Unsaturated Fatty Acid-mediated Decreases in Sterol Regulatory Element-mediated Gene Transcription Are Linked to Cellular Sphingolipid Metabolism*

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A major physiological feedback mechanism of cholesterol in transcription of a number of lipid metabolism-related genes is mediated by sterol regulatory elements (SREs) and their binding proteins (SREBPs). Polyunsaturated free fatty acids alone, as well as synergistically with sterols, decrease SRE-mediated gene expression up to 80% in a dose-dependent manner by decreasing levels of the active transcription factor SREBP. We investigated potential mechanisms for this effect. We hypothesized that free fatty acids reduce SREBP-mediated gene transcription by increasing intracellular cholesterol content through the hydrolysis of cellular sphingomyelin, which has a high affinity for free cholesterol. We also questioned whether the lipid second messenger ceramide, a product of sphingomyelin hydrolysis, can decrease SRE-mediated gene transcription. First we investigated the effect of fatty acids on sphingomyelin hydrolysis. Incubation of [3H]choline-labeled cells with unsaturated (but not saturated) fatty acids induced hydrolysis of [3H]choline-labeled sphingomyelin. Also, incubation of cell extracts from fatty acid-treated cells with [3H]sphingomyelin increased generation of [3H]ceramide compared with control cells in vitro. We found that addition of ceramide analogs alone and additively with fatty acids decreased SRE expression and that ceramide analogs reduced levels of the transcriptionally active forms of SREBP-1 and SREBP-2. Increasing intracellular ceramide levels by exogenous sphingomyelinase or inhibition of ceramidase decreased SRE-mediated gene expression. None of the above conditions induced apoptosis. Incubation with U18666A, a compound that inhibits intracellular cholesterol movement, increased SRE-mediated gene transcription. C2-ceramide abrogated the effect of U18666A on SRE-mediated gene transcription, suggesting cholesterol-independent regulation of SREBP. We provide evidence that sphingomyelin hydrolysis and intermediates of sphingomyelin metabolism (in addition to cholesterol and fatty acids) contribute to regulation of SRE-mediated gene transcription.

Fatty acids and cholesterol interact at a number of metabolic levels. Both contribute to the composition of circulating lipoproteins and cellular membranes. Clinically, higher intakes of polyunsaturated free fatty acids are linked to lower levels of low density lipoprotein cholesterol, whereas higher intakes of saturated and trans-fatty acids correlate with increased low density lipoprotein cholesterol and decreased high density lipoprotein cholesterol as well as increased risk for cardiovascular disease (1). At the cellular level, cholesterol and fatty acids are linked to the regulation of intracellular cholesterol levels by activation of acyl-CoA:cholesterol acyltransferase (2, 3). Of relevant interest, genes regulating both cholesterol and fatty acid metabolism contain sterol regulatory elements (SREs)1 in their promoter regions. Still, little information is available on possible interactions between fatty acids and cholesterol in the regulation of gene transcription.

SRE-binding proteins (SREBPs) are transcription factors that are post-transcriptionally regulated. SREBP in its precursor form is located in the endoplasmic reticulum, where it is bound at the C-terminal end to the SREBP cleavage-activating protein. In sterol depletion, both proteins are translocated to the Golgi apparatus (4, 5). Sequential cleavage by two proteases, site-1 protease and site-2 protease, releases transcriptionally active mature SREBP (mSREBP). In the nucleus, mSREBP binds to SREs, cis-acting elements in the promoters of genes of cholesterol and fatty acid synthesis (6). Cholesterol exerts a negative feedback mechanism on its own synthesis by inhibiting the proteolytic cleavage of SREBP by site-1 protease.

SREBP-mediated regulation has been demonstrated for a number of key enzymes in the synthesis of polyunsaturated fatty acids (7). We (9) and others (8, 10) have shown that mono- and polyunsaturated fatty acids decrease SRE-mediated gene transcription by decreasing the amount of transcriptionally active mSREBP and that fatty acids exert this effect alone and synergistically with cholesterol. Fatty acid-mediated regulation of SREBP has also been demonstrated to occur at the transcriptional (8, 11, 12) and post-transcriptional (10) levels. Our earlier data showed that the effect of fatty acids is dependent on changing SREBP cleavage. Fatty acids do not affect the mSREBP protein once it has been cleaved from the precursor protein. For example, fatty acids did not reduce SRE-mediated gene expression in mutant cells overexpressing the mSREBP protein (9).

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1 The abbreviations used are: SRE, sterol regulatory element; SREBP, SRE-binding protein; mSREBP, mature SREBP; CHO, Chinese hamster ovary; BSA, bovine serum albumin; d-MAPP, (1S,2R)-(−)-erythro-2-(N-myristoylamo)-1-phenyl-1-propanol; HMG-CoA synthase, hydroxymethylglutaryl-CoA synthase.
In this study, we tested the hypothesis that one contributing pathway whereby fatty acids modulate intracellular cholesterol movement is by affecting cellular sphingomyelin. Sphingomyelin, a major plasma membrane component, has a higher affinity for free cholesterol than for other phospholipids (13–15). Sphingomyelinase, an enzyme that hydrolyzes sphingomyelin, has been shown to be activated by fatty acids (16, 17). We anticipated, as shown by others (18–20), that decreasing plasma membrane sphingomyelin content would induce more free cholesterol to partition to other intracellular membranes (e.g. the endoplasmic reticulum), resulting in decreased SREBP cleavage. Our data support this hypothesis. Furthermore, we found that a product of sphingomyelin hydrolysis, ceramide, independently and reversibly decreases SRE expression.

Our data provide new evidence for sterol- and non-sterol-related effects of fatty acids on the maturation cascade of SREBP.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Choline (75 Ci/mmol) was purchased from ICN Biochemicals, Inc. (Irvine, CA). [3H]Sphingomyelin (N-palmitoyl) was obtained from Dupontoma, Ltd. (Maidenhead, England) and was synthesized as previously described (21). It contains the 3H label on the palmitic acid, allowing identification of ceramide obtained after sphingomyelin hydrolysis. Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Manassas, VA). Sphingomyelinase (Bacillus cereus), ethanol, oleate, stearate, arachidonic acid, fatty acid-free bovine serum albumin (BSA), cholesterol, 25-hydroxycholesterol, and U18666A (3-β-(2-diethylaminoethoxy)androsten-5-en-17-one) were obtained from Sigma. (1S,2R)-N-erythro-2-(N-myristoyl)-1-phenyl-1-propanol (n-MAPP), C12-ceramide (N-erythro-N-acetylphosphogamine), C16-ceramide (N-erythro-N-octanoylphosphogamine), and C16-dihydroceramide (N-erythro-N-hexanoyldihydrophosphogamine) were obtained from BIOSOL Research Labs Inc. (Plymouth Meeting, PA). All cell culture reagents, LipofectAMINE, and neomycin (G418) were obtained from Invitrogen. All organic solvents were purchased from Fisher (Springfield, NJ).

**Plasmids**—The pSyn-SRE plasmid contains a generic TATA-box and three SREs (bp 326 to 235) of the hamster hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) promoter fused to the luciferase pGL2-Basic vector (Promega). Madin-Darby canine kidney cells (MDCK) were cultured in medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (150 μg/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B). Cells were plated on day 1 in regular growth medium (2% FBS, 1% CoA synthase (HMG-CoA synthase) promoter fused to the luciferase

**3 weeks.** Pooled clones were analyzed for luciferase expression. Experiments were repeated in at least 2 independent experiments with similar results.

**Protein Determination and Cell Viability**—The amount of cell protein was determined by the Bio-Rad method and BSA was used as a standard. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide thiazolyl blue test (22). In this assay, the ability of cells to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide to a soluble formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes is evaluated and used to measure cell viability, cell number, and cytotoxicity. The percentage of apoptotic cells was determined by flow cytometric analysis of isolated nuclei stained with propidium iodide using a method previously described (23). Cells were pelleted at 200 x g and fixed in 2 ml of cold 70% ethanol at 4 °C for 60 min. The cells were then centrifuged, washed with 1 ml of phosphate-buffered saline, and resuspended in 0.5 ml of phosphate-buffered saline. To a 0.5-ml cell sample was added 0.5 ml of RNase (1 mg/ml) and 10 μl of propidium iodide solution was added. The solution was kept at 4 °C in the dark until measured by FACS can flow cytometry (Becton Dickinson, San Jose, CA).

**Sphingomyelinase Assay**—The sphingomyelinase activity of cell extracts was measured as described (21). CHO cells were plated in 35-mm plates and incubated for 18 h with 0.3 mM oleate, 10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol, the control conditions containing 1% BSA, or 10 microunits/ml sphingomyelinase. After the incubation, cells were washed twice with ice-cold 0.25 μm sucrose and scraped into 0.3 ml of sucrose solution/35-mm plate. The scraped cells of two plates were pooled and disrupted by sonication on ice using three 5-s bursts (Branson Model 450 sonifier). An aliquot of 15 μg of total protein was pelleted at 50 000 x g for 30 min. The supernatant (0.25 ml) was added; vortexed; and spun at 8000 x g for 10 min. The upper phase was discarded, and the lower phase was washed with 3 x 2 ml of 0.3 M NaCl plus 1 mM HCl. Lipids were then dried under N2. Alkaline hydrolysis was performed to remove [3H]phosphatidylcholine. Dried lipid was incubated in 2 ml of 0.1 N KOH in methanol at 37 °C for 1 h. Lipids were then re-extracted by adding 2 ml of chloroform and 1.2 ml of balanced salt solution (135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM Heps, pH 7.2) containing 100 mM EDTA (1.08 mM). Lipids were re-extracted by adding 2 ml of chloroform and 0.5 ml of 1 N NaOH. The lower phase was dried under N2 (27, 30). The extracted lipids were then dissolved in 50 μl of chloroform/methanol (1:1), spotted onto TLC plates (Sigma Gel 60, Merck, Darmstadt, Germany), and chromatographed with chloroform, methanol, and 0.22% aqueous CaCl2 (60:35:8, v/v) (31). Ceramide and sphingomyelin (dissolved at 1 μg/ml) were run as standards. The lipids were identified according to their Rf values after visualization in an iodine vapor tank. The TLC plate was cut at the corresponding lipid spots, mixed with scintillation fluid (Ultima Gold, Packard Instrument Co.), and analyzed in a Wallac 1409 scintillation counter (Perkin Elmer Wallac, Gaithersburg, MD). Results are expressed in dpm/mg of protein as a percentage of total counts.

**Western Blot Analysis**—Cells were plated on day 1 in regular growth medium. On day 2, cells were incubated in control medium (1% fatty acid-free BSA) or under the respective conditions. Two hours before harvesting, all cells received 25 μg/ml N-acetyl-leucyl-leucyl-norleucinal to inhibit proteolysis of SREBP by the proteasome. After 8 h, cells were scraped and pelleted at 1000 x g. The pellet was resuspended in Western lysis buffer (100 μg/ml Tris-Cl, 100 mM NaCl, and 1% SDS, pH 7.6 containing protease inhibitors (Complete[trademark] Roche Molecular Biochemicals). Cells were lysed by sonication for 20 passes through a 22-gauge needle. An aliquot of each sample (30 μg of protein) was subjected to electrophoresis on a 7.5% denaturing SDS-polyacrylamide gel. The monoclonal antibodies against SREBP-1 and SREBP-2 (BD Biosciences, San Jose, CA) and peroxidase-labeled anti-mouse IgG (NIF 824; Amersham Biosciences, Inc.) were used for Western blot analysis according to the manufacturers’ instructions. Detection was performed with ECL detection system (Amersham Biosciences). Membrane was visualized by autoradiography. The bands were quantified using a Bio-Rad GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA).

**Free Fatty Acid Addition**—Fatty acids were dissolved in 100% ethanol and complexed to BSA at a molar ratio of 2:1 as described (22). Equal amounts of ethanol (<0.1% of the medium volume) were added under control conditions and had no effect on reporter gene and other assays.

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RESULTS

Fatty Acids Decrease Cellular Sphingomyelin in Cultured Cells—Most cellular cholesterol is located within the plasma membrane (14), where it has a high affinity for sphingomyelin (13, 32). We investigated the ability of different fatty acids to enhance hydrolysis of sphingomyelin, a mechanism that would promote the release of cholesterol from the plasma membrane.

CHO cells were incubated for 1 h with myristic acid, palmitate, stearate, oleate, or arachidonate. Before addition of fatty acids, the CHO cells were incubated with [3H]choline for 48 h to label cellular sphingomyelin. The results in Fig. 1 show that treatment with a 0.3 mM concentration of the unsaturated fatty acid oleate or arachidonate reduced the amount of [3H]choline-labeled sphingomyelin by one-third compared with that obtained under control conditions. Neither saturated fatty acids nor cholesterol plus 25-hydroxycholesterol decreased the amount of [3H]choline-labeled sphingomyelin. Treatment with sphingomyelinase (shown as a positive control) reduced [3H]choline-labeled sphingomyelin to about half of control values (Fig. 1).

Cell Extracts from Fatty Acid-treated Cells Have Increased Sphingomyelinase Activity—To examine the ability of oleate to increase sphingomyelin hydrolysis after longer incubations, an in vitro assay for sphingomyelinase activity was established. Cell extracts from cells treated for 18 h with 0.3 mM oleate were incubated for 1 h with [3H]sphingomyelin. The [3H]sphingomyelin that was used is labeled on the fatty acid moiety, allowing measurement of [3H]ceramide resulting from [3H]sphingomyelin hydrolysis. Cell extracts from oleate-treated cells converted one-half of the total sphingomyelin to ceramide compared with controls or cells incubated with cholesterol, which both converted only one-fourth of [3H]sphingomyelin to [3H]ceramide (Fig. 2).

Sphingomyelinase Decreases SRE-mediated Gene Transcription—It has been shown that short-term incubations (90–150 min) with exogenous sphingomyelinase decrease the amount of mSREBP protein (33). To examine the effect of sphingomyelin hydrolysis over a longer period of time, cells that stably express the SRE linked to a luciferase reporter gene were incubated in the presence of sphingomyelinase for 8 h. Sphingomyelinase decreased SRE-mediated gene transcription to one-third after 8 h. Incubation of sphingomyelinase together with 0.3 mM oleate did not further decrease SRE-mediated luciferase expression (Fig. 3).

Ceramide Decreases SRE-mediated Gene Transcription Alone and Additively with Fatty Acids—Sphingomyelin hydrolysis decreases the capacity to bind cholesterol within the plasma membrane (34–36) and results in the generation of phosphocholine and ceramide. Ceramide is a second messenger implicated in multiple cellular pathways such as cell growth, differentiation, regulation of transcription factors, and apoptosis (37, 38). By several lines of evidence, we determined that fatty acids or ceramide analogs did not induce apoptosis or cell death under our experimental conditions. First, the 3,4,5-trimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide mitochondrial uptake assay (23) showed no differences between...
control and treatment groups up to 24 h. Second, propidium iodide uptake of nuclei analyzed by FACScan (24) showed no difference between control and treatment groups (data not shown). Third, the effects of ceramide were reversible and did not affect expression of another reporter gene (see below). Fourth, protein levels were not decreased by ceramide treatment.

The effect of ceramide on SRE-mediated gene expression was investigated next. Fig. 4 shows that the ceramide analog C\textsubscript{2}-ceramide decreased SRE-mediated gene transcription by 5 h and that this effect was synergistic with oleate. Incubation for 16 h with 0.3 mM oleate alone caused the previously described decrease in SRE-mediated gene expression (9). After 16 h, the effect of ceramide alone on SRE expression apparently disappeared; but in the presence of 0.3 mM oleate, a substantial decrease persisted. Addition of fresh C\textsubscript{2}-ceramide at 8 h sustained the decrease in SRE-mediated reporter gene expression, suggesting instability of the compound over an extended period of time.

Because C\textsubscript{2}-ceramide can serve as a substrate for the synthesis of sphingomyelin, we also tested the effects of the longer chain C\textsubscript{6}- and C\textsubscript{8}-ceramides, which are not substrates for sphingomyelin synthesis. Incubation for 8 h with C\textsubscript{6}- or C\textsubscript{8}-ceramide similarly decreased SRE-mediated gene transcription to 46 ± 8 and 43 ± 7%, respectively.

We determined next whether the effect of ceramide analogs on SRE-mediated gene transcription is reversible. Cells were incubated with C\textsubscript{2}-ceramide for 5 h, sufficient time to substantially decrease SRE-mediated reporter gene transcription, as shown in Fig. 4. The medium was then changed to control medium (1% BSA) for 3 h for a total incubation time of 8 h. Fig. 5 shows that SRE-mediated gene transcription returned to control levels, indicating that the effects of ceramide are reversible.

We then determined the protein levels of SREBP at 8 and 16 h in the presence of C\textsubscript{2}-ceramide. The levels of mSREBP-1 and SREBP-2 decreased at 8 h (Fig. 6A). Parallel to the reporter gene assays (Fig. 4), mSREBP levels returned to control values at 16 h, in keeping with the concept of instability of ceramide over an extended period of time (Fig. 6B). This indicates that the effects of ceramide on decreasing mSREBP levels are also reversible.

It is noteworthy, that the effect of dihydroceramide analogs on SRE-mediated gene transcription were indistinguishable from those of ceramide analogs (Fig. 7A). Ceramide differs from dihydroceramide by the presence of a 4,5-trans-double bond in the sphingoid backbone; but unlike ceramides, which have a role in the induction of cell signaling of apoptosis, dihydroceramides do not induce apoptosis (39, 40). Addition of C\textsubscript{2}-ceramide analogs did not affect the expression of a β-galactosidase control reporter gene (Fig. 7B). These results further indicate that decreased SRE expression in response to ceramides is not related to apoptotic pathways or other "toxic" or irreversible pathways.

Increased Endogenous Ceramide Decreases SRE Transcription—Ceramide analogs might have different metabolic properties compared with endogenous cellular ceramide. Thus, we investigated the effect of increased endogenous ceramide levels on SRE-mediated gene transcription. Cells were incubated for 16 h in the presence or absence of 0.3 mM oleate with a 30 μM concentration of the alkaline ceramidase inhibitor D-MAPP (41, 42). It has been shown that D-MAPP increases cellular ceramide levels (41, 42). Incubation with D-MAPP reduced SRE-mediated gene transcription and HMG-CoA synthase mRNA levels by half compared with controls (Figs. 8 and 10). This effect was further enhanced in the presence of oleate (Fig. 8). Thus, increasing endogenous ceramide levels by oleate (Figs. 1 and 2), addition of exogenous sphingomyelinase (Fig. 3), addition of exogenous ceramides (Figs. 4–7), and inhibition of endogenous ceramide hydrolysis (Fig. 8) all have a similar effect on reducing SRE-mediated gene transcription.

Fatty Acids and Ceramide Decrease SRE Expression during Inhibition of Intracellular Cholesterol Transport—We previously reported that the effects of oleate on decreasing SRE-mediated gene transcription are dependent on cleavage of precursor SREBP within cells and thus are likely associated with changes in intracellular cholesterol trafficking (9). We next questioned whether the observed effects of ceramide are related to cholesterol transfer within the cell. Cholesterol regulates the activity of SREBP cleavage-activating protein and SREBP translocation to the Golgi apparatus, where a sterol-regulated protease initiates the release of the transcriptionally active mature form (4, 5). Because cholesterol partitions between the plasma membrane and other compartments within...
S.D. of two different experiments, each performed in triplicate.

or in the presence of C2-ceramide (20 μM). On day 2, cells were incubated for 8 h with 1% BSA (control) and mSREBP-1 and mSREBP-2 after 8 h. B, on day 1, CHO cells were plated in regular growth medium. On day 2, cells were incubated for 16 h with 1% BSA (control) or in the presence of C2-ceramide (20 μM). Whole cell extracts (30 μg of protein) were loaded on a 4–14% continuous gradient SDS-polyacrylamide gel. P and M denote the precursor (168 kDa) and mature (68 kDa) forms of SREBP-1, respectively, in a representative experiment. C2-ceramide decreased mSREBP-1 and mSREBP-2 after 8 h. B, on day 1, CHO cells were plated in regular growth medium. On day 2, cells were incubated for 16 h with 1% BSA (control) or in the presence of C2-ceramide (20 μM). Whole cell extracts (30 μg of protein) were loaded on a 4–14% continuous gradient SDS-polyacrylamide gel. P and M denote the precursor (168 kDa) and mature (68 kDa) forms of SREBP-2, respectively, in a representative experiment. At 16 h, there was no difference in the levels of mSREBP-1 in the presence of C2-ceramide compared with controls.

FIG. 5. Effect of C2-ceramide on SRE-mediated gene transcription is reversible. On day 1, CHO cells stably transfected with an SRE promoter construct linked to the luciferase reporter gene were plated at 80% confluency. On day 2, cells were incubated for 8 h in the presence of C2-ceramide (C2-Cer, 20 μM). In parallel, other cells were incubated for 5 h with C2-ceramide (20 μM), and the medium was then switched to medium containing 1% BSA for 3 h for the remainder of a total incubation time of 8 h. Cells were harvested, lysed, and analyzed for luciferase activity (measured in relative light units (RLU)) and protein content after 8 h. Data are expressed as a percentage of the control and represent the means ± S.D. of two different experiments, each performed in triplicate.

FIG. 6. C2-ceramide decreases cellular mSREBP-1 and SREBP-2 levels. A, on day 1, CHO cells were plated in regular growth medium. On day 2, cells were incubated for 8 h with 1% BSA (control) or in the presence of C2-ceramide (20 μM). Whole cell extracts (30 μg of protein) were loaded on a 4–14% continuous gradient SDS-polyacrylamide gel. P and M denote the precursor (168 kDa) and mature (68 kDa) forms of SREBP-1, respectively, in a representative experiment. C2-ceramide decreased mSREBP-1 and mSREBP-2 after 8 h. B, on day 1, CHO cells were plated in regular growth medium. On day 2, cells were incubated for 16 h with 1% BSA (control) or in the presence of C2-ceramide (20 μM). Whole cell extracts (30 μg of protein) were loaded on a 4–14% continuous gradient SDS-polyacrylamide gel. P and M denote the precursor (168 kDa) and mature (68 kDa) forms of SREBP-2, respectively, in a representative experiment. At 16 h, there was no difference in the levels of mSREBP-1 in the presence of C2-ceramide compared with controls.

We next examined whether the results obtained with SRE reporter gene assays reflect changes in the regulation of HMG-CoA synthase mRNA (Fig. 10), a gene known to be sensitively regulated by the SRE (46). C2-ceramide and n-MAPP decreased the amount of HMG-CoA synthase mRNA by half. Exogenous sphingomyelinase reduced the HMG-CoA synthase mRNA levels to 30% compared with controls. Thus, the effects of ceramide on SRE-mediated gene expression are reflected in the transcription of HMG-CoA synthase, a gene recognized to be closely regulated by the SRE (46).

DISCUSSION

We investigated potential mechanisms by which fatty acids can decrease SRE-mediated gene transcription. Previously, we have shown that fatty acids regulate SRE-mediated gene transcription by decreasing mSREBP in the absence of cholesterol as well as synergistically with cholesterol (9). We now demonstrate that fatty acids can affect SRE-mediated gene transcription by two interdependent mechanisms. Fatty acids can induce sphingomyelin hydrolysis, which can result in the intracellular displacement of membrane cholesterol. Sphingomyelin hydrolysis also results in the generation of ceramide, which can decrease levels of transcriptionally active mSREBP and SRE-mediated gene transcription.

We first investigated the hypothesis that unsaturated fatty acids decrease SRE-mediated gene expression by pathways that would lead to increased intracellular cholesterol levels. Cholesterol and sphingomyelin contents of cellular membranes are unequally distributed within the cell. Most cholesterol and sphingomyelin are found in the plasma membrane, whereas the endoplasmic reticulum contains much smaller amounts (14, 47, 48). Incubation with U18666A (2.5 μM) increases SRE-mediated reporter gene expression 2-fold and HMG-CoA synthase mRNA levels 1.7-fold, consistent with an inability of cellular cholesterol to reach and inhibit SREBP cleavage-activating protein regulatory compartment. Addition of ceramide almost completely abolished the increase in SRE-mediated gene transcription induced by U18666A (Fig. 9). Therefore, ceramide-mediated reduction of SRE-mediated gene transcription is in part independent of intracellular cholesterol movement.

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myelinase hydrolysis, whereas cell extracts from cholesterol-treated cells had no effect (Fig. 2). We then showed that exogenous sphingomyelinase resulted in persistently decreased SRE-mediated reporter gene expression and HMG-CoA reductase levels for up to 8 h (Fig. 3). High concentrations of bacterial sphingomyelinase have been demonstrated to reduce cellular levels of mSREBP within 30 min (33).

Sphingomyelin hydrolysis results not only in the release of cholesterol bound to sphingomyelin, but also in the generation of ceramide. Ceramide has multiple cellular roles, from induction of apoptosis to cell growth and differentiation (49). Experimentally, short chain ceramide and dihydroceramide analogs both promote cell growth and differentiation, but only ceramide analogs (not dihydroceramide analogs) can induce apoptosis (39, 40). Several lines of evidence indicate that the induction of apoptosis had no role in the regulation of SRE-mediated gene transcription investigated under our experimental conditions. 1) Ceramide analogs did not induce changes in the cell cycle or mitochondrial function under our experimental conditions. 2) The effect of ceramide analogs and dihydroceramide analogs were indistinguishable (Fig. 7) (of note, dihydroceramide analogs have no role in the induction of apoptosis (39)). 3) Contrary to our results, mSREBP levels have been reported to be increased in apoptosis (50), but we observed a decrease. 4) The effect of ceramide analogs on SRE-mediated gene transcription was reversible (Fig. 5). 5) Ceramide analogs had no adverse

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Fig. 10. Northern blot analysis of HMG-CoA synthase. CHO cells were incubated for 8 h with control medium (1% BSA; lane 1), C2-ceramide (C2-Cer; 20 μM; lane 2), d-MAPP (30 μM; lane 3), or bacterial sphingomyelinase (SMase; 10 micromunits/ml; lane 4). Total RNA (30 μg) was loaded per lane, electrophoresed on a formaldehyde-containing 1.2% agarose gel, and transferred to a nylon membrane. The membrane was hybridized with 32P-labeled probes for HMG-CoA synthase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described under “Experimental Procedures.” The HMG-CoA synthase mRNA level relative to control values was calculated after quantitative PhosphorImager analysis and correction of loading differences determined by the glyceraldehyde-3-phosphate dehydrogenase signal.

However, several lines of evidence support the conclusion that SRE-mediated gene transcription is reduced by ceramides. Increasing ceramide through exogenous sphingomyelinase (Fig. 3), addition of ceramide analogs (Figs. 4–7), and inhibition of endogenous ceramide by d-MAPP (Fig. 8) all resulted in decreased SRE-mediated reporter gene expression. In parallel, ceramides decreased cellular levels of mSREBP-1 and mSREBP-2 and HMG-CoA synthase mRNA (Figs. 6 and 10). Schock et al. (33) have reported that exogenous ceramides have no effect on SREBP proteolysis within a time period of 150 min. Perhaps longer exposure to ceramide may be required because we noted that the ceramide-mediated decrease in SRE-mediated luciferase expression appeared after 4 h (data not shown).

In our previous study (9), we have shown that oleate and other polyunsaturated fatty acids decrease SRE-mediated gene transcription alone as well as synergistically with exogenous cholesterol and concluded that the effect is not dependent on exogenous cholesterol, but is likely related to endogenous cellular cholesterol movement. Our present work links fatty acid-induced sphingomyelin hydrolysis to decreasing sphingomyelin levels coupled with the concomitant generation of ceramide from sphingomyelin hydrolysis. To investigate whether ceramide-mediated effects on SRE-mediated gene transcription depend on cholesterol movement, we used U18666A, a substance well characterized to decrease intracellular cholesterol trafficking and cholesterol content of the endoplasmic reticulum (43, 51). We showed that U18666A increased SRE-mediated reporter gene expression and HMG-CoA synthase mRNA levels. Ceramide analogs decreased SRE-mediated gene transcription despite the presence of U18666A (Fig. 9), suggesting that the effects of ceramide on SRE-mediated gene transcription are partly independent of intracellular cholesterol trafficking. Although the exact mechanisms whereby ceramide inhibits SREBP cleavage are not yet defined, it is possible that ceramide affects SREBP proteolysis or intracellular trafficking (52).

Fatty acids are also known to decrease SREBP-1c mRNA transcription by antagonizing ligand-dependent activation of liver-X-receptors and by decreasing the half-lives of hepatic SREBP-1a and SREBP-1c though a cycloheximide-sensitive process (12, 53). Both mechanisms result in decreased precursor protein levels. Because the levels of precursor SREBP-1 and SREBP-2 were not decreased in the presence of ceramide analogs, this potential regulatory mechanism was not further investigated (but cannot be excluded) in our study.

Sphingomyelin and cholesterol levels are coordinately regulated, and both are important metabolically in a large number of physiological and biochemical pathways. Both are constituents of lipid rafts, and sphingomyelin synthesis and levels determine the capacity of membranes to absorb cholesterol (54). Our results indicate that sphingomyelin hydrolysis contributes to these homeostatic changes by initiating two separate regulatory mechanisms of SREBP proteolysis: a ceramide-mediated decrease in SRE-mediated gene transcription by decreasing plasma membrane cholesterol and a ceramide-mediated effect that occurs even in the presence of inhibitors of cellular cholesterol movement. Ceramide, a product of sphingomyelin hydrolysis, thus joins cholesterol and fatty acids as a regulator of SRE-mediated gene transcription.

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