

Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway

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Abstract In this report, we show for the first time that ceramide-1-phosphate (C1P) stimulates the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) pathway, which is a major mechanism whereby growth factors promote cell survival. Also, C1P induced IκB phosphorylation, and enhanced the DNA binding activity of the transcription factor NF-κB. Apoptotic macrophages showed a marked reduction of Bcl-X_L levels, and this was prevented by C1P. These findings suggest that C1P blocks apoptosis, at least in part, by stimulating the PI3-K/PKB/NF-κB pathway and maintaining the production of antiapoptotic Bcl-X_L. Based on these and our previous observations, we propose a working model for C1P in which inhibition of acid sphingomyelinase and the subsequent decrease in ceramide levels would allow cell signaling through stimulation of the PI3-K/PKB pathway to promote cell survival.

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1. Introduction

Ceramide-1-phosphate (C1P) is emerging as a new bioactive molecule [1] capable of regulating vital pathophysiological functions including cell proliferation [2,3], apoptosis [4], phagocytosis [5], and inflammation [6,7]. Although the existence of C1P and its metabolizing enzymes, ceramide kinase and C1P phosphatase, in mammalian tissues have been known

for over a decade [8–10], current understanding of the metabolic and signaling pathways that are affected by this bioactive sphingolipid is incomplete. We first reported that synthetic short-chain acetyl- (C₂) and octanoyl (C₈)-C1P [2], or natural long-chain C1P [3] stimulated DNA synthesis and cell division in rat or mouse fibroblasts. These effects were accompanied by increases in the levels of proliferating cell nuclear antigen. Synthetic C₈-C1P was also able to stimulate the growth of chick otic vesicle explants that were maintained in culture [11]. Nonetheless, unlike other mitogenic phospholipids such as sphingosine-1-phosphate (S1P), lysophosphatidic acid, or phosphatidic acid, in fibroblasts C1P does not induce Ca²⁺ mobilization, does not affect the activity of extracellular regulated kinase 1-2 (ERK1-2) or phospholipase D (PLD), and does not induce the expression of the early genes *c-fos* or *c-myc* [2,3]. C1Ps also failed to induce Ca²⁺ mobilization in neutrophils [12], and A549 cells [7]. However, C₂-C1P has been reported to induce Ca²⁺ mobilization in calf pulmonary artery endothelial cells [13], thyroid FTRL-5 cells [14], and GH4C1 pituitary cells [15], and induction of ERK2 phosphorylation has been observed in osteoblasts [16]. The reason for these discrepancies is unknown at present, but it may be that the effects of C1P are cell type specific.

Two major findings regarding the biological actions of C1P have recently been reported. First, we demonstrated that natural C1P blocks apoptosis of primary bone marrow-derived macrophages (BMDM) incubated in the absence of growth factors. This action involved direct inhibition of acid sphingomyelinase (A-SMase) and blockade of the caspase-9/caspase-3 pathway [4]. Second, Pettus and co-workers [7] have shown that C1P can stimulate calcium-dependent cytosolic phospholipase A₂ (cPLA₂) thereby leading to arachidonic acid release and generation of eicosanoids in lung carcinoma A549 cells. These two reports are the first to demonstrate that A-SMase and cPLA₂ are direct intracellular targets of C1P, and place C1P as a central molecule in the regulation of apoptosis and inflammatory responses. A major mechanism whereby growth factors promote cell survival is the activation of the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) (Akt) pathway [17]. Activation of PI3-K leads to phosphatidylinositol (3,4,5) trisphosphate (PI (3,4,5) P3) formation, and this results in the recruitment of pleckstrin homology domain containing proteins such as PKB. Recruitment of PKB to the cell membrane results in its phosphorylation and activation, and this allows it to phosphorylate downstream effectors.

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Abbreviations: A-SMase, acid sphingomyelinase; BMDM, bone marrow-derived macrophages; C1P, ceramide-1-phosphate; C₂-, acetyl; C₈-, octanoyl; CAPE, caffeic acid phenylethyl ester; EMSA, electromobility shift assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 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)-2H-tetrazolium, inner salt]; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; PI (3,4,5) P3, phosphatidylinositol (3,4,5) trisphosphate; PKB, protein kinase B; PLD, phospholipase D; cPLA₂, calcium-dependent cytosolic phospholipase A₂; PMS, phenazine methosulfate; PTX, pertussis toxin; S1P, sphingosine-1-phosphate; TBS, Tris-buffered saline; TLC, thin-layer chromatography

A target of PKB that is known to regulate apoptosis is the transcription factor NF- κ B. The latter is an inducible heterodimeric factor responsible for the transcription of many important antiapoptotic molecules including Bcl-X_L. The p65 subunit of NF- κ B is normally sequestered in the cytoplasm by I κ B; upon cell stimulation, I κ B is activated causing phosphorylation of I κ B at serines 32 and 36. Phosphorylated I κ B dissociates from p65 and is targeted for ubiquitination and degradation by the 26S proteasome pathway. The p65 subunit can then dimerize with p50, translocate to the nucleus, and promote the transcription of NF- κ B dependent target genes. Activation of this pathway is sufficient to prevent death from a variety of apoptotic stimuli including tumor necrosis factor, UV, Trail, and Fas. Aberrant activation of this pathway is also commonly seen in cancer cells [18].

We demonstrate here that C1P stimulates PI3-K activity leading to PKB phosphorylation, activation of NF- κ B, upregulation of Bcl-X_L, and inhibition of apoptosis in BMDM. These findings, together with our prior observations that C1P inhibits A-SMase and the subsequent generation of ceramides and caspase 9/caspase 3 activation indicate that C1P is an important regulator of cell survival.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, C1P (from bovine brain, containing predominantly stearic and nervonic acids), PI, PI (3,4,5) P3, phenazine methosulfate (PMS), and ceramide were from Sigma/Aldrich Canada (Oakville, ON). Defined fetal bovine serum (FBS) was from Hyclone (Logan, UT). Fisher Scientific (Edmonton, AB) supplied 3-(4,5-dimethylthiazol-2-yl) 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Antibody to the p85 subunit of PI3-K was from Upstate Biotechnology, Inc. (Lake Placid, NY). [³²P]orthophosphate was from ICN (Costa Mesa, CA). [γ -³²P]ATP (6000 Ci/mmol) was from Dupont-NEN Research Products (Boston, MA). Antibodies to ERK1-2, phospho-ERK1-2, phospho-I κ B, PKB, phospho-PKB (Ser 473), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technologies (Mississauga, ON), and antibodies to Bcl-X_L were supplied by BDPharmingen (Mississauga, ON). Goat anti-rabbit IgG and goat anti-mouse IgG, horseradish peroxidase secondary anti-bodies, PD98059, UO126, LY294002, wortmannin, SC-514, and caffeic acid phenyl ethyl ester (CAPE) were from Calbiochem supplied by VWR Canlab (Mississauga, ON).

2.2. Cell culture

BMDMs were isolated from femurs of 6- to 8-week-old female CD-1 mice as described [19]. Cells were plated in RPMI 1640 medium containing 10% FBS and 10% L-cell conditioned medium as the source of macrophage colony stimulating factor (M-CSF) [19]. After 24 h, non-adherent cells were harvested and cultured in the above medium until about 80% confluence was reached (4–6 days) prior to use in the experiments.

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 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 [19].

2.4. Detection and quantitation of intracellular PI (3,4,5) P3

Radioactivity in PI (3,4,5) P3 was determined essentially as described [20] after labeling BMDM with 200 μ Ci/ml of [³²P]orthophosphate overnight in phosphate-free RPMI 1640 with 10% FBS

and 10% L-cell conditioned medium as the source for M-CSF [19]. Cells were then washed twice with RPMI 1640 without label or M-CSF. The macrophages were stimulated with C1P for different times. Cells were then washed twice with ice-cold calcium-free phosphate buffered saline and scraped into 0.5 ml of methanol. They were then 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 H₃PO₄. Chloroform phases were dried down under N₂ and lipids were separated by thin-layer chromatography (TLC) using oxalate-treated silica Gel 60-coated glass plates. The plates were developed to the top in chloroform/acetone/methanol/acetic acid/water (80:30:26:24:14, by vol) [21], then dried and exposed to autoradiography film. The spot corresponding to ³²P-labeled PI (3,4,5) P3 in extracts was located by co-migration with a standard produced by *in vitro* phosphorylation of PI (4,5) P2 by PI3-K [20]. The PI (3,4,5) P3 spots were scraped from the TLC plate and quantified by liquid scintillation counting. Values were normalized to total ³²P dpm in the extract.

2.5. *In vitro* determination of PI3-K activity

The activity of PI3-K was determined in immunoprecipitates essentially as described previously [22]. Briefly, the cells were lysed in solubilization buffer (50 mM Tris, pH 7.7, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 40 μ g/ml PMSF, 1 μ M pepstatin, 15 μ g/ml leupeptin, 0.2 μ M NaVO₄) and 360 μ g of whole cell lysate of each sample was incubated with 7.2 μ l of anti-PI3-K p85 monoclonal antibody (Upstate Biotechnology) overnight at 4 °C. Samples were immunoprecipitated with 100 μ l of Protein G Sepharose for 2 h at 4 °C. The beads were washed twice with solubilization buffer, three times with 10 mM Tris, pH 7.4, in 0.1 mM NaVO₄, and then twice with the assay dilution buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 mM NaVO₄). To each sample, 10 μ g of PI as substrate, 5 μ l of 100 mM MgCl₂, 4 mM ATP, and 10 μ Ci of [γ -³²P]ATP were added and then incubated at 37 °C for 15 min. Reactions were stopped with 100 μ l of 1 M HCl and then 200 μ l of chloroform/methanol (1:1, v/v). Phases were separated and the lower phase was retained for lipid analysis. Lipids were separated by TLC in oxalate-treated silica gel 60 plates that were run in chloroform/methanol/water/28% NaOH (90:70:15:5, by vol). The plates were stained with iodine and the PI3-P spots were scraped from the TLC plate and quantitated by liquid scintillation counting.

2.6. Nuclear preparations and electromobility shift assay

Cells were grown in 100 mm tissue culture plates. Isolation of nuclei and radioactive labeling of electromobility shift assay (EMSA) NF- κ B probe was performed as previously described [23]. Nuclear extracts (10 μ g) were preincubated for 15 min in binding buffer (20 mM HEPES, pH 7–9, 100 mM KCl, 10% glycerol, 1 mM dithiothreitol) containing 1 μ g of poly(dIdC) (Amersham). The probe (20 000 counts/min) was then added and the reaction mixture incubated at room temperature for 30 min before electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.25 \times TBE Tris 89 mM, boric acid 89 mM, EDTA 2 mM (TBE) at 200 V for 1.5 h. The gel was subsequently dried for 45 min and imaged using a Bio-Rad FX phosphor-imager.

2.7. Western blotting

Macrophages were harvested and lysed in ice-cold homogenization buffer as described [19]. Aliquots of protein (40–50 μ g) from each sample were loaded and separated by SDS-PAGE, using 10% or 15% separating gels. Proteins were transferred onto 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, ON).

2.8. Statistical analysis

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

3. Results

3.1. C1P stimulates the PI3-K/PKB pathway

We and others have previously shown that BMDM undergo apoptosis when they are incubated in the absence of M-CSF [4,19,24,25]. We have also reported that M-CSF withdrawal causes stimulation of A-SMase activity in BMDM, and have suggested that this is one of the mechanisms whereby these cells become apoptotic [4,19,25]. In addition, we recently found that C1P blocks apoptosis in BMDM at least in part through a mechanism involving inhibition of A-SMase and, as a consequence, a decrease of ceramide levels [4]. However, phosphatidic acid (PA), which is a glycerolipid analogue of C1P [2], and lyso-PA failed to inhibit apoptosis in these cells ([25], and data not shown). The present studies were done to determine whether the antiapoptotic effect of C1P also involves stimulation of the PI3-K/PKB pathway, which plays a central role in the regulation of cell survival and proliferation. To test for PI3-K activation, BMDM were incubated for varying times with C1P and then PI3-K activity was immunoprecipitated from whole cell lysates, and assayed *in vitro* using ^{32}P -labeled PI. As shown in Fig. 1 (upper panel), C1P stimulated the formation of ^{32}P -PI (3) P in a time-dependent manner. The activity of PI3-K was also determined using an *in vivo* approach, by measuring the formation of PI (3,4,5) P₃ in live cells that were pre-labeled overnight with ^{32}P orthophosphate. Fig. 1 (lower panel) shows that C1P increased the formation of PI (3,4,5) P₃ in intact cells. In these experiments, an aqueous dispersion of C1P was prepared by sonication, and the optimal concentration of C1P was 30 μM . When C1P is dissolved in a mixture of 49:1 methanol/dodecane or ethanol/dodecane, dispersion is improved and effects on cell proliferation in fibroblasts or arachidonic acid release in A549 cells have been reported at concentrations as low as 2.5 μM [3,6,7]. In the present work, we found that although 2.5 μM C1P was effective in promoting macrophage survival when added in methanol/dodecane, the solvent mixture alone caused an increase of about 18% on macrophage survival, and therefore it was decided not to use methanol/dodecane in subsequent experiments.

A downstream target of PI3-K that is involved in promoting cell survival is PKB. We have previously demonstrated that stimulation of PKB is critical for macrophage survival [19,25], and this is also the case for many other cell types [26]. Therefore, we tested to see whether PI3-K activation by C1P could lead to phosphorylation of PKB. Immunoblot analysis using a specific antibody against the Ser 473 phosphorylation site of PKB demonstrated that C1P was able to induce rapid phosphorylation of this kinase (Fig. 2), and this occurred within the same time frame of PI3-K activation and PI (3,4,5) P₃ formation (Fig. 1). The mitogen-activated protein kinases (MAPKs), ERK1-2 have also been reported to play a central role in cell survival [27–29]. In particular, ERK2 has been shown to be phosphorylated by stimulation of osteoblastic

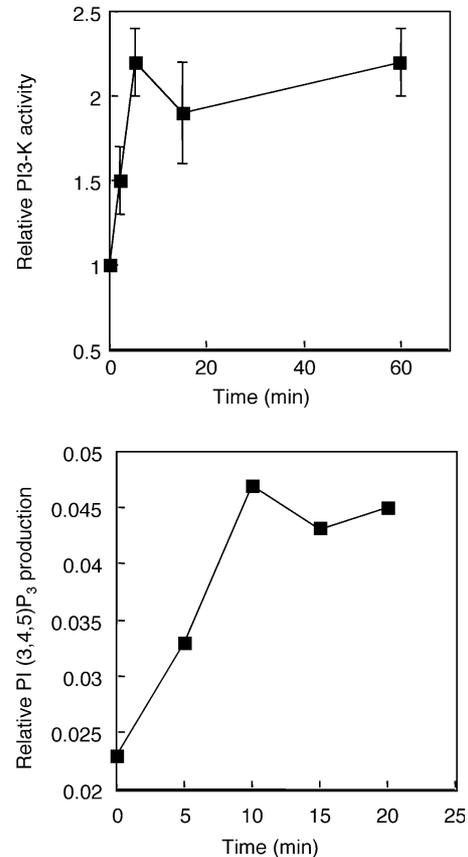


Fig. 1. Ceramide-1-phosphate stimulates PI3-K activity. Upper panel: BMDM were treated as indicated in Section 2, and incubated with C1P (30 μM) at various times, as indicated. Whole cell lysates were subjected to immunoprecipitation with anti-PI3-K p85 monoclonal antibody. PI3-K activity was assayed in the immunoprecipitates using PI as the substrate. ^{32}P -labeled PI-3-P from TLC plates was excised and quantitated in scintillation counter. Results are expressed as the fold stimulation relative to incubations in the absence of C1P, and they are the means \pm S.E.M. of three independent experiments. Lower panel: ^{32}P -labeled BMDM were stimulated for various times with C1P (30 μM). PI (3,4,5) P₃ in BMDM was located using an authentic standard that was synthesized enzymatically [20]. The PI (3,4,5) P₃ spot was excised and ^{32}P quantitated by scintillation counting. The results were calculated as a percentage of the radioactivity present in ^{32}P -PI (3,4,5) P₃ compared to that in total ^{32}P -labeled lipids. Results are the mean of two independent experiments. The values for each independent time point were: 0.022/0.024, 0.029/0.037, 0.042/0.052, 0.038/0.048, 0.040/0.050, for 0, 5, 10, 15, and 20 min of incubation with C1P, respectively.

cells with short-chain C₈-C1P [16]. However, we found that C1P failed to induce phosphorylation of ERK1-2 in BMDM (not shown), which is in agreement with our previous work in rat or mouse fibroblasts [2,3].

3.2. Involvement of the PI3-K/PKB pathway in the antiapoptotic effect of C1P

To evaluate whether the PI3-K pathway was involved in the inhibition of apoptosis by C1P, we tested the effects of selective inhibitors on cell survival in the presence of C1P. As shown in Fig. 3 (upper panel), the PI3-K inhibitors LY294002 (5 μM) and wortmannin (100 nM) blocked the effect of C1P on macrophage survival, whereas the MAPK/ERK kinase (MEK) inhibitors PD98059 (10 μM) and UO126 (2 μM) did not, even though both of these inhibitors blocked ERK1/

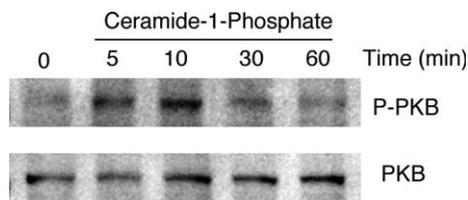


Fig. 2. Ceramide-1-phosphate induces PKB phosphorylation. BMDM were seeded at 5×10^6 cells/100 mm dish and preincubated in RPMI 1640 without M-CSF and serum for 4 h. C1P (30 μ M) was then added to the cells for various times, as indicated. Phosphorylation of PKB was examined by immunoblotting as described in Section 2 with antibodies specific to PKB phospho-Ser 473 and total PKB. Similar results were obtained in each of two replicate experiments. PKB, total protein kinase B protein; P-PKB, phospho-PKB protein.

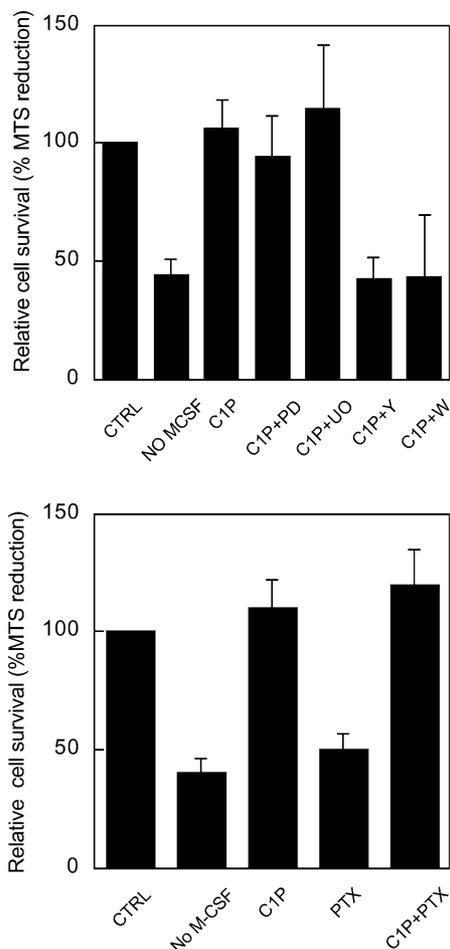


Fig. 3. Effect of MAPK inhibitors, PI3-K inhibitors, and PTX on C1P-mediated macrophage survival. BMDM were seeded at 25×10^3 cells/well in 96-well plates. They were then preincubated with vehicle, or with the MEK inhibitors PD98059 (PD) at 10 μ M and UO126 (UO) at 2 μ M, or with the PI3-K inhibitors LY294002 (LY) at 5 μ M and wortmannin (W) at 100 nM for 1 h before treatment with 30 μ M C1P, as indicated (wortmannin was added back to the cells at 5 and 10 h after stimulation with C1P so as to compensate for its relatively rapid degradation in tissue culture medium) (upper panel). Cells were preincubated for 18 h with vehicle, or with 100 ng/ml PTX before treatment with C1P, as indicated (lower panel). CTRL 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 and are means \pm SEM of three independent experiments performed in quadruplicate.

ERK2 phosphorylation in these cells [30]. The prosurvival effect of C1P was unaffected by pretreatment of the macrophages with pertussis toxin (PTX) (100 ng/ml) (Fig. 3, lower panel). Higher PTX concentrations (up to 1 μ g/ml) also failed to inhibit the prosurvival effect of C1P. However, these PTX concentrations were not used in further experiments because they increase cell viability by an unknown mechanism. In addition, C1P-induced PKB phosphorylation was also unaltered in the presence of PTX (not shown). Taken together these observations suggest that the PI3-K/PKB signaling cascade, but not the MAPK (ERK) pathway, is involved in C1P-stimulated macrophage survival, and that the effect of C1P is independent of interaction with Gi/o coupled receptors.

3.3. The prosurvival effect of C1P requires the activation of I κ B/NF- κ B, and the expression of Bcl-X_L

An important target of PKB is the NF- κ B transcription factor. Therefore, we next tested the ability of C1P to activate this pathway. The activation of NF- κ B requires the phosphorylation of I κ B by I κ K, so we examined the phosphorylation status of I κ B in C1P-treated cells using immunoblot analysis. C1P was able to stimulate the phosphorylation of I κ B in a time-dependent fashion (Fig. 4A). To complement this, we examined the ability of C1P to induce DNA binding of activated NF- κ B, using nuclear extracts and EMSA. Fig. 4B shows that there is minimal basal binding of NF- κ B to DNA in apoptotic macrophages. Of interest, NF- κ B binding to DNA was significantly increased on exposure of the cells to C1P. This effect of C1P was as robust as the lipopolysaccharide (LPS)-induced NF- κ B response, indicating that C1P is a potent activator of NF- κ B in murine macrophages.

There is convincing evidence that the PI3-K/PKB signaling cascade can affect Bcl-2 family members, such as Bcl-X_L. We recently reported that M-CSF withdrawal caused a selective decrease in the level of antiapoptotic Bcl-X_L protein [19]. Also, it is known that Bcl-X_L expression is dependent upon NF- κ B activation, and that this is a requirement for the antiapoptotic effect of oxidized LDL [19]. Since we observed a strong activation of NF- κ B by C1P, we next tested whether its downstream target Bcl-X_L would also be upregulated. Upon examination of Bcl-X_L by Western blotting, we found that it was downregulated in apoptotic BMDM after 30 h of incubation in the absence of M-CSF, and that C1P completely restored Bcl-X_L expression in these cells (Fig. 4C). This observation is consistent with the antiapoptotic effect of C1P. Taken together, these findings demonstrate for the first time that C1P is able to regulate the NF- κ B pathway, and the subsequent expression of antiapoptotic Bcl-X_L.

3.4. Involvement of NF- κ B in the antiapoptotic effect of C1P

To evaluate whether NF- κ B was required for the inhibition of apoptosis by C1P, we tested the effects of selective inhibitors on cell survival in the presence of C1P. As shown in Fig. 5, the NF- κ B inhibitors CAPE and SC-514 blocked the prosurvival effect of C1P in the macrophages, suggesting that NF- κ B is required for this action of C1P.

3.5. Working model of the mechanism whereby C1P promotes cell survival

Based on the findings presented in this study and our recently published work [4], the working model that we propose

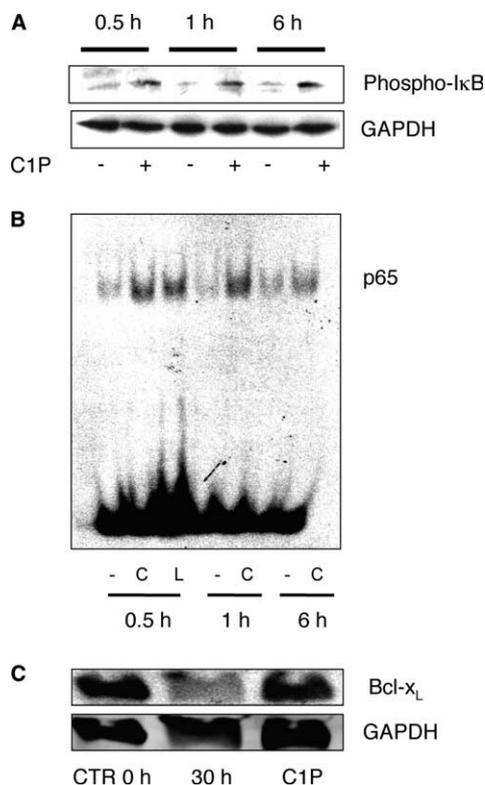


Fig. 4. Ceramide-1-phosphate induces I κ B phosphorylation, NF- κ B activation and Bcl- X_L expression. Panel A: BMDM were seeded and incubated as in Fig. 3. C1P (30 μ M) was added to the cells for various times, as indicated. Phosphorylation of I κ B was examined by immunoblotting as described in Section 2. Similar results were obtained in each of two replicate experiments. Panel B: BMDM were seeded and incubated as in Fig. 3. Nuclear NF- κ B DNA binding activity was determined by EMSA, after stimulation of the cells at various times with C1P (C) (30 μ M) or LPS (L) (200 ng/ml), as described in Section 2. Similar results were obtained in each of two replicate experiments. Panel C: BMDM were seeded and treated as in Fig. 4. Bcl- X_L and GAPDH levels were examined by immunoblotting as described in Section 2. Similar results were obtained in each of three replicate experiments. CTR 0 h, control at 0 h; 30 h, time of incubation in the absence of M-CSF.

for the mechanism involved in C1P-mediated macrophage survival is outlined in Fig. 6. This suggests that C1P prevents macrophage apoptosis by stimulating the PI3-K/PKB pathway, and by inhibiting A-SMase activation and the subsequent generation of ceramide. Ceramide is known to inhibit PKB activity and therefore inhibition of its formation would facilitate signal transduction through the PI3-K/PKB pathway to promote cell survival.

4. Discussion

In previous studies, we have shown that apoptosis of BMDM induced by M-CSF withdrawal involves stimulation of A-SMase and ceramide accumulation [19,25]. A-SMase activation seems to be essential for apoptosis in the macrophages because BMDM that were obtained from A-SMase knockout mice (generously provided by Dr. Richard N. Kolesnick) were resistant to apoptosis upon M-CSF withdrawal (A. Gómez-Muñoz, unpublished work). In addition, desipramine, a sphingomyelinase inhibitor, prevented ceramide accumulation and

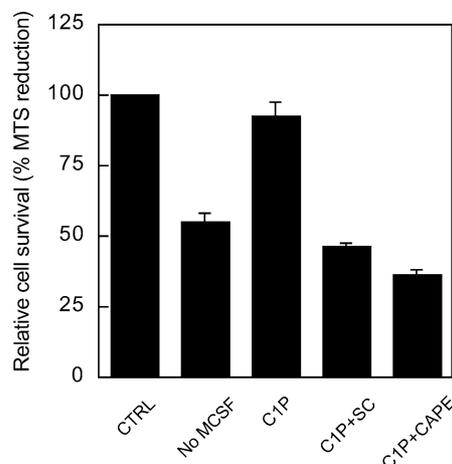


Fig. 5. NF- κ B inhibitors block C1P-mediated macrophage survival. BMDM were seeded at 25×10^3 cells/well in 96-well plates. They were then preincubated with vehicle, or with the NF- κ B inhibitors CAPE (12.5 μ g/ml) or SC-514 (50 μ M) for 1 h before treatment with 30 μ M C1P, as indicated. CTRL indicates 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 and are means \pm SEM of three independent experiments performed in quadruplicate.

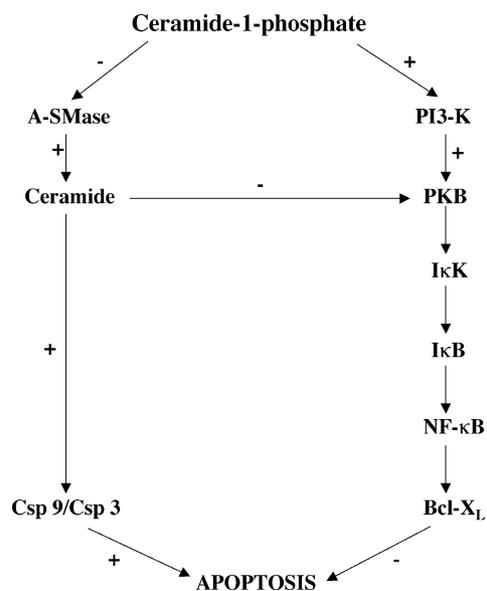


Fig. 6. Working model for the induction of cell survival by C1P. C1P prevents macrophage apoptosis by at least two primary mechanisms: (1) by inhibiting A-SMase and the subsequent generation of ceramide and caspase 9/ caspase 3 activation and (2) by activating the PI3-K/PKB (Akt) pathway. PKB-mediated phosphorylation of I κ B leads to activation of NF- κ B, which then maintains expression of Bcl- X_L thereby promoting cell survival.

blocked cell death [19], whereas incubation of the macrophages with the cell-permeable C_2 -ceramide rendered the cells apoptotic [25].

In a more recent study, we have demonstrated that natural C1P blocks apoptosis in primary BMDM. The mechanism whereby C1P exerts this action probably involves direct inhibition of A-SMase, as C1P was able to completely inhibit this enzyme activity in cell homogenates [4]. In addition, C1P inhibited DNA fragmentation, the stimulation of caspases 3

and 9, and PARP cleavage [4]. Incubation of BMDM with S1P in the absence of M-CSF also led to inhibition of A-SMase, but unlike C1P, S1P did not inhibit A-SMase directly [25]. The physiological relevance of the pro-survival effect of C1P is emphasized by our finding that the intracellular levels of C1P are decreased in apoptotic macrophages [4]. The mechanism for the depletion of C1P levels in apoptotic cells has not been completely defined but it may be explained, at least in part, by a 30% increase in the activity of C1P phosphatase that we have observed in apoptotic BMDM (unpublished observation).

The main finding of the present studies is that C1P stimulates the PI3-K/PKB pathway, which is a major mechanism by which growth factors promote cell survival. The effects of C1P on inhibition of A-SMase and activation of PI3-K are consistent with previous observations by Testai et al. [31] who have recently demonstrated that inhibition of PI3-K leads to A-SMase activation in oligodendrocytes.

The importance of the PI3-K/PKB pathway in C1P-induced cell survival was demonstrated using the two selective PI3-K inhibitors, LY294002 and wortmannin in the presence of C1P. Both of these inhibitors completely abolished the antiapoptotic effect of C1P, suggesting that the PI3-K/PKB pathway is involved in the pro-survival effect of C1P. However, C1P does not affect the phosphorylation state of ERK1-2 in the macrophages, and therefore the MAPK pathway does not seem to be essential for the pro-survival effect of C1P.

Another important observation in this work was that C1P induced activation of the PKB downstream target NF- κ B, supporting our previous finding that PI3-K is an important regulator of NF- κ B activation in BMDM [19]. As expected from these results, C1P enhanced the ability of NF- κ B to bind to DNA, suggesting that activation of this transcription factor is an important component of the signaling pathway leading to inhibition of apoptosis by C1P. In the present work, we also show that C1P upregulates antiapoptotic Bcl-X_L, which is a downstream target of NF- κ B. This parallels the effect of oxidized LDL in BMDM, which also causes upregulation of Bcl-X_L via activation of PI3-K/PKB and inhibition of A-SMase [19]. Inhibition of NF- κ B activation by the selective inhibitors CAPE, or SC-514 abolished the antiapoptotic effect of C1P suggesting that this transcription factor was required for C1P-mediated survival in the macrophages. The above results provide the first evidence for a novel biological role of C1P in the regulation of cell survival by the PI3-K/PKB/NF- κ B pathway. Furthermore, the development of drugs that inhibit C1P formation or that stimulate its degradation may have potential applications in inflammatory diseases, as well as cancer, in which NF- κ B is upregulated.

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