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Inhibition of CTP:Phosphocholine Cytidylyltransferase by C_2-Ceramide and Its Relationship to Apoptosis

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ABSTRACT

Apoptosis induced by antitumor phospholipid analogs takes place after the inhibition of the CTP:phosphocholine cytidylyltransferase (CCT; EC 2.7.7.15) catalyzed step of phosphatidylcholine (PtdCho) biosynthesis. Exposure of cells to synthetic short-chain ceramide analogs also triggers apoptosis concomitant with decreased PtdCho biosynthesis, and the present study was undertaken to ascertain whether C_2-ceramide inhibition of PtdCho synthesis is direct or secondary to other ceramide-mediated cellular responses. The exposure of COS-7 cells to either C_2-ceramide, ET-18-OCH_3, or farnesol resulted in time- and dose-dependent apoptotic cell death. Cells treated with C_2-ceramide or ET-18-OCH_3 selectively and immediately accumulated phosphocholine, whereas CDP-choline increased with farnesol treatment. In vitro assays of CCT activity demonstrated that C_2-ceramide directly inhibited CCT. Comparison of different *N*-linked sphingosine derivatives suggests an inverse relationship between the length of the *N*-linked carbon chain and the derivatives ability to trigger apoptosis and inhibit CCT. Taken together, our results suggest CCT as a primary target for C_2-ceramide inhibition that accounts for its cytotoxic effects.

Regulation of phosphatidylcholine (PtdCho) metabolism is a vital facet of cell biology, with implications in the control of cell proliferation and programmed cell death or apoptosis (Lykidis and Jackowski, 2000). Biosynthesis of PtdCho occurs via the CDP-choline pathway (Kennedy and Weiss, 1956) and involves three enzymatic reactions. Choline is first phosphorylated to phosphocholine, which is activated by CTP to yield cytidinediphosphocholine, and PtdCho is synthesized by transferring the phosphocholine moiety of cytidinediphosphocholine to diacylglycerol. These reactions are catalyzed by choline kinase, CTP:phosphocholine cytidylyltransferase (CCT; EC 2.7.7.15), and CPT, respectively (Kennedy and Weiss, 1956; Kent, 1995). The rate-limiting step in this pathway is the CCT-catalyzed reaction and the activity of this enzyme is highly regulated. CCT is potently controlled by lipid regulators that bind to the amphipathic helical domain (Yang et al., 1995) or the carboxyl-terminal domain of CCT (Lykidis et al., 2001) to modulate activity.

An emerging body of evidence connects the inhibition of PtdCho biosynthesis with apoptosis in a variety of experimental systems (Voziyan et al., 1993; Haug et al., 1994; Boggs et al., 1995; Miquel et al., 1998; Anthony et al., 1999; Yen et al., 1999), and the Kennedy pathway is a direct target for cytotoxic drugs. For instance, the inhibition of CCT both in vivo and in vitro by the antitumor phospholipid analogs, ET-18-OCH_3 (or edelfosine) and hexadecylphosphocholine (or miltefosine), are well characterized (Voziyan et al., 1993; Boggs et al., 1998). The apoptogenic isoprenoids farnesol and geranylgeraniol inhibit CPT activity, and similar results have been obtained with other compounds, such as camptothecin, etoposide, and chelerythrine (Voziyan et al., 1993; Haug et al., 1994; Miquel et al., 1998; Anthony et al., 1999). The inhibitory action on PtdCho synthesis, either at the CCT- or the CPT-catalyzed steps, correlates with the cytotoxic properties of these drugs, although farnesol may also induce apoptosis independent of its effect on PtdCho synthesis (Wright et al., 2001). The observations that choline deficiency induces apoptosis (Yen et al., 1999, 2001), that either CCT overexpression or PtdCho supplementation rescues cells from apoptosis induced by antineoplastic phospholipids (Baburina and Jackowski, 1998; Boggs et al., 1998), and that transfer of a mutant cell line with a thermosensitive CCT to

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ABBREVIATIONS: ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; C_2-ceramide, *N*-acetyl-D-*erythro*-sphingosine; C_2-dihydroceramide, *N*-acetyl-D-dihydro-*erythro*-sphingosine; C_6-ceramide, *N*-hexanoyl-D-*erythro*-sphingosine; C_{16}-ceramide, *N*-palmitoyl-D-*erythro*-sphingosine; Cho, choline; PtdCho, phosphatidylcholine; CCT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2,-diacylglycerol phosphotransferase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

the nonpermissive temperature triggers apoptosis (Cui et al., 1996) strongly support the hypothesis.

Ceramide is an intracellular signaling molecule implicated in the induction of apoptosis (Hannun and Luberto, 2000). *N*-linked, short carbon-chain derivatives of sphingosine are widely used as cell-permeable analogs of ceramide to mimic its cellular actions (Luberto and Hannun, 2000). Ceramide analogs inhibit PtdCho synthesis (Bladergroen et al., 1999; Allan, 2000; Ramos et al., 2000; Vivekananda et al., 2001), and the pattern of metabolic intermediate accumulation in cells exposed to C_2-ceramide points to CCT as the target (Allan, 2000; Vivekananda et al., 2001), whereas C_6-ceramide may inhibit CPT (Bladergroen et al., 1999). It is not known whether the effects of ceramide analogs are caused by direct inhibition of Kennedy pathway enzymes, as is the case for ET-18-OCH_3, or may be more complex and secondary to other ceramide-mediated responses, as proposed recently (Awasthi et al., 2001).

We show that C_2-ceramide induces apoptosis in COS-7 cells and inhibits CCT activity in both whole cells and in vitro enzyme assays, and we uncover a relationship between the ability of different *N*-linked carbon chain ceramide analogs to inhibit CCT and their cytotoxic properties.

Materials and Methods

Materials. Dulbecco's modification of Eagle's minimal essential medium (DMEM) was purchased from BioWhittaker (Walkersville, MD). Trypan blue, trypsin, phosphate-buffered saline (PBS), penicillin/streptomycin, LipofectAMINE reagent, and Geneticin (G418) were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) was from Atlanta Biologicals (Norcross, GA). [*methyl*-^{3}H]Choline chloride (80 Ci/mmol) and cytidine diphospho-[*methyl*-^{14}C]choline (55 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Phospho-[*methyl*-^{14}C]choline (58 mCi/mmol) was supplied by Amersham Biosciences (Piscataway, NJ). C_2-ceramide, C_2-dihydroceramide, C_6-ceramide, C_{16}-ceramide, ET-18-OCH_3, and farnesol were all obtained from Calbiochem-Novabiochem (San Diego, CA). Diolein and oleic acid were supplied by Avanti Polar Lipids (Birmingham, AL) or Sigma-Aldrich (St. Louis, MO). In situ cell death detection kit was purchased from Roche Applied Science (Indianapolis, IN). Silica gel thin-layer chromatography plates were supplied by Analtech (Newark, DE). All other chemicals and supplies were reagent grade or better. COS-7 cells were obtained from the laboratory of J. Ihle (St. Jude Children's Research Hospital, Memphis, TN).

Cell Culture. COS-7 cells were routinely grown in DMEM supplemented with 10% (v/v) FCS, fresh 2 mM glutamine, penicillin (50 units/ml), and streptomycin (50 μg/ml), and maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C. Before treatment, cells were trypsinized, counted, and seeded in complete DMEM supplemented with 0.5% (v/v) FCS, and incubated at least 2 h to allow cells to attach. After attachment, C_2-ceramide (in ethanol), ET-18-OCH_3 (in ethanol), or farnesol (in ethanol) were added at the indicated concentration. Ethanol was added to control cells so that the final concentration of vehicle was $<0.2\%$ in control and treated cultures. After incubation for the indicated times, adherent and floating cells were collected for analysis.

For CCT assays, COS-7 cells were transiently transfected with pcDNA3.1 vector containing the rodent CCTα cDNA (Lykidis et al., 2001). Briefly, cells at 80% confluence were exposed to LipofectAMINE reagent complexed with DNA and incubated for 12 h in medium without serum, after which time the medium was replaced with fresh medium containing 10% FCS. Cells were harvested 24 h later, washed with PBS, and cell pellets were stored at −20°C up to 2 weeks. Cells were also cultured in medium containing 500 μg/ml G418 and stable resistant clones were selected. Ten independent clones were screened for overproduction of PtdCho by metabolic labeling with [*methyl*-^{3}H]choline as described below. Two independent clones were found which overexpressed CCT activity to the same extent as in transiently transfected cells and one was used for enzyme assays.

Viability Determinations. Cells were seeded at a density of 6.125×10^5 cells in 35-mm culture dishes. After 18 h of incubation, cells were collected, 0.2% (v/v) trypan blue was added, and cells were counted in a hemocytometer. Total cell numbers were calculated from the average of four different fields with at least 100 cells each. Cells that excluded the vital dye were divided by the total number of cells and multiplied by 100 to calculate the percentage viable.

Analysis of Cellular DNA Content. After 18 h of treatment, floating cells were collected from the medium by centrifugation and adherent cells were trypsinized, washed with PBS, and combined. Washed cells were resuspended in 0.05 mg/ml propidium iodide, 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 at a concentration of 10^6 cells/ml. Cells were treated 30 min with 0.2 mg/ml ribonuclease in 10 mM Tris-HCl, pH 7.5, 15 mM NaCl, and filtered through nylon mesh with a 40-μm pore size. Particulate DNA was quantitated by flow cytometry at the St. Jude Flow Cytometry facility.

Electron Microscopy. Cells were seeded at 1.75×10^6 cells in DMEM containing 0.5% FCS, in 60-mm culture dishes, and allowed to attach for 2 h. Cells were then incubated 18 h with 40 μM C_2-ceramide or ethanol, then rinsed with PBS and fixed with 2% (v/v) glutaraldehyde in PBS. After fixation, cells were scraped and pelleted by centrifugation. Cell pellets were postfixed with a solution containing 1% (v/v) osmic acid in PBS and stained with 2% uranyl acetate in ethanol. Pellets were dehydrated in graded ethanol (50 to 100%) and embedded in Spurr's resin. Thin sections were poststained with uranyl acetate and Reynolds lead citrate. Samples were examined with a JEOL 1200 Ex microscope.

Quantitation of Apoptosis. Apoptotic cells were detected by terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) as described previously (El Mouedden et al., 2000). Trypsinized cells and media were centrifuged, and pelleted cells were rapidly rinsed with PBS containing 1% bovine serum albumin and fixed in 4% formaldehyde for 1 h. After centrifugation and rinsing with PBS, cells were transferred to 96-well plate and treated with 0.3% H_2O_2 in methanol for 10 min to quench endogenous peroxidase activity. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated extension of 3′-hydroxyl ends of fragmented DNA, using fluorescein-labeled dUTP as a precursor, according to the instructions from the supplier. DNA-bound fluorescein was detected by reaction with antifluorescein antibody conjugated to peroxidase. Peroxidase activity in immunocomplexes was visualized by reaction of diaminobenzidine in H_2O_2. Cells were resuspended in PBS, spread on poly(lysine)-coated slides and allowed to air dry. Cells were then counterstained with methyl green, rinsed with distilled water, and the preparations were mounted using permanent medium. Quantitation of apoptotic nuclei was made on slides from each experiment, using an E600 Nikon light microscope with a 50× objective and a 10× eyepiece. We counted all nuclei exhibiting a frank brown labeling. These nuclei most often displayed typical alterations such as pyknosis, crescent-like condensation of chromatin, or segregation into apoptotic bodies. The incidence of apoptotic nuclei was given as the percentage relative to total nuclei. At least 100 cells were counted for each determination.

Metabolic Labeling of Cells. Cells were seeded at a density of 8.75×10^5 cells in 60-mm dishes in complete medium and incubated for 24 h. The medium was then replaced with medium containing 0.5% serum, and [*methyl*-^{3}H]choline was added (10 μCi/ml), at the same time as C_2-ceramide or other agents at the indicated concentrations and times. After incubation, the medium was removed and cells were washed twice with ice-cold PBS. Cells were harvested on ice, washed with 1 ml of PBS, and then pelleted for extraction of

lipids (Bligh and Dyer, 1959). Briefly, the pellet was resuspended in 0.1 ml water, and 0.24 ml of methanol and 0.15 ml of chloroform were added. After 10 min at room temperature, 0.15 ml of chloroform and 0.12 ml of water were added. The tubes were capped, shaken vigorously and then centrifuged to clearly separate the two phases. Total radioactivity in the upper (aqueous) and lower (organic) phases was quantified by scintillation counting. To separate the water-soluble [^{3}H]choline metabolites, 0.2-ml aliquots of the upper phase were evaporated, resuspended in 40 μl of water, and spotted onto preadsorbent Silica Gel G thin-layer chromatography plates, which were developed in 95% ethanol/2% NH_4OH (1:1, v/v). Identification of radiolabeled choline, phosphocholine, and CDP-choline was made by comigration with authentic standards. The radiolabeled lipid in the lower phase was >95% PtdCho at the time points in this study, as verified by thin-layer chromatography on Silica Gel G plates developed in chloroform/acetic acid/methanol:water (5:2:4:1, v/v). Quantitation was done by scraping into liquid scintillation vials the silica gel from regions corresponding to migration of the standards.

CCT Assay. COS-7 cells overexpressing CCT were lysed, and CCT protein was partially purified and delipidated essentially as described previously (Vance et al., 1980). Briefly, the lysis buffer contained 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, and 1 μM Na_3VO_4. After a 30-min incubation on ice to allow cell swelling under the hypotonic conditions, cells were disrupted by sonication in a sonifier (Misonix, Inc., Farmingdale, NY) equipped with a cup horn three times for 30-s intervals. Cell debris was removed by low-speed centrifugation. The supernatant was loaded onto a 1-ml DEAE-Sepharose column, which was washed in succession with 2 ml of lysis buffer, 1.5 ml of lysis buffer plus 1% Triton X-100, to remove endogenous lipid, 2 ml of lysis buffer to remove Triton X-100, and finally 1.5 ml of 0.25 M NaCl in lysis buffer. CCT activity, which eluted in the 0.25 M NaCl fraction, was determined in an in vitro assay by measuring the incorporation of phospho-[*methyl*-^{14}C]choline into cytidine diphospho-[*methyl*-^{14}C]choline using a method essentially as described previously (Awasthi et al., 2001). The assays contained 150 mM bis-Tris-HCl, pH 6.5, 10 mM $MgCl_2$, 2 mM CTP, 40 μM PtdCho/oleic acid (1:1), 1 mM [^{14}C]phosphocholine (specific activity, 4.2 mCi/mmol), and 5 to 20 μg of delipidated lysate protein. C_2-ceramide, C_2-dihydroceramide, C_6-ceramide, C_{16}-ceramide, (in ethanol), ET-18-OCH_3 (in ethanol), or farnesol (in ethanol) were prewarmed to 37°C and added to the assays. Control assays contained solvent alone, which did not significantly alter enzyme activity. Assays (50 μl) were started by the addition of [^{14}C]phosphocholine, incubated for 15 min at 37°C, and stopped by addition of 5 μl of 0.5 M EDTA. Aliquots (40 μl) of each assay were spotted onto preadsorbent Silica Gel G thin-layer plates and developed in 95% ethanol/2% NH_4OH (1:1, v/v). Identification of CDP-choline product was made on the basis of comigration with authentic standard, and quantitation was performed using an Imaging Detector BioScan (Washington, DC). Assays were done in duplicate and the experiment was repeated twice.

Results

In the first series of experiments, we studied the effect of C_2-ceramide on COS-7 cell viability, and compared it with that of the known inhibitors of PtdCho biosynthesis, ET-18-OCH_3 and farnesol. C_2-Ceramide induced cell death in a dose-dependent fashion, as did inhibitors of PtdCho synthesis (Fig. 1). The half-maximal effective concentration of C_2-ceramide was about 20 μM, whereas those of ET-18-OCH_3 and farnesol were 7 μM and 25 μM, respectively.

Further experiments were designed to establish the apoptotic nature of the cell death induced by C_2-ceramide in COS-7 cells. Analysis of the cell DNA content by flow cytometry revealed a clear increase of subdiploid DNA nuclei (denoted as M1 in Fig. 2) in cells treated with C_2-ceramide for 18 h (Fig. 2B). A similar incidence of cells with fragmented DNA appeared after treatment with ET-18-OCH_3 or farnesol (Fig. 2, C and D). Interestingly, cells treated with C_2-ceramide or ET-18-OCH_3 had an increased number of tetraploid nuclei, indicating significant accumulation of cells in G_2/M phases (Fig. 2, B and C). These data indicate a delay, or possibly a block in cell cycle progression, associated with C_2-ceramide or ET-18-OCH_3 treatment, but not with farnesol treatment (Fig. 2, D). Ultrastructural analysis of COS-7 cells treated with C_2-ceramide displayed typical apoptotic features such as cell shrinkage, vesiculation, and nuclear chromatin condensation compared with control cells (data not shown). We also detected the appearance of DNA strand breaks by TUNEL staining (data not shown), indicative of intranucleosomal DNA fragmentation and consistent with the induction of apoptosis by C_2-ceramide. The incidence of apoptosis increased with C_2-ceramide concentration and with the duration of incubation.

A quantitative assessment of the time course of appear-

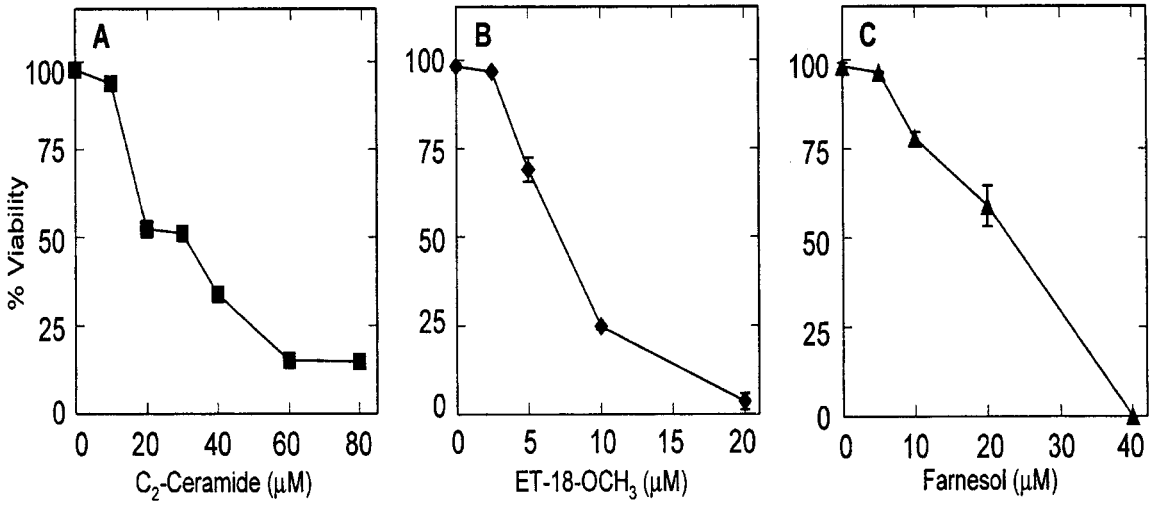

Fig. 1. C_2-ceramide, ET-18-OCH_3, and farnesol reduce COS-7 cell viability. Cells were treated with the indicated concentrations of C_2-ceramide (A), ET-18-OCH_3 (B), or farnesol (C) for 18 h. Viability was determined by trypan blue exclusion. Data represent the mean ± range of duplicate determinations from a representative experiment, which was repeated three times with essentially the same results.

ance of TUNEL-positive nuclei revealed that apoptosis was not evident in the cell population until 4 h after addition of C_2-ceramide and was not complete until after 20 h. Maximum response to C_2-ceramide was obtained with 40 μM ceramide both in the induction of cell death and in the appearance of TUNEL-positive nuclei. Similar data were obtained with ET-18-OCH_3 and farnesol treatment of cells (Fig. 1, B and C), supporting the idea that the toxic effects of C_2-ceramide were caused by programmed cell death, and in this regard, the effects are comparable with those of ET-18-OCH_3 and farnesol.

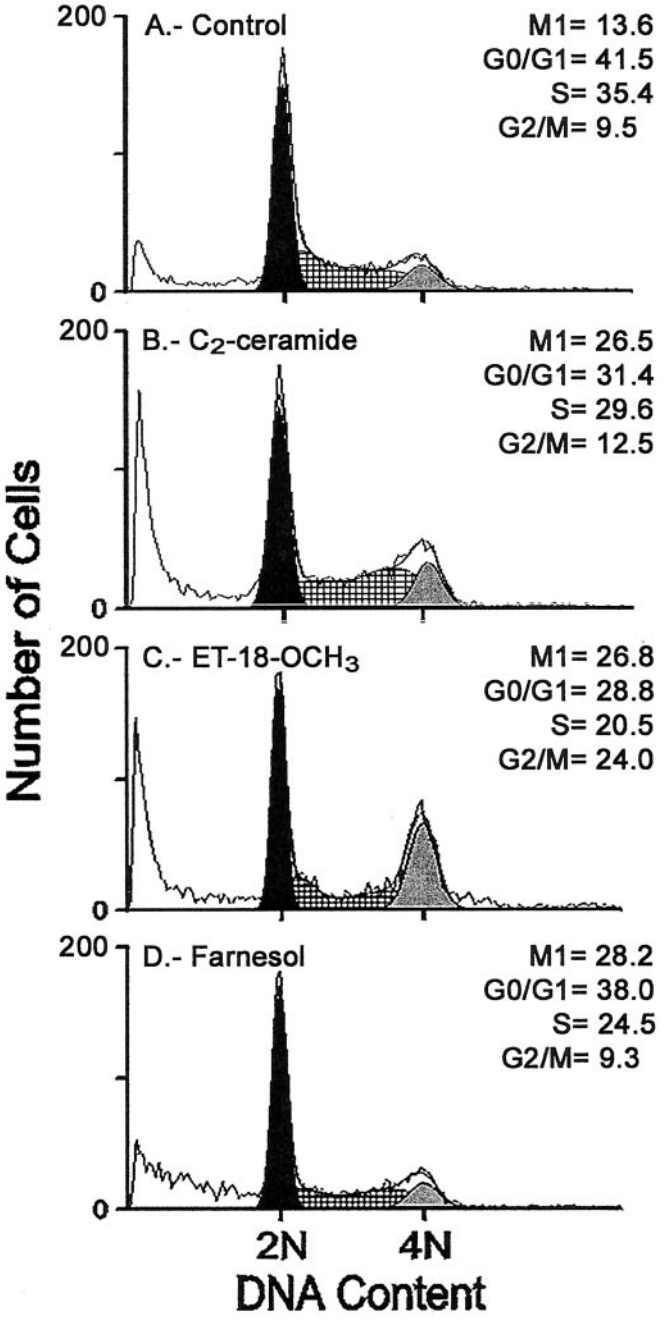

Fig. 2. Analysis of DNA content of COS-7 cells after treatment with C_2-ceramide, ET-18-OCH_3, and farnesol. Cells were treated with 40 μM C_2-ceramide (B), 4 μM ET-18-OCH_3, (C) 15 μM farnesol (D), or vehicle (A) for 18 h. DNA per cell nucleus was quantitated by flow cytometry as described under *Materials and Methods*. The results are representative of two independent experiments.

The next experiments investigated the inhibitory effect of C_2-ceramide on PtdCho biosynthesis. The total uptake of [^{3}H]choline by cells was not affected by C_2-ceramide (Fig. 3, A), demonstrating that inhibition of PtdCho synthesis was not caused by reduced [^{3}H]choline transport. In agreement with previous reports (Bladergroen et al., 1999; Allan, 2000; Ramos et al., 2000), we observed a significant block in PtdCho labeling with [^{3}H]choline within 2 h after addition of 40 μM C_2-ceramide (Fig. 3C). The incorporation of [^{3}H]choline into lipid was inhibited about 75% by C_2-ceramide throughout the time course up to 6 h. A reciprocal accumulation of the total water-soluble PtdCho precursors accompanied the block in PtdCho biosynthesis and was also evident within 2 h (Fig. 3B) and continued through to 6 h, by which time the intracellular pools of PtdCho precursors were equilibrated with [^{3}H]choline as indicated by the plateau in the labeling kinetics. The individual PtdCho precursors labeled by [^{3}H]choline were quantitated and the data revealed that both intracellular choline and phosphocholine increased after C_2-ceramide treatment (Fig. 4, A and B), whereas the CDP-choline level was essentially unchanged (Fig. 4C). Phosphocholine, a substrate for the CCT reaction, was the largest pool and was about three times larger in ceramide-treated cells that in control cells. These results led us to conclude that the reaction catalyzed by CCT was inhibited by C_2-ceramide in cells. We then compared the distribution of PtdCho precursors after C_2-ceramide treatment with the distribution of precursors resulting from treatment with ET-18-OCH_3 or with farnesol (Fig. 5). ET-18-OCH_3 inhibits CCT (Boggs et al., 1995) and farnesol inhibits CPT (Miquel et al., 1998), the subsequent step in PtdCho biosynthesis. Exposure of COS-7 cells to either ET-18-OCH_3 or farnesol resulted in the inhibition of [^{3}H]choline incorporation into lipid (Fig. 5A), similar to treatment with C_2-ceramide. However, ET-18-OCH_3 elicited the selective accumulation of phosphocholine (Fig. 5B), as did C_2-ceramide, whereas farnesol induced a clear increase in CDP-choline without affecting phosphocholine levels (Fig. 5C). Therefore, our results show that C_2-ceramide effects on PtdCho metabolism in COS-7 cells are caused by the selective inhibition of CCT. This CCT inhibition could be due to either a direct effect on the enzyme or to other metabolic actions or signaling cascades affected by C_2-ceramide.

To determine whether inhibition of CCT was a direct effect of C_2-ceramide, we overexpressed CCT in COS-7 cells and performed in vitro assays of CCT activity in partially purified cell extracts. The CCT activity was totally dependent on lipid addition to the assay and both C_2-ceramide and ET-18-OCH_3 inhibited the lipid activation of CCT (Fig. 6). The ranges of effective concentrations of these inhibitors were remarkably similar to those required for the induction of apoptosis (Fig. 1), whereas farnesol did not affect CCT activity. On the other hand, C_2-ceramide and ET-18-OCH_3, at concentrations up to those maximally effective for the induction of apoptosis, did not inhibit CPT activity in vitro as measured in microsomes prepared from COS-7 cells (data not shown).

Previous reports agree that the short-chain analogs C_2-ceramide, C_6-ceramide, and C_8-ceramide inhibit to different extents the rate of PtdCho synthesis (Bladergroen et al., 1999; Allan, 2000; Vivekananda et al., 2001), whereas the longer chain C_{16}-ceramide does not seem to have any significant effect on this pathway (Bladergroen et al., 1999). It has

been proposed that C_2-ceramide would inhibit the CCT-catalyzed step (Allan, 2000; Vivekananda et al., 2001), whereas C_6-ceramide might act on CPT (Bladergroen et al., 1999). Regardless of these apparently conflicting reports, a clear relationship between inhibition of enzymes of the Kennedy pathway and the induction of apoptosis by ceramides has not been established yet. To address this question, we assayed CCT activity in vitro in the presence of ceramides with different chain-lengths. We found that C_2-ceramide was approximately twice as effective as C_6-ceramide in the inhibition of CCT activity, whereas C_{16}-ceramide or dihydro-C_2-ceramide did not have any significant effect (Fig. 7, A). The rank order of efficacies for CCT inhibition was $C_2 > C_6 > C_{16}$ = dihydroC_2 in a 15-min assay and was closely mirrored by the abilities of the different ceramides to induce apoptosis as quantified by the TUNEL technique after a 20-h treatment (Fig. 7B). The lack of inhibition by dihydro-C_2-ceramide shows the requirement for the 4–5-*trans* double bond both to inhibit CCT and to induce apoptosis, whereas the lack of inhibition by the C_{16}-ceramide argues against inhibition of CCT by the naturally occurring long-chain ceramides. These data also identify CCT as a primary intracellular target for the short-chain C_2- and C_6-ceramides that can account for their cytotoxic effects.

Discussion

Our data point to the direct inhibition of PtdCho synthesis at the CCT step as the underlying mechanism for C_2-ceramide-induced apoptosis. The overall characteristics of the cellular responses to C_2-ceramide were the same in our experimental system as reported previously in other cell lines. Based on the reduction of cell viability, detection of DNA fragmentation by propidium iodide/flow cytometry, electron microscopy, and the TUNEL staining technique, our results demonstrate that exposure of COS-7 cells to C_2-ceramide induces cell death through an apoptotic mechanism, which is in agreement with the emerging consensus in the field (Hannun and Luberto, 2000; Luberto and Hannun, 2000). Furthermore, our data showing reduction in cell viability and DNA fragmentation in cells treated with either ET-18-OCH_3 or farnesol are also in accordance with the established apoptogenic properties of these compounds (Boggs et al., 1998; Kent and Carman, 1999). Metabolic [^{3}H]choline-labeling experiments extend previous observations on the C_2-ceramide induced inhibition of [^{32}P]orthophosphate incorporation into neuronal PtdCho (Ramos et al., 2000) to show the selective accumulation of phosphocholine. Based on the similarity to the effects of ET-18-OCH_3 on Cho metabolism, we hypothesized that C_2-ceramide inhibited the same reaction in the pathway, CCT. This possibility was considered previously (Allan, 2000; Vivekananda et al., 2001). In this regard, the inhibitory effect of C_2-ceramide on PtdCho synthesis has been proposed to be secondary to the action of sphingomyelin synthase, which may form C_2-sphingomyelin (Allan, 2000), a compound known to inhibit CCT (Wieder et al., 1995). Although not necessarily in conflict with this report, our data

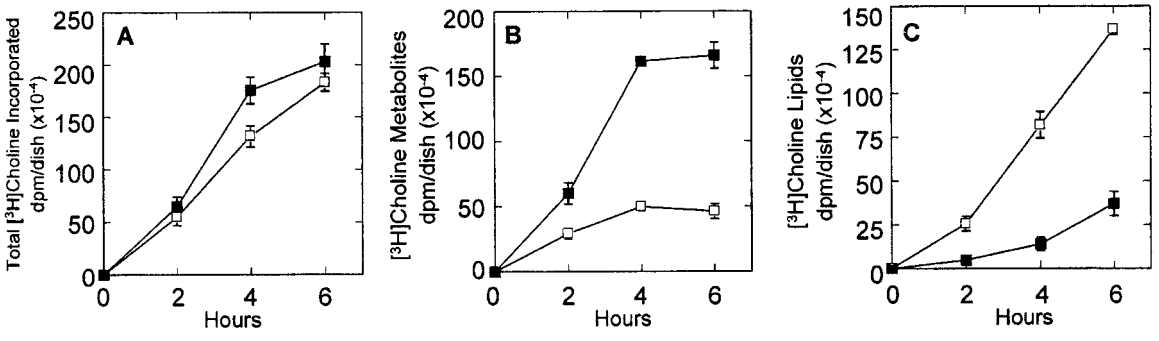

Fig. 3. C_2-ceramide inhibits PtdCho biosynthesis. Cells were incubated up to 6 h in the presence (■) and absence (□) of 40 μM C_2-ceramide in medium supplemented with [*methyl*-^{3}H]choline (5 μCi/ml). Cells were harvested at the indicated times and extracted as described under *Materials and Methods*. A, total radioactivity incorporated by cells was determined in aliquots of the harvested cell suspensions. B, the aqueous [^{3}H]choline-containing metabolites, representing precursors of PtdCho, were quantitated. C, [^{3}H]choline-labeled lipids in the organic phase, representing ≥95% PtdCho, were quantitated. The data are means ± range from duplicate determinations in a representative experiment. Three independent experiments were performed with similar results.

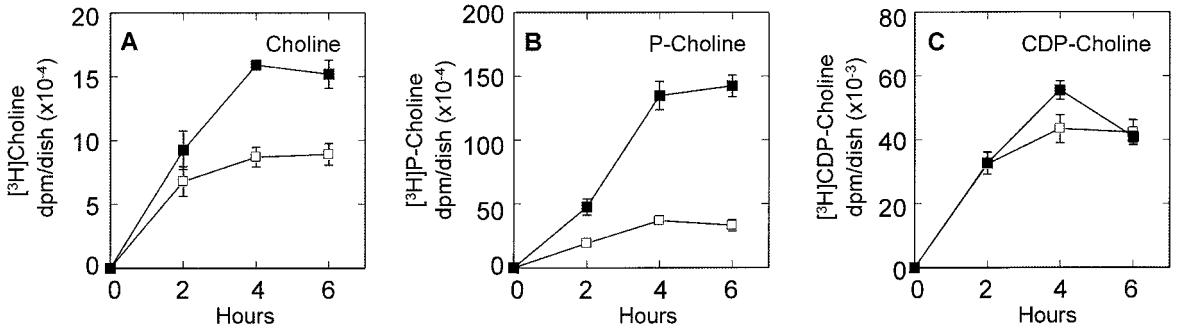

Fig. 4. Accumulation of PtdCho precursors after C_2-ceramide treatment. The aqueous [^{3}H]choline-labeled metabolites from cells incubated up to 6 h in the absence (□) or presence (■) of 40 μM C_2-ceramide were separated and quantitated after thin-layer chromatography as described under *Materials and Methods*. The data represent radioactivity comigrating with authentic choline (A), phosphocholine (B), or CDP-choline (C) and are means ± range of duplicate samples in a representative experiment. Three independent experiments confirmed the same results.

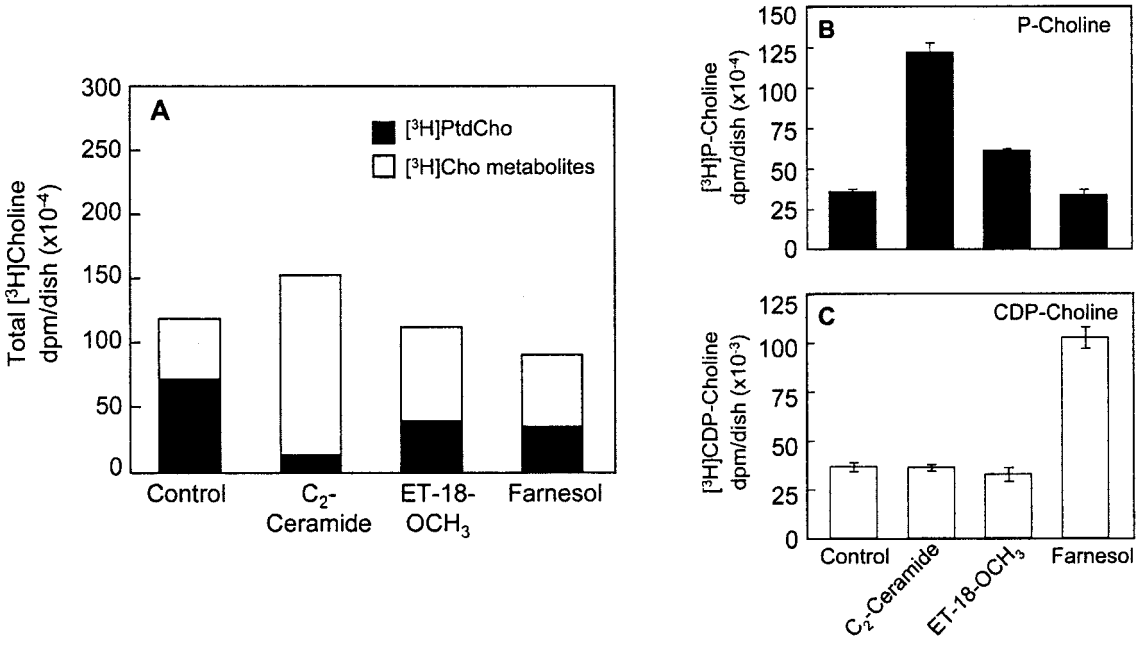

Fig. 5. C_2-ceramide inhibits PtdCho biosynthesis in a way similar to ET-18-OCH_3 but different from farnesol. Cells were treated with 40 μM C_2-ceramide, 4 μM ET-18-OCH_3, 20 μM farnesol, or vehicle in medium supplemented with [*methyl*-^{3}H]choline (5 μCi/ml) for 4 h, then the radioactivity in aqueous and organic phases was quantitated (A). Aqueous choline metabolites were separated by thin-layer chromatography, identified as choline, phosphocholine (B), or CDP-choline (C) and quantitated as described under *Materials and Methods*. The data are means ± range of duplicate samples from a representative experiment that was performed twice with similar results.

provide a simpler explanation, as they demonstrate for the first time direct inhibition of CCT enzyme activity by C_2-ceramide.

The conclusion that CCT is a primary target accounting for the cytotoxic effects of the cell permeable ceramides is consistent with the increasing body of evidence supporting the idea that disturbance of the PtdCho biosynthetic pathway is in itself an insult capable of initiating the apoptotic cascade (Lykidis and Jackowski, 2000). Conversely, facilitation of PtdCho synthesis constitutes a promising cytoprotective strategy in a variety of pathological conditions (Adibhatla et al., 2002). Several pieces of data support this hypothesis: (1) C_2-ceramide inhibits CCT by a mechanism similar to that of the well-characterized ether lysophospholipid analog ET-18-OCH_3; (2) CCT inhibition is an early event in C_2-ceramide action; and (3) the effective C_2-ceramide concentrations that inhibit CCT correspond to those that induce apoptosis.

The mechanisms whereby inhibition of PtdCho synthesis leads to apoptosis are not well understood. In this regard, the generation of endogenous ceramide could be considered as a possible link. Choline deficiency, which induces apoptosis in a variety of cells (Kent and Carman, 1999; Yen et al., 2001), is associated with elevated ceramide levels, although the late onset of ceramide accumulation during the apoptotic process argues against a cause-effect relationship in this particular paradigm (Yen et al., 2001). Based on the observation that fumonisin B_1, an inhibitor of ceramide synthase, blocks the induction of apoptosis by the anticancer, CCT-inhibiting drug hexadecylphosphocholine, it was proposed that ceramide generated after the blockade of PtdCho synthesis may constitute the missing link mediating apoptosis (Wieder et al., 1998). Finally, the exposure of H441 lung cells to C_2-ceramide increases sphingomyelinase activity (Vivekananda et al., 2001). Interestingly, similar effects were observed after addition of sphingosine, a molecule that inhibits CCT (Sohal and Cornell, 1990) and induces apoptosis (Ohta et al., 1995; Jarvis et al., 1996). It is tempting to speculate, therefore, that sphingosine cytotoxicity is caused by direct CCT inhibition.

A challenging aspect of ceramide research is to recapitulate cellular functions of ceramide. The cell-permeable cer-

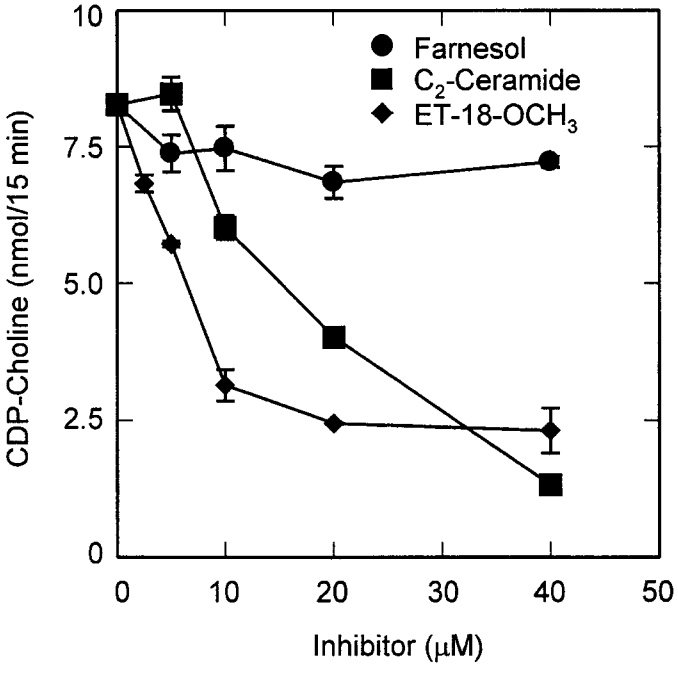

Fig. 6. Inhibition of choline cytidylyltransferase (CCT) in vitro. COS-7 cell lysates overexpressing CCTα were delipidated as described under *Materials and Methods*. CCTα activity was assayed in the presence of increasing concentrations of C_2-ceramide, ET-18-OCH_3, or farnesol. Values are means ± S.D. from two independent experiments.

amide analogs, such as C_2-ceramide, were first employed to provide direct evidence for the biological effects of natural ceramides (Luberto and Hannun, 2000). However, the abilities of different *N*-linked sphingosine derivatives to directly inhibit CCT activity and PtdCho synthesis (whereas the natural ceramides do not) challenge the wisdom of trusting short-chain ceramide analogs to exactly mimic the effects of their natural, long-chain counterparts. Recent evidence indicates that these analogs indirectly generate natural ceramide after recycling of their sphingosine backbone via deacylation/reacylation (Ogretmen et al., 2002). Comparison of the abilities of C_2-, C_6-, and C_{16}-ceramides to modify CCT activity in vitro suggests an inverse relationship between the length of the *N*-linked carbon chain and the inhibitory efficacy, which closely mirrors the relative cytotoxic effects of the different ceramides. These observations are consistent with results on the global inhibition of PtdCho synthesis by ceramide analogs in whole cells (Bladergroen et al., 1999) and support our proposal that CCT inhibition is a primary effect of C_2-ceramide that eventually results in apoptosis. The structural similarities among C_2-ceramide, lysoPtdCho, and ET-18-OCH_3 suggest that all of these compounds inhibit CCT in the same manner (Boggs et al., 1995), which is understood in light of the curvature elastic stress hypothesis for CCT regulation (Attard et al., 2000). The data also suggest that the primary effects of C_2-ceramide on cells are more similar to those of sphingosine, a CCT inhibitor, than those of the naturally occurring long-chain ceramides, which do not inhibit CCT (Sohal and Cornell, 1990).

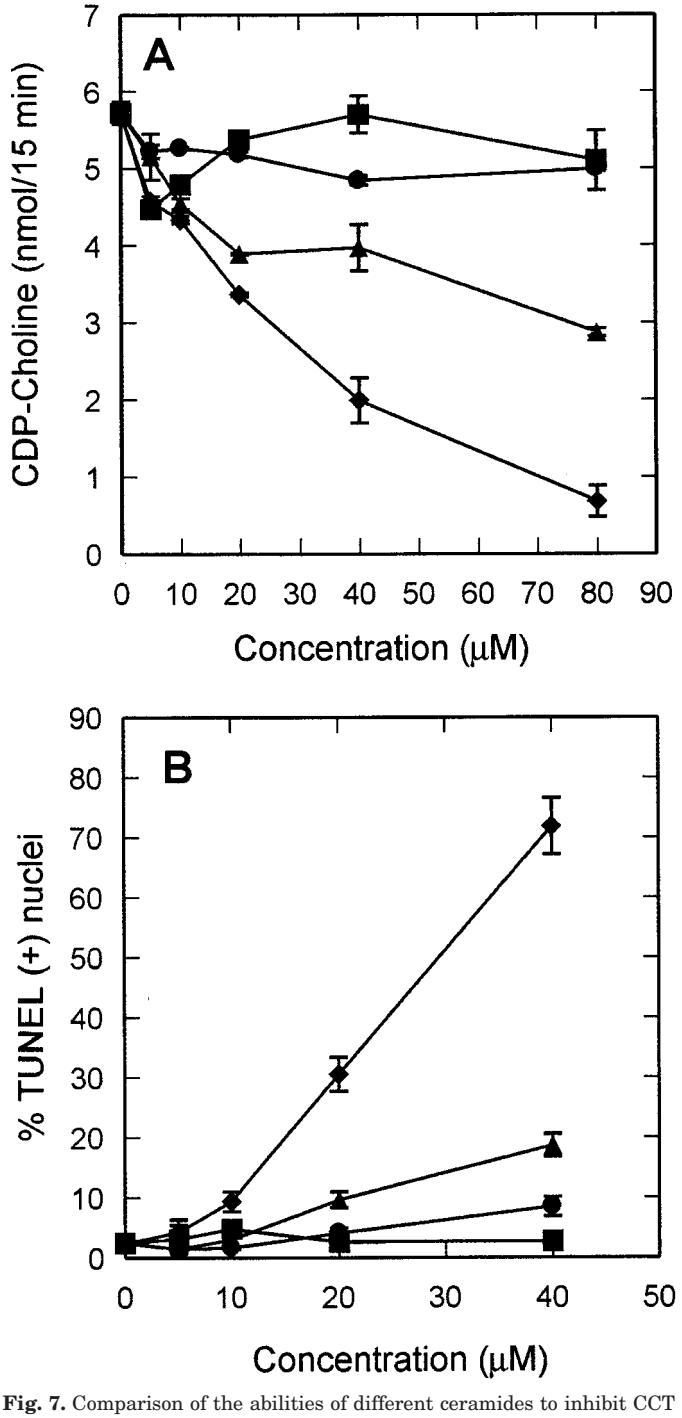

Fig. 7. Comparison of the abilities of different ceramides to inhibit CCT activity in vitro to their apoptotic effect. A, COS-7 cell lysates overexpressing CCTα were delipidated as described under *Materials and Methods*, and CCTα activity was assayed in the presence of increasing concentrations of C_2-ceramide (♦), dihydro-C_2-ceramide (■), C_6-ceramide (▲), or C_{16}-ceramide (●). Values are means ± range of duplicate determinations from a representative experiment that was performed twice with similar results. B, cells were treated with C_2-ceramide, dihydro-C_2-ceramide, C_6-ceramide, or C_{16}-ceramide for 20 h. Apoptosis was quantified by counting TUNEL-positive nuclei (or clusters of apoptotic bodies) and expressed as percentage of total nuclei. At least 100 nuclei were counted in each field. Values are means ± S.D. of two experiments.

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References

Adibhatla RM, Hatcher JF, and Dempsey RJ (2002) Citicoline: neuroprotective mechanisms in cerebral ischemia. *J Neurochem* **80:**12–23.

Allan D (2000) Lipid metabolic changes caused by short-chain ceramides and the connection with apoptosis. *Biochem J* **345:**603–610.

Anthony ML, Zhao M, and Brindle KM (1999) Inhibition of phosphatidylcholine biosynthesis following induction of apoptosis in HL-60 cells. *J Biol Chem* **274:** 19686–19692.

Attard GS, Templer RH, Smith WS, Hunt AN, and Jackowski S (2000) Modulation of CTP:phosphocholine cytidylyltransferase by membrane curvature elastic stress. *Proc Natl Acad Sci USA* **97:**9032–9036.

Awasthi S, Vivekananda J, Awasthi V, Smith D, and King RJ (2001) CTP: phosphocholine cytidylyltransferase inhibition by ceramide via PKC-α, p38 MAPK, $cPLA_2$, and 5-lipoxygenase. *Am J Physiol* **281:**L108–L118.

Baburina I and Jackowski S (1998) Apoptosis triggered by 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine is prevented by increased expression of CTP: phosphocholine cytidylyltransferase. *J Biol Chem* **273:**2169–2173.

Bladergroen BA, Bussiere M, Klein W, Geelen MJ, van Golde LM, and Houweling M (1999) Inhibition of phosphatidylcholine and phosphatidylethanolamine biosynthesis in rat-2 fibroblasts by cell-permeable ceramides. *Eur J Biochem* **264:**152–160.

Bligh EG and Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37:**911–917.

Boggs KP, Rock CO, and Jackowski S (1995) Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. *J Biol Chem* **270:**7757–7764.

Boggs KP, Rock CO, and Jackowski S (1998) The antiproliferative effect of hexadecylphosphocholine toward HL60 cells is prevented by exogenous lysophosphatidylcholine. *Biochim Biophys Acta* **1389:**1–12.

Cui Z, Houweling M, Chen MH, Record M, Chap H, Vance DE, and Tercé F (1996) A genetic defect in phosphatidylcholine biosynthesis triggers apoptosis in chinese hamster ovary cells. *J Biol Chem* **271:**14668–14671.

El Mouedden M, Laurent G, Mingeot-Leclercq MP, and Tulkens PM (2000) Gentamicin-induced apoptosis in renal cell lines and embryonic rat fibroblasts. *Toxicol Sci* **56:**229–239.

Hannun YA and Luberto C (2000) Ceramide in the eukaryotic stress response. *Trends Cell Biol* **10:**73–80.

Haug JS, Goldner CM, Yazlovitskaya EM, Voziyan PA, and Melnykovych G (1994) Directed cell killing (apoptosis) in human lymphoblastoid cells incubated in the presence of farnesol: effect of phosphatidylcholine. *Biochim Biophys Acta* **1223:** 133–140.

Jarvis WD, Fornari FA, Traylor RS, Martin HA, Kramer LB, Erukulla RK, Bittman R, and Grant S (1996) Induction of apoptosis and potentiation of ceramide-

mediated cytotoxicity by sphingoid bases in human myeloid leukemia cells. *J Biol Chem* **271:**8275–8284.
Kennedy EP and Weiss SB (1956) The function of cytidine coenzyme in the biosynthesis of phospholipids. *J Biol Chem* **222:**193–214.
Kent C (1995) Eukaryotic phospholipid biosynthesis. *Annu Rev Biochem* **64:**315–343.
Kent C and Carman GM (1999) Interactions among pathways for phosphatidylcholine metabolism, CTP synthesis and secretion through the Golgi apparatus. *Trends Biochem Sci* **24:**146–150.
Luberto C and Hannun YA (2000) Use of short-chain ceramides. *Methods Enzymol* **312:**407–420.
Lykidis A and Jackowski S (2000) Regulation of mammalian cell membrane biosynthesis. *Prog Nucleic Acid Res Mol Biol* **65:**361–393.
Lykidis A, Jackson P, and Jackowski S (2001) Lipid activation of CTP: phosphocholine cytidylyltransferase α: Characterization and identification of a second activation domain. *Biochemistry* **40:**494–503.
Miquel K, Pradines A, Terce F, Selmi S, and Favre G (1998) Competitive inhibition of choline phosphotransferase by geranylgeraniol and farnesol inhibits phosphatidylcholine synthesis and induces apoptosis in human lung adenocarcinoma A549 cells. *J Biol Chem* **273:**26179–26186.
Ogretmen B, Pettus BJ, Rossi MJ, Wood R, Usta J, Szulc Z, Bielawska A, Obeid LM, and Hannun YA (2002) 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. *J Biol Chem* **277:**12960–12969.
Ohta H, Sweeney EA, Masamune A, Yatomi Y, Hakomori S and Igarashi Y (1995) Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. *Cancer Res* **55:**691–697.
Ramos B, Salido GM, Campo ML, and Claro E (2000) Inhibition of phosphatidylcholine synthesis precedes apoptosis induced by C2-ceramide: protection by exogenous phosphatidylcholine. *Neuroreport* **11:**3103–3108.
Sohal PS and Cornell RB (1990) Sphingosine inhibits the activity of rat liver CTP: phosphocholine cytidylyltransferase. *J Biol Chem* **265:**11746–11750.
Vance DE, Trip EM, and Paddon HB (1980) Poliovirus increases phosphatidylcholine biosynthesis in HeLa cells by stimulation of the rate-limiting reaction catalyzed by CTP:phosphocholine cytidylyltransferase. *J Biol Chem* **255:**1064–1069.
Vivekananda J, Smith D, and King RJ (2001) Sphingomyelin metabolites inhibit sphingomyelin synthase and CTP:phosphocholine cytidylyltransferase. *Am J Physiol* **281:**L98–L107.
Voziyan A, Goldner CM, and Melnykovych G (1993) Farnesol inhibits phosphatidylcholine biosynthesis in cultured cells by decreasing cholinephosphotransferase activity. *Biochem J* **295:**757–762.
Wieder T, Orfanos CE, and Geilen CC (1998) Induction of ceramide-medicated apoptosis by the anitcancer phospholipid analog, hexadecylphsphocholine. *J Biol Chem* **273:**11025–11031.
Wieder T, Perlitz C, Wieprecht M, Huang RTC, Geilen CC, and Orfanos CE (1995) Two new sphingomyelin analogues inhibit phosphatidylcholine biosynthesis by decreasing membrane-bound CTP: phosphocholine cytidylyltransferase levels in HaCaT cells. *Biochem J* **311:**873–879.
Wright MM, Henneberry AL, Lagace TA, Ridgway ND, and McMaster CR (2001) Uncoupling farnesol-induced apoptosis from its inhibition of phosphatidylcholine synthesis. *J Biol Chem* **276:**25254–25261.
Yang W, Boggs KP, and Jackowski S (1995) The association of lipid activators with the amphipathic helical domain of CTP:phosphocholine cytidylyltransferase accelerates catalysis by increasing the affinity of the enzyme for CTP. *J Biol Chem* **270:**23951–23957.
Yen C-LE, Mar M-H, and Zeisel SH (1999) Choline deficiency-induced apoptosis in PC12 cells is associated with diminished membrane phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacylglycerol and activation of a caspase. *FASEB J* **13:**135–142.
Yen CL, Mar MH, Meeker RB, Fernandes A, and Zeisel SH (2001) Choline deficiency induces apoptosis in primary cultures of fetal neurons. *FASEB J* **15:**1704–1710.

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