Regulation of Mitogen-Activated Protein Kinases by Sphingolipid Products in Oligodendrocytes

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Sphingolipid products such as ceramide (cer), sphingosine (sph), and sphingosine-1-phosphate (SPP) are implicated in the regulation of cell growth and apoptosis. We have recently shown that cer, sph, and SPP differentially modulate ionic events in cultured oligodendrocytes (OLGs). Cer but not sph or SPP inhibits the inward rectifier (I_{Kir}) in OLGs. To further investigate the role of sphingolipid products in OLGs, we studied the effect of cer, sph, and SPP on OLG survival and on the regulation of mitogen-activated protein kinases (MAPKs). We found that cer, sph, and SPP differentially modulate OLG survival and activation of MAPK members. Cer causes OLG apoptosis, sph causes OLG lysis, and SPP does not affect OLG survival. Cer induces a preferential activation of p38 α , whereas sph and SPP

induce a preferential activation of extracellular signal-regulated kinase 2 (ERK2) in OLGs. In addition, the effect of cer on p38 α activity is mimicked by the inhibition of I_{Kir} with Ba²⁺. In contrast, exposure to cer results in increased activity of ERK2 but not of p38 α in astrocytes. Cer-induced OLG apoptosis is attenuated by a p38 inhibitor, SB203580, and by expression of a p38 α dominant negative mutant. We conclude that p38 α is the mediator in cer-induced OLG apoptosis and that cer-induced I_{Kir} inhibition may contribute to the sustained activation of p38 α in OLGs.

Key words: ceramide; glial cells; apoptosis; protein kinases; lipid mediators; signal transduction

Sphingolipids are derivatives of sphingoid bases that are implicated in the regulation of cell growth, differentiation and apoptosis (Spiegel and Merrill, 1996). Activation of sphingomyelinases (SMases) by extracellular factors such as tumor necrosis factor- α (TNF- α) or Fas ligand leads to the formation of ceramide (cer), sphingosine (sph), and sphingosine-1-phosphate (SPP). Sph and SPP stimulate mitogenesis, mobilize intracellular Ca²⁺ (Ca_i) stores, and activate phospholipase D in fibroblasts (Zhang et al., 1991; Desai et al., 1992; Gomez-Munoz et al., 1994; Olivera et al., 1994). In contrast, cer reverses SPP-stimulated mitogenesis in fibroblasts (Gomez-Munoz et al., 1994) and induces apoptosis in many cell types, including oligodendrocytes (OLGs) and the human oligodendroglioma (HOG) cell line (Casaccia-Bonnel et al., 1996b; D'Souza et al., 1996; Larocca et al., 1997; Scurlock and Dawson, 1999). OLGs are known to be susceptible to cytokine-mediated injury (Selmaj and Raine, 1988; Soliven et al., 1991; Eitan et al., 1992; Louis et al., 1993; Mayer and Noble, 1994; D'Souza et al., 1996). Treatment of HOG cells and CG4 cells with cytokines such as TNF- α or interleukin-1 β (IL-1 β) results in increased cer levels, although a correlation with apoptosis is evident only with TNF- α (Brogi et al., 1997; Scurlock and Dawson, 1999). TNF- α also induces activation of SMases in myelin (Chakraborty et al., 1997). Whether increased cer is required for the commitment to apoptosis or simply reflects membrane deg-

radation as a consequence of apoptosis remains to be clarified (Hofmann and Dixit, 1998).

The role of cer as a second messenger is recently challenged by evidence that the diacylglycerol kinase assay used to measure endogenous cer is flawed (Watts et al., 1997), and by data demonstrating membrane-destabilizing properties of exogenous cer (Simon and Gear, 1998). Perhaps not all biological effects of exogenous cer are attributable to its detergent action. Although both cer and sph exert membrane-destabilizing action, we found that cer and sph (and SPP) differentially modulate the Ca_i, resting membrane potential, and K⁺ currents in OLGs (Hida et al., 1998b). Furthermore, glial cell subtypes exhibit differential susceptibility to cer-induced apoptosis (Casaccia-Bonnel et al., 1996a). The goals of this study were (1) to delineate the modulatory actions of cer, sph, and SPP on cell survival and on activation of mitogen-activated protein kinases (MAPKs) in OLGs, and (2) to investigate the relationship between cer-induced inward rectifier (I_{Kir}) inhibition and activation of MAPK cascades. Mammalian MAPKs include extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs), and p38 subgroups. Activation of ERKs is associated with cell growth, whereas activation of JNK/p38 induces apoptosis (Kyriakis et al., 1994; Xia et al., 1995; Chen et al., 1996). We found that both cell survival and activation of various MAPK members are differentially regulated by cer, sph, and SPP in OLGs. Cer-induced increase in p38 α activity is mimicked by I_{Kir} inhibition with Ba²⁺. In addition, modulation of MAPK cascades by cer differs between OLGs and astrocytes, suggesting that the relative susceptibility of glial cell subtypes to cer is linked to the differential regulation of MAPK cascades.

MATERIALS AND METHODS

Neonatal rat OLG cultures

Primary glial cultures were established from 3- to 5-d-old Holtzman rat pups (Harlan Sprague Dawley, Madison, WI) as previously described

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Figure 1. Effect of cer, sph, and SPP on MTT reduction in OLGs. Neonatal OLGs were treated with cer (0.1–25 μ M), sph (10 μ M), or SPP (10 μ M) for 5 or 24 hr. Results are expressed in optical density $\times$ 1000. Data points represent averages from four to nine experiments for the concentration dependence of cer action, whereas the rest were averages from five to six experiments. For cer concentrations, $p < 0.05$ for untreated versus 1–5 μ M cer; $p < 0.0001$ for untreated versus 10–25 μ M cer. For 24 hr treatment, $p < 0.001$ for CTRL versus cer; $p < 0.00001$ for CTRL versus sph; and $p > 0.05$ for CTRL versus SPP. Statistical analysis was performed using ANOVA followed by Scheffé's F test.

(McCarthy and de Vellis, 1980) and were maintained in DMEM (1.0 gm/l D-glucose) supplemented with 10% FCS plus 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). Bipolar progenitor cells were detached from mixed glial cultures after 10–14 d by overnight

shaking (200 rpm) at 37°C, collected and plated on poly-L-lysine-coated coverslips (CS) or dishes. After 24 hr, the culture medium was changed to DMEM plus 0.5% FCS and 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenium (ITS; Sigma, St Louis, MO). The medium was changed every other day up to days 4–6 when experiments were initiated.

Figure 2. Phase and fluorescence micrographs of neonatal OLGs, either untreated (Ctrl) or treated with cer, sph, or SPP. Concentration of cer, sph, or SPP: 10 μ M. Duration of treatment: 4.5 hr. PI was added to cultures for 5 min before fixation. Failure to exclude PI was observed only in sph-treated OLGs, although both cer- and sph-treated OLGs exhibited decreased number of processes. Scale bar, 30 μ m.

Transient transfection experiments

Plasmids encoding wild-type p38 α (pCMV-Flag-p38) and dominant negative p38 α mutant [pCMV-Flag-p38 (AGF)] were generous gifts from Dr. Roger Davis (University of Massachusetts). The latter had Thr¹⁸⁰ and Tyr¹⁸² replaced with Ala and Phe, respectively (Raingeaud et al., 1995). Plasmids encoding green fluorescent protein (pEGFP-N1) were purchased from Clontech Laboratories (Palo Alto, CA). Co-transfection experiments were initially carried out in cultured BHK21 cells to demonstrate the concomitant expression of GFP and p38 α dominant negative mutant.

Cultured OLGs on coverslips were co-transfected with the above plasmids using activated dendrimers (Superfect; Qiagen, Chatsworth, CA) following the manufacturer's instructions. The transfection reagent contained a constant total dose of 250 ng of DNA/18 mm CS of cultured OLGs. After 2 hr incubation in the transfection mixture, cells were maintained in fresh culture medium for another 48 hr. Efficiency of transfection determined by GFP expression ranged from 3 to 5%.

Cell survival assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was dissolved in PBS at 5 mg/ml, filtered, and added to the cultures at 1:10 dilution for 2 hr at 37°C. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product. For MTT microelisa assay, cells in 96 well plates were incubated with MTT for 3 hr at 37°C, followed by replacement of medium with 50 μ l of DMSO for 30 min at room temperature for color development, and then assayed by measuring the optical density (OD) at 562 λ – OD at 650 λ .

Quantification of OLG lysis and apoptosis

Propidium iodide (PI) exclusion. For detection of lysis and necrosis, live OLGs were incubated for 5 min at room temperature with PI (4.5 μ g/ml) and examined with a standard epi-illumination microscope (450 λ). Cells with membrane disruption take up propidium iodide, which intercalates with DNA to yield a red fluorescence and represent nonviable cells (lysis).

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end-labeling method. OLG apoptosis was detected using the Apoptag *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD), which is based on the terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-dUTP nick end-labeling (TUNEL) procedure. Briefly, fixed OLGs with their endogenous peroxidases inactivated by 2% H₂O₂ were incubated with TdT enzyme for 1 hr at 37°C. DNA nicking was detected by peroxidase-conjugated anti-digoxigenin antibody and subsequent

Figure 3. Phase and bright-field micrographs demonstrating DNA nicking (TUNEL⁺) in Ctrl OLGs and OLGs treated with cer or sph. Concentration of cer or sph: 10 μ M. Duration of treatment: 4.5 hr. Scale bar, 30 μ m.

Table 1. Effect of sphingolipid products and depolarizing agents on OLG survival

Conditions	TUNEL ⁺ cells (%)	PI exclusion (% failure)
Ctrl	7.8 $\pm$ 0.5 (<i>n</i> = 4)	1.6 $\pm$ 0.1 (<i>n</i> = 3)
cer (10 μ M)	14.4 $\pm$ 0.5 (<i>n</i> = 4)*	2.4 $\pm$ 0.7 (<i>n</i> = 3)
sph (10 μ M)	Not analyzed	90.3 $\pm$ 0.7 (<i>n</i> = 4)**
SPP (10 μ M)	8.7 $\pm$ 0.4 (<i>n</i> = 4)	2.1 $\pm$ 0.6 (<i>n</i> = 4)

*For TUNEL experiments, *p* < 0.0001 for cer versus Ctrl.

**For PI exclusion experiments, *p* < 0.0001 for sph versus Ctrl. Statistical analysis was performed using ANOVA followed by Scheffé's *F* test.

color development with DAB (Sigma). Scoring of PI⁺ cells or TUNEL⁺ cells was accomplished by examining 8–10 randomly selected, nonoverlapping microscopic fields (300–500 cell nuclei) on one CS. Results from three or four independent experiments were averaged.

PI nuclear staining. The TUNEL method cannot be used in transfection experiments, because GFP fluorescence is irreversibly destroyed by 1–2% H₂O₂, a necessary step in the TUNEL procedure described above. Therefore, we switched to PI nuclear staining for the assessment of apoptosis in transfection experiments. Fixed OLGs were incubated with PI (4.5 μ g/ml) for 5 min. The percentage of GFP⁺ OLGs with abnormal nuclei was counted (~200 GFP⁺ cells per sample). This method underestimates the degree of apoptosis, because cells with equivocal nuclear fragmentation were considered negative. Both TUNEL and PI methods are less sensitive than the MTT microelisa assay, because floating dead cells are not included in the analysis in the former.

Immunocomplex kinase assay

Cells were harvested in freshly made whole-cell extract (WCE) buffer (25 mM HEPES-HCl, pH 7.7, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20 mM β -glycerophosphate, 0.1% Triton X-100, 0.1 mM Na₃VO₄, 2 μ g/ml leupeptin, 100 μ g/ml PMSF, and 0.5 mM dithiothreitol). ERK2 and p38 α were immunoprecipitated using 1 μ g of rabbit polyclonal anti-ERK2 antibody and rabbit polyclonal anti-p38 α antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Immunoprecipitates were washed twice with WCE buffer and twice with kinase assay buffer (40 mM HEPES-HCl, pH 7.8, 10 mM MgCl₂, 0.1 mM EGTA, and 2 mM dithiothreitol). Kinase activity was assayed for 30 min at 30°C in a buffer containing 0.33 mg/ml myelin basic protein (MBP), 20 μ M ATP, and 0.5 μ Ci of [γ -³²P]ATP (Amersham, Arlington Heights, IL). The reaction was stopped by Laemmli buffer followed by boiling for 5 min. Proteins were resolved by 12% SDS-PAGE, and autoradiograms were analyzed with a Visage 110 densitometer (BioImage, Ann Arbor, MI). For the JNK1 assay, JNK1 was immunoprecipitated with 2 μ g of rabbit polyclonal anti-JNK1 antibody (Santa Cruz), and GST-cJun(1–79) (Santa Cruz) was used as substrate instead of MBP.

Results are expressed as mean $\pm$ SEM with the number of experiments in parentheses. Unless otherwise specified, data were analyzed with ANOVA, followed by Scheffé's *F* test.

Materials

Drugs or agents used in this study were obtained from the following sources: C2-cer and dihydro-cer, Calbiochem (San Diego, CA); sph and rabbit brain MBP, Sigma; SPP, Biomol (Plymouth Meeting, PA); SB203580, Upstate Biotechnology (Lake Placid, NY). Cer and dh-cer were dissolved in ethanol, whereas sphingosine and SB203580 were dissolved in DMSO. Aliquots of stock solutions were stored at –20°C

Figure 4. Differential activation of ERK2, p38α, and JNK1 by sphingolipid products in OLGs. *A*, Examples of autoradiograms of labeled MBP or GST-cJun from immunocomplex kinase assay. Concentration of cer, sph, or SPP: 10 μM; duration of treatment: 20 min. *B*, Summarized data. Results from densitometric analysis were expressed as percent CTRL substrate phosphorylation. *For ERK2 activity, $p < 0.002$ for overall ANOVA; $p < 0.003$ for cer versus sph; $p < 0.03$ for cer versus SPP; for p38α activity, $p < 0.02$ for overall ANOVA; $p < 0.05$ for cer versus sph or SPP; for JNK1 activity, $p > 0.05$ for cer versus sph or SPP.

and diluted to the final concentrations on the day of experiment. SPP was prepared according to the manufacturer's instructions (Biomol).

RESULTS

Differential actions of SM-derived products on OLG survival

We first screened for the effect of sphingolipid-derived messengers on OLG survival using the MTT microelisa assay. MTT reduction, an index of viability, was decreased in cultured neonatal rat OLGs (4–5 d *in-vitro*) exposed to C2-cer for 5–24 hr, compared with untreated OLGs. The effect of cer was dependent on both concentration and duration of exposure. Figure 1 shows MTT reduction measured as OD (mean ± SD) under different conditions. Cer induced a decrease in MTT reduction at concentrations ≥ 1 μM. Next, we compared the effect of 24 hr incubation with 10 μM cer, 10 μM sph, or 10 μM SPP on MTT reduction. OD was 89.0 ± 7.1 ($n = 5$) in untreated cultures, 45.5 ± 5.2 ($n = 6$) in cer-treated cultures, 10.2 ± 3.1 ($n = 6$) in sph-treated cultures, and 79.2 ± 4.1 ($n = 5$) in SPP-treated cultures [$p < 0.001$ for control (CTRL) vs cer; $p < 0.00001$ for CTRL vs sph; $p > 0.05$ for CTRL vs SPP]. Thus, cer and sph but not SPP caused a decrease in MTT reduction in OLGs. Treatment of OLGs for 24 hr with

0.2% DMSO or 0.2% ethanol had no effect on MTT reduction ($n = 3$ each).

Decreased MTT reduction as described above could reflect either cell death resulting in decreased cell number or overall decrease in reducing capacity in OLGs treated with cer or sph. Microscopically, we did not observe uniformly decreased reducing capacity in all cells but, rather, absence of blue formazan product in cells that appeared rounded up or nonviable (data not shown). Next, the TUNEL method and PI exclusion were used to delineate the mechanism of cell death (apoptosis vs necrosis). Failure to exclude PI indicates cell lysis and necrosis, whereas TUNEL demonstration of DNA nicking indicates apoptosis. Because almost total cell death was seen in OLGs treated for 24 hr with sph, these experiments were performed in cultures treated for a shorter period (4.5 hr) with these agents. Figure 2 shows examples of PI uptake in control and cer-, sph-, and SPP-treated OLGs. Phase micrographs revealed the decrease in the number of processes, rounding up of cell bodies, and cell shrinkage in OLGs treated with 10 μM cer but not in control cultures. In sph-treated cultures, groups of cells underwent cell death. In contrast, SPP-treated cells remained phase-bright with extensive networks of processes. Fluorescence micrographs revealed failure to exclude

Figure 5. Examples of autoradiograms of labeled MBP or GST-cJun illustrating the differential regulation of ERK2, p38 α , and JNK1 activity by high K⁺ and Ba²⁺. OLGs were exposed to high K⁺ (20 mM K⁺) or Ba²⁺ (1 mM) for 20 min.

PI in OLG cultures exposed for 4.5 hr to 10 μ M sph but not in untreated (Ctrl), cer-treated, or SPP-treated cells, indicating that only sph causes OLG lysis and necrosis.

Figure 3 shows examples of TUNEL demonstration of DNA nicking in OLGs treated for 4.5 hr with cer (10 μ M) or with sph (10 μ M). An increase in TUNEL⁺ cells was observed in cer-treated cultures, compared with Ctrl cultures (Ctrl, 7.8 $\pm$ 0.5%; n = 4; cer, 14.4 $\pm$ 0.5%; n = 4; p < 0.0001). Note that TUNEL labeling in sph-treated cells was often diffuse, although TUNEL labeling restricted to nuclei could occasionally be observed. Because of possible nonspecific staining as a result of sph-induced cell swelling and lysis, data from sph-treated OLGs were not included in the analysis of percent apoptotic cells (Table 1). Thus, cer caused OLG apoptosis, whereas sph caused predominantly OLG lysis and necrosis. Treatment with SPP had no effect on OLG survival.

Activation of MAPK members by SM-derived products and OLG survival

To determine the downstream events that are involved in the action of sphingolipid products on OLG survival, we investigated whether cer, sph, and SPP differentially modulate the dynamic balance between the ERK and JNK-p38 kinases that may determine whether a cell survives or undergoes apoptosis (Xia et al., 1995; Chen et al., 1996). Cultured OLGs were exposed to 10 μ M cer, sph, or SPP for 20 min. Cell extracts were immunoprecipitated with anti-ERK2, anti-p38 α , or anti-JNK1 antibodies. These

immune complexes were assayed for kinase activity using MBP or GST-cJun (1–79) as the substrate, respectively. For ERK2 and p38 α activity, phosphorylation of 18, 16, and 15.5 kDa MBP isoforms was detected. Changes in MBP phosphorylation appeared to extend to all isoforms. For simplicity, only results from densitometric analysis of the 18 kDa isoform will be presented. For JNK1 activity, phosphorylation of a 37 kDa protein was observed. Examples of autoradiograms of labeled MBP or GST-cJun (1–79) are shown in Figure 4, illustrating that ERK2 activity was enhanced by sph and SPP, whereas p38 α activity was increased by cer. Results from densitometric analysis were expressed as percentage of CTRL substrate phosphorylation. ERK2 activity was 205 $\pm$ 27% (n = 5) in sph-treated OLGs, 181 $\pm$ 16% (n = 4) in SPP-treated OLGs, and 101 $\pm$ 12% (n = 7) in cer-treated OLGs (p < 0.003 for cer vs sph; p < 0.03 for cer vs SPP). On the other hand, p38 α activity was 178 $\pm$ 38% (n = 6) in cer-treated OLGs, 75 $\pm$ 9% (n = 5) in sph-treated OLGs, and 71 $\pm$ 9% (n = 5) in SPP-treated OLGs (p < 0.05 for cer vs sph or SPP). There was no difference in JNK1 activity among cer-, sph-, and SPP-treated OLGs. Treatment for 20 min with 0.2% DMSO or 0.2% ethanol had no effect on ERK2, p38 α , or JNK1 activity (n = 2 each).

Because we have previously found that cer induced OLG depolarization via inhibition of the $I_{K_{ir}}$ (Hida et al., 1998b), we investigated whether depolarization contributes to the preferential activation of p38 α by cer. The effect of depolarizing agents such as high K⁺ or Ba²⁺ on the activity of ERK2, p38 α , and JNK1 was studied. Inhibition of $I_{K_{ir}}$ with Ba²⁺ (1 mM) resulted in increased p38 α activity (168 $\pm$ 16%; n = 4) but not ERK2 or JNK1 activity (n = 5 each). Depolarization with high K⁺ (20 mM) had no effect on ERK2, p38 α , or JNK1 activity (n = 6 each). Examples of autoradiograms of labeled MBP and GST-cJun (1–79) under depolarizing conditions are shown in Figure 5.

Effect of cer on the survival and activation of MAPK cascades in astrocytes

Astrocytes exhibit decreased susceptibility to cer-induced apoptosis when compared with OLGs (Casaccia-Bonnet et al., 1996a). Treatment of cultured astrocytes with cer (10 μ M) in 0.5% FCS plus ITS supplements for 4–16 hr did not induce apoptosis (Hida et al., 1998a). Brief exposure of astrocytes to cer (10 μ M) induced activation of ERK2 but not p38 α . At 20 min exposure to cer (10 μ M), ERK2 activity was 178.8 $\pm$ 23.6% (n = 7); JNK1 activity was 129.0 $\pm$ 7.0% (n = 9); and p38 α activity was 80.4 $\pm$ 5.9% (n = 9) of control values. Comparison of the time course of activation of ERK2, p38 α , and JNK1 by 10 μ M cer in astrocytes and OLGs is depicted in Figure 6. Cer-induced activation of ERK2 in astrocytes was confirmed by Western blot analysis of cell lysates with a polyclonal phospho-ERK antibody (n = 2; data not shown). These findings suggest that the relative susceptibility of OLGs and astrocytes to cer-induced apoptosis correlates with the differential regulation of p38 α versus ERK2 activity.

Role of p38 α in cer-induced OLG apoptosis

We examined the effect of a p38 inhibitor, SB203580, on cer-induced OLG apoptosis assessed by the TUNEL method (see Fig. 8A). The inhibitory action of SB203580 (10 μ M) on p38 α activity was confirmed using an *in vitro* kinase assay (n = 3; data not shown). OLG cultures were pretreated with SB203580 (10 μ M) for 1 hr before and during 4.5 hr incubation with 10 μ M cer. OLG apoptosis was decreased in cultures treated with SB203580 $\pm$ cer compared with those treated with cer alone (ctrl,

Figure 6. Comparison of the pattern and time course of activation of ERK2, p38 α and JNK1 by cer in OLGs and astrocytes. *A*, Examples of autoradiograms of labeled MBP or GST-cJun in OLGs and astrocytes treated with cer (10 μ M). Duration of treatment: lane 1 (untreated), 0 min; lane 2, 5 min; lane 3, 20 min; lane 4, 1 hr; lane 5, 2 hr. *B*, Summarized data illustrating the time course of activation of ERK2, p38 α , and JNK1 in OLGs (open squares) and astrocytes (closed circles). $n = 4$ –9 for each data point; ERK2 activity at 20 min, $p < 0.01$ for OLGs versus astrocytes; p38 α activity at 20 min, $p < 0.03$ for OLGs versus astrocytes.

$8.2 \pm 0.7\%$; $n = 4$; cer, $15.5 \pm 1.0\%$; $n = 4$; SB203580, $8.7 \pm 1.3\%$; $n = 2$; SB203580 + cer, $9.7 \pm 0.6\%$; $n = 4$; $p < 0.002$ for cer vs SB203580 + cer).

To confirm the role of p38 α in cer-induced OLG apoptosis, we transfected OLG cultures with plasmids encoding p38 α wild type (p38 α -wt) or p38 α dominant negative mutant (p38 α -dn). Control experiments consisted of cultures transfected with empty vectors. Transfected cells were detected by cotransfection with plasmids encoding green fluorescent protein (pEGFP-N1; Clontech). Initial experiments confirmed that OLGs expressing GFP also showed immunoreactivity to the Flag epitope, as shown in Figure 7*A*. OLGs transfected with empty vectors or with plasmids encoding p38 α -wt or p38 α -dn were treated with either vehicle (0.2% ethanol) or 10 μ M cer for 12 hr, fixed, and stained with PI. Examples of GFP $^{+}$ OLGs with and without apoptotic nuclei are shown in Figure 7*B*. These results were summarized in Figure 8*B*. The percentage of OLG apoptosis in Figure 8*B* was lower than that depicted in Figure 8*A* because of the following: (1) only GFP $^{+}$ cells were counted in Figure 8*B*; and (2) GFP $^{+}$ cells with equivocal nuclear fragmentation as detected by PI nuclear staining were considered negative. Cer-induced OLG apoptosis was attenuated by the expression of p38 α -dn ($p < 0.03$ for empty + cer vs p38 α -dn + cer; $p < 0.01$ for empty + cer vs p38 α -wt + cer;

$p < 0.001$ for p38 α -wt + cer vs p38 α -dn + cer). Transfection of OLGs with plasmids encoding dominant negative c-Jun (pCMV-TAM67) did not attenuate cer-induced OLG apoptosis ($n = 3$) (data not shown). These results indicate that p38 α rather than JNK1 plays an important role in cer-induced OLG apoptosis.

DISCUSSION

This study demonstrates that cer, sph, and SPP differentially modulate OLG survival and the activation of MAPK members, although these sphingolipid products are interconvertible. Cer is deacylated to form sph, which is then phosphorylated to form SPP. The forward reactions are catalyzed by ceramidase and sph kinase, whereas the reverse reactions are catalyzed by phosphatidate phosphohydrolase and cer synthase, respectively. We found that cer causes OLG apoptosis, in agreement with the work by other investigators (Casaccia-Bonnel et al., 1996a,b; Larocca et al., 1997). In contrast, sph causes predominantly OLG lysis and necrosis, whereas SPP has no effect on OLG survival. Sphingolipid products are implicated in the regulation of cell survival by cytokines and growth factors. TNF- α and γ -interferon induce early and reversible SM hydrolysis in HL-60 cells, which results in increased cer levels (Kim et al., 1991). NGF activates SM hydrolysis in T9 glioma cells (Dobrowsky et al., 1994). In cells of OLG

Figure 7. Photomicrographs of OLGs co-transfected with plasmids encoding GFP and p38 α dominant negative mutant (p38 α -dn). *A*, Phase and fluorescence micrographs showing OLGs with concomitant GFP expression and immunoreactivity to the FLAG epitope (p38-dn expression). Fixed OLGs were incubated with monoclonal anti-FLAG antibody followed by TRITC-conjugated goat anti-mouse IgG antibody. *B*, Phase and fluorescence micrographs depicting nuclear staining with PI in GFP⁺ cells from untreated cultures (*CTRL*) and cultures treated with cer (10 μ M) for 12 hr. Fixed OLGs were stained with PI for the assessment of nuclear morphology. Nuclear shrinkage or fragmentation indicates apoptosis. Note that nuclei overstained with PI can be seen through the fluorescein filter.

lineage, accumulation of cer is induced by binding of NGF, TNF- α , or IL-1 β to their receptors (Casaccia-Bonnel et al., 1996b; Brogi et al., 1997; Singh et al., 1998; Scurlock and Dawson, 1999), as well as by receptor-independent mechanisms such as hypoxia and glutathione depletion (Kendler and Dawson, 1990; Singh et al., 1998). On the other hand, sphingolipid products are also implicated in signal transduction by platelet-derived growth factor (PDGF), a known mitogen for OLG progenitors. Fatatis and Miller (1996) reported that sph and SPP appear to be responsible for PDGF-induced oscillatory and nonoscillatory Ca²⁺ responses, respectively. There is evidence that cer and SPP exert opposing actions on cell survival in other cell types (Cuvillier et al., 1996) and that SMase and ceramidase constitute important sites of regulation by growth factors and proinflammatory cytokines (Coroneos et al., 1995).

The mechanisms involved in the regulation of cell growth and survival by sphingolipid products are not completely understood. We have recently shown that cer and SPP cause OLG depolarization, whereas sph elicits OLG hyperpolarization. Sph induces Ca_i increases in OLGs consistently, whereas Ca_i responses are observed infrequently with cer or SPP. In addition, we found that inhibition of OLG $I_{K_{ir}}$ underlies cer-induced depolarization but

not SPP-induced depolarization (Hida et al., 1998b) (also summarized in Table 2). Both cer and SPP induce OLG depolarization, yet OLG apoptosis is enhanced only by cer. In this study, we examined whether downstream effectors such as MAPK members play a role in determining whether conditions are permissive to apoptotic stimuli. JNK and ERK2 are differentially regulated by sphingolipid products in airway smooth muscle cells and rat mesangial cells, supporting the concept that the dynamic balance between ERK2 and JNK/p38 cascades is important in determining cell survival (Coroneos et al., 1996; Cuvillier et al., 1996; Pyne et al., 1996). We observed similar although not identical results in OLGs. We found that p38 α was activated by cer only, whereas ERK2 was activated by sph and SPP. There was no difference in the JNK1 activity in cer-, sph-, and SPP-treated OLGs. One interpretation would be that cer-induced activation of p38 α , but not of ERK2, is permissive to OLG apoptosis; conversely, SPP-induced ERK2 activation, but not p38 α activation, is not permissive. In addition, we found that the effect of cer on p38 α activity was mimicked by Ba²⁺, a known $I_{K_{ir}}$ blocker, but not by high K⁺, suggesting that $I_{K_{ir}}$ inhibition rather than depolarization per se is a contributory signal to the differential activation of MAPK members. Failure of SPP to inhibit $I_{K_{ir}}$, despite its depolarizing action, correlates with the absence of p38 α induction and absence of apoptosis in SPP-treated cells. Based on MAPK cascades activated by sph, sph should not induce cell death in OLGs. However, sph also causes sustained Ca_i increases in OLGs, which can lead to cell death (Hida et al., 1998b). Hence, the mechanisms underlying sph-induced OLG lysis and necrosis differ from those of cer-induced OLG apoptosis.

In general, JNK and p38 kinase pathways are considered key mediators of the inflammatory response and are activated by both Fas and TNF receptor oligomerization or other stressful stimuli (Brenner et al., 1997; Juo et al., 1997); however, their respective role in apoptosis remains controversial. The p38 subfamily consists of at least four isoforms; p38 α (also known as p38) and p38 β but not p38 γ and p38 δ are inhibited by pyridinyl imidazole compounds such as SB203580 (Young et al., 1997). Activation of p38 α induces apoptosis in Jurkat T cells and cardiac myocytes, whereas activation of p38 β inhibits apoptosis or induces a hypertrophic response (Nemoto et al., 1998; Wang et al., 1998). We found that cer causes sustained activation of p38 α in OLGs; cer-induced apoptosis is inhibited by SB203580 and by p38 α dominant negative mutant, indicating that activated p38 α mediates cer-induced OLG apoptosis. In view of the uniform, modest activation of JNK1 by cer, sph, and SPP, the role of JNK1 in cer-induced OLG apoptosis in our study appears to be less significant than p38 α . Other stimuli that activate JNK in OLGs include NGF, TNF- α , IL, UV light, and heat shock (Casaccia-Bonnel et al., 1996b; Zhang et al., 1996). Studies from Jurkat T cells and other cell lines suggest that JNK activation is associated with apoptosis (Kyriakis et al., 1994; Chen et al., 1996). But other investigators have stressed that activation of JNK alone is not sufficient to induce apoptosis (Gardner and Johnson, 1996). Transfection with c-Jun dominant negative mutant or with SEK1 dominant negative mutant protects neurons against apoptosis induced by withdrawal of trophic factors and protects U937 cells against cer-induced apoptosis (Ham et al., 1995; Verheij et al., 1996; Eilers et al., 1998) but does not protect Jurkat T cells or human breast carcinoma cells against Fas- or TNF-induced apoptosis (Liu et al., 1996; Lenczowski et al., 1997). It is plausible that the role of JNK1 versus p38 α in apoptosis depends on the cell type and the apoptotic trigger. In murine fibroblast cell line

Figure 8. Inhibition of cer-induced OLG apoptosis by p38 inhibitor SB203580 (*A*) and by p38 α -dn (*B*). The TUNEL method was used in *A*; PI nuclear staining was used in *B*. Concentration: 10 μ M for cer and SB203580; duration: 4.5 hr in *A* and 12 hr in *B*. In *A*, $p < 0.0003$ for overall ANOVA; $p < 0.0004$ for ctrl versus cer; $p < 0.002$ for cer versus cer + SB203580. In *B*, $p < 0.0005$ for overall ANOVA; $p < 0.03$ for empty + cer versus p38 α -dn + cer; $p < 0.01$ for empty + cer versus p38-wt + cer; $p < 0.001$ for p38 α -wt + cer versus p38 α -dn + cer.

Table 2. Differential actions of sphingolipid products on ionic events, MAPK cascades, and cell survival in OLGs

	Cer	Sph	SPP
$I_{K_{ir}}$ inhibition	++	–	–
Depolarization	++	–	++
Ca_i increases	+/-	++	+/-
Hyperpolarization	–	++	–
MAPK activation	$\uparrow$ p38 α	$\uparrow$ ERK2	$\uparrow$ ERK2
Outcome	Apoptosis	Lysis/necrosis	Survival

++, Consistent response; –, no effect; +/-, infrequent response; $\uparrow$, increased activity.

L9290cyt16, neither JNK nor p38 α appears to be required for Fas- or TNF-induced apoptosis (Roulston et al., 1998).

In contrast to p38 α and JNK1, activation of ERK1/2 is generally associated with cell proliferation or differentiation, depending on whether activation is sustained or transient (Kaplan and Stephens, 1994). ERK1 and ERK2 are activated by mitogenic factors (PDGF and basic FGF) and phorbol esters in OLGs and progenitors (Bhat and Zhang, 1996; Stariha et al., 1997). Stariha et al. (1997) found that OLGs treated with PD098059 had a limited number of processes, suggesting a role of ERKs in process extension. We found that ERK2 activity is transiently enhanced by cer in astrocytes but not in OLGs, whereas p38 α is

enhanced by cer in OLGs but not in astrocytes. These results are in agreement with the concept that cell survival is regulated by opposing actions of ERK and p38/JNK pathways (Xia et al., 1995; Cuvillier et al., 1996). On the other hand, simultaneous activation of both ERK1/2 and p38 cascades appears to be required for maximal endotoxin-induced astroglial cell activation (Bhat et al., 1998) and for NGF-induced neuronal differentiation of PC12 cells (Morooka and Nishida, 1998). One interpretation of the apparent discrepancy would be that the pattern of activation of MAPK members is a crucial, but not the sole determinant of cell survival, activation, and differentiation. Other important factors that influence cell survival include Bcl2, BAD, and other related mitochondrial proteins, intracellular glutathione content, and ionic fluxes.

We have recently shown that cer inhibits $I_{K_{ir}}$ via a ras- and raf-1-dependent pathway in cultured OLGs (Hida et al., 1998b). Yet, cer activates p38 α instead of ERK2. The experiments with Ba^{2+} indicate that $I_{K_{ir}}$ inhibition may contribute to cer-induced activation of p38 α and perhaps prevent an increase in ERK2 activity as well. A working model whereby proinflammatory cytokines, hypoxia, or other apoptotic stimuli lead to OLG apoptosis is shown in Figure 9. Although cer-induced $I_{K_{ir}}$ inhibition and increased p38 α activity may constitute two simultaneous independent signals required for OLG apoptosis, we propose that $I_{K_{ir}}$ inhibition contributes to the cer-induced sustained activation of

Figure 9. Schematic diagram showing the proposed model for the role of p38 α and $I_{K_{ir}}$ inhibition in cer-induced OLG apoptosis.

p38 α , perhaps via activation of caspases. Cer-induced $I_{K_{ir}}$ inhibition, by reducing K^+ influx, leads to diminished $[K^+]_i$, a condition linked to caspase activation and apoptosis (Hughes et al., 1997; Yu et al., 1997; Dallaporta et al., 1998). Interleukin 1 β -converting enzyme (ICE) family proteases are required for activation of p38 by Fas but not by sorbitol or etoposide (Juo et al., 1997). Our studies support the concept that sphingomyelin cycle is an important regulator of cell survival and that the ultimate cellular outcome depends on the integration of multiple signals, including activation of MAPK members and modulation of ionic events at the plasma membrane.

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