25-Hydroxycholesterol stimulates sphingomyelin synthesis in Chinese hamster ovary cells

Neale D. Ridgway

Atlantic Research Centre, and Departments of Pediatrics and Biochemistry, Dalhousie University, 5849 University Avenue, Halifax, Nova Scotia Canada B3H 4H7

Abstract In several experimental and pathological conditions the cellular concentrations of cholesterol and sphingomyelin (SM) change coordinately. In an effort to identify factors mediating co-regulation, a class of suppressors of cholesterol synthesis, generically termed oxysterols, were tested for effects on sphingolipid synthesis in Chinese hamster ovary (CHO) cells. 25-Hydroxycholesterol was found to stimulate [^{3}H]serine, [1-^{3}H]palmitate, and [methyl-^{3}H]choline incorporation into sphingomyelin 2- to 3-fold and increase sphingomyelin mass significantly, but did not influence synthesis of other major phospholipids. Maximal labeling of sphingomyelin by [^{3}H]serine was observed 4–6 h after oxysterol addition, and coincided with inhibition of transcription of sterol-regulated genes and activation of cholesteryl ester synthesis. 25-Hydroxycholesterol dose-response curves for activation of sphingomyelin synthesis, suppression of sterol-regulated transcription, and activation of cholesteryl ester synthesis were also similar. Stimulation of SM and glucosylceramide synthesis was observed only with 25-hydroxycholesterol; other oxysterols and cholesterol were ineffective or inhibitory. The effects of 25-hydroxycholesterol on sphingolipid synthesis could not be reproduced by low density lipoprotein (LDL), whole serum, or non-lipoprotein cholesterol in the medium, and stimulation by 25-hydroxycholesterol was evident irrespective of a cholesterol source in the medium. 25-Hydroxycholesterol-treated CHO cells displayed enhanced conversion of [^{3}H]sphinganine-labeled ceramide into sphingomyelin. Sphingomyelin synthesis from *N*-hexanoyl [3-^{3}H]ceramide and *N*-hexanoyl [3-^{3}H]dihydroceramide was also increased significantly. Consistent with enhanced ceramide conversion to sphingomyelin, ceramide mass was reduced by 20–40% in 25-hydroxycholesterol-treatment. However, in vitro activity of sphingomyelin synthase (assayed with short-chain ceramide) was not increased in membranes from oxysterol-treated cells. Stimulation of sphingolipid synthesis by 25-hydroxycholesterol is temporally related to effects of this oxysterol on cholesterol metabolism, and is the result of enhanced conversion of ceramide to SM.—**Ridgway, N. D.** 25-Hydroxycholesterol stimulates sphingomyelin synthesis in Chinese hamster ovary cells. *J. Lipid Res.* 1995. **36:** 1345–1358.

Supplementary key sphingomyelin • ceramide • oxysterols • sphingomyelin synthase

There is a long-standing observation that the cellular concentrations of sphingomyelin (SM) and cholesterol are positively correlated in several pathological and experimental conditions (reviewed in ref. 1). In atherosclerosis, there is abnormal deposition of cholesterol, cholesteryl esters, and SM in artery wall lesions. Accumulation of SM occurs primarily in the intima and may account for 70–80% of the total phospholipid in advanced lesions (2, 3). SM and cholesterol accumulate in intima as a function of aging (4, 5), and experimentally induced hypercholesterolemia in rabbits (5, 6). Accumulation of cholesterol and sphingolipids is also observed in tissues from individuals with Niemann-Pick disease (7). In the type I disorder, SM accumulation in tissues, due to a lysosomal sphingomyelinase deficiency, is paralleled by increased cholesterol and other lipids. In the type II disorder, cholesterol accumulation, due to defective cellular cholesterol trafficking, is accompanied by a modest increase in SM. Whether this is the result of increased synthesis, decreased catabolism in response to cholesterol loading, or deposition of lipoprotein-derived SM is unknown. Furthermore, the mechanism and factors responsible for coordinately elevating cholesterol and SM in cells have not been identified.

Co-localization of SM and cholesterol is evident at the cellular level, with both lipids being enriched in the plasma membrane compared to interior membranes (8, 9). The importance of plasma membrane SM to cholesterol homeostasis was shown in experiments using bacterial sphingomyelinase: hydrolysis of SM resulted in movement of cholesterol to the interior of the cell where it was esterified, and repressed HMG-CoA reductase activity and sterol synthesis (10, 11). In other studies, artificially increasing cell SM content with liposomes stimulated cholesterol synthesis and repressed esterifica-

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; C_6-ceramide, *N*-hexanoyl sphingosine; C_6-dihydroceramide, *N*-hexanoyl sphinganine; CHO, Chinese hamster ovary; FCS, fetal calf serum; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; SM, sphingomyelin; SPT, serine palmitoyltransferase.

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tion (12, 13). How the plasma membrane enrichment in SM and cholesterol is maintained is unclear. This could be achieved by a preferential physical interaction between cholesterol and SM as demonstrated in model membranes (6, 14).

One mechanism for coordinate changes in SM and cholesterol is via dual regulation of the biosynthetic or degradative pathways. While this is an attractive hypothesis, a recent study using CaCo-2 cells showed that compounds that alter cholesterol synthesis do not influence serine palmitoyltransferase (SPT) activity and long-chain base incorporation into total sphingolipids (15). Previously, LDL was shown to modestly inhibit (16–18) synthesis of SM in cultured cells. Increased cholesterol synthesis in response to SM liposomes (12, 13, 15) and decreased sphingolipid and cholesterol synthesis in cholesterol-loaded cells (15) is difficult to reconcile in light of the apparent lack of regulation of the rate-limiting enzymes SPT and HMG-CoA reductase. It is possible that other steps in sphingolipid metabolism are affected by cholesterol, that a derivative of cholesterol is the true regulator, or that coordinate regulation is sensitive to absolute membrane concentrations of SM and cholesterol, which may not be altered during short-term, acute suppression or induction of cholesterol or SM synthesis.

In this study, a role for oxysterols in regulating SM synthesis was investigated. Oxysterols are a class of polar cholesterol derivatives that, like lipoprotein-derived cholesterol, inhibit cholesterol synthesis (19) and stimulate cholesterol esterification (20). Although still unproven, oxysterols could represent a class of endogenous regulatory molecules that mediate feed-back repression of cholesterol synthesis. One of the more efficacious of these oxysterols, 25-hydroxycholesterol, was tested in CHO cells for regulation of SM synthesis in parallel with inhibition of transcription of sterol-regulated genes and activation of cholesteryl ester synthesis. The results show that 25-hydroxycholesterol reduces endogenous ceramide and stimulates SM synthesis by a novel mechanism that involves enhanced conversion of ceramide to SM.

MATERIALS AND METHODS

Cholesterol, 22(R)-hydroxycholesterol and 25-hydroxycholesterol were purchased from Steraloids Inc., Wilton, NH. All other oxysterols, palmitoyl CoA, D-*erythro*-sphingosine, DL-*erythro*-sphinganine, and ceramide (from bovine brain SM) were from Sigma Chemical Co., St. Louis, MO. [1-^{3}H]palmitate, [^{3}H(G)]serine, NaB[^{3}H]$_4$, [*methyl*-^{3}H]choline, [1-^{14}C]oleate, [γ-^{32}P]ATP, and [α-^{32}P]dATP were from DuPont-New England Nuclear. Silica Gel 60 thin-layer chromatography plates were from E. Merck, Darmstadt, Germany. All other chemicals were of reagent grade.

Cell culture

CHO-KI cells, obtained from American Type Culture Collection (ATCC CCL 61), were grown in monolayers at 37°C in an atmosphere of 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 34 μg proline/ml (medium A). Cells were seeded on day 0 in 60-mm dishes (175,000 cells) or 100-mm dishes (400,000 cells) in 3 or 8 ml medium A, respectively. In some experiments, cells were switched to Dulbecco's modified Eagle's medium containing 5% delipidated fetal calf serum and proline (medium B) on day 3. All experiments were started on day 4, 18–24 h after addition of medium B. Oxysterols were prepared as 2.5 mg/ml stock solutions in ethanol and added to warm medium (ethanol concentrations in medium did not exceed 0.2%).

Synthesis of radiolabeled short-chain ceramide and dihydroceramide

C_6-[3-^{3}H]ceramide and C_6-[3-^{3}H]dihydroceramide (220 dpm/pmol) were prepared as previously described (21, 22). Briefly, D-*erythro*-sphingosine or DL-*erythro*-sphinganine were acylated with hexanoic anhydride followed by oxidation of the 3-hydroxyl with chromic anhydride in pyridine. The 3-keto ceramide was dissolved in methanol, reduced with 2-fold molar excess of NaB[^{3}H]$_4$ on ice for 30 min, and the mixture of *erythro* and *threo* isomers was separated by preparative thin-layer chromatography (22). Only the DL-erythro isomers were used in these studies. [3-^{3}H]sphinganine (220 dpm/pmol) was prepared by acid hydrolysis of C_6-[3-^{3}H]dihydroceramide in 0.5 M HCl in acetonitrile-water 9:1 (v/v) for 1 h at 75°C (23) and purified by thin-layer chromatography in a solvent system of chloroform–methanol–2 N NH_4OH 40:10:1 (v/v).

Extraction and analysis of phospholipids and sphingolipids

Monolayers of CHO cells (in 60-mm dishes) were incubated with the amount and activity of radioactive precursor indicated in figure legends. At the end of the pulse period, medium was removed and cells were washed once with ice-cold phosphate-buffered saline, scraped in 1 ml of methanol–water 5:4 (v/v) and transferred to a screw-cap vial. Each culture dish was rinsed with 1 ml of methanol–water, extracts were combined, and 5 ml of chloroform–methanol 1:2 (v/v) was added to each tube. After addition of 4 ml 0.58% NaCl, extracts were mixed and phases were separated by centrifugation at 2,000 *g* for 5 min. The organic phase was washed twice with 2 ml methanol–0.58% NaCl–chloroform 45:47:3 (v/v) and dried over anhydrous sodium sulfate. Aliquots of lipid extract were hydrolyzed in 0.1 M KOH in methanol at 37°C for 1 h; lipids were extracted as described above af-

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ter neutralization with HCl, and separated by thin-layer chromatography in chloroform–methanol–water 65:25:4 (v/v). Radiolabeled SM, glucosylceramide, and ceramide were identified by fluorography and quantitated by scraping and liquid scintillation counting. Total lipids were resolved by thin-layer chromatography in chloroform–methanol–acetic acid–water 60:40:4:1 (v/v), visualized by exposure to iodine vapor, and PtdSer, PtdEtn and SM were scraped from plates and radioactivity was quantitated.

Metabolic products of C_6-[3-^{3}H]ceramide, C_6-[3-^{3}H]dihydroceramide, and [3-^{3}H]sphingosine were extracted from cells as described above. Lipids were resolved by thin-layer chromatography in chloroform–methanol–15 mM $CaCl_2$ 65:35:8 (v/v) and subjected to fluorography for 24–72 h. Bands corresponding to C_6-SM, C_6-glucosylceramide, and C_6-ceramide were identified, using the fluorogram as a template, scraped from plates, and radioactivity was measured.

Quantitation of mRNA

Total RNA was isolated from CHO cells by centrifugation through CsCl according to the method of Chirgwin et al. (24). mRNA for HMG-CoA reductase and LDL receptor was measured by S1 nuclease protection assays as described previously (25). HMG-CoA synthase was quantitated by primer extension analysis using an oligonucleotide corresponding to nucleotides +61 to +90 of the hamster cDNA (25). Ribosomal S17 protein mRNA was quantitated by primer extension (25) and used as an internal mRNA load control for quantitating other messages. Autoradiograms were analyzed on a Macintosh Apple OneScanner and relative amounts of RNA were quantitated using the NIH Image software package (version 1.47).

Enzyme assays

CHO cell membranes were prepared in the following manner. Cells were rinsed twice with 2 ml of phosphate-buffered saline, scraped in the same buffer, and collected by centrifugation at 2,000 *g* for 5 min. Cells were homogenized in 20 mM Tris-HCl (pH 7.7) and 10 mM EDTA (buffer A) by 10 passages through a 22-gauge needle. The homogenate was centrifuged at 100,000 *g* for 1 h and the membrane fraction (pellet) was resuspended in buffer A to a final concentration of approximately 2 mg protein/ml. SM synthase was assayed by a modification of the method of Futerman and Pagano (26). Assays were for 10 min in a volume of 0.5 ml containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 0.5 mM EDTA, and 100–150 μg of membrane protein. The assay was initiated by the addition of 10 nmol C_6-[3-^{3}H]ceramide, prepared as a 1/1 (mol/mol) complex with bovine serum albumin. Sphinganine *N*-acyltransferase was assayed as described by Wang et al. (27). Assays were for 10 min and contained 10 μM [3-^{3}H]sphinganine, 200 μM palmitoyl CoA, 200 μM bovine serum albumin, and 25 mM potassium phosphate (pH 7.4). Products of the assay were resolved by thin-layer chromatography in chloroform–methanol–2 N ammonium hydroxide 40:10:1 (v/v), and *N*-palmitoyl [^{3}H]dihydroceramide was identified by fluorography and comigration with an authentic standard. SPT was assayed as previously described (18). Lipid extracts were resolved by thin-layer chromatography, and 3-keto sphinganine was identified by fluorography and comparison to an authentic standard. ACAT activity in monolayers of CHO cells was measured as previously described (28) by monitoring conversion of [1-^{14}C]oleate (7,000–8,000 dpm/nmol) into cholesteryl[1-^{14}C]oleate during a 1 h incubation.

Other methods and analysis

Cellular ceramide and diglyceride mass were quantitated using the bacterial diglyceride kinase assay and [γ-^{32}P]ATP as previously described (29). Bovine brain ceramide (derived from sphingomyelin) was used as a standard. Protein was determined by the method of Lowry et al. (30) using bovine serum albumin as standard. Lipid phosphorus was measured by the method of Rouser, Siakatos, and Fleisher (31). Delipidated serum was prepared by ultracentrifugation at a density of 1.21 g/ml and dialyzed against phosphate-buffered saline (28). Human LDL (d 1.019–1.063 g/ml) was prepared as previously described (28). The unpaired Student's *t*-test (two-tailed) was used to determine significant differences between data sets.

RESULTS

Stimulation of SM synthesis by 25-hydroxycholesterol

Oxysterols, in particular 25-hydroxycholesterol, have been shown to potently inhibit cholesterol synthesis by suppressing transcription of HMG-CoA reductase, HMG-CoA synthase, and LDL receptor, and stimulating the degradation of HMG-CoA reductase protein (32). Concomitant with suppression of cholesterol synthesis and uptake, oxysterols increase cholesteryl ester synthesis by activation of ACAT (20). To determine whether 25-hydroxycholesterol also affected the synthesis of sphingolipids, CHO cells were treated with oxysterol for 4 h and pulse-labeled with [^{3}H]serine for up to 2 h to measure sphingolipid synthesis (**Fig. 1**). Ceramide, an intermediate in sphingolipid synthesis, was rapidly labeled and incorporation appeared to approach a maximum by 2 h (Fig. 1A). Incorporation into ceramide was not significantly increased by 25-hydroxycholesterol treatment. In contrast, 25-hydroxycholesterol stimulated the incorporation of [^{3}H]serine into SM by 2- to 3-fold and

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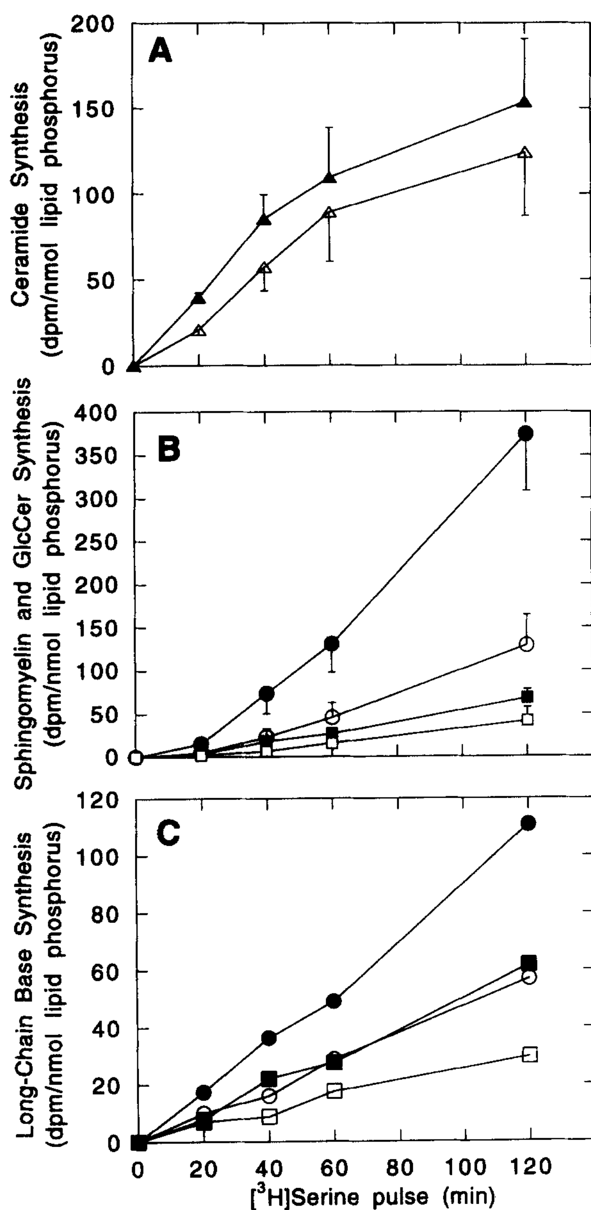

Fig. 1. Stimulation of sphingolipid and long-chain base synthesis by 25-hydroxycholesterol. CHO cells were treated with 25-hydroxycholesterol (2.5 μg/ml, closed symbols) or ethanol (open symbols) in medium A. After 4 h, fresh serine-free medium A was added containing [^{3}H]serine (7.5 μCi/ml) and cells were incubated for the indicated times and harvested. A and B: incorporation of [^{3}H]serine into ceramide (△, ▲), glucosylceramide (□, ■), and SM (○, ●) was measured after base hydrolysis of lipid samples. Results are the means of three experiments ± standard deviation. C: incorporation of [^{3}H]serine into sphingosine (○, ●) and sphinganine (■, □) of total sphingolipids was measured after acid hydrolysis. Results are the means of duplicate determinations from a representative experiment.

glucosylceramide by 1.8-fold (Fig. 1B). There was a distinct lag in [^{3}H]serine incorporation into both SM and glucosylceramide, indicative of a precursor-product relationship with ceramide. Lipid samples from experiments shown in Fig. 1A and B were subjected to acid hydrolysis and the sphingosine and sphinganine component of [^{3}H]serine-labeled sphingolipids quantitated (Fig. 1C). 25-Hydroxycholesterol treatment increased [^{3}H]serine incorporation into both long-chain bases by 2-fold, indicating that de novo long-chain base synthesis, and not *N*-acylation of [^{3}H]serine-labeled fatty acid into sphingolipids, was stimulated.

As mentioned above, oxysterols, particularly 25-hydroxycholesterol, are known to elicit a series of well-defined regulatory responses in cultured cells that culminate in inhibition of de novo cholesterol synthesis. The time course and dose response for transcriptional suppression and ACAT activation by 25-hydroxycholesterol were reexamined in the context of stimulated SM synthesis. In **Fig. 2**, the time course for 25-hydroxycholesterol-mediated transcriptional suppression and ACAT activation was compared to effects on SM synthesis. As expected, 25-hydroxycholesterol activated ACAT by 4–6 h (Fig. 2B) and fully suppressed transcription (Fig. 2C). [^{3}H]serine incorporation into SM (Fig. 2A) also peaked during this time and showed a gradual decline similar to that seen for cholesteryl ester synthesis. The decline in cholesteryl ester synthesis probably reflects a decrease in substrate availability as cholesterol synthesis is suppressed and exogenous non-lipoprotein cholesterol was not added with 25-hydroxycholesterol. mRNA suppression, cholesterol esterification, and SM synthesis were also measured over a range of 25-hydroxycholesterol concentrations (**Fig. 3**). mRNA for LDL receptor, HMG-CoA synthase, and HMG-CoA reductase were maximally suppressed at 1 μg/ml oxysterol in the culture medium (Fig. 3B). HMG-CoA synthase mRNA was more sensitive to 25-hydroxycholesterol as displayed by 90% suppression at 0.5 μg/ml compared to $<$ 25% suppression for LDL receptor and HMG-CoA reductase mRNA. SM and cholesteryl ester synthesis increased rapidly up to 1 μg/ml 25-hydroxycholesterol and then displayed a more gradual rise in activity up to a 5 μg/ml (Fig. 3A and C). The aforementioned treatments with 25-hydroxycholesterol had no significant effect on synthesis of PtdSer, PtdEtn, and triacylglycerol.

25-Hydroxycholesterol has been shown to be one of the more efficacious oxysterols for suppression of HMG-CoA reductase activity in cultured cells (19). Of the oxysterols tested here, 25-hydroxycholesterol was the most potent in activating ACAT and suppressing mRNAs for sterol-regulated genes (**Fig. 4B and C**), and only 25-hydroxycholesterol stimulated [^{3}H]serine incorporation into SM and glucosylceramide (Fig. 4A). Interestingly, [^{3}H]serine-labeling of ceramide was not significantly increased by 25-hydroxycholesterol or any other oxysterol (Fig. 4A). 20-Hydroxycholesterol, 19-hydroxycholesterol, and 7-ketocholesterol, which have approximately one-half the biological activity for activation of cholesterol esterification (Fig. 4B) and suppression of mRNA for HMG-CoA reductase, HMG-CoA synthase, and LDL receptor (Fig. 4C), did not stimulate sphingolipid synthesis. Treatment

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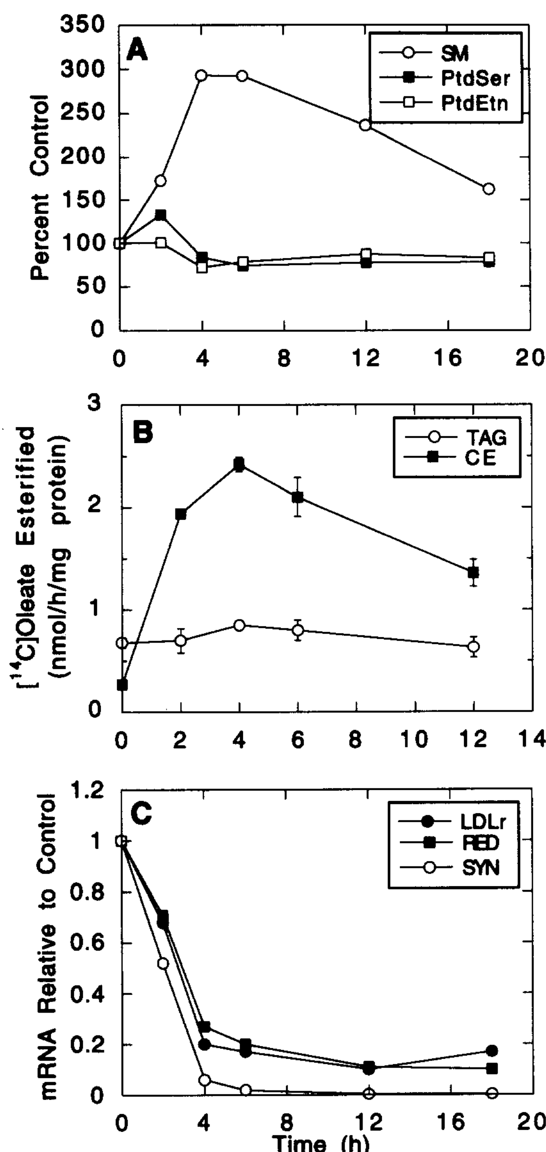

Fig. 2. Temporal relationship for activation of SM synthesis (A) and cholesterol esterification (B), and suppression of transcription (C) by 25-hydroxycholesterol. CHO cells were cultured in medium B for 12 h prior to replacement with medium B containing 25-hydroxycholesterol (2.5 μg/ml) or ethanol (control) for the indicated periods of time. Addition of sterol was staggered so cells were harvested simultaneously. A: cells were incubated with oxysterol or ethanol for the indicated period of time and synthesis of SM, PtdSer, and PtdEtn assessed by a 1-h pulse with [^{3}H]serine (7.5 μCi/ml). Results are expressed as a percentage of control values from cells that received ethanol alone for the same times as treated cells. B: cholesteryl esterification was measured by a 0.1 mM [1-^{14}C]oleate pulse for the last 2 h of each treatment. C: mRNA levels were quantitated as described in Materials and Methods and expressed relative to controls that received ethanol for 18 h. Results are from representative experiments performed in duplicate ([^{3}H]serine labeling and mRNA quantitation) or triplicate (cholesterol esterification assays). Abbreviations are; CE, cholesteryl ester; TAG, triacylglycerol; LDLr, LDL receptor; RED, HMG-CoA reductase; SYN, HMG-CoA synthase.

of CHO cells with 22(S)-hydroxycholesterol was found to inhibit SM, ceramide, and glucosylceramide labeling by about 50%. None of the sterols tested perturbed [^{3}H]serine incorporation into PtdSer or PtdEtn indicating that the effects are not due to alterations in serine uptake or metabolism in cells.

Effect of culture conditions on 25-hydroxycholesterol-stimulation of SM synthesis

Stimulation of SM labeling by 25-hydroxycholesterol was observed in experiments performed in delipidated se-

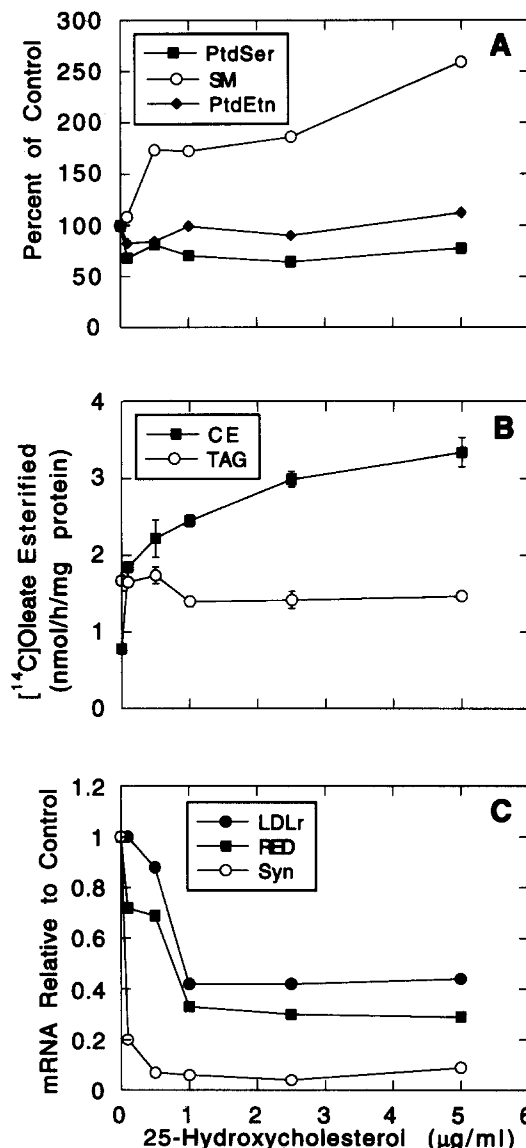

Fig. 3. 25-Hydroxycholesterol dose-response curves for activation of SM synthesis (A) and cholesterol esterification (B), and suppression of transcription of sterol-regulated genes (C). CHO cells were cultured in medium B for 12 h, followed by medium B containing the indicated concentrations of 25-hydroxycholesterol or ethanol for 6 h. A: synthesis of SM, PtdSer, and PtdEtn was measured by a 1-h pulse with [^{3}H]serine (7.5 μCi/ml) and expressed relative to control cells that received an equivalent volume of ethanol for 6 h. B: cholesterol esterification was assessed by a 0.1 mM [1-^{14}C]oleate pulse for the last hour of each treatment. C: mRNA for LDL receptor, HMG-CoA reductase, and HMG-CoA synthase was assayed as described and expressed relative to cells that received ethanol alone. Results are from a representative experiment performed in duplicate ([^{3}H]serine labeling and mRNA quantitation) or triplicate (ACAT assays). Abbreviations are the same as Fig. 2.

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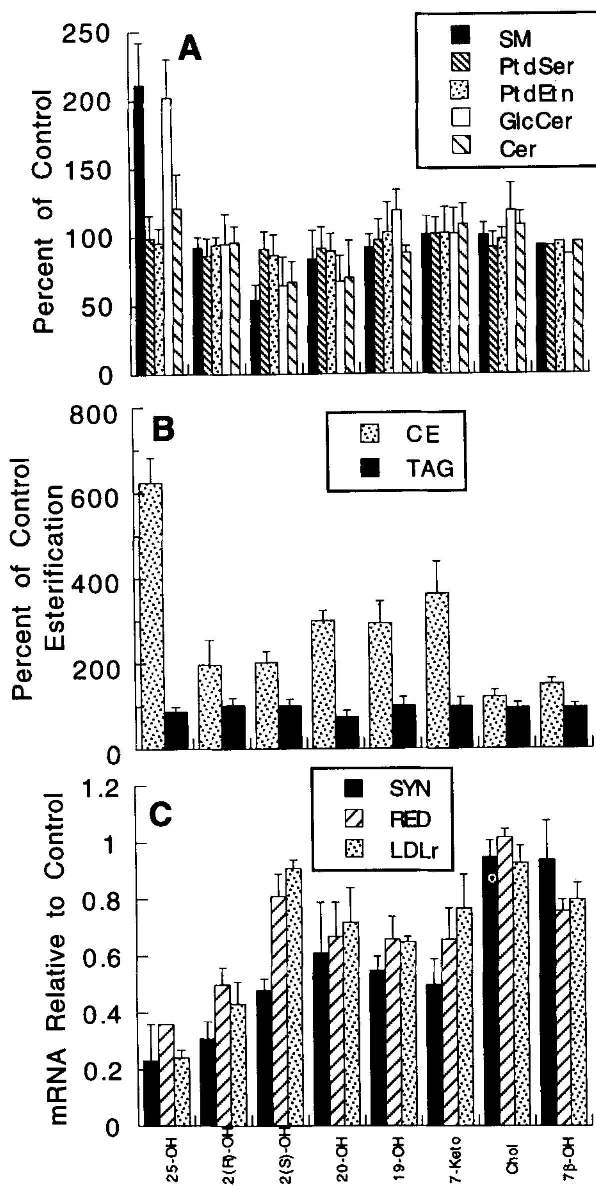

Fig. 4. Effects of various oxysterols on [^{3}H]serine incorporation into phospholipids and sphingolipids (A), cholesterol esterification (B), and transcriptional suppression (C). A: CHO cells (grown in medium A) received 2 ml of medium A containing 2.5 μg/ml of the indicated sterol for 4 h. For the last 2 h of the 4-h incubation with sterol, cells were pulsed with [^{3}H]serine (7.5 μCi/ml) and label incorporation into PtdSer, PtdEtn, and sphingolipids was measured. Results are the mean ± standard deviation of four or two (7β-hydroxycholesterol) separate experiments each done in duplicate. B: cells were cultured in medium B for 18 h prior to addition of sterols (2.5 μg/ml) in medium B for 4 h. Cholesteryl ester and triacylglycerol synthesis was assayed by a 1-h pulse with 0.1 mM [1-^{14}C]oleate. Results are the mean ± standard deviation of three experiments each done in duplicate. C: Cells were cultured in medium B and steady-state mRNA levels for HMG-CoA reductase (RED), LDL receptor (LDLr), and HMG-CoA synthase (SYN) were determined in cells treated with oxysterol (2.5 μg/ml) in medium B for 6 h. Results are the mean ± standard deviation of three separate experiments. Results in all three panels are expressed relative to control values for cells that received ethanol for 4 or 6 h. The following abbreviations were used: 25-OH, 25-hydroxycholesterol; 22(R)-OH, 22(R)-hydroxycholesterol; 22(S)-OH, 22-(S)-hydroxycholesterol; 20-OH, 20-hydroxycholesterol; 19-OH, 19-hydroxycholesterol; 7-keto, 7-ketocholesterol; Chol, cholesterol; 7β-OH, 7β-hydroxycholesterol; GlcCer, glucosylceramide; Cer, ceramide.

rum (Figs. 2 and 3) and whole serum (Fig. 1 and 4). A more complete comparison of culture conditions that alter cholesterol metabolism and related effects on sphingolipid synthesis is shown in **Table 1.** Growth of cells in whole serum (fetal calf serum) tended to suppress labeling of SM compared to culturing in delipidated serum (LPDS), and this could be partially reproduced with human LDL and nonlipoprotein cholesterol. A similar pattern of suppression was seen for glucosylceramide and ceramide labeling. When cells cultured in the four different media conditions for 16 h were treated with 25-hydroxycholesterol for 4 h, the specific activity of SM was similar. However, cells grown in medium containing nonlipoprotein cholesterol displayed a blunted stimulation relative to the other conditions. The absolute increment in SM labeling induced by 25-hydroxycholesterol was greater for cells grown in LDL (2.6-fold) and whole serum (3-fold) compared to delipidated serum simply because the synthetic rate in the absence of 25-hydroxycholesterol was lower in the former two conditions. This is also true for glucosylceramide labeling, where culturing in medium containing lipoprotein suppressed synthesis compared to delipidated serum, but the specific activity of sphingolipid after stimulation by 25-hydroxycholesterol was similar. PtdSer and PtdEtn labeling varied by no more than 10% for all the growth conditions shown in Table 1. Relative ACAT activity was also assessed under the different culture conditions as an indicator of the cellular pool of cholesterol available for regulation and esterification. Clearly, lipoprotein and nonlipoprotein cholesterol elevated ACAT activity to the same degree as 25-hydroxycholesterol treatment of cells grown in delipidated serum, but sphingolipid synthesis was not increased proportionally in the former two cases. Addition of 25-hydroxycholesterol to FCS or LDL-treated cells resulted in a slight increase in ACAT activity, but produced the greatest stimulation in SM and glucosylceramide synthesis.

The effect of 25-hydroxycholesterol treatment on the mass of SM in CHO cells grown in fetal calf serum (medium A) was measured (**Table 2**). Based on lipid phosphorus determinations, SM was the only phospholipid that significantly increased (50%) in mass after an 18-h treatment with 25-hydroxycholesterol. The PtdCho content of 18-h oxysterol-treated cells decreased; however, this was not significant.

Mechanism of 25-hydroxycholesterol stimulation of SM synthesis

Several radiolabeled precursors of sphingolipids were tested to determine which step in the SM synthetic pathway was affected by 25-hydroxycholesterol. The final step in SM synthesis involves the transfer of phosphocholine from PtdCho to the 1-hydroxy group of ceramide, with the resultant generation of diacylglycerol (33). Synthesis was assayed by pulse-labeling PtdCho and SM with

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TABLE 1. Effect of medium conditions on stimulation of sphingolipid synthesis by 25-hydroxycholesterol

Medium Conditions	Sphingomyelin	Glucosylceramide	Ceramide	Relative ACAT Activity
	dpm/nmol lipid phosphorus			
LPDS	97.5 ± 20.6	47.2 ± 18.7	90.8 ± 16.6	1.0
LPDS + 25-OH	147.0 ± 30.8[a]	42.2 ± 15.0	89.1 ± 18.7	6.4
LPDS + LDL	68.1 ± 21.0	26.0 ± 4.3	55.2 ± 5.2	5.7
LPDS + LDL + 25-OH	172.9 ± 12.9[d]	51.4 ± 13.3[b]	68.7 ± 16.2	6.8
LPDS + Chol	69.3 ± 8.9	26.0 ± 3.9	70.6 ± 4.2	5.1
LPDS + Chol + 25-OH	115.8 ± 13.3[c]	27.2 ± 8.7	66.2 ± 10.4	7.6
FCS	52.7 ± 10.0	25.2 ± 5.2	52.5 ± 9.5	5.0
FCS + 25-OH	154.7 ± 25.4[d]	44.7 ± 9.7[a]	75.2 ± 19.5	8.9

Cells were cultured for 16 h in lipoprotein-deficient serum (LPDS), LPDS with human LDL (50 μg/ml), LPDS with cholesterol (Chol, 10 μg/ml) or fetal calf serum (FCS) prior to addition of medium A with 25-hydroxycholesterol (2.5 μg/ml) or equivalent volume of ethanol for 4 h. Cells were pulsed with [;s3H]serine (7.5 μCi/ml) for the last hour of oxysterol or ethanol treatment, harvested, and lipids were isolated as described in Materials and Methods. Results are the mean ± standard deviation of four or five separate experiments done in duplicate. ACAT activity was measured by a 1-h [1-^{14}C]oleate pulse and is expressed relative to values for cells grown in LPDS without 25-hydroxycholesterol (average of three experiments). Statistical comparisons were between 25-hydroxycholesterol-treated and control cells.
[a]$P < 0.05$, [b]$P < 0.025$, [c]$P < 0.005$, [d]$P < 0.001$.

[*methyl*-^{3}H]choline during treatment with 25-hydroxycholesterol (2.5 μg/ml). This pulse-labeling protocol generated 100-fold greater incorporation of [*methyl*-^{3}H]choline into PtdCho compared to SM, probably due to a lack of preference in using newly synthesized versus existing PtdCho for SM synthesis. Treatment of CHO cells with 25-hydroxycholesterol for 2–6 h afforded a 1.3- to 1.6-fold increase in incorporation of [*methyl*-^{3}H]choline into SM without influencing absolute PtdCho labeling (**Table 3**).

The effect of 25-hydroxycholesterol of PtdCho and SM catabolism was assessed by pulse-chase studies. CHO cells, labeled to equilibrium for 48 h with 1 μCi/ml [*methyl*-^{3}H]choline, showed no effect of 25-hydroxycholesterol on catabolism of [^{3}H]choline-labeled SM or PtdCho relative to controls over a 12-h chase period in choline-containing medium (data not shown). Similarly, 25-hydroxycholesterol did alter SM metabolism during a 10-h chase period after a 2-h pulse with [^{3}H]serine. Thus, increased [^{3}H]choline and [^{3}H]serine incorporation into SM was not in response to rapid 25-hydroxycholesterol-mediated SM degradation and ensuing resynthesis, and effects of oxysterol on SM catabolism appear to be minor.

25-Hydroxycholesterol also stimulated incorporation of [^{3}H]palmitate in SM and glucosylceramide of CHO cells (**Fig. 5A**). Incorporation of [^{3}H]palmitate into SM and glucosylceramide was saturated at 100 μM and stimulated 2-fold by 25-hydroxycholesterol over the entire concentration range. 25-Hydroxycholesterol did not increase [^{3}H]palmitate labeling of ceramide, and ceramide labeling was not fully saturated up to 200 μM palmitate in the medium. Similar to Fig. 1, 25-hydroxycholesterol increased [^{3}H]palmitate-labeling of long-chain bases in total sphingolipids by 2-fold (results not shown). 25-Hydroxycholesterol stimulated labeling of SM and glucosylceramide by greater than 2-fold at [^{3}H]serine concentrations up to 1 mM in the culture medium (Fig. 5B). If the data from Figs. 5A and B are replotted in double reciprocal form, lines were found to intersect on or close to the x-axis, and the y-intercept value was 2-fold greater for oxysterol-treated cells. Clearly, 25-hydroxycholesterol stimulated the absolute capacity (V_{max}) of cells to synthesize SM and glucosylceramide and not the apparent dissociation constants (K_m) for uptake or incorporation into sphingolipids. It should be noted that [^{3}H]palmitate labeling was carried out in the presence of 430 μM serine, the concentration in Dulbecco's modified Eagle's medium.

Experiments with radioactive precursors showed that 25-hydroxycholesterol stimulates de novo SM and

TABLE 2. Phospholipid mass in 25-hydroxycholesterol-treated cells

Treatment	SM	PtdCho	PtdEtn	PtdSer
	nmol phospholipid/mg cell protein			
6 h NA	2.9 ± 1.2	63.6 ± 4.7	26.0 ± 2.7	8.5 ± 0.7
6 h 25-OH	3.4 ± 0.8	64.7 ± 6.2	27.2 ± 3.0	9.6 ± 1.4
18 h NA	2.7 ± 0.7	64.7 ± 5.8	28.6 ± 2.9	9.6 ± 1.3
18 h 25-OH	4.1 ± 1.0[a]	59.1 ± 5.2	26.8 ± 3.2	9.3 ± 0.5

CHO cells were treated with 25-hydroxycholesterol (2.5 μg/ml, 25-OH) or ethanol (NA) for 6 or 18 h. Phosphorus content of the four major lipid classes was determined after separation by thin-layer chromatography. Results are the mean ± standard deviation of five separate experiments each done in duplicate or triplicate.
[a]$P < 0.05$ versus control cells at 18 h.

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TABLE 3. Stimulation of [*methyl*-^{3}H]choline labeling of SM by 25-hydroxycholesterol

	Treatment	2 h	4 h	6 h
			dpm/nmol lipid phosphorus	
SM	− 25-OH	55.6 ± 5.2	68.5 ± 7.5	65.6 ± 2.7
SM	+ 25-OH	76.4 ± 5.4	83.9 ± 1.48	101.6 ± 6.0
PtdCho	− 25-OH	6035.5 ± 188.5	6110.2 ± 281.0	6611.6 ± 295.0
PtdCho	+ 25-OH	5873.5 ± 585.4	5840.2 ± 293.1	6385.4 ± 373.3
			[methyl-^{3}H]choline-labeled SM/PtdCho ($\times 10^{-2}$)	
	− 25-OH	0.92	1.12	0.99
	+ 25-OH	1.30	1.44	1.59

CHO cells were cultured in choline-deficient medium A for 1 h prior to the start of the experiment. Cells then received 25-hydroxycholesterol (2.5 μg/ml) or ethanol in choline-deficient medium A and were incubated for 2, 4, or 6 h. Cells received [*methyl*-^{3}H]choline (2 μCi/ml) during the last 2 h of each incubation. Results are the mean of triplicate determinations ± standard deviation from a representative experiment.

glucosylceramide synthesis in CHO cells. However, studies of incorporation of the first and final precursors of SM did not indicate where 25-hydroxycholesterol affected synthesis. Results with [^{3}H]serine and [^{3}H]palmitate incorporation suggested that ceramide is a steady state intermediate in the pathway and a substantial pool becomes labeled within cells. The possibility that ceramide synthesis or conversion to sphingolipids were the regulated steps was tested by incubating the precursor of ceramide, [3-^{3}H]sphinganine, with CHO cells pretreated with 25-hydroxycholesterol (**Fig. 6**). Conversion of [3-^{3}H]sphinganine to ceramide, SM, and glucosylceramide was monitored during a 1-h pulse. Initially, label rapidly appeared in ceramide and declined slightly during the 1-h incubation in control cells (Fig. 6A). During this time SM and glucosylceramide synthesis was linear (Fig. 6B). In 25-hydroxycholesterol-treated cells, less ceramide was synthesized but this was compensated by increased SM synthesis, indicative of enhanced conversion of ceramide to SM. Interestingly, this relationship did not hold for glucosylceramide, although 25-hydroxycholesterol stimulated synthesis of this glycosphingolipid as measured by serine or palmitate incorporation (Fig. 5). Compared to results at 1 h, decreased ceramide labeling in 25-hydroxycholesterol-treated cells at earlier time points could not be completely accounted for by increased SM production. The magnitude of 25-hydroxycholesterol stimulation of SM synthesis was less using [3-^{3}H]sphinganine as a precursor compared to [^{3}H]serine (1.5-fold compared to 2- to 3-fold, respectively). Finally, results from Fig. 6A and B were not due to differential uptake of the precursor as [3-^{3}H]sphinganine levels in cells and medium were the same in control and oxysterol treated cells (Fig. 6C).

Results from Fig. 6 suggest that 25-hydroxycholesterol stimulates the activity of SM synthase, delivery of ceramide to the synthase, or increases the specific activity of newly synthesized ceramide. To test whether these poten-

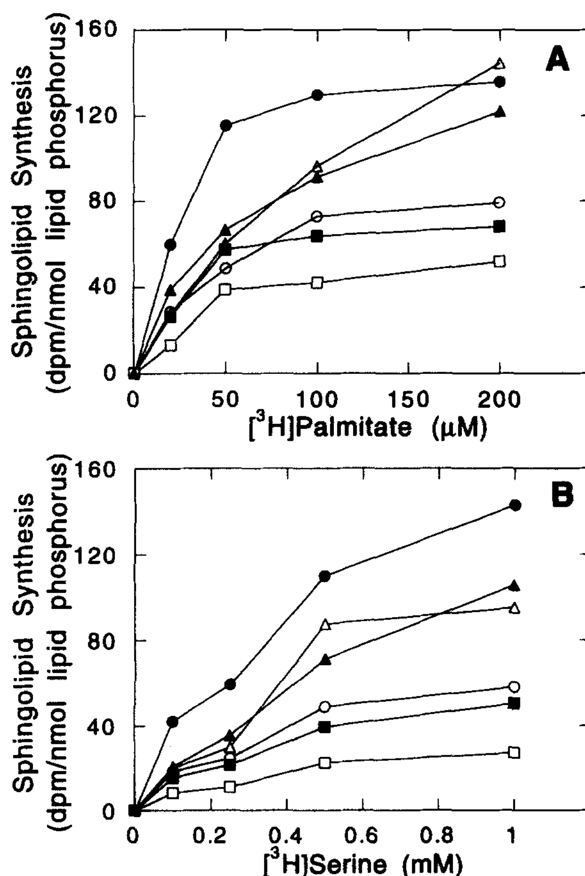

Fig. 5. 25-Hydroxycholesterol stimulates sphingolipid synthesis at saturating concentrations of [^{3}H]palmitate (A) and [^{3}H]serine (B). CHO cells were cultured in medium A prior to the start of each experiment. Cells received medium A containing 25-hydroxycholesterol (2.5 μg/ml, closed symbols) or ethanol (open symbols) for 6 h. Cells were pulsed with the indicated concentrations of [^{3}H]palmitate (A) or [^{3}H]serine (B) during the last 2 h of oxysterol treatment. The specific activity of [^{3}H]serine and [1-^{3}H]palmitate (1:1 mol/mol complex with BSA) were held constant at 50 dpm/pmol and 44 dpm/pmol, respectively. SM (○, ●), glucosylceramide (□, ■), and ceramide (▲, △) were extracted and analyzed as described in Materials and Methods. Results are the means of two separate experiments each done in duplicate.

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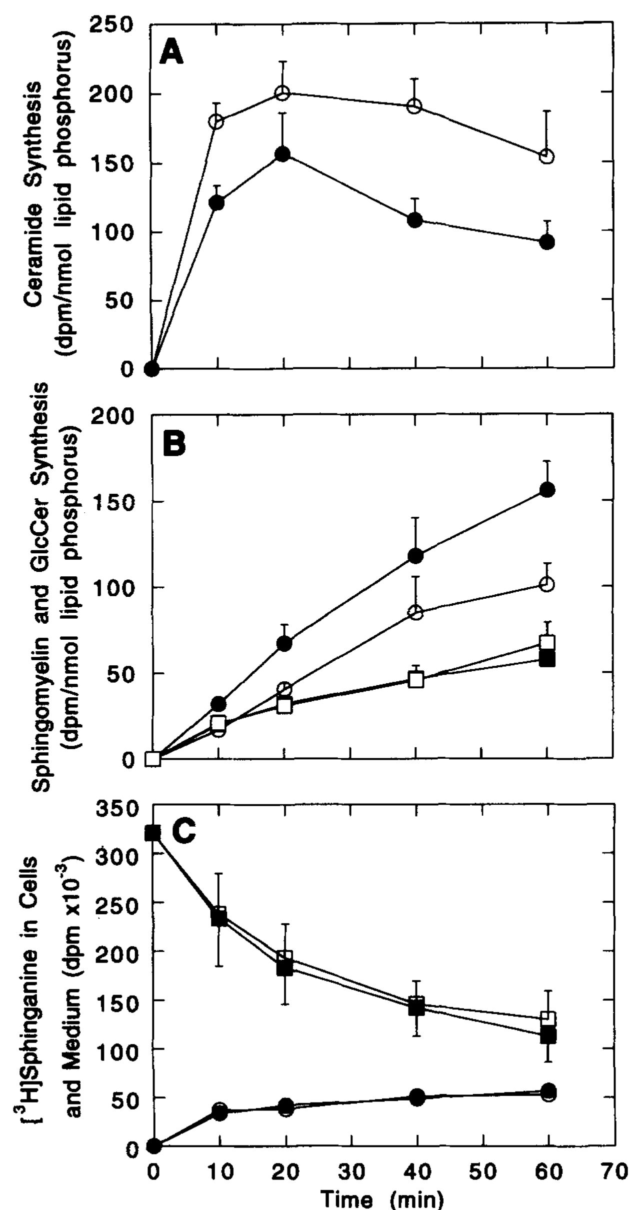

Fig. 6. 25-Hydroxycholesterol stimulates synthesis of sphingomyelin from [3-^{3}H]sphinganine in CHO Cells. CHO cells were cultured in medium A with (closed symbols) or without (open symbols) 25-hydroxycholesterol (2.5 μg/ml) for 4 h. After this time, cells received 1 ml of medium A containing 1 μM [3-^{3}H]sphinganine with or without 25-hydroxycholesterol (2.5 μg/ml). Cells were incubated for the indicated times, medium and cells were harvested, and lipids were extracted and separated in a solvent system of chloroform-methanol-2 N NH_4OH 65:25:4 (v/v). A: incorporation of radioactivity into ceramide (○, ●). B: incorporation into SM (○, ●), glucosylceramide (□, ■). C: [3-^{3}H]sphinganine remaining in cells (○, ●) and medium (□, ■). Results are the mean of three separate experiments ± standard deviation.

tial effects on endogenous ceramide metabolism could be mimicked using short-chain cell permeable ceramide analogues, CHO cells were treated with 25-hydroxycholesterol for 4 h, treated for 20 min with C_6-[3-^{3}H]ceramide or C_6-[3-^{3}H]dihydroceramide (5 μM), and C_6-SM and C_6-glucosylceramide synthesis was measured (**Table 4**). 25-Hydroxycholesterol caused a minor, but significant increase in cellular C_6-SM synthesized from C_6-[3-^{3}H]ceramide and C_6-[3-^{3}H]dihydroceramide. Total C_6-SM synthesis from C_6-ceramide or C_6-dihydroceramide (cellular and medium C_6-SM combined) was also increased significantly ($P < 0.1$) by 25-hydroxycholesterol; however, secretion of short-chain SM released into the medium was not. Similar to results with [3-^{3}H]sphinganine, there was no effect on synthesis of short-chain glucosylceramide using either substrate. Also, uptake of short-chain ceramide and dihydroceramide into cells was not altered by oxysterol treatment.

Enhanced conversion of ceramide to SM could result in changes in cellular ceramide mass. This result may not be obvious from precursor labeling experiments because of changes in the size and specific activity of the endogenous ceramide pool. Ceramide mass in control cells was found to decrease slightly during the first 3 h after medium addition, but recovered to initial values by 6 h (**Table 5**). In contrast, 25-hydroxycholesterol-treated cells displayed significantly lower ceramide levels at 3 and 6 h, but returned to control values at 18 h. Diglyceride was also reduced by approximately 10–20% after medium exchange on control and treated cells; however, diglyceride mass was not significantly altered by 25-hydroxycholesterol treatment.

To determine whether results for precursor labeling studies could be due to direct effects on sphingolipid biosynthetic enzymes, the activities of SPT, SM synthase, and sphinganine *N*-acyltransferase were examined in cell membranes isolated from CHO cells treated with 25-hydroxycholesterol for up to 6 h (**Table 6**). No increase in activity of SPT or SM synthase was observed relative to control membranes, and sphinganine *N*-acyltransferase activity actually decreased significantly over the time course. Also, the specific activity of sphinganine *N*-acyltransferase was 6- to 7-fold greater than the other two enzymes. In other experiments it was found that 25-hydroxycholesterol (1 μg/ml) added directly to assays did not alter SPT or SM synthase activity. Neither were enzyme activities altered in whole cell homogenates from 25-hydroxycholesterol-treated cells.

DISCUSSION

The biological activity of oxysterols is dependent on a C3-hydroxyl, C17-hydrocarbon side chain, intact sterol ring structure, and an additional oxygen group on the sterol nucleus or side chain (19). Oxysterols are present in various cultured cells and tissues, and the concentrations of several oxysterols, including 24-hydroxycholesterol, 25-hydroxycholesterol, 32-hydroxylanosterol, and 32-oxolanosterol, are increased by mevalonate or cholesterol loading

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TABLE 4. Conversion of C_6-ceramide and C_6-dihydroceramide to short-chain sphingolipids in 25-hydroxycholesterol-treated cells

Substrate and Treatment	Cells			Medium	
	C_6-SM	C_6-GluCer	C_6-Cer/DHC	C_6-SM	C_6-Cer/DHC
			dpm/nmol lipid phosphorus		
C_6-Cer	115.2 ± 11.3	146.4 ± 52.7	2531 ± 693	32.3 ± 14.1	3618 ± 1206
C_6-Cer +25-OH	140.3 ± 23.1[a]	148.7 ± 46.2	2451 ± 681	43.9 ± 19.5	3827 ± 390
C_6-DHC	12.8 ± 3.1	5.5 ± 1.9	867 ± 260	9.5 ± 4.9	8867 ± 1624
C_6-DHC +25-OH	20.5 ± 7.2[a]	6.1 ± 2.6	898 ± 323	15.2 ± 9.8	8393 ± 683

Cells were grown in medium A containing 25-hydroxycholesterol (2.5 μg/ml) or ethanol for 4 h prior to the addition of 5 nmol [3-^{3}H]C_6-ceramide (C_6-Cer) or [3-^{3}H]C_6-dihydroceramide (C_6-DHC) in 1 ml of medium A. After a 20-min incubation with the radiolabeled substrates, cells and medium were harvested, and short-chain SM (C_6-SM), glucosylceramide (C_6-GlcCer), and ceramide or dihydroceramide (C_6-Cer/DHC) were isolated and quantitated. Results are the means ± standard deviation of five experiments.
[a] $P < 0.1$ versus control cells.

(34–36). This observation, coupled with the potency of some oxysterols in suppressing cholesterol synthesis, has led to the hypothesis that these compounds mimic endogenous regulators of cholesterol metabolism. In this study, 25-hydroxycholesterol was used to perturb sterol balance in cultured CHO cells and a hitherto undiscovered stimulation of sphingolipid metabolism was observed. 25-Hydroxycholesterol increased SM mass, decreased endogenous ceramide, and stimulated de novo synthesis of SM. Stimulation of SM synthesis by 25-hydroxycholesterol was not a side effect of chronic cholesterol starvation of cells since increased SM synthesis occurred in unison with activation of cholesterol esterification and transcriptional suppression of sterol-regulated genes.

Why would sphingolipid synthesis be elevated in response to oxysterols? If oxysterols are the intracellular signal that the cholesterol concentration is above acceptable levels, then increased SM synthesis is in response to a perceived cholesterol load. If, as mentioned earlier, SM has a role in stabilizing or promoting cholesterol absorption at the plasma membrane, more SM might buffer the cell against increased unesterified cholesterol. Alternatively, SM could be required during cholesteryl ester synthesis for deposition or stabilization of ester droplets. However, it would appear that the effects of 25-hydroxycholesterol on sphingolipid synthesis are independent of cholesterol and cholesterol esterification in CHO cells. It was observed that stimulation of SM synthesis by 25-hydroxycholesterol occurred whether cells were grown in medium containing delipidated serum, whole serum, LDL, or nonlipoprotein cholesterol; a clear demonstration that oxysterol and not cholesterol stimulates sphingolipid synthesis. In a previous study using CHO cells (18), fetal calf serum caused a slight suppression of SM synthesis. In contrast, LDL inhibited SM synthesis in fibroblasts (16) and proximal tubular cells (17) by 70–80%. However, caution should be taken when inter-

TABLE 5. Ceramide and diglyceride mass in 25-hydroxycholesterol-treated CHO cells

Treatment	0 h	1 h	3 h	6 h	18 h
			pmol ceramide/nmol lipid phosphorus		
NA	6.19 ± 0.44	4.99 ± 0.86	5.06 ± 0.42	7.93 ± 1.17	7.34 ± 0.08
25-OH		4.64 ± 1.06	4.07 ± 0.42[a]	5.06 ± 0.24[b]	6.89 ± 0.61
			pmol diglyceride/nmol lipid phosphorus		
NA	64.3 ± 5.74	57.7 ± 9.9	51.2 ± 4.0	48.5 ± 5.5	53.0 ± 3.9
25-OH		52.7 ± 7.9	45.8 ± 4.6	44.3 ± 3.8	51.4 ± 7.4

Ceramide and diglyceride levels were assayed in lipid extracts cells treated with 25-hydroxycholesterol (25-OH, 2.5 μg/ml) or ethanol solvent (no addition, NA) for the indicated times. Results are the means ± standard deviation of three or four separate experiments each done in duplicate.
[a] $P < 0.025$ versus untreated cells.
[b] $P < 0.005$ versus untreated cells.

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TABLE 6. Activity of sphingomyelin biosynthetic enzymes in membranes from 25-hydroxycholesterol-treated CHO cells

	NA	2 h	4 h	6 h
		pmol/min/mg protein		
Serine palmitoyltransferase	52.6 ± 14.6	60.7 ± 10.9	53.6 ± 4.7	48.4 ± 9.0
SM synthase	44.4 ± 11.9	46.0 ± 7.3	49.7 ± 15.4	39.7 ± 7.6
Sphinganine *N*-acyltransferase	368.6 ± 37.4	287.1 ± 39.4[b]	319.0 ± 19.5[a]	255.8 ± 27.8[c]

Enzyme activities were assayed in membranes isolated from CHO cells cultured in medium A with 25-hydroxycholesterol (2.5 μg/ml) for 2, 4, or 6 h as described in Materials and Methods. No addition (NA) controls were grown for 6 h in medium containing an equivalent volume of ethanol. Results are the means of three separate experiments (done in duplicate) ± standard deviation.

[a] $P < 0.1$; [b] $P < 0.025$; [c] $P < 0.01$ versus control.

preting these results. Catabolism of LDL by cells would increase the cellular concentration of fatty acids and long-chain bases, both of which are known to alter sphingolipid synthesis (37, 38). Also, LDL cholesterol rapidly down-regulates the LDL receptor and cholesterol synthesis, and stimulates cholesterol esterification, resulting in little or no change in the cellular cholesterol concentration (39). Recently, a careful comparison of SM and cholesterol content was made in macrophages incubated with acetyl-LDL (40). This atherogenic lipoprotein more than doubled the unesterified cholesterol content and significantly increased SM in J774.A1 mouse macrophages during a 2-day treatment. This response was potentiated by co-treatment with the ACAT inhibitor 58-035, which elevated unesterified cholesterol 8-fold. Although the source of the SM that accumulated in cells was not determined, the results suggest that cholesterol promotes the accumulation of SM. Acetyl-LDL has also been shown to transiently increase SM synthesis in mouse peritoneal macrophages (41). Thus, results shown here with 25-hydroxycholesterol may not be reproduced by cholesterol under conditions where the cell can regulate cholesterol uptake and esterification, but increased de novo synthesis of SM might occur if cellular cholesterol content is elevated for an extended period. Interestingly, PtdCho also accumulated in the cholesterol-loaded mouse macrophage due in part to up-regulation of the rate-limiting enzyme in PtdCho synthesis, CTP:phosphocholine cytidylyltransferase (42). Whether SM biosynthetic enzymes are similarly affected by cholesterol loading has not been reported.

Given the broad range of potencies of the various oxysterols for regulating cholesterol metabolism, it is surprising that 25-hydroxycholesterol was the only oxysterol tested that stimulated SM synthesis. This could be a threshold phenomenon whereby only maximal changes in cholesterol homeostasis result in increased sphingolipid synthesis. In this regard, 25-hydroxycholesterol was the most potent sterol in suppressing transcription and increasing cholesterol esterification. It is also possible that SM synthesis is exclusively activated by 25-hydroxycholesterol. Another oxysterol, 22(S)-hydroxycholesterol, inhibited sphingolipid and ceramide synthesis independent of effects on cholesterol regulation. This suggests another mechanism for oxysterol regulation of sphingolipid metabolism that acts prior to ceramide synthesis.

Precursor labeling experiments and mass measurements show that 25-hydroxycholesterol has a complex affect on SM synthesis. Concomitant with increased synthesis is a reduction in endogenous ceramide. This has several important implications when interpreting the radioactive precursor labeling experiments. Ceramide, newly synthesized from [^{3}H]serine, would have a greater specific activity in 25-hydroxycholesterol-treated cells. An isotope dilution effect of this type could account for increased SM and glucosylceramide labeling. However, this seems unlikely as SM mass and [^{3}H]choline incorporation into SM were also increased. If isotope dilution did occur, the amount of labeled ceramide in control cells should have been greater than oxysterol-treated cells due to the larger endogenous pool. This was not the case; [^{3}H]serine-labeled ceramide was unchanged or slightly increased in oxysterol-treated cells. Reduced endogenous ceramide and enhanced conversion of [^{3}H]sphinganine-labeled ceramide and short-chain ceramide to SM in 25-hydroxycholesterol-treated cells indicates that the final step in the synthetic pathway is probably affected. Conversion of ceramide to sphingolipids is a logical site of regulation for several reasons. First, it is a branch point for synthesis of SM and the complex glycosphingolipids. Competition for ceramide amongst the glycosyltransferases and SM synthase might be predicted. Second, based on pulse-labeling studies with [^{3}H]serine, [^{3}H]palmitate, and [3-^{3}H]sphinganine, it appears that ceramide is not immediately converted to SM and glycosphingolipids, but reaches a steady-state level in the cell. The size and rate of metabolism of this ceramide pool will influence sphingolipid synthesis. Third, the enzymes involved in ceramide synthesis and those that convert ceramide to SM and glycosphingolipids are in separate organelles (43).

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This creates the need for ceramide transport from the endoplasmic reticulum to sites of sphingolipid synthesis in other membranes. Recent discoveries on topology of ceramide and sphingolipid synthesis have led to a sequential model of synthesis that connects the endoplasmic reticulum, Golgi apparatus, and plasma membrane (44–46).

If SM synthase is substrate limited, than the size of an endoplasmic reticulum pool of ceramide and rate of transport to the Golgi would regulate SM synthesis. 25-Hydroxycholesterol could target steps in this part of the pathway by enhancing transport of ceramide to the site of SM synthesis or by directly stimulating SM synthase activity. Results presented here could not define in absolute terms which part of the process was affected. Lack of effect of 25-hydroxycholesterol on SM synthase activity in vitro could be the result of using short-chain ceramide analogues as substrates. However, conversion of these same compounds to short-chain SM was stimulated (albeit to a lesser degree then that observed for de novo synthesis) by 25-hydroxycholesterol in intact cells. This implies that correct membrane topology is required, and co-localization of these ceramide analogues with SM synthase is stimulated by 25-hydroxycholesterol. Studies with fluorescent short-chain ceramides (C_6-NBD-ceramide) have demonstrated specific localization of these analogues to the *trans* Golgi (47). Although cholesterol deprivation of cells reduced the fluorescent staining of the Golgi apparatus by C_6-NBD-ceramide (48), it is not clear whether cholesterol or oxysterols actually alter affinity of ceramide for the Golgi apparatus.

25-Hydroxycholesterol did not stimulate glucosylceramide synthesis when sphinganine or short-chain ceramide and dihydroceramide were substrates in vivo. Although SM synthesis from [3-^{3}H]sphinganine was stimulated, it should be noted this was about 50% less than that observed with [^{3}H]serine. It is possible that ceramide made from exogenously added sphinganine is distinct from that made de novo, and the source of ceramide dictates its biosynthetic fate. Alternatively, SM and glucosylceramide synthesis could occupy different subcellular compartments, as suggested by localization of glucosyltransferase to endoplasmic reticulum and pre-Golgi fractions (49) compared to *cis/medial* Golgi localization of SM synthase in rat liver (50, 51). In CHO cells, SM synthesis and transport to the plasma membrane is via the Golgi apparatus (44, 45, 52), but insensitivity to brefeldin A and reduced temperature has led to the conclusion that most glucosylceramide is transported to the plasma membrane via a non-Golgi pathway (52). Thus, ceramide in the endoplasmic reticulum could be targeted to different membranes resulting in conversion to SM or glucosylceramide, and the ceramide pool destined for conversion to SM under regulation by oxysterol.

One feature of a model that proposes ceramide conversion to SM as a rate-limiting and regulated step is the required stimulation of de novo long-chain base synthesis to replenish the ceramide pool. In this study, 25-hydroxycholesterol stimulated long-chain base synthesis as indicated by enhanced incorporation of [^{3}H]serine-labeled sphinganine and sphingosine into sphingolipids. It is feasible that synthesis of long-chain base is regulated by ceramide, and when the ceramide pool in the endoplasmic reticulum is depleted, long-chain base synthesis is up-regulation. As evidence of this, [^{3}H]sphinganine-labeled ceramide was actually reduced by oxysterol (the result of conversion to SM), while endogenous ceramide synthesized de novo from [^{3}H]serine was the same or increased. This implies that de novo ceramide production is stimulated to replace that converted to SM, but up-regulation of sphinganine acylation does not occur. Clearly, the affected step is not sphinganine acylation as sphinganine *N*-acyltransferase activity and conversion of [^{3}H]sphinganine to ceramide were slightly inhibited by 25-hydroxycholesterol. The reduction in sphinganine *N*-acyltransferase activity might not be expected to alter ceramide or sphingolipid synthesis as the specific activity is still 5- to 7-fold greater than SPT or SM synthase. 25-Hydroxycholesterol did not alter SPT activity measured in vitro. More work is need to identify what role, if any, ceramide plays in regulating SPT activity (or other enzymatic steps prior to ceramide synthesis) and whether 25-hydroxycholesterol stimulates SPT.

Ceramide has recently been shown to mediate the action of a variety of cytokines on cell growth and differentiation (reviewed in ref. 53). The primary source of ceramide is via receptor-activation of a neutral sphingomyelinase and hydrolysis of plasma membrane SM. It will be interesting to determine whether 25-hydroxycholesterol has specific effects on cell proliferation related to changes in de novo ceramide biosynthesis and increased production of SM.

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