Phosphorylated cis-4-Methylsphingosine Mimics the Mitogenic Effect of Sphingosine-1-phosphate in Swiss 3T3 Fibroblasts*

Gerhild van Echten-Deckert‡, Andreas Schick§, Thomas Heinemann¶, and Barbara Schnieders†

From the Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, 53121 Bonn, Germany, and the Institut für Klinische Chemie der Universität Köln, 50924 Köln, Germany

The phosphorylated derivative of sphingosine, sphingosine-1-phosphate, is a short-living metabolite of ultimate ceramide degradation and was shown to act as an intracellular signaling molecule, stimulating cell proliferation in quiescent Swiss 3T3 fibroblasts and inducing the release of calcium from intracellular stores (Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) J. Cell. Biol. 114, 155–167). In the present study, 24-h treatment of Swiss 3T3 fibroblasts with the synthetic sphingosine analogue cis-4-methylsphingosine resulted in proliferation of quiescent Swiss 3T3 fibroblasts that was 3-fold stronger than that of equimolar sphingosine-1-phosphate. The phosphorylated derivative of cis-4-methylsphingosine accumulated drastically in the cells. Simultaneous treatment with the sphingosine kinase inhibitor L-threo-sphinganine reduced both the amount of phosphorylated cis-4-methylsphingosine and cell proliferation induced by this compound by about 50%, indicating that the phosphorylated derivative mediated the proliferative stimulus. The mitogenic effect of cis-4-methylsphingosine was associated with a mobilization of intracellular calcium in Swiss 3T3 fibroblasts that was similar to that induced by sphingosine-1-phosphate.

The results demonstrate that the phosphorylated derivative of cis-4-methylsphingosine mimics the previously reported mitogenic action of sphingosine-1-phosphate in Swiss 3T3 cells, and the stronger effect most likely corresponds to the unusual accumulation of this compound.

Sphingolipids (SL), i.e. glycosphingolipids and sphingomyelin, are structural molecules of the plasma membrane of eukaryotic organisms. Glycosphingolipids are involved in intercellular and cell-matrix interactions and membrane binding of antibodies, bacteria, and viruses (1). Sphingolipid catabolism converges on ceramide, which is consecutively degraded to dihydrosphingosine (14). The rapid turnover of SPP provides an appropriate rationale for the phosphorylation of sphingosine by sphingosine kinase (12, 13) and rapid ultimate cleavage into ethanolamine and the corresponding aldehyde by sphingosine-phosphate lyase (14). The rapid turnover of SPP provides an appropriate requirement for a signaling second messenger (10); however, it makes SPP-mediated signaling difficult to examine.

Recent results from our laboratory suggest that the phosphorylated derivative of the sphingosine analogue cis-4-methylsphingosine might act as a metabolically stable mimetic of SPP (15). Like sphingosine, cis-4-methylsphingosine is efficiently phosphorylated in primary cultured mouse cerebellar neurons. In contrast to SPP, the corresponding phosphorylated derivative is, however, not subject to rapid degradation but was demonstrated to accumulate intracellularly. To further determine the potential role of cis-4-methylsphingosine phosphate as a mimetic of SPP, the effect of cis-4-methylsphingosine on cell growth regulation was examined in Swiss 3T3 fibroblasts. As previously shown in primary cultured neurons (15), we demonstrate here a substantial accumulation of cis-4-methylsphingosine phosphate in Swiss 3T3 cells. cis-4-Methylsphingosine treatment mimicked the mitogenic effect of SPP, but resulted in a much stronger proliferative response. The decrease of both proliferation and phosphorylation by inhibition of sphingosine kinase indicates that cis-4-methylsphingosine phosphate mediates the proliferative stimulus.

EXPERIMENTAL PROCEDURES

Materials—cis-4-Methylsphingosine was synthesized and purified as described (15). L-threo-C14-sphinganine was synthesized in our laboratory according to Clasen (16). SPP was obtained from Biomol (Hamburg, Germany). All culture media were obtained from Life Technologies, Inc. (Karlsruhe, Germany). All other chemicals and supplies were purchased as described previously (15).

Cell Culture—Swiss 3T3 fibroblasts, obtained from Prof. A. H. Merrill Jr. (Emory University, Atlanta, GA), were cultured in DMEM,

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† To whom correspondence should be addressed: Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Straße 1, 53121 Bonn, Germany. Tel.: 49-228-73-2703; fax: 49-228-73-7778; E-mail: echter@chemie.uni-bonn.de.
‡ Present address: Hoechst Marion Roussel Deutschland GmbH, 65812 Bad Soden am Taunus, Germany.
§ Supported by a fellowship from Görres-Gesellschaft, Germany.
¶ The abbreviations used are: SL, sphingolipid; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; SPP, sphingosine-1-phosphate; L-threo-sphinganine, L-threo-C14-sphinganine (the term sphinganine is used for dihydrosphingosine).
supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum as well as penicillin (100 units/liter) and streptomycin (100 mg/liter).

For experiments, cells were subcultured in 8-cm² Petri dishes. Medium was renewed every 48 h until confluency was reached. Swiss 3T3 fibroblasts were kept 5 days confluent and quiescent before use.

Labeling of Cells with 3H-Thymidine—Cells were washed with phosphate-free DMEM and subsequently incubated with this medium containing 3H-thymidine (40 μCi/ml) for 24 h as described (8). The cells were treated with 10 μM of the respective sphingoid for either 1 or 24 h. For inhibition studies, sphingosine-1-phosphate (SPP) and sphingosine (10 μM) were added simultaneously. Sphingosine phosphates were extracted following the method of Yatomi et al. (17), as described in detail previously (15). Phosphorylated sphingosines were resolved by thin layer chromatography in 1-butanol/methanol/acetic acid/water (80:20:10:20, by volume), visualized by autoradiography, and identified by their RF value.

Assay of DNA Synthesis—DNA synthesis was estimated by incorporation of tritiated thymidine into trichloroacetic acid-precipitable cellular material as described previously (8). Quiescent cultures were washed three times with DMEM to remove residual serum and incubated for 12 h with 1 ml of DMEM/Waymouth medium (1:1) supplemented with 20 μg/ml bovine serum albumin (BSA) and 5 μg/ml transferrin. Cells were treated with the respective sphingoid added as a complex with BSA. After 18 h, cells were pulsed with 1 μCi of [3H]thymidine for 6 h. The incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described by Spiegel and Panagiotopoulous (18).

Measurement of Cytoplasmic Free Ca²⁺ Concentrations—Cytoplasmic free calcium concentrations [Ca²⁺], were determined with the fluorescent calcium indicator dye Fura-2 AM in a Perkin-Elmer LS-5B spectrofluorimeter equipped with a fast filter device as described previously (19). Briefly, confluent and quiescent Swiss 3T3 cells were harvested by trypsinization, and the cell pellet was resuspended in a buffer (pH 7.4) containing 1 μM Fura-2 AM, 137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 6.5 mM Na₃HPO₄, 1.5 mM KH₂PO₄, 1 mg/ml BSA, and 1 mg/ml glucose (0.5–1.0 × 10⁶ cells/ml). After 1 h at 37°C, cells were washed twice, resuspended in fresh buffer and immediately used for fluorescence measurements. To remove dye leaked into the medium, aliquots were pelleted in a microfuge, resuspended in prewarmed buffer without BSA, and transferred to a thermostatted cuvette (37°C) in the spectrofluorimeter. 10 μM of the respective sphingosine derivatives were added as complexes with BSA in phosphate-buffered saline. Excitation was alternating at 340 and 380 nm, and emission read at 495 nm. Fluorescence data were converted into calcium concentration using software supplied by the manufacturer.

Analysis of Apoptosis—DNA fragmentation was determined by using “Cell Death Detection ELISA™ Plus” test kit according to the instructions given by the manufacturer (Boehringer Mannheim, Mannheim, Germany). This photometric enzyme immunoassay allows for the quantitative in vitro measurement of histone-associated DNA fragments (mono and oligonucleosomes) in the cytoplasmic fraction of cell lysates. Cells were harvested and lysed 24 h after addition of the respective sphingosine derivative. The cytoplasmic nucleosome enrichment factor was calculated from the ratio of absorbance measured in treated cells versus untreated control cells, thus illustrating the specific enrichment of mono- and oligonucleosomes released into the cytoplasm of treated cells.

In addition, chromatin fragmentation was determined by quantitative nuclear fluorescence morphology assay using bisbenzimide (Hoechst stain H 33258, obtained from Sigma, Deisenhofen, Germany). Cells were incubated with 10 μM of the respective sphingosine derivative for 24 h, trypsinized, washed with phosphate-buffered saline, and fixed in phosphate-buffered saline containing 3% paraformaldehyde. Cells were stained with bisbenzimide for 15 min at room temperature, transferred to glass slides, and examined by fluorescence microscopy. 300 cells per experiment were scored on chromatin fragmentation. The presence of at least three chromatin fragments per cell was considered as apoptosis, and the ratio to cells with intact nuclei was calculated.

RESULTS

cis-4-Methylsphingosine Exhibits a Strong Proliferative Effect in Swiss 3T3 Fibroblasts—To examine the effect of cis-4-methylsphingosine on proliferation of quiescent Swiss 3T3 fibroblasts, [3H]thymidine incorporation into DNA was assayed and compared with the effect of SPP and sphingosine, respectively, as shown in Fig. 1. Relative to untreated control cells, incubation of cells with cis-4-methylsphingosine resulted in an ~10-fold increase of [3H]thymidine incorporation, whereas incorporation was 3-fold and nearly 2-fold after treatment of cells with SPP and sphingosine, respectively.

To further differentiate whether cis-4-methylsphingosine or its endogenously phosphorylated derivative caused the marked effect on proliferation in Swiss 3T3 fibroblasts, sphingosine kinase was blocked with the competitive inhibitor l-threo-sphinganine (12). As shown in Fig. 1, when l-threo-sphinganine was combined with cis-4-methylsphingosine, the proliferative effect of this compound was significantly reduced by about 50%. l-threo-sphinganine also reduced the mitogenic effect of sphingosine, however, to a lesser extent. These results suggest that the induction of the proliferative response relied on the phosphorylated derivative of cis-4-methylsphingosine.
The Mitogenic Effect of cis-4-Methylsphingosine Relies on the Intracellular Accumulation of Its Phosphorylated Derivative—

Fig. 2 depicts the in vivo phosphorylation of cis-4-methylsphingosine in Swiss 3T3 fibroblasts prelabeled with $^{32}$P. Relative to the amount of endogenously labeled SPP of control cells (100%), the amount of phosphorylated cis-4-methylsphingosine was found to be elevated about 13-fold after 1 h of treatment, rising to about 45-fold after 24 h of treatment.

When sphingosine kinase was inhibited with L-threo-sphinganine (20 μM), the elevated intracellular amount of phosphorylated cis-4-methylsphingosine was reduced by about 15 and 50% after 1 and 24 h, respectively (Fig. 2). Thus the decrease of cis-4-methylsphingosine-induced cell proliferation observed upon simultaneous treatment with L-threo-sphinganine is paralleled by its inhibitory effect on phosphorylation.

The Mitogenic Effect of cis-4-Methylsphingosine in Swiss 3T3 Fibroblasts Is Accompanied by Intracellular Calcium Mobilization—The SPP-induced stimulation of DNA synthesis was shown to correlate well with an SPP-triggered mobilization of calcium from intracellular stores, suggesting that the mitogenic response is mediated, at least in part, by intracellular calcium release (8). The effect of 10 μM cis-4-methylsphingosine, SPP, and sphingosine on mobilization of intracellular calcium is shown in Fig. 3. The release of free calcium was similar when cells were incubated with SPP and cis-4-methylsphingosine, respectively. After cis-4-methylsphingosine treatment, the amplitude of free calcium was less pronounced compared with SPP, and the decrease of free calcium to baseline levels was somewhat delayed. These results, however, suggest that both compounds trigger analogous mechanisms in cellular metabolism. In contrast, less calcium release was induced by sphingosine, which correlates with the reduced effect of this compound on cell proliferation.

cis-4-Methylsphingosine Is Not Apoptotic in Swiss 3T3 Fibroblasts—Sphingoid bases have been demonstrated to induce apoptosis in a variety of cell types (20). Also, SPP (20 μM) was demonstrated to induce apoptosis in human hepatoma cells (21). To rule out that cis-4-methylsphingosine alone or in combination with L-threo-sphinganine exhibits apoptotic effects in Swiss 3T3 cells, apoptosis was quantified by determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of the cell lysates in vitro by a photometric enzyme immunoassay (Boehringer Mannheim). In addition, apoptosis was assessed by nuclear fluorescence.
TABLE I

Determination of apoptosis in Swiss 3T3 fibroblasts

Apoptosis was determined in quiescent Swiss 3T3 cells by assessment of DNA fragmentation after 24 h of treatment with the indicated compounds. Concentrations used were 10 μM mitoxantrone, 20 μM L-threo-sphinganine, and 10 μM all other sphingoids, respectively. The left column represents the cytoplasmic nucleosome enrichment factor obtained from the Cell Death Detection ElisaPLUS test kit (Boehringer Mannheim), which was calculated from the ratio of absorbance measured in treated versus untreated control cells. The right column represents the percentage of cells showing at least three chromatin fragments, as determined by a quantitative nuclear fluorescence morphology assay from a total of 300 cells. Results are means of at least three different experiments. cis-4-MeSo, cis-4-methylsphingosine; L-t-Sa, L-threo-sphinganine. ND, not determined as the color of this drug interferes with the absorbance measurement.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytoplasmic enrichment of mono- and oligonucleosomes (fold increase)</th>
<th>Percentage of apoptotic cells (chromatin fragments ≥ 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>ND</td>
<td>41.6 ± 5.2</td>
</tr>
<tr>
<td>Sphingosine-1-phosphate</td>
<td>1.10 ± 0.10</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>1.23 ± 0.08</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>cis-4-Methylsphingosine</td>
<td>1.09 ± 0.11</td>
<td>2.9 ± 1.3</td>
</tr>
<tr>
<td>L-threo-sphinganine</td>
<td>1.36 ± 0.07</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>cis-4-MeSo + L-t-Sa</td>
<td>1.30 ± 0.10</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>

morphology. Mitoxantrone was used as positive control. As given in Table I, although treatment with 10 μM mitoxantrone caused significant cell death, neither of the other compounds alone or in combination did so, as assessed with both methods.

**DISCUSSION**

SPP, the phosphorylated degradation product of sphingosine, was shown previously to function as a second messenger in sphingolipid-mediated signaling, inducing cell proliferation in Swiss 3T3 cells (6, 8, 22). In the present study, treatment of Swiss 3T3 fibroblasts with cis-4-methylsphingosine resulted in a much stronger proliferative effect compared with SPP. The same set of enzymes catalyzing sphingosine degradation via SPP is supposed to metabolize cis-4-methylsphingosine as well. As demonstrated in the present study, the compound is indeed efficiently phosphorylated, however, whereas the half-life of SPP is relatively short because of rapid ultimate degradation (23, 24), phosphorylated cis-4-methylsphingosine accumulates substantially in the cells, as was also shown previously in primary cultured cerebellar neurons (15). Thus, as both cell types have little physiological resemblance, the delayed degradation of this compound appears to be related to the altered molecular structure, which may reduce the rate of either sphingosine lyase-catalyzed degradation or phosphatase-catalyzed dephosphorylation, or both.

Besides the striking similarity of the effects of cis-4-methylsphingosine and SPP treatment on proliferation in Swiss 3T3 cells, evidence for the phosphorylated derivative being the mitogenic agent was obtained in the present study by blocking sphingosine kinase with the competitive inhibitor L-threo-sphinganine (13). This compound was shown to decrease DNA synthesis in Swiss 3T3 cells induced by platelet-derived growth factor or serum by 45% and 55%, respectively (25). L-threo-sphinganine decreased the proliferative effect of cis-4-methylsphingosine to a similar extent. Moreover, strong support for the involvement of phosphorylation of cis-4-methylsphingosine in the proliferative effect comes from direct determination of phosphorylation in cultured cells in the absence and presence of L-threo-sphinganine. When sphingosine kinase was inhibited, 32P-labeling of cis-4-methylsphingosine was reduced by about 50% after 24 h compared with noninhibited cells. This reduction caused by L-threo-sphinganine was clearly not because of an apoptotic effect of this compound, neither when applied alone nor in combination with cis-4-methylsphingosine (Table I).

The proliferative effect of SPP in Swiss 3T3 cells was demonstrated to be associated with mobilization of calcium from intracellular stores (8). This concept is supported by our data demonstrating that SPP and cis-4-methylsphingosine, both compounds inducing proliferation, similarly triggered substantial calcium release. Treatment with sphingosine, which exhibits less proliferative effect, resulted in an only moderate calcium increase. In cultured cells, the unique calcium mobilization by exogenous SPP was demonstrated to be mediated by high affinity interaction with G-protein-coupled membrane receptors, whereas sphingosine and other sphingolipids appear to be poor ligands to these receptors as judged by their calcium releasing effect (26). However, high affinity binding of SPP to G-protein-coupled cell membrane receptors in human (EDG-3 (27) as well as EDG-1 (28)) and rat (H218) (27) mediates a subset of the cellular actions of SPP but not calcium mobilization (29). It appears, however, unlikely that the proliferative effect of endogenous SPP, which is thought to serve as short living mitogenic signaling molecule, exclusively relies on the activation of receptors located on the cell membrane. A substantial part of the SPP-induced cell proliferation might be mediated by putative intracellular ligand-gated Ca2+-channels (7, 30, 31). In this concept, efficient calcium mobilization requires phosphorylation of the respective sphingosine derivative which could explain the weak calcium release induced by exogenous sphingosine compared with SPP, and the much higher effect of intracellularly accumulated cis-4-methylsphingosine-phosphate. Further work needs to clarify these mechanisms.

The results of this study demonstrate a striking similarity of the effects of SPP and phosphorylated cis-4-methylsphingosine on cell growth regulation in quiescent Swiss 3T3 fibroblasts. The stronger effect of cis-4-methylsphingosine on cell proliferation compared with SPP most likely corresponds to the unusual intracellular accumulation of this compound, which might result in sustained stimulation of signaling pathways.

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