Ceramide Enhances Cholesterol Efflux to Apolipoprotein A-I by Increasing the Cell Surface Presence of ATP-binding Cassette Transporter A1*

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It is widely accepted that functional ATP-binding cassette transporter A1 (ABCA1) is critical for the formation of nascent high density lipoprotein particles. However, the cholesterol pool(s) and the cellular signaling processes utilized by the ABCA1-mediated pathway remain unclear. Sphingomyelin maintains a preferential interaction with cholesterol in membranes, and its catabolites, especially ceramide, are potent signaling molecules that could play a role in ABCA1 regulation or function. To study the potential role of ceramide in this process, we treated a variety of cell lines with 20 μM C2-ceramide and examined apolipoprotein-mediated cholesterol efflux to lipid-free apoA-I. We found that cell lines expressing ABCA1 displayed 2-3-fold increases in cholesterol efflux to apoA-I. Cell lines not expressing ABCA1 were unaffected by ceramide. We further characterized the cholesterol efflux effect in Chinese hamster ovary cells. Ceramide treatment did not cause significant cytotoxicity or apoptosis and did not affect cholesterol efflux to non-apolipoprotein acceptors. Raising endogenous ceramide levels increased cholesterol efflux to apoA-I. Using a cell surface biotinylation method, we found that the total cellular ABCA1 and that at the plasma membrane were increased with ceramide treatment. Also ceramide enhanced the binding of fluorescently labeled apoA-I to Chinese hamster ovary cells. These data suggest that ceramide may increase the plasma membrane content of ABCA1, leading to increased apoA-I binding and cholesterol efflux.

The importance of circulating plasma high density lipoprotein (HDL) is implicit from several population studies showing an inverse correlation between plasma HDL concentration and cardiovascular disease risk (1, 2). A major function of HDL is to transport excess cholesterol from extrahepatic tissues back to liver for excretion into the bile (3). This process is often referred to as “reverse cholesterol transport” (4). It is clear that functional ABCA1 is critical for the formation of HDL in vivo as patients with Tangier disease or familial HDL deficiency have vastly reduced plasma HDL values (5–10). Consequently ABCA1 has been studied extensively as a target for promoting excess cholesterol release from cells.

The first step in HDL formation involves a specific interaction of lipid-poor apoA-I, the major protein component of HDL, with cell surface ABCA1. There is evidence that apoA-I binds to ABCA1 directly (11–13); however, it has also been suggested that apoA-I may bind to a lipid domain created by ABCA1 activity (14). Regardless of the interaction, the end result is a nascent HDL particle containing apoA-I, phospholipids, and cholesterol. It remains unclear whether apoA-I obtains phospholipids and cholesterol together (15, 16) or in a two-step process involving apoA-I receiving phospholipids before obtaining cholesterol (10, 17).

Two possibilities exist for the source of cholesterol utilized by ABCA1: plasma membrane or intracellular. Evidence exists that ABCA1 may utilize plasma membrane cholesterol from isolated lipid rafts (18) or caveolae (19, 20), although one study showed that ABCA1 does not utilize detergent (Triton-)isolated raft cholesterol (21). It was known well before the discovery of ABCA1 that apoA-I can obtain cholesterol from intracellular sources (22–24). More recently, a study looking specifically at ABCA1-mediated cholesterol efflux showed that an endosomal/lysosomal pool is a preferred source (25).

A complete mechanism for the regulation and transport of cholesterol to ABCA1-accessible pools has not been established. Several signaling molecules have been implicated in apolipoprotein- or ABCA1-mediated cholesterol efflux including phospholipases C and D (26, 27), protein kinase C (28–30), nuclear orphan receptor ligands (31–35), and cAMP (36, 37). In the case of phospholipase C, phospholipase D, and protein kinase C, it is thought that these molecules control cholesterol transport to ABCA1.

Another potential tactic the cell can utilize to regulate cholesterol availability to ABCA1 is with membrane sphingomyelin content. Evidence suggests that cholesterol maintains a preferential contact with sphingomyelin in cellular membranes (38–40). By modulating sphingomyelin content with sphingomyelinases, cholesterol could either be made available to or sequestered from ABCA1. The majority of sphingomyelin exists in the plasma membrane; however, roughly 25% of cellular sphingomyelin is contained in the lysosomes, endosomes, and Golgi combined (41). These cellular locations of sphingomyelin coincide with hypothesized sources of ABCA1-accessible cholesterol pools (18–20, 25). The digestion of sphingomyelin with...
sphingomyelinases yields ceramide, a potent lipid signaling molecule shown to potentiate a variety of cellular events including cell growth, apoptosis, cell differentiation, and the stress response (42–45). Therefore, as cholesterol is released from an interaction with sphingomyelin by sphingomyelinase activity, the released ceramide could be envisioned to activate a pathway that transports or makes available cholesterol to an ABCA1-accessible pool.

The current study was designed to test the hypothesis that ceramide may play a role in ABCA1-mediated cholesterol efflux. To test this, we treated several cell types with C2-ceramide. It was found that ceramide treatment increased cholesterol efflux in cells expressing ABCA1. This effect is further characterized in Chinese hamster ovary (CHO) cells. The major findings were that ceramide treatment increases total and cell surface levels of ABCA1 protein, leading to increased apolipoprotein binding and cellular cholesterol efflux.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human aortic smooth muscle cells (aSMCs) and SmGM-2 Bullet Kit medium were from BioWhittaker (Walkersville, MD). All other cell culture media and supplements were from Invitrogen. CHO, HeLa, and RAW 264.7 mouse macrophage cells were from American Type Culture Collection (ATCC, Manassas, VA). C2-ceramide (N-ethyldihydroceramide, N-ctetyl-N-acetyldihydropyrophosphino) was from Calbiochem. MAPP (15,2R)-N-ethrythro-2-N-myristoylamine-1-phényl-1-propanol was from BIOMOL (Plymouth Meeting, PA). Methylcyclohexatin, propionamide iodide, RNase A, and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) were from Sigma. Egg phosphatidylcholine was acquired from Avanti Polar Lipids (Birmingham, AL). SDS-polyacrylamide gels (Redi-gels) were from Bio-Rad. All other chemical reagents were of the highest grade from Fisher.

**Cell Culture**—aSMCs were grown in smooth muscle basal medium (SmBM) with SmGM-2 Bullet Kit supplements and maintained according to the manufacturer’s instructions. HeLa cells and RAW macrophages were grown in DMEM with 10% fetal bovine serum, 10% dialysed fetal bovine serum albumin (BSA) were from Calbiochem. MAPP (15,2R)-N-ethyldihydroceramide, (N-ctetyl-N-acetyldihydropyrophosphino), sulfo-NHS-SS-biotin, and fatty acid-free bovine serum albumin (BSA) were from Calbiochem. MAPPP (15,2R)-N-ethyldihydroceramide, (N-ctetyl-N-acetyldihydropyrophosphino) was from Calbiochem. Egg phosphatidylcholine was acquired from Avanti Polar Lipids (Birmingham, AL). SDS-polyacrylamide gels (Redi-gels) were from Bio-Rad. All other chemical reagents were of the highest grade from Fisher.

**Cholesterol Efflux Experiments**—Cells were grown to 90% confluency in 12-well plates in their respective growth medium as described above. Monolayers were then labeled with 1 μCi/ml [3H]cholesterol in the appropriate serum-free basal medium supplemented with 0.2% BSA (designated DMEMe, HAMSe, or SmBMe). After 24 h, the labeling medium was removed, and cells were washed twice with phosphate-buffered saline (PBS) with 0.2% BSA and once with appropriate basal medium containing 0.2% BSA; Cholesterol acceptors (in basal medium with 0.2% BSA) were added to yield the following final concentrations: 10 μg/ml human plasma apoA-I, 15 μg/ml phospholipid vesicles, and 0.5 mM methylcyclohexatin. Sphingolipid treatments were added as described in figure legends with untreated cells receiving equivalent volumes of ethanol vehicle. A 150-μl sample of efflux medium was obtained at the times designated and passed through a 0.45-μm filter to remove any floating cells. Monolayers were washed twice in PBS, and cellular lipids were extracted with isopropanol. Medium and cell-associated [3H]cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: (Treatment value - media with vehicle value)/0.1% Triton X-100 value - media with vehicle value) × 100. A lactate dehydrogenase release assay was performed according to the manufacturer’s instructions (Roche Applied Science).

**Cellular ABCA1 Content and Cell Surface Biotinylation**—Cellular ABCA1 content and cell surface biotinylation was performed as described previously (46) with slight modifications. CHO cells were grown in T-75 flasks to confluence. Medium was replaced with HAMSe with or without 20 μM C2-ceramide and incubated for 16 h. Cells were then washed with ice-cold PBS. Surface proteins were biotinylated with 1 mg/ml sulfo-NHS-SS-biotin (in PBS) for 30 min at 4 °C on a platform rotator. Cells were then washed twice with ice-cold quench buffer (50 mM Tris, 0.1% EDTA, 150 mM NaCl) and once with ice-cold PBS. Cells were scraped into PBS and then pelleted by centrifugation. Pellets were resuspended in 100 μl of lysis buffer (50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.2% NaN3, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Triton X-100, 0.5% sodium deoxycholate) and incubated on ice for 30 min. Cellular debris were pelleted by centrifugation at 4 °C. Cleared supernatant was added back to the supernatant was taken for protein concentration determination. 55 μg of protein cell was set aside to examine total cellular ABCA1 content. 200–250 μg of cell protein (final volume of 200 μl in lysis buffer) was added to 75 μl of UltraLink Plus immobili- zed streptavidin gel (Pierce) and incubated overnight on a platform mixer at 4 °C. The gel was pelleted and washed five times with lysis buffer. 55 μl of loading dye (80 mM Tris–Cl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue) with 350 mM β-mercaptoethanol was added to the gel pellet and incubated at 37 °C for 30 min. 30 μl was run on a 4–15% SDS-polyacrylamide gel, and Western blotting was performed as described below.

**Western Blotting**—Cell extracts (55 μg of total cell protein or 30 μl of purified biotinylated proteins) were loaded on 7.5% or 4–15% SDS-polyacrylamide gels and run for 1 h at 200 V. Proteins were transferred to nitrocellulose and blocked for 2 h at 4 °C in TBS-T (20 mM Tris, 137 mM NaCl, 1% Tween 20, pH 7.6) with 10% dried milk. Blots were incubated with 1:400 anti-ABCA1 (Upstate Cell Signaling Solutions, Waltham, MA) overnight at 4 °C, washed, and then incubated with 1:5000 horseradish peroxidase-conjugated anti-rabbit secondary antibody (Amersham Biosciences) for 1 h at room temperature. After washing cell extracts, blots were subsequently stripped, blocked in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) with 10% dried milk, and probed with 1:2000 anti-actin (Novus Biologicals, Littleton, CO) for 1 h at room temperature. Blots were developed with an ECL kit (Am- ersham Biosciences) according to the manufacturer’s instructions.

**Binding of Fluorescently Labeled APOA-I (ALEXAX488-ApoA-I) to CHO Cells**—A cysteine mutant of apoA-I (Cys@93) was expressed, purified, and labeled with ALEXA-488 (Molecular Probes, Eugene, OR) as described previously (49). CHO cells expressing GFP-ABCA1 were grown to near confluence on glass cell culture plates. 135121, Nalg Nunc International, Rochester, NY) in the maintenance media described above. Cells were then pretreated with or without 20 μM C2-ceramide in CHOoe for 16 h. After one quick wash with CHOoe, fresh CHOe containing 10 μg/ml ALEXA488-ApoA-I was added to the wells, and the slides were incubated on ice for 2 h. Cells were washed four times with ice-cold PBS and then fixed with 5% paraformaldehyde, 2% sucrose on ice for 30 min. Cells were then coveredslipped and immediately examined with a Leica TCS 4D confocal microscope (Heidelberg, Germany) using an Omnichrome krypton-argon laser (Chino, CA).

**Other Methods**—The ABCA1-GFP construct (a gift from Dr. Richard Lawn) in the mammalian expression vector pEGFP-N1 (Clontech) was stably expressed in CHO cells using the LipofectAMINE Plus kit (Invitrogen) following the manufacturer’s instructions. Protein concentrations were determined by the Markwell modification of the Lowry assay (50). Phospholipid concentrations were determined by the method of Sokoloff et al. (51). Student’s t test was used to determine statistical differences.
RESULTS

C2-ceramide Treatment Increases Cholesterol Efflux to ApoA-I in Cells Expressing ABCA1—As stated above, the intimate relationship between sphingomyelin and cholesterol led us to hypothesize that sphingomyelin breakdown products might play a role in the cellular regulation of cholesterol efflux. For our initial studies of ABCA1-dependent cholesterol efflux in vitro, we chose CHO cells and primary human aSMCs. Both cell types were exchange labeled with 1 μCi of [14C]cholesterol in serum-free media for 16 h. The cells were washed, and 10 μg/ml apoA-I with 0–20 μM ceramide or vehicle was added in appropriate serum-free media. Efflux of [14C]cholesterol to apoA-I was measured after 24 h. Percentage of total cholesterol present at the beginning of the efflux incubation that appeared in the media was calculated as described under “Materials and Methods.” Cell incubations lacking ceramide contained an identical volume of vehicle (ethanol). CHO cells (filled circles) and aSMCs (open circles) are shown. The error bars represent 1 sample standard deviation from measurements made in triplicate.

Since ceramide showed a clear and consistent effect in both cell types, we treated both cell types with C2-ceramide, a cell-permeant ceramide analog. Fig. 1 shows a clear dose-dependent increase in cholesterol efflux to lipid-free apolipoprotein A-I with ceramide treatment in both cell types. We also tested two other sphingomyelin metabolites, sphingosine and sphingosine phosphate, but no consistent effect on cholesterol efflux to apoA-I was observed with either compound (not shown).

Since ceramide showed a clear and consistent effect in both cell types, we treated RAW 264 macrophages (without cAMP stimulation) and HeLa cells with 20 μM C2-ceramide. Both of these cell lines have been shown to lack detectable levels of ABCA1 (12, 53). These previous findings were confirmed by Western blotting (data not shown). For comparison, parallel experiments were done with WT CHO cells, CHO cells stably transfected with a human GFP-ABCA1 construct, and HeLa cells stably transfected with a human GFP-ABCA1 construct. As shown in Fig. 2, RAW 264 macrophages and WT HeLa cells failed to display the ceramide induction of cholesterol efflux to lipid-free apoA-I. In contrast, all ABCA1-expressing cell lines displayed ceramide-induced efflux with the ABCA1-transfected CHO and HeLa cells showing a significant increase over the respective WT controls. Thus, pre-existing ABCA1 expression is required for the ceramide induction of cholesterol efflux.

Ceramide Treatment Does Not Cause Significant Apoptosis or Toxicity in CHO Cells under the Experimental Conditions Used—Because of their ease of use, we chose to further characterize the ceramide induction of cholesterol efflux using the CHO cells. Since ceramides are well known to act as stimulators of cellular apoptosis in some cell lines (42–45), a possible explanation for the results in Fig. 1 is that ceramide caused cytotoxicity or induced apoptosis, and the cells released cholesterol during death. To address this possibility, we used three different approaches: the MTT test (54) to measure overall metabolic activity of the cell, a lactate dehydrogenase release assay as an index of plasma membrane integrity, and hypodiploid nuclei analysis to assess apoptosis. Table I shows that none of the three tests revealed significant cytotoxicity (MTT and lactate dehydrogenase release) or apoptosis (hypodiploid) with ceramide treatment. These data are in agreement with previous studies showing that C2-ceramide does not cause significant cytotoxicity or apoptosis in CHO cells at ≥20 μM (55, 56).

We also investigated whether treatment with C2-dihydroceramide, an analog of C2-ceramide that does not cause apoptosis (57), could stimulate cholesterol efflux. C2-dihydroceramide differs from C2-ceramide by the absence of a 4–5 trans double bond. Cells are known to take up both compounds to similar extents over 24 h (57). Fig. 3 shows that C2-dihydroceramide treatment causes ceramide-induced cholesterol efflux to levels equivalent to C2-ceramide. Taken with the data in Table I, this result suggests that ceramide-induced cholesterol efflux is not caused by an apoptotic mechanism or general cell cytotoxicity.

Ceramide-induced Efflux in CHO Cells Demonstrates Specificity to Apolipoprotein Acceptors, Is Fully Reversible, and Can Result from Increases in Endogenous Ceramide Levels—In addition to ABCA1-mediated cholesterol efflux to lipid-free apoA-I, cholesterol is also known to leave the cell via the relatively nonspecific aqueous diffusion mechanism (58) in the presence of phospholipid-containing acceptors (59, 60). To determine whether ceramide affects this pathway, we studied the effect of ceramide on the efflux of cholesterol from CHO cells to small unilamellar vesicles of phospholipid. Fig. 4 shows that ceramide had no effect on this transfer. We also found that ceramide did not affect the ability of methylcyclodextrins to

![Fig. 1. Effect of ceramide on cholesterol efflux from human aSMCs and CHO cells.](Image)

![Fig. 2. Effect of ceramide on cell lines expressing different levels of ABCA1.](Image)
Inhibiting alkaline ceramidase and results in up to a 3-fold increase in intracellular ceramide levels (62). Including MAPP in the media with apoA-I resulted in an 80–100% increase in cholesterol efflux to lipid-free apoA-I (Fig. 6). By contrast, MAPP treatment did not affect cholesterol efflux to phospholipid-containing small unilamellar vesicles (not shown). This suggests endogenous ceramides may play a role in the regulation of cholesterol efflux by ABCA1. In addition, increased cholesterol efflux due to C2-ceramide treatment is not likely the result of properties intrinsic to this relatively soluble form of ceramide.

Ceramide Treatment Leads to Increased Cell Surface Presence of ABCA1 and Binding of ApoA-I—Having established that ceramide is a specific modulator of ABCA1-mediated cholesterol efflux, we next determined the effect of ceramide on ABCA1 expression in CHO cells. Western blot analysis on whole cell protein extracts showed that total ABCA1 protein was modestly elevated by ceramide treatment (Fig. 7A). Densitometry scanning showed that the ratio of ABCA1/actin was elevated about 1.5–2-fold after treatment with ceramide. Similar treatment of RAW macrophages (unstimulated with cAMP analogs) and WT HeLa cells did not increase ABCA1 expression (data not shown). Since previous studies have proposed that ABCA1 may be most active at the plasma membrane, presumably to allow interactions with extracellular apolipoproteins (12, 46), we nonspecifically biotinylated all CHO cell surface proteins in the absence and presence of ceramide and then isolated them with streptavidin immobilized on agarose beads. The biotinylation was performed at 4 °C with sulfo-NHS-SS-biotin to prevent potential biotinylation of intracellular proteins. The isolated cell surface proteins were then analyzed by Western blot probed with a polyclonal antibody to ABCA1. Fig. 7B shows that ceramide treatment increases the amount of ABCA1 present at the cell surface. Densitometry quantitation indicated the increase was about 1.75–2-fold that of untreated cells. We also examined the binding of fluorescently labeled apoA-I to CHO cells expressing GFP-ABCA1 with or without 20 μM ceramide pretreatment. The confocal microscope images in Fig. 8 demonstrate a significantly increased degree of bound fluorescent apoA-I (red) at the surfaces of the cells in the presence of ceramide versus untreated cells.

### Table I

| Condition          | LDH% | MTT% | Hypodiploid nuclei |
|--------------------|------|------|-------------------|
| C2- Ceramide       |      |      |                   |
| 0                  | 0    | 100  | 3.8 ± 1.1         |
| 20 μM              | 3.8 ± 1.4 | 102.7 ± 0.4 | 4.1 ± 0.9 |

* Lactate dehydrogenase. The lactate dehydrogenase assay is an index of plasma membrane integrity. When membrane is disrupted, lactate dehydrogenase is released into the medium. The control arbitrarily set to 0% release, and 100% (maximal possible) release is measured in the presence of 0.1% Triton X-100 in Ham’s media. At 20 μM ceramide, over 96% of lactate dehydrogenase is retained in the cell.

* The MTT assay measures mitochondria function. Intact mitochondria cleave MTT, whereas disrupted ones do not. Control cells were arbitrarily set to 100% cleavage. 0% cleavage was set by cells incubated with 0.1% Triton X-100.

* Each test was performed after 24 h of treatment with or without 20 μM C2- ceramide in HAMSe media under the same conditions as for Fig. 1. The control incubation contained vehicle only.

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**Fig. 3. Effect of C2-dihydroceramide, a ceramide analog that is not associated with apoptosis, on cholesterol efflux from CHO cells.** Cells were labeled with 1 μCi of [3H]cholesterol in HAMSe for 24 h. Washed monolayers were treated with 10 μg/ml apoA-I with vehicle (open bar), 20 μM C2- ceramide (Cer) (hatched bar), or 20 μM C2- dihydroceramide (Dihydro) (striped bar). Efflux of [3H]cholesterol was measured after 24 h. The error bars represent 1 sample standard deviation from measurements made in triplicate. The * indicates a significant difference from the non-ceramide-treated condition by Student’s t test (p < 0.05).

**Fig. 4. Effect of ceramide on the aqueous diffusion mode of cholesterol efflux to phospholipid-containing small unilamellar vesicles.** The cholesterol efflux experiments were performed on WT CHO cells as in Fig. 2. Washed monolayers were treated with HAMSe containing 10 μg/ml apoA-I (right side) or 15 μg/ml phospholipid egg yolk phosphatidylycholine vesicles (left side) and 20 μM C2- ceramide (hatched bars) or vehicle (open bars). [3H]Cholesterol efflux was measured after 24 h. The error bars represent 1 sample standard deviation from measurements made in triplicate. The * indicates a significant difference from the non-ceramide-treated condition by Student’s t test (p < 0.05).
This indicates that the increased ABCA1 at the cell surface results in an increased propensity for apoA-I binding to the cell.

**DISCUSSION**

Most of the data in the literature describe a transcriptional control of cellular ABCA1 content and subsequent cholesterol efflux by cAMP (36, 37) or orphan receptor (31–35)-mediated pathways. However, it was recently shown that post-translational factors such as phosphorylation (63) or unsaturated fatty acid treatment (64) can affect ABCA1 functionality and trafficking. In the present study, we demonstrate that levels of ceramide can also be a modulator of the ABCA1-mediated cholesterol efflux pathway.

Our data clearly demonstrates that C₂-ceramide can induce
Cholesterol efflux from a variety of cell types. This is true in transformed rodent cells such as CHO and in primary human cells such as the aSMCs. We have also measured a similar effect in mouse peritoneal macrophages. Thus, the effect is generally applicable and not due to peculiarities within a particular cell type. Interestingly, ceramide only induced cholesterol efflux in cells actively expressing ABCA1 and did not affect cholesterol efflux via the diffusional pathway. These observations strongly indicate that the effect is specific for ABCA1-mediated cholesterol efflux. The effects of C2-ceramide in this study were not due to cytotoxicity or apoptosis as shown by three different assays and the ability of C2-dihydroceramide to promote the effect. We also found that the effects of C2-ceramide are fully reversible, further arguing against a toxicity or cell death mechanism. Finally, pharmacological treatments known to increase endogenous levels of ceramide showed an increase in cholesterol efflux to apoA-I. In terms of the mechanism of action, ceramide-induced efflux appeared to be caused by an increase in cellular ABCA1 content. This increase led to an elevated plasma membrane content of ABCA1 and enhanced binding of fluorescently labeled apoA-I. Overall, these findings suggest a link between the ABCA1/apoA-I cholesterol efflux and sphingolipid pathways.

We were intrigued that ceramide increased cholesterol efflux after a relatively long period of 8–10 h of treatment. One possibility for this delay is that ceramide (or a metabolite of ceramide) must reach a critical concentration in a particular cellular compartment before it exerts an effect on cholesterol efflux. Although this study did not examine the effects of ceramide on other proteins involved in cholesterol trafficking or efflux to lipoproteins, it is unlikely that ceramide increases ABCA1 expression via a transcriptional mechanism. Two different cell lines, RAW macrophages (untreated with cAMP analogs) and HeLa, do not display any increase in ABCA1 expression or cholesterol efflux. Thus, ABCA1 must already be expressed to mediate any ceramide-induced effect. This supports a post-translational mechanism for ABCA1 regulation.

Ceramide may affect the ABCA1-mediated pathway in a number of ways. The physical properties of ceramides are such that they affect vesicle/membrane fusion events. Therefore, ceramide could be envisioned to act by altering the trafficking of ABCA1 itself. Using GFP-ABCA1 and time lapse photography, it was shown that GFP-ABCA1 shuttles between the plasma membrane and endocytic compartment.

Movement of ABCA1 may be integral to the process of cholesterol efflux and could feasibly be modulated by ceramide by affecting ABCA1 cellular distribution. Based on our current data, two possible mechanisms can be envisioned: (a) increased shuttling of ABCA1 to the plasma membrane from the interior or (b) delayed shuttling of ABCA1 from the plasma membrane to the interior. In support of both, one study has found that short-chain ceramide treatment decreases the rate of endocytosis in CHO cells (65). This effect led to decreases in low density lipoprotein (and, presumably, low density lipoprotein receptor) internalization and trafficking to the lysosome. A similar mechanism could function in the current study where the internalization and subsequent degradation of ABCA1 is significantly slowed allowing for increased apoA-I interaction and cholesterol removal. Alternatively, ceramide may inhibit an element of a degradation pathway used to regulate cellular ABCA1 content. Current studies are aimed at distinguishing between these possibilities.

Another aspect of ABCA1-mediated cholesterol efflux that may be affected by ceramide is the transport of cholesterol to ABCA1-accessible pools. The release of cholesterol from interactions with sphingomyelin may depend on the breakdown of sphingomyelin. Several studies have correlated membrane sphingomyelin content as an important determinant for cholesterol desorption (40, 66–68). Interestingly, it was found that ceramide can have a positive feedback effect on acid sphingomyelinase expression. Increased acid sphingomyelinase activity in CHO cells may result in increased cholesterol release or availability from the lysosomal compartment. We have found that ceramide-induced cholesterol efflux to apoA-I is defective in a Niemann-Pick C CHO cell line and can be blocked with lysosomal cholesterol trafficking blocking agents in WT CHO cells. However, the formation of ceramide from sphingomyelin breakdown may have additional effects such as activation of specific cell signaling pathways related to cellular cholesterol trafficking. For example, ceramide has been implicated in the activation of certain isoforms of protein kinase C (45), which are known to affect the translocation of intracellular cholesterol for apolipoprotein-mediated efflux (28, 29). Also, a recent study found that the presence of apoA-I increases vesicular transport from the Golgi to the plasma membrane, which requires functional ABCA1 (70). Therefore, it is possible ceramide complements this process thereby increasing cholesterol movement via the Golgi to the plasma membrane for efflux.

In summary, increasing cellular ceramide levels stimulate cholesterol efflux via the ABCA1 pathway. Although the specific function(s) of ceramide in relation to cholesterol efflux remain to be determined, the mechanism involves a net increase in cellular ABCA1 protein levels. The findings reported here, particularly the effectiveness of C2-dihydroceramide, may have therapeutic implications in the treatment of cardiovascular diseases where enhanced cellular cholesterol removal by apolipoproteins would be beneficial.

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