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Ceramide enhances cholesterol efflux to apolipoprotein A-I by increasing the cell surface presence of ATP-binding cassette transporter A1

Scott R. Witting, J. Nicholas Maiorano and W. Sean Davidson*

*To whom correspondence should be addressed:

Department of Pathology and Laboratory Medicine, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, Ohio 45267-0529 USA

Telephone: (513) 558-3707 Fax: (513) 558-2289 E-mail: Sean.Davidson@UC.edu

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ABSTRACT

It is widely accepted that functional ABCA1 is critical for the formation of nascent high-density lipoprotein (HDL) 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 two to three fold increases in cholesterol efflux to apoA-I. Cell lines not expressing ABCAI were unaffected by ceramide. We further characterized the cholesterol efflux effect in Chinese hamster ovary (CHO) 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 was increased with ceramide treatment. Also, ceramide enhanced the binding of fluorescently-labeled apoA-I to CHO cells. These data suggest that ceramide may increase the plasma membrane content of ABCA1 – leading to increased apoA-I binding and cholesterol efflux.

INTRODUCTION

The importance of circulating plasma high density lipoprotein (HDL) is implicit from several population studies showing a 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 if 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 (PLC) and D (PLD) (26;27), protein kinase C (PKC) (28-30), LXR/RXR ligands (31-35), and cyclic AMP (cAMP) (36;37). In the case of PLC, PLD, and PKC, it is thought that these molecules control cholesterol transport to ABCA1.

Another potential tactic the cell can employ 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 to apoA-I only in cells expressing ABCA1. This effect was 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 (aSMC) and SmGM-2 Bullet Kit media were from BioWhittaker (Walkersville, MD). All other cell culture media and supplements were from Invitrogen (Carlsbad, CA). Chinese hamster ovary (CHO), HeLa, and RAW 264.7 mouse macrophage cells were from American Type Culture Collection (ATCC, Manassas, VA). C2-ceramide (D-*erythro*-N-acetylsphingosine), C2-dihydroceramide (D-*erythro*-N-acetyldihydrosphingosine), NHS-SS-biotin, fatty acid free bovine serum albumin (BSA) were from Calbiochem (San Diego, CA). D-MAPP ((1S, 2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol) was from BIOMOL (Plymouth Meeting, PA). Methyl-cyclodextrin, propidium iodide, Rnase A, and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) were from Sigma (St. Louis, MO). Egg phosphatidylcholine was acquired from Avanti Polar lipids (Birmingham, AL). SDS-PAGE gels (Redi-gels) were from Bio-Rad (Hercules, CA). All other chemical reagents were of the highest grade from Fisher (Pittsburgh, PA).

Cell Culture – SMCs were grown in smooth muscle basal medium (SmBM) with SmGM-2 BulletKit supplements and maintained as per manufacturer instructions. HeLa cells and RAW macrophages were grown in DMEM with 10% FBS, 10 μg/ml gentamycin (DMEMg). CHO cells were maintained in Ham's F-12 with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin (HAMSg). GFP-ABCA1 expressing CHO cells were grown in HAMSg supplemented with 2 mg/ml of G418. GFP-ABCAI expressing HeLa cells were maintained as described previously (46). All cells were maintained at 37^{o} C in a humidified 5% CO_2 atmosphere (cell culture incubator).

Sphingolipids were added from stock ethanolic solutions with the final media concentration of ethanol always less then 0.5%.

Cholesterol Efflux Experiments – Cells were grown to 90% confluency in 12-well plates in their respective growth media as described above. Monolayers were then labeled with 1 μCi/ml of [^{3}H]cholesterol in the appropriate serum-free basal media supplemented with 0.2% BSA (designated DMEMe, HAMSe, or SmBMe). After 24 h, the labeling media was removed and cells were washed twice with phosphate buffered saline (PBS) with 0.2% BSA and once with appropriate basal media containing 0.2% BSA. Cholesterol acceptors (in basal media with 0.2% BSA) were added to yield the following final concentrations: 10 μg/ml human plasma apoA-I, 15 μg/ml phosholipid vesicles, and 0.5 mM methylcyclodextrin. Sphingolipid treatments were added as described in figure legends with untreated wells receiving equivalent volumes of ethanol vehicle. A 150 μL sample of efflux media 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. Media and cell associated [^{3}H]cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by the following equation: [total media counts/(total cellular counts + total media counts)]*100.

Cytotoxicity Tests – Cells were grown in microtiter plates and subjected to the experimental culture conditions and treatments as described for efflux experiments (without radiolabel). 0.5 mg/ml MTT was added to each well and incubated for 4 h in the cell culture incubator. Solubilization buffer (10% SDS in 0.01 M HCl) was added to each well and incubated in cell culture incubator overnight. Absorbance was measured at

550 nm on a mirotiter plate reader. Percent MTT cleavage was determined as follows: [(treatment value – media with vehicle value)/(0.1% Triton-X value - media with vehicle value)] *100. LDH release assay was performed according to manufacturer's instructions (Roche, Indianopolis, IN).

Hypodiploid Nuclei Determination – Performed as described previously (47). Briefly, cells were grown in T-75 culture flasks and subjected to the experimental culture conditions treatments as described for efflux experiments (without radiolabel). Cells were trypsinized, washed in PBS, and then fixed with 70% ethanol at -20^{o} C overnight. After washing in PBS, cells were incubated with DNA staining reagent (0.1 mM EDTA, 0.05 mg/ml Rnase A, 50 ug/ml Propidium Iodide) for 15 min. Stained cells were analyzed by flow cytometry. Percent apoptotic cells were defined as the sub G_0/G_1 cell cycle peak.

Cellular ABCA1 Content and Cell Surface Biotinylation – Performed as described previously (48) with slight modifications. CHO cells were grown in T-75 flasks to confluency. Media was replaced with HAMSe with or without 20 uM C2-ceramide and incubated for 16 h. Cells were put on ice and then washed twice with ice cold PBS. Surface proteins were biotinylated with 1 mg/ml NHS-SS-biotin (in PBS) for 30 min at 4^{o} C on a platform rotator. Cells were then washed twice with ice cold quench buffer (50 mM Tris, 0.1 mM 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 (50mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.2% NaN_3, 0.1% SDS, 100 µg/ml PMFS 1µg/ml aprotonin, 1% Triton-100, 0.5% sodium deoxycholate) and incubated on ice for 30 min. Cellular debris was pelleted by centrifugation at 4^{o} C.

Cleared supernatant was transferred to a fresh tube and aliquots were taken for protein concentration determination. 55 μg of cell protein 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 immobilized streptavidin gel (Pierce, Rockford, IL) and incubated overnight on a platform mixer at 4^{o} C. Gel was pelleted and washed 5 times with lysis buffer. 55 μl of loading dye (60mM Tris-Cl (pH 6.8), 25% glycerol, 2% SDS, 0.1% bromophenol blue) with 350 mM of beta-mercaptoethanol was added to the gel pellet and incubated at 37^{o} C for 30 min. 30 μl was run on a 4-15% SDS-PAGE 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-PAGE gels and run for 1 h at 200 V. Proteins were transferred to nitrocellulose and blocked for 2 h at 4^{o} 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^{o} C, washed, and then incubated with 1:5000 HRP-conjugated anti-rabbit secondary (Amersham-Pharmacia) for 1 h at room temperature. For total cell extracts, blots were subsequently striped, blocked in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) with 10% dried milk, and reprobed with 1:2000 anti-actin (Novus Biologicals, Littleton, CO) for 2 h at room temperature and then 1:3000 HRP-conjugated anti-rabbit secondary (Amersham-Pharmacia) for 1 h at room temperature. Blots were developed with an ECL kit (Amersham-Pharmacia) as per manufacturer instructions.

Binding of Fluorescently-labeled apoA-I (ALEXA488-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-ABCAI were grown to near confluency on slide well plates (#138121, Nalge Nunc International, Rochester, NY) in the maintenance media described above. Cells were then pretreated with or without 20 μM C2 ceramide in CHOe for 16 h. After one quick wash with CHOe, fresh CHOe containing 10 μg/ml of 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 then fixed with 3% paraformaldehyde/2% sucrose on ice for 30 minutes. Cells were again washed four times with ice cold PBS. Slides were then coversliped 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 of Dr. Richard Lawn) in the mammalian expression vector pEGFP-N1 (Clontech, Palo Alto, CA) was stably expressed in Chinese hamster ovary (CHO) cells using the Lipofectamine Plus kit (Invitrogen, Carlsbad, CA) 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 aortic smooth muscle cells (aSMCs). Both cell lines contain relatively high levels of ABCA1 (52) and span the spectrum between easy to manipulate transformed cell models (CHO) and primary human cells that are relevant to the atherosclerotic disease process in the arterial wall (aSMC) (17). The presence of ABCA1 in these cells was confirmed by western blot analysis (data not shown). For this study, we intentionally avoided the use of pharmacological induction of ABCA1 expression (such as cAMP induction of ABCA1 in RAW macrophages) that would undoubtedly confound any conclusions drawn about ABCA1 regulation. To examine the potential effect of ceramide on cholesterol efflux, we treated both cell types with C2 ceramide, a cell permeant ceramide analog. Figure 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 Figure 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 ABCAI 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, an LDH release assay as an index of plasma membrane integrity, and hypodiploid nuclei analysis to assess apoptosis. Table 1 shows that none of the three tests revealed significant cytotoxicity (MTT, LDL 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). Figure 3 shows that C2-dihydroceramide treatment causes ceramide-induced cholesterol efflux to levels equivalent to C2-ceramide. Taken with the data in Table 1, 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 non-specific aqueous diffusion mechanism (58) in the presence of phospholipid-containing acceptors(59;60). To determine if ceramide affects this pathway, we studied the effect of ceramide on the efflux of cholesterol from CHO cells to small unilamellar vesicles (SUV) of phospholipid. Figure 4 shows that ceramide had no effect on this transfer. We also found that ceramide did not affect the ability of methylcyclodextrins to remove cholesterol by the same mechanism (data not shown). This indicates that the effect of ceramide is specific for the ABCA1 mediated pathway and argues against the possibility that the ceramide effects observed in Figs. 1-3 are due to a non-specific perturbation of the plasma membrane.

We next examined the kinetics of ceramide-induced cholesterol efflux to apoA-I. The time course experiment in Figure 5A shows that cells began to exhibit significantly increased cholesterol efflux at approximately 8-10 h after exposure to ceramide. We then measured how long the effect persists in ceramide-stimulated cells after ceramide is removed from the medium. There was a progressive loss of cholesterol efflux ability over time with efflux levels returning to baseline by 16 - 24 h (Fig. 5B). The continuous presence of ceramide in the media, in contrast, maintained enhanced cholesterol efflux.

These data show that the ceramide effect on cholesterol efflux takes several hours to manifest and is a fully reversible phenomenon.

Although C2-ceramide can be found in cellular membranes (61), longer chain ceramides are likely the physiological mediators of most ceramide signaling events in the cell. Therefore, to examine the effects of increasing endogenous ceramide levels, CHO cells were treated with the inhibitor D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol or MAPP. This compound prevents the breakdown of intracellular ceramide by 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 PL SUV (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 to 2 fold after treatment with ceramide. Similar treatment of RAW macrophages (unstimulated with cAMP analogs) and WT HeLa cells did not increase ABCAI 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 non-specifically 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^{o} C with NHS-X-X-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. Figure 7b shows that ceramide treatment increases the amount of ABCA1 present at the cell surface. Densitometry quantitation indicated the increase was about 1.75 to 2 fold that of untreated cells. We also examined the binding of fluorescently-labeled apoA-I to CHO cells expressing GFP-ABCAI with or without 20 μM ceramide pre-treatment. The confocal microscope images in Figure 8 demonstrate that a significantly increased degree of bound fluorescence at the surfaces of the cells in the presence of ceramide vs. untreated cells. This indicates that the increased ABCAI 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 LXR/RXR (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 (Du and Witting, unpublished result). 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 a caused by an increase in cellular ABCA1 content. This increase led to an elevated plasma membrane content of ABCAI and enhanced binding of fluorescently-labelled 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 hours 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 ABCAI expression via a transcriptional mechanism. Two different cell lines, RAW macrophages (untreated with cAMP analogs) and HeLa, do not display any increase in ABCAI expression or cholesterol efflux. Thus, ABCAI 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 (44). 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 (46). This 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 ABCAI *from* the plasma membrane to the interior. In support of "B", one study has found that short-chain ceramide treatment decreases the rate of endocytosis in CHO cells (65). This effect led to decreases in LDL (and, presumably, LDL-receptor) internalization and trafficking to the lysosome (65). A similar mechanism could function in the current study where the internalization and subsequent degradation of ABCAI 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 ABCAI 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 (69). 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 (Witting et al, manuscript in preparation). 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 PKC (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 stimulates 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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FOOTNOTES

[1]Abbreviations: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; CHO, Chinese hamster ovary; PLC, phospholipase C; PLD, phospholipases D; PKC, protein kinase C; cAMP, cyclic AMP; aSMC,aortic smooth muscle cells; BSA, bovine serum albumin; D-MAPP, (1S, 2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol; MTT, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; GFP, green fluorescent protein; LDH, lactate dehydrogenase; PMSF, phenylmethyl sulfonyl fluoride; FC, free (unesterified) cholesterol; HDL, high density lipoprotein; SUVs, small unilamellar vesicles; pAI, plasma apoA-I; PS, phosphatidyl serine.

TABLES

Table 1: The effect of ceramide on CHO cell toxicity and apoptosis.

C-2 Ceramide (μM)[A]	LDH (%)[B]	MTT (%)[C]	Hypodiploid Nuclei (%)
0	0	100	3.8 ± 1.1
20	3.8 ± 1.4	102.7 ± 0.4	4.1 ± 0.9

A: Each test was performed after 24 h of treatment with or without 20 μM C-2 ceramide in HAMSe media under the same conditions as for Fig. 1. The control incubation contained vehicle only.

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

C: 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.

FIGURE LEGENDS

Figure 1. Effect of ceramide on cholesterol efflux from human aortic smooth muscle cells (aSMC) and CHO cells. aSMCs and CHO cells were exchange labeled with 1 μCi of ^{3}H-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 ^{3}H-cholesterol to apoA-I was measured after 24 h. Percent of total cholesterol present at the beginning of the efflux incubation that appeared in the media was calculated as described in "Materials and Methods". Cell incubations lacking ceramide contained an identical volume of vehicle (ethanol). CHO cells (filled circles) and aSMC (open circles) are shown. The error bars represent 1 sample standard deviation from measurements made in triplicate.

Figure 2. Effect of ceramide on cell lines expressing different levels of ABCA1. Cells were labeled with 1 μCi of ^{3}H-cholesterol in DMEMe (RAW 264, HeLa) or HAMSe (wt-CHO, ABCA1-CHO) for 16 h. After washing, the appropriate media containing 10 μg/ml apoA-I and 20 μM ceramide (hatched bars) or vehicle alone (open bars) was added. Efflux of ^{3}H-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 students t-test ($P<0.05$).

Figure 3. The 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 ^{3}H-cholesterol in HAMSe for 24 h. Washed monolayers were treated with 10 μg/ml apoA-I with vehicle (open bar), 20 μM C2 ceramide (hatched), or 20 μM C2 dihydroceramide (striped). Efflux of ^{3}H-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 students t-test ($P<0.05$).

Figure 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 PC vesicles (left side) and 20 μM C2 ceramide (hatched) or vehicle (open). ^{3}H-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 students t-test ($P<0.05$).

Figure 5. Time dependence of the ceramid-induced cholesterol efflux effect. A) Time required for effect to appear in the presence of ceramide. CHO cells were labeled with 1 μCi ^{3}H-cholesterol in HAMSe for 16 h. Washed monolayers were treated with HAMSe containing 10 μg/ml apoA-I with (open circles) or without (filled circles) 20 μM C2 ceramide. ^{3}H-cholesterol efflux was measured at the indicated times. B) Time required for effect to "die away" when cells that had been pre-stimulated with ceramide are incubated in its absence. CHO cells were labeled as in A. The monolayers were then

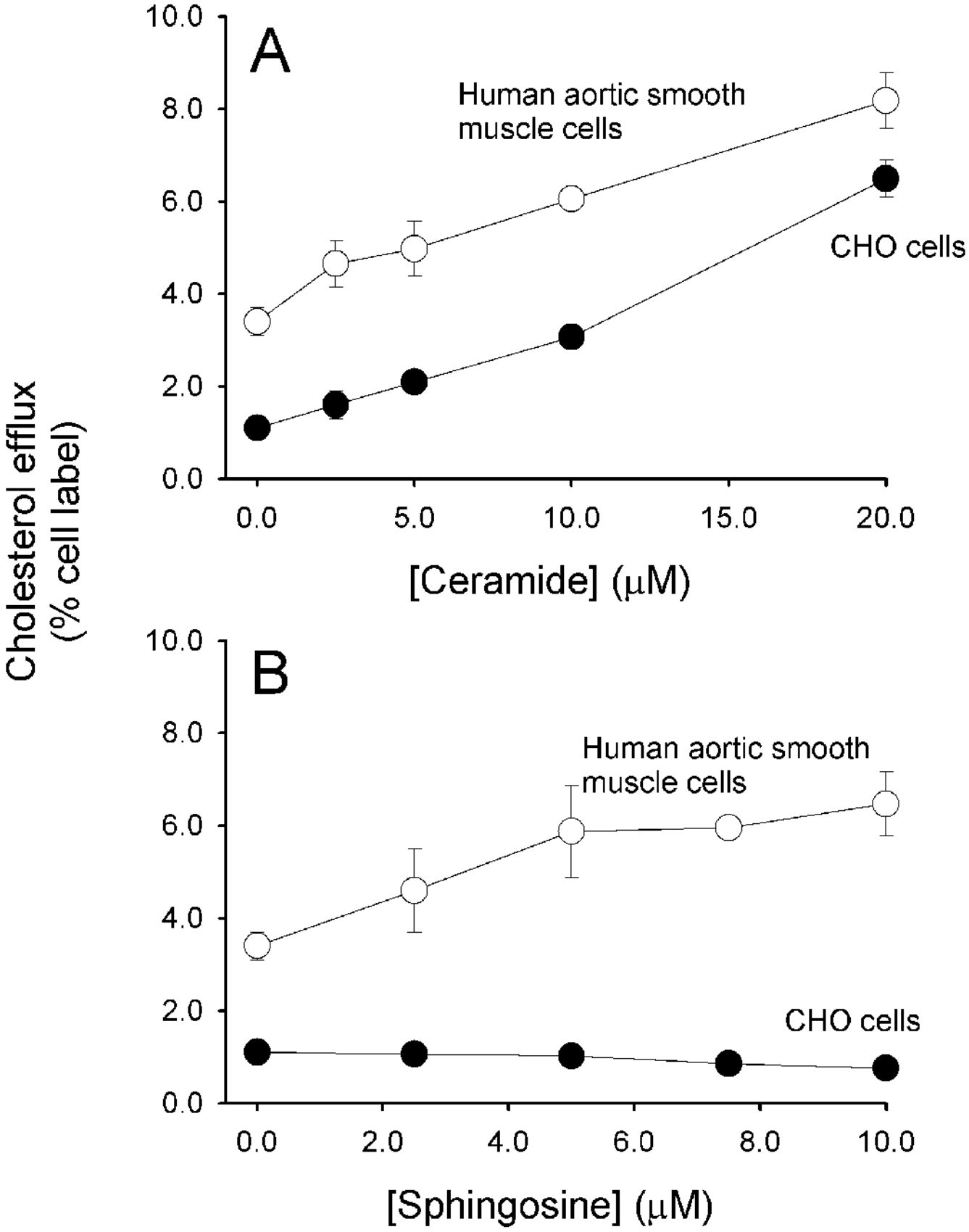
pre-treated with 20 μM ceramide (+) for 16 h to fully activate the cholesterol efflux effect or vehicle only (-) as a control (Pretreatment). The cells were washed and incubated in a secondary incubation containing fresh HAMSe without ceramide (-) or with ceramide (+) as a control (Post treatment). At the indicated time points, the post treatment was interrupted and the cells were washed and allowed to efflux cholesterol to 10 μg/ml apoA-I in the presence of ceramide for 4 h. The error bars represent 1 sample standard deviation from measurements made in triplicate. The * indicates a significant difference between the open and filled squares by students t-test ($P<0.05$).

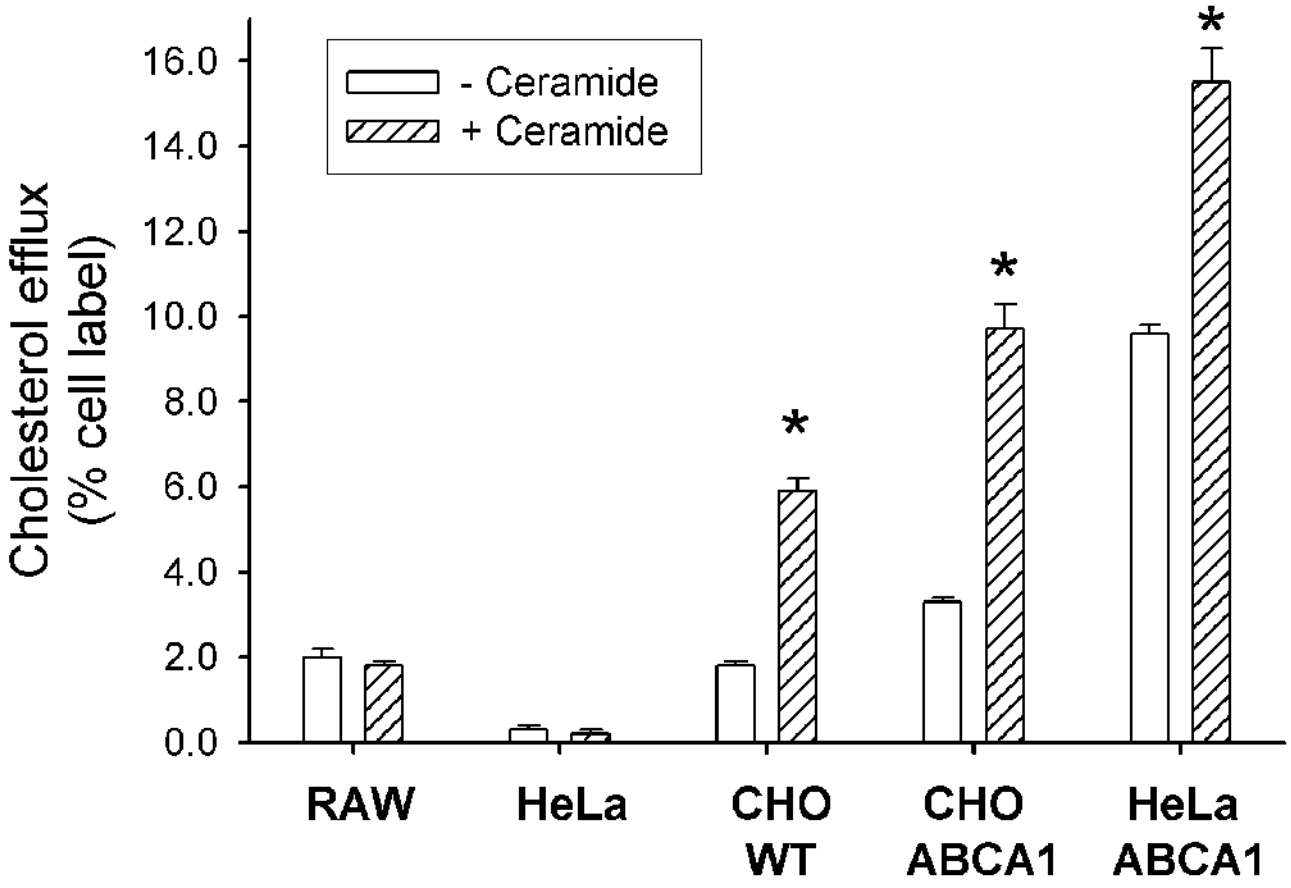
Figure 6. Effect of MAPP manipulation of endogenous ceramide on cholesterol efflux to apoA-I. The experiments were carried out as in Fig. 1. Washed CHO monolayers were treated with 10 μg/ml apoA-I with either vehicle (open bars) or 30 μM MAPP (striped bars). Efflux of ^{3}H-cholesterol was measured after 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 students t-test ($P<0.05$).

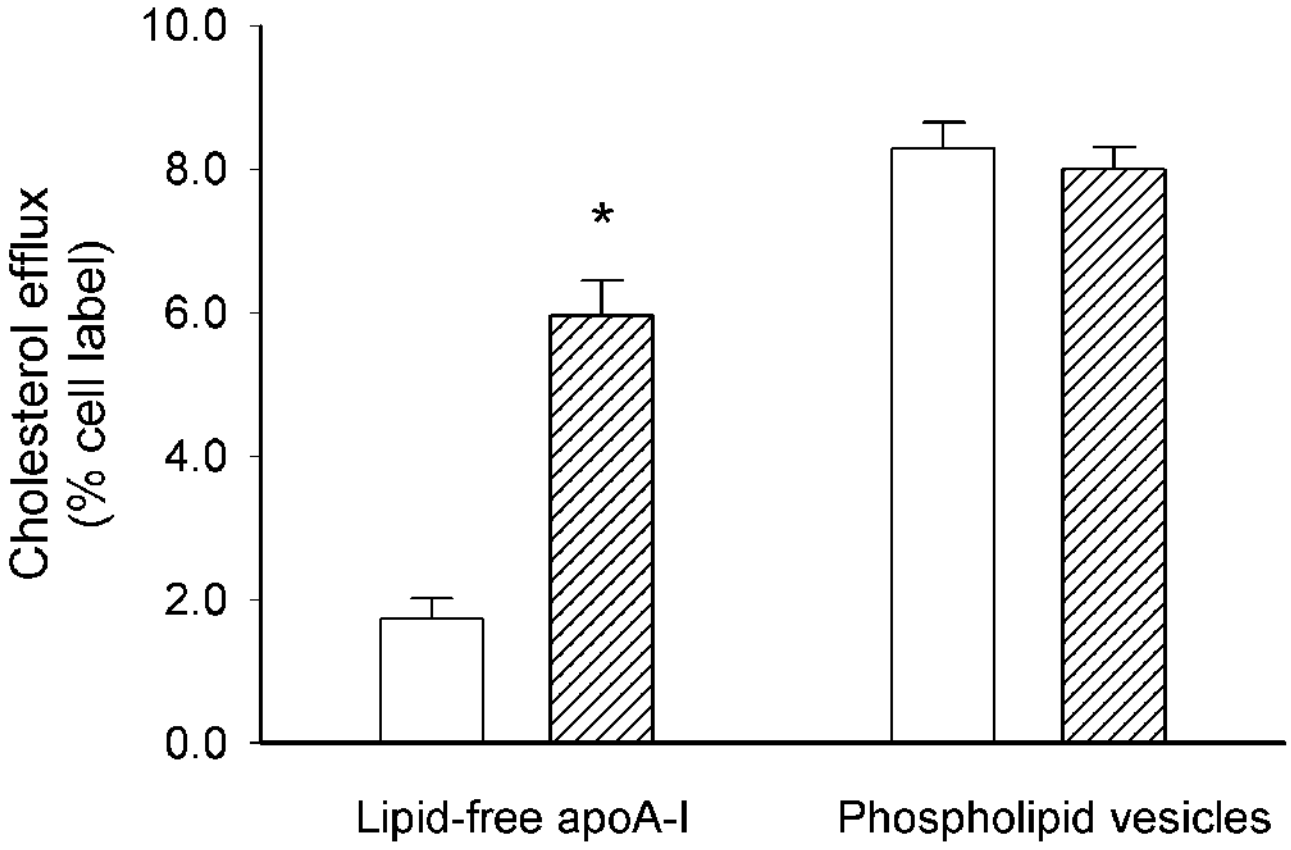
Figure 7. Effect of ceramide on the expression and cell surface localization of ABCA1 in CHO cells. Confluent monolayers of CHO cells were incubated for 16 h in the presence or absence of 20 μM C2-ceramide. Before harvesting, cell surface proteins were biotinylated at 4^{o} C for 30 min. Cells were then lysed and protein concentration was determined. **A)** 55 μg aliquots of the cell extract for *total cellular ABCA1 content* determination by Western blot analysis using anti-ABCA1 polyclonal antibody. Cellular

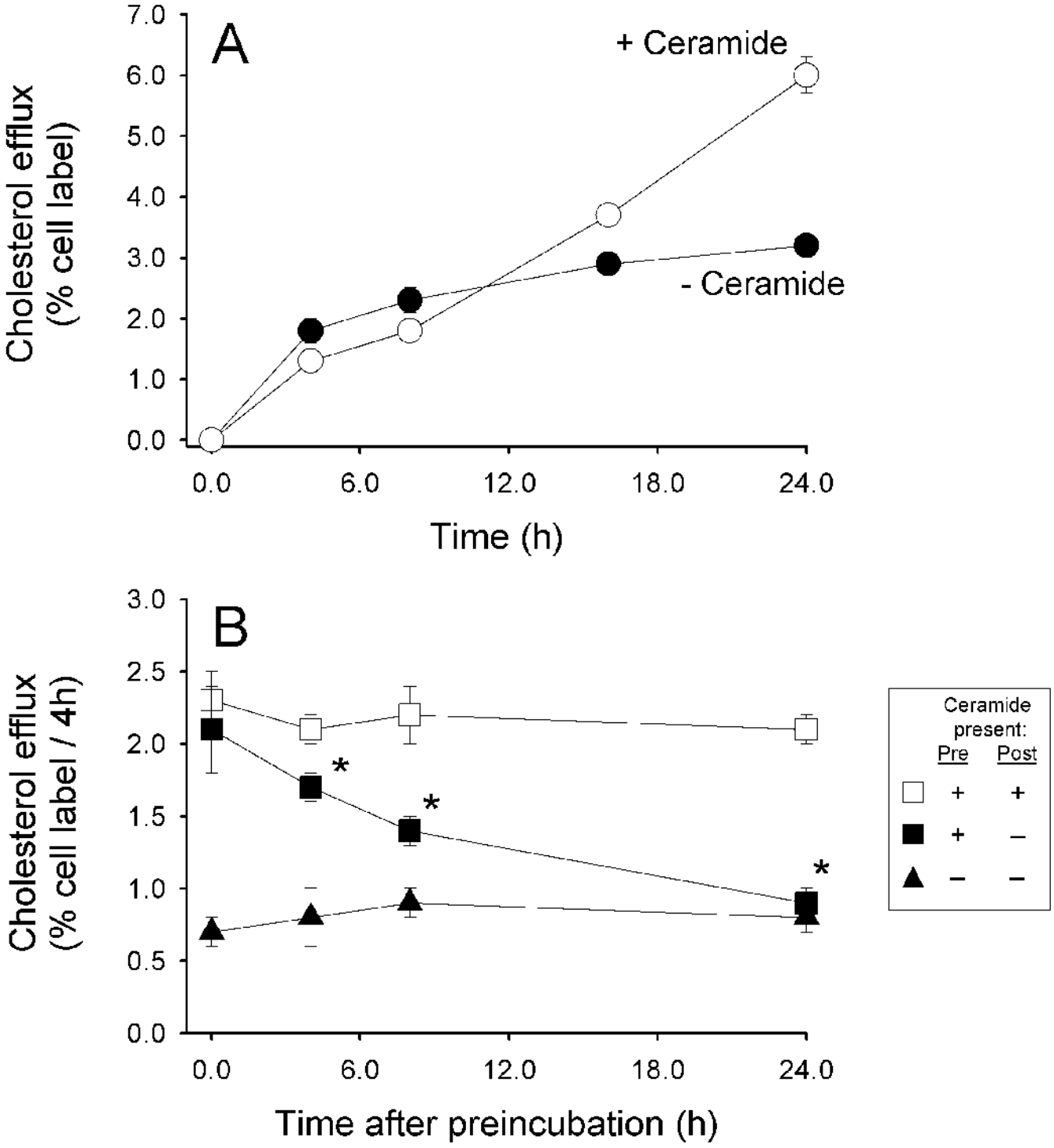
extract from RAW macrophages stimulated with cAMP to express ABCA1 was run as a positive control. Both bands represent ABCA1 (possibly different glycosylation states). **B)** 200 μg aliquots of the CHO cell extracts were incubated with streptavidin beads to isolate biotinylated proteins for determination of *cell surface ABCA1*. Western blot for ABCA1 was performed with the extracts from A.

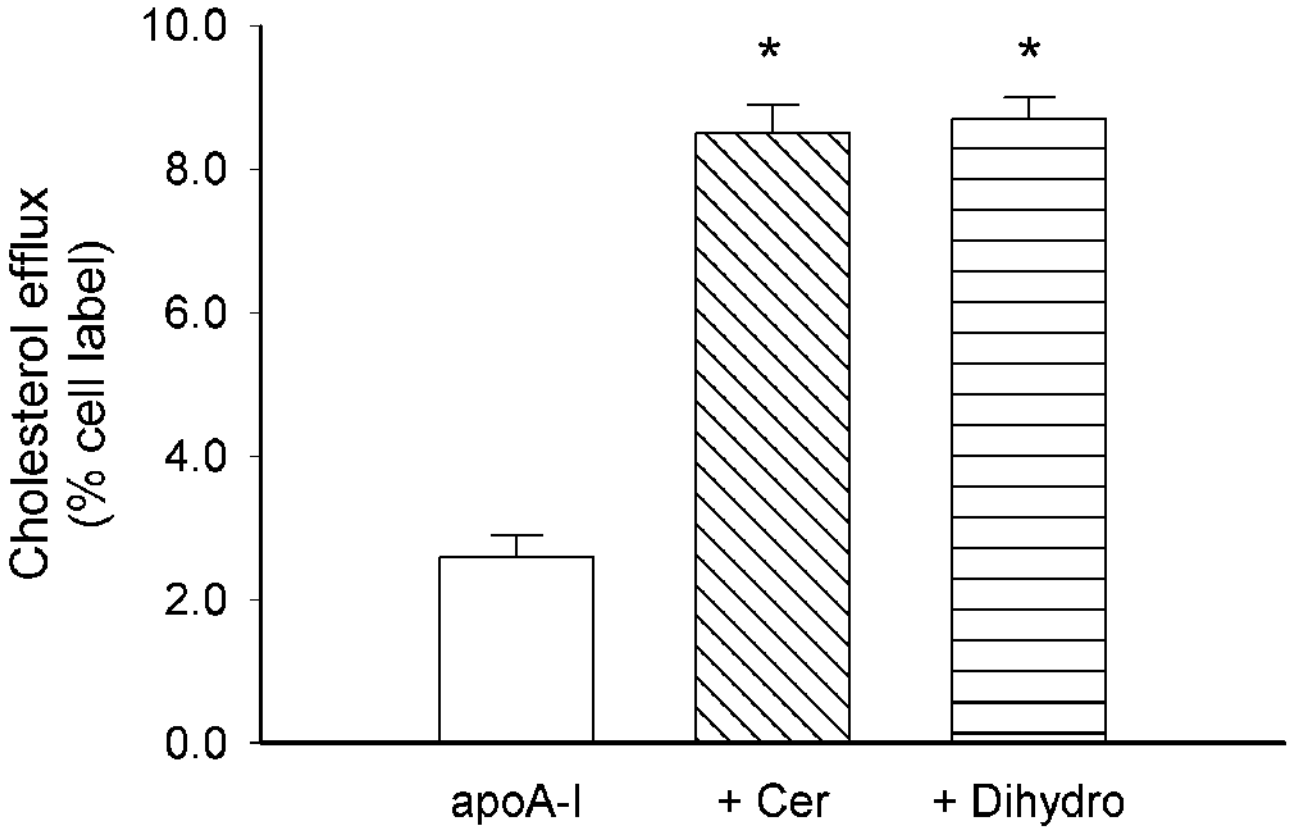
Figure 8. Binding of ALEXA-apoA-I to CHO cells expressing GFP-ABCAI. Confluent monolayers of CHO cells expressing GFP-ABCAI in slide-well chambers were incubated with (B) or without (A) 20 μM ceramide for 16 h. ALEXA-apoA-I (10 μg/ml) was added to each well and the slide-wells were incubated on ice for 2 h. Monolayers were then washed, fixed, and coverslipped for imaging on a confocal microscope. Images are representative fields from the same experiment. Each image was taken within minutes of each other with identical microscope settings.

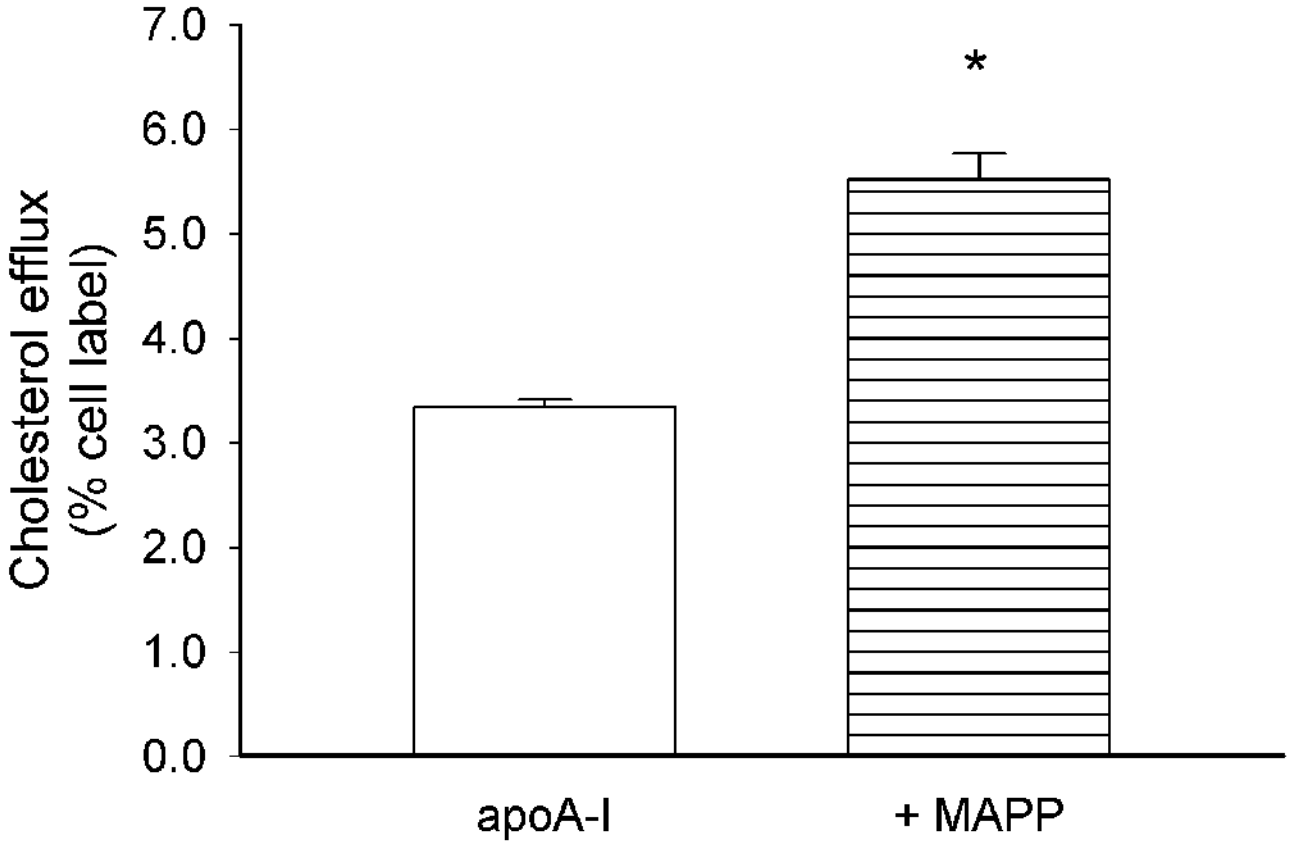

A
Human aortic smooth muscle cells
CHO cells
10.0
8.0
6.0
4.0
2.0
0.0
0.0
5.0
10.0
15.0
20.0
[Ceramide] (μM)
B
Human aortic smooth muscle cells
CHO cells
10.0
8.0
6.0
4.0
2.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
[Sphingosine] (μM)
Cholesterol efflux (% cell label)

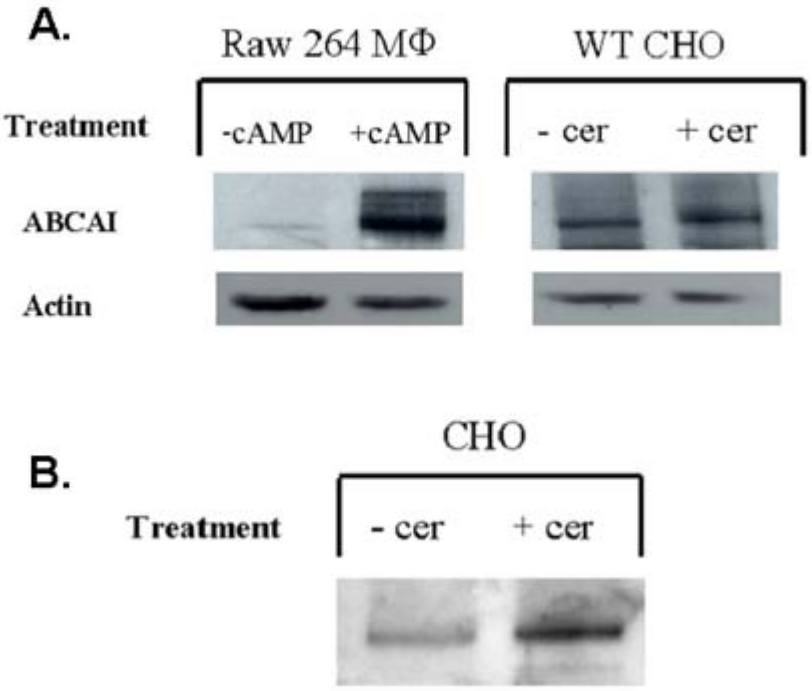

Cholesterol efflux
(% cell label)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
- Ceramide
+ Ceramide
*
*
*
RAW
HeLa
CHO
WT
CHO
ABCA1
HeLa
ABCA1

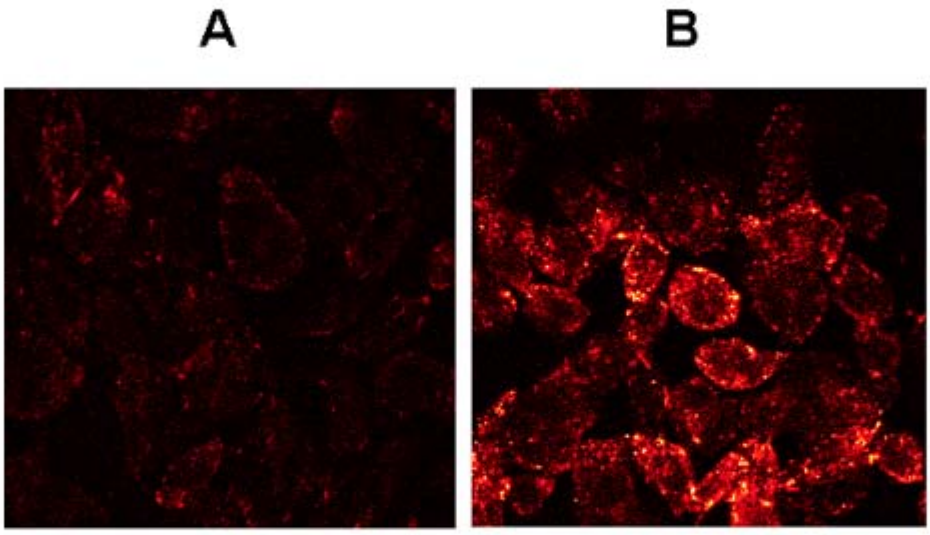

10.0
8.0
6.0
4.0
2.0
0.0
Cholesterol efflux
(% cell label)
*
Lipid-free apoA-I
Phospholipid vesicles


A
+ Ceramide
- Ceramide
Cholesterol efflux
(% cell label)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.0
6.0
12.0
18.0
24.0
Time (h)
B
Cholesterol efflux
(% cell label / 4h)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
0.0
6.0
12.0
18.0
24.0
Time after preincubation (h)
Ceramide present:
Pre
Post
+
+
+
–
–
–


Cholesterol efflux
(% cell label)
10.0
8.0
6.0
4.0
2.0
0.0
*
*
apoA-I
+ Cer
+ Dihydro


Cholesterol efflux
(% cell label)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
apoA-I
+ MAPP


A.
Raw 264 MΦ
WT CHO
Treatment
-cAMP
+cAMP
- cer
+ cer
ABCAI
Actin
B.
CHO
Treatment
- cer
+ cer


A
B