Activation of p38 Mitogen-Activated Protein Kinase Is Required for Death Receptor–Independent Caspase-8 Activation and Cell Death in Response to Sphingosine

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Abstract

Sphingosine induces activation of multiple signaling pathways that play critical roles in controlling cell death. However, the precise molecular mechanism of cell death induced by sphingosine remains to be clarified. In this study, we show that sphingosine induces death receptor–independent caspase-8 activation and apoptotic cell death via p38 mitogen-activated protein kinase (MAPK) activation and that suppression of the MAPK/extracellular signal–regulated kinase (ERK) kinase/ERK pathway by protein phosphatase 2A (PP2A) is required for p38 MAPK activation. Treatment of cells with sphingosine induced suppression of ERK and activation of p38 MAPK. Inhibition of p38 MAPK led to the marked suppression of death receptor–independent caspase-8 activation and subsequent cell death induced by sphingosine. Interestingly, pretreatment with phorbol 12-myristate 13-acetate or transfection of MAPK/ERK kinase/ERK resulting in ERK activation completely attenuated sphingosine-induced p38 MAPK activation. PP2A activity was additionally elevated on sphingosine treatment. Small interfering RNA targeting of PP2A effectively attenuated sphingosine-induced p38 MAPK activation through restoration of ERK activity, suggesting PP2A-mediated opposing regulation of ERK and p38 MAPK. Our findings clearly imply that activation of p38 MAPK promotes death receptor–independent activation of caspase-8 and apoptotic cell death pathways, thus providing a novel cellular mechanism for the anticancer activity of sphingolipid metabolites.

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Introduction

Sphingolipid metabolites, such as ceramide, sphingosine, and sphingosine 1-phosphate (S1P), are key regulators of diverse cellular processes, including apoptosis, cell proliferation, differentiation, and inflammation (1). Considerable attention has been focused on ceramide as a potential endogenous mediator of apoptosis in response to cytokines, antigens, anticancer drugs, or environmental stress (2, 3). Indeed, these diverse exogenous stimuli rapidly enhance intracellular levels of ceramide through sphingomyelinase-mediated hydrolysis of sphingomyelin. Ceramide is further metabolized by ceramidase to generate sphingosine during the early stages of apoptosis. Moreover, sphingosine itself is capable of triggering apoptosis when added exogenously to a variety of leukemic cells or solid cancer cell lines (4). These findings have led to the proposal that sphingosine plays a key role in apoptosis signaling.

Accumulating evidence suggests a role of mitogen-activated protein kinase (MAPK) in sphingosine-induced apoptosis. For instance, sphingosine treatment results in complete inhibition of extracellular signal–regulated kinase (ERK) activity in leukemic and solid cancer cells (5-7), indicating that suppression of this pathway is required for sphingosine-induced apoptosis. Moreover, sphingosine is suggested to function as a mediator of stress responses, leading to the activation of c-jun NH2-terminal kinase (JNK) or p38 MAPK (8-11). However, the molecular mechanism by which apoptotic cell death occurs in response to sphingosine has been widely explored but not precisely deciphered.

A close relationship exists between the ERK pathway and the p38 MAPK or JNK pathway in a variety of eukaryotic cells (8-11). The concomitant inactivation of survival signals may be necessary for JNK- and p38 MAPK–mediated cell death (11). Interestingly, deprivation of neurotrophic factors or UV irradiation not only activates stress kinase cascades but also leads to dramatic inhibition of the ERK pathway (12). In addition, ERK is required for survival signaling in response to cellular growth factors (13). Furthermore, overexpression of...
ERK in NIH 3T3 cells largely impairs the UV-induced apoptotic response (14). Thus, it seems that the cell fate (i.e., death or survival) is decided by a critical balance between the ERK pathway and the JNK or p38 MAPK pathway.

Regulation of MAPKs involves dynamic interplay between kinases and phosphatases. ERKs are activated by phosphorylation of both conserved threonine and tyrosine residues and inactivated on dephosphorylation by tyrosine and serine-threonine kinases and phosphatases, respectively.
phosphatases (15-20). Serine-threonine protein phosphatase 2A (PP2A) dephosphorylates MAPK/ERK kinase (MEK) and ERK family kinases in vitro (19). Moreover, inhibition of PP2A leads to the activation of MEK and ERK (21, 22). Recent studies show that ERK activity is down-regulated by serine-threonine phosphatase during p38 MAPK activation induced by arsenite in NIH 3T3 fibroblasts (23). Moreover, p38 MAPK regulates activation of the JNK pathway in human neutrophils via PP2A (24).

In the present study, we investigate the signaling pathway that triggers apoptotic cell death in response to sphingosine. Activation of p38 MAPK contributes to death receptor–independent caspase-8 activation, and suppression of ERK activity exerts positive effects on p38 MAPK signaling. We additionally present evidence that PP2A activity is necessary for sphingosine-induced suppression of ERK activity. The molecular signaling pathways leading to sphingosine-induced apoptotic cell death may be applied to improve chemotherapeutic approaches for human malignancies.

**Results**

**Caspase-Dependent Apoptotic Cell Death in Response to Sphingosine Treatment**

To investigate the kinetics of apoptotic cell death induced by sphingosine, we treated Jurkat T cells with different doses of sphingosine for various times and analyzed apoptotic cell death by Hoechst 333258 staining. Figure 1A depicts a dose- and time-dependent increase in the proportion of apoptotic cells after sphingosine treatment. Treatment of cells with sphingosine also caused activation of caspase-8 and caspase-3 (Fig. 1B). Requirement of caspase activity for sphingosine-induced apoptosis was additionally examined using a broad-spectrum caspase inhibitor, z-VAD-fmk, and a caspase-8 inhibitor, z-IETD-fmk. Treatment with the caspase inhibitors prevented sphingosine-induced apoptosis (Fig. 1C), supporting the theory that the cell death occurs in a caspase-8–dependent manner. We next examined the changes in intracellular levels of ceramide and S1P after treatment with sphingosine. Intracellular level of ceramide was significantly increased following sphingosine treatment, but the level of S1P was subtly increased (Fig. 1D). To rule out the effects of ceramide and S1P on apoptotic cell death after sphingosine treatment, we pretreated cells with fumonisin B1, an inhibitor of ceramide synthase, or small interfering RNA (siRNA) for sphingosine kinase. As expected, fumonisin B1 and si-sphingosine kinase completely inhibited induction of ceramide and S1P, respectively, in response to sphingosine (Fig. 1E).

However, fumonisin B1 or si-sphingosine kinase failed to affect caspase-8 and caspase-3 activations in response to sphingosine treatment (Fig. 1F). Moreover, pretreatment with fumonisin B1 and si-sphingosine kinase did not affect caspase-8 and caspase-3 activations in response to sphingosine treatment (Fig. 1G and H).

**Sphingosine Stimulates Caspase-8 Activation Independently of the Death Receptor**

Several studies suggest that caspase-8 activation is dependent on its oligomerization, which is stimulated by association with the adaptor molecule, Fas-associated death domain (FADD), via the death effector domains of the two molecules. This step requires either direct or indirect interactions of FADD with surface receptors possessing death domains, such as Fas or tumor necrosis factor receptor (25, 26). To determine the involvement of the death receptor in sphingosine-induced apoptosis, we immunoprecipitated FADD, followed by immunoblotting for caspase-8. As shown in Fig. 2A, caspase-8 activation and interactions between FADD and caspase-8 were detected following treatment with Ch11, a soluble Fas ligand. However, whereas the addition of sphingosine led to caspase-8 activation, no interactions between caspase-8 and FADD were detected after sphingosine treatment. To further confirm these results, we examined caspase-8 activation in FADD-deficient Jurkat T cells in response to sphingosine. Despite FADD deficiency, sphingosine effectively induced caspase-8 activation (Fig. 2B) and apoptotic cell death (Fig. 2C). In addition, no changes in death receptor levels were observed after sphingosine treatment (data not shown). Our results establish that sphingosine-induced caspase-8 activation occurs in a death receptor–independent manner.

**FIGURE 1.** Sphingosine induces caspase-8–dependent apoptotic cell death in human Jurkat T cells. **A.** Jurkat cells were treated with various concentrations of sphingosine for the indicated times. Cells were stained with Hoechst 333258, and apoptosis was measured optically under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number measured. Columns, mean from three independent experiments; bars, SE. **B.** Jurkat cells were treated with 15 μM/L sphingosine for the indicated time periods. Total cell extracts were subjected to Western blot analysis with anti–caspase-8, anti–caspase-3, and anti–z-actin antibodies. **C.** Jurkat cells were treated with 15 μM/L sphingosine for the indicated times. Concentrations of sphingosine (SP) and S1P in cell pellets were determined by HPLC as described in Materials and Methods. Columns, mean from six independent experiments; bars, SD. **D.** Jurkat cells were treated with 15 μM/L sphingosine for 3 h in the presence or absence of 30 μM/L of z-VAD-fmk or z-IETD-fmk. Cells were stained with Hoechst 333258 and observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number measured. Columns, mean from three independent experiments; bars, SE. **E.** Jurkat cells were treated with 15 μM/L sphingosine in the presence or absence of fumonisin B1 (F81). Concentrations of ceramide in cell pellets were determined by HPLC. Columns, mean from three independent experiments; bars, SD. **F.** Cells were treated with 15 μM/L sphingosine in the presence or absence of 10 μM/L of fumonisin B1 or sphingosine kinase siRNA. After 3 h, cells were stained with Hoechst 333258 and observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number measured. Columns, mean from three independent experiments; bars, SD. **G.** Jurkat cells were treated with 15 μM/L sphingosine in the presence or absence of 10 μM/L fumonisin B1. After 3 h, cell lysates were subjected to Western blot analysis with anti–caspase-8, anti–caspase-3, and anti–z-actin antibodies. **H.** Jurkat cells were transfected with control-siRNA or sphingosine kinase siRNA, and then cells were treated with 15 μM/L sphingosine for 3 h. Cell lysates were subjected to Western blot analysis with anti–caspase-8, anti–caspase-3, and anti–z-actin antibodies. **I.** Actin was used as a loading control. The data represent a typical experiment conducted at least thrice with similar results.


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Sphingosine Induces Rapid Activation of p38 MAPK and Suppression of ERK, and p38 MAPK Activity Is Required for Sphingosine-Induced Caspase-8 Activation

MAPKs play a pivotal role in regulating apoptosis in response to various stimuli (5-11). To investigate whether the MAPK signaling pathway is involved in sphingosine-induced cell death, we initially examined the MAPK activities and changes in protein levels following sphingosine treatment. As shown in Fig. 3A, the addition of sphingosine led to a dramatic increase in phosphorylated p38 MAPK. Increased p38 MAPK activity was initially detectable at 1 hour and gradually increased until 6 hours. However, sphingosine induced marked suppression of phosphorylated ERK1/2. Phosphorylated ERK started to diminish within 1 hour of sphingosine treatment and was sustained for 6 hours. The phosphorylated JNK level after sphingosine treatment was constant over time-course studies. In addition, pretreatments with fumonisin B1 and si-sphingosine kinase did not affect sphingosine-induced suppression of phosphorylated ERK1/2 and increase in phosphorylated p38 MAPK (Fig. 3B and C). These results suggest that sphingosine triggers rapid activation of p38 MAPK and suppression of ERK activity in Jurkat T cells.

A number of studies show that p38 MAPK acts at an early step, before caspase activation during the apoptotic process induced by specific stimuli (27, 28). In contrast, under certain circumstances, p38 MAPK activation is dependent on caspase activation (29). Therefore, we examined the effects of p38 MAPK inhibition on caspase-8 activation in response to sphingosine. Retroviral expression of dominant-negative p38 MAPK (MFG-Flag-DN-p38, T180A/Y182F) or pretreatment with PD169316 effectively blocked sphingosine-induced caspase-8 activation (Fig. 3D) and apoptotic cell death (Fig. 3E). However, ERK activity was not affected by p38 MAPK inhibition. Moreover, pretreatment with PD169316 clearly attenuated sphingosine-induced caspase-8 activation in FADD-deficient Jurkat cells (Supplementary Fig. S1). These results suggest that p38 MAPK activity is required for death receptor–independent caspase-8 activation in response to sphingosine.

Suppression of MEK/ERK Signaling Is Required for p38 MAPK–Mediated Caspase-8 Activation during Sphingosine-Induced Apoptosis

To determine whether suppression of ERK activity is required for sphingosine-induced apoptosis, we transfected cells with constitutive active forms of MEK1 and examined apoptotic cell death. Overexpression of constitutively active forms of MEK1 (CA-MEK1 and S217E/S221E) attenuated sphingosine-induced decrease of phosphorylated ERK and activation of caspase-8 (Fig. 4A). Moreover, CA-MEK1 markedly suppressed apoptotic cell death in response to sphingosine treatment (Fig. 4B). Interestingly, overexpression of CA-MEK1 effectively attenuated sphingosine-induced p38 MAPK activation. To further clarify the relationship between ERK activation and sphingosine-induced cell death, we used U0126, a highly selective inhibitor of MEK. Simultaneous treatment with U0126 and sphingosine of cells overexpressing CA-MEK1 effectively reversed the inhibitory effect of MEK1 on sphingosine-induced activation of p38 MAPK and caspase-8 (Fig. 4C) and apoptotic cell death.
Discussion

Despite extensive characterization, the precise molecular mechanism of anticancer activity of sphingolipid metabolites remains to be elucidated. In this investigation, we analyzed the basis for linkage between signaling pathways and apoptotic cascades during sphingosine-induced cell death. Our data show that sphingosine induces apoptotic cell death via p38 MAPK–mediated, death receptor–independent caspase-8 activation, and that suppression of the MEK/ERK pathway by PP2A is required for p38 MAPK activation in response to sphingosine.

p38 MAPK and/or JNK is positively associated with apoptosis induction in response to various cellular stimuli (8-11). Moreover, the p38 MAPK pathway plays an important role in sphingosine-induced apoptosis. Sphingosine treatment induced rapid phosphorylation/activation of p38 MAPK. This rapid activation suggests that p38 MAPK plays a key role in the early events of sphingosine-induced apoptosis. Consistent with this theory, inhibition of p38 MAPK completely attenuated sphingosine-induced caspase-8 activation and apoptotic cell death. Under specific conditions, caspases regulate the activation of p38 MAPK as in Fas-treated Jurkat T cells (30). However, activation of p38 MAPK by sphingosine was insensitive to the broad-spectrum caspase inhibitor z-VAD-fmk (data not shown), suggesting that this step occurs upstream of the caspase cascade during sphingosine-induced apoptosis.

Accumulating evidence suggests a role of ERK in sphingosine-induced apoptosis. For instance, sphingosine treatment results in complete inhibition of ERK activity in leukemic and solid cancer cells (5-7). Indicating that suppression of this pathway is required for sphingosine-induced apoptosis. However, involvement of the apoptotic target machinery of the ERK pathway in sphingosine-induced cell death has not been established to date. We provide evidence in this study that suppression of the MEK/ERK pathway is required for p38 MAPK activation, caspase-8 activation, and apoptotic cell death in response to sphingosine. A relationship between the ERK pathway and the p38 MAPK or JNK pathway has been reported in a variety of eukaryotic cells (8-11). It seems that the ability of a cell to die or survive is decided by a critical balance between the ERK pathway and the p38 MAPK or JNK pathway. Deprivation of neurotrophic factors or UV irradiation not only activates stress kinase cascades but also leads to dramatic inhibition of the ERK pathway (13). Furthermore, ERK expression in NIH 3T3 cells impairs the majority of the UV-induced apoptotic response (14). The prosurvival function of ERK was confirmed in a recent report showing that inhibition of ERK signaling leads to increased sensitivity to cisplatin (cis-diammine-dichloroplatinum) in ovarian cancer cells (31). Conversely, activation of the MEK/ERK signaling pathway in activated Jurkat T cells suppresses tumor necrosis factor-related apoptosis-inducing ligand–mediated apoptosis in a similar manner to Fas-mediated apoptosis (30). These studies support the general view that activation of the ERK pathway delivers a survival signal that counteracts the proapoptotic effects associated with p38 MAPK or JNK activation.

Regulation of MAPKs involves a dynamic interplay between kinases and phosphatases. ERKs are activated by phosphorylation of both conserved threonine and tyrosine residues and inactivated on dephosphorylation by specific phosphatases (15-20). Serine-threonine PP2A can dephosphorylate MEK- and ERK-family kinases in vitro (21, 22). In this study, we also found that inhibition of PP2A clearly restored sphingosine-induced suppression of MEK and ERK. Moreover, inhibition of PP2A effectively reduced p38 MAPK, caspase-8 activation, and apoptotic cell death in response to sphingosine. From these results, we suggest that activation of PP2A induces p38 MAPK activation—mediated, caspase-8–dependent apoptotic cell death induced by sphingosine via suppression of the MEK/ERK pathway.

Our results conclusively indicate that activation of p38 MAPK is required for death receptor–independent caspase-8 activation, and that suppression of the MEK/ERK pathway by PP2A is associated with p38 MAPK activation. An improved understanding of the mechanisms involved in sphingosine metabolite–induced apoptosis may ultimately provide novel strategies of intervention of specific signaling pathways to favorably alter therapeutic efficacy in the treatment of human malignancies.

Materials and Methods

Plasmids and Cell Transfection

The method of constructing the MFG retroviral vector by replacing the GFP sequence of MFG.GFP.IRES.puro was used to construct MFG-Flag-DN-p38 MAPK (T180A/Y182F). The MFG.GFP.IRES.puro itself was used as a negative control throughout the experiment. The retroviral plasmids were introduced into T293 retrovirus packaging cell line by transient transfection with Lipofectamine 2000 (Invitrogen). After 72 h, the supernatants were harvested and used for retroviral infection. The virus titers, measured in NIH 3T3 cell line by puromycin-resistant colony formation, were between 10^5/mL.
FIGURE 3. Activation of p38 MAPK is required for caspase-8 activation and cell death in response to sphingosine. A. Jurkat cells were treated with 15 μmol/L sphingosine for the indicated time periods. After 3 h, cell lysates were subjected to Western blot analysis with anti–phospho-ERK1/2, anti–phospho-p38 MAPK, anti–phospho-JNK, anti-ERK, anti–p38 MAPK, and anti-JNK antibodies. The data represent a typical experiment conducted at least thrice with similar results. B. Jurkat cells were treated with 15 μmol/L sphingosine in the presence or absence of 10 μmol/L fumonisin B1. After 3 h, cell lysates were subjected to Western blot analysis with anti–phospho-ERK1/2, anti–phospho-p38 MAPK, anti-ERK, and anti–p38 MAPK. The data represent a typical experiment conducted at least thrice with similar results. C. Jurkat cells were transfected with control-siRNA or sphingosine kinase siRNA, and then cells were treated with 15 μmol/L sphingosine for 3 h. Cell lysates were subjected to Western blot analysis with anti–phospho-ERK1/2, anti–phospho-p38 MAPK, anti-ERK, and anti–p38 MAPK antibodies. The data represent a typical experiment conducted at least thrice with similar results. D. Jurkat cells were treated with 15 μmol/L sphingosine for 3 h in the presence or absence of retroviral Flag-tagged dominant-negative p38 MAPK (MFG-Flag-DN-p38, T180A/Y182F) or PD169316 (5 μmol/L). Cell lysates were prepared and Western blot analysis was done with anti–phospho-ERK1/2, anti–phospho-p38 MAPK, anti–phospho-ERK1/2, anti–phospho-p38 MAPK, anti–phospho-JNK, anti–phospho-p38 MAPK, and anti–p38 MAPK antibodies. J-Actin was used as a loading control. The data represent a typical experiment conducted thrice with similar results. E. Cells were stained with Hoechst 33258, and apoptosis was observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as a percentage of the total cell number counted. Columns, mean from three independent experiments; bars, SE. *, P < 0.001, versus control.
and $5 \times 10^5$/mL. Target cells were infected with 1 mL of MFG-Flag-DN-p38 supernatants in the presence of 8 μg/mL polybrene (Sigma Chemical Co.). Constitutively active forms of MEK1 (S217E/S221E) were purchased from Cell Biolabs. The plasmids were introduced into target cells by transient transfection with Lipofectamine 2000 (Invitrogen).

**Cell Culture**

Jurkat, human T-cell lymphoma (type II), and FADD-deficient Jurkat T cells were obtained from American Type Culture Collection. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin at 37°C in a humidified incubator with 5% CO₂.

**Materials**

Sphingosine was purchased from Sigma. Polyclonal antibodies specific for caspase-8, phospho-JNK, JNK, p38 MAPK, and PP2A and monoclonal anti–phospho-ERK1/2 and anti-ERK antibodies were from Santa Cruz. Polyclonal anti–β-actin antibody was obtained from Sigma. Polyclonal antibodies for cleaved caspase-3, MEK1, and phospho-p38 MAPK were from Cell Signaling Technology. The broad-spectrum caspase inhibitor z-VAD-fmk, caspase-8 inhibitor z-IETD-fmk, MEK-specific inhibitor PD98059, and p38 MAPK–specific inhibitor PD169316 were acquired from Calbiochem. Constitutively active forms of MEK1 (S217E/S221E) were purchased from Cell Biolabs.

**Hoechst 33258 Staining**

Hoechst 33258 staining was done as described previously (32). Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed once with PBS. Hoechst 33258 (50 ng/mL) was added to fixed cells, which were incubated for 30 min at room temperature and then washed with PBS. Cells were mounted and examined by

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**FIGURE 4.** Suppression of ERK activity is necessary for the activation of p38 MAPK and caspase-8 during sphingosine-induced cell death. **A.** Jurkat cells were transfected with constitutively active forms of MEK1 (CA-MEK1 and S217E/S221E) and then treated with 15 μmol/L sphingosine. After 3 h, cell lysates were subjected to Western blot analysis with anti-MEK1, anti-phospho-ERK1/2, anti-ERK1/2, anti–caspase-8, and anti–β-actin. β-Actin was used as a loading control. The data represent a typical experiment conducted at least thrice with similar results. **B.** After 3 h, cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as percentages of the total cell numbers counted. Columns, mean from three independent experiments; bars, SE. *, P < 0.001, versus control. **C.** Jurkat cells were transfected with CA-MEK1 and then treated with 15 μmol/L sphingosine in the presence or absence of U0126. After 3 h, cell lysates were subjected to Western blot analysis with anti-MEK1, anti–phospho-ERK1/2, anti-ERK1/2, anti–phospho-p38 MAPK, anti–p38 MAPK, anti–caspase-8, and anti–β-actin antibodies. β-Actin was used as a loading control. The data represent a typical experiment conducted thrice with similar results. **D.** After 3 h, cells were stained with Hoechst 33258 and observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin fragments were scored and expressed as percentages of the total cell numbers counted. Columns, mean from three independent experiments; bars, SE. *, P < 0.001, versus control.
fluorescence microscopy. Apoptotic cells were identified by condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated from the ratio of apoptotic cells to total cells counted. A minimum of 500 cells were counted for each treatment.

Reverse Transcription-PCR Assay

The cells were washed twice with PBS. Reverse transcription-PCR was done. In brief, total RNA of the cells was isolated from cells by Trizol (Invitrogen). cDNA was obtained by reverse transcription of 2 μg of total RNA, and amplification of the respective cDNA region was conducted by PCR. PCR primers used were, for sphingosine kinase, CTCTGGTGGTCATGTCTGGA (sense) and CAGGTG-TCTTGGAACCCACT (antisense). β-Actin cDNA was used as an internal standard. The PCR consisted of 30 cycles (94°C for 1 min, 58°C for 1 min, and 72°C for 1 min). PCR products were analyzed by electrophoresis on 1% agarose gels.

Measurement of Cellular PP2A Activity

PP2A was immunoprecipitated and its activity determined using a nonradioactive kit (Upstate Biotechnologies). Jurkat cell lysates were prepared in 20 mmol/L imidazole-HCl, 2 mmol/L EDTA, 2 mmol/L EGTA (pH 7.0), with aprotinin, benzamidine, and 4-(2-aminoethyl)benzenesulfonylfluoride (Sigma-Aldrich). Total protein was immunoprecipitated with an anti-PP2A catalytic subunit antibody (Upstate) and protein...
A-Sepharose beads (Sigma-Aldrich). Equivalent immunoprecipitation of PP2A from all samples was confirmed by Western blot analysis. Immunoprecipitated PP2A was examined for activity in a 10-min reaction at 37°C in which phosphopeptide (K-R-pT-I-R-R) dephosphorylation was assayed spectrophotometrically at 650 nm using malachite green. PP2A activity was determined for all samples relative to control or a phosphate standard curve.

Ceramide Measurement

The lipids from cell pellets added to internal standard C17 sphingosine–based ceramide (C17 ceramide) were extracted with chloroform/methanol (1:2, v/v), and the extract residue was dissolved in methanol, spotted on TLC silica gel plates (Merck), and developed in chloroform/methanol/29% NH4OH (40:10:1, v/v) to half of the plate length. After drying, the plate was rechromatographed in heptane/disopropylether/acetic acid (60:40:3, v/v/v). Lipids were visualized by dipping into 10% sulfuric acid and drying. The band of ceramides corresponding to the band of standard C17 ceramide was scraped off and eluted with methanol. Ceramide was used to release sphingosine from ceramide by deacylation. The released sphingosine was derivatized with o-phthalaldehyde reagent. High-performance liquid chromatography (HPLC) analysis was done using a Shimadzu model LC-10AT pump and SIL-10AXL autoinjector. Nova-Pak C18 column was equilibrated with a mobile phase (92% methanol, 0.1% triethylamine) at a flow rate of 1 mL/min. The fluorescence detector (RF-10AXL) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Measurement of Sphingosine and S1P

Concentrations of sphingosine and S1P in cell pellets were determined by HPLC following a previously described procedure with modification (33). Cell pellets were lysed with 0.2 N NaOH for the determination of protein content. For the determination of sphingosine content, chloroform/methanol (1:2, v/v) and C17 So as internal standard were added to cell lysates and the sphingolipids were extracted and analyzed by HPLC. For the determination of S1P content, chloroform/methanol-1 mol/L NaCl (1:1, v/v), 3N-NaOH, and C17 So as internal standard were added to cell lysates and the sphingolipids were extracted and analyzed by HPLC. For the determination of S1P content, chloroform/methanol-1 mol/L NaCl (1:1, v/v), 3N-NaOH, and C17 S1P as internal standard were added to cell lysates and incubated for 5 h. The aqueous fraction of S1P extract was mixed with methanol, spotted on TLC silica gel plates (Shimadzu model LC-10AT pump and SIL-10AXL autoinjector). Nova-Pak C18 column was equilibrated with a mobile phase (92% methanol, 0.1% triethylamine) at a flow rate of 1 mL/min. The fluorescence detector (RF-10AXL) was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm.

Statistical Analysis

Data were analyzed using one-way ANOVA. Bonferroni’s posttest multiple comparison procedure was used to determine statistical significance. Data are represented as mean ± SD.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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