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. Martín, L. O’Brien, H-S. Byun, R. Bittram, 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), increased ceramide levels, and a decrease in intracellular Cer-1-P. 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 2 A 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). 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 Futera (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-(3-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\(^{2+}\) mobilization in fibroblasts, and that it did not alter the expression of the early genes c-fos or c-myc (21, 22). Failure of C\(_{24}\)-Cer-1-P to induce intracellular Ca\(^{2+}\) mobilization has been confirmed recently in neutrophils (20). However, Gijbers et al. (24) and Hogback et al. (25) reported that C\(_{24}\)-Cer-1-P caused fast and transient intracellular rises in Ca\(^{2+}\) in both call pulmonary artery endothelial cells and thyroid FRTL-5 cells, respectively, an action that might be related to the mitogenic effect of C\(_{24}\)-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, phosphatidylserine, phosphatidylinositol, glycerol 3-phosphate, phenazine methosulfate, and SM (from bovine brain) were from Avanti Polar Lipids (Alabaster, AL). [\(3^2\)P]orthophosphate, and radiolabeled bovine SM (choline\(^{14}\)C)methyl) were from Mandel Scientific (Guelph, Ontario, Canada). Antibodies to caspase-3 proenzyme were from Stressgen (Victoria, British Columbia, Canada), and antibodies to active caspases 3 and 9 were supplied by BD-Pharmingen (Mississauga, Ontario, Canada). PD08059, I2994002, 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 CD1 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 \(\sim\)80% confluency 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 \(\mu\)Ci/ml [\(^3\)H]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\(_2\)PO\(_4\). Chloroform phases were dried down under \(N_2\) 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) -diethylther-acetic acid (60:40:1; v/v/v). The position of ceramides was identified after staining with I\(_2\) 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,
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 10⁴ 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 C₅-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, lysophosphatidic 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). After withdrawal of M-CSF for 30 h, 14 ± 2% of cells showed 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 lysophosphatidic 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).
In agreement with recent work (15, 26), M-CSF deprivation caused a 4.4 ± 0.9-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). Likewise, C2-Ceramide, but not its inactive analog, dihydro-C2-ceramide, blocked Cer-1-P-induced mac

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 mac

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Fig. 7. Cer-1-P inhibits A-SMase activation in cell homogenates. BMDMs were incubated without M-CSF for 30 h to stimulate A-SMase. Upper panel: cells were homogenized, and replicate aliquots were mixed with labeled sphingomyelin substrate together with the indicated concentrations of long-chain Cer-1-P (circles), C8-Cer-1-P (diamonds), C2-Cer-1-P (squares), or Sph-1-P (triangles). Results represent means ± SEM of three independent experiments. Lower panel: cells were treated as in the upper panel and incubated with 30 μM concentrations of Cer-1-P (C1P), phosphatidic acid (PA), lysophosphatic acid (LPA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), or glycerol-3-phosphate (G3P). Results represent means ± range of two independent experiments.

Fig. 8. Time-dependent decrease of Cer-1-P in BMDMs deprived of M-CSF. Cells were labeled for 24 h with 200 μCi/ml [32P]H3PO4. The label was then removed, and the cells were washed twice with RPMI. They were then incubated in the absence of M-CSF for the indicated times. Lipids were extracted and Cer-1-P was quantified as indicated in Materials and Methods. Cer-1-P levels are expressed as a percentage of total phospholipids. Data represent the means ± range of two different experiments performed in duplicate.

Fig. 9. C2-ceramide prevents Cer-1-P-mediated macrophage survival. BMDMs were seeded as indicated in Fig. 1 and then preincubated with 10 μM C2-ceramide (C2) or 10 μM dihydro-C2-ceramide (H2C) for 1 h before treatment with 30 μM long-chain Cer-1-P (CL-1-P). Macrophage viability was measured after 30 h by the MTS assay. Results are expressed relative to control cells at 0 h. Data represent means ± SEM of five independent experiments.
roperation (Fig. 9), thereby emphasizing the importance for cells to maintain an appropriate balance in the levels of intracellular ceramide and Cer-1-P.

**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- and C18-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 oviduct explants. In that study, C8-Cer-1-P also decreased cell death in the explants caused by serum withdrawal, suggesting that Cer-1-P might have cytoprotective or anti-apoptotic effects (31). The mechanism of action of Cer-1-P appears to differ from that of other phospholipid metabolites, such as Sph-1-P and lysophosphatidic acid, in that Cer-1-P does not modify the activity of MAPK (ERK1/2), PLD, or adenyl cyclase, which are all enzymes involved in the regulation of cell proliferation (10, 12, 13, 21, 22, 32, 33). In addition, we now show that Cer-1-P blocks DNA fragmentation and caspase activation in macrophages, suggesting that its prosurvival effect in these cells is due to inhibition of apoptosis.

In a previous study, we showed that apoptosis of BMDMs induced by M-CSF withdrawal involves stimulation of A-SMase activity and the accumulation of ceramides (15). Sphingomyelinase activation appears to play a significant role in apoptosis in these cells, because desipramine, a sphingomyelinase inhibitor, prevents ceramide accumulation and blocks apoptosis (26). More direct evidence that A-SMase activation and ceramide generation are essential for apoptosis after M-CSF withdrawal was obtained in BMDMs from A-SMase knockout mice (generously provided by Dr. Richard Kolesnick). Preliminary results with A-SMase−/− BMDMs indicate that these cells are strikingly resistant to apoptosis after M-CSF withdrawal (A. Gómez-Muñoz, unpublished observations).

In the present work, we demonstrate that Cer-1-P potently inhibits A-SMase activation in BMDMs, thereby preventing the accumulation of ceramides. This inhibitory effect of Cer-1-P probably involves a direct physical interaction with the enzyme, because Cer-1-P can also inhibit A-SMase activity in cell homogenates. This observation also suggests that the effect of Cer-1-P on intact cells is not mediated through receptor interaction. It is unlikely that Cer-1-P acts through metabolism to Sph-1-P, because there is little or no conversion of Cer-1-P to Sph-1-P (17, 21, 22, 34). Furthermore, we demonstrated that Sph-1-P does not inhibit A-SMase activity in cell homogenates, suggesting that it does not directly affect A-SMase in the manner that Cer-1-P does.

The physiological relevance of the prosurvival effect of Cer-1-P is underscored by our finding that the intracellular levels of Cer-1-P are substantially decreased in the macrophages after M-CSF withdrawal. This could release A-SMase from inhibition, thereby triggering ceramide generation and apoptotic cell death.

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