Title: Effect of sphingomyelin on the lipid-export activities of ABCA1 and ABCB4

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Effect of sphingomyelin on the lipid-export activities of ABCA1 and ABCB4

(ABCA1 と ABCB4 の脂質排出活性に対する
スフィンゴミエリンの影響)

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2015
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INTRODUCTION

Lipids are necessary nutrients for human-beings because they are one of the basic components of cells and form the main framework of bio-membranes. However, excessive intakes of lipids over necessities are harmful for health, causing diseases such as arteriosclerosis. Thus, it is important to keep lipid homeostasis.

ATP-Binding Cassette (ABC) Transporters are a family of proteins in living cells mediating vital transport processes including the cross-membrane transport of lipids. Several ABC transporters play key roles in the metabolism of lipids. For example, ABCA1 removes excessive cholesterol from peripheral cells and transfers cholesterol to apolipoprotein A-I (apoA-I) during the formation of nascent high-density lipoprotein (HDL). Nascent HDL then receives cholesterol and sphingomyelin (SM) transported by ABCG1 to mature and returns cholesterol to liver. In liver, cholesterol is converted to bile salts and then secreted by ABCB11. Bile salts are strong surfactants and bring damage to the membrane of bile canaliculi. In the canalicular membrane, ABCB4 is expressed to secrete phosphatidylcholine (PC) to canaliculi to dilute nascent bile in order to protect the canalicular membrane of hepatocytes. In this way, ABC transporters take part in the metabolism of lipids, and help the balance of lipid homeostasis in human body.

In this thesis, the author focused on the lipid efflux activities of ABCA1 and ABCB4 as well as the impact of membrane environment on the activities of the two transporters. In human body,
ABCA1 is expressed in most tissues exporting cholesterol and PC as substrates. In contrast, expression of ABCB4 is limited to canalicular membrane of hepatocytes and ABCB4 is known to export PC to nascent bile physiologically (Figure I-1). However, it is found that both ABCA1 and ABCB4 are able to export PC and cholesterol \textit{in vitro}, and bile salts are available to serve as lipid accepters for the both.

Lipids in the plasma membrane are not distributed homogeneously but form microdomains with different compositions and properties. Some of these microdomains are characterized by the resistance to non-ionic detergent solutions and are called Detergent Resistance Membranes (DRM) or rafts. DRMs are main locations for various proteins to function, and contribute to intracellular signaling. Also DRMs are known to contain higher cholesterol and SM content than non-DRMs.

ABC transporters localize in distinguished membrane microdomains to function properly, especially those whose transport substrates are lipids. What’s more, the alteration in lipid components in the plasma membrane is known to affect the activities of these ABC transporters as well. For example, ABCA1 is known to localize in membrane sensitive to Triton X-100. When cellular SM content is decreased, the lipid efflux activity of ABCA1 is enhanced. In contrast to ABCA1, ABCG1 functions inside of Triton X-100 DRM and its lipid efflux activity is depressed when cellular SM is reduced.

Similar to ABCA1, ABCB4 is recovered from Triton X-100-sensitive membrane and transports PC and cholesterol. Therefore the author predicted that when cellular SM content is reduced, the lipid efflux activity of ABCB4 should be enhanced as ABCA1 does. However, to the author’s surprise, it was discovered that the lipid efflux activity of ABCB4 was reduced after SM inhibitor treatment. To clarify the reason for the opposite reactions to SM reduction between ABCA1 and ABCB4, the author determined the membrane environment where the two
transporters localize and compared the impact of membrane environmental change to the lipid efflux activities of the two transporters.

In Chapter I, the author examined the lipid efflux activity of ABCA1 in HEK293 as well as BHK cells and confirmed the effect of SM deletion on the lipid export activity of ABCA1 by using sphingolipid synthesis inhibitors. In Chapter II, the lipid efflux activity of ABCB4 expressed in HEK293 and BHK cells was analyzed. Then, the membrane distributions of ABCA1 and ABCB4 were compared and the impacts of membrane environment on the two transporters were also examined. From these results, it is shown that although ABCA1 and ABCB4 function similarly in transporting PC and cholesterol out of cells, they response to SM deletion in opposite ways. This probably arises from an evolutionary adaption to the membrane environment in which these transporters function.

REFERENCES


CHAPTER I

Depletion of SM enhances
ABCA1-dependent Lipid Export
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Depletion of SM enhances ABCA1-dependent Lipid Export

ABCA1 plays a key role in maintaining cholesterol homeostasis in human body. It transports cholesterol and phosphatidylcholine (PC) to extracellular lipid acceptors such as apolipoprotein A-I (apoA-I) during the formation of high-density lipoproteins (HDL) (1). The molecular biological mechanism of the process of HDL formation is being studied in recent years.

Proteins and lipids are not distributed in a homogeneous way in the plasma membrane. Specific microdomains rich in cholesterol and sphingomyelin (SM) are formed (2). These microdomains are considered to be platforms for various proteins to function and interact with each other. It was found that ABCA1 localizes out of these SM-rich microdomains (3). Another kind of ABC transporters, ABCG1, is reported to function in SM-rich microdomains, loading cholesterol and SM to nascent HDL (4,5). These results suggest that specific membrane environment may be necessary for ABC transporters to function.

Nagao et al. reported that CHO cells, defect in SM synthesis due to a mutation in ceramide transfer protein (CERT), exhibited enhanced ABCA1-mediated cholesterol and PC export activity compared to the parent CHO cells(3). However, ABCG1-mediated cholesterol and SM export activity was reported to be reduced in SM deficient CHO cells(4). These results suggest that the lipid export activities of ABC transporters are affected by cellular SM content.

SM synthesis in cells is a multistep process and is catalyzed by a series of enzymes. Firstly, L-serine and palmitoyl-CoA are condensed into 3-ketosphingasine by serine palmitoyl transferase. After a string of reduction, N-acylation and desaturation, 3-ketosphingasine is converted into ceramide. These reactions are considered to occur on the membrane of
endoplasmic reticulum (ER). Then, ceramide is transported by to the \textit{trans}-Golgi apparatus, where ceramide is converted to SM by the catalysis of SM synthase\(^{(6)}\).

In this study, two different SM synthesis inhibitors, myriocin and HPA-12, were used for SM reduction. Myriocin inhibits the reaction of L-serine and palmitoyl-CoA, and HPA-12 mimics ceramide and associates with CERT at a high affinity. With these inhibitors, the author reduced cellular SM level in HEK293 and BHK cells, and tried to confirm the alteration of PC and cholesterol export activity of ABCA1 under the SM-reduced conditions.
MATERIAL AND METHODS

Materials

The rat anti–human ABCA1 monoclonal antibody KM3073 was generated against the first extracellular domain (amino acids 45–639) of human ABCA1, as described previously (7). Anti–green fluorescent protein (GFP) antibody was purchased from Santa Cruz Biotechnology. Recombinant apoA-I was prepared as reported previously (8). The CERT inhibitor (1R,3S)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide (HPA-12) (9) and its inactive stereoisomer were kindly provided by Dr. Hanada (10). All other chemicals were purchased from Sigma-Aldrich, Life Technologies, Nacalai Tesque, Serva Feinbiochemica, and Dojindo.

Cell Culture

Both HEK293 and BHK cells were grown in a humidified incubator with 5% CO$_2$ at 37°C in DMEM supplemented with 10% heat-inactivated FBS. HEK293 cells stably expressing human ABCA1 fused with GFP at the C terminus were established as described (11). BHK/ABCA1 cells (12) were a kind gift from the late Dr. John Oram.

Measurement of SM content

Cells were washed with phosphate-buffered saline (PBS) and then air-dried. Then, membrane lipids were extracted with hexane:2-propanol (3:2), and SM content was determined as described (13).

Cellular SM and cholesterol/PC efflux assay

Cells were subcultured in poly-L-lysine–coated 6-well plates at a density of $5 \times 10^5$ cells per
well in DMEM containing 10% FBS. After incubation for 24 h, cells were washed with DMEM, and then incubated in DMEM containing 0.02% BSA containing an SM inhibitor and mifepristone in the presence of 1 mM NaTC or 5 µg/ml recombinant apoA-I. After incubation for 16 or 24 h, lipids in the medium was extracted with chloroform/methanol (2:1) and the amounts of cholesterol and PC were determined by colorimetric enzyme assays as described (14). NaTC (1mM) treatment for 24 h did not show significant cytotoxicity to BHK/ABCA1 and BHK/ABCB4 cells even after myriocin treatment at 80 µM (in this study myriocin was used at 20 or 40 µM) either in the absence or presence of mifepristone. Cellular protein concentration was measured using a BCA protein assay kit (Pierce).

Western blotting

Proteins were separated on 5–20% gradient SDS polyacrylamide gels (Atto) and immunodetected with the indicated antibodies. Blots were analyzed and quantitated using an LAS-3000 imaging system and software (Fujifilm).

Cell viability assay

Cell viability was estimated by measuring the lactate dehydrogenase (LDH) activity in media and total cells using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega).

Statistical Analysis

All experiments were repeated at least twice. Each replication of quantitative experiments was performed in triplicate. Values are presented as means ± S.E. The statistical significance of differences between mean values was analyzed using the non-paired t-test. Multiple comparisons were performed using the Dunnett test following ANOVA. A value of p < 0.05
was considered statistically significant.
RESULTS

Depletion of SM in HEK293 and BHK cells

To reduce the cellular SM level in HEK293 and BHK cell lines, the author employed myriocin, an inhibitor for the first step of sphingolipid biosynthesis, the condensation of L-serine and palmitoyl-CoA, catalyzed by serine palmitoyl transferase(9). Myriocin treatment reduced cellular SM in HEK293 or BHK cells by 20-25% (Figure 1-1 A, B). And this was not affected by expression of ABCA1 (Figure 1-1 C) or ABCB4 (Data not shown). Although myriocin was reported to be cytotoxic to some cells, lactate dehydrogenase release tests demonstrated the amount of myriocin applied in this study (20 µM for HEK293 cells or 40 µM for BHK cells) did not bring significant cytotoxicities to HEK293 or BHK cells (Figure 1-1 D and E).

Besides myriocin, HPA-12, a synthesized analog of ceramide was also employed to suppress SM production(9). HPA-12 interacts with ceramide transfer protein, which translocates ceramide from ER to the Golgi apparatus. Comparing with myriocin, HPA-12 has the following advantages: 1) HPA-12 does not block the synthesis of ceramide or glycosylceramide, which is known as a component of the plasma membrane. 2) Inactive stereoisomers of HPA-12 are also available as a negative control (Figure 1-2 A). Application of HPA-12 to BHK cells reduced cellular SM by 25%, while its stereoisomers (inactive HPA) did not reduce cellular SM level (Figure 1-2 B). In the lactate dehydrogenase release test, 10µM HPA-12 utilized in this study did not show significant cytotoxicity to BHK cells (Figure 1-2 C).
Figure 1-1 Effect of SM synthesis inhibitor myriocin. (A) HEK cells were treated by 0 or 20 µM myriocin for 24 h. Cellular lipids were extracted and analyzed. (B, C) BHK cells without (B) or with (C) induced ABCA1 expression were treated with 0 or 40 µM myriocin for 24 h. Cellular lipids were extracted and analyzed. (D, E) Lactate dehydrogenase (LDH) release from HEK (D) or BHK (E) cell lines treated with indicated concentrations of myriocin for 24 h. Open bars cells without ABCA1 expression; filled bars, cells with induced ABCA1 expression. Cytotoxicity (%) is shown as the ratio of the released LDH relative to the total (cellular and released) LDH.
Figure 1-2 Effect of SM synthesis inhibitor HPA-12. (A) The chemical structure of HPA-12 as well as its inactive stereoisomers. In this study, the inactive stereoisomers were applied to cells in the form of mixture. (B) BHK cells were treated with 10 µM HPA-12 or inactive HPA isomers for 48 h. Cellular SM was then extracted and measured. (C) Lactate dehydrogenase release from BHK cells treated with indicated concentrations of HPA-12 for 24 h. Cytotoxicity (%) is shown as the ratio of the released LDH relative to the total (cellular and released) LDH.
**Impact of SM synthesis inhibitors on expression of ABCA1**

In order to inspect the lipid efflux activity of ABCA1, ABCA1 was expressed in both HEK293 and BHK cell lines. In HEK293 cells, ABCA1 was expressed stably as described in previous report(11). In BHK cells, the author employed GeneSwitch system to create a mifepristone-induced expression(15). By treatment with 10nM mifepristone for 24 h, substantial amount of ABCA1 was expressed in BHK cells (Figure 1-3 A). To confirm the impact of SM synthesis inhibitors on the expression of ABCA1, the author treated HEK or BHK cells expressing ABCA1 with myriocin or HPA-12 at indicated concentrations. Neither myriocin nor HPA-12 treatment significantly affected ABCA1 expression in HEK nor BHK cells (Figure 1-3 B, C). These results implied that ABCA1 expression is not affected by SM reduction.

**Effect of SM reduction on ABCA1-dependent choline phospholipids and cholesterol efflux**

ABCA1 removes cellular PC and cholesterol from the plasma membrane and transports them to extracellular acceptors such as apoA-I. Treatment with myriocin enhanced ABCA1-dependent lipid efflux to apoA-I in both HEK293 and BHK cells. 20μM of myriocin treatment of HEK/ABCA1 cells for 24 h increased PC and cholesterol efflux for 18% and 37% respectively (Figure 1-4 A). PC and cholesterol efflux from BHK/ABCA1 cells treated with 40 μM myriocin for 16 h were also enhanced by 18% and 16%, respectively (Figure 1-4 B).
Figure 1-3 Impact of SM synthesis inhibitors on ABCA1 expression. (A) ABCA1 expression in BHK cells with 0 or 10nM mifepristone induction for 24 h. (B) ABCA1 expression in HEK/ABCA1 cells with 0 or 20µM myriocin treatment for 24 h. (C) ABCA1 expression in BHK/ABCA1 cells were induced by mifepristone. ABCA1 expression was analyzed after treatment with 0 or 40µM myriocin for 24 h (Left) or 0 or 10 µM for 48 h (Right). In B and C, the lower panels showed the relative expression of ABCA1. Vinculin was used as a loading control.
Figure 1-4 Enhancements of ABCA1-mediated apoA-I-dependent lipid efflux activity by SM reduction. (A) HEK/ABCA1 cells were incubated with 0 or 20 µM myriocin together with apoA-I as a lipid acceptor for 24 h, then lipids in culture solution were extracted and PC and cholesterol contents were measured. (B) BHK/ABCA1 cells were incubated with 0 or 10 µM mifepristone in the absence or presence of 40 µM myriocin together with apoA-I for 16 h. Then lipids in culture solution were extracted and PC and cholesterol contents were measured. Open bars, cells cultured without apoA-I; filled red bars, cells incubated together with 10µg/ml apoA-I.
Figure 1-5 Enhancements of ABCA1-mediated NaTC-dependent lipid efflux activity by SM reduction
(A) HEK/ABCA1 cells were incubated with 0 or 20 µM myriocin in the presence of NaTC for 24 h,
then lipids in culture solution were extracted and PC and cholesterol contents were measured. (B)
BHK/ABCA1 cells were incubated with 0 or 10 µM mifepristone in the absence or presence of 40 µM
myriocin for 16 h. Then lipids in culture solution were extracted and PC and cholesterol contents
were measured. Open bars, cells cultured without NaTC; filled red bars, cells incubated with 1mM
NaTC.
It was found that *in vitro* ABCA1 was also able to export PC and FC to in the presence of bile salts such as sodium taurocholate (NaTC) as ABCB4 does (11). To confirm the result with apoA-I, lipid efflux activities of HEK/ABCA1 and BHK/ABCA1 treated by SM synthesis inhibitors were also measured in the presence of NaTC. After treatment with 20 µM myriocin for 24 h, PC efflux from HEK/ABCA1 cells was enhanced by 18%, and FC efflux was enhanced by 109% (Figure 1-5 A). In the experiments using BHK/ABCA1 cells, 40 µM myriocin treatment enhanced PC efflux by 11% and FC efflux by 24% (Figure 1-5 B). In addition, treatment with 10µM HPA-12 for 48 h showed similar enhancement of PC efflux by 42% and FC efflux by 26% from BHK/ABCA1 cells (Figure 1-6).

**Figure 1-6** Enhancement of ABCA1-mediated NaTC-dependent lipid efflux by HPA-12 treatment. BHK/ABCA1 cells were pretreated without or with 10 µM HPA-12 or its inactive stereoisomers in the absence or presence of 10 nM mifepristone for 24 h. Then, cells were incubated in the absence or presence of 1 mM NaTC for 24 h. Lipids in the medium were extracted, and PC and cholesterol contents were analyzed.
DISCUSSION

It was reported that SM depletion in a CHO mutant cell line enhanced ABCA1-mediated cholesterol and PC export (3). To confirm the effect of SM depletion, the author employed HEK293 and BHK cell lines for the expression of ABCA1 and reduced cellular SM contents by using two different SM synthesis inhibitors.

To reduce cellular SM content, the author employed two kinds of SM synthesis inhibitors, myriocin and HPA-12. The first step of SM biosynthesis is the conjugation of L-serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase. This reaction occurs on the membrane of ER (17). Myriocin blocks this first step of SM synthesis by reacting with serine palmitoyltransferase to form an adduct that resembles the natural intermediate of the catalytic reaction (18). Different to myriocin, HPA-12 inhibits SM synthesis in another mechanism. HPA-12 structurally mimics ceramide, a precursor of SM synthesized on ER membrane and transported to the trans-Golgi by CERT, and associates with CERT at high affinity (19,20). Thus, application of HPA-12 could effectively blocks SM synthesis without affecting the synthesis of glucosylceramide, an important component of the plasma membrane (9,19,21). The inactive HPA, which is a mixture of stereoisomers of HPA-12, did not show any effect on SM reduction, indicating that the inhibition of CERT by HPA-12 was highly specific (9).

In this chapter, the author confirmed that reduction of cellular SM enhanced ABCA1-mediated lipid export. In the author’s experiments, cellular SM was reduced in both HEK293 and BHK cell lines, and two different SM synthesis inhibitors with distinguished inhibition mechanisms were employed for SM reduction. Thus, these results indicated that the enhancement of ABCA-mediated lipid export by SM reduction is not dependent on cell types or mechanism of SM reduction. What’s more, bile salts, which were known as the physiological
lipid acceptors for ABCB4, was also confirmed to work as lipid acceptors for ABCA1. NaTC-dependent PC and cholesterol export was also enhanced by SM reduction.
REFERENCES


CHAPTER II

ABCB4 exports Lipid in a Sphingomyelin-dependent Manner
CHAPTER II

ABCB4 exports Lipid in a Sphingomyelin-dependent Manner

ABCB4 is an ABC transporter expressed in canalicular membrane of hepatocytes and secretes PC to bile canaliculus \textit{in vivo} (1). By associating with PC, bile salts in canaliculi are diluted so that they will not bring severe damage to canalicular membrane due to their detergent activities (2). In vitro, ABCB4 is also found to efflux cholesterol to bile salts (3).

As described in Chapter I, ABCA1 transports cholesterol and PC to the extracellular lipid acceptor—apoA-I. In previous studies of the author’s group, it was discovered that ABCA1 was able to transport cholesterol and PC to bile salts as effectively as to apoA-I(4). These results suggested that the author could compare the lipid efflux activities of ABCA1 and ABCB4 under the same conditions and determine the effect of the decrease of cellular SM content. The author predicted that ABCB4-dependent lipid efflux activity should be enhanced by SM depletion as the situation of ABCA1.

In the plasma membrane, lipids are not distributed homogenously. They form various specific microdomains such as lipid rafts, which are known to be rich in SM and cholesterol. These microdomains are dynamically organized and reorganized. Various cellular functions such as signal transduction are known to be generated in these microdomains. Usually these microdomains are resistant to cold non-ionic detergents such as Triton X-100 at low temperature and thus are called detergent resistant membrane (DRM). ABCG1 localizes in these SM-enriched DRMs and exports SM as a substrate during the formation of mature HDL. In contrast, ABCA1 exports PC as a substrate in the process of nascent HDL generation and it functions out of DRMs (5,6). ABCB4 was reported to be distributed in Triton-X-100-soluble microdomains rather than
-resistant microdomains (7). These results suggest that the membrane localizations of ABC transporters are related to their functions.

In this chapter, the author employed HEK293 cells stably expressing ABCB4 as well as BHK cells with an inducible ABCB4 expression system, and reduced cellular SM level in these cells by using SM synthesis inhibitors. Under these conditions, the impact of SM reduction on ABCB4-dependent lipid efflux activity was analyzed. In addition, the author compared the distribution of ABCA1 and ABCB4 in the plasma membrane as well as the lipid content in membrane microdomains where ABCA1 and ABCB4 were recovered.
Material and Methods

Materials

Monoclonal antibody C219, which recognizes human ABCB4, was purchased from Centocor. The rat anti–human ABCA1 monoclonal antibody KM3073 was generated against the first extracellular domain (amino acids 45–639) of human ABCA1, as described previously (8). Anti–green fluorescent protein (GFP) antibody was purchased from Santa Cruz Biotechnology. Recombinant apoA-I was prepared as reported previously (9). Sodium taurocholate (NaTC) was obtained from Wako Pure Chemicals Industries. Myriocin was purchased from Enzo Life Sciences. The CERT inhibitor (1R,3S)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)dodecanamide (HPA-12) (10) and its inactive stereoisomer were kindly provided by Dr. Hanada (11). All other chemicals were purchased from Sigma-Aldrich, Life Technologies, Nacalai Tesque, Serva Feinbiochemica, and Dojindo.

Cell Culture

Both HEK293 and BHK cells were grown in a humidified incubator with 5% CO₂ at 37°C in DMEM supplemented with 10% heat-inactivated FBS. HEK293 cells stably expressing human ABCA1 fused with GFP at the C terminus were established as described (4). HEK293 cells stably expressing human ABCB4 were established as described (3). BHK/BCA1 cells (12) were a kind gift from the late Dr. John Oram. BHK cells in which expression of ABCB4 can be induced by mifepristone were established in this study.

Generation of BHK cells expressing human ABCB4

BHK cells expressing human ABCB4 were generated using the mifepristone-inducible
GeneSwitch system (Invitrogen). BHK cell line transfected with the pSwitch plasmid was generously provided by late Dr. Oram (13). Human ABCB4 cDNA was inserted into pGene/V5-HisA(blasticidin), in which original zeocin resistance gene was replaced by blasticidin resistance gene. Cells were transfected with pGene/V5-HisA(blasticidin)/ABCB4 and stably transfected cells were selected with 350 µg/mL of hygromycin and 5 µg/mL of blasticidin. BHK/ABCB4 cell line, in which ABCB4 expression was highly induced by mifepristone, was isolated. Control BHK/mock cell line was established by transfecting pGene/V5-HisA(blasticidin).

**Cellular SM and cholesterol/PC efflux assay**

Cells were subcultured in poly-L-lysine–coated 6-well plates at a density of $5 \times 10^5$ cells per well in DMEM containing 10% FBS. After incubation for 24 h, cells were washed with DMEM, and then incubated in DMEM containing 0.02% BSA containing an SM inhibitor and mifepristone in the presence of 1 mM NaTC or 5 µg/ml recombinant apoA-I. After incubation for 16 or 24 h, lipids in the medium were extracted with chloroform/methanol (2:1) and the amounts of cholesterol and PC were determined by colorimetric enzyme assays as described (14). NaTC (1mM) treatment for 24 h did not show significant cytotoxicity to BHK/ABCA1 and BHK/ABCB4 cells even after myriocin treatment at 80 µM (in this study we used myriocin at 20 or 40 µM) either in the absence or presence of mifepristone. Cellular protein concentration was measured using a BCA protein assay kit (Pierce).

**Preparation of detergent-soluble and detergent-insoluble membrane fractions**

Cells were harvested, suspended in MES-buffered saline (protease inhibitor, 0.15 M NaCl, 25 mM MES, pH 6.5) containing 1% detergent. The suspension was kept on ice for 20 min, and
then centrifuged at 14,000 × g for 20 min. The supernatant was removed and used as the detergent-soluble fraction. Precipitate (detergent-insoluble fraction) was resuspended by sonication in HEPES-buffered saline (protease inhibitor, 0.15 M NaCl, 25 mM HEPES, pH 7.4) containing 1% detergent.

**OptiPrep gradient fractionation**

Harvested BHK cells were suspended in TNE (Tris-NaCl-EDTA) buffer, and a single-cell suspension was generated by repeatedly drawing the sample through a 26G needle. After centrifuge at 860 × g for 5 min, supernatant was transferred to an equal volume TNE/2% CHAPS buffer to yield a final concentration of 1% CHAPS. After incubation on ice for 30 min, the samples were adjusted with 60% iodixanol to a final concentration of 40% iodixanol. The mixture was overlaid with 30% iodixanol in TNE, and finally with TNE. The samples were centrifuged at 166,000 × g for 4 h. Proteins in each fraction were precipitated with cold TCA.

**Western blotting**

Proteins were separated on 5–20% gradient SDS polyacrylamide gels (Atto) and immunodetected with the indicated antibodies. Blots were analyzed and quantitated using an LAS-3000 imaging system and software (Fujifilm).

**Liquid chromatography (LC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS)**

The LC-ESI-MS/MS analysis was performed on a Shimadzu Nexera ultra high performance liquid chromatography (UHPLC) system (Shimadzu, Kyoto, Japan) coupled with QTRAP 4500 hybrid triple quadrupole linear ion trap mass spectrometer (AB SCIEX, Framingham, MA, USA).
Chromatographic separation was performed on an Acquity UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm, Waters, Milford, MA, USA) maintained at 40°C using mobile phase A (water/methanol 50:50 (v/v) containing 10 mM ammonium acetate and 0.2% acetic acid) and mobile phase B (isopropanol/acetone 50:50 (v/v)) in a gradient program (0-20 min: 30% B → 70% B; 20-24 min: 90% B; 24-28 min: 30% B) with a flow of 0.5 mL/min. A neutral loss scan of 74 Da in the negative ion mode was used for detecting phosphatidylcholine (PC) and sphingomyelin (SM). The instrument parameters were as follows (arbitrary units if not specified): Curtain Gas = 10 psi; Collision Gas = 7; IonSpray Voltage = -4500 V; Temperature = 700 °C; Ion Source Gas 1 = 40 psi; Ion Source Gas 2 = 80 psi; Declustering Potential = -105 V; Entrance Potential = -10 V; Collision Energy = -32 V; Collision Cell Exit Potential = -19 V. Product ion analysis in the negative ion mode was performed to determine the fatty acid composition of each PC species. Quantification was performed by integration of the peak area of the extracted ion chromatograms for each phospholipid species. Although ion peaks from a triple quadrupole mass spectrometer do not allow for direct comparison between phospholipid species, SM and PC standards showed the comparable peak area under the experimental conditions employed in this study.

**Statistical Analysis**

All experiments were repeated at least twice. Each replication of quantitative experiments was performed in triplicate. Values are presented as means ± S.E. The statistical significance of differences between mean values was analyzed using the non-paired t-test. Multiple comparisons were performed using the Dunnett test following ANOVA. A value of p < 0.05 was considered statistically significant.
RESULTS

The Impact of SM synthesis inhibitors on ABCB4 expression

Similar to HEK/ABCA1 and BHK/ABCA1 described in Chapter I, the author established HEK cells with stable ABCB4 expression and BHK cells with mifepristone-inducible ABCB4 expression with the GeneSwitch System (Figure 2-1 A). Either myriocin or HPA-12 was applied to these cells and the impacts on the expression of ABCB4 were analyzed by Western Blotting. Addition of myriocin did not cause significant alteration in the expression level in HEK/ABCB4 cells. However, treatment of myriocin and HPA-12 both brought slight but significant increase in ABCB4 expression in BHK/ABCB4 cells compared with untreated cells (Figure 2-1 B and C).
Figure 2-1 Impact of SM synthesis inhibitors on ABCB4 expression (A) ABCB4 expression in BHK cells induced with or without 10 nM mifepristone for 24 h. (B) ABCB4 expression in HEK/ABCA1 cells with or without 20 µM myriocin treatment for 24 h. (C) ABCB4 expression in BHK/ABCA1 cells induced by mifepristone. ABCB4 expression was analyzed after treatment with or without 40 µM myriocin for 24 h (Left) or with or without 10 µM HPA-12 for 48 h (Right). In B and C, the relative ABCB4 expression was shown. Vinculin was used as a loading control.
ABCB4-dependent lipid efflux was suppressed by SM depletion

To investigate the effect of SM depletion on ABCB4-dependent lipid efflux, 20 µM myriocin was applied to HEK/ABCB4 cells and 1 mM NaTC was used as a lipid acceptor. As described in Chapter I, treatment of SM synthesis inhibitors enhanced ABCA1-dependent lipid efflux in both HEK and BHK cells (Figure 1-3 to 1-6). Thus it was expected that lipid efflux activity of ABCB4 was also enhanced by myriocin treatment. However, to the author’s surprise, PC efflux from HEK/ABCB4 cells was reduced by 31% when cells were treated with 20 µM myriocin for 24 h (Figure 2-2 A).

For further confirmation, BHK cells with inducible expression of ABCB4 were treated with 40 µM myriocin or 10 µM HPA-12. Treatment of 40 µM myriocin for 24 h of BHK/ABCB4 cells reduced PC efflux by 28 % and cholesterol efflux by 34 % (Figure 2-2 B and C). And similar results were obtained when treating cells with 10 µM HPA-12 for 48 h. HPA-12-treated ABCB4-expressing cells showed lower lipid effluxes (27 % in PC efflux and 29 % in FC efflux) compared to untreated cells. In contrast, the same amount of inactive HPA (mixture of stereoisomers of HPA-12) treatment did not affect the lipid efflux activity significantly (Figure 2-2 D and E).

These results indicated that cellular SM reduction reduced the lipid efflux activity of ABCB4, though the same treatment enhanced ABCA1-dependent lipid efflux. Although ABCB4 is functionally similar to ABCA1, the responses to SM depletion are different between the two ABC transporters. The reason for the distinguished responses to SM depletion may rely on the different membrane distributions of the two transporters.
Figure 2-2 Reduction of ABCB4-dependent lipid efflux activity by SM reduction (A) HEK/ABCB4 cells were incubated with 0 or 20 μM myriocin with or without 1mM NaTC for 24 h, then lipid in culture solution was extracted and PC and cholesterol content was measured. (B, C) BHK/ABCA1 cells were incubated with 0 or 10μM mifepristone and 0 or 40 μM myriocin together with or without 1mM NaTC for 24 h. Then lipid in culture solution was extracted and PC and cholesterol content were measured. (D, E) BHK/ABCB4 cells were precultured with or without 10μM HPA-12 or its stereoisomers for 48 h before harvest. Last 24 h before harvest, culture medium was added with 1mM NaTC or 10nM mifepristone as indicated. For A-C, open bars refer to cells cultured without NaTC, filled blue bars refers to cells incubated together with NaTC.
**ABCB4 localizes in a specific membrane microdomain which is rich in SM**

Some proteins localize in specific microdomains in the plasma membrane and can be discovered in detergent soluble and resistant fractions according to the solubility after treatment of non-ionic detergents at low temperature. In this section, the author treated BHK cells with induced ABCB4 or ABCA1 expression with several detergents including 1% Triton X-100, 1% Lubrol WX or 1% CHAPS. Then the soluble and insoluble fractions were recovered, the relative amount of ABC proteins in each fraction were analyzed by Western blotting. Caveolin-1 was used as marker protein for detergent-insoluble fractions.

In BHK/ABCA1 cells, ABCA1 was recovered from soluble fraction when treated with 1% Triton X-100 or 1% CHAPS, and was recovered from insoluble fraction under 1% Lubrol WX treatment (Figure 2-3 A). Similarly to ABCA1, ABCB4 was also recovered from Triton X-100 soluble fraction and Lubrol WX insoluble fraction. However, in contrast to ABCA1, ABCB4 was recovered mainly in insoluble fraction under 1% CHAPS treatment (Figure 2-3 B). To further examine the distribution of ABCB4 in CHAPS insoluble fraction, OptiPrep gradient experiment was also carried out. ABCB4 was found to float on top of the OptiPrep gradient, and was co-recovered with caveolin-1 (Figure 2-3 C), indicating that ABCB4 mainly exists in CHPAS-resistant membrane fractions.
Figure 2-3 Different distribution of ABCB4 and ABCA1 in detergent-soluble and -insoluble fractions. BHK/ABCB4 (A) and BHK/ABCA1 cells (B) were solubilized with 1% Triton X-100, 1% Lubrol WX, or 1% CHAPS. Proteins from the soluble (S) and insoluble (IS) fractions were separated on 5–20% gradient SDS polyacrylamide gels, and ABCB4 and ABCA1 were immunodetected. Caveolin was detected as a marker for detergent-insoluble fractions. (C) OptiPrep gradient fractions were separated on 5–20% gradient SDS polyacrylamide gels. ABCB4 and caveolin-1 were detected with specific antibodies.
Lipid contents or phospholipids species do not differ significantly between Triton X-100 and CHAPS insoluble fractions

Since the lipid efflux activity of ABCB4 is sensitive to SM content, the author speculated that lipid content, especially the ratio of PC versus SM, might be the reason why ABCB4 distributes in CHAPS-insoluble fraction rather than in Triton-insoluble fraction. So the author measured the content of PC and SM, as well as cholesterol, contained in fractions recovered by both 1% Triton X-100 and 1% CHAPS from BHK cells. The ratio of PC/SM of Triton X-100 soluble and insoluble fractions were $14.7 \pm 2.0$ and $2.9 \pm 0.53$, respectively. Unexpectedly there was no significant difference from those of fractions recovered by CHAPS, which were $20.6 \pm 5.3$ for soluble and $4.5 \pm 0.87$ for insoluble fractions. There was no significant difference in cholesterol content, either (data not shown). Thus, it was not likely the difference in lipid contents was the reason for the specific distribution of ABCB4 to CHAPS insoluble fraction.

Next, the author focused on the species of SM and PC, such as the length and the degree of unsaturation, of their fatty acid chains of Triton X-100 and CHAPS insoluble fraction recovered from BHK cells by mass spectrometry. However, neither the species nor the relative amount of PC and SM showed clear difference between the two fractions (Figure 2-4).
Figure 2-4 Mass spectrometry analysis of PC and SM species. Mass spectra of \([M+CH_3COO]\) ions of PC and SM by scanning for neutral loss of 74 Da. (A) The peaks of standard sample PC and SM utilized for measurement. (B) Lipid extracts (0.7 nmol of total phospholipids) of CHAPS and Triton X-100-insoluble fractions were subjected to LC-ESI-MS/MS analysis. Assigning specific phospholipid species to m/z values was based on their calculated theoretical monoisotopic masses and verified by MS/MS. The prefix “d” represents sphingoid base and the suffixes “e” and “p” indicate a chain with an alkyl ether linkage and a chain with a vinyl ether linkage, respectively. (B) The amount of each molecular species of SM and PC was quantified by integration of the peak area of the extracted ion chromatograms for the molecular species of SM and PC. PC molecular species are indicated by the total number of carbons and the number of double bonds in the fatty acyl chain. The prefix “O-“ indicates an ether-type phospholipid.
DISCUSSION

SM depletion is reported to affect the lipid export activities of ABCA1 and ABCG1 (15,16), indicating that membrane environment alteration may affect the lipid export activities of ABC transporters. However, SM is one of the transport substrate of ABCG1, and depletion of SM in the plasma membrane potentially alters the concentration of PC and cholesterol, the substrates of ABCA1. Therefore the effects of SM depletion on ABC transporters could be also due to the alteration of transport substrate concentrations. Because both ABCB4 and ABCA1 are known to transport PC and cholesterol to bile salts (3,17), the effect of SM depletion on transport substrates for the two transporters can be neglected.

In this chapter, ABCB4 was expressed in both HEK293 and BHK cell lines, and cellular SM in these cells was reduced with two types of inhibitors as described in Chapter I. For the analysis of membrane environment where ABCA1 and ABCB4 function, their membrane distributions were compared by fractionating membrane with different detergent solutions.

The author found that SM depletion depressed ABCB4-mediated lipid efflux activity in vitro. This is similar to the previous reports that ABCG1-mediated SM and cholesterol export activity declines in a SM-depleted CHO cell lines(16). Previous studies of the author’s group demonstrated by mass spectrometry that ABCB4 mediates PC efflux rather than SM efflux in HEK293 cell lines(3). Furthermore, the lipid composition of bile contains PC at 90-95%, and SM only exists at a trace ratio in vivo, suggesting that SM is not a transport substrate for ABCB4 (18). Thus, the depression of ABCB4-dependent lipid efflux was not due to the reduction of transport substrate, but was likely due to the alteration in membrane environment brought by SM depletion. In other words, the findings in this study indicate that the membrane environment is important for the lipid export activities of ABC transporters.
In vivo, ABCB4 is limitedly expressed in canalicular membrane of hepatocytes. The lipid composition of canalicular membrane is quite different from that of basolateral membranes, because canalicular membrane has to tolerate the powerful detergent activity of bile salts. It has been reported that canalicular membrane is richer in SM than capillary membrane(19). This is consistent with the result the author obtained in vitro that ABCB4 localizes in a relative SM-rich microdomain in membrane and has depressed activities when cellular SM level is lowered. However, it is unclear that how ABCB4 localizes in CHAPS-insoluble fraction rather than in Triton X-100-insoluble fraction even though their lipid composition and species are quite similar with each other.

In this chapter, the author demonstrated that SM content in the plasma membrane affects the lipid efflux activity of ABC transporters. SM depletion reduced ABCB4-dependent lipid efflux activity while the same treatment enhanced ABCA1-dependent lipid efflux activity. The author also discovered that ABCB4 and ABCA1 localize in different membrane microdomains with each other.
REFERENCES


CONCLUSIONS

The purpose of the study described in this thesis is to understand the impact of membrane environment on the lipid efflux activity of two ABC transporters, ABCA1 and ABCB4. The lipid efflux activities of the two transporters were measured and compared under the conditions that cellular SM content was reduced by SM synthesis inhibitors. The membrane distributions of the two ABC transporters were also compared by membrane fractionation, and the lipid content in the fractions recovered by detergents were also measured and compared.

In Chapter I, the author employed two different types of sphingomyelin synthesis inhibitors, myriocin and HPA-12, to reduce cellular SM level in HEK/ABCA1 and BHK/ABCA1. With these treatments, cellular SM was reduced to around 75% in both HEK293 and BHK cells. Under these conditions, ABCA1-dependent PC and cholesterol efflux from HEK/ABCA1 was enhanced. In BHK cells with induced ABCA1 expression, PC and cholesterol efflux was also increased. These results are consistent with the previous studies that in SM-deficient CHO cells ABCA1-dependent lipid efflux was enhanced. The application of myriocin and HPA-12 provided new ways to analyze the effect of the reduced cellular SM on the activities of ABC transporters. The author also compared the change in the lipid efflux activity of ABCA1 in HEK293 and BHK cells when NaTC served as a lipid accepter, and obtained similar results with apoA-I-mediated lipid export. These results confirmed that alteration in membrane environment, such as depletion of SM, affects the activity of ABCA1 transporter.

In Chapter II, In contrast to ABCA1, PC efflux as well as cholesterol efflux was suppressed in ABCB4-expressing cells after myriocin treatment. And HPA-12 treatment caused similar decline in lipid efflux activities. Although ABCB4 functioned similarly to ABCA1 in removing cellular PC and cholesterol \textit{in vitro}, the response to SM reduction of lipid efflux activities was
opposite. Then the author investigated about the membrane distribution of ABCA1 and ABCB4 in BHK cells by treating cells with different detergents. Both ABCA1 and ABCB4 were found to localize in Triton X-100 soluble fractions. However, ABCB4 existed in CHAPS-insoluble fractions while ABCA1 localized in CHAPS-soluble fractions. The author further attempted to find difference between CHAPS-insoluble fraction and Triton-X-100-insoluble fractions by comparing lipid composition and species of choline phospholipids of the two fractions. Both CHAPS- and Triton-X-100-insoluble fractions were rich in SM and no significant difference in lipid species was found between the two fractions.

From these results, the author concluded that SM content affects lipid efflux activity of ABC transporters, such as ABCA1 and ABCB4. SM is necessary for ABCB4 to function as a transporter to remove PC to extracellular acceptors. ABCB4 is distributed in a SM-rich microdomain in the plasma membrane (Figure C-1). Physiologically, ABCB4 is expressed in the canalicular membrane of hepatocytes, which is known to be rich in cholesterol and SM. These high levels of SM and cholesterol are probably necessary for bile canaliculi to tolerate high concentrations of bile salts, which are powerful detergents. We speculate that ABCB4 has been evolved to be maximally active in the SM-rich membrane environment of canalicular membrane, where it transports PC as its physiological transport substrate.

![Figure C-1](image.png) ABCA1 functions in membrane with low SM content, while ABCB4 localizes in SM-enriched membrane.
LIST OF PUBLICATION

A. Yu Zhao, Masato Ishigami, Kohjiro Nagao, Kentaro Hanada, Nozomu Kono, Hiroyuki Arai, Michinori Matsuo, Noriyuki Kioka, and Kazumitsu Ueda

ABCB4 exports phosphatidylcholine in a sphingomyelin-dependent manner


RELATED PUBLICATION

A. Kohjiro Nagao, Yu Zhao, Kei Takahashi, Yasuhisa Kimura and Kazumitsu Ueda

Sodium taurocholate-dependent lipid efflux by ABCA1: effects of W590S mutation on lipid translocation and apolipoprotein A-I dissociation

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