Dual Actions of Sphingosine-1-Phosphate: Extracellular through the G\textsubscript{i}-coupled Receptor Edg-1 and Intracellular to Regulate Proliferation and Survival

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Abstract. Sphingosine-1-phosphate (SPP), a bioactive lipid, acts both intracellularly and extracellularly to cause pleiotropic biological responses. Recently, we identified SPP as a ligand for the G protein–coupled receptor Edg-1 (Lee, M.-J., J.R. Van Brocklyn, S. Thangada, C.H. Liu, A.R. Hand, R. Menzeleev, S. Spiegel, and T. Hla. 1998. Science. 279:1552–1555). Edg-1 binds SPP with remarkable specificity as only sphinganine-1-phosphate displaced radiolabeled SPP, while other sphingolipids did not. Binding of SPP to Edg-1 resulted in inhibition of forskolin-stimulated cAMP accumulation, in a pertussis toxin–sensitive manner. In contrast, two well-characterized biological responses of SPP, mitogenesis and prevention of apoptosis, were clearly unrelated to binding to Edg-1 and correlated with intracellular uptake. SPP also stimulated signal transduction pathways, including calcium mobilization, activation of phospholipase D, and tyrosine phosphorylation of p125\textsubscript{FAK}, independently of edg-1 expression. Moreover, DNA synthesis in Swiss 3T3 fibroblasts was significantly and specifically increased by microinjection of SPP. Finally, SPP suppresses apoptosis of HL-60 and pheochromocytoma PC12 cells, which do not have specific SPP binding or expression of Edg-1 mRNA. Conversely, sphinganine-1-phosphate, which binds to and signals via Edg-1, does not have any significant cytoprotective effect. Thus, SPP is a prototype for a novel class of lipid mediators that act both extracellularly as ligands for cell surface receptors and intracellularly as second messengers.

Key words: sphingolipids • mitogenesis • G proteins • apoptosis • signal transduction

Sphingolipid metabolites, ceramide, sphingosine, and sphingosine-1-phosphate (SPP),¹ are emerging as members of the lipid rheostat that acts as second messengers (Kolesnick and Fuks, 1995; Spiegel and Milstien, 1995; Hannun, 1996). Ceramide, formed by receptor-coupled activation of sphingomyelinase, has been associated with cell growth arrest and is an important component of stress responses and apoptosis (Hannun, 1996), whereas SPP, a further metabolite of ceramide, is mitogenic in diverse cell types (Zhang et al., 1991; Bornfeldt et al., 1995; Gomez-Munoz et al., 1995; Pyne et al., 1996) and opposes ceramide-mediated apoptosis (Cuvillier et al., 1996; Edsall et al., 1997). Thus, we have proposed that the relative intracellular levels of these two sphingolipid metabolites is an important factor that determines whether cells will survive or die (Cuvillier et al., 1996). More recently, it has been demonstrated that this ceramide/SPP rheostat is an evolutionarily conserved stress regulatory mechanism influencing growth and survival of yeast (Mandala et al., 1997). Various stimuli, including PDGF and serum (Olivera and Spiegel, 1995; Bornfeldt et al., 1995), NGF (Edsall et al.,...
1997), activation of protein kinase C (Mazurek et al., 1994; Buehrer et al., 1996), and cross-linking of the FceRI receptor by antigens (Choi et al., 1996), increase cellular levels of SPP by activation of sphingosine kinase, the enzyme that catalyzes the phosphorylation of sphingosine. Competitive inhibitors of sphingosine kinase eliminate formation of SPP and selectively block cellular proliferation induced by PDGF and serum (Olivera and Spiegel, 1993; Rani et al., 1997), as well as FceRI-mediated calcium signaling (Choi et al., 1996) and the cytoprotective effects of cytokines (Cuvillier et al., 1996) and NGF (Edsall et al., 1997), further supporting a role for endogenous SPP.

Although many studies indicate an intracellular site of action of SPP, some of its biological effects when added exogenously may be due to binding to cell surface receptors. SPP is stored in high concentrations and is released from human platelets upon activation by physiological stimuli (Yatomi et al., 1995), and it is present at high levels in serum (Yatomi et al., 1997b). Previously, we have shown that pertussis toxin–sensitive G proteins are involved in some of the signaling pathways activated by SPP, suggesting that it might activate a receptor coupled to a Gi50-protein (Goodemote et al., 1995). In agreement, low concentrations of SPP activate Gi protein–gated inward rectifying K+ channels only when applied at the extracellular face of atrial myocytes (van Koppen et al., 1996). Moreover, sodium channels only when applied at the extracellular face of

**Materials and Methods**

**Materials**

SPP, dihydrosphingosine-1-phosphate (dihydro-SPP), sphingosine, N,N-dimethylsphingosine, and N-acetyl sphingosine (C2-ceramide) were purchased from BIOMOL Research Laboratory, Inc. (Plymouth Meeting, PA). N-octanoyl ceramide-1-phosphate (C8-ceramide-1-P) was from Calbiochem (La Jolla, CA). Other lipids were purchased from Avanti Polar Lipids (Birmingham, AL). SPP-phosphonate was kindly provided by Dr. Richard R. Schmidt (University of Konstanz, Konstanz, Germany). [methyl-3H]Thymidine (83 Ci/mmol) and [γ-32P]ATP (3,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Fura-2/aceatoxyl methyl ester (fura-2/AM) was from Molecular Probes, Inc. (Eugene, OR). Serum and medium were obtained from Biofluids, Inc. (Rockville, MD). Pertussis toxin was from List Biological Labs (Campbell, CA).

**Cell Culture**

Human embryonic kidney cells (HEK293, ATCC CRL-1573 [American Type Culture Collection, Rockville, MD]) stably transfected with epitope-tagged human edg-1 cDNA in the pCDNANeo expression vector or pCDOCKER control vector and NIH 3T3 fibroblasts (ATCC CRL-1658) stably transfected with pMEXNeo vector or pMEXNeo containing FLAG-tagged edg-1 were grown in DME containing 10% fetal bovine serum, 0.25 g/liter G418 sulfate (Biofluids, Inc.) as previously described (Lee et al., 1996, 1998). Swiss 3T3 cells (ATCC CCL-92) were subcultured at a density of 1.5 × 105 cells/cm2 in DME supplemented with 2 mM glutamine and 10% calf serum, refed with the same medium after 2 d, and used 5 d later when the cells were confluent and quiescent (Olivera and Spiegel, 1993). Rat pheochromocytoma PC12 cells (ATCC CRL-1721) were maintained in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Edsall et al., 1997). Human promyelocytic HL-60 cells (ATCC CCL-240) were grown in RPMI 1640 containing 10% fetal bovine serum as previously described (Cuvillier et al., 1996).

**Sphingosine-1-Phosphate Binding Assay**

[32P]SPP was synthesized enzymatically using partially purified sphingosine kinase as previously described (Olivera et al., 1994). The specific activity of [32P]SPP was 1.28 × 108 cpm/nmol. Cells were incubated with 1 nM [32P]SPP (200,000 cpm) in 200 μl binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 15 mM NaF, 2 mM deoxyxypiridoxine, 0.2 mM PMSF, 1 μg/ml aprotinin and leupeptin) for 30 min at 4°C. Unlabeled lipid competitors were added as 4 mg/ml fatty acid-free BSA complexes (Zhang et al., 1991). Cells were washed twice with 200 μl ice-cold binding buffer containing 0.4 mg/ml fatty acid-free BSA and resuspended in PBS, and bound [32P]SPP was quantitated by scintillation counting.

**Measurement of Cyclic AMP Levels**

Cells were incubated for 15 min at 37°C in DME containing the phosphodiesterase inhibitor IBMX (0.5 mM), in the absence or presence of 10 μM forskolin, and the indicated concentration of SPP or vehicle. Medium was then aspirated, cells were washed with PBS, and cAMP was extracted by sonication in 4 mM EDTA. After centrifugation, samples were boiled for 5 min, pulsed with 1.0 μCi of [3H]cAMP, and [3H]cAMP was measured using the Biotrak [1H]cAMP assay system (Amersham Corp.) according to the manufacturer's instructions.

**Measurement of DNA Synthesis**

Cells were grown to confluence in 24-well tissue culture plates, washed with serum-free DME containing 5 μg/ml transferrin and 20 μg/ml BSA, stimulated by the addition of the indicated concentration of SPP, and after 16 h, pulsed with 1.0 μCi of [3H]thymidine for 8 h. Incorporation of [3H]thymidine into trichloroacetic acid-insoluble material was measured as described (Olivera et al., 1992). Values are the means of triplicate determinations, and standard deviations were routinely less than 10% of the mean.

**Northern Hybridization**

Total RNA from various cell lines was extracted by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). RNA (10 μg) was electrophoresed on 1% agarose/formaldehyde gels and transferred to Zeta-probe membranes (Bio-Rad Laboratories, Hercules, CA). Mouse edg-1 cDNA probe was labeled with [α-32P]dCTP using a random primer labeling kit (Amersham Corp.). Membranes were hybridized as described previously (Liu and Hla, 1997) and exposed to x-ray film for autoradiography.

**Measurement of Uptake and Metabolism of SPP**

Cells were washed with PBS, incubated in DME at 4 or 37°C for 30 min in the presence of 10 μM [32P]SPP (64,000 cpm/nmol) (Van Veldhoven and Mannaerts, 1994), washed with DME containing 0.4 mg/ml fatty acid-free BSA, and then incubated in DME for the indicated time. After washing with cold DME, cells were trypsinized, and cellular lipids were extracted as described (Olivera and Spiegel, 1993). Extracts were applied to silica gel 60 G plates, which were developed with chloroform/methanol/25 mM NaH2PO4, (60:35:8, vol/vol), and then exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 2 h. Radioactive bands were quantified using a Storm densitometer with the ImageQuant program (Molecular Dynamics). Radioactivity in aliquots from each sample was also quantitated.
by liquid scintillation counting for measurement of SPP uptake. Sphingosine production was measured as described (Lavie et al., 1994).

**Determination of Tyrosine Phosphorylation of p125FAK**

HEK293 cells were treated with SPP added as a complex with 4 mg/ml BSA for the indicated times, washed twice with PBS, and then lysed by addition of 0.5 ml lysis buffer (50 mM Hepes, pH 7.9, 1% Triton X-100, 100 mM NaCl, 10 mM EDTA, 4 mM sodium pyrophosphate, 10 mM NaF, 1 mM PMSF, 2 mM Na3VO4, and 2 μg/ml aprotonin and leupeptin). p125FAK was immunoprecipitated from samples containing equal amounts of protein with anti-p125FAK monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Immunoprecipitates were washed three times with lysis buffer and separated by 7% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and probed with monoclonal antiphosphotyrosine 4G10 (Upstate Biotechnology, Inc.). Bands were visualized with Super Signal chemiluminescent reagent (Pierce Chemical Co., Rockford, IL).

**Measurement of Calcium Concentrations**

Cells were grown on 35-mm plastic tissue culture dishes and loaded with the fluorescent calcium-sensitive dye, fura-2/AM (2 μM) for 45 min at 37°C in DME supplemented with 60 μg/ml BSA. Subsequently, cells were washed with Locke’s buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl2, 0.93 mM CaCl2, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, 1 mg/ml glucose, 1 mg/ml BSA, pH 7.4) and mounted in a 35-mm holder maintained at 30°C. Changes in fura-2 fluorescence were measured in single cells by dual excitation imaging using an Attofluor Digital Fluorescence Microscopy System (Atto Instruments Inc., Rockville, MD). 

**Staining of Apoptotic Nuclei**

Cells were cultured in serum-free medium in the absence or presence of the indicated sphingolipids for 5 h at 37°C. Cells were then fixed with 4% paraformaldehyde for 10 min. After washing with PBS, fixed cells were then incubated with biotinylated trihydrochloride (24 μg/ml in 30% glycerol/PBS; Hoechst #33258, Calbiochem) for 10 min. Stained cells were analyzed using a Zeiss Axioskop fluorescent microscope (Zeiss, Germany). Staining of apoptotic nuclei was performed using the Fluorescein Isothiocyanate-conjugated Apoptosis Detection Kit (Roche, Germany). Apoptotic cell nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, OR) using a Hoechst 33258 Kit (Molecular Probes). Cells were then examined under a fluorescent microscope. Apoptotic nuclei were identified by condensation and fragmentation of chromatin.

**Results**

**Specificity of SPP Binding to Edg-1**

We recently identified the G protein–coupled receptor Edg-1 as a receptor for SPP (Lee et al., 1998). In an attempt to identify other potential agonists or antagonists of Edg-1, we examined the effects of structurally related lipids on binding of [32P]SPP to human embryonic kidney 293 fibroblasts stably expressing FLAG epitope–tagged Edg-1 (HEK293/Edg-1). Only sphinganine-1-phosphate (dihydro-SPP), which lacks the double bond at the 4 position, blocked binding in a dose-dependent manner nearly as potently as did unlabeled SPP (Kd = 15 nM) (Fig. 1A). In contrast, other related lipids, including sphingosine, the nonhydrolyzable analogue, SPP-phosphonate (Tarnowski et al., 1997), a short chain SPP analogue (C8-SPP), N,N-dimethylsphingosine, C2-ceramide, sphingosylphosphorylcholine (SPC), and N-octanoyl ceramide-1-phosphate (C8-cer-1-P), did not compete with SPP for binding to Edg-1 (Fig. 1B).

**SPP Decreases cAMP Levels via the Gi-coupled Receptor Edg-1**

Biochemical evidence and the yeast two-hybrid system indicate that Edg-1 is capable of interaction with Gαi1 and Gαi3 (Lee et al., 1996). Moreover, Edg-2 and the cannabinoid receptors, which are related to the edg family, are known to be linked to Gi signaling pathways leading to decreased levels of cAMP (Howlett, 1995; Hecht et al., 1996). Therefore, functional coupling of the Edg-1 receptor was investigated in HEK293 cells in response to its ligand SPP was investigated. The effect of SPP on cAMP accumulation was examined after stimulation of adenylyl cyclase with forskolin in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) to ensure that changes in cAMP levels were not mediated by effects on phosphodiesterase activity. Binding of SPP to Edg-1 markedly inhibited forskolin-stimulated cAMP accumulation in a dose-dependent manner (Fig. 2A), which correlated closely with binding, as the Kd of Edg-1 for SPP is 8 nM (Lee et al., 1998). Although lower concentrations of SPP had no effect on cAMP accumulation induced by forskolin in vector-transfected cells (HEK293-vector) de-
void of Edg-1 expression and specific SPP binding, much higher concentrations slightly reduced cAMP levels, albeit to a lesser extent than in cells that express Edg-1 (Fig. 2A). Thus, the additional decrease in cAMP seen at higher SPP concentrations may be due to non–receptor-mediated effects. Similarly to SPP, dihydro-SPP, which also binds to Edg-1, decreased cAMP levels (Fig. 2B). In contrast, other structurally related lipids that do not bind to Edg-1, including SPC, sphingosine, C8-SPP, C8-cer-1-P, and particularly SPP-phosphonate, were without effect (Fig. 2B). Moreover, pretreatment with N,N-dimethylsphingosine (10 μM), at a concentration that markedly inhibits sphingosine kinase and production of endogenous SPP, had no effect on the decreased cAMP accumulation induced by binding of SPP to Edg-1 (data not shown).

The SPP-induced cAMP decrease in HEK293-edg-1 cells was completely blocked by pretreatment with pertussis toxin, which ADP ribosylates and inactivates G₁ and G₀ proteins (Fig. 2C). Therefore, binding of SPP to the serpentine receptor Edg-1 on the cell surface activates a pertussis toxin–sensitive G₁ protein.

Figure 1. Effects of SPP analogues and other lipids on specific binding of [³²P]SPP to Edg-1. Competition of SPP binding by related lipids. HEK293–edg-1 cells were incubated in the presence of 1 nM [³²P]SPP with increasing concentrations of unlabeled SPP, dihydro-SPP, sphingosine, or C2-ceramide (A) or in the presence of 100 nM of the indicated lipids (B), and binding was measured as described in Materials and Methods. Specific binding of SPP to HEK293–edg-1 (total binding minus binding in the presence of 100-fold excess unlabeled SPP) was 32 fmol/10⁶ cells. Results are means ± standard deviations of triplicate determinations.

Figure 2. SPP decreases cAMP levels through the Edg-1 G₁-coupled receptor. (A) HEK293-vector (open bars) or HEK293–edg-1 cells (filled bars) were stimulated with 10 μM forskolin in the presence of 0.5 mM IBMX and the indicated concentrations of SPP for 15 min, and levels of cAMP were then measured. The levels of cAMP in untreated and forskolin-treated cells were 20 ± 1.7 and 420 ± 30 pmol/10⁶ cells and 10 ± 1.5 and 820 ± 60 pmol/10⁶ cells for vector and edg-1–overexpressing cells, respectively. Results are means ± SD of triplicate determinations. (B) Effects of SPP analogues on cAMP accumulation. HEK293–edg-1 cells were treated with lipids or SPP analogues (100 nM), and forskolin-stimulated cAMP accumulation was determined as described in A. (C) Pertussis toxin inhibits the effect of SPP on cAMP accumulation. Cells were pretreated in the absence (filled bars) or presence (open bars) of 200 ng/ml pertussis toxin (PTX) for 3 h and then were treated for 15 min with forskolin (10 μM) and/or SPP (100 nM), or combinations, as indicated, and cAMP accumulation was measured.
Recently, it was shown that the decrease in cAMP resulting from binding of the endogenous ligand anandamide to the CB-1 receptor resulted in tyrosine phosphorylation of focal adhesion kinase (p125FAK) (Derkinderen et al., 1996). Moreover, we previously showed that SPP enhances phosphorylation of p125FAK in Swiss 3T3 cells (Wang et al., 1997). Surprisingly, we found that SPP-induced tyrosine phosphorylation of p125FAK was independent of Edg-1 receptor activation, as the response was greater in cells lacking the receptor and was even decreased in cells overexpressing edg-1 (Fig. 3A). Furthermore, tyrosine phosphorylation of p125FAK was increased even more at concentrations of SPP higher than those that saturate Edg-1 binding (Lee et al., 1998).

In many cell types, SPP has been shown to activate several signal transduction pathways (Desai et al., 1992; Chao et al., 1994; Ghosh et al., 1994; Mattie et al., 1994; Nataraajan et al., 1994; Bornfeldt et al., 1995; Goodemote et al., 1995; Choi et al., 1996; Blakesly et al., 1997; Fatatis and Miller, 1997; Okajima et al., 1997), particularly mobilization of calcium and activation of phospholipase D, which in certain cell types are activated by low concentrations of SPP. Thus, it was of interest to determine whether these responses were dependent or independent of activation of the cell surface receptor Edg-1. As illustrated in Fig. 3B, low concentrations of SPP (100 nM) had no effect on calcium mobilization in either vector-transfected or edg-1-overexpressing HEK293 cells, whereas treatment with 10 μM SPP led to identical increases in [Ca$^{2+}$]$_i$, suggesting that this response to SPP is not mediated by Edg-1. The calcium responses were also identical in vector- and edg-1-transfected NIH 3T3 cells (data not shown). In agreement, only high, mitogenic concentrations of SPP release calcium from internal sources in Swiss 3T3 fibroblasts (Mattie et al., 1994). Similarly, mobilization of calcium from internal sources by cannabinoid agonists was also shown to be independent of activation of the cannabinoid receptor (Felder et al., 1992). The effects of SPP on activation of phospholipase D were also Edg-1 receptor independent and were even attenuated by edg-1 overexpression in NIH 3T3 fibroblasts (Fig. 3C). Thus, several mitogenic signal transduction pathways that are thought to be involved in SPP-induced proliferation are clearly Edg-1 receptor independent.

**SPP Stabilizes Non–receptor-mediated Signal Transduction Pathways**

Because the most well-established biological response to SPP is the stimulation of cell growth (Zhang et al., 1991; Olivera and Spiegel, 1993; Su et al., 1994; Gomez-Munoz et al., 1995; Goodemote et al., 1995; Wu et al., 1995; Pyne et al., 1996; Blakesly et al., 1997; Rani et al., 1997), we examined the effect of SPP on DNA synthesis in several cell lines expressing different levels of edg-I: SPP is a powerful

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**Figure 3.** SPP stimulates non-Edg-1 receptor-mediated signal transduction pathways. (A) SPP stimulates tyrosine phosphorylation of p125FAK independently of Edg-1 expression. HEK293–edg-1 and vector-transfected HEK293 cells were treated with the indicated concentrations of SPP for various times, and cell lysates were immunoprecipitated with anti-p125FAK mAb and analyzed by Western blotting with anti-Tyr(P) antibody. The arrow indicates the migration p125FAK. (B) SPP-induced changes in intracellular free calcium. Vector and edg-1 stably transfected HEK293 cells were loaded with fura-2/AM, washed, and incubated at 37°C in Locke’s buffer. At the indicated times, SPP (100 nM or 10 μM) was added, and [Ca$^{2+}$]$_i$ was determined by fura-2 imaging (Mattie et al., 1994). Subsequent addition of ionomycin always caused increased [Ca$^{2+}$], suggesting that this response to SPP is not mediated by Edg-1. The calcium responses were also identical in vector- and edg-1-transfected NIH 3T3 cells (data not shown). In agreement, only high, mitogenic concentrations of SPP release calcium from internal sources in Swiss 3T3 fibroblasts (Mattie et al., 1994). Similarly, mobilization of calcium from internal sources by cannabinoid agonists was also shown to be independent of activation of the cannabinoid receptor (Felder et al., 1992). The effects of SPP on activation of phospholipase D were also Edg-1 receptor independent and were even attenuated by edg-I overexpression in NIH 3T3 fibroblasts (Fig. 3C). Thus, several mitogenic signal transduction pathways that are thought to be involved in SPP-induced proliferation are clearly Edg-1 receptor independent.
mitogen for Swiss 3T3 fibroblasts (Zhang et al., 1991; Desai et al., 1992; Su et al., 1994; Rani et al., 1997; Wang et al., 1997) and is also mitogenic for NIH 3T3 fibroblasts, albeit less potent (Blakesly et al., 1997). In agreement with previous studies (Zhang et al., 1991; Desai et al., 1992; Su et al., 1994; Rani et al., 1997; Wang et al., 1997), 10 μM SPP induced marked stimulation of DNA synthesis in Swiss 3T3 fibroblasts (Fig 4 A). SPP was less mitogenic for control, vector-transfected NIH 3T3 fibroblasts (NIH 3T3/pMEX.2), and least mitogenic for edg-1–transfected NIH 3T3 cells (NIH 3T3/pMFE1.2), which express human Edg-1.

Northern analysis of HEK293–edg-1 and HEK293–vector cells using a human edg-1 cDNA probe revealed, as expected, a prominent band in HEK293–edg-1 cells and no band in HEK293–vector cells (Fig. 4 B). As edg-1 expression was expected to be low in mouse fibroblasts, a mouse edg-1 probe that detects mouse edg-1 more sensitively than the human probe was used to analyze NIH 3T3 and Swiss 3T3 cells. Swiss 3T3 cells express the lowest levels of endogenous edg-1 mRNA of the three fibroblast lines tested (Fig. 4 B), which was confirmed by reverse transcriptase PCR analysis (data not shown). NIH 3T3 cells stably transfected with human edg-1 show the presence of both mouse and human edg-1 (2.9 and 1.8 kb, respectively; Fig. 4 B). In agreement, Swiss 3T3 cells showed a low level of specific binding (Fig. 4 C), whereas NIH–edg-1 cells have much higher specific [32P]SPP binding (Fig. 4 C). Thus, it seems that there is no correlation between edg-1 expression and the mitogenic response to SPP (compare Fig. 4 A, and B and C). In agreement, SPP was not mitogenic for HEK293 cells regardless of whether or not they expressed Edg-1 (Fig. 4 A).

Although low levels of SPP bound specifically to Swiss 3T3 fibroblasts at 4°C, no specific binding could be detected at any concentration of [32P]SPP (up to 10 μM) in Swiss 3T3 fibroblasts at 37°C. However, there was a large, cell-associated fraction of [32P]SPP (278 pmol per 10⁶ cells) detected when Swiss 3T3 fibroblasts were incubated with 10 μM [32P]SPP at 37°C. This is due to uptake, as there was extensive intracellular metabolism catalyzed by SPP lyase and SPP phosphatase, both located in the endoplasmic reticulum (Van Veldhoven and Mannaerts, 1994; Mandala et al., 1997), and 57% of the cell-associated SPP was metabolized within 1 h while after 15 h, only 18% of the SPP still remained intact (data not shown).

To further substantiate the notion that the mitogenic actions of SPP observed at high concentrations was due to intracellular actions rather than binding to a cell surface receptor, we took advantage of our previous observation that mitogenesis induced by SPP only required a short exposure time (Wang et al., 1997). In agreement, exposure of Swiss 3T3 fibroblasts to SPP for 30 min at 37°C, followed by washing and replacement of the media, resulted in 50% of maximum DNA synthesis induced by 24 h of treatment (Fig. 5 A). In contrast, when Swiss 3T3 fibroblasts were incubated with SPP for 30 min at 4°C, to allow binding but significantly less uptake (Fig. 5 B), there was much less stimulation of DNA synthesis, suggesting that SPP must be taken up by cells to produce a mitogenic effect. Furthermore, in another approach to increase uptake and delivery of SPP into cells, SPP was mixed with the cationic lipid, lipofectamine, which forms liposomes that are readily

Figure 4. SPP-stimulated DNA synthesis does not correlate with Edg-1 expression. (A) Swiss 3T3 fibroblasts (filled bars), vector-transfected NIH 3T3 cells (hatched bars), NIH 3T3 cells stably transfected with edg-1 (open bars), HEK293–vector (stippled bars), and HEK293–edg-1 cells (gray bars) were grown to confluence in 24-well tissue culture plates, washed with serum-free DMEM containing 5 μg/ml transferrin and 20 μg/ml BSA, and then stimulated with the indicated concentration of SPP. After 16 h, cells were pulsed with 1.0 μCi of [3H]thymidine for 8 h, and incorporation of [3H]thymidine into trichloroacetic acid–insoluble material was measured. Values are the means of triplicate determinations, and standard deviations were routinely less than 10% of the mean. (B) Northern analysis of Edg-1 expression was performed on total RNA from the indicated cells as described in Materials and Methods. A probe corresponding to the mouse edg-1 gene (Liu and Hla, 1997) was used to detect the low level of edg-1 mRNA present in Swiss 3T3 cells. For analysis of HEK293–edg-1 and HEK293–vector cells, a probe corresponding to the human edg-1 gene was used (Hla and Maciag, 1990). (C) Specific [32P]SPP binding to various cell types. Nonspecific (open bars) and total binding (filled bars) of 1 nM [32P]SPP to Swiss 3T3 fibroblasts, vector-transfected NIH 3T3 cells, NIH 3T3 cells stably transfected with edg-1, vector-transfected HEK293 cells, and edg-1–transfected HEK293 were measured as described in Materials and Methods.
internalized and has been widely used to deliver DNA, RNA, and protein (Felgner et al., 1987). In this liposome form, the dose-response for stimulation of DNA synthesis by SPP was shifted to much lower concentrations (Fig. 5 C) with a corresponding increase in [32P]SPP uptake (Fig. 5 D). Collectively, these results strongly suggest that the mitogenic effects of SPP are independent of receptor-mediated actions. In agreement, the nonhydrolyzable SPP-phosphonate, which does not bind to Edg-1 or stimulate the Gt signaling pathway, is as potent a mitogenic agent for Swiss 3T3 fibroblasts as is SPP (data not shown).

To conclusively demonstrate that SPP stimulates DNA synthesis intracellularly, we microinjected SPP into the cytoplasm of quiescent Swiss 3T3 fibroblasts together with IgG to identify microinjected cells and examined the incorporation of BrdU into nascent DNA 24 h later. Using double immunofluorescence to visualize injected cells and BrdU incorporation, it is evident that DNA synthesis is increased after microinjection of SPP, as 6% of injected cells were positive for BrdU incorporation compared with 1% of adjacent, noninjected cells in the same field (Fig. 6). It should be noted that after exogenous treatment of Swiss 3T3 fibroblasts with optimum mitogenic concentrations of SPP or vehicle alone, 10 and 1% of the cells showed BrdU staining, respectively. Microinjection of rabbit IgG alone had no significant effect on DNA synthesis. Moreover, in contrast to the mitogenic effect of SPP, C8-ceramide-1-P, which has no effect on [3H]thymidine incorporation when added exogenously, also had no significant effect on BrdU incorporation when microinjected into quiescent Swiss 3T3 fibroblasts. It is noteworthy that similarly to the synergistic effect between insulin and SPP on [3H]thymidine incorporation (Zhang et al., 1991), microinjected SPP also potentiated the stimulation of BrdU incorporation by insulin (Fig. 6 H). Thus, induction of DNA synthesis by SPP does not require interaction with a cell surface receptor to activate the cellular machinery needed for proliferation. In addition, DNA synthesis stimulated by microinjected SPP was insensitive to pertussis toxin treatment, whereas 70% of that caused by exogenous SPP was blocked by pertussis toxin (Fig. 6 H), in agreement with our previous results (Goedemote et al., 1995).

To examine whether the cytoprotective effects of SPP can also be separated from its binding to Edg-1, we used two cell lines, HL-60 and PC12 cells, in which SPP is known to have a strong survival effect (Cuvillier et al., 1996; Edsall et al., 1997). In agreement with the lack of detectable edg-1 mRNA in HL-60 or PC12 cells (Fig. 4 B), no specific binding of SPP could be detected in either cell line (Fig. 7 A). SPP-phosphonate, which lacks the oxygen atom at the 1 position and does not compete for binding to Edg-1 (Fig. 1 B), was at least as potent as SPP in suppressing ceramide-mediated apoptosis in HL-60 cells (Fig. 7 B) and was as potent as SPP in prevention of DNA fragmentation due to serum deprivation in PC12 cells (Fig. 7 C). These protective effects were specific, because dihydro-SPP, which lacks the trans double bond present in SPP and binds to Edg-1 (Fig. 1), did not significantly prevent apoptosis either in HL-60 or in PC12 cells.

Discussion

Appareantly contradictory reports describe intracellular and extracellular actions of SPP in diverse cell types (Spie...
gel and Milstien, 1995; Spiegel et al., 1996; Moolenaar et al., 1997). This study demonstrates that SPP indeed has dual actions, acting as a second messenger to stimulate cell growth and prevent apoptosis, and as a first messenger through its cell surface receptor Edg-1 (Lee et al., 1998). Similar to the effects of the ligands for other closely related G protein–coupled receptors, such as CB-1 (Bouaboula et al., 1995; Felder et al., 1995) and Edg-2 (Hecht et al., 1996), binding of SPP to Edg-1 markedly inhibits cAMP accumulation (Fig. 2) and activates the mitogen-activated protein (MAP) kinase, ERK-2 (Lee et al., 1998), in a pertussis toxin–sensitive manner. Although the biological function of Edg-1 is not completely understood, it was cloned as an immediate early gene involved in endothelial differentiation (Hla and Maciag, 1990). Binding of SPP to Edg-1 also induces morphogenetic differentiation of HEK293–edg-1 cells and P-cadherin expression by a Rho-dependent mechanism (Lee et al., 1998). Thus, SPP might play a role in angiogenesis since the $K_d$ for SPP binding to Edg-1 is 8 nM (Lee et al., 1998) and SPP is a serum-borne component, where its concentration is 480 nM (Yatomi et al., 1997b). However, because edg-1 expression has been detected in many tissues, including brain, spleen, heart, lung, placenta, muscle, liver, uterus, and kidney (Liu and Hla, 1997), both Edg-1 and SPP might regulate diverse signaling events.

Specific binding of [3H]SPP has also been detected in platelets (Yatomi et al., 1997a) and in F10 melanoma cells (Yamamura et al., 1997). However, the SPP receptors in these cells have characteristics distinct from Edg-1. In both cases, the affinity for SPP ($K_d > 110$ nM) was much lower than the affinity of Edg-1 for SPP ($K_d = 8$ nM), and lyso-

Figure 6. Microinjected SPP stimulates DNA synthesis. Serum-starved Swiss 3T3 cells were microinjected with rabbit IgG together with vehicle (A–C) or SPP (D–F). Cells were visualized by Texas red fluorescence (red), demonstrating immunoglobulin G localization after microinjection (A and D). BrdU incorporation into injected and uninjected cells on the same coverslips was visualized by green FITC fluorescence (B and E). C and F are superimposed images visualized using a triple band pass filter. Yellow-orange color indicates colocalization of microinjected SPP with BrdU staining. In G, stimulation of DNA synthesis in cells injected (open bars) with IgG in the absence or presence of SPP or C8- cer-1-P, as well as in adjacent uninjected cells (filled bars), was assessed by BrdU incorporation. Values (means ± SD) are the percentage of cells positive for BrdU staining and correspond to the average of three measurements in which 100 cells were scored. At least 400 cells were microinjected and scored in each experiment. For comparison, BrdU staining of Swiss 3T3 fibroblasts treated with vehicle (cross-hatched bar) or with 10 μM exogenous SPP (dotted bar) was also determined. (H) Swiss 3T3 cells were serum-starved 24 h in the presence of 5 μg/ml insulin and then treated with or without 200 ng/ml pertussis toxin for 3 h. Cells were then microinjected with rabbit IgG together with vehicle or SPP, or treated with 10 μM exogenous SPP and BrdU incorporation was assessed. In the experiment where SPP was microinjected, 14.5% of adjacent, uninjected cells were positive for BrdU incorporation.
Figure 7. Effects of SPP and analogues on suppression of apoptosis. (A) Lack of specific \[^{32}\text{P}]\text{SPP binding to HL-60 and PC12 cells. Nonspecific (open bars) and total binding (filled bars) of 1 nM \[^{32}\text{P}]\text{SPP to HL-60 and PC12 cells was measured. (B)}\) Inhibition of sphingomyelinase-induced DNA fragmentation by SPP in HL-60 cells. HL-60 cells were incubated with \[^{3}\text{H}]\text{thymidine (1 \(\mu\text{Ci/ml}) for 24 h to label DNA, washed, and then treated without or with 100 mM Staphylococcus aureus sphingomyelinase in the presence of vehicle or with the indicated concentrations of SPP (filled squares), SPP-phosphonate (open squares), or dihydro-SPP (filled triangles), added as BSA complexes. After 5 h, DNA fragmentation was determined from the ratio of unfragmented/fragmented DNA (Cuvillier et al., 1996). Data are expressed as percent inhibition of DNA fragmentation. Percent protection from apoptosis = 100 \times \left[\frac{\text{[fragmentation induced by SMase}}{\text{[fragmentation induced by SMase in the presence of cytotoxic protective agents}}} - \frac{\text{[fragmentation induced by SMase}}{\text{[fragmentation induced by SMase}} - \frac{\text{[fragmentation of untreated controls]}}{\text{[fragmentation of untreated controls]}}\right]. (C) SPP and its hydrolysis-resistant analogue suppress apoptosis in PC12 cells induced by trophic factor withdrawal. PC12 cells were incubated in serum-free medium in the absence or presence of 5 \(\mu\text{M SPP, dihydro-SPP, SPP-phosphonate, or NGF (100 ng/ml) for 15 h, fixed in 3.7\% formaldehyde, and then stained with bisbenzimide trihydrochloride (24 \(\mu\text{g/ml in 50\% glyceral/PBS; Hoechst \#33258, Calbiochem} (Edsall et al., 1997). Cells with chromatin condensation or segmentation of nuclei into three or more fragments were considered to be apoptotic. A minimum of 2,000 cells in each field was scored. Values are means \pm SD of triplicate determinations. Percent protection from apoptosis = 100 \times \left[\frac{\text{[number of cells with fragmented nuclei)}}{\text{[number of cells with fragmented nuclei + number of intact cells]}}\right].

SPP, SPP-phosphonate, or NGF (100 ng/ml) for 15 h, fixed in 3.7\% formaldehyde, and then stained with bisbenzimide trihydrochloride (24 \(\mu\text{g/ml in 50\% glyceral/PBS; Hoechst \#33258, Calbiochem} (Edsall et al., 1997). Cells with chromatin condensation or segmentation of nuclei into three or more fragments were considered to be apoptotic. A minimum of 2,000 cells in each field was scored. Values are means \pm SD of triplicate determinations. Percent protection from apoptosis = 100 \times \left[\frac{\text{[number of cells with fragmented nuclei)}}{\text{[number of cells with fragmented nuclei + number of intact cells]}}\right].
The possible role of Edg-1 in these pathways was addressed by examining ceramide levels and mitotic effect of SPP. We found that injection of SPP into cultured cells produces a significant increase in ceramide, indicating that ceramide is a key mediator of SPP-induced effects.

Ceramide is a product of sphingolipid metabolism and plays a critical role in cell signaling and apoptosis. Our results indicate that ceramide levels are elevated in response to SPP injection, suggesting that SPP may be activating ceramide metabolism in these pathways. This is consistent with previous studies showing that ceramide levels are elevated in response to a variety of stimuli, including growth factors, cytokines, and stress signals.

In summary, our results suggest that SPP acts via ceramide metabolism to regulate cell proliferation and apoptosis. This mechanism may be important for the regulation of cell growth in a variety of contexts, including development and disease. Further studies are needed to understand the molecular mechanisms underlying SPP-induced ceramide metabolism and its role in cell signaling.

Received for publication 4 February 1998 and in revised form 6 May 1998.

We thank Dr. R.R. Schmidt for providing sphingolipid analogues, and Dr. Susette C. Mueller, director of the Lombardi Cancer Center Microscopy/Imaging shared resources (supported by U.S. Public Health Service Grant 1P30-CA-51008), for providing use of microinjection and microscopy facilities.

This work was supported by Research Grants from the National Institutes of Health (GM43880) to S. Spiegel and (DK45659) to T. Hla.
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