown of sphingomyelin (SM) produces bioactive sphingolipid metabolites, some of which are believed to act as second messengers that control critical cellular functions. For example, N-deacylation of SM generates sphingosine phosphocholine, which is mitogenic for fibroblasts (1). Stimulation of SMase activity produces ceramides, which can inhibit cell proliferation and are potent inducers of apoptosis (2–4). Ceramides have been shown to regulate several protein kinases, including ceramide-activated protein kinase (5, 6) and protein kinase C (7), or protein phosphatases of the 2A family (8). In addition, ceramides are potent inhibitors of phospholipase D, both in cultured cells (9, 10) and in cell-free systems (11). Ceramides can be degraded by ceramidases to sphingosine, and this, in turn, can be phosphorylated by sphingosine kinase to produce sphingosine-1-phosphate (Sph-1-P) (16, 17). Both sphingosine and Sph-1-P have been implicated in the regulation of cell proliferation and death (12–15).

Another important ceramide metabolite that can be generated through the action of ceramide kinase is ceramide-1-phosphate (Cer-1-P) (16, 17). Boudker and Futerman (18) characterized a phosphatase that specifically hydrolyzes Cer-1-P in plasma membranes, suggesting that ceramide and Cer-1-P can be interconverted in cells. More recently, Riboni et al. (19) observed that Cer-1-P can also be produced from the recycling of sphingosine produced from ganglioside catabolism, and Rile et al. (20) reported that Cer-1-P can be formed intracellularly in neutrophils. Critical biological functions have been attributed to Cer-1-P. We first found that short-chain acetyl (C2)- and octanoyl (C8)-Cer-1-P, as well as natural long-chain Cer-1-P, stimulated the incorporation of [3H]thymidine into DNA in fibroblasts, and that this action did not involve conversion of Cer-1-P to Sph-1-P (21, 22). More recently, it was found that Cer-1-P can be generated during the phagocytosis of antibody-coated erythrocytes through stimulation of SMase activity in neutrophils, and that it plays an important role.

Abbreviations: A-SMase, acid sphingomyelinase; BMDMs, bone marrow-derived macrophages; Cer-1-P, ceramide-1-phosphate; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; M-CSF, macrophage colony-stimulating factor; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(5-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; Sph-1-P, sphingosine-1-phosphate.

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in liposome fusion (23). To date, little is known about the metabolic pathways that may be modulated by Cer-1-P. We observed that Cer-1-P, at concentrations within the micromolar range, did not affect phospholipase D (PLD), mitogen-activated protein kinase (MAPK), adenylyl cyclase, or Ca<sup>2+</sup> mobilization in fibroblasts, and that it did not alter the expression of the early genes c-fos or c-myc (21, 22). Failure of Ca<sup>2+</sup>-Cer-1-P to induce intracellular Ca<sup>2+</sup> mobilization has been confirmed recently in neutrophils (20). However, Gijbers et al. (24) and Hogback et al. (25) reported that Ca<sup>2+</sup>-Cer-1-P caused fast and transient intracellular rises in Ca<sup>2+</sup> in both calf pulmonary artery endothelial cells and thyroid FRTL-5 cells, respectively, an action that might be related to the mitogenic effect of Ca<sup>2+</sup>-Cer-1-P.

In recent studies, we found evidence of sphingomyelinase activation and ceramide generation in bone marrow-derived macrophages (BMDMs) induced to undergo apoptosis by growth factor withdrawal (26). We also showed that inhibition of SMase activity with desipramine completely prevented apoptosis in BMDMs, indicating that in this model of apoptosis, ceramides play a causal role (26). The objective of the present work was to determine whether Cer-1-P could inhibit cell death in BMDMs. In this report, we demonstrate that Cer-1-P inhibits apoptosis in macrophages and show that the mechanism whereby Cer-1-P exerts this effect involves inhibition of SMase activity, thereby preventing formation of ceramides.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, Cer-1-P (from bovine brain, contains predominantly stearic and nervonic acids), phosphatidic acid, lysophosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylycerine, phosphatidylinositol, glycero 3-phosphate, phenazine methosulfate, and SM (from bovine brain) were from Sigma/Aldrich Canada (Oakville, Ontario, Canada). Defined fetal bovine serum (FBS) was from Hyclone (Logan, UT). Fisher Scientific (Edmonton, Alberta, Canada) supplied [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] (MTS), Ca<sup>2+</sup>-Cer-1-P, Ca<sup>2+</sup>-Cer-1-P, Ca<sup>2+</sup>-ceramide, dihydroC<sub>2</sub>-ceramide, sphingosine, and Sph-h-P were from Avanti Polar Lipids (Alabaster, AL). [3H]palmitate, [32P]orthophosphate, and radiolabeled bovine SM (choline[14C]methyl) were from Mandel Scientific (Guelph, Ontario, Canada). Antibodies to caspase-3 proenzyme were from Stressgen (Victoria, British Columbia, Canada), and antibodies to active caspsases 3 and 9 were supplied by BD-Pharmingen (Mississauga, Ontario, Canada). PD098059, LY294002, and wortmannin were from Calbiochem products supplied by VWR Canlab (Mississauga, Ontario, Canada).

Cell culture

Bone marrow macrophages were isolated from femurs of 6–8-week-old female CD-1 mice as described (27). Cells were plated for 24 h in RPMI 1640 medium containing 10% FBS and 10% L cell-conditioned medium as the source of macrophage colony-stimulating factor (M-CSF) (28). The nonadherent cells were removed and cultured in the above medium until ~80% confluence was reached (4–6 days) prior to use in the experiments.

Cell viability assay

Macrophages were seeded at 25,000 cells/well in 96-well plates and incubated overnight in RPMI 1640 with 10% FBS and 10% L cell-conditioned medium as a source of M-CSF. The medium was then replaced by fresh RPMI 1640 medium in the presence or absence of agonists and/or inhibitors as appropriate. Cell viability was estimated by measuring the rate of reduction of the tetrazolium dye MTS as described (28).

Ceramide determination

Radioactivity in ceramide was determined after labeling BMDMs with 5 µCi/ml [3H]palmitate for 24 h in RPMI 1640 with 10% FBS and 10% L cell-conditioned medium as the source for M-CSF as described (9, 10). The radioactive medium was aspirated, and the cells were washed twice with nonradioactive RPMI 1640 without M-CSF. The macrophages were then incubated in this same medium in the absence or in the presence of agonist for 30 h. Cells were then washed twice with ice-cold calcium-free phosphate buffer saline and scraped into 0.5 ml methanol. The cells were washed with a further 0.5 ml methanol, and the two methanol samples were combined and mixed with 0.5 ml chloroform. Lipids were extracted by separation of phases with a further 0.5 ml chloroform and 0.9 ml of a solution containing 2 M KCl and 0.2 M H<sub>3</sub>PO<sub>4</sub>. Chloroform phases were dried down under N<sub>2</sub> and lipids were separated by thin-layer chromatography using Silica Gel 60-coated glass plates. The plates were developed for 50% of their lengths with chloroform-methanol-acetic-acid (9:1:1; v/v/v) and then dried. They were then developed for their full length with petroleum ether (boiling point 40°C to 60°C) -diethylth ether-acetic-acid (60:40:1; v/v/v). The position of ceramides was identified after staining with I<sub>2</sub> vapor by comparison with authentic standards. Radioactivity was quantified by scraping the ceramide spots from the plates by liquid scintillation counting.

Measurement of DNA fragmentation

DNA fragmentation was determined by using flow cytometry as previously described (28). Briefly, cells were harvested by scraping,

**Fig. 1.** Ceramide-1-phosphate (Cer-1-P) promotes macrophage survival. Bone marrow-derived macrophages (BMDMs) were seeded at 25 x 10<sup>3</sup> cells/well in 96-well plates and incubated in RPMI 1640 with 10% fetal bovine serum (FBS) but without macrophage colony-stimulating factor (M-CSF), in the absence (empty symbols) or in the presence of 30 µM Cer-1-P (filled symbols) for the times that are indicated. Cell viability was determined by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] (MTS) assay as described in Materials and Methods. Results are expressed relative to control cells at 0 h. Data represent means ± SEM of three different experiments performed in quadruplicate.
fixed in ice-cold 70% ethanol for 1 h at −20°C, washed three times with ice-cold calcium-free phosphate buffer saline, and resuspended in hypotonic fluorochrome buffer consisting of 0.1% Triton X-100, 0.1% sodium citrate, RNase (25 μg/ml), and propidium iodide (50 μg/ml). Fluorescence was measured with an Epics XL-MCL fluorescence-activated cell sorter (Beckman Coulter, Fullerton, CA). Subdiploid DNA content analysis was performed on singlet populations using WinMDI 2.8 (J. Trotter, Scripps Research Institute, La Jolla, CA). At least 10^4 cellular events were counted.

**Sphingomyelinase assay**

The activities of acid sphingomyelinase (ASMase) and neutral sphingomyelinase were determined as described by Liu and Hanun (29) using (choline-[14C]methyl) SM as the substrate. SMase activities were proportional to the amount of protein added to the assay, and the incubation time was adjusted so that <10% of the substrate was consumed. The reaction rate was proportional to the time of incubation and to protein concentration up to at least 50 μg of enzyme preparation for 120 min. ASMase activity in cell homogenates was determined in a similar manner (9).

**32P labeling of BMDMs and determination of intracellular Cer-1-P levels**

Radioactivity in Cer-1-P was determined after labeling the cells with 200 μCi/ml [32P]orthophosphate for 24 h in phosphate-free RPMI 1640 with 10% FBS and 10% L cell-conditioned medium as the source for M-CSF. The radioactive medium was aspirated, and cells were washed twice with nonradioactive RPMI 1640 without M-CSF and FBS. The macrophages were then incubated in this same medium, as required. Lipids were extracted as indicated above and separated by thin-layer chromatography using oxalate-impregnated high-performance plates that were developed as reported (23). The corresponding spots for Cer-1-P were identified by comigration with authentic standards and by autoradiography, and scraped and quantitated by liquid scintillation.

**Western blotting**

Macrophages were harvested and lysed in ice-cold homogenization buffer as described (28). Protein (40–50 μg) from each sample was loaded and separated by SDS-PAGE using 10% or
15% separating gels. Proteins were transferred to nitrocellulose paper and blocked for 1 h with 4% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN₃ and 0.1% Tween 20, and then incubated overnight with the primary antibody in TBS-0.1% Tween 20 at room temperature. After three washes with TBS-0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized using enhanced chemiluminescence and recorded with a Fluorochem 8000 imaging system (Canberra Packard Canada, Mississauga, Ontario, Canada). Densitometric values for bands were calculated using the NIH Image analysis program that was developed at the Research Services Branch of the National Institutes of Health.

Statistical analysis
Results are expressed as means ± SEM of three independent experiments performed in triplicate or quadruplicate, unless indicated otherwise. Statistical analysis was done using Student’s t test with level of significance set at P < 0.05.

RESULTS
It is well known that removal of growth factors from cultures of hemopoietic cells causes cell death via apoptosis. One well-established experimental model is BMDMs, which typically undergo apoptosis within 24–48 h of M-CSF withdrawal (28, 30), even in the presence of serum. We chose this model to evaluate the effects of Cer-1-P on macrophage survival. Figure 1 shows that Cer-1-P increases macrophage viability after M-CSF withdrawal in a time-dependent manner. The maximal increase in cell viability was attained at about 30 h of incubation of macrophages with 30 μM Cer-1-P (Figs. 1, 2). Synthetic short-chain C₂-Cer-1-P and C₆-Cer-1-P also increased the viability of the macrophages after M-CSF withdrawal, although to a lesser extent than did natural long-chain Cer-1-P (Fig. 3). Of note, maximal cell survival with C₂-Cer-1-P was attained at 10 μM, and higher concentrations were less effective or resulted in toxicity (not shown). One would expect greater uptake of C₂-Cer-1-P than of longer-chain Cer-1-P. However, the failure of C₂-Cer-1-P to promote viability at higher concentrations does not necessarily indicate direct toxicity from Cer-1-P, because dephosphorylation could result in ceramide levels above a toxic threshold. As expected from recent work (15), Sph-1-P was also able to induce macrophage survival, whereas its glycerolipid analog, lysophosphatic acid, was ineffective (Fig. 3). To evaluate whether the prosurvival effect of Cer-1-P was due to inhibition of apoptosis or to effects on necrosis, which also occurs under these conditions, cells were analyzed for DNA fragmentation by flow cytometry (mean ± SEM of three independent experiments), and this was decreased to 2 ± 0.5% by 30 μM Cer-1-P (P < 0.05) (Fig. 4). Similar concentrations of lysophosphatic acid or sphingosine were ineffective (not shown). In addition, Cer-1-P prevented the activation of caspase 9 and the cleavage of the 116 kDa poly (ADP-ribose) polymerase substrate by active caspase 3 (Fig. 5).

Fig. 5. Cer-1-P prevents activation of the caspase-9/caspase-3 cascade. BMDMs were treated as in Fig. 4. Caspase 9, caspase 3 and poly (ADP-ribose) polymerase (PARP) were assayed by immunoblot as described in Materials and Methods. Normalized densitometric values (NIH Image 1.62) for the active caspase 3 bands were 34, 100, and 37, respectively. Values for PARP were 100, 22, and 93. Those for active caspase 9 were 0.2, 100, and 40. Similar results were obtained in each of two replicate experiments.
Inhibition of apoptosis by ceramide-1-phosphate

In agreement with recent work (15, 26), M-CSF deprivation caused a 4.4-fold increase in ceramide levels in macrophages (mean ± SEM of three independent experiments), and this was decreased to 1.7 ± 0.2-fold by 30 μM Cer-1-P or to 1.7 ± 0.4-fold by 10 μM C2-Cer-1-P (mean ± SEM of three independent experiments). This observation suggests that Cer-1-P might block cell death by inhibiting ceramide production.

We previously found that both acidic and neutral SMase activities were stimulated in BMDMs by M-CSF deprivation, but more than 95% of the total SMase activity was attributable to the acidic form of the enzyme (15, 26). Figure 6 presents the interesting observation that Cer-1-P inhibits the activation of A-SMase in intact cells, thereby blocking the generation of ceramides. We previously reported that Sph-1-P also inhibits A-SMase activity in intact cells (15). However, these two ceramide metabolites evidently act on A-SMase by different mechanisms, because Cer-1-P potently inhibited A-SMase activation in cell homogenates, whereas Sph-1-P did not (Fig. 7, upper panel). This suggests that the inhibition of A-SMase by Sph-1-P must be indirect, whereas inhibition by Cer-1-P may involve a direct physical interaction with the enzyme. The inhibitory effect of Cer-1-P was specific, because other structurally related phospholipids had little or no effect on A-SMase activity (Fig. 7, lower panel).

A critical observation was that M-CSF deprivation was associated with a significant decrease in the levels of endogenous Cer-1-P (Fig. 8). This indicates that our finding of antiapoptotic effects of exogenous Cer-1-P in BMDMs may be of physiological relevance and that the decrease in Cer-1-P could account, at least in part, for the activation of A-SMase that occurs in these cells after M-CSF withdrawal.

We previously reported that Cer-1-P and ceramides are antagonistic signals. In particular, it was demonstrated that short-chain or natural long-chain Cer-1-P-induced DNA synthesis was inhibited by cell-permeable ceramides (21, 22). Likewise, C2-Ceramide, but not its inactive analog, dihydro-C2-ceramide, blocked Cer-1-P-induced macropHage survival.

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DISCUSSION

The ability of Cer-1-P to stimulate cell proliferation was first reported by Gómez-Muñoz et al. (21) using synthetic short-chain C₈-Cer and C₈-Cer-1-P (21), or natural long-chain Cer-1-P (22). These effects were accompanied by an increase in the levels of proliferating cell nuclear antigen (22). More recently, Frago et al. (31) showed that C₈-Cer-1-P caused a modest increase in the size of cultured chick otic vesicle explants. In that study, C₈-Cer-1-P also decreased macrophage survival (Fig. 9), thereby emphasizing the importance for cells to maintain an appropriate balance in the levels of intracellular ceramide and Cer-1-P.

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