Chinese hamster ovary cells overexpressing the oxysterol binding protein (OSBP) display enhanced synthesis of sphingomyelin in response to 25-hydroxycholesterol

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Abstract **25-Hydroxycholesterol negatively regulates cholesterol synthesis and activates cholesterol esterification in a variety of cultured cells. Concurrent with these effects, 25-hydroxycholesterol also stimulates the synthesis of sphingomyelin in Chinese hamster ovary (CHO)-K1 cells. The role of oxysterol binding protein (OSBP), a high affinity receptor for 25-hydroxycholesterol, in activation of SM synthesis was assessed by overexpression in CHO-K1 cells. When compared to mock transfected controls, three CHO-K1 clones overexpressing OSBP by 10- to 15-fold displayed a 2- to 3-fold enhancement of [^{3}H]serine incorporation into sphingomyelin when treated with 25-hydroxycholesterol. Closer examination of one of these clones (CHO-OSBP cells) revealed a >8.5-fold stimulation of sphingomyelin synthesis after a 6-h treatment with 25-hydroxycholesterol compared to 3.5-fold in controls, slightly higher basal levels of sphingomyelin synthesis, and a more rapid response to 25-hydroxycholesterol. [^{3}H]serine incorporation into phosphatidylserine, phosphatidylethanolamine, ceramide, or glucosylceramide was affected by <15%. Synthesis of sphingomyelin from exogenous [^{3}H]sphinganine-labeled ceramide was enhanced in overexpressing cells treated with 25-hydroxycholesterol. However, in vitro activities of sphinganine *N*-acyltransferase, sphingomyelin synthase, and serine palmitoyltransferase were not affected by OSBP overexpression or 25-hydroxycholesterol. Overexpression of OSBP or 25-hydroxycholesterol did not significantly affect the ceramide content of Golgi-enriched fractions from control or overexpressing cells. However, diglyceride mass was reduced in Golgi-enriched fractions from overexpressing cells and by treatment with 25-hydroxycholesterol. Results from overexpressing cells show that OSBP potentiates the stimulatory effects of 25-hydroxycholesterol on sphingomyelin synthesis. 25-Hydroxycholesterol promotes translocation of OSBP to the Golgi apparatus where it appears to stimulate conversion of ceramide to sphingomyelin.—**Lagace, T. A., D. M. Byers, H. W. Cook, and N. D. Ridgway. **Chinese hamster ovary cells overexpressing the oxysterol binding protein (OSBP) display enhanced synthesis of sphingomyelin in response to 25-hydroxycholesterol. *J. Lipid Res.* 1999.** 40: **109–116.**

Supplementary key words sphingomyelin • 25-hydroxycholesterol • oxysterol binding protein • ceramide

Similar intracellular localization (1, 2), coordinate changes in several lipid storage diseases and atherosclerosis (3), and apparent coordinate metabolism in experimental systems (4–7) has led to the proposal that cholesterol and sphingomyelin (SM) may share a common regulatory pathway. While interaction between cholesterol and SM has been demonstrated at the physical level (8) and in terms of tissue and cell distribution, it is unclear what mechanism(s) are responsible for coordinately sensing and maintaining the ratios of these two important membrane constituents.

Evidence suggests that plasma membrane sphingomyelin is critical for maintaining normal cholesterol distribution and regulation. Hydrolysis of plasma membrane SM with bacterial sphingomyelinase results in increased cholesterol esterification (9) and reduced synthesis (5). Increasing plasma membrane sphingomyelin has the opposite effect (4, 6). Recent evidence suggests that cholesterol and SM, along with components of signal transduction pathways, are sequestered in plasma membrane structures called caveolae (10). SM and cholesterol are required for structural integrity of caveolae, and SM in these structures may serve as a source of ceramide for signal transduction (11). Caveolae also serve as sites for cholesterol efflux to extracellular acceptors such as high density lipoproteins (12) and delivery of newly synthesized cholesterol (13). Cholesterol and sphingolipid are also concentrated in detergent insoluble "rafts" that are involved in delivery of proteins, sphingolipids, and cholesterol from the trans-Golgi network to the plasma membrane. Sphingolipid-

Abbreviations: BFA, brefeldin A; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GlcCer, glucosylceramide; HMG, 3-hydroxy-3-methylglutaryl; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; OSBP, oxysterol binding protein; PAGE, polyacrylamide gel electrophoresis; PNS, post-nuclear supernatant; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; SPT, serine palmitoyltransferase; SM, sphingomyelin.

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rich membrane rafts are required for targeted delivery of some proteins and lipids to apical surfaces in polarized cells (14) and for axonal sorting in neurons (15).

The metabolism of plasma membrane cholesterol and SM is intimately related, but the mechanism(s) responsible for coordinating metabolism are unknown. Sphingolipid synthesis was shown to be inhibited by low density lipoprotein (LDL) in fibroblasts (16) and proximal renal tubular cells (17), but not in CHO cells (18). In CaCo2 cells, the activities of serine palmitoyltransferase (SPT) or HMG-CoA reductase were not affected by acute changes in cholesterol or SM synthesis, respectively (7). Similarly, mRNA for cholesterol and fatty acid biosynthetic enzymes, but not a SPT isoform, were coordinately regulated in human keratinocytes (19). However, when cholesterol or sphingomyelin content of cells was altered by exogenous supplementation, synthetic rates changed in parallel (4, 6, 7). This suggests that cells may sense the absolute amounts of cholesterol and SM in membrane domains and alter synthesis or catabolism to maintain appropriate ratios. How this is achieved and the signalling molecules and enzymes involved are unknown. The ratio of cholesterol and SM in caveolae and sphingolipid "rafts" could be a key factor in mediating these changes in metabolism.

We previously investigated 25-hydroxycholesterol as a regulator of both cholesterol and SM metabolism in CHO-K1 cells (20). In CHO-K1 cells, 25-hydroxycholesterol potently suppressed de novo cholesterol biosynthesis and stimulated cholesterol esterification, and also stimulated SM synthesis with time- and dose-dependence that was similar to effects on cholesterol homeostasis (20). 25-Hydroxycholesterol seemed to stimulate the conversion of ceramide to SM, suggesting either a direct affect on SM synthase or ceramide transport to this enzyme. The presumed site of this effect is the Golgi apparatus as ceramide would have to be transported from the endoplasmic reticulum to the Golgi where SM synthase is localized (21–23). Interestingly, a high affinity receptor for oxysterols, the oxysterol binding protein (OSBP), was found to undergo 25-hydroxycholesterol-mediated translocation from a cytosolic/vesicular compartment to the Golgi apparatus (24). Early findings of a positive correlation between the affinity of oxysterols for OSBP and down-regulation HMG-CoA reductase indirectly implicated OSBP in oxysterol suppression of cholesterol synthesis (25). This conclusion is supported by recent studies in CHO-K1 cells overexpressing OSBP (26). These cells display constitutive down-regulation of ACAT mRNA and activity, as well as elevated cholesterol synthesis and mRNA for sterol-regulated genes. How OSBP mediates these effects on cholesterol regulation is unknown, but it could involve altered lipid and sterol trafficking through the Golgi/vesicular pathway.

Our initial finding that 25-hydroxycholesterol stimulated sphingomyelin synthesis prompted us to investigate whether this response was modified in CHO-K1 cells overexpressing OSBP. Here we report that overexpressing cells had enhanced 25-hydroxycholesterol-mediated stimulation of SM synthesis compared to mock transfected control cells. Similar to control cells, the point of stimulation was ceramide conversion to SM.

MATERIALS AND METHODS

25-Hydroxycholesterol was purchased from Steraloids Inc., Wilton, NH. Brefeldin A (BFA) and diglyceride kinase were from Calbiochem, La Jolla, CA. BFA was stored as a 1 mg/ml stock in ethanol at −20°C. [^{3}H(G)]serine and [γ-^{32}P]ATP were from DuPont-New England Nuclear. Tissue culture reagents and medium were from Gibco-BRL. Uniformly labeled [^{3}H]sphinganine was a gift from Dr. Christopher McMaster, Dalhousie University, NS. Silica gel 60 thin-layer chromatography plates were from E. Merck, Darmstadt, Germany. All other chemicals were of reagent grade. Lipoprotein-deficient serum (LPDS) and human LDL were prepared as previously described (27).

Cell culture

CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS) and 34 μg proline/ml (medium A) at 37°C in a 5% CO_2 atmosphere. Cells were stably transfected with the rabbit OSBP cDNA as previously described (24, 26). The stability and proportion of overexpressing cells (>95%) was routinely monitored by immunoblotting and immunofluorescence, respectively. Stock cultures of transfected cells were maintained in DMEM with 5% FCS and 300 μg/ml G418, but for experiments cells were seeded in the same medium without G418. Cells were routinely seeded at 150,000/60-mm dishes in 3 ml of medium A and experiments were started 3 days later. Stock solutions of 25-hydroxycholesterol (2.5 mg/ml) were prepared in ethanol (ethanol concentrations in the medium did not exceed 0.1%).

Labeling, extraction, and analysis of phospholipids and sphingolipids

Cells were labeled with 7.5 μCi [^{3}H]serine/ml for the times indicated in figure and table legends. After labeling, cells were washed once with cold phosphate-buffered saline (PBS), scraped in 1 ml methanol–water 5:4 (v/v) and transferred to screwcap tubes. Lipids were extracted as previously described (20) and a portion was resolved by thin-layer chromatography in chloroform–methanol–acetic acid–water 60:40:4:1 (v/v). PtdSer, PtdEtn, and SM were identified by iodine staining, scraped into vials, and radioactivity was quantitated by liquid scintillation counting. Another aliquot of the lipid extract was subjected to base hydrolysis and nonsaponified sphingolipids were separated by thin-layer chromatography in a solvent system of chloroform–methanol–water 65:25:4 (v/v). SM, ceramide, and glucosylceramide were identified by fluorography, and radioactivity was quantitated by scintillation counting. The metabolic products of [^{3}H]sphinganine (SM, ceramide, and GlcCer) were extracted from cells as described above, resolved by thin-layer chromatography in chloroform–methanol–water 65:25:4 (v/v), identified by fluorography, and quantitated by scintillation counting.

Enzyme assays

CHO-OSBP and control cells were harvested by scraping into ice-cold PBS and collected by centrifugation at 2,000 *g* for 5 min. The cell pellet was homogenized in 20 mm Tris-HCl (pH 7.7) and 10 mm EDTA by 10 passages through a 22-gauge needle, centrifuged at 100,000 *g* for 1 h, and the membrane/particulate fraction (pellet) was resuspended in Tris-EDTA buffer. SPT (18), SM synthase (28), and sphinganine *N*-acyltransferase (29) were assayed in the membrane/particulate fraction with the previously described modifications (20).

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Quantitation of ceramide and diglyceride in Golgi-enriched fractions

A Golgi-enriched fraction was isolated from the 10,000 *g* post-mitochondrial supernatant (PNS) of control and overexpressing CHO-K1 cell homogenates on discontinuous sucrose gradients (30). The Golgi fraction was enriched 25- to 40-fold for SM synthase, a marker of the *cis*/*medial* cisternae (22). NADPH cytochrome c reductase activity was only enriched 2.5-fold in Golgi membranes relative to the PNS. Lipids were extracted from the PNS and Golgi-enriched fractions as described above, and diglyceride and ceramide mass was quantitated by the diglyceride kinase assay (31).

Other methods

Endogenous and overexpressed OSBP was quantitated by immunoblotting using a polyclonal antibody (AB104) prepared against a GST-OSBP fusion protein expressing amino acids 201–309 of the rabbit protein (32). Briefly, cells were extracted in 10 mm sodium phosphate (pH 7.4), 150 mm NaCl, 2 mm EDTA, 2 mm EGTA, 10 mm NaF, 1 mm sodium pyrophosphate, 1 mm β-glycerophosphate, 100 μm phenylmethanesulfonyl fluoride, 2 μg aprotinin/ml, 2.5 μg leupeptin/ml, and 0.3% (w/v) Triton X-100 (buffer A) on ice for 15 min and a soluble fraction was isolated by centrifugation at 4°C for 15 min at 10,000 *g* in a microcentrifuge. Triton X-100 extracts prepared in this manner contained all detectable OSBP and were resolved by SDS/6% polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. The nitrocellulose filter was incubated with AB104 in 20 mm Tris-HCl (pH 7.4), 150 mm NaCl, 5% (w/v) skim milk powder, and 0.1% (v/v) Tween 20 for 1 h followed by a goat anti-rabbit secondary antibody coupled to horseradish peroxidase. OSBP was detected by the chemiluminescence method according to the manufacturer's instructions (DuPont-New England Nuclear). Protein was determined by the method of Lowry et al. (33) and lipid phosphorus by the method of Rouser, Siakatos, and Fleisher (34).

RESULTS

Analysis of sphingomyelin synthesis in CHO cells overexpressing OSBP

Three clonal CHO-K1 cell lines stably overexpressing rabbit OSBP and three cell lines harboring the empty vector were assayed for stimulation of SM synthesis by 25-hydroxycholesterol. **Figure 1** (panel A) shows immunoblot analysis of endogenous hamster OSBP in three mock transfected cell lines (M1–M3) and rabbit OSBP in three overexpressing cell lines. Endogenous OSBP was evident as a protein of approximately 100 kDa, while the overexpressed protein appeared as a doublet of 97 and 100 kDa. The upper band in OSBP is the result of reduced mobility in SDS-PAGE due to extensive phosphorylation (32). OSBP was overexpressed approximately 15-fold in CHO-OSBP 7 and 16, and 10-fold in CHO-OSBP 18. [^{3}H]serine incorporation into SM under basal conditions was similar between control and overexpressing cells (Fig. 1B). Mock transfected cells treated with 25-hydroxycholesterol for 4 h displayed a 1.6- to 2.2-fold increase in [^{3}H]serine incorporation into SM compared to their untreated conditions. In contrast, SM synthesis in overexpressing cells was stimulated 2.8- to 3.4-fold by 25-hydroxycholesterol.

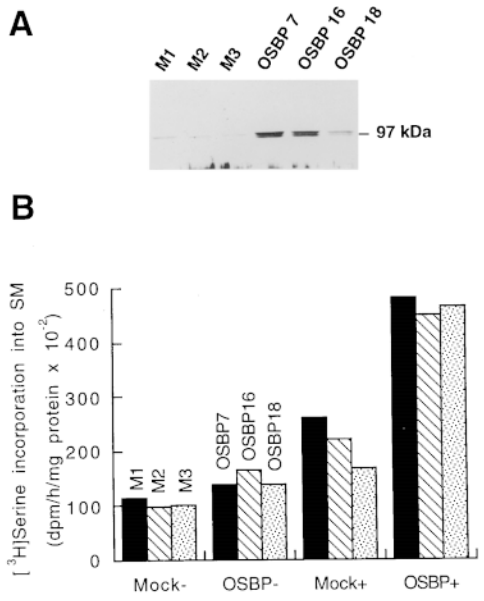

Fig. 1. OSBP expression and SM biosynthesis in CHO-K1 cells overexpressing the rabbit oxysterol binding protein. A: three CHO-K1 cell lines overexpressing OSBP and three mock transfected cell lines expressing empty vector (pCMV) were analyzed for OSBP expression by immunoblotting using polyclonal antibody AB104 and chemiluminescence detection. B: cells were treated with 25-hydroxycholesterol (2.5 μg/ml) in DMEM containing 5% FCS for 4 h. SM synthesis was assessed by [^{3}H]serine labelling (7.5 μCi/ml) for the final 2 h of oxysterol treatment. [^{3}H]SM was isolated and quantitated as described in Materials and Methods. Results are the average of duplicate determinations from a representative experiment that was repeated twice with similar results.

As the enhanced response to 25-hydroxycholesterol was similar in the three overexpressing cell lines, we chose one (CHO-OSBP 16, referred to hereafter as CHO-OSBP cells) to examine in more detail. A time course of 25-hydroxycholesterol treatment was performed and SM synthesis assessed by [^{3}H]serine incorporation (**Fig. 2**). SM synthesis increased in a time-dependent manner in both control and overexpressing cells. However, CHO-OSBP cells had a marked stimulation in response to 25-hydroxycholesterol compared to controls; this was most evident prior to 2 h, and stimulation persisted for 6 h. Overexpression did not affect [^{3}H]serine incorporation into PtdSer, but did have a slight stimulatory effect (20%) on PtdEtn that was evident 2 h after 25-hydroxycholesterol treatment.

[^{3}H]serine incorporation into ceramide and glucosylceramide was examined in control and overexpressing cells exposed to 25-hydroxycholesterol for 4 h (**Table 1**). Incorporation into ceramide and GlcCer was increased slightly in overexpressing cells and by 25-hydroxycholesterol treatment, but this was not significant. Under the same conditions, 25-hydroxycholesterol stimulated [^{3}H]serine incorporation into SM in control and overexpressing cells by 3- and 6-fold, respectively. Untreated, overexpressing cells displayed a slight increase (40%) in SM synthesis (as shown in Figs. 1 and 2), but this was not significant.

OSBP overexpression and 25-hydroxycholesterol stimu-

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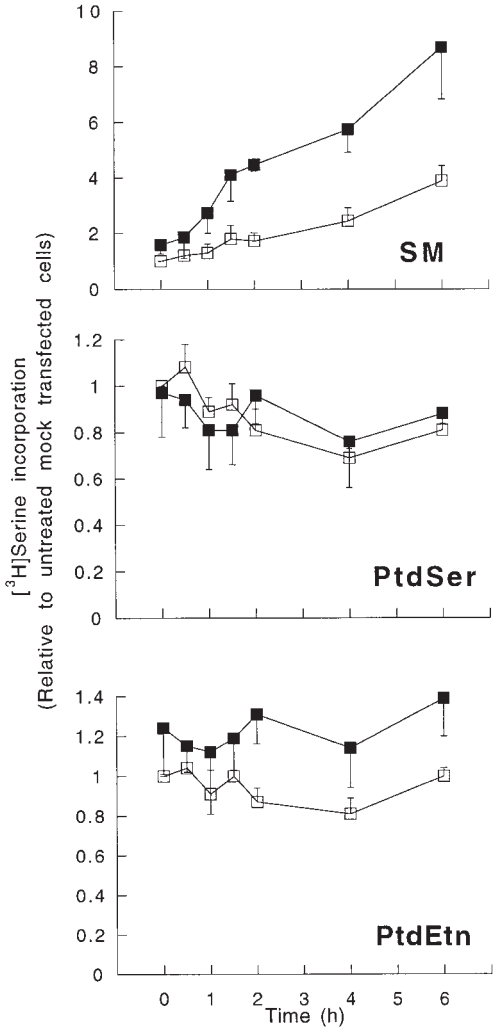

Fig. 2. Time course for the activation of SM synthesis by 25-hydroxycholesterol in CHO-OSBP and mock transfected cells. CHO-OSBP (clone OSBP 16, ■) and mock transfected (clone M3, □) cells were cultured in serine-free DMEM with 5% FCS and treated with 25-hydroxycholesterol (2.5 μg/ml) for the indicated times. During the last 30 or 60 min of oxysterol treatment, cells were pulsed with [^{3}H]serine (7.5 μCi/ml) and incorporation into SM, PtdSer, and PtdEtn was quantitated as described in Materials and Methods. Results are expressed relative to [^{3}H]serine incorporation into SM (59.1 dpm/min per mg protein), PtdSer (1167.6 dpm/min per mg protein), and PtdEtn (242.6 dpm/min per mg protein) in untreated mock cells, and are the mean and standard deviation of three to six experiments.

lated [^{3}H]serine incorporation into the long-chain base of sphingolipids. The distribution of [^{3}H]sphingosine and [^{3}H]sphinganine in total lipid extracts closely paralleled the incorporation of [^{3}H]serine into SM. 25-Hydroxycholesterol stimulated [^{3}H]serine incorporation into sphingosine and sphinganine of control and overexpressing cells by 2- and 2.5-fold, respectively. When compared to 25-hydroxycholesterol-treated control cells, [^{3}H]serine incorporation into sphinganine and sphingosine of total sphingolipids of CHO-OSBP cells was increased by 40% and 100%, respectively (results not shown). Increased incorporation of [^{3}H]serine into long-chain bases of 25-

TABLE 1. [^{3}H]serine incorporation into sphingomyelin, ceramide, and glucosylceramide in CHO-K1 cells overexpressing OSBP

	SM	GluCer	Ceramide
		dpm/h/mg protein $\times 10^{-2}$	
Mock NA	18.3 ± 3.6	11.9 ± 3.3	34.7 ± 11.9
Mock +25-OH	56.3 ± 16.8[a]	17.6 ± 7.0	38.2 ± 14.0
CHO-OSBP NA	25.6 ± 8.0	14.6 ± 5.0	45.3 ± 11.2
CHO-OSBP +25-OH	132.8 ± 32.2[b,c]	22.2 ± 7.1	58.2 ± 16.0

Mock and overexpressing cells were cultured in DMEM with 5% FCS and 25-hydroxycholesterol (2.5 μg/ml) or ethanol solvent (no addition, NA) for 4 h. During the last 2 h of this treatment, cells were labelel with 7.5 μCi [^{3}H]serine/ml, harvested, and isotope incorporation into sphingomyelin (SM), glucosylceramide (GluCer), and ceramide was quantitated as described in Materials and Methods. Results are the mean and standard deviation of four or six experiments.

[a] $P < 0.005$.
[b] $P < 0.001$ versus untreated mock or CHO-OSBP cells, respectively.
[c] $P < 0.001$ versus 25-hydroxycholesterol-treated mock cells.

hydroxycholesterol-treated cells was reported previously in CHO-K1 cells (20).

Stimulation of SM synthesis by 25-hydroxycholesterol was blunted when CHO-K1 cells were grown in LPDS compared to FCS or LDL-supplemented LPDS (20). We examined whether SM synthesis in overexpressing cells was also dependent on lipoprotein-replete medium (**Fig. 3**). When cells were grown in FCS, 25-hydroxycholesterol stimulated SM synthesis in mock and overexpressing cells by 1.5- and 3.2-fold, respectively. However, cells cultured in LPDS had increased basal SM synthesis and no significant response to 25-hydroxycholesterol.

In an effort to determine which step in SM synthesis was affected by OSBP overexpression, the incorporation of [^{3}H]sphinganine into ceramide and SM was measured (**Table 2**). The rationale for examining this step in the biosynthetic pathway can be found in our previous obser-

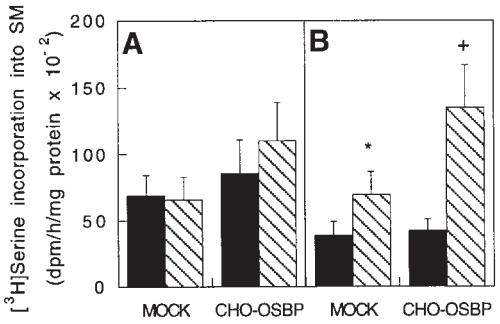

Fig. 3. Effect of culture conditions on activation of SM synthesis by 25-hydroxycholesterol in mock and overexpressing cells. Mock or OSBP transfected cells were cultured for 18 h in DMEM containing either (A) 5% LPDS or (B) 5% FCS. Cells were then switched to identical medium (serine-free) for 6 h and cells were treated with 25-hydroxycholesterol (2.5 μg/ml, striped bar) or ethanol (solid bar) for 2 h. During the final 1 h of oxysterol treatment, cells were labeled with 7.5 μCi [^{3}H]serine/ml and incorporation into SM was quantitated as described in Material and Methods. Results are the mean and standard deviation of four experiments. *$P < 0.025$, +$P <$ 0.005 versus mock cells.

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TABLE 2. Incorporation of [^{3}H]sphinganine into ceramide, glucosylceramide, and sphingomyelin in OSBP overexpressing CHO-K1 cells

	SM	GlcCer	Ceramide
	pmol/h/mg protein		
Mock NA	28.2 ± 4.2	11.4 ± 1.2	85.2 ± 19.2
Mock +25-OH	42.2 ± 5.2[a]	12.6 ± 1.8	70.2 ± 15.0
CHO-OSBP NA	28.2 ± 2.4	11.4 ± 1.4	86.4 ± 6.0
CHO-OSBP +25-OH	52.8 ± 2.8[b,d]	12.6 ± 2.4	61.8 ± 7.8[c]

Mock and overexpressing cells were treated with 2.5 μg 25-hydroxycholesterol/ml in DMEM and 5% FCS for 4 h. During the last hour, [^{3}H]sphinganine (1200 dpm/pmol) dissolved in ethanol was added to cells. Cells were harvested and incorporation into sphingomyelin (SM), glucosylceramide (GlcCer), and ceramide was quantitated. Results are the mean and standard deviation from three experiments.

[a] $P < 0.05$.

[b] $P < 0.001$.

[c] $P < 0.025$ versus untreated mock or CHO-OSBP cells, respectively.

[d] $P < 0.05$ versus 25-hydroxycholesterol-treated mock cells.

vation that 25-hydroxycholesterol stimulated the conversion of [^{3}H]sphinganine-labeled ceramide to SM, and caused decreased ceramide mass in CHO-K1 cells (20). When similar experiments were performed in control and overexpressing CHO-K1 cells, 25-hydroxycholesterol stimulated [^{3}H]sphinganine incorporation into SM with a reduction in the level of [^{3}H]ceramide. Oxysterol-treated CHO-OSBP cells showed a significantly greater increase in SM labeling and decreased ceramide labeling compared to controls. Isotope incorporation into GlcCer was unaffected by oxysterol or OSBP overexpression.

While oxysterol treatment and OSBP overexpression stimulated [^{3}H]sphinganine incorporation into SM, we were unable to detect changes in the in vitro activity of sphinganine *N*-acyltransferase (52.1 ± 7.2 versus 50.1 ± 6.0 pmol/min per mg [n = 3] in control and overexpressing cells, respectively) and SM synthase (17.8 ± 1.56 versus 23.6 ± 0.85 pmol/min per mg [n = 3] in control and CHO-OSBP cells, respectively) in membrane fractions after treatment of cells with 25-hydroxycholesterol for 4 h. SPT activity was also unaffected by OSBP overexpression and oxysterol treatment (40.0 ± 5.0 versus 37.4 ± 0.9 pmol/min per mg [n = 3] in 25-hydroxycholesterol treated control and CHO-OSBP cells, respectively).

Effect of OSBP overexpression on SM synthesis and ceramide levels in the Golgi apparatus

Conversion of ceramide to SM requires transport to SM synthase in the *cis*/*medial* elements of the Golgi apparatus (21–23). The endoplasmic reticulum must be a relatively rich source of ceramide since mixing of the Golgi apparatus and ER by BFA resulted in 2- to 4-fold increase in SM synthesis (35, 36). It is presumed that increased ceramide availability to SM synthase accounted for increased SM synthesis. If 25-hydroxycholesterol also increased ceramide availability for the synthase, albeit not by physically merging the two organelles, the effects of BFA and 25-hydroxycholesterol on SM synthesis should not be additive. BFA (1 μg/ml) stimulated [^{3}H]serine incorporation into SM to the same degree in control and overexpressing

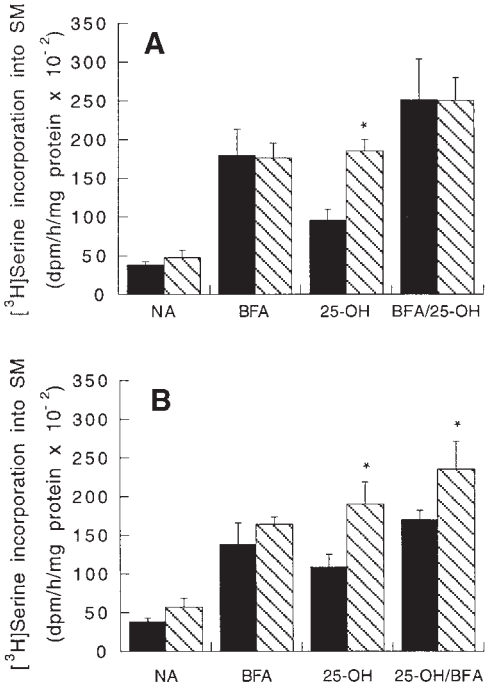

Fig. 4. Effect of brefeldin A on stimulation of SM synthesis by 25-hydroxycholesterol. Mock transfected (solid bars) and CHO-OSBP cells (striped bars) were cultured in DMEM with 5% FCS. A: cells either received no addition (NA, ethanol solvent), BFA (BFA, 1 μg/ml) for 2 h, 25-hydroxycholesterol (25-OH, 2.5 μg/ml) for 2 h, or BFA addition 15 min prior to 25-hydroxycholesterol addition for 2 h (BFA/25-OH). B: cells either received no addition (ethanol solvent), BFA (1 μg/ml) for 75 min, 25-hydroxycholesterol (2.5 μg/ml) for 3 h, 25-hydroxycholesterol addition for 2 h prior to BFA addition for 75 min. SM synthesis was measured by [^{3}H]serine (7.5 μCi/ml) labeling for the final 1 h of each treatment. Results are the mean and standard deviation for three experiments. *$P < 0.025$ versus mock transfected cells.

cells (3.5- to 4-fold, **Figs. 4A** and **4B**), and the magnitude of increase in SM labeling was similar to that afforded by 25-hydroxycholesterol in overexpressing cells. When cells were treated with 25-hydroxycholesterol after pretreatment with BFA (Fig. 4A), SM synthesis in control and overexpressing cells was similar. The effect of BFA and 25-hydroxycholesterol was additive only for the mock transfected cells, but synthesis was not significantly different from values for BFA alone. Under conditions where cells were pretreated with 25-hydroxycholesterol followed by BFA, SM synthesis was greater in CHO-OSBP cells by 40% (Fig. 4B).

Stimulation of SM synthesis by 25-hydroxycholesterol could also affect ceramide and diglyceride (substrate and product of SM synthase, respectively), particularly in the Golgi apparatus (37). We examined whether 25-hydroxycholesterol and OSBP overexpression resulted in changes in diglyceride and ceramide levels in the Golgi-enriched fraction of cells cultured in 5% FCS or LPDS, with or without 25-hydroxycholesterol for 4 h (**Table 3**). Increased SM synthesis in 25-hydroxycholesterol-treated CHO-OSBP and mock cells paralleled a consistent 10–25% reduction

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TABLE 3. Diglyceride and ceramide content of PNS and Golgi-enriched fractions from mock and OSBP transfected CHO-K1 cells

Cell line	Post-Nuclear Supernatant		Golgi-Enriched Fraction	
	Ceramide	Diglyceride	Ceramide	Diglyceride
	pmol/mg protein			
In 5% FCS				
Mock-	0.54 ± 0.11	5.07 ± 1.01	1.88 ± 0.36	18.82 ± 3.32
Mock + 25-OH	0.42 ± 0.12	4.33 ± 0.82	1.70 ± 0.56	16.63 ± 1.19
CHO-OSBP	0.52 ± 0.10	5.39 ± 0.46	1.89 ± 0.62	17.00 ± 3.32
CHO-OSBP + 25-OH	0.40 ± 0.06	3.98 ± 0.64[a]	1.46 ± 0.44	14.31 ± 3.58
In 5% LPDS				
Mock	0.43 ± 0.22	7.92 ± 2.01	2.68 ± 0.32	42.11 ± 2.37
Mock + 25-OH	0.39 ± 0.20	5.97 ± 2.27	2.14 ± 0.33	29.77 ± 2.72[c]
CHO-OSBP	0.51 ± 0.23	8.92 ± 2.35	1.83 ± 0.30[e]	30.74 ± 4.01[d]
CHO-OSBP + 25-OH	0.40 ± 0.24	6.40 ± 2.24	1.45 ± 0.11[b]	24.11 ± 5.86

Post-nuclear supernatant and Golgi-enriched fractions were isolated from mock transfected and overexpressing cells cultured in DMEM with 5% FCS or 5% LPDS with or without 25-hydroxycholesterol (2.5 μg/ml) for 4 h. Ceramide and diglyceride mass was measured as described in Materials and Methods. Results are the mean and standard deviation of three or five experiments.

[a] $P < 0.005$ versus untreated CHO-OSBP cells.

[b] $P < 0.05$ versus 25-hydroxycholesterol treated mock cells.

[c] $P < 0.005$; [d] $P < 0.025$; [e] $P < 0.05$ versus untreated mock cells.

in ceramide mass in PNS and Golgi-enriched fractions, but only in CHO-OSBP cells grown in LPDS was this significnat. Similarily, diglyceride levels were also reduced, but only significantly for mock cells grown in LPDS. In addition to effects related to oxysterol treatment, the diglyceride and ceramide content of the Golgi fraction from untreated CHO-OSBP cells was significantly reduced compared to mock cells grown in LPDS. These differences were not evident in cells cultured in FCS where we observed the major effects of 25-hydroxycholesterol and OSBP expression on SM synthesis (Fig. 3).

DISCUSSION

Our previous finding that 25-hydroxycholesterol stimulated SM synthesis has been extended to show that overexpression of the high affinity oxysterol receptor, OSBP, enhanced this response. 25-Hydroxycholesterol treatment of cell lines overexpressing OSBP resulted in increased SM synthesis relative to controls, but had little effect on [^{3}H]serine incorporation into GluCer, PtdSer, PtdEtn, or ceramide. The relative lack of effect of 25-hydroxycholesterol or OSBP overexpression on GluCer synthesis is probably the result of localization of GlcCer synthase to heavy *cis*/*medial* elements and vesicle fractions (38) or distal Golgi elements (39), which are relatively devoid of SM synthase activity. Also, GluCer synthase and SM synthase active sites are localized to the cytoplasmic and luminal surfaces of membranes (38, 39), respectively, suggesting that they are exposed to different ceramide pools. Thus, 25-hydroxycholesterol/OSBP could enhance ceramide availability to SM synthase with minimal stimulation of GluCer synthesis.

The CHO-OSBP cells used in this study also displayed alterations in cholesterol and cholesteryl ester synthesis and regulation (26). CHO-OSBP cells grown in the absence of 25-hydroxycholesterol had increased cholesterol synthesis and mRNA for sterol-regulated genes, reduced ACAT mRNA and activity, and a normal or slightly enhanced suppression of mRNA and cholesterol synthesis by 25-hydroxycholesterol. Thus, effects of OSBP overexpression on cholesterol and SM synthesis are manifested under different conditions; SM synthesis was stimulated by 25-hydroxycholesterol, while cholesterol synthesis was elevated in the absence of oxysterol. The following scenario could explain this metabolic relationship of OSBP and 25-hydroxycholesterol to SM and cholesterol metabolism. When cells are treated with exogenous 25-hydroxycholesterol, a signal to the cell of increased cholesterol loading, cholesterol synthesis and uptake via the LDL receptor is down-regulated. At the same time there is a compensatory increase in SM synthesis and mass (20), a response that is enhanced when OSBP is overexpressed. Presumably, SM is increased in order to maintain appropriate cholesterol/SM ratio in membranes and prevent cytotoxic effects of a expanded cholesterol pool. In untreated CHO-OSBP cells grown in LPDS, both SM and cholesterol synthesis were elevated relative to controls. In this case we cannot say with certainty whether the primary effect of overexpression is on SM or cholesterol synthesis. However, increased cholesterol synthesis in these cells could produce a sterol intermediate that binds OSBP and activates SM synthesis. OSBP may not be the only receptor involved because at least two other OSBP-related proteins with 40–60% amino acid identity are present in cDNA data bases (ref. 40 and N.D. Ridgway, unpublished results). Whether these homologues bind oxysterols or interact with OSBP is unknown.

A more complete explanation of the effect of 25-hydroxycholesterol and lipoproteins on SM synthesis requires that we consider the intracellular localization of OSBP. We recently found that endogenous OSBP in CHO-K1 cells cultured in FCS or LDL-supplemented LPDS is primarily cytosolic or in small vesicles (41). Under these

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conditions 25-hydroxycholesterol caused OSBP translocation to the Golgi apparatus. However, OSBP in cells cultured in LPDS was associated with the Golgi apparatus, and 25-hydroxycholesterol had no further effect on localization (41). The most plausible interpretation is that cells cultured in LPDS actively synthesize cholesterol, and a biosynthetic intermediate in the pathway binds to OSBP and promotes translocation to the Golgi apparatus. Under this condition, basal SM synthesis is elevated and poorly stimulated by exogenous 25-hydroxycholesterol (ref. 20, Fig. 3). In cells cultured in LDL or FCS, cholesterol biosynthesis is suppressed, the endogenous OSBP ligand is not produced, and OSBP is cytosolic. In this situation, basal SM synthesis is reduced but fully activated by exogenous 25-hydroxycholesterol (ref. 20, Fig. 3). Thus it appears that OSBP stimulation of SM synthesis requires active cholesterol synthesis and is dependent on translocation to the Golgi apparatus. Alternative possibilities are that OSBP localization and effects on SM synthesis are: *i*) differentially regulated by cholesterol (or a cholesterol product) derived from de novo synthesis or LDL, or *ii*) effects are related to cholesterol content of specific cellular compartments such as the Golgi apparatus. Furthermore, care should be taken when interpreting results of [^{3}H]serine incorporation experiments in cells cultured in LDL, as products of LDL-SM catabolism could cause significant isotope dilution of [^{3}H]serine-labeled intermediates. This could explain inhibition of basal [^{3}H]serine incorporation into SM in FCS compared to LPDS (16, 17), but not the robust activation of SM synthesis by 25-hydroxycholesterol in FCS.

Oxysterols and OSBP could mediate their effects on SM and cholesterol metabolism by regulating movement through the Golgi/vesicular pathway, as this is a site for SM synthesis (21–23) and a delivery route for sterols (42, 43) to the plasma membrane. OSBP overexpression and 25-hydroxycholesterol stimulated ceramide conversion to SM, but a lack of effect on the in vitro activity of SM biosynthetic enzymes implied that substrate presentation or product removal was involved (20). BFA, an agent that promotes Golgi and ER mixing, stimulated SM synthesis by abrogating the ceramide transport step from ER to Golgi (35, 36). BFA and 25-hydroxycholesterol caused similar activation of SM synthesis in CHO-OSBP cells, but stimulation was not additive in cells treated with both agents. It could be argued that either BFA and 25-hydroxycholesterol had a similar mechanism of action or SM synthesis had reached a maximum. Contrary to this, SM synthesis in mock transfected cells pretreated with BFA followed by 25-hydroxycholesterol was additive. It appears that in control cells, BFA pretreatment enhanced the effects of 25-hydroxycholesterol, perhaps by positioning SM synthase close to ceramide pools in the ER prior to activation by oxysterol. Consistent with this interpretation, significantly lower SM synthesis was observed in control cells pretreated with 25-hydroxycholesterol prior to BFA. This implies that once 25-hydroxycholesterol stimulated ceramide conversion to SM, the effect of BFA become redundant.

Analysis of Golgi-enriched fractions from control and overexpressing cells revealed few significant differences in ceramide mass, indicating that increased SM synthesis did not affect substrate levels in this organelle. There are several reasons why changes may not have been apparent: *i*) mechanisms may exist that conserve Golgi ceramide levels because of its potential importance in the function of this organelle (44–46), *ii*) rapid consumption of ceramide by the SM synthase at the Golgi complex precluded any accumulation, or *iii*) the contribution to changes in the ceramide and diglyceride pool in the Golgi apparatus from SM synthase could be minor and below the level of detection. It is also possible that our isolation technique was not sufficiently rapid to conserve or maintain the lipid composition of an organelle subject to enormous changes in lipid flux.

If 25-hydroxycholesterol and OSBP overexpression enhanced conversion of ceramide to SM at the Golgi apparatus, we would predict diglyceride accumulation. Instead, diglyceride was slightly reduced in PNS and Golgi fractions. Because of its important role in vesicular transport in the Golgi complex (47, 48), diglyceride is likely regulated within a narrow range and mass contributions from SM synthesis may not be obvious. It is feasible that 25-hydroxycholesterol and OSBP could mediate the removal of diglyceride from the Golgi apparatus thus alleviating product inhibition of SM synthase (49). However, this was not directly correlated with stimulation of SM synthesis as the largest reduction of diglyceride was observed in Golgi membranes of cells grown in LPDS.

In summary, our results from overexpressing cells demonstrate that 25-hydroxycholesterol stimulation of SM synthesis is mediated by OSBP. Stimulation of SM synthesis was correlated with active de novo cholesterol synthesis and OSBP localization to the Golgi apparatus.

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