Sphingomyelin Turnover Induced by Vitamin D₃ in HL-60 Cells

ROLE IN CELL DIFFERENTIATION

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Sphingolipid metabolism was examined in human promyelocytic leukemia HL-60 cells. Differentiation of HL-60 cells with 1α,25-dihydroxyvitamin D₃ (vitamin D₃; 100 nm) was accompanied by sphingomyelin turnover. Maximum turnover of [³H]choline-labeled sphingomyelin occurred 2 h following vitamin D₃ treatment, with sphingomyelin levels decreasing to 77 ± 6% of control and returning to baseline levels by 4 h. Ceramide and phosphorylcholine were concomitantly generated. Ceramide mass levels increased by 55% at 2 h following vitamin D₃ treatment and returned to baseline levels by 4 h. The amount of phosphorylcholine produced equaled the amount of sphingomyelin hydrolyzed, suggesting the involvement of a sphingomyelinase. Vitamin D₃ treatment resulted in a 90% increase in the activity of a neutral sphingomyelinase from HL-60 cells. The inferred role of sphingomyelin hydrolysis is the induction of cell differentiation, as was investigated using an exogenous sphingomyelinase. When a bacterial sphingomyelinase was added at concentrations that caused a similar degree of sphingomyelin hydrolysis as 100 nM vitamin D₃, it enhanced the ability of subthreshold levels of vitamin D₃ to induce HL-60 cell differentiation. This study demonstrates the existence of a "sphingomyelin cycle" in human cells. Such sphingolipid cycles (Hannun, Y., and Bell, R. (1989) Science 243, 500-507) may function in a signal transduction pathway and in cellular differentiation.

Sphingolipids and sphingolipid breakdown products are emerging as a new class of bioactive molecules that affect cell regulation, secretion, cell differentiation, and oncogenesis (1–3). Recently, we hypothesized the existence of a "sphingolipid cycle," analogous to the phosphatidylinositol cycle (1). This hypothesis led us to examine whether sphingolipid turnover occurred during human promyelocytic leukemia HL-60 cell differentiation induced by 1α,25-dihydroxyvitamin D₃ (vitamin D₃)

In this report, vitamin D₃ is reported to induce the turnover of sphingomyelin (SM). ¹ Phosphorylcholine and ceramide are the products of SM turnover, which occurs secondary to the activation of an endogenous neutral sphingomyelinase (SMase). Addition of bacterial neutral SMase enhanced the action of subthreshold vitamin D₃ in inducing HL-60 cell differentiation. These results suggest that SM hydrolysis and its breakdown products may play a previously unrecognized role in cell differentiation.

EXPERIMENTAL PROCEDURES

Materials

1α,25-Dihydroxyvitamin D₃ was a kind gift from Dr. Milan Uskokovic (Hoffmann-La Roche). SM and phosphorylcholine (PC) were from Avanti Polar Lipids, Inc., and ceramide was from Supelco, Inc. Choline, phosphorylcholine, CDP-choline, glycerol 3-phosphorylcholine, inulin, transferrin, SMase from Streptomyces species, Triton X-100, nitro blue tetrazolium, and naphthyl acetate were from Sigma. [³H]Palmitic acid, [methyl-³H]choline chloride, [¹⁴C]methyl iodide, and [γ-³²P]ATP were from Du Pont-New England Nuclear. RPMI 1640 medium was from Gibco. Human promyelocytic leukemia HL-60 cells were a kind gift from Dr. J. Niedel (Duke University).

Methods

Labeling of HL-60 Cells—HL-60 cells were grown in RPMI 1640 medium containing 10% fetal calf serum in 5% CO₂ at 37°C. The cells were washed three times with phosphate-buffered saline and incubated with [³H]palmitic acid (10 μM, 1 μCi/ml; specific activity: 200 mCi/mmol) for 12 h or with [³H]choline chloride (0.5 μCi/ml; specific activity: 80 Ci/ml) for at least 48 h in serum-free RPMI 1640 medium containing insulin (5 μg/liter) and transferrin (5 μg/liter). The cells were then washed three times with phosphate-buffered saline and incubated in serum-free medium in the presence or absence of 100 nm vitamin D₃.

Lipid Extraction and Identification—After harvesting the cells at the indicated times, the lipids were extracted by the method of Bligh and Dyer (4). The samples were dried down under N₂ gas and dissolved in 100 μl of chloroform; then, 20 μl were applied on Silica Gel 60 TLC plates (Merck), and 40 μl were used to measure phospholipid phosphate (duplicate measurements) (5). To identify SM and PC, TLC plates were developed in chloroform/methanol/acetic acid/H₂O (65:25:4; solvent system A) or chloroform/methanol/CH₃OH (65:35:5; solvent system B) or chloroform/methanol/H₂O (65:25:4; solvent system C). The combination of solvent systems A and B or A and C was also used for two-dimensional TLC separation. SM was further identified on TLC by alkaline hydrolysis. Chloroform extracts of cells were saponified in methanolic NaOH (0.1 N) at 37°C for 1 h to eliminate ester-containing glycerolipids and then alkaline-hydrolyzed in methanolic NaOH (1 N) at 120°C for 20 h to remove the N-acyl chains yielding sphingosylphosphorylcholine, which co-migrated with pure standard. The SM and PC spots were scraped and counted in 4 ml of Safety-Solve (Research Products International Corp.) using a scintillation counter (Pharmacia LKB, Biotechnology Inc., 1209 RACKBETA). Radioactivity was corrected for the amount of phospholipids.

Sphingomyelin and Phosphatidylcholine Quantitation—Phospholipids were isolated and separated on TLC as described above. The sphingomyelin and phosphatidylcholine spots were scraped, and the lipids were eluted from silica gel in chloroform/methanol (2:1). SM and PC were then quantitated by measuring their phosphate content (6).

Ceramide Quantitation—Ceramide levels were measured enzymatically using DAG kinase as described (6, 22). Base-line ceramide
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RESULTS AND DISCUSSION

Detection of Sphingomyelin Turnover—Initially, we examined whether changes in sphingolipids occurred during the early phase of vitamin D₃-induced HL-60 cell differentiation. Cells were labeled with [³H]palmitic acid and then treated with 100 nM vitamin D₃, an optimal concentration for induction of differentiation. Among the labeled sphingolipids, SM showed significant changes in labeling during the first 4 h after addition of vitamin D₃. SM levels decreased to 77 ± 6% of control 2 h after treatment and then returned to control levels by 4 h (Fig. 1A). SM metabolism was also followed by labeling with [³H]choline. With [³H]choline, only three lipids (PC, SM, and lysosphingomyelin) were detected (data not shown). PC and SM were identified by co-migration with cold standards and by two-dimensional thin layer chromatography. Again, SM decreased to 76 ± 8% of control 2 h after treatment and then returned to control levels by 4 h (Fig. 1B). The percent change of [³H]choline-labeled SM was nearly identical to that seen with [³H]palmitic acid-labeled SM. These results show that vitamin D₃ induces SM turnover in the early phase of HL-60 cell differentiation.

Metabolic Pathways Involved in Sphingomyelin Hydrolysis—SM turnover could result from a number of biochemical reactions (Fig. 2A). These include a sphingomyelinase (Fig. 2A, reaction I), phospholipase D-type hydrolysis (reaction II), N-deacylation (reaction III), and reaction by phosphorylcholine exchange enzyme (SM + diacylglycerol → ceramide + PC) (reaction IV).

To elucidate which enzymatic activity was involved in SM turnover, the levels of PC, ceramide, phosphocholine, and lysosphingomyelin were determined. When the cells were labeled with [³H]palmitic acid, [³H]choline, or [³H]lysine, no changes in lysosphingomyelin, ceramide phosphate, choline, CDP-choline, and glycerol 3-phosphocholine were detected (data not shown). Thus, it is unlikely that either SM N-deacylation (reaction III) or phospholipase D-type SMase (reaction II) was involved in SM breakdown. The mass of cellular ceramide showed significant increases. Ceramide levels peaked at 2 h and returned to base-line levels by 4 h (Fig. 2B). Ceramide levels were 25.2 ± 1.36 pmol/nmol of phospholipids. The solvent system to separate phosphatidic acid and ceramide phosphate was chloroform/pyridine/formic acid (60:30:8, v/v).

Phosphorylcholine Determination—Phosphorylcholine in the aqueous phase was dried down by vacuum centrifugation at 40 °C and dissolved in 50 μl of 50% ethanol. 20 μl were applied on Silica Gel 60 TLC plates and developed in methanol, 0.5% NaCl, ammonia (100:100:2, v/v). Rf values of choline, phosphorylcholine, CDP-choline, and glycerol 3-phosphorylcholine were 0.15, 0.32, 0.52, and 0.63, respectively. Phosphorylcholine was identified by co-migration of cold phosphorylcholine and degradation of phosphorylcholine to choline by alkaline phosphatase.

Preparation of [methyl-³C]Sphingomyelin—[methyl-³C]SM was synthesized according to the method of Stoffel et al. (8). [methyl-³C]SM was distilled with unlabeled SM to a specific activity of 10,000 cpm/nmol.

Assay of Neutral Sphingomyelinase—HL-60 cells were harvested, washed twice with phosphate-buffered saline, and suspended in 0.5 ml of 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, and 0.1% Triton X-100 after treatment with 100 nM vitamin D₃ or with vehicle. The cells were homogenized by 30 strokes in a Dounce glass homogenizer and centrifuged at 100,000 × g for 5 min to separate the two phases. The clear upper phase (0.4 ml) was removed and placed in a glass scintillation vial. Ten ml of 10 mM MgCl₂, 0.1% Triton X-100, and 50-300 μg of enzyme in a final volume of 0.1 ml. Incubation was carried out at 37 °C for 30 min. The reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1). Then, 0.2 ml of double distilled water was added to the tubes and vortexed. The tubes were centrifuged at 1,000 × g for 5 min to separate the two phases. The clear upper phase (0.4 ml) was removed and placed in a glass scintillation vial. Ten ml of scintillation fluid (Safety Solve) were added. After shaking, the vials were counted. Control tubes contained boiled enzyme. Protein was measured by the method of Lowry et al. (9) with bovine albumin as a standard.

Analysis of Cell Differentiation—Nitro blue tetrazolium-reducing ability and nonspecific esterase activity were quantified as previously described (10).
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**Figure 2.** Products of SM hydrolysis in vitamin D<sub>3</sub>-treated HL-60 cells. A, structure of SM showing possible pathways involved in its metabolism. Reaction I, SMase activity (SMase) yielding ceramide and phospho-
rylcholine; reaction II, D-type SMase resulting in ceramide phosphate and choline; reaction III, N-deacylation resulting in lysosphingomyelin and fatty acid; reaction IV, phosphatidylcholine:ceramide cholinephosphotransferase (exchange enzyme) resulting in the transfer of phosphorylcholine head groups between SM and PC. SM synthesis may occur through reaction IV or V (CDP-choline:ceramide cholinephosphotransferase). B and C, changes in levels of ceramide and PC in HL-60 cells treated with vitamin D<sub>3</sub>, respectively. PC and ceramide levels were standardized against total cellular phospholipids by correcting for phospholipid phosphate. The data are shown as percent of control (in the absence of vitamin D<sub>3</sub>). The results were obtained from six (B) and three (C) different experiments. Bars represent 1 S.D.

**Production**

Production is consistent with activation of either SMase (reaction I) or an exchange enzyme (reaction IV). [3H]Choline-labeled PC did not show any significant change during the same time interval (Fig. 2C). Therefore, it seems unlikely that the exchange enzyme was involved in SM turnover. This is further supported by the concomitant changes in the level of phosphorylcholine, a product of sphingomyelinase reaction.

1. Total cellular PC levels (400-500 pmol/nmol of phospholipids) were 10-fold greater than total SM levels as measured by head group phosphates. Therefore, changes in PC levels which could account for significant changes in SM levels could not be accurately assessed by choline labeling. Evidence against reaction IV is obtained by the changes in phosphorylcholine and from the detection of SMase of the phospholipase C-type in crude extracts of HL-60 cells treated with vitamin D<sub>3</sub>, but not of the exchange reaction (Fig. 3). Moreover, the amount of SM breakdown (e.g. 251 ± 13 cpn/nmol of phospholipids at 2 h) corresponded to that of phosphorylcholine generation in the cells (228 ± 56 cpn/nmol of phospholipids at 2 h) at the same time points (Fig. 3). Therefore, these results suggest activation of a SMase by vitamin D<sub>3</sub>. The mass of hydrolyzed SM was then compared to the mass of generated ceramide. Vitamin D<sub>3</sub> treatment of HL-60 cells resulted in a maximum decrease of SM (32.9 ± 4.0%), which corresponds to hydrolysis of 17 ± 0.43 pmol of SM/nmol of phospholipids at 2 h. This was accompanied by the generation of 14 ± 2.8 pmol of ceramide/nmol of phospholipids. These results quantitatively demonstrate that ceramide is the predominant product of SM hydrolysis. Other potential minor products, such
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Fig. 3. Changes in SM and phosphorycholine in HL-60 cells treated with vitamin D3. The cells were labeled with [3H]choline and incubated in the presence or absence of 100 nM vitamin D3. Radioactivity was corrected for phospholipid phosphate. The data are shown as the differences of corrected radioactivity of SM (D) or phosphorycholine (●) in cells incubated in the presence or absence of 100 nM vitamin D3. The results were obtained from two different experiments. Bars represent 1 S.D.

Fig. 4. Increase of activity of neutral sphingomyelinase in HL-60 cells treated with vitamin D3. HL-60 cells were treated in the presence or absence of 100 nM vitamin D3. The activity was measured as described under "Experimental Procedures." The results were obtained from two different experiments. Bars represent 1 S.D.

as ceramide phosphate or lysosphingomyelin, were not detected.

SM mass levels returned to base-line levels (51 ± 5.9 pmol/nmol of phospholipids) by 4 h, indicating a resynthesis phase of SM accompanied by decreases in the levels of ceramide. These results are therefore consistent with the existence of a sphingomyelin cycle in response to vitamin D3 action.

Activation of Neutral Sphingomyelinase by Vitamin D3—Because of the inherent limitations of metabolic labeling studies, we next investigated the presence of endogenous SM hydrolyzing activity. Detergent extracts of HL-60 cells contained acid and neutral sphingomyelinase. Treatment of HL-60 cells with vitamin D3 resulted in a time-dependent increase in the neutral SMase activity (Fig. 4) which peaked at 1.5–2 h (no SM N-deacylase or D-type SMase was detected). These data show that the predominant effect of vitamin D3 is the induction of SMase activity.

Role of Sphingomyelin Hydrolysis in Cell Differentiation—To examine whether the observed hydrolysis of SM plays a role in HL-60 cell differentiation, the effect of the addition of SMase from a Streptomyces species on HL-60 cell differentiation was investigated. Optimal concentrations of SMase were determined. Treatment of HL-60 cells with various concentrations of SMase (0–100 milliunits/ml) for 4 h resulted in a time- and dose-dependent hydrolysis of SM (data not shown). SM levels decreased by 15 ± 1% 2 h after treatment with 100 milliunits/ml SMase and by 25 ± 2% 4 h after treatment. Additional experiments showed that, in the range used, SMase

The recovery of SM and ceramide levels by 4 h suggests that SM resynthesis occurs either by reaction IV or through de novo synthesis (reaction V).
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did not induce DAG accumulation.⁵
Exogenous (bacterial) SMase acted in synergy with vitamin D₃ in inducing HL-60 differentiation. Simultaneous addition of 100 milliunits/ml SMase and 1 nM vitamin D₃, a subthreshold concentration which barely induces differentiation, caused partial partial differentiation (Fig. 5). Concomitant with cell differentiation, there was a significant decrease in cell proliferation (Fig. 5, inset). Two parameters of differentiation showed significant changes. Nitro blue tetrazolium-reducing ability increased from 4 ± 2 to 19 ± 6%, and nonspecific esterase activity increased from 0 ± 1 to 26 ± 3% after 4 days. The synergy between vitamin D₃ (1 nM) and SMase (1–100 milliunits/ml) was dose-dependent (Fig. 5). SMase itself did not show any induction of HL-60 cell differentiation in the absence of vitamin D₃ (data not shown).⁶ Therefore, hydrolysis of SM by exogenous SMase synergistically enhanced the action of a subthreshold concentration of vitamin D₃ in inducing HL-60 cell differentiation.

CONCLUDING REMARKS

Increases in SM levels have been observed in dexamethasone-treated neutrophils (11) and 3T3-L1 cells (12) in HL-60 cells treated with phorbol 12-myristate 13-acetate (13). The functional significance of these changes, however, has not been determined. In addition, it has been shown that high concentrations of DAG stimulate SM hydrolysis in GH₃ pituitary cells (14). This effect was not reproduced by phorbol esters and occurred in cells where protein kinase C was down-regulated, suggesting that SM hydrolysis occurred independently of protein kinase C activation. In similar studies (15), it was shown that exogenous bacterial SMase reduced membrane-associated protein kinase C activity, suggesting a role for SM hydrolysis in inhibiting protein kinase C activation. SMase treatment of HL-60 cells also caused an inhibition of phorbol 12-myristate 13-acetate-induced differentiation of HL-60 cells (16).

In this study, we show the induction of SM hydrolysis by vitamin D₃ in HL-60 cells. This is accompanied by the generation of ceramide and phosphorylcholine. A neutral sphingomyelinase, detected in extracts of HL-60 cells, was induced by vitamin D₃ treatment. SM, ceramide, and phosphorylcholine levels returned to base-line levels by 4 h, suggesting a resynthesis phase of SM, thus completing a sphingomyelin cycle. We also demonstrate a role for SM hydrolysis in enhancing HL-60 differentiation.

This observed turnover of SM may indicate the operation of a sphingomyelin cycle with a function in cell regulation. Unlike the phosphatidylinositol cycle, SM turnover occurred over a longer period and may be involved in longer term cell changes such as shown in this study with cell differentiation.

This study raises important questions as to how vitamin D₃ regulates SMase activity. Vitamin D₃ belongs to the steroid hormone family whose cellular actions are mediated through interaction with intracellular receptors (17–19). These receptors appear to mediate the action of steroid hormones by enhancing/suppressing gene activity (19). Preliminary studies suggest that the effects of vitamin D₃ on the induction of SMase are inhibited by cycloheximide.

Another major question raised by this study relates to the mechanism by which SM hydrolysis and the generation of ceramide and phosphorylcholine enhance cell differentiation. Studies (20) with exogenous SMase suggest an important role for ceramide, either as a second messenger or as a precursor for sphingosine or other metabolites. This latter possibility suggests a link between SM hydrolysis and regulation of protein kinase C since sphingosine inhibits protein kinase C in HL-60 cells (21). Further experiments are required to define the biochemical pathways leading from SM hydrolysis to cell differentiation.

A sphingomyelin cycle and its role in cellular regulation are being defined by this and other studies.

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