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COMMENTARY

Sphingosine-1-phosphate: Extracellular Mediator or Intracellular Second Messenger?

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ABSTRACT. Sphingosine-1-phosphate (SPP), a polar sphingolipid metabolite, has received much attention recently as an extracellular mediator and an intracellular second messenger. It regulates a wide range of biological responses such as cell growth, death, differentiation, and migration. Recent identification of plasma membrane receptors and the cloning of SPP metabolizing enzymes have increased our understanding of the biology of SPP synthesis and action. However, controversy exists regarding the mode of action of this molecule. EDG-1 and related G-protein-coupled receptors were identified recently as plasma membrane receptors for SPP. In light of this recent discovery, many of the functions of SPP previously thought to be due to intracellular second messenger action should be reevaluated. In addition, signaling properties and functions of the three known receptors for SPP need to be fully delineated. The structures and the evolutionary conservation of SPP metabolizing enzymes from yeast to mammals support the hypothesis that SPP also plays a role as an intracellular second messenger. However, definitive assignment of the intracellular role of SPP awaits purification/molecular cloning of elusive intracellular receptors. Better knowledge of the molecular basis of SPP action is needed to assess the physiological and pathophysiological significance of this bioactive lipid mediator. BIOCHEM PHARMACOL **58**;2:201–207, 1999. © 1999 Elsevier Science Inc.

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In recent years, increasing attention has been paid to bioactive lipid mediators generated from membrane phospholipids. Sphingomyelin is an abundant component of biologic membranes, and analogous to glycerolipids it is degraded by a complex metabolic pathway to generate highly potent mediators [1]. For historical reasons and by analogy with glycerolipids, such metabolites generally are thought to function as intracellular second messengers. Indeed, many studies have been conducted by administering sphingomyelin metabolites to cells, and the resulting effects have been assumed to be due to a second messenger mode of action [2]. However, it appears that many lysophospholipids act via specific, high-affinity plasma membrane receptors to mediate a wide variety of biologic responses [3, 4]. In particular, SPP† appears to be a broad-spectrum signaling molecule [5]. In this commentary, biological actions of SPP will be reviewed in light of the recent discovery of high-affinity SPP receptors. The reader is encouraged to refer to other recent reviews on the biology and biochemistry of this sphingolipid [5, 6].

BIOSYNTHESIS AND METABOLISM OF SPP

SPP is generated from the metabolism of sphingomyelin upon cellular activation; specifically, sphingomyelinase degrades sphingomyelin to ceramide, which is then degraded by ceramidase to sphingosine, which is converted subsequently by the enzyme SPK to SPP [5]. Alternatively, *de novo* synthesis of ceramide via the fumonisin B1-sensitive ceramide synthase pathway also may contribute to SPP levels [5]. In cultured cells, cell-associated SPP is present at low levels under basal conditions; however, upon stimulation with growth factors, a transient increase of SPP levels is detected [5, 7]. Once formed, SPP is degraded rapidly by a specific phosphatase [8] as well as a lyase [9]. However, due to the hydrophobic nature of SPP, extracellular export is difficult to detect, and these data do not rule out export and loose association with the plasma membrane components. Platelets lack the SPP degradative activity, and thus high concentrations of SPP have been detected in these cells after activation [10]. Indeed, activation of platelets with prothrombotic stimuli results in the extracellular "secretion" of SPP, causing SPP levels to rise from approximately 100 nM in plasma to 500 nM in serum [11].

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† *Abbreviations*: SPP, sphingosine-1-phosphate; SPK, sphingosine kinase; DMS, *N*,*N*′-dimethylsphingosine; LBP, long chain base phosphatase; MAP kinase, mitogen-activated protein kinase; LPA, lysophosphatidic acid; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; and cAMP, cyclic AMP.

The enzymes that metabolize SPP have been characterized recently. SPP can be degraded by a specific lyase, which cleaves the C2—3 bond, resulting in the formation of phosphoethanolamine and hexadecanal [9]. It is a microsomal pyridoxal-5′-phosphate-dependent enzyme that is expressed in most cell types, except platelets [9]. The lyase enzyme was first cloned in the yeast *Saccharomyces cerevisiae* and has been cloned recently in the mouse [9, 12]. A single putative transmembrane motif is present in the N-terminus of SPP lyase. The role of this enzyme in SPP metabolism and signaling, and the subcellular topology of the active site of the enzyme, remain to be established. However, evolutionary conservation of this enzyme from yeast to mammals suggests that control of SPP levels may be important in a variety of organisms, both unicellular and multicellular.

Another critical enzyme in the formation of SPP is SPK [13]. SPK activity is stimulated by extracellular factors such as phorbol esters, platelet-derived growth factor, and Fc receptors [5]. SPK activation and subsequent formation of SPP correlate with cell growth and survival [14]. In addition, inhibition of SPK by DMS inhibits cell growth and promotes apoptosis [5, 14]. These data suggest that SPK is regulated acutely to produce SPP in the cell. SPK activity is detected in the cytosolic as well as the membrane fraction [13]. Recently, the yeast SPK enzyme was cloned and the homologous murine cDNAs encoding the first mammalian SPK were isolated [15, 16]. The SPK protein sequences from yeast, worms, and mammals exhibit a high level of sequence conservation [15, 16]. The sequences are not similar to known protein or lipid kinases; however, several motifs with significant sequence similarity to diacylglycerol kinase ζ were found [16]. Diacylglycerol kinase ζ was shown recently to be regulated by reversible nuclear translocation upon phosphorylation [17]. It will be of interest if SPK is also regulated similarly, and if so, this would suggest a nuclear function of SPP. SPK possesses several potential phosphorylation sites and calcium/calmodulin binding sites, suggesting that enzyme activity may be modulated by intracellular calcium levels and phosphorylation. The fate of intracellular SPP is not known; once it is produced by SPK activation, it could be degraded by the SPP phosphatase or the lyase, or it could act on intracellular receptors, or it could be secreted to act on extracellular receptors in an autocrine manner.

SPP phosphatase was cloned from yeast as a LBP [8, 18]. It converts long chain sphingoid base phosphates (phytosphingosine and dihydrosphingosine phosphates) to their dephosphorylated derivatives [8, 18]. The sequence of this cDNA did not suggest similarities to other known proteins. Deletion of this gene by homologous recombination in yeast results in strains that show enhanced resistance to heat shock. These data suggest that SPP homologues in yeast may be involved in cell survival after heat shock-induced stress [8, 18]. The mammalian homologues of this gene have not been isolated. The LBP gene product was found associated with the endoplasmic reticulum [8]. The regulation of this protein in relation to the SPK and SPP lyase enzyme remains to be determined.

BIOLOGICAL ACTIONS OF SPP

Sphingosine was originally described as an inhibitor of protein kinase C [1]. However, Spiegel and colleagues found that exogenously added sphingosine promoted the growth of NIH3T3 cells and that it was readily converted to SPP [2, 19]. SPP was more potent as a mitogen than sphingosine, suggesting that it is the bioactive mediator [2, 5, 19]. The authors suggested that exogenously added SPP traversed the membrane and acted as a second messenger to induce mitogenesis [5]. Indeed, exogenously added SPP induced the transcription factor AP-1 binding to DNA, MAP kinase activation, and cyclin-dependent kinase activation, consistent with its action as a mitogenic mediator [20–22]. However, SPP-mediated MAP kinase activation and proliferation are blocked by pertussis toxin, strongly suggesting that G_i-protein-coupled receptors are required [21].

Igarashi and colleagues [23, 24] showed that exogenously added SPP potently inhibited migration of melanoma cells and vascular smooth muscle cells. The mode of action of SPP in this phenomenon is not well understood; however, modulation of actin nucleation may be involved [23, 24]. Moolenaar and colleagues [25] showed that SPP in nanomolar concentrations rapidly induced G_i-protein-independent, small GTPase Rho-dependent neurite retraction in neuroblastoma cells. The authors postulated that this action of SPP is likely to be via a cell surface receptor, similar to LPA and thrombin, other agonists that act on G-protein-coupled, Rho-dependent receptors [26].

Consistent with its role as an activator of MAP kinase, SPP promotes cell survival [14]. Specifically, Spiegel's group showed that ceramide- or sphingomyelinase-induced apoptosis of monocytic cells is inhibited by exogenous SPP. In addition, tumor necrosis factor-α-induced JNK activation also was blocked by exogenous SPP. Furthermore, DMS, which blocks the SPK enzyme, induces apoptosis [14]. Thus, intracellular production of SPP may somehow induce MAP kinase activation and cell survival. The same authors recently showed that caspase-dependent death pathways are antagonized by SPP [27]. Equally likely is that endogenous production of SPP followed by secretion and autocrine signaling via the extracellular receptors induces cell survival and inhibits apoptosis.

Addition of SPP to cultured cells results in induction of stress fibers and focal adhesion plaques [28]. These effects are shared between SPP and LPA, which is better characterized with respect to effects on the cytoskeleton [29]. It is thought that LPA activation of the Rho-coupled seven-transmembrane receptor results in changes in the activity of kinases that modulate the cytoskeletal architecture and focal adhesion assembly [30]. Similarly, SPP-induced effects on the cytoskeleton may be related to plasma membrane receptor signaling and activation of the small GTPase Rho

family members. The focal adhesion plaque is the site of cell anchorage to extracellular matrix molecules and is important in cell proliferation and migration [30]. In addition, exogenous LPA and SPP activation regulates fibronectin matrix assembly via a Rho-coupled pathway [31, 32]. Thus, SPP-induced proliferation and survival may be influenced critically by the modulation of cell–matrix interactions.

Recently, cell–cell contact sites, typified by adherens junctions, have been shown to be regulated by extracellular agents [33]. Adherens junctions are held together by homotypic adhesion of cadherin molecules, which associate with cytosolic catenins and the actin cytoskeleton [34]. Microinjection studies have shown that the activities of the small GTPases Rac and Rho are critical for the assembly of adherens junctional complexes [35]. In human embryonic kidney fibroblasts, SPP and LPA signaling via the EDG-1 G-protein-coupled receptor results in adherens junction assembly, up-regulation of P- and E-cadherin levels, cell–cell aggregation, and morphogenetic differentiation [36]. Cadherin-dependent cell–cell contacts are essential for normal tissue homeostasis and are disrupted in epithelial–mesenchymal transitions and carcinogenesis [33, 34]. Thus, the function of SPP in normal physiological conditions may be to maintain epithelial and endothelial structure via the regulation of cell–cell junctions.

As stated previously, SPP is produced in abundance from activated platelets [10, 11]. Extracellular addition of SPP to non-activated platelets induces shape change and aggregation. However, the effect of SPP is cross-desensitized by LPA, and radioligand binding of SPP to platelets is potently competed by LPA. Thus, SPP may act on the platelet LPA receptor to induce platelet aggregation [37].

Various effects of SPP were discovered in tissue culture systems after exogenous application to the medium. Due to the lipophilicity of this mediator, it was assumed that SPP accessed the intracellular compartments and acted as a second messenger to modulate components of intracellular signaling pathways. Indeed, such a role exists for ceramide, a precursor of SPP [38]. Many signaling pathways are modulated by extracellular SPP administration. For example, inhibition of ligand-stimulated and forskolin-stimulated cAMP levels has been observed [5]. In addition, in many systems, calcium transients are induced after SPP addition. The ability of SPP to induce calcium transients is inhibited by pertussis toxin, suggesting that a G_i-linked SPP receptor is involved [39]. It was reported recently that muscarinic G-protein-coupled receptor activation of calcium transients was inhibited by the SPK inhibitor DMS [40], suggesting a direct intracellular activation of calcium channels by SPP. However, indirect toxic effects of DMS may be involved, and therefore evidence for intracellular action of SPP is circumstantial at present. In addition, MAP kinases, particularly the ERK family, are induced by extracellular SPP addition and are suppressed in part by pertussis toxin [21]. These data suggest that G_i-coupled SPP receptors may be involved. Various other signaling pathways such as phospholipase D as well as NFκB-dependent transcription factor binding to cognate sequences are induced by extracellular SPP administration [5, 41].

Thus, SPP appears to act as a broad-spectrum bioactive lipid that affects a wide variety of cells. The mechanism and mode of action of this lipid, for example, either intracellular or extracellular, have not been defined completely. In addition, the physiological significance of these findings remains to be determined.

THE EDG-1 FAMILY OF G-PROTEIN-COUPLED SPP RECEPTORS

The EDG-1 cDNA was originally isolated as a phorbol ester-inducible immediate early transcript from vascular endothelial cells [42]. Since phorbol esters induce morphogenetic differentiation of vascular endothelial cells into capillary-like tubules, EDG-1 protein was assumed to play a role in endothelial cell differentiation and angiogenesis [42]. Sequence analysis of EDG-1 indicated that it encoded a polypeptide of 380 residues, which possessed seven hydrophobic domains and significant sequence similarity to the G-protein coupled receptor superfamily [42]. Dendrogram analysis indicated high similarity ($\sim$ 25%) to the cannabinoid receptor subfamily, suggesting that the EDG-1 ligand may be a lipid. Subsequent to the cloning of EDG-1, several receptors with significant sequence identity ($>$ 37%) were described as orphan receptors. These include EDG-2 [43], AGR16/H218/EDG-5 [44, 45], EDG-3 [46], and EDG-4 [47].

We took a biochemical approach to define the signaling properties of EDG-1 [48]. The third intracellular loop (i_3) of EDG-1 was fused to the glutathione *S*-transferase polypeptide and used as an affinity matrix to characterize the associated G-proteins. We found that the heterotrimeric G-proteins of the G_i family associate with the i_3 domain and that overexpressed EDG-1 could be found associated with $G_{i\alpha 1}$ and $G_{i\alpha 3}$ polypeptides. These studies indicated that EDG-1 is capable of coupling to the G_i pathway. Indeed, overexpression of EDG-1 induced ERK-2 activation, which was blocked by pertussis toxin treatment, suggesting that EDG-1 regulates MAP kinase activity by coupling to the G_i protein [48]. The use of the ERK-2 pathway as a readout to discover the EDG-1 ligand, however, was not successful.

Various cell lines were tested for EDG-1 expression, and we found a subclone of HEK293 cells that expressed extremely low levels of EDG-1 mRNA. These cells were transfected, and the EDG-1-overexpressing clones were selected. High levels of mRNA and protein for EDG-1 were expressed by stable transfectants but not in vector or parental cells. The EDG-1 overexpressing cells exhibited a unique morphology; cells were aggregated and formed network-like structures, which resembled tubular networks of differentiated endothelial cells. This phenomenon, termed morphogenetic differentiation, was dependent on EDG-1 expression and the presence of serum-borne lipids [36]. Using this assay, two structurally related serum-borne

lipids, SPP and LPA, were identified as potential EDG-1 ligands. SPP induced EDG-1-dependent morphogenetic differentiation potently, whereas LPA was less potent. All other lipids tested were ineffective in this assay, suggesting specificity [36]. To further demonstrate that EDG-1 and SPP interacted directly, a highly specific [^{32}P]SPP binding assay was used [36]. SPP bound to EDG-1 with high affinity (apparent K_d = 8.1 nM) and specificity. High-affinity binding was competed effectively by SPP and dihydrosphingosine-1-phosphate [49]. LPA bound to EDG-1 with an apparent K_d of 2.3 μM and competed weakly for SPP binding, suggesting that it is a low affinity agonist [50]. Consistent with the receptor affinity, nanomolar concentrations of SPP induced ERK-2 activation, depressed cAMP levels, stimulated receptor phosphorylation, and induced receptor trafficking into intracellular vesicles [36]. These data establish that EDG-1 is a high-affinity receptor for SPP.

Although the G_i/ERK-2 pathway was induced by SPP activation of EDG-1, morphogenetic differentiation did not require this signaling pathway. EDG-1 activation also resulted in formation of adherens junctions and enhanced cell–cell aggregation and up-regulation of P- and E-cadherin polypeptide levels [36]. SPP-induced morphogenetic differentiation and P-cadherin induction were inhibited completely by the C3 exotoxin, a specific inhibitor of the small GTPase Rho, which is known to regulate stress fibers and the formation of focal adhesions [29]. The Rho family of GTPases were implicated recently in the formation of adherens junctions [35]. Furthermore, Rac and Cdc42 were shown to regulate the structures of lamellopodia and filopodia, respectively [29]. Recently, SPP- and LPA-induced T lymphoma cell migration was shown to require the activity of Rho as well as Rac signaling [51]. Although the upstream heterotrimeric G-protein involved in Rho and Rac activation is not clear at present, the $G_{12/13}$ family have been implicated [52, 53]. Interestingly, deletion of the G_{13} gene in mice results in endothelial cell defects in the embryo and developmental failure of the embryonic cardiovascular system [54]. Together, these data suggest that EDG-1 couples to the Rho pathway to induce adherens junction formation and morphogenesis [36]. Indeed, recent observations suggest that EDG-1 regulates morphogenesis and angiogenesis of endothelial cells (Lee M-J and Hla T, unpublished observations).

Van Brocklyn *et al.* [49] reported that, in the HEK293 cell system, several SPP-induced signaling events occurred independent of EDG-1 expression. For example, extracellular SPP administration induced calcium transients, focal adhesion kinase, and paxillin phosphorylation independent of EDG-1 expression. Moreover, extracellular SPP-induced stimulation of cell growth and inhibition of apoptosis were also unrelated to EDG-1 expression in 3T3 fibroblasts and PC12 pheochromocytoma cells. However, microinjection of SPP into Swiss 3T3 fibroblasts induced a modest increase in DNA synthesis [49]. It was concluded that intracellular action of SPP is important for cell proliferation and apoptosis regulation. These data are, however, indirect, since they do not rule out the existence of additional SPP receptors on the plasma membrane that were not detected by ligand binding assays. Moreover, these data are consistent with the concept that EDG-1 couples to G_i and not to G_q, and that other EDG-1 family receptors that are coupled to G_q and other G-proteins mediate the effects on signal transduction events, cell growth, and apoptosis in these cells.

Independent of the EDG-1 work, Chun and colleagues cloned the mouse edg-2 homolog, which was named VZG-1, from the developing ventricular zone of the cerebral cortex. They showed that VZG-1 transfection induced LPA-dependent cell rounding and suppression of intracellular cAMP levels and concluded that VZG-1 is a high-affinity receptor for LPA [55]. Subsequent work from the same group showed that VZG-1 is capable of activating multiple G-proteins in response to low doses of LPA [56]. Other work has also provided data showing that VZG-1 indeed encodes a functional LPA receptor [57, 58]. However, binding constants of LPA to VZG-1 have not been defined. A related receptor, termed EDG-4, was cloned recently and was shown to be a LPA receptor as well [47]. The same group characterized EDG-3 and AGR16/H218/EDG-5 receptors [59]. Both of these receptors were activated by nanomolar concentrations of SPP when expressed in *Xenopus* oocytes and induced calcium fluxes, suggesting that they are capable of coupling to the G_q pathway [59]. In addition, upon expression in Jurkat T cells, SPP activated EDG-3 and EDG-5 to induce SRE-dependent transcription [59]. Binding studies of SPP to EDG-3 and -5 have not been reported. EDG-1, -3, and -5 are highly related in sequence (>50% identity), whereas EDG-2 and -4 are more divergent (35–40% sequence identity). Thus, the available data support the concept that EDG-1, -3, and -5 are high-affinity SPP receptors whereas EDG-2 and -4 are high-affinity LPA receptors.

FUTURE DIRECTIONS AND CONCLUSIONS

The discovery of the EDG-1 family of G-protein-coupled SPP and LPA receptors should stimulate research on the signal transduction and physiology of bioactive lipid mediators. The expression of these receptors appears to be wide-spread; most cells respond to either SPP or LPA or both. It is possible that these lipids play a fundamental role in the growth and survival of cells, since most cells express receptors of this family. Gene deletion studies of the EDG-1 family of receptors should yield interesting developmental and physiological insights. An important immediate question is why multiple receptors exist for both SPP and LPA. Each receptor subtype may couple to distinct signaling pathways and thus may regulate a unique biologic function. In support of this concept, SPP and LPA induce cell rounding in parental HEK293 cells independently of EDG-1 expression. However, morphogenetic differentiation was induced by these ligands only when EDG-1 was

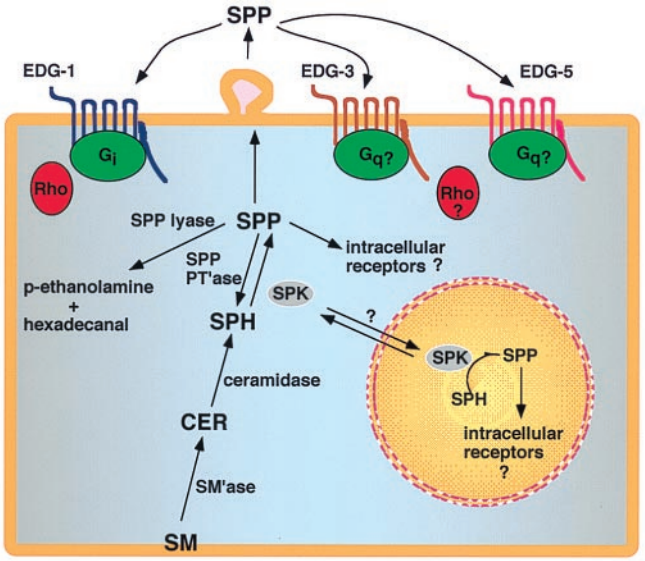

FIG. 1. A working model of SPP synthesis and action. The formation of SPP likely occurs as a result of the activation of the enzymes by extracellular mediators. Once formed, SPP can activate putative intracellular receptors or be exported out of the cell. It is tempting to speculate that by analogy with diacylglycerol kinase ζ, SPK may also be translocated into the nucleus. The mechanism of SPP export is not known; a hypothetical model of exocytic microvesicle shedding is proposed. This mechanism was shown to occur in platelets after activation and may release LPA [60]. Once secreted, SPP activates its high-affinity G-protein-coupled receptors EDG-1, -3, and -5. Coupling of EDG-1 to G_i is established and results in ERK-2 activation and decrease of cAMP levels. In addition, EDG-1 may couple to the Rho pathway. EDG-3 and -5 signaling is not well defined, but may couple to the G_q pathway [59]. Abbreviations: SM, sphingomyelin; CER, ceramide; SPH, sphingosine; SM'ase, sphingomyelinase; SPK, sphingosine kinase; and PT'ase, phosphatase.

overexpressed [50]. It would be important to define specific signaling pathways and biologic actions induced by each receptor subtype in receptor null cells. Furthermore, it is critical to address whether many of the previously described actions of SPP are mediated by the EDG-1 family of receptors. Important actions of SPP, namely regulation of cell growth, survival, migration, and differentiation, may be regulated by distinct members of the EDG-1 family of receptors. Alternatively, as yet unidentified G-protein-coupled receptors or intracellular receptors may mediate some of these actions. A related issue is whether SPP and LPA are secreted by cells. Recent studies have shown that LPA is indeed "exported" by adipocytes and in exocytic microparticles after cell activation [60, 61]. Secretion of SPP and LPA may allow autocrine signaling via the EDG-1 family of receptors. Indeed, we observed that endogenously formed SPP is capable of activating EDG-1 in an autocrine manner (Liu CH and Hla T, unpublished observations). EDG-1 is internalized rapidly into intracellular vesicles after SPP stimulation [36]. It will be of interest to determine the fate of the internalized SPP. In addition, putative intracellular SPP receptors need to be purified and cloned. Finally, coordinate regulation of endogenous SPP synthesis relative to receptor activation needs to be characterized to define the situations in which this lipid mediator acts as an autocrine or paracrine mediator. Our current understanding of SPP synthesis and action is schematically depicted in Fig. 1.

In conclusion, SPP is a highly potent lipid mediator with a broad spectrum signaling activity. However, controversy exists as to whether it acts as an extracellular mediator or an intracellular second messenger or both. Recent identification of plasma membrane-localized receptors for SPP and recent cloning of SPP metabolizing enzymes in organisms from yeast to mammals have illustrated the potential multiplicity of SPP action. These studies undoubtedly will stimulate further research on the biology of SPP action and will allow a critical assessment of physiological and pathophysiological relevance of this mediator.

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