Sphingomyelin depletion in cultured cells blocks proteolysis of sterol regulatory element binding proteins at site 1
(neutral sphingomyelinase/cholesterol biosynthesis/cholesteryl ester formation/endoplasmic reticulum/transcription)

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ABSTRACT The current studies explore the mechanism by which the sphingomyelin content of mammalian cells regulates transcription of genes encoding enzymes of cholesterol synthesis. Previous studies by others have shown that depletion of sphingomyelin by treatment with neutral sphingomyelinase causes a fraction of cellular cholesterol to translocate from the plasma membrane to the endoplasmic reticulum where it expands a regulatory pool that leads to down-regulation of cholesterol synthesis and up-regulation of cholesterol esterification. Here we show that sphingomyelinase treatment of cultured Chinese hamster ovary cells prevents the nuclear entry of sterol regulatory element binding protein-2 (SREBP-2), a membrane-bound transcription factor required for transcription of several genes involved in the biosynthesis and uptake of cholesterol. Nuclear entry is blocked because sphingomyelinase treatment inhibits the proteolytic cleavage of SREBP-2 at site 1, thereby preventing release of the active NH2-terminal fragments from cell membranes. Sphingomyelinase treatment thus mimics the inhibitory effect on SREBP processing that occurs when exogenous sterols are added to cells. Sphingomyelinase treatment did not block site 1 proteolysis of SREBP-2 in 25-RA cells, a line of Chinese hamster ovary cells that is resistant to the suppressive effects of sterols, owing to an activating point mutation in the gene encoding SREBP cleavage-activating protein. In 25-RA cells, sphingomyelinase treatment also failed to down-regulate the mRNA for 3-hydroxy-3-methylglutaryl CoA synthase, a cholesterol biosynthetic enzyme whose transcription depends on the cleavage of SREBPs. Considered together with previous data, the current results indicate that cells regulate the balance between cholesterol and sphingomyelin content by regulating the proteolytic cleavage of SREBPs.

Interactions between cholesterol and sphingomyelin are important in regulating cholesterol homeostasis in animal cells. Membranes such as the plasma membrane that are enriched in cholesterol are also enriched in sphingomyelin. This is especially true in caveolae, which are “rafts” in the plasma membrane that are highly enriched in cholesterol and sphingomyelin (1–3). Whenever sphingomyelin overaccumulates in cells, sphingomyelinase treatment causes a measurable amount of plasma membrane cholesterol to translocate into intracellular membranes, where it expands the regulatory pool that down-regulates HMG CoA reductase and up-regulates the acyl-CoA cholesterol acyltransferase (ACAT) enzyme that esterifies cholesterol. Lange and Steck (8) confirmed and extended these findings, showing directly that sphingomyelinase treatment of intact cells increases the pool of endoplasmic reticulum (ER) cholesterol that is accessible to ACAT. The mechanism by which an expanded ER cholesterol pool down-regulates cholesterol biosynthesis and uptake is beginning to be elucidated.

Recently, our laboratory described a family of ER proteins, designated sterol regulatory element binding proteins (SREBPs) that play integral roles in the feedback pathway by which cholesterol suppresses transcription of genes encoding HMG CoA reductase and other enzymes of cholesterol biosynthesis as well as the low density lipoprotein (LDL) receptor (reviewed in ref. 11). The SREBPs are transcription factors of ~1,150 amino acids in length that are bound to the ER and nuclear envelope by virtue of two membrane spanning regions. When these membranes are depleted of sterols, a two-step proteolytic process releases the NH2-terminal segments of the SREBPs, which then travel to the nucleus and activate transcription of multiple genes in the cholesterol biosynthetic pathway and the gene for the low density lipoprotein receptor (11). The first cleavage of SREBPs occurs at site 1, which is in the lumen of the ER. This generates a membrane-bound intermediate that is then cleaved at site 2, which is in the first transmembrane segment. This cleavage generates soluble NH2-terminal fragments that migrate on SDS/PAGE with apparent molecular masses of ~68 kDa and are designated as the mature forms of SREBPs. Under certain conditions the mature SREBPs also stimulate transcription of genes encoding enzymes in the pathway of fatty acid biosynthesis such as acetyl CoA carboxylase and fatty acid synthase (11–13). When cells are overloaded with sterols, such as by incubation with low density lipoprotein or with 25-hydroxycholesterol, cleavage of SREBPs at site 1 is inhibited. The proteins remain bound to membranes, and transcription of the target genes declines (11).

Abbreviations: ACAT, acyl-CoA cholesterol acyltransferase; CHO cells, Chinese hamster ovary cells; ER, endoplasmic reticulum; HMG CoA, 3-hydroxy-3-methylglutaryl CoA; SREBP, sterol regulatory element binding protein; SCAP, SREBP cleavage-activating protein.
Recent evidence indicates that sterols alter site 1 proteolysis by interacting with another protein of the ER designated SREBP Cleavage-Activating Protein (SCAP) (11, 14). SCAP is a polytopic membrane protein with up to eight membrane-spanning regions followed by a COOH-terminal domain consisting of “WD” repeats. Gain-of-function point mutations in the membranous domain of SCAP enhance SREBP cleavage and prevent sterols from suppressing proteolysis of SREBPs (14, 15). For this reason, SCAP is proposed to act as the sterol sensor in the pathway by which sterols achieve feedback regulation of gene transcription (11).

In the current studies we have attempted to unite the observations on SREBPs and the observations on the regulatory effects of sphingomyelinase treatment of cultured cells. The data demonstrate that sphingomyelinase treatment reduces the amounts of the mature NH2-terminal fragments of SREBPs that are found in the cell nucleus, apparently by reducing the proteolytic cleavage of SREBPs. Inasmuch as sphingomyelinase treatment is already known to elevate the cholesterol content of ER membranes, these studies provide further evidence that the cleavage of SREBPs is regulated by ER cholesterol and that inhibition of SREBP cleavage is responsible for the inhibition of transcription produced by sphingomyelinase treatment of animal cells.

**MATERIALS AND METHODS**

**Materials.** We obtained neutral sphingomyelinase (*Staphylococcus aureus*) from Sigma (catalog no. S-8633); compound 58-035 from Sandoz; N-acetyl-leucyl-leucyl-norleucinal from Calbiochem; and other protease inhibitors from Boehringer Mannheim. Newborn calf lipoprotein-deficient serum (d > 1.215 g/ml) was prepared by ultracentrifugation (16). Protein concentrations were determined using the BCA protein assay reagent (Pierce) with BSA as a standard.

**Cell Culture and Cell Fractionation.** Stock cultures of CHO-7 cells (17) and 25-RA cells (18) (kindly provided by T. Y. Chang, Dartmouth Medical School, Hanover, NH) were grown in monolayer culture at 37°C in an atmosphere of 8–9% CO2 as described (14). On day 0, cells were set up at a density of 7 × 105 cells per 100-mm dish in medium A (1:1 mixture of Ham’s F-12 medium and DMEM (GIBCO/BRL) containing 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 5% (vol/vol) newborn calf lipoprotein-deficient serum). On day 2, the cells were washed twice with 5 ml of PBS and once with medium B (1:1 mixture of Ham’s F-12 medium and DMEM) followed by the addition of 5 ml of medium B supplemented with various additions as indicated in the legends. After incubation for the indicated time, the cells were washed once with 4 ml of ice-cold PBS and then resuspended in 2 ml of PBS containing a mixture of protease inhibitors (0.1 mM Pefabloc/2 μg/ml aprotinin/10 μg/ml leupeptin/5 μg/ml pepstatin A/25 μg/ml N-acetyl-leucyl-leucyl-norleucinal). The cells from three dishes were pooled, collected by centrifugation at 800 × g for 10 min at 4°C, and incubated for 15 min on ice in 0.45 ml of buffer B (19). A nuclear extract and membrane fraction (105 × g pellet) were prepared as described (20) except that the nuclei were extracted for 1 h at 4°C and then centrifuged for 30 min at 105 × g.

**Immunoblot Analysis.** Aliquots of the nuclear extract and membrane fractions were solubilized in SDS loading buffer (21) and then subjected to SDS/PAGE on 7% gels and transferred to Hybond-C extra nitrocellulose (Amersham). Gels were calibrated with prestained molecular weight markers (Bio-Rad). Immunodetection was carried out with 5 μg/ml IgG-7D4, a mouse mAb directed against the NH2-terminus of hamster SREBP-2 (amino acids 32–250) (ref. 22). Bound antibody was visualized either by incubation with peroxidase-conjugated sheep anti-mouse IgG (Amersham) using the Enhanced Chemiluminescence Western Blotting Detection kit (Amersham) as previously described (20) or by incubation with 4 × 106 cpm/ml of [32P]-labeled sheep anti-mouse IgG (Amersham). The filters were exposed for the indicated time, respectively, to Reflection film (DuPont/NEB) at room temperature or to X-Omat AR film (Kodak) with an intensifying screen at −70°C.

**RNA Blot Analysis.** Total RNA was prepared from CHO-7 cells using RNA STAT-60 (TEL-Test “B,” Friendswood, TX). For Northern gel analysis, aliquots of total RNA (20 μg) were denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose/0.6% formaldehyde gel, and transferred to Hybond-N+ nitrocellulose membranes (Amersham) for hybridization. A 32P-labeled cDNA probe for hamster HMG Co synthase (nucleotides 271–910) (ref. 23) was prepared with Prime-It II Random Primer Labeling kit (Stratagene). The filters were hybridized with the 32P-labeled probe (2 × 106 cpm/ml) for 2 h at 65°C using Rapid-hyb buffer (Amersham), washed twice with 0.1% (wt/vol) SDS/0.1× standard saline citrate (0.15 M sodium chloride/0.015 M sodium citrate, pH 7) at 65°C for 20 min, and exposed to X-Omat AR film (Kodak) with an intensifying screen at −70°C. For quantification, the dried filters were analyzed with a Bio-Imaging analyzer using MacBAS software (Fuji) and a Bas 1000 Fuji PhotoImager screen. The results were normalized with regard to the signal generated from glyceraldehyde-3-phosphate dehydrogenase mRNA (obtained from the same filter). Films for glyceraldehyde-3-phosphate dehydrogenase were exposed at −70°C with an intensifying screen.

**Analysis of [3H]Serine-Labeled Lipids.** CHO-7 cells were set up as described above at a density of 5 × 105 cells per 100-mm dish. On day 1, cells received medium A containing 25 μCi/ml (1 Ci = 37 GBq) L-[3-3H]serine (Amersham). On day 3, cells were washed three times with PBS and three times with medium A. After further incubation at 37°C for 1 h in medium A, the cells were washed twice with PBS and once with medium B. We then added 5 ml of medium B supplemented with various additions as indicated in the legends. After treatment for the indicated time, the cells were washed once with 5 ml ice-cold PBS and then resuspended in 0.2 ml chloroform/methanol (5:4, vol/vol) per dish. Each dish was rinsed with 0.5 ml methanol/water. Cells from duplicate dishes were combined, and 5 ml of chloroform/methanol (1:2, vol/vol) was added. After vigorous shaking, 4 ml of 0.58% NaCl was added, and the extracts were centrifuged for 5 min at 2,500 × g. The organic phase was transferred twice with 2 ml of 0.58% NaCl/methanol/chloroform (47:45:3, vol/vol/vol), dried under nitrogen, and resuspended in 0.2 ml chloroform/methanol (1:1, vol/vol). Aliquots of each sample (0.1 ml) were separated by thin-layer chromatography on Polygram Sil G plates (Macherey & Nagel) in chloroform/methanol/water (65:25:4, vol/vol/vol), and the labeled lipids were visualized by exposure to Kodak BioMax MS film using a BioMax TranScan-LE intensifying screen for 14 h at −70°C. Radioactivity in sphingomyelin and ceramide bands was quantified by scintillation counting and normalized with regard to the recovery of [N-methyl-14C]sphingomyelin (Amersham), which was added to the cell suspension before the chloroform/methanol extraction.

**RESULTS AND DISCUSSION**

The current experiments were designed to test the hypothesis that sphingomyelinase treatment, which is known to cause cholesterol to translocate to the ER, would reduce the proteolytic cleavage of SREBPs, thereby depriving the mature NH2-terminal fragments from the nucleus. In designing the experiments, we took into consideration the previous observation that the cholesterol that translocates to the ER undergoes esterification, thereby removing it from the regulatory pool. We therefore performed our experiments in the presence of compound 58-035, an inhibitor of ACAT (24, 25), which
should prevent esterification, thereby increasing the buildup of free cholesterol.

Fig. 1 shows an experiment in which CHO cells were incubated with varying concentrations of sphingomyelinase for 90 min in the absence or presence of compound 58–035. Nuclear extracts and membrane fractions were subjected to electrophoresis and blotted with an antibody against the NH2-terminal segment of SREBP-2, one of the two isoforms of SREBP that are produced in CHO cells. Fig. 1A, shows that in the presence of compound 58–035 sphingomyelinase treatment reduced the amount of mature SREBP-2 in nuclear extracts without affecting the amount of the SREBP-2 precursor in cell membranes. Fig. 1B shows that sphingomyelinase treatment depleted the mature nuclear form of SREBP-2, even in the absence of the ACAT inhibitor (lane 10). However, the effect was greater when the ACAT inhibitor was present (lane 10). Even though the ACAT inhibitor was not necessary to observe the effect of sphingomyelinase, we found that the results were more consistent when the ACAT inhibitor was present. For this reason, in subsequent experiments we included the ACAT inhibitor along with sphingomyelinase.

In the experiment of Fig. 1 and in all other experiments with sphingomyelinase, we never observed that sphingomyelinase caused a buildup of the intermediate form of SREBP in cell membranes (data not shown). These findings indicate that sphingomyelinase treatment blocks cleavage of SREBPs at site 1, which is the same step that is blocked when sterols accumulate in cells.

Fig. 2 shows a time course of treatment with sphingomyelinase at 100 milliunits/ml. After sphingomyelinase treatment, mature SREBP-2 declined noticeably after 30 min and decreased further through 150 min of incubation.

The mature nuclear forms of SREBPs are required to maintain high level transcription of genes encoding several enzymes in the cholesterol biosynthetic pathway, including HMG CoA synthase (11). In wild-type CHO cells, sterols suppress cleavage of SREBPs at site 1, and this interrupts transcription of the gene for HMG CoA synthase and genes encoding several other proteins required for cholesterol synthesis and low density lipoprotein uptake. Sterols do not suppress SREBP cleavage or gene transcription in 25-RA cells, a line of CHO cells with a D443N mutation in SCAP (14). In the experiment of Fig. 3, treatment of wild-type CHO cells with sphingomyelinase blocked processing of SREBP-2 as expected, and this led to a 90% fall in the level of mRNA for HMG CoA synthase (lanes 1 and 2). In the mutant 25-RA cells, sphingomyelinase treatment did not significantly block processing of SREBPs nor did it produce a significant decline in the HMG CoA synthase mRNA (lanes 3 and 4). The resistance of 25-RA cells to sphingomyelinase strongly supports the notion that sphingomyelinase inhibits processing of SREBPs by increasing the regulatory pool of cholesterol in ER membranes.

The experiment of Fig. 4 was conducted to determine whether the effect of sphingomyelinase is reversible. CHO cells were incubated for 48 h with 1[3H]serine, which was incorporated into various lipids. The cells were then treated with sphingomyelinase for 2 h in the presence of the ACAT inhibitor, and the radioactive lipids were analyzed by thin layer chromatography and autoradiography. Sphingomyelinase treatment caused a 70% drop in the content of sphingomyelin, which migrated as two bands on the thin layer sheets (Fig. 4A, 0 h as compared with untreated cells). There was a corre-
The filters were exposed to film for 15 s. PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

The conclusion that sphingomyelinase treatment of cells mediate in part by a reduction in the activity of HMG CoA reductase (5–10). The current results demonstrate that sphingomyelinase treatment also reduces the mature nuclear form of SREBP-2 and that this is accompanied by a fall in the cholesterol content of the ER, thereby inhibiting the proteolytic processing of SREBPs. The current paper describes measurements only of SREBP-2, largely because our antibodies against hamster SREBP-1 cannot reliably detect endogenous levels of this protein on immunoblots of nontransfected cells. However, we have repeated this crucial experiment in human fibroblasts and have observed that sphingomyelinase treatment down-regulates the processing of both SREBP-1 and SREBP-2 (data not shown).

The conclusion that sphingomyelinase treatment of cells inhibits proteolytic cleavage of membrane-bound SREBP-2 at
site 1 is based on previous observations (11, 26) that show that increasing the sterol content of cells inhibits this proteolytic reaction. This conclusion is supported by the experiment of Fig. 3 and other similar experiments (not shown), which demonstrate that this inhibition does not occur in sterol-resistant 25-RA cells.

Considered together, all of the data on sphingomyelinase treatment indicate that cells use the SREBP pathway to help achieve an optimal ratio of cholesterol to sphingomyelin. The current studies show that sphingomyelin depletion inhibits SREBP processing and thereby decreases cholesterol synthesis and uptake. It seems likely that the converse is also true, i.e., sphingomyelin excess sequesters cholesterol and up-regulates SREBP cleavage and hence cholesterol synthesis. If so, these findings would explain the cholesterol overaccumulation that occurs when sphingomyelin accumulates in cells as a result of sphingomyelinase deficiency (4).

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