Ceramide Synthesis Correlates with the Posttranscriptional Regulation of the Sterol-Regulatory Element-Binding Protein

Tilla S. Worgall, Rebecca A. Juliano, Toru Seo, Richard J. Deckelbaum

Objective—Sterol-regulatory element-binding proteins (SREBPs) regulate transcription of genes of lipid metabolism. Ceramide decreases transcriptionally active SREBP levels independently of intracellular cholesterol levels. Mechanisms of the ceramide-mediated decrease of SREBP levels were investigated.

Methods and Results—Experiments were performed in Chinese hamster ovary cells. Inhibition of ceramide synthesis with myriocin, cycloserine, or fumonisins decreases levels of transcriptionally active SREBP and reduces SRE-mediated gene transcription. When ceramide synthesis is increased through exogenous sphingosine or inhibition of sphingosine kinase, SRE-mediated gene transcription is increased. The important role of ceramide synthesis in SRE-mediated gene transcription is confirmed in LY-B cells that do not synthesize ceramide de novo. LY-B cells fail to increase SRE-mediated gene transcription in sterol depletion.

Conclusions—Ceramide synthesis correlates with the generation of transcriptionally active SREBP and SRE-mediated gene transcription. Inhibition of ceramide synthesis decreases levels of transcriptionally active SREBP and SRE-mediated gene transcription. It is hypothesized that the process of ongoing ceramide synthesis contributes to the physiological processing of SREBP, perhaps affecting ER-to-Golgi trafficking. Taken together, modification of ceramide synthesis could be a novel target for drug development in the pharmacologic modification of SRE-dependent pathways. (Arterioscler Thromb Vasc Biol. 2004;24:943-948.)

Key Words: SREBP ■ ceramide ■ myriocin ■ LY-B ■ sphingosine

The sterol-regulatory element-binding proteins (SREBPs) are important transcription factors of genes of lipid metabolism. In sterol depletion, precursor SREBP is translocated from the endoplasmic reticulum by vesicular trafficking to the Golgi apparatus,1,2 where the transcriptionally active mature SREBP is generated. Mature SREBP binds to sterol-regulatory elements (SRE), cis-acting elements in the promoters of genes of cholesterol and fatty acid synthesis.3

Cholesterol and unsaturated fatty acids are known regulators of transcriptional and posttranscriptional processing of SREBP. We recently reported that unsaturated fatty acid-mediated decreases in SRE-mediated gene transcription are linked to cellular sphingolipid metabolism.4 Ceramide, a metabolite of sphingomyelin hydrolysis, also regulates mature SREBP levels. Regulation occurs even in the presence of inhibitors of intracellular cholesterol trafficking, suggesting a cholesterol-independent effect.4

Ceramide has multiple roles ranging from lipid second messenger to the induction of apoptosis, cell growth, and differentiation.5,6 Cellular ceramide levels are generated either by de novo synthesis from serine and palmitoyl-CoA or through a recycling pathway of sphingolipid hydrolysis. Ceramide also has a role in intracellular protein trafficking, can inhibit coated vesicle formation and exocytosis in Chinese hamster ovary (CHO) cells,7 and can modulate endocytosis in mammalian cells.8 In yeast, ongoing ceramide synthesis is critical in the vesicular ER-to-Golgi transport of GPI-anchored proteins.9–11

We investigated the effect of ceramide synthesis on SREBP levels and SRE-mediated gene transcription. Because increased ceramide levels decrease mature SREBP protein levels and SRE-mediated gene transcription,4 it was anticipated that inhibition of ceramide synthesis should increase mature SREBP levels and SRE-mediated gene transcription. Contrary to this hypothesis, inhibition of ceramide synthesis decreases SRE-mediated gene transcription. Increasing ceramide synthesis correlates with increased mature SREBP levels and SRE-mediated gene transcription. Consequently, the effect of ceramide on its own synthesis was investigated and shown to be inhibitory, providing an explanation that increased ceramide levels and decreased ceramide synthesis inhibit SRE-mediated gene transcription. The role of ceramide synthesis in SRE-mediated gene transcription is supported by experiments in CHO cells that lack ceramide de
novel synthesis (LY-B cells) because of a mutation in a subunit of serine-palmitoyl transferase. LY-B cells fail to increase SRE-mediated gene transcription when they are cholesterol-depleted. Ceramide increases levels for precursor SREBP but decreases levels for mature SREBP, suggesting a block in the generation of mature SREBP. Our data provide evidence that ceramide synthesis is an important regulatory factor in the maturation cascade of SREBP.

**Methods**

H-serine (555 Gbq-1.48 TBq; 0.1 mCi/mL) and H-sphingosine (555 Gbq-1.11 TBq; 0.1 mCi/mL) were purchased from Perkin Elmer (Boston, Mass). CHO cells were obtained from American Type Culture Collection (Rockville, Md). LY-B cells were provided by Dr K. Hanada, National Institutes of Infectious Diseases, Tokyo, Japan. Ethanol, fatty acid free bovine serum (BSA), cholesterol, 25-hydroxycholesterol (25-OH cholesterol), and fumonisin B1 were obtained from Sigma, (St. Louis, Mo). D-MAPP (1S,2R)-D-methylenecyclopentylphosphinosine, D-25-hydroxycholesterol (25-OH cholesterol), and fumonisin B1 were obtained from Sigma, (St. Louis, Mo). D-MAPP (1S,2R)-D-methylenecyclopentylphosphinosine, D-25-hydroxycholesterol (25-OH cholesterol), and fumonisin B1 were obtained from Sigma, (St. Louis, Mo).

**Plasmids**

The pSyn-SRE luciferase reporter plasmid originates from the hamster HMG-CoA synthase promoter and contains three SRE elements (−326 to −225 bp) and has been described before. The pWLNeo plasmid was obtained from Stratagene (La Jolla, Calif).

**Cell Culture and Stable Transfections**

Cells were grown in F12-nutrient mixture medium containing 10% fetal bovine serum, 1% glutamine (v/v), 1% penicillin/streptomycin (v/v), and 10% fetal bovine serum (v/v) at 37°C in humidified CO₂ (5%). The generation of stable transfectants has been previously described.

**Enzyme Assays**

Luciferase activity was analyzed in aliquots of cell extracts using a luminometer (Berthold LB 9501; Wallac, Gaithersburg, Md). Luciferase activity was analyzed in aliquots of cell extracts using a luminometer (Berthold LB 9501; Wallac, Gaithersburg, Md). Results were expressed in dpm/mg protein as a percentage of total counts.

**Protein Determination**

Cellular protein was determined by the Biorad method. BSA was used as a standard.

**Cell Survival**

In all conditions, cell survival was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion. Cytotoxicity was assessed by measuring LDH release using a colorimetric assay (Roche, Nutley, NJ). Two hours before harvesting, all cells had received 25 μg/mL N-acetyl-leucyl-leucyl-norleucinal (ALLN). An aliquot (30 μg of protein) was electrophoresed on a denaturing 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The monoclonal antibodies against SREBP-1 (BD Biosciences, San Jose, Calif) and actin (Sigma, St. Louis, Mo) and the peroxidase-labeled anti-mouse IgG (Amersham, Arlington Heights, Ill) were run as standards. The TLC plate was cut at the corresponding lipid spots, mixed with scintillation fluid (Ultima Gold; Packard Instrument Company, CT), and analyzed in a scintillation counter (Perkin Elmer Wallac, Gaithersburg, Md). Results were expressed in dpm/mg protein as a percentage of total counts.

**Northern Blot Analysis**

Cells were incubated in control media (1% BSA) or with the respective conditions. After 8 hours, cells were scraped and pelleted at 1000 g and resuspended in lysis buffer C (100 mMol/L Tris-Cl, 100 mMol/L NaCl, 1% SDS, pH 7.6) containing protease inhibitor Complete (Roche Pharmaceuticals, Nutley, NJ). Two hours before harvesting, all cells had received 25 μg/mL N-acetyl-leucyl-leucyl-norleucinal (ALLN). An aliquot (30 μg of protein) was electrophoresed on a denaturing 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The monoclonal antibodies against SREBP-1 (BD Biosciences, San Jose, Calif) and actin (Sigma, St. Louis, Mo) and the peroxidase-labeled anti-mouse IgG (Amersham NIF 824) were used for Western blot analysis using the ECL method (Amersham, Arlington Heights, Ill).

**Data Analysis**

Statistical significance was calculated by paired t tests. Results are given as mean±SD. All experiments were repeated on different days at least 3 times and each time in triplicate.

**Results**

We previously showed that increasing levels of cellular ceramide decreases mature SREBP and SRE-mediated gene transcription. We investigated potential mechanisms for this effect.

**Increased Exogenous and Endogenous Ceramide Decreases Ceramide Synthesis**

Cells were incubated for 8 hours in the presence of C6-ceramide or C8-ceramide (20 μmol/L), DH-C6-ceramide (20 μmol/L), D-MAPP (20 μmol/L), D-MAPP (20 μmol/L), an inhibitor of alkaline ceramidase, or PPMP (20 μmol/L), an inhibitor of glucosylceramide synthesis. As a negative control, cells were incubated with NB-DNJ (40 μmol/L), an inhibitor of glucosylceramide synthesis that does not increase ceramide levels. For the last 1.5 hours of incubation, H-serine was added as a label to determine ceramide synthesis. All conditions, except incubation with NB-DNJ,
Figure 1. Exogenous and endogenous ceramides, dihydroceramides, decrease incorporation of 3H serine into de novo synthesized ceramide. CHO cells, stable transfectants for an SRE-reporter gene, were treated for 8 hours with control conditions (1% BSA) (negative control) and myriocin (1 μmol/L) (positive control), C6-Cer (20 μmol/L), or NB-DNJ (40 μmol/L). After 6.5 hours, 3H serine (1 μL/mL) was added to each condition to measure incorporation of label into de novo synthesized ceramide within 1.5 hours. All experimental conditions, except NB-DNJ, (which does not increase endogenous ceramide levels) significantly decrease 3H-serine incorporation into ceramide (P<0.05).

Figure 2. C6-ceramide increases precursor SREBP levels. CHO cells, stable transfectants for an SRE-reporter gene, were incubated for 4 and 8 hours with 1% BSA (control) or C6-ceramide (20 μmol/L). Whole-cell extracts (20 μg protein) were loaded on a 4% to 14% continuous gradient SDS-PAGE gel. P indicates the precursor (125 kd) form of SREBP-1. C6 ceramide increases the precursor form of SREBP-1 and decreases the mature form of SREBP-1. Probing for actin demonstrates equal loading of the samples.

Figure 3. Inhibition of ceramide synthesis and SRE-mediated gene transcription. Decreased ceramide synthesis correlates with decreased levels of SRE-mediated gene transcription and mature SREBP. CHO cells, stable transfectants for an SRE-reporter gene, were incubated for 8 hours in the presence of control conditions (1% BSA), cholesterol and 25-OH cholesterol (10 μg/mL and 1 μg/mL), myriocin (1 μmol/L), cycloserine (500 mmol/L), fumonisins B1 (20 μmol/L), PPMP (20 μmol/L), or NB-DNJ (negative control) (40 μmol/L); n=4 separate experiments. Compared with control, all conditions except NB-DNJ reduce luciferase expression (P<0.05) measured as relative light units (RLU).

Inhibition of Ceramide de Novo Synthesis Decreases SRE-Mediated Gene Transcription

The effect of decreased ceramide synthesis on SRE-mediated gene transcription was investigated next (Figure 3). CHO cells, stable transfectants for SRE-regulated reporter gene, were incubated for 8 hours with myriocin (1 μmol/L), a specific inhibitor of serine-palmitoyl transferase,25 cycloserine (500 mmol/L), another inhibitor of serine-palmitoyl transferase,26,27 or fumonisins B1 (10 μmol/L), an inhibitor of ceramide synthase.28 All 3 inhibitors significantly reduced SRE-mediated gene transcription by 8 hours (Figure 3). Cells were also incubated with PPMP (20 μmol/L), a glucosyltransferase inhibitor that increases intracellular ceramide levels and decreases ceramide synthesis as measured by incorporation of 3H-serine (Figure 1). As a negative control, cells were incubated with 40 μmol/L NB-DNJ, an inhibitor of glucosylceramide synthase that does not increase ceramide levels24 (Figure 3). The effects of myriocin were dose-dependent (Figure I, available online at http://atvb.ahajournals.org) and reversible within 8 hours and did not decrease the expression of a β-gal control reporter gene (data not shown). All inhibitors shown did not affect cell survival at the concentrations used.

Increased Ceramide Synthesis Increases SRE-Mediated Gene Transcription

Sphingosine can increase ceramide synthesis.29–32 Sphingosine levels can also be increased by DMS, an inhibitor of sphingosine-1-phosphate kinase.33,34 Cells were incubated with sphingosine (1.5 μmol/L) or DMS (1.5 to 5 μmol/L) for up to 6 hours. DMS dose-dependently increases SRE-mediated gene transcription (Figure 4) and levels of mature SREBP (Figure 4 inset). Sphingosine increases SRE-mediated gene transcription up to 2-fold (Figure 4). DMS dose-dependently increases incorporation of 3H-sphingosine label into ceramide by 70% within 5 hours (Figure II, available online at http://atvb.ahajournals.org).
LY-B Cells That Do Not Synthesize Ceramide de Novo Fail to Increase SRE-Mediated Gene Transcription in Sterol Depletion

The role of ongoing ceramide synthesis on SRE-mediated gene transcription was investigated in LY-B cells. 12 In LY-B cells, a mutation in the LCB1 subunit of serine-palmitoyl transferase results in a complete lack of enzyme activity and inability to de novo synthesize any sphingolipid species. SRE-mediated gene transcription was first suppressed by incubation for 16 hours in the presence of cholesterol (10 μg/mL) and 25-OH cholesterol (1 μg/mL). Then cells were switched for 6 hours to medium containing 1% BSA. Control cells increased SRE-mediated gene transcription but LY-B cells failed to do so (Figure 5). Control experiments were performed to demonstrate that LY-B cells are able to increase SRE-mediated gene transcription once a precursor for ceramide synthesis is supplied. This time medium was switched to 1% BSA containing 5 μmol/L DMS after previous incubation with cholesterol. Within 6 hours, cells significantly increased SRE-mediated gene transcription. This indicates that when the block in ceramide synthesis is bypassed by DMS, SRE-mediated gene transcription increases as expected, reflecting the physiological response to cellular sterol depletion.

Inhibition of Ceramide de Novo Synthesis Decreases Levels of HMG-CoA Synthase mRNA

Northern blot analysis of HMG-CoA synthase was performed to confirm results obtained with SRE-reporter gene assays. HMG-CoA synthase is sensitively regulated by SREBP. 13 We have previously shown that ceramide and D-MAPP decrease HMG-CoA synthase mRNA levels. 14 Incubation with myriocin for 16 hours decreases HMG-CoA synthase mRNA levels to less than half (Figure 6).

Discussion

In summary, the present study set out to investigate mechanisms how ceramide decreases transcriptionally active SREBP levels. Several lines of evidence demonstrate a prominent role for ongoing ceramide synthesis in the post-transcriptional regulation of SREBP and that ceramide reduces SREBP processing by decreasing de novo ceramide synthesis. Inhibition of ceramide synthesis results in accumulation of precursor SREBP and decreased mature SREBP.

Figure 4. Increased ceramide synthesis and SRE-mediated gene transcription. Increased ceramide synthesis correlates with increased SRE-mediated gene transcription. CHOs, stable transfectants for an SRE-reporter gene, were incubated for 6 hours in the presence of DMS (1.5 to 5 μmol/L) or with sphingosine (1.5 μmol/L). Inset, Western blot analysis. CHO cells were incubated for 6 hours with control medium (1% BSA) or DMS (5 μmol/L). Whole-cell extracts (30 μg protein) were loaded on a 4% to 14% continuous gradient SDS-PAGE gel. P and M indicate the precursor (125 kd) and mature (68 kd) forms of SREBP-1 in a representative experiment.

Figure 5. LY-B cells fail to recover SRE-mediated gene transcription in cholesterol depletion but recover SRE-mediated gene transcription in the presence of DMS. LY-B and control CHO-cells, stably transfected with an SRE-reporter gene, were incubated for 16 hours with 1% BSA (control) or in the presence of cholesterol (10 μg/mL) and 25-OH cholesterol (1 μg/mL) to decrease SRE-mediated gene transcription. Then, medium was switched to 1% BSA or DMS (5 μmol/L) for 6 hours. Cholesterol decreases SRE-mediated gene transcription significantly stronger in LY-B cells (white bars) compared with control cells (black bars) (P<0.05). Incubation for 6 hours in the presence of 1% BSA fails to increase SRE-mediated gene transcription in LY-B cells compared with controls (P<0.05). Incubation with DMS (5 μmol/L) significantly (P<0.05) increases SRE-mediated gene transcription in LY-B and controls.

Figure 6. Myriocin decreases levels of HMG-CoA synthase mRNA. CHO cells were incubated for 8 hours with control medium (1% BSA) or myriocin (1 μmol/L). Then 30 μg of total RNA were electrophoresed on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized with 32P-labeled probes for HMG-CoA synthase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lane 1 shows control (1% BSA); lane 2, myriocin (1 μmol/L). The level of HMG-CoA synthase mRNA relative to control values was calculated after quantitative phosphorimager analysis and correction of loading differences determined by the glyceraldehyde-3-phosphate dehydrogenase signal.
protein leading to decreased SRE-dependent gene transcription.

We had previously shown that increasing cellular levels of ceramide decreases SRE-mediated gene transcription independent of cellular cholesterol levels, the classical feedback inhibitor of SRE-mediated gene transcription. Therefore, the initial hypothesis was that decreased ceramide synthesis should increase SRE-mediated gene transcription. On the contrary, inhibition of ceramide synthesis decreases SRE-mediated gene transcription (Figure 3) and levels of mature SREBP. Myriocin, a very specific inhibitor of serine-palmitoyl transferase, inhibits SRE-mediated gene transcription even more than cholesterol (Figure 3). Synthesis of ceramide occurs de novo through serine-palmitoyl transferase or through a recycling pathway via sphingosine. SRE-mediated gene transcription and mature SREBP levels are increased when ceramide synthesis is increased by sphingosine either added exogenously or increased endogenously through inhibition of sphingosine kinase (Figure 4). Taken together, the data suggest that increasing the flux into ceramide synthesis either de novo or through a recycling pathway modifies SREBP proteolysis and SRE-mediated gene transcription. The role of both pathways is demonstrated in LY-B cells. These cells, mutated in a subunit of serine-palmitoyl transferase and unable to synthesize sphingolipids de novo, fail to increase SRE-mediated gene transcription in sterol depletion but recover this ability when sphingosine is present in the incubation medium (Figure 5). Our data do not implicate sphingosine 1-phosphate in SREBP proteolysis because inhibition of sphingosine kinase (DMS) increases SREBP proteolysis, and phosphatidylethanolamine, a metabolite of sphingosine 1-phosphate lyase, does not affect SRE-mediated gene transcription (data not shown).

To explain the finding that decreasing ceramide synthesis (this article) or increasing cellular ceramide levels both decrease SRE-mediated gene transcription, we questioned whether ceramide inhibits its own synthesis as shown for short-chain ceramides, dihydrceramides and dihydrceramide analogues. The data demonstrate that all ceramide analogues and inhibitors that increase intracellular cellular levels (i.e., DMAPP, an inhibitor of ceramidase that increases cellular ceramide levels or PPMP, an inhibitor of glucosyl-ceramide synthase) decrease ceramide de novo synthesis (Figure 1). Importantly, NB-DNJ, a glucosylceramide synthase inhibitor shown not to increase ceramide levels, equally does not affect ceramide de novo synthesis (Figure 1) or SRE-mediated gene transcription (Figure 3). These data support the role of ceramide in SRE-mediated gene regulation and furthermore suggests that glucosylceramide does not regulate SRE-mediated gene transcription. Therefore, inhibition of its own synthesis can be a mechanism of ceramide-mediated decrease of SRE-mediated gene expression.

Our data show that addition of exogenous ceramide increases levels of precursor SREBP and decreases levels of mature SREBP (Figure 2). This could suggest that the processing of precursor SREBP to mature SREBP is inhibited, potentially at multiple cellular sites. Ceramide synthesis has been shown to be obligatory in the ER to Golgi trafficking in yeast. Of relevance, increased levels of ceramides inhibit the formation of coated vesicles in CHO cells, glycoprotein traffic through the secretory pathway, and decrease endocytosis in mammalian cells. These observations also make it unlikely that the effect of ceramide on SREBP trafficking is unique.

We previously reported that ceramide decreases mature SREBP levels and SRE-mediated gene transcription even in the presence of inhibitors of intracellular cholesterol movement. Further evidence of a cholesterol-independent regulation of SREBP is found in Drosophila melanogaster, where SREBP levels are only regulated by palmitic acid and phosphatidylcholine but not by cholesterol or unsaturated fatty acids. Palmitic acid determines the rate of long-chain sphinganine synthesis, which can be further metabolized to ceramide. Hence, ceramide synthesis may also contribute to SREBP regulation in Drosophila. In mammalian cells, SREBP formation and cleavage occur by a number of metabolic pathways that can be modified by diet or by therapeutic agents. These “regulators” include cholesterol fatty acids, and fatty acids, and, as we show herein, modification of ceramide synthesis.

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**Fig I: Myriocin dose-dependently decreases SRE-mediated gene transcription**

Incubation for 8 h in the presence of control conditions (1% BSA) or increasing levels of myriocin (0.25 µM -1 µM) significantly reduce luciferase expression (p<0.05) measured as relative light units (RLU).

**Fig. II: DMS increases ceramide de-novo synthesis.**

CHO cells were treated for 3.5 h either with control conditions (1% BSA) or DMS (2.5-5 µM). Then, $^3$H-sphingosine (1 µl/ml) was added to each condition to measure incorporation of label into de-novo synthesized ceramide for 1.5 h. After lipid extraction and alkaline hydrolysis and TLC radioactivity was determined in ceramide spots.
Fig. I

![Bar chart showing RLU/Protein (percent of control) for different concentrations of Myriocin. The chart compares Control, 0.25 uM, 0.5 uM, and 1 uM Myriocin against RLU/Protein.](image-url)
Fig. II

\( ^3\text{H}-\text{sphingosine label incorporation into Ceramide (dpm/protein) \% control} \)