Subcellular Origin of Sphingosine 1-Phosphate Is Essential for Its Toxic Effect in Lyase-deficient Neurons

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Cerebellar granule cells from sphingosine 1-phosphate (S1P) lyase-deficient mice were used to study the toxicity of this potent sphingolipid metabolite in terminally differentiated postmitotic neurons. Based on earlier findings with the lyase-stable, semi-synthetic, cis-4-methylsphingosine phosphate, we hypothesized that accumulation of S1P above a certain threshold induces neuronal apoptosis. The present studies confirmed this conclusion and further revealed that for S1P to induce apoptosis in lyase-deficient neurons it must also be produced by sphingosine-kinase2 (SK2). These conclusions are based on the finding that incubation of lyase-deficient neurons with either sphingosine or S1P results in a similar elevation in cellular S1P; however, only S1P addition to the culture medium induces apoptosis. This was not due to S1P acting on the S1P receptor but to hydrolysis of S1P to sphingosine that was phosphorylated by the cells, as described before for cis-4-methylsphingosine. Although the cells produced S1P from both exogenously added sphingosine as well as sphingosine derived from exogenous S1P, the S1P from these two sources were not equivalent, because the former was primarily produced by SK1, whereas the latter was mainly formed by SK2 (as also was cis-4-methylsphingosine phosphate), based on studies in neurons lacking SK1 or SK2 activity. Thus, these investigations show that, due to the existence of at least two functionally distinct intracellular origins for S1P, exogenous S1P can be neurotoxic. In this model, S1P accumulated due to a defective lyase, however, this cause of toxicity might also be important in other cases, as illustrated by the neurotoxicity of cis-4-methylsphingosine phosphate.

Sphingosine 1-phosphate (S1P) is a potent lipid mediator that has been shown to regulate a wide range of physiological processes, including proliferation, differentiation, motility, cytoskeleton rearrangements, and calcium homeostasis (1, 2). There is convincing experimental evidence that this bioactive sphingolipid can act both extracellularly, as a ligand for a family of five specific G protein-coupled receptors, and inside the cells, as a second messenger (3, 4). In most cell types described so far, S1P and its metabolic precursor ceramide exert antagonistic effects on cell survival with S1P being generally regarded as a survival signal, whereas ceramide and sphingosine are generally toxic (5, 6). Interestingly, generation of sphingosine and S1P is generally thought to be dependent on the availability of ceramide (7), however, relatively high amounts of S1P are also present in blood, lymph, and cerebrospinal fluid (8, 9) and may serve as additional sources for some cells.

More than a decade ago, we introduced the synthetic sphingosine analog cis-4-methylsphingosine as a tool for studies of sphingoid base metabolism and function (10). When added to the culture medium, this analog is taken up and mainly phosphorylated to the respective cis-4-methylsphingosine phosphate, which accumulates intracellularly, because it is poorly cleaved (if at all) by S1P lyase (10). Intriguingly, this compound promoted proliferation of quiescent Swiss 3T3 fibroblasts (11), as does S1P (12), but induced apoptosis in postmitotic terminally differentiated primary cultured neurons (13).

Despite the fact that neither S1P nor sphingosine were able to induce apoptosis in neurons, we proposed that cis-4-methylsphingosine is phosphorylated by cells yielding a metabolically stable analog of S1P. This prediction was based on experimental results indicating that the different physiological effects, apoptosis in the case of the accumulating metabolically stable synthetic compound versus no apoptosis in the case of the short living S1P, rely only on nuances of impact (13). Both sphingoid phosphates affected similar pathways. However, the effect of the synthetic accumulated compound was more pronounced and persistent when compared with the more transient and less pronounced effect of the short living physiological counterpart (13). We therefore assumed that conditions that allow sufficient accumulation of S1P in primary cultured neurons should end up in neuronal apoptosis.

To explore this hypothesis, which might be relevant to neurodegenerative processes, we attempted to elevate intracellular S1P using siRNAs directed to S1P lyase (encoded by the Sgpl1 gene). However, suppression of lyase by ~70% did not result in...
an accumulation of endogenous S1P in primary cultured neurons (14).

The central aim of the present study was to evaluate the hypothesis that endogenous S1P induces neuronal apoptosis when it exceeds a certain threshold by a more effective method for lyase activity suppression. We thus used primary cultured neurons prepared from cerebella of 6-day-old lyase-deficient mice (15). The present studies not only confirmed that elevation of S1P induced cell death but also revealed that the origin of the S1P was important. Intriguingly, neuronal apoptosis was induced only by S1P derived from exogenous S1P that was dephosphorylated and then resynthesized to S1P by sphingosine kinase 2 (SK2). Interestingly, we then found that this is also the kinase responsible for synthesis of cis-4-methylsphingosine phosphate. In addition, our data document that the pro-apoptotic effect of S1P is independent of cellular ceramide content.

EXPERIMENTAL PROCEDURES

Cell Culture—Granule cells from cerebella of 6-day-old wild-type, Sgp111/+, or Sgp11−−/− pups, inbred in a Swiss Webster genetic background, were cultured as described before (10). Note that the heterozygote Sgp111/+-non-inbred mouse was generated from gene-trapped ES cells (OST 58278) by Lexicon Inc. (The Woodlands, TX) on a fee basis (15). Recently Schmahl et al. reported also on a S1P lyase deficiency in mice (16). Briefly, cells were isolated by mild trypsinization (0.05% w/v) and dissociated by repeated passage through a constricted Pasteur pipette in a DNase solution (0.05% w/v) and detached with a cell lifter in the respective medium and centrifuged (2000 g, 10 min, 4 °C), thus collecting both adherent and detached cells. Cell pellets were then resuspended in 500 l of reaction buffer, containing dithiothreitol (10 mM) and DEVD-p-nitroanilin substrate (50 lM), samples were incubated for 1 h at 37 °C in the absence of light. Absorbance was measured at 405 nm. Effector caspase activities are expressed relative to untreated wild-type controls.

Western Blotting—Western blot analysis following SDS-PAGE was performed as described before (13) using anti-cyclin D1 antibody (A-12), anti-cyclin E antibody (M-20), and anti-α-tubulin antibody (H-300) from Santa Cruz Biotechnology (Santa Cruz, CA).

Labeling of Cells with 32P—Cells were rinsed with phosphate-free minimal essential medium (Promocell, Heidelberg, Germany) and then incubated in the same basic medium supplemented with 30 μCi/ml 32P, for 2 h. Cells were then treated with 10 μM of the indicated sphingolipid or vehicle. After 1 h S1P was extracted as described in detail previously (10) and resolved by TLC in 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v). Radioactively labeled S1P was visualized by autoradiography and quantified using the bioimaging analyzer FUJIX Bas 1000 and Software TINA 2.09 (Raytest).

siRNA Transfections—The following siRNA Duplexes were used to silence the S1P receptors: S1P1, GGAAUUAGCCG-CAGGAAAUU; S1P2, CGACUUUCCUGGGUUAAUU; and S1P3, GUAGGUCAUGCUCAGUAAUU. Transfections of cells with siRNA molecules were performed using Lipofectamin RNAiMAX and Opti-MEM (Invitrogen) as described by the manufacturer.

Cells were treated with siRNAs for 24 h prior to the addition of S1P. Knockdown of target gene transcription was confirmed by real-time PCR.

RNA Isolation and Real-time PCR—Around 1 μg of total RNA (isolated using a RNA isolation kit from Qiagen) was used in reverse transcription with the Super Script III First-Strand Synthesis System (Invitrogen) and random primers (Invitrogen) as recommended by the manufacturer. The resulting total cDNA was then used in real-time PCR to measure mRNA. The RNA levels of 18 S rRNA were used as internal controls. The following primers and Universal ProbeLibrary probes (Roche Applied Science) were used for real-time PCR amplification: S1P1 (probe #3), 5’-CTACACAACCGGGACACAG-CAACAG (for) and 5’-CCCCGGATGAGGGAGAT (rev); S1P2 (probe #78), 5’-CAGGATCTACTCTTGTGTCAGC (for) and 5’-GAGATGTCTTGTGCCGAGGT (rev); and S1P3 (probe #17), 5’-CCCAACTCCGGGACATAGA (for) and 5’-ACAGCCACTTGTGTTT (rev). The reactions were performed at 95 °C for 10 min, 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s.

Mass Spectrometry of Sphingolipids—Cells were incubated with sphingosine (10 μM), S1P (10 μM), or vehicle, respectively, for 24 h and harvested in the respective medium as described above. Sphingolipids (sphingosine, S1P, and (dihydro)ceramide species) were quantified by liquid chromatography electrospray ionization mass spectrometry as described previously (18).

Presentation of Data and Statistics—All experiments were repeated at least three times with consistent results. Data are expressed as means ± S.D. and normalized as indicated. Statistical analysis was performed using Student’s t test. Results presented as DNA or protein gels correspond to data obtained with at least three different cell preparations.
Subcellular Origin of S1P Determines Its Neurotoxic Effect

RESULTS

Elevation of the Amount of S1P, Sphingosine, and Ceramide in Lyase-deficient Cerebellar Neurons—The content of bioactive sphingolipids, including S1P, sphingosine, and ceramide, was analyzed in cerebellar neurons from wild-type, heterozygous, and lyase-deficient mice using liquid chromatography electrospray ionization tandem mass spectrometry as described under “Experimental Procedures.” Data (sphingosine, dashed bars; S1P, black bars) are averages of triplicate determinations and expressed relative to wild-type cells treated with vehicle (A) or as nanomoles/mg of cell protein (B). * significantly different from +/+ neurons treated with vehicle (p < 0.05).

and sphingosine, respectively, the sphingosine content in cells lacking lyase activity increased considerably (amounting to 20 nmol/mg and 33 nmol/mg, respectively), it remained below the content of S1P, which reached >33 nmol/mg and 35 nmol/mg, respectively (Fig. 1B). Note that the amount of free sphingosine usually exceeds that of S1P (19) as evident in wild-type and also in heterozygous cells.

As illustrated in Fig. 2A, cerebellar granule cells exhibit several molecular species of ceramide, the most abundant being those containing stearic acid (C18:0) and the second most abundant those with palmitic acid (C16:0) in amide linkage to the sphingosine backbone. Despite a significant increase of sphingosine and S1P in lyase-deficient neurons, no significant changes in the amount of cellular ceramide could be detected in these neurons. However, upon addition of S1P to the culture medium a significant increase in total cellular ceramide was observed in wild-type, heterozygous as well as in lyase-deficient neurons (Fig. 2B). Similarly, addition of sphingosine to the culture medium brought about a significant increase in the ceramide content of heterozygous and lyase-deficient neurons.
Note that this increase affected mainly the C16- and C18-ceramide subspecies and was highest in lyase-deficient neurons, amounting to 200–250% of the respective untreated cells (Fig. 2A). A considerable increase in total cellular ceramide (by 1.5-fold) was also found in wild-type and heterozygous neurons (Fig. 2B). In conclusion, exogenous sphingosine and S1P provoke similar changes in cellular content of bioactive sphingolipids in lyase-deficient neurons.

Morphological Changes of Lyase-deficient Neurons in the Presence of S1P—Light microscopy revealed no morphologic differences among wild-type, heterozygous, and lyase-deficient cerebellar granule neurons (Fig. 3, A–C). Cerebellar neurons from all three genotypes were fully differentiated and developed a rich network of fine fibers. Incubation with either sphingosine (Fig. 3, G and H) or S1P (Fig. 3, D and E) did not cause any striking differences in cell morphology of wild-type and heterozygous cells, respectively. Interestingly, there were considerable changes in cell morphology of lyase-deficient neurons incubated with S1P (Fig. 3F), but not when sphingosine was added (Fig. 3I). As illustrated in Fig. 3F, cell aggregates connected by radial running neurites were formed during 24-h incubation of lyase-deficient neurons in the presence of S1P (10 μM). Note that similar morphological changes have been reported to be induced by cis-4-methylsphingosine in wild-type neurons (10). In summary, exogenously added S1P, but not sphingosine, induces morphological changes in terminally differentiated lyase-deficient neurons, although both sphingoid bases similarly affected the content of bioactive sphingolipids in these neurons.

S1P but Not Sphingosine Induces Caspase-dependent Apoptosis in Primary Cultured Lyase-deficient Cerebellar Neurons—When the viability of neurons was assessed after 24 h of incubation with sphingosine or S1P (10 μM each), a considerable decrease in cell viability (by ~40%) was observed only in S1P treated lyase-deficient neurons (Fig. 4A). To determine if the decrease in cell viability by S1P was associated with induction of apoptosis, the activities of effector caspases as well as the integrity of genomic DNA were examined.

Caspase activity was increased by ~2.5-fold in S1P-treated lyase-deficient neurons (Fig. 4B), whereas no changes of caspase activity were found in wild-type and heterozygous cells, treated with S1P. Caspase activation was accompanied by
readily visible oligonucleosomal fragmentation of DNA in lyase-deficient neurons incubated with S1P (Fig. 4C). In contrast, sphingosine did not affect cell viability, caspase activity, and DNA integrity (Fig. 4D).

The effect of S1P on the viability of lyase-deficient neurons was compared with the previously described neuronal apoptosis induced by cis-4-methylsphingosine (via cis-4-methylsphingosine phosphate) (13). As depicted in Fig. 5, the decrease in viability and time courses were similar for cis-4-methylsphingosine in wild-type cells (Fig. 5A) and lyase-deficient cells treated with S1P (Fig. 5B). Treatment of wild-type neurons with S1P did not affect the viability of wild-type neurons, whereas cis-4-methylsphingosine was similarly toxic for both wild-type and heterozygous neurons (results not shown).

**S1P Partially Reactivates the Cell Cycle in Lyase-deficient Neurons**—We have shown previously that cis-4-methylsphingosine phosphate-induced neuronal apoptosis involves an inappropriate reactivation of the cell cycle (13). To determine whether S1P-induced apoptosis in lyase-deficient neurons involves a similar mechanism, we analyzed the expression of cyclin D1 and cyclin E. Cyclin D1 and its associated kinase, cyclin-dependent kinase 4, are major regulators of early steps in cell cycle progression, e.g. re-entry of quiescent cells into the cell cycle and start of G1 phase (25). Cyclin D1 has been reported to be involved in the induction of apoptosis in cerebellar granule cells and other postmitotic neurons (26, 27). Expression of cyclin E, on the other hand, denotes that a cell has overcome the restriction point in G1 and is committed to pass onto S phase.

As illustrated in Fig. 7, a sustained and significant up-regulation of cyclin D1 expression was observed in lyase-deficient neurons incubated with S1P, whereas expression of cyclin E was not induced by S1P.

**S1P Enhancement in Lyase-deficient Neurons following Addition of Sphingosine and S1P Is Ascribed to SK1 and SK2, Respectively**—As shown above exogenous S1P induces in lyase-deficient neurons effects that correspond to those reported for cis-4-methylsphingosine in wild-type neurons (10, 13). Most intriguing is the fact that sphingosine, the naturally occurring sphingoid base for which cis-4-methylsphingosine was prepared as an analog (10) does not. Different origins of intracellular S1P following addition of sphingosine and S1P, respectively, could explain this apparent contradiction. To evaluate this possibility, we performed phosphorylation experiments using neurons prepared from mice lacking either SK1 or SK2 activity. Fig. 8 depicts to what extent SK1 and SK2, respectively, catalyze the formation of intracellular S1P. In neurons lacking SK1 activity the formation of S132P following addition of sphingosine is reduced by ∼70% when compared with wild-type neurons treated alike, whereas formation of S132P following addition of S1P is only slightly affected being reduced by ∼20% compared with wild-type cells. Note that phosphorylation of cis-4-methylsphingosine is also not affected in SK1-deficient neurons indicating that this synthetic sphingosine is primarily phosphorylated by SK2. By contrast, in neurons lacking SK2 activity the formation of S132P following addition of sphingosine is only marginally affected, whereas formation of S132P following S1P addition is reduced by ∼60% compared with wild-type cells. Also phosphorylation of cis-4-methyl-
sphingosine is drastically reduced by ~80% in SK2-deficient neurons compared with wild-type neurons.

**DISCUSSION**

The central aim of the present study was to evaluate the hypothesis that endogenous S1P induces neuronal apoptosis when it accumulates above a certain threshold, thus constituting a potential hitherto missing link in neurodegeneration. To eliminate degradation of S1P we used primary cultured neurons prepared from cerebella of 6-day-old lyase-deficient mice. Incubation of the lyase-deficient cells with either S1P or sphingosine resulted in equal elevations in S1P (referred to as S1P_{S1P} and S1P_{Sph} for that derived from S1P or sphingosine, Sph, respectively), however, only the elevation in S1P_{S1P} induced neuronal apoptosis, and the additional changes in cell morphology and aberrant cell cycle reactivation, whereas S1P_{Sph} did not. Notably the S1P_{S1P} effects were similar to what has been observed previously in wild-type neurons incubated with cis-4-methylsphingosine that was metabolized to the phosphate (10, 13). This intriguing result is unlikely to be explained by an elevated ceramide content in lyase-deficient neurons incubated with S1P, because ceramide contents were equally increased also upon sphingosine addition to the culture medium. It thus appears that at least in postmitotic terminally differentiated neurons ceramide is not causative for the apoptotic processes observed. It is of interest that elevation of endogenous sphingoid base phosphates following elimination of lyase and phosphatase pathways have been reported to kill yeast cells as well (28).

An important finding of the present study is that, in addition to a certain threshold, the origin of the S1P is essential for its apoptotic effect. Thus, S1P_{S1P} and S1P_{Sph} are distinguished by the sphingosine kinase that produces them: with SK2 mainly generating S1P_{S1P} (and cis-4-methylsphingosine phosphate) and SK1 primarily producing S1P_{Sph} most probably at distinct sub-cellular sites. Hence our data argue in favor of the assumption that signaling and regulatory functions of bioactive sphingolipids are closely associated with the sub-cellular compartment(s) where they are generated (19). With respect to the production of S1P by the two SK isoenzymes, it is well known that, despite some similarity in amino acid sequence (29, 30), these isoenzymes differ in kinetic properties and sub-cellular localization as well as in developmental and tissue expression (30), suggesting distinct physiological functions. SK1 is located in the cytosol (and translocates to the plasma membrane upon activation) and is associated with protection of cells against apoptosis (31), whereas SK2 resides mainly in the endoplasmic reticulum (ER) and is associated with inhibition of cell growth and induction of apoptosis (32, 33). Hence, our findings regarding the production of S1P_{S1P} by primarily SK2 (and the same
Subcellular Origin of S1P Determines Its Neurotoxic Effect

FIGURE 8. Sphingosine kinase (SK) isozymes contribute to a different extent to the formation of intracellular S1P following administration of sphingosine and S1P, respectively. Cerebellar neurons from wild-type (+/+), SK1 (SK1 –/–) or SK2 (SK2 –/–)-deficient mice were rinsed with phosphate-free minimal essential medium and incubated in the same medium containing [32P]labeled Sph (30 μCi/ml) for 2 h. Then vehicle (gray bars) or 10 μM of sphingosine (Sph, dotted bars), S1P (black bars), or cis-4-methylsphingosine (cis) were added. After 1 h cells were harvested, and sphingoid phosphates (S1P and cis-4-methylsphingosine phosphate, cisP) were extracted, separated by TLC, and quantified as described under “Experimental Procedures.” Data are averages from at least three separate experiments and were normalized to wild-type controls treated alike. *, significantly different from cells treated alike. **, significantly different from cells treated alike (p < 0.05).

FIGURE 9. Scheme of the proposed two different sources generating functionally distinct intracellular S1P. The apoptotic effect of cis-4-methylsphingosine phosphate (cisP) has been reported in the past (10, 13). Subcellular localization of sphingosine kinases (SK1 and SK2) has been proposed by Sarah Spiegel’s group (33). PM, plasma membrane; S1Pcis, S1P generated after sphingosine addition; S1Pcis, S1P generated after S1P addition; SPP, sphingosine-phosphate phosphatase.

for phosphorylation of cis-4-methylsphingosine) suggest their formation is occurring in the ER (Fig. 9). The lower specificity of SK2 with respect to the sphingoid base substrate (30) was confirmed by phosphorylation of FTY720 (34) and is reflected also in phosphorylation of cis-4-methylsphingosine in this study. Based on these findings, we propose the working model in Fig. 9, wherein exogenous S1P is taken up by the cells by an unknown mechanism, followed by dephosphorylation and rephosphorylation by SK2 to generate S1Pcis, whereas S1Pspht is generated mainly by SK1 in the plasma membrane and possibly cytosol. This is consistent with the recent report that, for several lysosome-deficient cell types (35), exogenous S1P is initially cleared most probably by the S1P phosphatase activity associated with ER membranes (36). Accordingly, one would predict that cis-4-methylsphingosine phosphate is also produced in the ER, similar to the S1Pspht formed in lysosome-deficient neurons incubated with S1P. On the other hand, the S1Pspht would be expected apart from the ER, most probably at the plasma membrane (Fig. 9).

Thus, our results suggest that S1P (and cis-4-methylsphingosine phosphate) are cytotoxic when generated by SK2 in lysosome-deficient neurons (and wild-type cells for cis-4-methylsphingosine phosphate) and reaching a certain threshold, consistent with our earlier model (13). In contrast, S1P generated by SK1 failed to induce apoptosis, although it accumulated to the same extent as the SK2-derived S1P in lysosome-deficient neurons.

Note that exogenous S1P brought about the formation of radioactively labeled S1P not only in SK1 but also in SK2-deficient neurons albeit to a significantly lower extent. This result suggests that a minor, yet significant amount of exogenous S1P, might be dephosphorylated already on its way to the ER, most probably by lipid phosphate phosphatases (37). The sphingosine fraction generated in this way is most probably rephosphorylated by SK1.

Collectively, our results highlight the importance of evaluation of the origin and localization of S1P production in interpreting the role of this bioactive compound on cells. They also illustrate the utility of studies using sphingoid base analogs such as cis-4-methylsphingosine and its cellular metabolite cis-4-methylsphingosine phosphate, which first uncovered that a sphingoid base 1-phosphate could have a pro-apoptotic effect in neurons (13).

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Subcellular Origin of S1P Determines Its Neurotoxic Effect

APRIL 24, 2009 • VOLUME 284 • NUMBER 17 • JOURNAL OF BIOLOGICAL CHEMISTRY

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