Sphingosine-1-phosphate inhibits acid sphingomyelinase and blocks apoptosis in macrophages

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Abstract Sphingosine-1-phosphate (Sph-1-P) regulates critical cellular functions including cell proliferation, differentiation, angiogenesis, and cell survival. However, its mechanisms of action are incompletely understood. Here, we show a novel biological effect of Sph-1-P: inhibition of acidic sphingomyelinase (A-SMase) activity in apoptotic bone marrow-derived macrophages. A-SMase catalyzes the conversion of sphingomyelin to ceramides, which are pro-apoptotic. This action of Sph-1-P prevents the accumulation of ceramides and blocks apoptosis, thereby promoting survival of the macrophages.

Key words: Sphingosine-1-phosphate; Sphingomyelinase; Ceramide; Apoptosis; Protein kinase B; Macrophage

1. Introduction

Sphingosine-1-phosphate (Sph-1-P) is a bioactive lipid that controls important biological functions. It was first described as a mitogen for cultured fibroblasts [1] and subsequently has been implicated in the regulation of other cellular processes including cell differentiation, migration, angiogenesis and cell survival [2,3]. There is evidence of dual messenger functions for Sph-1-P. First, Sph-1-P can be released into the blood stream upon platelet or mast cell activation, thereupon interacting with specific G protein-coupled receptors of the endothelial differentiation gene family. This action has been associated mainly with the regulation of cell migration, neovessel formation, neurite retraction, or cell survival [2–4]. Second, Sph-1-P has been shown to act intracellularly as a second messenger to regulate cell proliferation, suppression of apoptosis, and also cell survival [2,3]. In this regard, activation of sphingosine kinase and enhanced formation of Sph-1-P was shown to be induced by platelet-derived growth factor, but not by epidermal growth factor, leading to activation of the mitogen-activated protein kinase (MAPK) extracellular regulated kinase 1/2 (ERK1/2) [5]. In addition, Sph-1-P inhibits c-Jun N-terminal kinase (JNK) activation [6], which is relevant because the balance between ERK and JNK activation has been implicated in the control of apoptosis [7]. Furthermore, Sph-1-P counteracts the effects of ceramide, which is a sphingolipid metabolite that inhibits cell growth and induces apoptosis potently in different cell types [6,8]. The biological actions elicited by Sph-1-P involve activation of diverse signaling pathways. For example, effects of Sph-1-P on cytoskeletal rearrangement and cell motility are mediated by the small GTPases Rac and Rho, whereas stimulation of cell proliferation and cell survival by Sph-1-P involves intracellular Ca^{2+} mobilization and activation of MAPK, phospholipase D, protein kinase B (PKB), and transcription factors such as AP-1 [2,3].

We recently found evidence of sphingomyelinase (SMase) activation and ceramide generation in bone marrow-derived macrophages (BMDM) induced to undergo apoptosis by growth factor withdrawal (unpublished work). In the present study, we present the novel observation that Sph-1-P is a potent inhibitor of SMase activity and ceramide formation thereby promoting macrophage survival.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, lysophosphatidic acid (LPA), phenazine methosulfate, and sphingomyelin (from bovine brain) were from Sigma/Aldrich Canada (Oakville, ON, Canada). Defined fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Fisher Scientific (Edmonton, AB, Canada) supplied 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS). C_2-ceramide, dihydroC_2-ceramide, sphingosine and Sph-1-P were from Avanti Polar Lipids (Alabaster, AL, USA). [^{3}H]Palmitate and radiolabelled bovine sphingomyelin (choline-[^{14}C]methyl) were from Mandel Scientific (Guelph, ON, Canada). ERK1/2 (Thr-202/Tyr-204) and phospho-PKB antibodies were purchased from New England BioLabs (Beverly, MA, USA). Antibodies to PKB and caspase-3 proenzyme were from Stressgen (Victoria, BC, Canada). Antibodies to Bcl-X_L and active caspase-3 were supplied by BD-Pharmingen (Mississauga, ON, Canada). PD98059, LY294002, and wortmannin were from Calbiochem products supplied by VWR Canlab (Mississauga, ON, Canada).

2.2. Cell culture

Bone marrow macrophages were isolated from femurs of 6–8-week-old female CD-1 mice as described [9]. 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) [10]. The non-adherent cells were removed and cultured in the above medium until about 80% confluence was reached (5–7 days), for use in the experiments.

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Abbreviations: BMDM, bone marrow-derived macrophages; FBS, fetal bovine serum; LPA, lysophosphatidic acid; M-CSF, macrophage colony stimulating factor; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MEK, MAPK/ERK kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; Sph-1-P, sphingosine-1-phosphate

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2.3. 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 with 10% FBS 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 [10].

2.4. Ceramide determination

Radioactivity in ceramide was determined after labeling BMDM with 5 μCi/ml of [^{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 [8,11]. The radioactive medium was aspirated, and cells washed twice with non-radioactive RPMI 1640 containing 10% FBS, but without M-CSF. The macrophages were then incubated in this same medium in the absence or in the presence of agonist, as required. Cells were then washed twice with ice-cold calcium-free phosphate-buffered saline and scraped into 0.5 ml of methanol. The cells were washed with a further 0.5 ml of methanol, and the two methanol samples were combined and mixed with 0.5 ml of chloroform. Lipids were extracted by separation of phases with a further 0.5 ml of chloroform and 0.9 ml of a solution containing 2 M KCl and 0.2 M H_3PO_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) and then dried. They were then developed for their full length with petroleum ether, boiling point 40–60°C/diethylether/acetic acid (60:40:1, 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.

2.5. Measurement of DNA fragmentation

DNA fragmentation was determined using flow cytometry, as described [10].

2.6. SMase assay

The activities of acidic and neutral SMases (A-SMase and N-SMase, respectively) were determined as described by Liu and Hannun [12] using (choline-[^{14}C]methyl) sphingomyelin as the substrate. SMase activity in permeabilized macrophages was determined in a similar manner, but in the presence of 20 μg/ml digitonin [11].

2.7. Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [10]. 40–50 μg of protein 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% NaN_3 and 0.1% Tween 20, and then incubated overnight with the primary antibody in TBS/0.1% Tween 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, ON, Canada).

2.8. Statistical analysis

Results are expressed as means ± S.E.M. of three independent experiments performed in triplicate, unless indicated otherwise. Statistical analysis was done using ANOVA or Student's *t*-test as appropriate, with level of significance set at $P < 0.05$.

3. Results and discussion

It is generally believed that removal of growth factors from hemopoietic cells leads to cell death via apoptosis ([13] and references therein). One well-established experimental model is BMDM, which typically undergo apoptosis within 24–48 h of M-CSF withdrawal [10,13]. We chose this model to evaluate the effects of Sph-1-P on macrophage survival. As shown in Fig. 1 (upper panel), Sph-1-P significantly increased macrophage viability after M-CSF withdrawal whereas sphingosine or the structurally related lysophospholipid LPA had no effect. To evaluate whether the pro-survival effect of Sph-1-P was due to inhibition of apoptosis or to effects on primary or secondary necrosis (which also occur under these conditions), cells were analyzed for DNA fragmentation and caspase-3 activation. After withdrawal of M-CSF for 24 h, 23.7 ± 5.1% of cells had DNA fragmentation by flow cytometry (mean ± S.E.M. of four independent experiments), and this was decreased to 7.1 ± 3.1% by 30 μM Sph-1-P ($P < 0.05$). By contrast, LPA or sphingosine, at similar concentration, were ineffective. In agreement with other work [14,15], Sph-1-P also prevented the activation of caspase-3, which is an effector protein of apoptosis (Fig. 1, lower panel). Dying cells often show increased concentrations of ceramides, which are pro-apoptotic [16,17]. We observed that M-CSF deprivation caused a marked increase of ceramide levels in macrophages, and this was inhibited by 30 μM Sph-1-P (Fig. 2, upper panel). Therefore, we hypothesized that Sph-1-P might block cell

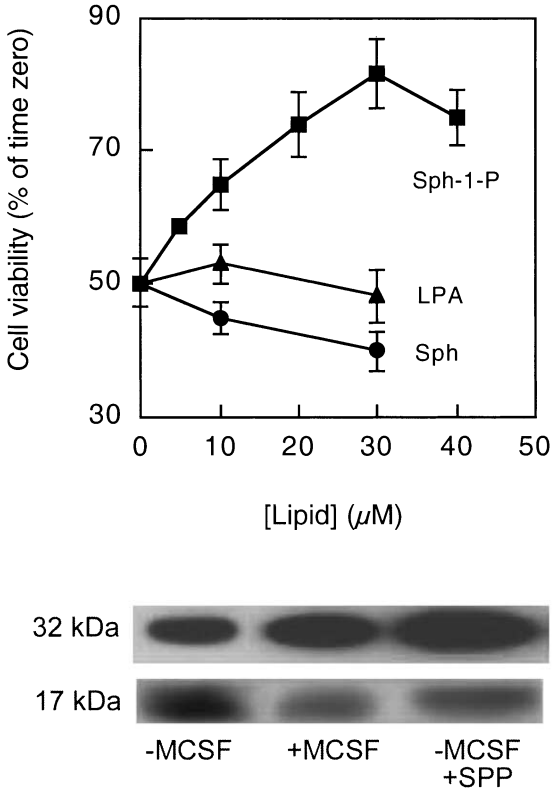

Fig. 1. Sph-1-P promotes macrophage survival. Upper panel: BMDM were seeded at 25×10^3 cells/well in 96-well plates and incubated in RPMI 1640 with 10% FBS, but without M-CSF, for 30 h in the absence or in the presence of increasing concentrations of Sph-1-P (squares), sphingosine (Sph, circles), or LPA (triangles). Cell viability was determined by the MTS assay as described in Section 2. Results are expressed relative to control cells at 0 h. Data represent means ± S.E.M. of four different experiments performed in quadruplicate. Lower panel: BMDM were seeded at 5×10^6 cells/100-mm dish and incubated in RPMI 1640 with 10% FBS, but with or without 10% L-cell conditioned medium (M-CSF) or Sph-1-P (30 μM) for 30 h, as indicated. The levels of the 32-kDa pro-enzyme of caspase-3 as well as the 17-kDa active fragment were assessed by immunoblotting as described in Section 2. Results shown are representative of three independent experiments.

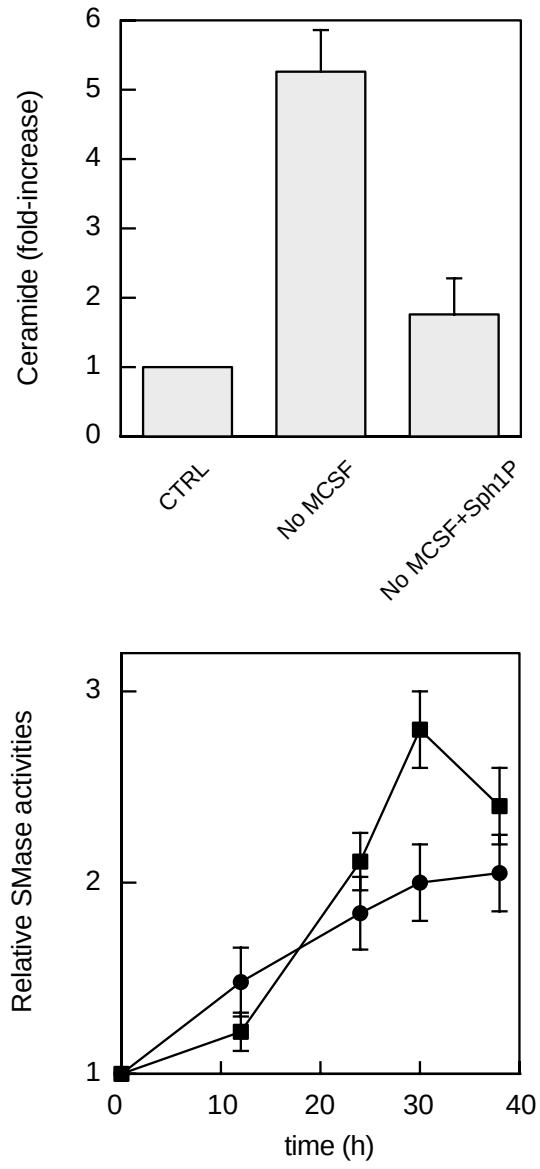

Fig. 2. M-CSF withdrawal increases ceramide levels through SMase activation. Upper panel: BMDM were seeded at 1×10^6 cells/well in 6-well plates and labeled with 5 μCi/ml of [^{3}H]palmitate as indicated in Section 2. Cells were then washed to remove unincorporated label and incubated for 38 h with or without 30 μM Sph-1-P, as indicated. Ceramide was isolated by thin-layer chromatography and quantitated as described in Section 2. Values are calculated as a percentage of the radioactivity present in [^{3}H]ceramide compared with that in total lipids, and then expressed as the fold stimulation relative to incubations at 0 h. Results are the mean ± S.E.M. of three independent experiments performed in triplicate. Lower panel: BMDM were seeded at 1×10^6 cells/well in 6-well plates and incubated in RPMI 1640 with 10% FBS for 0–38 h. Acid (squares) and neutral (circles) SMase activities were determined as indicated in Section 2. The specific activities of basal A-SMase and N-SMase are given in the text (Section 3). Results are expressed relative to control values at 0 h. Data represent means ± S.E.M. of three different experiments performed in duplicate.

death by inhibiting ceramide production. Although ceramides can be formed by de novo synthesis, one of the major pathways for generation of bioactive ceramide is the SMase pathway [16,17]. Thus, experiments were carried out to determine whether SMase activation was the cause for the production of ceramides. This was achieved by measuring A- and N-SMase activities after M-CSF withdrawal. We found that both A- and N-SMase activities were stimulated by M-CSF deprivation in a time-dependent manner, and that nearly all of the SMase activity (98%) was attributable to the acidic form of the enzyme. The extent of SMase activation varied among different experiments, but there was consistency in cell responsiveness for the macrophages of individual preparations. The specific activities of basal A-SMase and N-SMase were 39.8 ± 4.3 (mean ± S.E.M., $n=12$) and 0.9 ± 0.1 (mean ± S.E.M., $n=8$) nmol of substrate converted per hour per milligram of protein, respectively. Maximal activation of both A- and N-SMases was attained after about 30 h of M-CSF withdrawal (Fig. 2, lower panel). The N-SMase activity was totally dependent upon Mg^{2+} ions. Fig. 3 presents the key observation that Sph-1-P inhibited the activation of A-SMase in intact cells, thereby blocking the accumulation of ceramides. Sph-1-P also decreased the activation of N-SMase by about 28 ± 5% (mean ± range of two independent experiments). In-

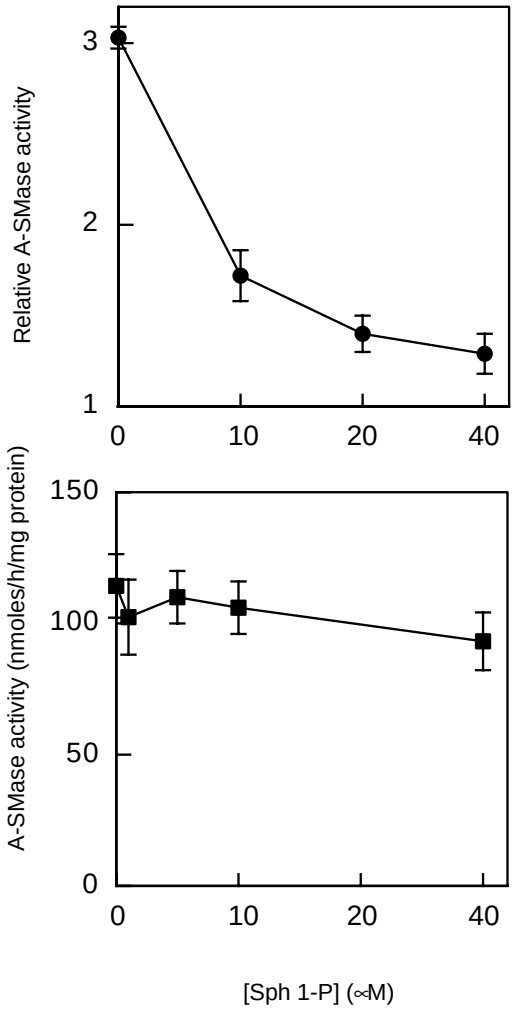

Fig. 3. Sph-1-P inhibits A-SMase activation in intact BMDM but not in cell homogenates. Intact cells (upper panel) were treated as in Fig. 2 and incubated for 30 h with increasing concentrations of Sph-1-P, as indicated. Results are calculated relative to control cells at 0 h and then expressed as mean ± S.E.M. of three independent experiments. Homogenates from cells incubated without M-CSF for 30 h to stimulate A-SMase (lower panel) were treated with increasing concentrations of Sph-1-P, as indicated. Results represent means ± S.E.M. of three independent experiments.

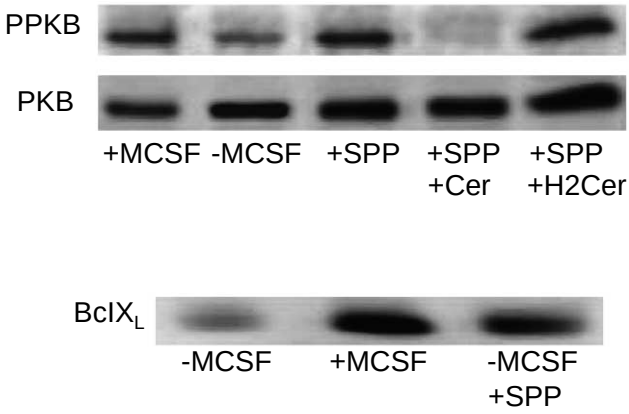

Fig. 4. Sph-1-P-mediated macrophage survival involves maintenance of PKB phosphorylation and increases Bcl-X_L levels. BMDM were seeded as indicated in Fig. 2. In the upper panel, cells in the first lane were incubated for 30 h with: 10% L-cell conditioned medium as source of M-CSF; all other lanes were without M-CSF with or without 30 μM Sph-1-P (SPP); 30 μM Sph-1-P plus 50 μM C_2-ceramide (SPP+Cer); or 30 μM Sph-1-P plus 50 μM dihydro-C_2-ceramide (SPP+H2Cer). Phospho-PKB (PPKB) and PKB levels were assessed by immunoblotting as described in Section 2. In the lower panel, cells were incubated without M-CSF, with 10% L-conditioned medium (M-CSF), or with 30 μM Sph-1-P (SPP) in the absence of M-CSF. Bcl-X_L levels were assessed by immunoblotting as described in Section 2. Similar results were obtained in each of two replicate experiments.

terestingly, the inhibition of A-SMase activity required cell integrity as Sph-1-P failed to inhibit SMase activation in cell homogenates (Fig. 3, lower panel). This observation suggests that the mechanism of A-SMase inhibition does not involve a direct physical interaction of Sph-1-P with the enzyme.

Although Sph-1-P can act intracellularly to modify signal transduction pathways, many of its biological effects when added exogenously are caused by binding to cell surface receptors that are coupled to pertussis toxin (PTX)-sensitive Gi/o proteins [3,18]. To determine if the inhibition of A-SMase by Sph-1-P was mediated by a receptor of this kind, BMDM were incubated for 30 h in the presence of PTX concentrations ranging from 10 to 100 ng/ml. This treatment failed to reverse the inhibitory effect of Sph-1-P on A-SMase activation. We noted that at concentrations greater than 100 ng/ml, PTX alone caused inhibition of A-SMase activity by an undetermined mechanism. Therefore, these were not used in the experiments. The possible implication of GTP-binding proteins in A-SMase activation was investigated further by treating permeabilized macrophages with GTPγS or GDPβS, which are non-hydrolyzable analogues of GTP and GDP, respectively. Incubation of permeabilized BMDM with concentrations of these nucleotides of up to 150 μM for various times (up to 60 min) did not alter A-SMase activity (data not shown). However, 25 μM GTPγS stimulated phospholipase D in rat fibroblasts under similar conditions [11]. Taken together, these results indicate that A-SMase activation by M-CSF withdrawal and the inhibitory effect of Sph-1-P on A-SMase activation are independent of GTP-binding proteins. In addition, A-SMase activation was independent of cAMP, as treatment with the adenylyl cyclase-activating agent forskolin, at concentrations shown to elevate cAMP, did not alter A-SMase activity in the macrophages (data not shown). These data are in agreement with previous studies suggesting that the cytoprotective effects of Sph-1-P are receptor-independent events [3].

It has been shown that Sph-1-P can activate ERK1/ERK2 or phosphatidylinositol 3-kinase (PI3-K)/PKB in different cell types [19–21]. In a previous study, we demonstrated that activation of the PI3-K/PKB pathway but not MAPK was required for the anti-apoptotic effect of oxidized low density lipoprotein in BMDM [10]. In the present work we found that Sph-1-P preserves PKB phosphorylation after M-CSF withdrawal (Fig. 4, upper panel). In addition, expression of anti-apoptotic Bcl-X_L, which is regulated by PI3-K, was also decreased by M-CSF withdrawal, and Sph-1-P partially restores this (Fig. 4, lower panel). To determine whether the PI3-K pathway was involved in the attenuation of apoptosis

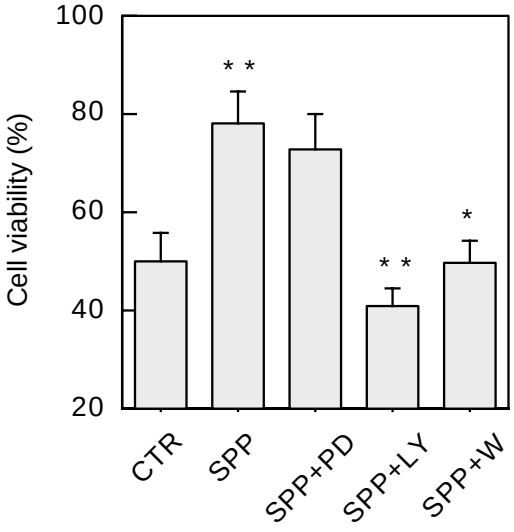

Fig. 5. PI3-K inhibitors block Sph-1-P-mediated macrophage survival. BMDM were seeded as indicated in Fig. 1. They were then pre-incubated with vehicle, or with the MEK inhibitor PD98059 (PD) at 10 μM, or with the PI3-K inhibitors LY294002 (LY) at 5 μM and wortmannin (W) at 100 nM for 1 h before treatment with 30 μM Sph-1-P (SPP), as indicated. CTR indicates conditions in the absence of agonist or inhibitors. Macrophage viability was measured after 30 h by the MTS assay. Results are expressed relative to control cells at 0 h. Data represent means ± S.E.M. of four independent experiments performed in quadruplicate (**$P < 0.01$, CTR vs. SPP value, and SPP value vs. SPP+LY value; *$P < 0.05$, SPP value vs. SPP+W value).

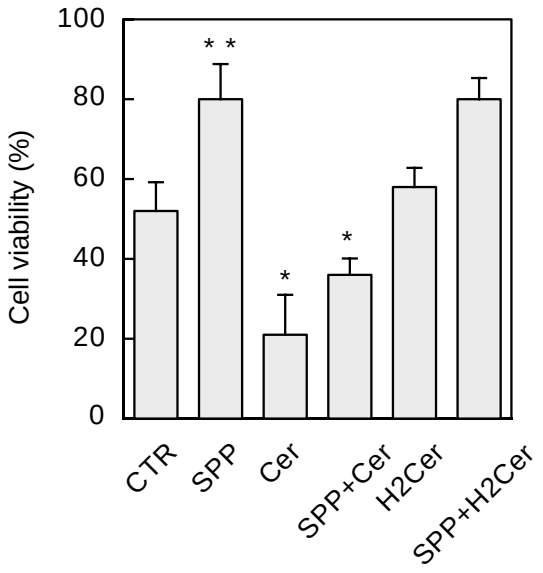

Fig. 6. C_2-ceramide prevents Sph-1-P-mediated macrophage survival. BMDM were seeded as indicated in Fig. 1. They were then preincubated with 50 μM C_2-ceramide (Cer) or 50 μM dihydro-C_2-ceramide (H2Cer) for 1 h before treatment with 30 μM Sph-1-P (SPP), as indicated. CTR indicates conditions in the absence of any addition. Macrophage viability was measured after 30 h by the MTS assay. Results are expressed relative to control cells at 0 h. Data represent means ± S.E.M. of three independent experiments performed in quadruplicate (**$P < 0.01$, CTR vs. SPP value; *$P < 0.05$, CTR vs. Cer value, and SPP value vs. SPP+Cer value).

by Sph-1-P, we tested the effects of selective inhibitors on cell survival in the presence of Sph-1-P. As shown in Fig. 5, the PI3-K inhibitors LY294002 and wortmannin blocked the effect of Sph-1-P on macrophage survival, whereas the MAPK/ERK kinase (MEK) inhibitor PD98059 did not, even though it blocked ERK1/ERK2 phosphorylation by immunoblot analysis. This finding is similar to our previous study of the anti-apoptotic effect of oxidized low density lipoprotein in BMDM, which was also blocked by PI3-K inhibitors but not MEK inhibitors [10]. These results suggest that the PI3-K/PKB signaling cascade, but not ERK1/ERK2, is involved in Sph-1-P-mediated macrophage survival. In agreement with other work [22,23] PKB phosphorylation was inhibited by the cell-permeable C_2-ceramide (*N*-acetylsphingosine), but not by its biologically inactive analogue *N*-dihydro-C_2-ceramide (Fig. 4, upper panel). Therefore, the inhibition of SMase activity and the subsequent decrease in ceramide levels by Sph-1-P could be important for maintaining PKB activation and macrophage survival. In this regard, we found that C_2-ceramide, but not dihydro-C_2-ceramide, blocked Sph-1-P-induced macrophage survival (Fig. 6), thereby emphasizing the importance for cells to maintain an appropriate balance in the levels of intracellular Sph-1-P and ceramides. These data are in agreement with our previous work showing that Sph-1-P and ceramides are antagonistic signals [8], and with that of Cuvillier et al. [6] who demonstrated that Sph-1-P blocks ceramide-induced apoptosis.

In conclusion, here we demonstrate for the first time that Sph-1-P inhibits SMase activity and the subsequent generation of ceramides, thereby promoting cell survival.

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