Ceramide 1-Phosphate, a Novel Phospholipid in Human Leukemia (HL-60) Cells

SYNTHESIS VIA CERAMIDE FROM SPHINGOMYELIN

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Prior studies demonstrated that conversion of sphingomyelin to ceramide via sphingomyelinase action resulted in the generation of free sphingoid bases and inactivation of protein kinase C in human leukemia (HL-60) cells (Kolesnick, R. N. (1989) J. Biol. Chem. 264, 7617-7623). The present studies define the novel phospholipid ceramide 1-phosphate in these cells and present evidence for formation of this compound by preferential utilization of ceramide derived from sphingomyelin. A ceramide 1-phosphate standard, prepared enzymatically via diacylglycerol kinase, was utilized for localization. In cells labeled to equilibrium with $^{32}$P, to label the head group of the molecule, the basal ceramide 1-phosphate level was $32 \pm 2 \text{ pmol} / \text{10}^6 \text{ cells}$. Generation of ceramide via the use of exogenous sphingomyelinase resulted in time and concentration-dependent formation of ceramide 1-phosphate. As little as $3.8 \times 10^{-2} \text{ units/ml}$ was effective and a 3-fold increase was observed with a maximal concentration of $3.8 \times 10^{-2} \text{ units/ml; \text{ED}_{50} \approx 2 \times 10^{-4} \text{ units/ml}}$. This effect was observed by 5 min and maximal at 30 min. Similarly, in cells labeled with [3H]serine to probe the sphingoid base backbone, the basal level of ceramide 1-phosphate was $39 \pm 5 \text{ pmol} / \text{10}^6 \text{ cells}$. Generation of ceramide 1-phosphate increased 2.5-fold with sphingomyelinase; \text{ED}_{50} \approx 5 \times 10^{-5} \text{ units/ml}. To determine the source of the phosphate moiety, studies were performed with cells short term labeled with $^{32}$P, and re-suspended in medium without radiolabel. Under these conditions, sphingomyelin was virtually unlabeled. Nevertheless, sphingomyelinase (3.8 x 10^{-2} \text{ units/ml}) induced a 12-fold increase in radiolabel incorporation, suggesting ceramide 1-phosphate formation occurred via ceramide phosphorylation. This event appeared specific for ceramide derived from sphingomyelin since ceramide from glycosphingolipids was not converted to ceramide 1-phosphate. In sum, these studies demonstrate the novel phospholipid ceramide 1-phosphate in HL-60 cells and suggest the possibility that a path exists from sphingomyelin to ceramide 1-phosphate via the phosphorylation of ceramide.

A renewed interest in the metabolism of sphingomyelin has developed over the past few years (1-10). This is based primarily on the observation that sphingomyelin metabolism may initiate a sequence of events resulting in the generation of free sphingoid bases, potential inhibitors of protein kinase C (11). Prior studies from this laboratory demonstrated that sphingomyelinase treatment of \text{GH}_{3} \text{pituitary cells resulted in the quantitative generation of ceramide from sphingomyelin (1)}$. A portion of the ceramide was subsequently converted to sphingoid bases. This correlated with inhibition of phorbol ester-mediated activation of protein kinase C (2). Sphingomyelinase treatment of HL-60 cells similarly stimulated the generation of free sphingoid bases and inactivated protein kinase C (7). Under these conditions, phorbol ester-induced differentiation of these cells into macrophages was also inhibited. These studies were interpreted as evidence for the concept that sphingomyelin metabolism might serve to initiate a feedback inhibitory pathway for protein kinase C.

Little is actually known of the regulation of ceramide metabolism. This compound not only serves as precursor to the generation of free sphingoid bases, but is of central importance in the formation of glycosphingolipids and sphingomyelin. Another possible path for the metabolism of ceramide would be to ceramide phosphate. This event has not been previously demonstrated in intact cells. However, the recent report of the existence of a specific ceramide kinase in rat brain synaptic vesicles suggests this event might be of physiological significance (12). The present studies demonstrate the existence of ceramide 1-phosphate in HL-60 cells and show that it may be generated via ceramide metabolism. Further, these studies suggest the possibility of a pathway from sphingomyelin to ceramide 1-phosphate via the generation of ceramide.

EXPERIMENTAL PROCEDURES

Materials—Sphingomyelin (bovine brain), ceramide (Type III), sphingomyelinase (Staphylococcus aureus), cardiolipin (bovine heart), n-octyl-$\beta$-D-glucopyranoside, dithiothreitol, diethylenetriaminepentaacetic acid, and HEPES were from Sigma. Diacylglycerol kinase was from Lipidex, Inc. Endoglycoceramidase (Rhodococcus) was from Genzyme Corp. Fetal bovine serum was from GIBCO. [3H]Serine (22.5 Ci/mmol), [3H]palmitic acid (30 Ci/mmol), and [y,32P]ATP (3000 Ci/mmol) were from Du Pont New England Nuclear. [3H]Orthophosphate (carrier-free) was from ICN Radiochemicals. Liquid scintillation solution (Liquiscint) was from National Diagnostics. Silica Gel TLC plates (L6K6D) were from Whatman. Reagents were HPLC grade and from Fisher.

Cell Culture—HL 60 cells were grown in suspension in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (10,000 units/ml), streptomycin (10,000 $\mu$g/ml), penicillin (10,000 $\mu$g/ml), and glutamine (10.7 $\mu$g/ml) as described (7).

Ceramide 1-Phosphate Standard—Ceramide 1-phosphate standard

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; BSS, balanced salt solution; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid.

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was prepared enzymatically as described by Schneider and Kennedy (13) and adapted by Preiss et al. (14). Briefly, Type III ceramide (0.5 mg) was solubilized in 5 mM cardiolipin, 7.5% octyl-$\beta$-glucopyranoside, 1 mM diethyletheraminepentacetic acid by bath sonication and resuspended in a reaction mixture (50 mM imidazole HCl, pH 6.6, 50 mM NaCl, 12.5 mM MgCl$_2$, 1 mM EDTA) containing diacylglycerol kinase (0.7 unit/ml). The reaction was started by addition of $\gamma$-$^{32}$P-ATP to a final concentration of 10 nM. After 90 min at room temperature, the reaction was stopped by extraction with chloroform:methanol (1:1, v/v), and resolved by TLC using chloroform:methanol:acetic acidwater (25:15:4, v/v/v) and chloroform:methanol:acetic acidwater (25:15:4:3.5, v/v/v/v) as solvent. Sphingosine phosphate was identified by autoradiography as a single spot at RF 0.25, eluted with chloroform:methanol (1:1, v/v) from the silica gel, and stored at -20°C under N$_2$ until use.

Cell Studies—For equilibrium-labeled studies, cells were incubated for 48-72 h in RPMI 1640 as above containing 75 $\mu$Ci/ml $^{32}$P, or 21 $\mu$M $[^3H]$serine. On the day of the experiment, cells were resuspended (1.67 X 10$^6$ cells/ml) in a balanced salt solution (BSS: 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2) without radiolabel containing sphingomyelinase in 0.5% glycerol phosphate buffer (diluent) or diluent alone. Reactions were stopped at the indicated times by extraction of cell pellets with 1 ml of chloroform:methanol:acetic acidwater (25:15:4:3.5, v/v/v/v) and 0.3 ml of BSS containing 10 mM EDTA. The organic phase was dried under N$_2$ and glycerophospholipids were removed by mild alkaline hydrolysis in 0.1 M methanolic KOH at 37°C for 1 h as described (I, 15). Mild alkaline hydrolysis did not directly generate ceramide phosphate. Ceramide phosphate was resolved by TLC using chloroform:methanol:acetic acidwater (25:15:4.5, v/v/v) and chloroform:methanol:acetic acidwater (25:15:4.3.5, v/v/v/v) as solvent, respectively, as previously described (8). Individual lipids were visualized by iodine vapor staining or autoradiography and quantified by liquid scintillation counting.

For short term labeling studies, cells were resuspended (35 X 10$^6$ cells/ml) in BSS containing 5 mCi/ml $^{32}$P. After 60 min, cells were resuspended in BSS without radiolabel and studied as above. Experiments involving endoglycoceramidase contained a 10 mM sodium acetate buffer with 0.003% bovine serum albumin, pH 6 (diluent) or diluent alone.

Identification of Ceramide 1-Phosphate—For these studies, cells were labeled with $^{32}$P, for 1 h and processed as above. After mild alkaline hydrolysis and resolution by TLC, ceramide phosphate was eluted from the silica gel, dried under N$_2$, and deacylated in 6 N HCl, 1-butanol (1:1, v/v) for 1 h at 100°C according to the method of Kaufman (10). Sphingosine phosphate was resolved by TLC using chloroform:methanol:acetic acidwater (25:15:4.3.5, v/v/v/v) as solvent. Recovery of ceramide phosphate as sphingosine phosphate was 65% via this procedure. Sphingosine phosphate was eluted from the silica gel and subjected to periodate oxidation (12). Briefly, the eluted material was dried under N$_2$ and resuspended in 0.30 ml of 0.03% methanolic Triton containing 0.03 m aqueous Na$_2$O$_3$. After 24 h at 22°C, products were extracted by the addition of 0.6 ml of methylene chloride and 0.15 ml of water. The aqueous phase was dried under reduced pressure and glycoaldehyde phosphate was resolved by TLC using 1-butanolacetic acidwater (6:2:2, v/v/v) as solvent (12). Recovery of sphingosine phosphate as glycoaldehyde phosphate was 70% via this procedure. A similar value was reported by Schneider and Kennedy (13). The organic phase was dried under N$_2$ and lipid products of this reaction were analyzed by TLC as above using chloroform:methanol:acetic acidwater (25:15:4.3.5, v/v/v/v) as solvent.

Other Procedures—Assuming "isotopic equilibrium," the specific activity of serine as determined from the radioactivity and phosphorus content of sphingomyelin (7, 17) was used to quantitate serine-containing compounds. A similar approach was used for $[^3H]$palmitate and $^{32}$P-labeled compounds.

Statistics—Statistical analysis was performed by t test and linear regression analysis by the method of least squares.

3 An alternative approach validated the results obtained with radiolabeled serine. These studies utilized the diacylglycerol kinase reaction to directly measure ceramide levels. Both methods yielded similar values. These were 180 ± 20 pmol/10$^6$ cells for $[^3H]$serine and 209 ± 29 pmol/10$^6$ cells for the radioenzymatic method.

RESULTS AND DISCUSSION

Initial studies determined the existence of ceramide 1-phosphate in HL-60 cells. These studies utilized cells labeled for 1 h with $^{32}$P. Fig. 1 compares the ceramide 1-phosphate generated enzymatically to a total $^{32}$P-labeled phospholipid extract of HL-60 cells. As seen in this autoradiogram (left lane), authentic ceramide 1-phosphate migrates as a single spot with an RF 0.25 in this system. A small portion of the total lipid extract (lane 1) co-migrates with the ceramide 1-phosphate standard. This compound is partially obscured by the mass of radiolabeled phospholipid. If this material is eluted from the silica gel, it co-migrates with authentic ceramide 1-phosphate in two additional solvent systems: chloroform:pyridine:formic acid (50:30:7, v/v; RF 0.25) and chloroform:methanol:acetic acid:water (25:15:4:3.5, v/v/v/v; RF 0.7). To separate this material from the mass of cellular phospholipid, the total lipid extract was subjected to mild alkaline hydrolysis (15). This removes the majority of the glycerophospholipids accounting for 95% of labeled phospholipids under these conditions. The material that co-migrates with ceramide 1-phosphate standard, however, was resistant to degradation (lane 2); recovery of material was 90%.

FIG. 1. Localization of ceramide 1-phosphate (C1P) in HL-60 cells by autoradiography. HL-60 cells (50 X 10$^6$/ml) were resuspended in BSS containing 5 mCi/ml $^{32}$P. After 60 min, cells were washed with BSS without radiolabel and centrifuged at 800 x g for 5 min. The cell pellet was extracted with 1 ml of chloroform:methanol:HCl (100:100:1, v/v/v) and 0.3 ml of BSS containing 10 mM EDTA. The organic phase was dried under N$_2$, resuspended in chloroform:methanol (1:1, v/v), and resolved by TLC using chloroform:methanol:acetic acid (65:15:5, v/v/v) as solvent as described (14). A portion of the organic phase was subjected to mild alkaline hydrolysis (0.1 m methanolic KOH at 37°C for 1 h)(1). Lane S, authentic ceramide 1-phosphate standard (3000 dpm) preplated enzymatically; lane 1, total cellular $^{32}$P-labeled phospholipid extract (1.5 X 10$^7$ cells); lane 2, cellular phospholipid extract after mild alkaline hydrolysis (3 X 10$^6$ cells).

S 1 2

Front Origin C1P
material from twice as many cells was applied to lane 2 as lane 1). Based on this evidence, the cellular material was tentatively identified as ceramide 1-phosphate.

To further define the cellular phospholipid as ceramide 1-phosphate, the material was first eluted from the silica gel and then subjected to acid butanol hydrolysis by the method of Kaller (16). This procedure deacylates ceramide 1-phosphate and its derivative sphingosine 1-phosphate (two left columns). In the solvent system utilized, chloroform:methanol:acetic acidwater (25:15:4:3.5, v/v), these sphingolipids migrate as doublets; ceramide 1-phosphate, 0.8; sphingosine 1-phosphate, 0.8. An identical pattern was obtained with the cellular material (two right columns). These studies clearly identify the phospholipid derived from HL-60 cells as ceramide 1-phosphate.

To establish the position of the phosphate group as either in the first or third position, [32P]sphingosine phosphate was subjected to periodate oxidation (12, 13). This procedure converts sphingosine 1-phosphate to glycoaldehyde phosphate, a hydrophilic molecule extracted into the aqueous phase. In contrast, this procedure converts ceramide 3-phosphate to radiolabeled long chain aldehydes which remain in the organic phase. In fact, 70% of the sphingosine phosphate derived either from the cellular material or authentic sphingosine 1-phosphate was converted to an aqueous extractable form. A similar result was obtained by Schneider and Kennedy (16). When this aqueous material was analyzed by TLC using 1-butanol:acetic acid:water (6:2:2, v/v) as solvent, the cellular

![Diagram of a typical experiment showing the effect of sphingomyelinase on ceramide 1-phosphate levels.](http://example.com/diagram.png)

**Fig. 3. Time course of the effect of sphingomyelinase on the level of ceramide 1-phosphate.** HL-60 cells were labeled for 48-72 h with [32P] (75 μCi/ml) and resuspended (1.67 × 10⁶ cells/ml) in BSS without radiolabel containing sphingomyelinase (3.8 × 10⁻⁶ units/ml) or 0.5% glycerol phosphate buffer (diluent) as described (7). At the indicated times, cell pellets were extracted and subjected to mild alkaline hydrolysis, and ceramide 1-phosphate was resolved by TLC using chloroform:methanol:acetic acid (65:15:5, v/v) as solvent as described in Fig. 1. Individual lipids were detected by autoradiography and quantified by liquid scintillation counting. Control incubations contained 1200 dpm/10⁶ cells of [32P]ceramide 1-phosphate. These data (mean ± S.E.) represent duplicate determinations from three experiments.
These studies were performed as described in Fig. 3 at 45 min of stimulation. The abbreviations are: S, ceramide 1-phosphate standard; C1P, ceramide 1-phosphate.

The specificity of the ceramide phosphorylation reaction for ceramide derived from sphingomyelin was evaluated. These studies compared the effect of sphingomyelinase and endoglycoceramidase on ceramide and ceramide 1-phosphate formation. The upper panel of Fig. 7 demonstrates that sphingomyelinase (2 x 10^-4-1 x 10^-3 units/ml) and endoglycoceramidase (3 x 10^-5-1 x 10^-3 units/ml) induce similar elevations in the level of ceramide as measured by the diacylglycerol kinase assay (14). In contrast, [32P] incorporation into sphingomyelin was minimal in basal incubations and did not change with sphingomyelinase stimulation. Hence, sphingomyelin does not serve as the direct precursor for the phosphate moiety of ceramide 1-phosphate.

In sum, the present studies describe the novel phospholipid phosphatidic acid and may represent a dialkyl form resistant to mild alkaline hydrolysis. Only ceramide 1-phosphate was consistently observed and responsive to sphingomyelinase in this system.

An additional set of studies was performed with cells radiolabeled with [3H]serine to preferentially label the sphingoid base backbone of the molecule. For these studies, cells were labeled to "isotopic equilibrium" with [3H]serine and resuspended in medium without radiolabel. Fig. 5 again shows that sphingomyelinase induced a concentration-dependent increase in the level of ceramide 1-phosphate at 45 min of stimulation. The basal level of ceramide 1-phosphate was 39 ± 5 pmol/10^6 cells. This value is very similar to that derived with cells labeled with [32P]. An increase in the level of ceramide 1-phosphate was detectable with as little as 3.8 x 10^-6 units/ml sphingomyelinase and a maximal effect to 86 pmol/10^6 cells was achieved with 3.1 x 10^-3 units/ml; ED50 ≈ 5 x 10^-5 units/ml. Similarly, sphingomyelinase induced a maximal 2.5-fold increase in ceramide 1-phosphate in studies (n = 2) performed with cells labeled to equilibrium with [3H]palmitate. [3H]Palmitate measures both the fatty acid moiety and the sphingoid base backbone (8). A basal level of 39 pmol/10^6 cells was again derived with this probe. Hence, similar basal and sphingomyelinase-stimulated levels of ceramide 1-phosphate were obtained with [3H]serine, [3H]palmitate, or [32P], probes that respectively measure the backbone, fatty acid moiety, and head group of the molecule.

To determine whether the phosphate moiety might be derived from sphingomyelin directly, perhaps via contamination of the sphingomyelinase preparation with a phospholipase D, or via phosphorylation of ceramide, studies were performed with cells short term labeled with [32P]. Under these conditions, sphingomyelin is virtually unlabeled and hence cannot contribute radiolabeled phosphate to ceramide 1-phosphate. Fig. 6 demonstrates the concentration-dependent effects of sphingomyelinase on [32P] incorporation into ceramide 1-phosphate and sphingomyelin. Sphingomyelinase-induced [32P] incorporation into ceramide 1-phosphate was detectable with as little as 3.8 x 10^-6 units/ml sphingomyelinase to 250 ± 60% of control and a maximal 12-fold increase in labeling was achieved with 3.8 x 10^-5 units/ml; ED50 ≈ 7 x 10^-5 units/ml. In contrast, [32P] incorporation into sphingomyelin was minimal in basal incubations and did not change with sphingomyelinase stimulation. Hence, sphingomyelin does not serve as the direct precursor for the phosphate moiety of ceramide 1-phosphate.

The origin and contained sphingomyelin and other lipids, and a faint spot at Rf 0.61. This latter spot co-migrated with phosphatidic acid and may represent a dialkyl form resistant to mild alkaline hydrolysis. Only ceramide 1-phosphate was consistently observed and responsive to sphingomyelinase in this system.
ceramide 1-phosphate in HL-60 cells. The compound was identified by use of a standard synthesized enzymatically by diacylglycerol kinase (13, 14). The material derived from HL-60 cells co-migrated with the standard in a variety of TLC systems and was converted to sphingosine phosphate by acidic butanol hydrolysis. Sphingosine phosphate was further oxidized to glycolydihydro phosphate by periodate treatment indicating the site of the phosphorylation as the first position. Additionally, elevation of ceramide levels from endogenous sphingomyelin stores resulted in ceramide 1-phosphate formation. Identical results were obtained whether the probe utilized measured the backbone or the head group of the molecule. Acute labeling studies with $^{32}$P, clearly demonstrated that the phosphate group was not derived directly from sphingomyelin but rather by phosphorylation of ceramide. This was anticipated since sphingomyelin degradation was achieved by sphingomyelinase action. Additionally, this effect appeared specific for ceramide derived from sphingomyelin since ceramide from glycosphingolipids was not converted to ceramide 1-phosphate. Thus, these studies suggest the possibility that a pathway exists from sphingomyelin to ceramide 1-phosphate via the generation of ceramide. Similarly, Slife et al (19) found that ceramide derived from sphingomyelin but not glycosphingolipid served as precursor for sphingoid base formation in rat liver plasma membranes. Although the present studies do not address the enzymatic mechanism involved in this process, preliminary studies suggest that HL-60 cells contain a specific ceramide kinase activity distinct from diacylglycerol kinase activity (12).

This series of events appears analogous to the initial arm of the phosphoinositide pathway. In both instances, phospholipid hydrolysis via a phospholipase C-like mechanism yields a compound of similar structure which is subsequently phosphorylated. Whether ceramide 1-phosphate may be synthesized in response to physiologic agonists such as $\alpha$-2,5 dihydroxvitamin D$_3$ (10) or may serve as a precursor to the formation of other phosphoglycerolipids (20) is presently under investigation.

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