Biochemical Mechanisms of the Generation of Endogenous Long Chain Ceramide in Response to Exogenous Short Chain Ceramide in the A549 Human Lung Adenocarcinoma Cell Line

ROLE FOR ENDOGENOUS CERAMIDE IN MEDIATING THE ACTION OF EXOGENOUS CERAMIDE*

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The sphingolipid ceramide has been shown to mediate anti-proliferative responses such as apoptosis, growth arrest, differentiation, and senescence (1). Ceramide is generated in response to various agents including Fas ligand, tumor necrosis factor-α, ionizing radiation, and chemotherapeutic agents (2, 3). Although some of these agents cause early and reversible generation of ceramide, they can also cause a delayed and sustained generation of ceramide, which, in most cases, is associated with its downstream biological activities. Ceramide generation occurs via agonist activation of either sphingomyelinase, which catalyzes the hydrolysis of sphingomyelin to ceramide and phosphocholine (4–6), or through the de novo pathway of sphingolipid biosynthesis (7, 8). Several studies have utilized inhibitors of the de novo pathway to establish a role for ceramide in apoptosis induced by several agents including daunorubicin, etoposide, tumor necrosis factor, camptothecin, IgM, and angiotensin II (7–12).

In many malignant cell lines (such as leukemia and breast carcinoma cells), ceramide rapidly and specifically induces apoptosis, whereas dihydroceramide and other related lipids are inactive (13). Ceramide has been shown to activate proteases of the ICE family (caspases), especially caspase-3, a PARP1-cleaving protease (14, 15). Importantly, activation of caspase-3 by ceramide and induction of apoptosis are inhibited by overexpression of bcl-2 (14), and Bcl-2 does not reduce the levels of ceramide produced in response to extracellular agents (16). Taken together, these results suggest that the generation of ceramide occurs upstream of the execution phase of apoptosis.

In addition to its role in apoptosis, ceramide has been shown to be involved in cell cycle arrest (17). Ceramide induces a G$_1$/G$_0$ cell cycle arrest, and this was mechanistically shown to be due to the induction of dephosphorylation of the retinoblas-toma gene product (Rb) (17). It has been reported also that the treatment of NIH 3T3 cells with a specific inhibitor of glucosylceramide synthase, which results in the accumulation of ceramide, causes a G$_1$/M cell cycle arrest, possibly mediated by ceramide-induced inhibition of the cyclin-dependent p34$^{cdk2}$ and Cdk2 kinases (18). More recent studies have shown that ceramide specifically inactivates the cyclin-dependent kinase Cdk2, but not Cdk4, through activation of a phosphatase (19).

Recently, we have demonstrated that both exogenous (with C$_6$-ceramide treatment) and endogenous ceramides (generated in response to bacterial sphingomyelinase overexpression or
daunorubicin treatment) inhibit mRNA synthesis of telomerase reverse transcriptase and telomerase activity via inactivation of c-Myc transcription factor in the A549 human lung adenocarcinoma cell line (20, 21). Moreover, this inhibition of telomerase activity mediated by ceramide has been shown to be independent of apoptosis but correlated with cell cycle arrest at G0/G1 (20). These results add to a growing body of literature implicating ceramide in senescence of human cells and aging yeast (22, 23).

It has been shown previously that the addition of short chain C8- and C10-ceramides to cells mimics many of the biological responses of agonists in mammalian and yeast cells, and this approach has been employed to suggest possible roles for endogenous ceramide in these processes (24).

In addition, there are results suggesting a mere direct relationship between exogenous and endogenous ceramides. It has been shown that treatment of Madin-Darby canine kidney cells with N-octanoyl-sphingosine liposomes caused about 10-fold increase in the total ceramide levels (25). Treatment of U937 human myeloid leukemia cells with 25 μM cell-permeable C8-ceramide triggered a sustained endogenous ceramide generation at 24 h, which was inhibited by fumonisin B1 (26). Also, it has been demonstrated that C8-ceramide triggers neutral sphingomyelinsae activation, sphingomyelin hydrolysis, and total ceramide accumulation in HL-60 and U937 cells at 10–30 min (26). Furthermore, it has been shown recently that treatment of human skin fibroblasts and peripheral blood mononuclear cells with C8-ceramide induces acid sphingomyelinase expression at the mRNA level that correlated with apoptotic cell death (27). Thus, there appear to be multiple mechanisms by which exogenous ceramide influences ceramide metabolism. These observations also raise the important question of whether downstream biological effects are mediated by short chain ceramide per se or if they are dependent on the generation of endogenous ceramide.

Therefore, in this study, we determined if exogenous short chain ceramide induces the generation of endogenous long chain ceramide in the A549 human adenocarcinoma cell line. We focused on identifying the mechanisms of this effect and its possible role in mediating cell responses. We show here, for the first time, that the sustained generation of long chain endogenous ceramide requires the biochemical recycling of the sphingosine backbone of C8-ceramide, which involves deacylation and reacylation of ceramide for the generation of endogenous long chain ceramide (mainly C16:0- and C24:1-ceramides), most likely by CoA-dependent ceramide synthase, which is inhibited by fumonisin B1. Moreover, the generation of long chain endogenous ceramide in response to C8-ceramide treatment is stereospecific, as shown by increased long chain ceramide generation by d-erythro-C8-ceramide but not l-erythro-C8-ceramide. In addition, evidence is provided that in A549 cells the generation of long chain endogenous ceramide mediates the effects of exogenous C8-ceramide on growth inhibition, cell cycle arrest, and the modulation of telomerase activity. Therefore, this study provides new insights into the mechanisms of generation of endogenous ceramide and its downstream biological roles in response to exogenous short chain ceramide.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The A549 human lung carcinoma cells were obtained from Dr. Alice Boylan (Medical University of South Carolina, Charleston, SC). The HL-60 human acute myeloid leukemia and MCF-7 human breast cancer cell lines were obtained from the ATCC (Manassas, VA). Cells were maintained in growth medium containing 10% fetal calf serum and 100 μg/ml of penicillin and streptomycin (Invitrogen) at 37 °C in 5% CO2. Cell-permeable and biologically active short chain ceramides and their radioactively labeled analogs were obtained from the Synthetic Lipid Core at the Department of Biochemistry and Molecular Biology (Medical University of South Carolina). Fumonisin B1 (FB1) was obtained from Alexis. Myriocin (MYR) was obtained from Sigma.

Exogenous short chain ceramides were dissolved in ethanol at a concentration of 100 mM and then directly added to the medium containing 10% fetal calf serum to obtain a final concentration of 1–20 μM. The final volume of ethanol in the medium was 0.02%, which had no effect on cell growth and/or survival.

Measurement of Total Endogenous Ceramide Levels—Total endogenous ceramide levels were measured using the diazacylglycerol phosphate kinase (DGK) method as described previously (28). In short, after total ceramide was extracted using the standard Bligh and Dyer protocol, they were dried under N2. The dried lipids were then resuspended and used for phosphate measurements and the Escherichia coli diazacylglycerol kinase assay as modified for ceramide. This assay depends on the phosphorylation of ceramide and diazacylglycerol, generating ceramide phosphate and phosphatidic acid, respectively, by diacylglycerol kinase in the presence of radiolabeled ATP. The radiolabeled products were visualized by thin layer chromatography, and the phosphorylated products of ceramide and diazacylglycerol were identified by comparison with known standards run on the same plate. In addition, the amounts of products that were scraped from the plates were quantitated by scintillation counting and normalized to internal phosphate levels.

The radiolabeled sphingolipids after treatments with [3-H]in ergo-ceramide or N-(1-13C) epoxy-C6-ceramide were extracted by the Bligh and Dyer method and separated by thin layer chromatography using a solvent system containing chloroform, methanol, and 15 mM CaCl2 (7.5:4.5:1).

Analysis of Ceramide Subspecies by Mass Spectrometry (MS)—The levels of endogenous ceramide species were analyzed by MS utilizing normal phase high performance liquid chromatography (HPLC) coupled to atmospheric pressure chemical ionization. Separations were performed using a ThermoFinnigan (Foster City, CA) LCQ ion trap mass spectrometer.2

[3H]Palmitate Labeling—Cells (1 × 106), grown in six-well plates, were treated in the presence of 1 μCi/ml [3H]palmitate (Amersham Biosciences) with or without 20 μM short chain ceramides at various time points. The lipids were extracted using Bligh and Dyer protocol and separated by thin layer chromatography (TLC) using a solvent system containing chloroform, methanol, and 2 N NH4OH. Various concentrations of total ceramide obtained from rat brain were used as controls.

Inhibition of Endocytosis—The effects of the inhibition of endocytosis on the generation of endogenous long chain ceramide in response to C6-ceramide were determined by palmitate labeling and the diacylglycerol kinase (DGK) assays as described above. Cells (1 × 106) grown in six-well plates were pretreated with known inhibitors of endocytosis such as nystatin (25 μg/ml), genistein (200 μM), and chlorpromazine (6 μg/ml) (30) and also with brefeldin A (10 μg/ml), which causes the disassembly of the Golgi (31, 32), for 30–60 min as described previously. Then cells were treated with 20 μM C6-ceramide for 60 min in the presence (for palmitate labeling) or absence (for the DGK assay) of 1 μCi/ml [3H]palmitate as described above. Ceramide measurements were performed as described above.

Measurement of Cell Survival by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or Trypan Blue Exclusion Assays—The concentrations of d-erythro-C6-ceramide or its stereoisomers that inhibited cell growth by 50% (IC50) were determined from cell survival plots obtained by MTT or trypan blue exclusion assays as described (20). In short, cells (5 × 103 cells/well) were plated into 96-well plates containing 100 μl of the growth medium in the absence or presence of increasing concentrations of C6-ceramide or its stereoisomers at 37 °C in 5% CO2 for 72 h. They were then treated with 25 μl of MTT for 4–5 h. After lysing the cells in 100 μl of the lysis buffer, the plates were read in a microplate reader (Dynatech, Chantilly, VA) at 590 nm. After that, the IC50 concentrations of the compounds were determined from cell survival plots as described (20). Triplicate wells were used for each treatment. The final concentration of ethanol (a solvent for ceramide analogs) in the growth medium was less than 0.1% (v/v), which had no effect on cell growth and survival.

To inhibit endocytosis, fumonisin B1 (FB1) (20), genistein (200 μM), chlorpromazine (6 μg/ml), and myriocin (200 μM) were added to the growth medium of (20), cells (100 × 103 cells/well) were grown in six-well plates with 4 ml of media in the absence or presence of increasing concentrations of C6-ceramide or its stereoisomers for 24 h. Then cells were trypsinized and then diluted in PBS.

2 B. J. Pettus, M. Busman, P. D. R. Moeller, B. J. Kroesen, Z. M. Szule, A. Bielawska, and Y. A. Hannun, unpublished data.
C16- and C24:1-ceramides were also examined using flow cytometry as described (20). Untreated cells were used as controls. The floating dead cells in the medium and cells attached to the plates were then counted using a hemacytometer in the presence of trypan blue solution at a 1:1 ratio (v/v) (Sigma) as described by the manufacturer.

Western Blotting—The degradation of PARP protein levels in cells was detected by Western blot analysis. In short, total proteins (50 μg/lane) were separated by 4–15% SDS-PAGE and blotted onto an Immobilon membrane, and PARP protein was detected using 1 μg/ml rabbit polyclonal anti-PARP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and peroxidase-conjugated secondary anti-rabbit antibody (1:2500) as described (20).

Analysis of Cell Cycle Profiles by Flow Cytometry—The effects of 20 μM C6-ceramide on the cell cycle profiles of A549 cells at 24 h were analyzed in the presence of DNase-free RNase and propidium iodine by flow cytometry as described (20). Untreated cells were used as controls.

Determination of Telomerase Activity—Telomerase activity in cell extracts was measured by the PCR-based telomere repeat amplification protocol (TRAP) using the TRAPeze kit (Intergen, Gaithersburg, MD), which includes a 36-bp internal control to allow quantification of activity as described by the manufacturer. Briefly, the cells, grown in six-well plates, were washed in phosphate-buffered saline and homogenized in 1× CHAPS lysing buffer for 30 min on ice. Then 50–100 ng of protein from each cell extract was analyzed in the TRAP reaction, as described (20, 21). The cell extracts were added directly to the TRAP reaction mixture containing dNTPs, TS primer (6 μM), reverse primer mix, and Taq polymerase. Then the extended telomerase products were amplified by two-step PCR (94 °C for 30 s, 60 °C for 30 s) for 27 cycles. The telomerase activity in each sample was quantitated by measuring the ratio of the 36-bp internal standard to the extended products as described by the manufacturer using Chemi-Imager (Alpha Innotech Corp., San Leandro, CA).

RESULTS

Generation of Endogenous Long Chain Ceramide in Response to Exogenous C6-ceramide—In order to examine the generation of endogenous long chain ceramide in response to exogenous C6-ceramide, A549 cells were grown in the absence or presence of 20 μM C6-ceramide at 24 h (lanes 1 and 2, respectively) were measured using the DGK assay as described under “Experimental Procedures.” The effects of FB1 (50 μM) and MYR (50 nM) on C6-ceramide-induced endogenous ceramide generation in A549 cells were also measured by the DGK assay (lanes 3 and 4, respectively). Also, the effect of FB1 on the basal ceramide levels is shown in lane 5. B, the levels of total endogenous long chain ceramides (C16–C26-ceramides) in response to 20 μM C6-ceramide at 24 h were detected using the HPLC/MS method in A549 cells as described under “Experimental Procedures.” C, the detection of species of endogenous long chain ceramide generated in response to C6-ceramide in these cells was performed using the HPLC/MS. D, the effects of C6-ceramide on the levels of endogenous dihydroceramide (C26-ceramides) were also examined by HPLC/MS. The lipid levels were normalized to the levels of inorganic phosphate levels (20, 21) and to a lesser extent C24:1-ceramide (Fig. 1B) compared with that of untreated controls (lane 1). In order determine the biochemical pathway(s) that are involved in the generation of endogenous long chain ceramide in response to C6-ceramide, A549 cells were treated with C6-ceramide (20 μM) in the presence or absence of 50 μM FB1, an inhibitor of C6-ceramide-dependent dihydroceramide/ceramide synthase, or 50 nM MYR, a specific inhibitor of serine palmitoyltransferase, and total ceramide levels were measured by the DGK assay. The results shown that FB1 prevented the generation of long chain endogenous ceramide levels in response to C6-ceramide, whereas MYR did not have any effect (Fig. 1A, lanes 3 and 4, respectively). The levels of C6-ceramide and diacylglycerol (DAG) were equal in these samples (Fig. 1A, lanes 2–4, lower and upper bands, respectively). The activity of MYR (50 nM) was confirmed in independent experiments in which it blocked the generation of endogenous ceramide in A549 cells after exposure to the chemotherapeutic agent gemcitabine.2 Also, measurement of the activity of neutral sphingomyelinase in response to C6-ceramide (20 μM) at 10 min to 24 h showed no significant changes in these cells (data not shown).

Similar results were obtained when total endogenous ceramide levels were measured by HPLC/MS in response to C6-ceramide treatment, in which long chain ceramide levels were significantly elevated (around 5-fold) in the presence of C6-ceramide (20 μM for 24 h) compared with that of untreated controls (Fig. 1B). This elevation was blocked completely by 50 μM FB1 but not by MYR (Fig. 1B). Moreover, the analysis of subspecies of endogenous ceramide levels generated in response to C6-ceramide by HPLC/MS revealed that C6-ceramide treatment resulted in increased levels of mainly C16:0-ceramide and to a lesser extent C24:1-ceramide in these cells (Fig. 1C). Importantly, there were no significant differences in the levels of dihydroceramide species in the absence or presence of C6-ceramide (Fig. 1D). The absolute mass of endogenous ceramide was about 0.8 and 5.1 pmol/nmol Pi in the absence and presence of 20 μM C6-ceramide at 24 h, respectively, in A549 cells as

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2 B. Ogretmen, M. J. Rossi, and R. Wood, unpublished data.
C6-ceramide was observed at 1 h, reached its maximum levels slightly at 1 h and reached its maximum levels at 6 h, and this into the long chain endogenous ceramide started to be detected.

The results also show that C6-ceramide served as a sphingosine backbone was initially incorporated into long chain derivatives after 24 h (lanes 1). The major derivatives were long chain sphingomyelin, glucosylceramide, lactosylceramide, and ceramide (35, 32, 9.8, and 9.2%, respectively). These results show that the generation of long chain endogenous ceramide, glucosylceramide, lactosylceramide, and sphingomyelin (no change in the activity of neutral sphingomyelinase and sphingomyelin levels).

To further investigate the biochemical pathways involved in ceramide formation in response to C6-ceramide, A549 cells were grown in the presence of unlabeled C6-ceramide (20 μM) at 0, 1, 3, 6, and 24 h. After extraction using the Bligh and Dyer method, total endogenous ceramide subtypes were analyzed using HPLC/MS method as described under “Experimental Procedures.” The results shown represent at least two independent experiments.

| [3H]C6-Cer (h) | C16:0-Cer | C16-Cer | C6-Cer | LC-GlcCer | C6-GlcCer | LC-LacCer | C6-LacCer | LC-SM | C6-SM |
|---------------|----------|---------|-------|-----------|---------|---------|----------|------|------|
| 0             |          |         |       |           |         |         |          |      |      |
| 1             |          |         |       |           |         |         |          |      |      |
| 3             |          |         |       |           |         |         |          |      |      |
| 6             |          |         |       |           |         |         |          |      |      |
| 24            |          |         |       |           |         |         |          |      |      |

**FIG. 2. Analysis of the time kinetics of the generation of endogenous long chain ceramide in response to [3-3H]C6-ceramide and unlabeled C6-ceramide in A549 cells.** A, time kinetics of the generation of endogenous ceramide in response to exogenous ceramide was examined by growing the cells in the presence of 1 μM [sphingosine-3-3H]C6-ceramide (1 μCi/ml) at 0, 1, 3, 6, and 24 h (lanes 1–5, respectively). The labeled lipids were extracted by the Bligh and Dyer method and analyzed by thin layer chromatography as described under “Experimental Procedures.” Purified C16- and C18-ceramides were used as standards. B, generation of endogenous ceramide at various time points was analyzed by growing cells in the presence of unlabeled C6-ceramide (20 μM) at 0, 1, 3, 6, and 24 h. After extraction using the Bligh and Dyer method, total endogenous ceramide subspecies were analyzed using HPLC/MS method as described under “Experimental Procedures.” The results shown represent at least two independent experiments.

measured using the DGK method. Therefore, these results show that the measurement of the levels of endogenous ceramide by the HPLC/MS method is highly comparable with the DGK assay. Taken together, these results also show that treatment of A549 cells with C6-ceramide results in a significant increase in the generation of endogenous long chain ceramide, via a pathway that is distinct from the de novo synthesis (resistance to myriocin) or hydrolysis of sphingomyelin (no change in the activity of neutral sphingomyelinase and sphingomyelin levels).

**Analysis of the Time Dependence of the Generation of Long Chain Endogenous Ceramide in Response to C6-ceramide—**To examine the kinetics of the generation of long chain endogenous ceramide in response to C6-ceramide, A549 cells were grown in the presence of 1 μM [sphingosine-3-3H]p-erythro-C6-ceramide (1 μCi/ml) at 0, 1, 3, 6, and 24 h, and then the labeled lipids were analyzed as described under “Experimental Procedures.” Results showed that the incorporation of the 3H label into the long chain endogenous ceramide started to be detected slightly at 1 h and reached its maximum levels at 6 h, and this increased ceramide generation was sustained at 24 h (Fig. 2A, lanes 1–5). There was also a marked increase in the levels of labeled short chain glucosylceramide, lactosylceramide, and sphingomyelin. The 3H label was also detected in long chain glucosylceramide, lactosylceramide, and sphingomyelin at 24 h (Fig. 2A, lane 5). These results show that the 3H label in the sphingosine backbone was initially incorporated into long chain ceramides followed by incorporation into complex sphingolipids. The results also show that C6-ceramide served as a direct substrate for SM synthase and glucosylceramide synthase.

Total endogenous long chain ceramide levels in response to 20 μM unlabeled ceramide at various time points were also measured by HPLC/MS. The accumulation of total long chain endogenous ceramides (mainly C16:0 and C24:1) in response to C6-ceramide was observed at 1 h, reached its maximum levels at 6 h, and was still detectable at 24 h (Fig. 2B). These results were highly comparable with the results obtained using 1 μM [sphingosine-3-3H]p-erythro-C6-ceramide as shown in Fig. 2A.

**Detection of the Biochemical Mechanisms for the Generation of Long Chain Endogenous Ceramide in Response to C6-ceramide—**To determine the biochemical mechanisms by which C6-ceramide caused increased generation of endogenous long chain ceramide, A549 cells were grown in the presence of 1 μM [sphingosine-3-3H]p-erythro-C6-ceramide (1 μCi/ml) for 24 h, and then labeled lipids were analyzed by thin layer chromatography following Bligh and Dyer extraction as described under “Experimental Procedures.” As Fig. 3A shows, 3H label was incorporated into the newly synthesized long chain ceramides (lane 1). Interestingly, the presence of FB1 caused a significant decrease in the incorporation of the 3H label into the long chain ceramide and caused some inhibitory effect on the incorporation of the label into the long chain glucosylceramide, lactosylceramide, and sphingomyelin (Fig. 3A, lane 2). The pretreatment of cells with MYR, however, did not have any significant effect on the generation of ceramide (Fig. 3A, lane 3). As seen in Fig. 3A, the majority (about 86%) of the [sphingosine-3-3H]p-erythro-C6-ceramide was metabolized to long chain derivatives after 24 h (lane 1). The major derivatives were long chain sphingomyelin, glucosylceramide, lactosylceramide, and ceramide (35, 32, 9.8, and 9.2%, respectively).

More importantly, when these cells were treated with 1 μM N-[N-hexanoyl-1-14C]p-erythro-C6-ceramide (1 μCi/ml) with the label on the fatty acid chain, the 14C label was not incorporated into the long chain endogenous ceramide, glucosylceramide, lactosylceramide, or sphingomyelin at 24 h, whereas it was readily detectable in their short chain forms in the absence or presence of FB1 (Fig. 3B, lanes 2 and 3). Taken together, these results show that the generation of long chain endogenous ceramide in response to C6-ceramide treatment is due to biochemical recycling of the sphingosine backbone of the short chain ceramide and not due to the elongation of its fatty acid chain in these cells.

**Detection of the Generation of Long Chain Endogenous Ceramide in Response to Exogenous C6-ceramide by 3H/Palmitate Labeling—**To further investigate the biochemical pathways involved in ceramide formation in response to C6-ceramide, studies were conducted in A549 cells grown in the presence of [3H]palmitate (1 μCi/ml) with or without unlabeled p-erythro-C6-ceramide (20 μM) for various time points. A significant increase in the incorporation of the [3H]palmitate into the endogenous C16:0-ceramide was observed at 1, 3, 6, and 24 h in the
presence of C6-ceramide when compared with untreated controls (Fig. 4A, lanes 1–8). Further analysis showed that the incorporation of palmitate into endogenous ceramide was detectable as early as 30 min (Fig. 4B, lanes 1–5). Moreover, the results also showed that the incorporation of the [3H]palmitate into endogenous ceramide in the presence of 20 μM C6-ceramide was prevented by FB1, but not MYR, at 3 h in these cells (Fig. 4C, lanes 4–6). Fig. 4C also shows that the basal levels of C16-ceramide in the absence of C6-ceramide were slightly inhibited by FB1 and MYR when compared with controls (lanes 2, 3, and 1, respectively). The inability of MYR to inhibit incorporation of [3H] label into ceramide demonstrates that palmitate was not introduced by serine palmitoyltransferase. On the other hand, inhibition by FB1 shows that the label was introduced at the level of dihydroceramide/ceramide synthase. Therefore, these results further demonstrate that the generation of endogenous long chain ceramide formation in response to C6-ceramide is not via the activation of the de novo pathway but involves the recycling of the sphingosine backbone following deacylation and reacylation, a process inhibited by FB1.

Since endocytosis was shown to be involved in the internalization of various sphingolipids (30), and exogenous short chain ceramide was implicated in the stability of the Golgi apparatus (33), the effects of inhibition of endocytosis and the disassembly of the Golgi on the generation of endogenous long chain ceramide were examined. Cells were treated with the inhibitors of endocytosis (nystatin and genistein for clathrin-dependent internalization) and chlorpromazine (for clathrin-independent internalization) (30) and also with brefeldin A, which is known to cause the disassembly of the Golgi (31, 32) as described under “Experimental Procedures.” Then, the levels of endogenous ceramide were measured by palmitate labeling and the DGK assay as described under “Experimental Procedures.” As seen in Fig. 4D, treatment of cells with the inhibitors of endocytosis resulted in a modest decrease (around 10–30%) in the incorporation of palmitate into endogenous C16-ceramide in response to C6-ceramide at 1 h. However, generation of endogenous ceramide in response to C6-ceramide was completely blocked in the presence of brefeldin A, suggesting a role for the Golgi in the metabolism of ceramide. Similar results were also obtained by the diacylglycerol kinase assay (data not shown).

Stereo specificity of Reacylation/Deacylation—Since the above results suggested deacylation followed by reacylation and since the currently known ceramidases and Ca2+-dependent ceramide synthase demonstrate high stereospecificity (34–36), it became important to evaluate the stereospecificity of the generation of the long chain ceramide in response to d-erythro-C6-ceramide. The approach used above with [3H]palmitate allowed us to examine the stereospecific effects of unlabeled stereoisomers (Fig. 5A) on the generation of ceramide. Interestingly, the data showed that [3H]palmitate incorporation into long chain ceramide was not detectable in the presence of l-erythro-C6-ceramide (20 μM at 3 h), whereas it was highly detectable in the presence of d-erythro-C6-ceramide (Fig. 5B, lanes 1–3). Moreover, d-erythro-C6-dihydroceramide did not result in increased incorporation of the labeled palmitate (Fig. 5B, lane 4). Similar results were obtained when d-erythro-C2- and d-erythro-C2-dihydroceramides were used in the presence of [3H]palmitate (Fig. 5B, lanes 5 and 6). These results were also confirmed by measuring the accumulation of total endogenous long chain ceramides in response to d-erythro- and l-erythro-C6-ceramides by HPLC/MS, which showed that d-erythro- but not l-erythro-C6-ceramide treatment resulted in a significant increase in the endogenous long chain ceramide levels in A549 cells (Fig. 5C). Therefore, these results indicate that the generation of endogenous ceramide in response to C6-ceramide via deacylation/reacylation is highly stereospecific, which was induced by d-erythro- but not by l-erythro-C6-ceramide in these cells.

Similar results were also obtained using the MCF-7 human breast cancer and HL-60 human acute myeloid leukemia cells, in which the generation of endogenous ceramide was induced in the presence of d-erythro-, but not l-erythro-C6-ceramide, as determined by [3H]palmitate labeling and HPLC/MS methods, respectively (Figs. 6, A and B).

Analysis of Roles of d-erythro- and l-erythro-C6-ceramides in Growth Inhibition, Cell Cycle Arrest, and Modulation of Telomerase Activity—We have shown previously that exogenous C6-ceramide and endogenous long chain ceramides generated by overexpression of bacterial sphingomyelinase or in response to daunorubicin treatment cause the inhibition of telomerase in A549 cells (24). The inhibition of telomerase in response to C6-ceramide treatment (20 μM at 3–24 h) has been shown to be independent of apoptosis but correlated with G1/G0 cell cycle arrest and inhibition of growth in these cells (20). To address the question of whether cell cycle arrest, growth, and telomerase inhibition in response to C6-ceramide are due to the intracellular accumulation of C6-ceramide per se or due to the generation of endogenous long chain ceramides, A549 cells were grown in the absence or presence of 20 μM d-erythro- or l-erythro-C6-ceramide for 24 h, and then cell survival, cell cycle...
profiles, and telomerase activity were measured as described under “Experimental Procedures.” The results showed that 20 μM \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide caused about 50% growth inhibition, whereas 20 μM \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramide had no significant effect at 24 h, as measured by MTT assay (Fig. 7A). Since MTT is a measure of living cells, and a decrease in MTT-positive cells can be either due to decreased growth or apoptosis, we performed trypan blue staining. Trypan blue is taken up by the cells with damaged plasma membrane due to apoptosis or necrosis. Consistent with our recent report (24), trypan blue staining was detected in only around 2–3% of the cell population after treatment with 20 μM \( \text{C}_{6} \)-ceramide at 24 h (data not shown), demonstrating that decreased number of MTT-positive cells in response to \( \text{C}_{6} \)-ceramide at 24 h is due to growth inhibition and not apoptotic cell death in these cells. Indeed, these results were confirmed with studies in which 20 μM \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide (at 24 h) caused G0/G1 cell cycle arrest; however, \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramide had no significant effect on cell cycle profiles in these cells (Fig. 7B). The results demonstrated also that telomerase activity was significantly inhibited in response to 20 μM \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide at 24 h, whereas 20 μM \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramide had no effect on telomerase activity when compared with untreated controls (Fig. 8A, lanes 1–3). In addition, telomerase inhibition due to \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide treatment was blocked in the presence of FB1 (Fig. 8B, lanes 1–3), which inhibited the formation of endogenous long chain ceramide, and not intracellular accumulation of \( \text{C}_{6} \)-ceramide, as shown in Figs. 1, 2, and 4.

Since A549 cells did not undergo apoptosis in response to usual concentrations ceramide (20), the effects of \( \delta \)-erythro- and \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramides on apoptosis were examined in HL-60 cells by trypan blue and PARP cleavage analyses as described under “Experimental Procedures.” Interestingly, results showed that both \( \delta \)-erythro- and \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramides were equally effective in the induction of apoptosis in these cells (Fig. 9A). The IC50 values of \( \delta \)-erythro- and \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramides were 11 and 13 μM at 24 h (Fig. 9A). These results were also confirmed by the detection of PARP cleavage in response to 10 μM \( \delta \)-erythro- and \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramides at 24 h in HL-60 cells (Fig. 9B). Taken together, these results suggest that the roles of endogenous long chain ceramide and exogenous short chain ceramide in apoptotic cell death might be distinct and/or cell line-specific.

**DISCUSSION**

This study shows that treatment of A549 cells with exogenous \( \text{C}_{6} \)-ceramide results in the generation of long chain (mainly \( \text{C}_{16:0} \) and \( \text{C}_{24:1} \)) ceramides, and this process involves recycling of the sphingosine backbone after deacylation/reacylation and is not due to the elongation of its \( \text{C}_{6} \) fatty acid chain. Our results also demonstrate that although \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide treatment results in the generation of endogenous long chain ceramides, treatment with its stereoisomer, \( \lambda \)-erythro-C6- \( \text{C}_{6} \)-ceramide, and its biologically inactive analog dihydroceramide did not cause the generation of endogenous ceramides in this cell line. In addition, the data provided in this study show that treatment of cells with brefeldin A, which causes Golgi disassembly, completely blocked the generation of endogenous long chain ceramide in response to \( \text{C}_{6} \)-ceramide. More interestingly, our results demonstrate for the first time that the generation of long chain endogenous ceramide correlates with the downstream biological roles of \( \delta \)-erythro-C6- \( \text{C}_{6} \)-ceramide in cell cycle arrest at G0/G1, growth, and telomerase inhibition, which were not affected by \( \lambda \)-erythro-C6- and dihydro-C6- \( \text{C}_{6} \)-ceramides.
The specific enzymes involved in the generation of long chain endogenous ceramide in response to C₆-ceramide are still unknown. However, our results showed that the generation of endogenous ceramide is inhibited by FB1, which inhibits CoA-dependent dihydroceramide/ceramide synthase (37, 38), but not by MYR, a specific inhibitor of serine palmitoyltransferase, the rate-limiting enzyme of the de novo pathway (39). These results, therefore, suggest a role of CoA-dependent ceramide synthase for the generation of endogenous long chain ceramide in response to C₆-ceramide treatment, which involves the reacylation of free sphingosine. This enhancement of formation of [³H]C₁₆-ceramide by C₆-ceramide could result from either a direct mechanism whereby C₆-ceramide is first deacylated to generate sphingosine or an indirect mechanism where C₆-ceramide stimulates the breakdown of complex sphingolipids, eventually resulting in more sphingosine. The former mechanism was confirmed by studies performed using [³H]-sphingosine-3-³H]-C₆- and N-[N-hexanoyl-1-¹⁴C]-d-erythro-C₆-ceramide, which showed that the sphingosine backbone of the labeled C₆-ceramide was reacylated by an FB1-inhibitable CoA-dependent ceramide synthase, following deacylation of C₆-ceramide probably by ceramidase(s), as summarized in Fig. 10.

Interestingly, the data presented here also showed that treatment of cells with d-erythro-C₆-ceramide and d-erythro-C₆-dihydroceramide did not cause the generation of endogenous ceramide, whereas d-erythro-C₆-ceramide was first deacylated to generate sphingosine or an indirect mechanism where C₆-ceramide stimulates the breakdown of complex sphingolipids, eventually resulting in more sphingosine. The former mechanism was confirmed by studies performed using [³H]-sphingosine-3-³H]-C₆-ceramide and N-[N-hexanoyl-1-¹⁴C]-d-erythro-C₆-ceramide, which showed that the sphingosine backbone of the labeled C₆-ceramide was reacylated by an FB1-inhibitable CoA-dependent ceramide synthase, following deacylation of C₆-ceramide probably by ceramidase(s), as summarized in Fig. 10.

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age of ceramide to give rise to sphingosine and free fatty acid (41). There are three types of ceramidases: acid, neutral, and alkaline. Acid ceramidase (40) is localized in lysosomes, and its dysfunction causes Farber disease, one of the sphingolipidoses. The human neutral ceramidase has recently been cloned and sequenced (42). It prefers ceramide over dihydroceramide and overexpressions (26). In the same study, it was also demonstrated that the sustained accumulation of endogenous ceramide in response to short chain ceramide treatment was inhibited by FB1 (26). The latter is consistent with the results presented in this study. However, our results showed no significant changes in the levels of neutral sphingomyelinase activity or SM hydrolysis in response to C6-ceramide in A549

It has been shown previously that treatment of U937 and HL60 myeloid leukemia cells and normal skin fibroblasts with cell-permeable short chain ceramides (C2- and C6-ceramides) causes the activation of neutral sphingomyelinase, sphingomyelin hydrolysis, and endogenous ceramide generation regardless of bcl-2 overexpression (26). In the same study, it was also demonstrated that the sustained accumulation of endogenous ceramide in response to short chain ceramide treatment was inhibited by FB1 (26). The latter is consistent with the results presented in this study. However, our results showed no significant changes in the levels of neutral sphingomyelinase activity or SM hydrolysis in response to C6-ceramide in A549
cells. Therefore, taken together, these results suggest that the downstream metabolic responses, such as activation of sphingomyelinase and hydrolysis of SM, due to short chain ceramide treatment might be cell line-specific.

Mechanistic studies have revealed that ceramide-induced apoptotic cell death is due to activation of effector caspases, and G2/M cell cycle arrest mediated by ceramide depends on activation of retinoblastoma gene product (Rb) due to its dephosphorylation (46). However, in cells that overexpress antiapoptotic proteins such as Bcl-2, or in which protein kinase-α has been activated, apoptosis can no longer be induced by ceramide, whereas it retains its ability to activate Rb and induce cell cycle arrest (16). These results also show that ceramide has a dual function to regulate apoptosis and cell cycle arrest and that ceramide is capable of regulating these two cellular events independently. Consistent with these findings, the results presented in this study showed that treatment of A549 cells with l-erythro-C₆-ceramide did not result in apoptosis but caused a significant inhibition of growth and telomerase activity and also induced G2/M cell cycle arrest, which is consistent with our previous results (20). However, l-erythro-C₆-ceramide and dihydro-C₆-ceramide, which did not increase the generation of long chain endogenous ceramides, did not have any effect on apoptosis, growth, telomerase activity, or cell cycle profiles in these cells. Therefore, these results indicate that the generation of endogenous long chain ceramide in response to C₆-ceramide treatment is important in growth inhibition, cell cycle arrest, and modulation of telomerase activity in A549 cells.

Interestingly, although l-erythro-C₆-ceramide did not induce the generation of endogenous ceramide in HL-60 cells, both d-erythro- and l-erythro-C₆-ceramides were equally effective in causing apoptotic cell death, as measured by trypan blue staining and PARP cleavage, in HL-60 cells. The IC₅₀ values of these two compounds were around 10 μM at 24 h in HL-60 cells. These results, therefore, suggest that the roles of endogenous long chain ceramide and exogenous short chain ceramide in apoptotic cell death might be distinct and/or cell line-specific. However, distinct roles of exogenous short chain and endogenous long chain ceramides in apoptotic cell death need to be further examined.

The inhibition of the generation of endogenous long chain ceramide from exogenous short chain by FB1 provides a mechanistic tool to study the specific downstream biological activities of endogenous long chain ceramide in growth inhibition, cell cycle arrest, and modulation of telomerase activity in A549 cells. The ³H and ¹⁴C labels are represented by * and †, respectively.

![Schematic model of the generation of endogenous ceramide in response to d-erythro-C₆-ceramide.](image)

**FIG. 10.** Schematic model of the generation of endogenous ceramide in response to d-erythro-C₆-ceramide. Biochemical mechanisms of generation of endogenous long chain ceramide in response to C₆-ceramide were examined using selectively labeled C₆-ceramides on the sphingosine backbone or on its fatty acid moiety, namely [sphingosine-3-³H]C₆-ceramide and N-[N-hexanoyl-1-¹⁴C]l-erythro-C₆-ceramide, respectively. The results shown in this study demonstrated that ³H label was incorporated into the newly synthesized long chain ceramides, which was prevented by the presence of FB1. The ³H label was also detected in the short chain ceramide, glucosylerceramide, lactosylerceramide, or sphingomyelin. However, when these cells were treated with N-[N-hexanoyl-1-¹⁴C]l-erythro-C₆-ceramide, the ¹⁴C label was not incorporated into the long chain endogenous ceramide, glucosylerceramide, lactosylerceramide, or sphingomyelin at 24 h, whereas it was readily detectable in their short chain forms. Taken together, these results show that the generation of long chain endogenous ceramide in response to C₆-ceramide treatment is due to biochemical recycling of the sphingosine backbone of the short chain ceramide, which involves deacylation (probably by a ceramidase) and reacylation (by FB1-inhibitable ceramide synthase), and is not due to the elongation of its fatty acid chain. More importantly, this study provides evidence for the downstream biological activities of endogenous long chain ceramide in growth inhibition, cell cycle arrest, and modulation of telomerase activity in A549 cells. The ³H and ¹⁴C labels are represented by * and †, respectively.
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