Ceramide structural features required to stimulate ABCA1-mediated cholesterol efflux to apolipoprotein A-I

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Abstract Ceramide is a component of the sphingomyelin cycle and a well-established lipid signaling molecule. We recently reported that ceramide specifically increased ABCA1-mediated cholesterol efflux to apolipoprotein A-I (apoA-I), a critical process that leads to the formation of cardioprotective HDL. In this report, we characterize the structural features of ceramide required for this effect. C2 dihydroceramide, which contains a fully saturated acyl chain and is commonly used as a negative control for ceramide apoptotic signaling, stimulated a 2- to 5-fold increase in ABCA1-mediated cholesterol efflux to apoA-I over a 0–60 μM concentration range without the cell toxicity apparent with native C2 ceramide. Compared with C2 ceramide, C6 and C8 ceramides with medium-length N-acyl chains showed a similar extent of efflux stimulation (a 2- to 5-fold increase) but at a higher onset concentration than the less hydrophobic C2 ceramide. In contrast, the reduced and methylated ceramide analogs, N,N-dimethyl sphingosine and N,N,N-trimethyl sphingosine, failed to stimulate cholesterol efflux. We found that changes in the native spatial orientation at either of two chiral carbon centers (or both) resulted in an ~50% decrease compared with native ceramide-stimulated cholesterol efflux. These data show that the overall ceramide shape and the amide bond are critical for the cholesterol efflux effect and suggest that ceramide acts through a protein-mediated pathway to affect ABCA1 activity.—Ghering, A. B., and W. S. Davidson. Ceramide structural features required to stimulate ABCA1-mediated cholesterol efflux to apolipoprotein A-I. J. Lipid Res. 2006. 47: 2781–2788.

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Ceramide, a critical intermediate in the sphingomyelin cycle, is well established as a lipid signaling molecule (1–4) involved in regulating apoptosis and cellular stress responses (1, 5–8). Many extracellular agents that induce apoptosis cause the accumulation of ceramide, including 1α,25-dihydroxyvitamin D3, tumor necrosis factor-α, Fas ligands, and nerve growth factor (9–12). Moreover, exogenous ceramide treatment induces internucleosomal DNA fragmentation, a hallmark of apoptosis onset (5). Because of this critical role for ceramide in inducing apoptosis, it is perhaps unsurprising that some tumors, particularly multi-drug-resistant tumors, exhibit reduced ceramide concentrations and convert ceramide to glucosylsphingolipids that do not activate the apoptotic program (13–15). Recently, ceramide dysregulation was also implicated in Alzheimer’s disease as a mediator of nerve degeneration (16).

In terms of novel ceramide functions, we recently found that treating certain cultured cell lines with ceramide significantly increased their ability to efflux cellular cholesterol (17). This increased cholesterol efflux was not an artifact of ceramide-induced apoptosis or cytotoxicity. Rather, ceramide upregulated cholesterol efflux only in cell lines expressing ABCA1, a membrane-associated ATPase that transfers cellular lipids to lipid-poor apolipoproteins such as apolipoprotein A-I (apoA-I) (18–20). Ceramide was found to specifically increase ABCA1 at the cell surface, with a corresponding increase in cellular apoA-I binding (17). As cardiovascular diseases stemming from atherosclerosis are a leading cause of death in industrialized countries (21), the ABCA1-apoA-I reaction is especially important as it leads to the formation of cardioprotective HDL. Unfortunately, most existing strategies for upregulating ABCA1 expression, such as cAMP treatment (22) and liver and retinoic acid X receptor agonists (23), have highly pleiotropic effects that may counteract the antiatherogenic benefits of increasing ABCA1. Thus, it is important to investigate the mechanism of the ceramide effect as a potential treatment strategy.

There are two general possibilities for how ceramide exerts its myriad signaling effects, all of which are believed to originate from membranes due to its extreme hydrophobicity (2). The first is that ceramide alters cellular signals by physically affecting membrane lipid packing to indirectly affect target proteins. Evidence that ceramide can act physically to affect signaling proteins comes from...
studies showing that ceramide can perturb phospholipid bilayers to alter protein kinase C activity (24). A second possibility is that ceramide can act through a biological mechanism and bind directly to target proteins associated with the membrane. Ceramide has been shown in vitro and in vivo to activate both phosphatase protein phosphatase 2A (PP2A) [ceramide-activated protein phosphatase (CAPP)] and Kinase Suppressor of Ras [ceramide-activated protein kinase (CAPK)] (25–29). Ceramide stimulation of CAPP or CAPK can then propagate signals through the protein kinase Co, c-Jun, and Bcl-2 or Ras, Raf, and extra-cellular signal-regulated kinase (ERK) signaling pathways, respectively (3).

To further characterize the actions of ceramide that lead to its effects on ABCA1, we set out to determine the structural features of ceramide that are required to enhance cholesterol efflux. This was accomplished by systematically modulating the chemical structure of ceramide and correlating those changes to the promotion of cholesterol efflux from cultured CHO cells. The data indicate that the ceramide-amide bond and overall molecular shape are critical for the cholesterol efflux effect.

**EXPERIMENTAL PROCEDURES**

Materials and equipment

CHO cells from the American Type Culture Collection (Manas-sas, VA) were used for all experiments. CHO cells were main-tained in growth medium, Ham’s F12 with 10% bovine serum, 100 U/ml penicillin, and 100 μg/ml streptavidin (all cell culture reagents were from Invitrogen, Carlsbad, CA) at 37°C in 5% CO2. [1α,2α(n-3)]cholesterol was used to radiolabel cells for cholesterol efflux experiments (GE Healthcare, Waukesha, WI). C2, C6, and C8 ceramides (N-acetyl, N-hexanoyl, and N-octanoyl sphingosine) and fatty acid-free BSA were from Calbiochem (San Diego, CA). C2 dihydroceramide (N-acetyl sphinganine) and t-erythro (2R,3S), t-threo (2R,3R), and t-threo (2S,3S) C2 ceramides were from Matreya (Pleasant Gap, PA). N,N-dimethyl sphingosine (DMS) and N,N,N-trimethyl sphingosine (TMS) were from Avanti Polar Lipids (Alabaster, AL). Scintiverse BD Cocktail, tolutene, and all other chemicals were of the highest grade from Fisher (Hampton, NH). Liquid scintillation counts were measured with the Perkin-Elmer 1900CA TriCarb Liquid Scintillation Analyzer.

Cholesterol efflux assay

CHO cells were grown to confluence on 12-well plates in growth medium and labeled with [3H]cholesterol by incubating with basal medium, Ham’s F12, and 0.2% BSA supplemented with [3H]cholesterol for 24 h (1 μCi/ml). Monolayers were then treated with sphingolipid (added as ethanol solution; final concentration, 0.5–60 μM) in basal medium for 12–16 h. After rinsing twice with basal medium, cells were incubated with apoA-I (10–15 μg/ml) in basal medium with sphingolipid (0.5–60 μM) for 4 h. Medium was collected and 0.45 μl was filtered (Millipore, Billerica, MA). Cellular lipids were extracted with isopropanol, which was then evaporated with positive air pressure and dissolved in toluene. Medium and cell-associated [3H]cholesterol were measured by adding aliquots of each sample to scintillation fluid followed by liquid scintillation counting. Fold change efflux (vs. unstimulated cholesterol efflux to apoA-I) was calculated as follows: fold change efflux = (((total media counts)/(total media counts + total cellular counts)) × 100)/(sphingolipid-treated cells + ((total media counts)/(total media counts + total cellular counts)) × 100)untreated cells efflux to apoA-I.

Purification of apoA-I

Human apoA-I was purified to homogeneity from plasma HDL (1.21 < density > 1.062 g/ml) as reported previously (30, 31).

Cell surface ABCA1 assay

ABCA1 present at the cell surface was measured using the biotin labeling-streptavidin capture method with slight modifications (17, 32). Briefly, CHO cells were grown to confluence in T75 flasks and treated for 12–16 h with sphingolipid in basal medium (final sphingolipid concentrations, 5 μM for DMS and TMS and 20 μM for ceramide and dihydroceramide; added as ethanol solution). Cells were chilled on ice in a cold room to 4°C and then washed five times with ice-cold PBS (1.06 mM potassium phosphate monobasic, 2.97 mM sodium phosphate dibasic, and 15.7 mM sodium chloride, pH 7.4; Invitrogen). Surface proteins were biotinylated through two 20 min incubations with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS on a platform orbital rotator at 4°C. Any remaining biotin reagent was quenched by two 5 min incubations with 50 mM Tris in PBS, pH 7.4, again on a platform orbital rotator at 4°C. Cells were then washed once with ice-cold PBS, scraped into 1 ml of PBS, and pelleted by centrifugation. Pellets were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml apro-tinin, and 1 μg/ml leupeptin) and lysed during 30 min of incubation on ice. Cellular debris was cleared by centrifugation at 4°C, and supernatant was transferred to a fresh Eppendorf tube. Protein concentration in the supernatants was determined using the Markwell-Lowry assay (33). Equal protein amounts (75–300 μg of protein) were added to 85 μl of Ultralink Plus immobilized streptavidin gel (Pierce) in Handee Spin Cups (Pierce) and incubated overnight with gentle shaking at 4°C. Eluate was removed from streptavidin gel by centrifugation at room temperature [eluant contains nonbiotinylated (i.e., intracellular) proteins]. The gel was washed five times with 300 μl of lysis buffer that was subsequently removed by centrifugation. Surface proteins were collected by adding 2-mercaptoethanol-enriched loading dye (30–35 μl; 60 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, and 350 mM 2-mercaptoethanol), incubating for 30 min at a 37°C water bath, followed by centrifugation. Proteins were separated by SDS-PAGE [20–30 μl loaded, 4–15% gel (Bio-Rad); 200 V, 60 min], transferred to a polyvinylidene difluoride (PVDF) membrane (35–50 V, 120 min), and visualized on an immunoblot as described below.

Western blotting

The PVDF membrane was blocked for 1 h in blocking buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 1% Tween-20, and 10% dried milk). Blocked blots were incubated for 2 h with 1:500 Novus rabbit ABCA1 antiserum at room temperature, washed, incubated with Pharmacia HR-conjugated anti-rabbit secondary antibody for 1 h at room temperature, and visualized using Pierce Super Signal reagents.

Determination of protein concentration

Sample protein concentration was determined using the Markwell modification of the Lowry protein assay (33).
RESULTS

Altering ceramide acyl chain saturation, N-acyl chain length, and amide functional groups

We chose CHO cells as the model system for this work because these cells express significant basal levels of ABCA1 without the need to artificially stimulate expression with cAMP analogs or nuclear receptor agonists. We first set out to compare efflux stimulated by C2 ceramide and C2 dihydroceramide, in which the double bond between carbons 4 and 5 of ceramide is fully saturated (Fig. 1). C2 dihydroceramide and C2 ceramide have similar cellular uptake properties, yet dihydroceramide does not exhibit the apoptotic or cytotoxic effects of ceramide and is often used as a negative control for ceramide apoptosis experiments (5, 27, 34, 35). As shown in Fig. 2, C2 dihydroceramide stimulated cholesterol efflux to lipid-free apoA-I to the same extent as C2 ceramide at concentrations up to ~20 μM. At the peak effective concentration for both compounds, cholesterol efflux to apoA-I increased 4-fold compared with that of untreated cells. Interestingly, C2 dihydroceramide continued to increase cholesterol efflux at higher concentrations, whereas for C2 ceramide the effect rapidly decreased. Further experiments revealed that this ceramide-mediated decline was a result of cell toxicity (i.e., many cells had detached from the cell wells) (data not shown).

In addition to varying the saturation of the ceramide double bond, we also manipulated the length of the N-acyl chain (Fig. 1). C2 ceramide is a water-soluble ceramide analog attributable to the near absence of an N-acyl chain, and its biological relevance has been debated versus longer chain ceramides that more closely resemble naturally occurring ceramides (25, 34, 36, 37). Figure 3 shows that

Fig. 1. Basic ceramide structure/stereochemistry and analogs used in cholesterol efflux experiments. The exogenous C2 version of the biologically native D-erythro ceramide (2S,3R) is shown largest with the stereochemically active carbons at the 2 and 3 positions indicated. Beneath the parent ceramide structure are ceramide analogs used in this study: left, the reduced and methylated N,N-dimethyl sphingosine (DMS) and N,N,N-trimethyl sphingosine (TMS); center, the varying N-acyl chain-length versions N-hexanoyl (C6) and N-octanoyl (C8) ceramides; right, the 4–5 double bond-saturated C2 dihydroceramide. Depicted at the top are the stereochemical permutations of the 2R,3S parent ceramide: L-erythro (2R,3S), D-threo (2R,3R), and L-threo (2S,3S) ceramides.
the C6 and C8 medium N-acyl chain lengths are also capable of stimulating cholesterol efflux. In contrast to C2 ceramide, which reached its peak effect at \( \sim 20 \mu M \) (Fig. 2), C6 ceramide reached a peak at \( 30 \mu M \) and C8 ceramide reached a peak at \( \sim 40 \mu M \). Also, the C2 treatment showed a more gradual increase in cholesterol efflux with increased concentration. The increased N-acyl chain length has been demonstrated to affect cellular uptake in breast cancer cell lines, with 3-fold more C6 ceramide appearing in cells than C16 ceramide (37). Such differences in uptake between the C6-C8 ceramides and the C2 ceramide could account for the higher effective concentration for the longer chain ceramides.

We also varied the structure of ceramide at the nitrogen functionality using methylated sphingosines, which closely resemble the sphingolipid sphingosine (Fig. 1). CHO cell monolayers were treated with DMS and TMS over the same concentration range as for the C2 ceramide treatment (Fig. 4A). We found no appreciable increase in cholesterol efflux over that stimulated by lipid-free apoA-I, and at DMS and TMS concentrations of 20–35 \( \mu M \), efflux had actually decreased to or below zero. This could be attributable to cytotoxic effects of the methylated sphingosines, as both DMS and TMS have been shown to inhibit cultured cell growth at 3–5 \( \mu M \) and the fluorescent TMS analog has been shown to induce significant amounts of apoptosis at 5 \( \mu M \) (38–40). Therefore, we performed another set of cholesterol efflux experiments at much lower concentrations of DMS and TMS (0–10 \( \mu M \) range). In this concentration regime below cytotoxic levels, both DMS and TMS failed to mediate an increase in cholesterol efflux and, in fact, inhibited efflux to apoA-I (Fig. 4B). Our previous studies demonstrated that ceramide-stimulated cholesterol efflux was accompanied by an increase in cell surface ABCA1 and a corresponding increase in cell surface-bound apoA-I (17). Consistent with the cholesterol efflux data, DMS or TMS treatment failed to stimulate the presence of ABCA1 at the cell surface like C2 ceramide (Fig. 4C).

**Ceramide stereoisomers and cholesterol efflux**

It is well known from organic chemistry that stereoisomers exhibit similar physical properties, such as solubility and molecular packing. For example, enantiomers of lipids have been shown to have identical TLC behavior (41), as have enantiomers of the ceramide mimic 2-amino-1-phenyl-1-propanol (42). Despite sharing these purely physical properties, enantiomers differ in spatial organization and have dissimilar shape-dependent properties, such as interaction with proteins. To determine whether ceramide stimulates cholesterol efflux by physically affecting membrane-packing properties or through direct interaction with partner protein(s), cholesterol efflux experiments were performed using the four ceramide stereoisomers: the naturally occurring enantiomer D-erythro (2\( \text{R},3\text{S} \)) (Fig. 1). Figure 5 shows that treatment of the cells with the natural isomer of C2 ceramide increased cholesterol efflux to lipid-free apoA-I by 4-fold versus untreated cells. Interestingly, when we changed from the native (2\( \text{S},3\text{R} \)) spatial orientation at either of two chiral centers to the D-threo (2\( \text{R},3\text{R} \)) or L-threo (2\( \text{S},3\text{S} \)) (Fig. 1). Figure 5 shows that treatment of the cells with the natural isomer of C2 ceramide increased cholesterol efflux to lipid-free apoA-I by 4-fold versus untreated cells. Interestingly, when we changed from the native (2\( \text{S},3\text{R} \)) spatial orientation at either of two chiral centers to the D-threo (2\( \text{R},3\text{R} \)) or L-threo (2\( \text{S},3\text{S} \)) enantiomer, we observed a \( \sim 50\% \) decrease in ceramide-stimulated cholesterol efflux. Yet, these compounds still stimulated cholesterol efflux over that of apoA-I alone. When the spatial orientation was opposite that of physiological D-erythro (2\( \text{R},3\text{S} \)) at both carbons in the L-erythro (2\( \text{R},3\text{R} \)) enantiomer, we observed a similar decrease in the ceramide-stimulated efflux effect (Fig. 5). Figure 5B shows the
dependence of cholesterol efflux on enantiomer concentration. It is clear that, even at higher concentrations, the enantiomers did not reach the level of cholesterol efflux stimulation exhibited by ceramide at 20 μM.

We performed biotin-streptavidin cell surface capture experiments with the four C2 ceramide stereoisomers and C2 dihydroceramide. Figure 6 shows that ABCA1 surface presence corresponded with increased cholesterol efflux. The ceramides that stimulated cholesterol efflux, D-erythro C2 ceramide and C2 dihydroceramide, stimulated a multi-fold increase in cell surface ABCA1. Furthermore, the ceramides that did not stimulate efflux to a large extent, L-erythro, D-threo, and L-threo ceramide, failed to increase cell surface ABCA1.

**DISCUSSION**

Ceramide is a potent lipid signaling molecule that participates in cellular responses to stress and, in particular, has a well-researched role in apoptosis (reviewed in Refs. 1, 6, 43). We recently broadened the known sphere of ceramide action, reporting that it stimulated apolipoprotein-mediated cholesterol efflux from cultured cells (17). This effect was not the result of either cytotoxicity or apoptosis causing nonspecific release of cholesterol from the cells, but rather a specific increase in ABCA1-mediated cholesterol release to apoA-I. We undertook to further characterize the structural and stereospecific aspects of ceramide that determine its ability to stimulate cholesterol efflux from cultured cells. This study produced the following results: 1) The presence or absence of an unsaturated bond between carbons 4 and 5 of ceramide does not affect the cholesterol efflux effect. 2) Longer ceramide acyl chains still promoted the cholesterol efflux effect, but at higher effective concentrations. 3) An acylated amide functionality is a requirement, as methylation completely blunted the effect. 4) The stereocchemistry at carbons 2 and 3 is important, as changes reduced the effectiveness for promoting ABCA1 cholesterol efflux. The importance of these results and their utility in determining the overall mechanism of the ceramide effect are discussed below.

Structurally, ceramide is a sphingoid base with a 4–5 unsaturated acyl chain and an additional acyl chain attached through an amide bond at carbon 2 (Fig. 1). The ceramide analog with the fully saturated acyl chain is called dihydroceramide. Dihydroceramide does not activate apoptosis and, thus, has been used as a negative control in ceramide signaling experiments (5, 34). We were surprised to find that C2 dihydroceramide functioned equally as well as C2 ceramide at increasing cholesterol efflux (Fig. 2) and continued to stimulate efflux over a broad concentration range. To our knowledge, this is the first time that dihydroceramide exhibited similar biological effects as the native ceramide. This observation that dihydroceramide stimulated cholesterol efflux without the apoptotic side effects of ceramide may be promising for the development of ceramide-based HDL-increasing drugs to combat cardiovascular disease. Although the magnitude of the ceramide effect is somewhat smaller than has been demonstrated for cholesterol loading of fibroblasts (44), or in macrophages by stimulation of the liver X receptor (45), or by cAMP treatment (22), the pleiotropic nature of these targets has limited enthusiasm for their potential therapeutic use. The pathway(s) through which ceramide mediates its effects on ABCA1 cellular location may offer new alternatives for potential exploitation.

Exogenous ceramides with varying N-acyl chain lengths have demonstrated relatively similar apoptosis signaling...
properties (25, 34, 36, 37), and this was also the case in their ability to stimulate cholesterol efflux. The C2 form has been used extensively because of its solubility and ease of introduction into cells. As ceramide N-acyl chain length is increased, cellular uptake decreases dramatically (37). Thus, one explanation for the higher onset concentration for C6 and C8 ceramides could be their lower uptake relative to C2 ceramide (37). Alternatively, ceramide may have to accumulate within a particular compartment membrane within the cell to exert its actions. It is possible that the longer acyl chains increase the time and/or concentration required to get ceramide into the active cellular compartment.

Past studies of ceramide action have led to two hypothesized mechanisms for its ability to alter cellular signals: 1) physical, by affecting lipid packing that indirectly affects target proteins; or 2) biological, by binding directly to target proteins (2, 6, 24–27). The spatial orientation at the chiral centers results in four stereoisomers that have highly similar physical properties, such as molecular packing, but these isomers should exhibit differential interactions with proteins. For example, of the four ceramide stereoisomers, only the native D-erythro ceramide activates CAPP, whereas the L-erythro, D-threo, and L-threo enantiomers do not (26).

We found that the orientation at these two carbons was important for mediating the optimal cholesterol efflux effect and for promoting the appearance of ABCA1 at the cell surface. Given the requirement for the native amide functionality, it can be generally stated that the sphingoid character of the lipid must be preserved to stimulate the cholesterol efflux effect, but changes to either acyl chain (be it saturation or length) can be tolerated. We suggest that the dependence of the effect on the molecular conformation argues that ceramide stimulates cholesterol efflux by interacting directly with one or more protein partners and not simply perturbing the structure of a given membrane. Given that dihydroceramide stimulates cholesterol efflux as well as ceramide, certain well-known ceramide targets, such as CAPP and CAPK, can be ruled out because dihydroceramide does not activate these enzymes (25, 27–29, 34). Other potential ceramide targets include proteins in the protein kinase C (46), phospholipase C (47), and phospholipase D (48) pathways, as ceramide is known to interact in these pathways and all are known to participate in regulating cholesterol efflux via ABCA1 (49–53). Current work in our laboratory is focusing on defining the operational pathway.

**Fig. 5.** Fold change in cholesterol efflux stimulated with the addition of each of the four C2 ceramide stereoisomers. A: This experiment was performed similarly to that in Fig. 2. Each of the four C2 ceramide stereoisomers, the biologically native D-erythro (2S,3R), L-erythro (2R,3S), D-threo (2R,3R), and L-threo (2S,3S), were incubated at 20 μM for 13 h on [3H]cholesterol-labeled CHO monolayers, and medium containing apoA-I from a 4 h treatment was collected to measure cholesterol efflux. Efflux (minus background efflux to BSA) is presented as fold change compared with efflux to lipid-free apoA-I (dashed line). Each point is the mean of three replicates ± SD. B: A separate concentration experiment performed under similar conditions as described for A. Each point is the mean ± SD of three replicate wells. Closed circles, biologically native D-erythro (2S,3R); open circles, L-erythro (2R,3S); closed triangles, D-threo (2R,3R); open triangles, L-threo (2S,3S). FC, free cholesterol.

**Fig. 6.** Immunoblot showing surface ABCA1 with the addition of each of the four C2 ceramide stereoisomers. CHO monolayers were treated with each of the four C2 ceramide stereoisomers, the biologically native D-erythro (2S,3R), L-erythro (2R,3S), D-threo (2R,3R), and L-threo (2S,3S), added overnight at 20 μM for 13 hours. Monolayers were surface-tagged with biotin, and equal protein amounts were applied to immobilized streptavidin to capture surface proteins. Captured proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and visualized with an anti-ABCA1 antibody.
REFERENCES


