Ceramide Conversion to Sphingosine-1-Phosphate is Essential for Survival in C3H10T½ Cells

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ABSTRACT Ceramide and sphingosine-1-phosphate (S1P) are important dietary lipids involved in cell growth, differentiation, apoptosis and cell survival. Treatment of C3H10T½ murine fibroblast cells (10T½) with ceramide did not induce apoptosis, a commonly observed effect of ceramide treatment. To determine whether the metabolism of ceramide played a role in this resistance to apoptosis, inhibitors of ceramidase and sphingosine kinase, two important enzymes in sphingolipid metabolism, were used. Treatment of 10T½ cells both without or with ceramide plus N-oleoyl-ethanolamine (NOE) and (1S,2R)-o-erythro-s-(N-myristoylamino)-1-phenol-1-propanol (MAPP), two ceramidase inhibitors, resulted in fourfold and eightfold increases, respectively, in apoptosis. Cells treated without or with ceramide plus N,N-dimethylsphingosine (DMS), a potent sphingosine kinase inhibitor, resulted in fourfold and sixfold increases, respectively, in apoptosis. In all treatments the induction of apoptosis was prevented by the addition of S1P. With the addition of S1P with NOE and MAPP as well as with ceramide, treatments reduced the apoptotic response by 30 and 35%, respectively; whereas the addition of S1P with the DMS only and ceramide with DMS treatments reduced the apoptotic response by 60 and 70%, respectively. Studies using labeled ceramide demonstrated ceramide was metabolized to S1P. In addition, a 14-fold increase in apoptosis occurred in cells treated with a nonhydrolyzable analog of ceramide, ceramine, compared with vehicle control. Because inhibiting the conversion of ceramide to S1P resulted in apoptosis, the lack of an apoptotic response to ceramide alone for C3H10T½ cells is attributable to the conversion of this pro-apoptotic sphingolipid to the anti-apoptotic metabolite S1P, which is essential for cell survival. J. Nutr. 131: 2826–2830, 2001.

KEY WORDS: • C3H10T½ cells • ceramide • sphingosine-1-phosphate • apoptosis

Sphingolipids are significant components of foods that have potentially powerful anticancer properties (1,2). Recently the sphingolipids, ceramide and sphingosine-1-phosphate (S1P)2, have been identified as important signaling molecules involved in cell growth, differentiation and apoptosis (3). The sphingolipid metabolites, ceramide, sphingosine and S1P, may all be derived from more complex sphingolipids, such as sphingomyelin (1). Sphingomyelin is a ubiquitous sphingolipid found in the plasma membrane of all mammalian cells (4). Upon appropriate stimuli, ceramide may be released from sphingomyelin by the action of distinct sphingomyelinases. There are a variety of agonists that stimulate sphingomyelin hydrolysis, such as 1,25-dihydroxyvitamin D3, tumor necrosis factor-α, interleukin-1β and ionizing radiation (5). Evidence from cell culture studies indicates ceramide activates many intracellular targets, such as specific kinases, phosphatases, and transcription factors that mediate a variety of cellular functions (6). The most noted cellular effect of ceramide is the induction of apoptosis, but growth arrest and cell differentiation have also been observed (7).

Ceramide is metabolized to sphingosine through the action of ceramidase, of which three isoforms, including an acidic, neutral and alkaline form, are currently known (8). Sphingosine, in turn, is phosphorylated by sphingosine kinase, producing S1P (9). Like ceramide, S1P exerts many actions within cells, such as cell proliferation and differentiation, as well as cell survival. Moreover, S1P suppresses ceramide-induced apoptosis (10,11). Ceramide initiates apoptosis through the induction of the stress-activated protein kinase (SAPK), a member of the mitogen-activated protein kinase (MAPK) family (12). S1P inhibits ceramide-induced apoptosis through the activation of the extracellular signal–regulated protein kinase (ERK), also a MAPK family member (10). Therefore, ceramide and S1P have opposing cellular effects mediated through activation of different MAPK family members producing different cellular outcomes (13).

The activities of several of the enzymes involved in sphingolipid metabolism, such as sphingomyelinase, ceramidase and sphingosine kinase, may be modulated by extracellular stimuli (14). For example, both ceramidase and sphingosine kinase may be activated by platelet-derived growth factor, fibroblast growth factor or other mitogenic compounds that produce sphingosine and S1P (15,16). Activation of these enzymes leads to the elevation of levels of one sphingolipid metabolite

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more than the other, and a change in the balance of ceramide to S1P dictates the cellular response. Therefore, the accumulation of ceramide via sphingomyelinase activation induces apoptosis, whereas the activation of sphingosine kinase produces increased levels of S1P and prevents apoptosis. In the present study we demonstrate that ceramide conversion to S1P is essential for cell survival in 3H11T1⁄2 (10T⁄2) cells.

MATERIALS AND METHODS

C8-ceramide, C8-ceramine, sphingosine-1-phosphate, N,N-di-methylsphingosine and (1S,2R)-(+)-erythro-2-(N-myristoyl)-1-phenol-1-propanol were purchased from Biomol (Plymouth Meeting, PA). N-ol-eoyl-ethanolamine was purchased from Sigma (St. Louis, MO), and Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum, and trypsin were purchased from Gibco (Rockville, MD). [14C]N-octanoyl-(-)-erythro-sphingosine (C8-ceramide) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Culture dishes (100 and 60 mm) were obtained from Falcon, and 15-mm polystyrene centrifuge tubes were purchased from Corning Scientific. Reagents used for fluorometric and apoptosis analysis were purchased from Boehringer Mannheim (Indianapolis, IN).

Cell culture. 10T⁄2 mouse fibroblast cells (CCL-226) were used in all experiments. Cells were grown in DMEM with 10% fetal bovine serum, 1.0 × 10^5 units/L penicillin and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% O₂. All experiments were performed with cells in linear growth.

Assessment of apoptosis. 10T⁄2 cells were plated at 105,000 cells/dish for 3 days in 100-mm culture dishes. A total of 48 h after plating, cells were treated with 20 μmol/L C8-ceramide, 20 μmol/L C8-ceramine (a nonhydrolyzable analog of ceramide) or 2.5 μmol/L S1P for 24 h. Upon harvesting, both adherent and nonadherent cells were collected and used for analysis. To harvest nonadherent cells, the media from each treatment were collected. Adherent cells were treated with 20 μmol/L C8-ceramide and 20 μmol/L C8-ceramine, 10 μmol/L C8-ceramide or 5 μmol/L S1P in one set of experiments and with 10 μmol/L C8-ceramide, 2.5 μmol/L S1P, 7.5 μmol/L dimethylsphingosine (DMS), 5 μmol/L N-ol-eoyl-ethanolamine (NOE) and 10 μmol/L (1S, 2R)-(+)-erythro-2-(N-myristoylamo)-1-phenol-1-propanol (MAPP) for another set of experiments. Cells were harvested as described above and apoptosis was assessed as directed by manufacturer’s instructions. This assay is a photometric enzyme-immunoassay based on the detection of in vitro cytoplasmic histone-associated DNA-fragments of mono- and oligonucleosomes. Data are expressed as absorbance at 405 nm of each sample over the vehicle control.

Metabolism of radio-labeled ceramide. 10T⁄2 cells were plated at 35,000 cells/dish in 35-mm dishes for 2 d. Two mg/mL [14C] C8-ceramide and 20 μmol/L unlabeled C8-ceramide were added for 2, 4, 6, and 8 h to the media of the cells. At the end of each short-term time point, media was removed and the cells were rinsed with CMF-PBS and scraped into 1 mL of 0.1mol/L HCl. Lipids were extracted by a chloroform:methanol:HCl (100:200:1, v/v) mixture for ceramide and sphingomyelin components and by a chloroform:methanol:Nacetile (2:1:2, v/v) mixture for sphingosine-1-phosphate. Sphingomyelin, ceramide, and sphingosine-1-phosphate components were determined by thin layer chromatography, as described by Olivera et al. (18) and Ruis et al. (19). Briefly, extracted lipids were sequentially separated using Silica gel coated thin layer chromatography plates in a chloroform:benzene:ethanol (80:40:75, v/v) solvent mixture, followed by a chloroform:methanol:28% ammonium hydroxide (65:25:5, v/v) solvent mixture for ceramide and sphingomyelin or by a 1-butanol:ethanol:acetic acid/water (8:2:1, v/v) mixture for sphingosine-1-phosphate. Lipids were visualized by iodine and samples were scanned with standards were scanned and radioactivity determined via scintillation counting. Data are expressed as cpm/μg protein.

Statistical analysis. Data were analyzed by one-way ANOVA followed by Duncan’s Multiple Range test (α = 0.05) with Statistical Analysis Software (SAS) (Cary, NC).

RESULTS

10T⁄2 cells are resistant to ceramide-induced apoptosis. Treatment of 10T⁄2 cells with ceramide, S1P or vehicle control did not induce apoptosis (Fig. 1). In addition, 10T⁄2 cells were treated with a higher concentration of ceramide, 50 μmol/L with no induction of apoptosis (data not shown).

Inhibition of ceramide metabolism induces apoptosis in 10T⁄2 cells. To determine if the metabolism of intracellular ceramide played a role in the resistance to ceramide-induced apoptosis, inhibitors to the enzyme ceramidase were employed. N-ol-eoyl-ethanolamine (NOE) is an inhibitor to the acidic form of ceramidase, and (1S, 2R)-(+)-erythro-2-(N-myristoylamo)-1-phenyl-1-propanol (MAPP) is an inhibitor to the alkaline form of ceramidase. Ceramide, S1P, NOE and MAPP did not stimulate an apoptotic response (Fig. 1). However, the combination of the two ceramide inhibitors, NOE and MAPP, induced an apoptotic response fourfold over control treatments (Fig. 1). The addition of ceramide to the inhibitors increased the apoptotic response eightfold over controls. However, when the ceramide metabolite S1P was present in the NOE/MAPP inhibitor treatment with or without ceramide, the apoptotic response was substantially reduced.

FIGURE 1 Induction of apoptosis by inhibiting the conversion of ceramide to sphingosine in 10T⁄2 cells. Cells were treated for 12 h with 10 μmol/L C8-ceramide or 5 μmol/L S1P with and without the ceramidase inhibitors, NOE or MAPP. Apoptosis was determined by the Cell Death Detection ELISA plus system as described in “Methods.” Data are the results of three independent experiments shown as means ± SE. Bars with different letters differ significantly, P < 0.05.
S1P reduced the apoptotic response of the NOE and MAPP group by 30% and by 35% in the ceramide plus NOE and MAPP group. Thus, inhibition of intracellular conversion of ceramide to sphingosine or S1P induced apoptosis, and this response was increased by exogenously added ceramide. In addition, exogenous addition of the end product of the metabolic conversion, S1P, protected the cells from apoptosis. These results suggest that the conversion of ceramide to S1P is essential for cell survival.

In addition to the ceramidase inhibitors, DMS, an inhibitor of sphingosine kinase, the enzyme responsible for phosphorylating sphingosine to produce S1P, was used. DMS alone induced an apoptotic response fourfold over ceramide and S1P controls, similar to the treatment with NOE and MAPP (Fig. 2). Likewise, the addition of exogenous ceramide with DMS enhanced the apoptotic response in 10T½ cells, with a sixfold increase in apoptosis over controls. When S1P was added to either the DMS-treated or DMS- and ceramide-treated cells, the apoptotic response was markedly reduced (60 and 70%, respectively) (Fig. 2). These results further demonstrate that the conversion of ceramide to S1P is essential for cell survival.

**Exogenous ceramide is metabolized to S1P.** To examine if exogenous ceramide is metabolized to S1P, we used [14C]-labeled ceramide. Exogenously added labeled ceramide is metabolized slowly in 10T½ cells (Fig. 3). Ceramide remains predominantly intact. However, labeled ceramide was metabolized to sphingosine (Fig. 3) and, to a lesser extent, converted to S1P (Fig. 3). In addition, <1% of labeled ceramide was incorporated into the more complex sphingolipids, such as sphingomyelin and gangliosides (data not shown). Nonetheless, though exogenously labeled ceramide primarily remains as intact ceramide, it is also converted to sphingosine and S1P in 10T½ cells.

**Ceramine, not ceramide, induces apoptosis in 10T½ cells.** To support evidence demonstrating that the metabolism of ceramide is critical to cell survival, cells were treated with the nonhydrolyzable ceramide analog, ceramine. As previously shown, treatments with either ceramide or S1P did not induce apoptosis in 10T½ cells (Fig. 4). However, treatment with ceramine produced a marked increase in the number of apoptotic cells, approximately sevenfold that of both ceramide and S1P treatments (Fig. 4). These results suggest that the metabolism of ceramide, potentially to S1P, in 10T½ cells prevents ceramide-induced apoptosis.

These results were confirmed using flow cytometric analysis. Ceramine resulted in a 74% apoptotic cell population (Fig. 5). These results also confirm the lack of apoptotic response to ceramide using flow cytometry to measure apoptosis.

**DISCUSSION**

It has been suggested that a ceramide/S1P rheostat exists within cells, dictating either an apoptotic fate or a survival response (10). The results of the present study demonstrate that 10T½ cells are resistant to ceramide-induced apoptosis. Furthermore, these results demonstrate that the inhibition of ceramide conversion to its metabolites results in an apoptotic response, which is prevented through the addition of the
metabolite S1P. Thus, these results demonstrate that the metabolism of ceramide to S1P is crucial for cell survival.

In support of the ceramide/S1P rheostat hypothesis, we found that when the enzymes ceramidase and sphingosine kinase are inhibited, an action which leads to decreased levels of sphingosine and S1P (21,22), the cells undergo apoptosis. Addition of exogenous S1P in conjunction with the inhibitors protects the cells from apoptosis. S1P is a potent inhibitor of ceramide-induced apoptosis (10,11). Similar to the findings observed in this study, DMS, a sphingosine kinase inhibitor, stimulates apoptosis in a number of cell lines (22). Edsall et al., (22) also demonstrated the prevention of apoptosis by the addition of S1P when pheochromocytoma PC12 cells were treated with DMS (23). Thus, inhibiting the formation of endogenously produced S1P, and thus shifting the balance from S1P to ceramide, in unstimulated cells reduces cell survival. These results demonstrate that regulation of the intracellular levels of ceramide and S1P within cells leads to either induction or prevention of apoptosis.

Ceramide treatment alone did not result in an apoptotic response; however, treatment with ceramine did induce apoptosis. Ceramine differs from ceramide in that the amide-linked fatty acyl group of ceramide is replaced with an amine-linked fatty alkyl group (20). The presence of this amine bond in the ceramide structure does not allow it to be metabolized by the enzyme ceramidase, which hydrolyzes the fatty acyl group off of ceramide-producing sphingosine. Hence the ceramide analog ceramine cannot be metabolized to either sphingosine or S1P. Therefore, in the cells treated with ceramine, the balance of ceramide to S1P is shifted to ceramide and the cells undergo apoptosis.

The intracellular levels of ceramide and S1P can be altered because of extracellular agents. Extracellular stimuli such as cytokines and growth factors can influence the enzymatic activity of several of the enzymes involved in sphingolipid metabolism, such as sphingomyelinase, ceramidase and sphingosine kinase (14). The increase in activity of one enzyme over another can lead to the accumulation of ceramide or the production of S1P, thus changing the cellular rheostat. Our data suggest that the activities of ceramidase and sphingosine kinase in 10T½ cells are maintained at levels that produce S1P. Muller et al. (24) observed the metabolism of [14C]palmitytoyceramide in U937 cells over a 2.5-h time period, and, similar to our results, most of the labeled ceramide remained intact. But in their study, a small amount of label also was incorporated into diacylglycerol and phosphorylcholine (24). In our studies, we were able to detect a proportion of the exogenously added radiolabel from ceramide incorporated into S1P (Fig. 3). S1P cellular levels are normally quite low but can become markedly increased after various stimuli (25,26). Thus, it was not surprising to find such a low level of radio-labeled S1P. Though small, the results of the current study demonstrate that the metabolism of ceramide to S1P is important for cellular survival.

Ceramide and S1P can function as lipid second messengers upon appropriate stimuli (27,28). However, in the absence of such stimuli, the intracellular levels of ceramide and S1P must be maintained at low levels to prevent the induction of apoptosis or cellular proliferation when such a response is not required. Therefore, in 10T½ cells, although ceramide treatment alone did not induce apoptosis, preventing the metabolism of ceramide to S1P markedly stimulated apoptosis. Furthermore, even though a small amount of labeled ceramide was metabolized to S1P, this amount was sufficient to prevent apoptosis. Therefore, treatment of 10T½ cells with ceramide does not result in apoptosis attributable to the conversion of ceramide to S1P, and S1P is required for cell survival in 10T½ cells.

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LITERATURE CITED


FIGURE 5

Induction of apoptosis in 10T½ cells treated with C8-ceramide as measured by the flow cytometry method described by Gorczyca et al. (17). Cells were treated for 24 h with (A) vehicle control, (B) 20 μmol/L C8-ceramide, (C) 2.5 μmol/L S1P or (D) 20 μmol/L C8-ceramide. Shaded areas represent the different phases of the cell cycle with black areas designated for G0/G1 and G2/M phases and the hatched areas representing S phase. In D, the gray area represents both S phase and G2/M phases. Sub G0 is indicated by the arrow on the x-axis. The y-axis represents cell number, and the x-axis represents DNA content.


