

(24*S*)-Hydroxycholesterol efflux from neuronal cells  
by  
ABC proteins

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## INTRODUCTION

The brain contains ~25% of the human body's total cholesterol, despite occupying only 2% of body mass. Some fraction of cholesterol is actively converted to (24*S*)-hydroxycholesterol (24-OHC) by cholesterol 24-hydroxylase, a cytochrome P450 (CYP46A1) highly expressed in a subset of neurons in the brain, and subsequently eliminated from brain tissue (1). About 0.02% of brain cholesterol in humans and 0.4% in mouse turns over each day (2). Disruption of the mouse CYP46A1 gene reduced the synthesis of new cholesterol in the brain by ~40%, indicating at least 40% of cholesterol turnover in the brain is dependent on the conversion to 24-OHC (3). This knockout mouse exhibited severe deficiencies in spatial, associative, and motor learning, as well as in hippocampal long-term potentiation (4), suggesting that cholesterol turnover via 24-hydroxylase is essential for brain functions, especially learning.

Because 24-OHC induces apoptosis of neuronal cells, it is vital to eliminate it rapidly from the cells. It is generally accepted that after its production in the brain, 24-OHC gains access to the circulation by spontaneous diffusion across cellular membranes (5) because hydroxylation of the side chain of cholesterol accelerates transfer across the lipid bilayer more than 1,000 times compared to unhydroxylated cholesterol (6). However, the elimination of 24-OHC from the brain is governed by a carrier-mediated process at the BBB and *oatp2* is involved in this brain-to-blood efflux (7). Therefore, the author hypothesized that efflux transporters are involved in the 24-OHC elimination from neuronal cells.

Although most of hydrophobic and amphipathic compounds pass freely through the lipid bilayer, some ABC proteins, such as ABCB1 (MDR1) and ABCG2, actively transport hydrophobic and amphipathic compounds, thereby playing important roles in

protecting our body by expelling such compounds into the intestinal lumen, into the bile from the liver, and into the urine from the kidney (8). ABCA1 and ABCG1 are involved in the efflux of cholesterol from peripheral cells. ABCA1 mediates the efflux of cholesterol to lipid-free apolipoprotein A-I (apoA-I), which serves as a lipid acceptor in the serum (9, 10); in the case of ABCG1, high-density lipoprotein (HDL) acts as the acceptor (11-14). Furthermore, ABCA1 and ABCG1 transport 25-hydroxycholesterol (15). Both ABCA1 and ABCG1 are expressed in neuronal cells (14, 16-18). Therefore, the author speculated that ABCA1 and ABCG1 may be involved in 24-OHC elimination from neuronal cells and apoptosis induced by 24-OHC is prevented by ABCA1 and ABCG1 if they transport 24-OHC in neuron.

In chapter 1, the author examined the efflux of 24-OHC from neuronal cells, using differentiated SH-SY5Y cells as a model. In chapter 2, the author revealed that 24-OHC is transported by ABCA1 and ABCG1 from cells to HDL as a lipid acceptor. The results described in chapter 3 suggested that expression of ABCA1 and ABCG1 protects neurons against toxicity of 24-OHC. From these results, the author proposes that ABCA1 and ABCG1 actively eliminate 24-OHC in the presence of HDL as a lipid acceptor and protect neuronal cells.

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## **CHAPTER 1**

### **Efflux mechanism of 24-OHC from SH-SY5Y cells**



## CHAPTER 1

### Efflux mechanism of 24-OHC from SH-SY5Y cells

(24S)-Hydroxycholesterol (24-OHC) is the main cholesterol metabolite in the brain (1). Following conversion from cholesterol in neuron, 24-OHC is eliminated from brain to the circulating blood (1-2). This is considered to be the main elimination route of brain cholesterol. 24-OHC is also a ligand for liver X receptor, and an LXR ligand has been reported to increase cholesterol release from primary glial cells associated with induction of ATP-binding cassette transporter (ABC) A1 and G1 (3). Therefore, 24-OHC would act as a regulator in the autoregulatory mechanism for CNS cholesterol homeostasis (4). From these findings, it is speculated that 24-OHC also regulates the expression of ABCA1 and ABCG1 in neuronal cells. However, it remains unclear if it is the case.

In peripheral cells, ABCA1 transfers cellular excess cholesterol to a lipid acceptor apolipoprotein A-I (apoA-I) (5-6) and ABCG1 transfers it to a lipid acceptor high-density lipoprotein (HDL) to maintain cellular cholesterol homeostasis (7). On the other hand, in neuronal cells, excess cholesterol is converted to 24-OHC by cytochrome P450 (CYP) 46A1 (1) to maintain cholesterol homeostasis. While ABCA1 and ABCG1 are expressed in neuronal cells (8-11), their functions are poorly understood. Because excess 24-OHC causes neuronal cell death (12-13), 24-OHC must be rapidly eliminated from neuronal cells. Therefore, the author hypothesized that ABCA1 and ABCG1 export cellular 24-OHC to lipid acceptors from neuronal cells and prevent 24-OHC accumulation in neuron.

In this chapter, the author analyzed whether ABCA1 and ABCG1 were induced by 24-OHC in neuron-like cells, SH-SY5Y. And then, the author examined whether

24-OHC is exported from SH-SY5Y cells and whether ABCA1 and ABCG1 are involved in 24-OHC efflux by using RNA silencing.

## **MATERIALS AND METHODS**

### ***Materials***

Anti-ABCA1 monoclonal antibody KM3110 was generated against the C-terminal 20 amino acids of ABCA1 in mice (14). Rabbit polyclonal anti-ABCG1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti- $\beta$ -actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). 24-OHC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). [22,23-<sup>3</sup>H] 24-OHC (specific activity: 50-60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). TO901317 was purchased from ALEXIS Biochemicals (San Diego, CA, USA). 9-*cis* retinoic acid (RA) was purchased from Sigma. HDL from human plasma was purchased from Athens Research & Technology (Athens, GA, USA). ApoA-I from human plasma was purchased from Calbiochem (San Diego, CA, USA). Human recombinant apoE3 and apoE4 were purchased from Wako Pure Chemical Industries (Osaka, Japan).

### ***Cell culture***

SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL benzylpenicillin potassium, 100  $\mu$ g/mL streptomycin sulfate, and 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. For differentiation of SH-SY5Y cells (15), 10  $\mu$ M all-trans retinoic acid was added to the culture medium a day after seeding, and cells were cultured for 5 days.

### ***Treatment with LXR and RXR ligands***

After differentiation, SH-SY5Y cells were incubated in serum-free media in the presence of TO901317 (5  $\mu$ M), a synthetic ligand of Liver X receptor (LXR), RA (5  $\mu$ M), a ligand of retinoid X receptor (RXR), or the indicated concentration of 24-OHC for 16 h at 37°C.

### ***Electrophoresis***

Cells were washed with PBS and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol and 1% Triton X-100) containing protease inhibitors (*p*-amidinophenyl)methanesulfonyl fluoride, 100  $\mu$ g/ml; leupeptin, 2  $\mu$ g/ml; and aprotinin, 2  $\mu$ g/ml). Electrophoresis was performed using SDS-polyacrylamide gradient gel (4–12%) (Invitrogen).

### ***[<sup>3</sup>H]24-OHC efflux from differentiated SH-SY5Y cells***

SH-SY5Y cells were first incubated with all-*trans* retinoic acid (10  $\mu$ M) for 3 days. Then, SH-SY5Y cells were loaded with [<sup>3</sup>H] 24-OHC (0.5  $\mu$ Ci/mL) and unlabeled 24-OHC (10  $\mu$ M) in DMEM containing 10% FBS and all-*trans* retinoic acid (10  $\mu$ M) for 24 h at 37°C. Cells were incubated with DMEM containing 0.1% BSA for 1 h to eliminate [<sup>3</sup>H] 24-OHC nonspecifically attached to the cell surface and dishes. Cells were washed once with PBS containing 0.1% BSA and once with PBS alone. Cells were then incubated in serum-free DMEM containing all-*trans* retinoic acid (10  $\mu$ M) with or without TO901317 (5  $\mu$ M) and RA (5  $\mu$ M) for 16 h to activate LXR/RXR pathway and induce the expression of ABCA1 and ABCG1. Next, cells were incubated for 4 h at 37°C with DMEM containing 0.02% BSA with or without HDL (50  $\mu$ g/mL), apoA-I (10  $\mu$ g/mL), apoE3 or apoE4 (10  $\mu$ g/mL). After 4 h incubation, the medium was collected and centrifuged at 3,000  $\times$  g for 3 min to remove cell debris.

Cells were washed twice with ice-cold PBS containing 0.1% BSA, once with PBS alone, and lipids were extracted with 3:2 (v/v) hexane:isopropanol.

### ***Reduction of ABCA1 or ABCG1 expression by RNA silencing***

Control small interfering RNA (siRNA; Stealth RNAi Negative Control Medium GC Duplex #2); two siRNAs targeting human ABCA1, ABCA1#1 (ABCA1-HSS100028) and ABCA1#2 (ABCA1-HSS-100029); and two siRNAs targeting human ABCG1, ABCG1#1 (ABCG1-HSS145233) and ABCG1#2 (ABCA1-HSS-190466) were purchased from Invitrogen. SH-SY5Y cells were plated in 24-well plates at  $1 \times 10^5$  cells/well, incubated for 24 h, and transfected with 10 nM siRNAs using Lipofectamine RNAiMAX (Invitrogen). In brief, siRNA and RNAiMAX, separately diluted in OptiMEM, were mixed and incubated for 10 min at room temperature. These mixtures were added to medium containing 10% FBS and 10  $\mu$ M all-trans retinoic acid, and cultured for 3 days while the cells differentiated; 24-OHC release assays were performed thereafter.

### ***Radio-HPLC analysis***

$^3\text{H}$ -compounds in cells and medium were analyzed using a Radio-HPLC system. The medium was extracted with 2 $\times$  volume of organic solvent (1:1 (v/v) acetonitrile:methanol). The cells were burst by addition of distilled water, and extracted with the 2  $\times$  volume of organic solvent. Supernatants were analyzed using the Radio-HPLC with a reversed-phase column (YMC-Pack, ODS-AM-302, 150 x 4.6 mm I.D., S-5  $\mu$ m, 120A): solvent, 15:85 (v/v) 0.1% formic acid:acetonitrile; flow rate, 1 mL/min.



### ***Data analysis***

Percent efflux was calculated by dividing the radioactivity in the medium by the sum of the radioactivity in the medium and cell lysate. Unless otherwise noted, all data are presented as the mean  $\pm$  SD. Experiments were done at least twice independently. 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**

### ***Induction of ABCA1 and ABCG1 expression in differentiated SH-SY5Y cells by 24-OHC***

The author used SH-SY5Y cells as a model to analyze the mechanism of 24-OHC elimination from neuron-like cells. SH-SY5Y cell line was established from human metastatic neuroblastoma tissue (16) and differentiates into neuron-like cells by the treatment with all-trans-retinoic acid (15, 17-18). Differentiated SH-SY5Y cells have been widely used to study neuronal functions.

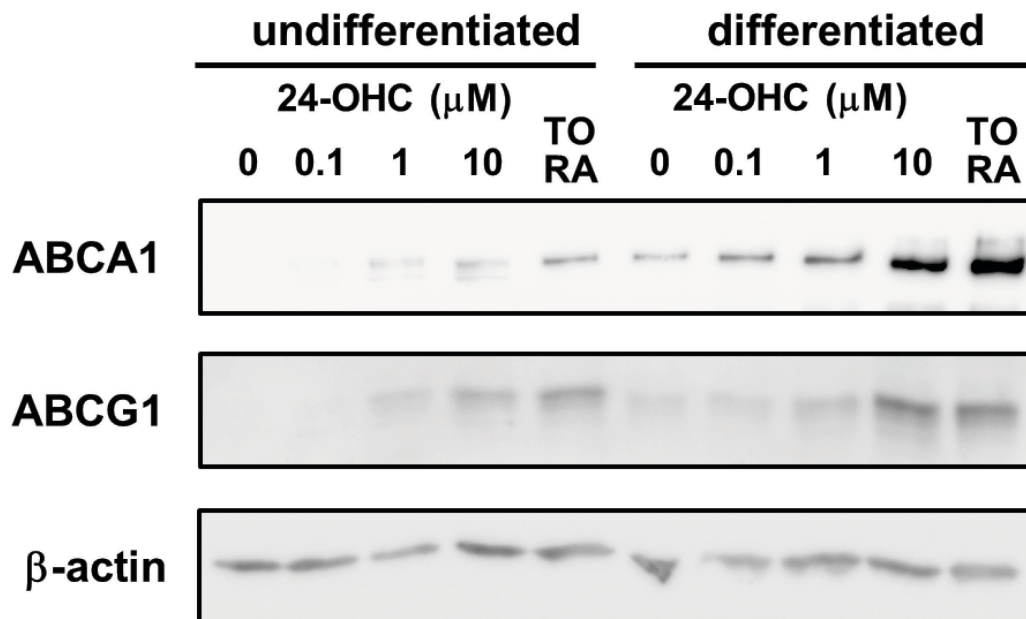
24-OHC, the major cholesterol metabolite in brain, is an endogenous ligand of the nuclear receptor LXR, and induces ABCA1 and ABCG1 gene transcription in macrophages (19-20). However, it was reported that ABCG1 is unresponsive to 24-OHC in undifferentiated SH-SY5Y cells (21). Therefore, the author first examined whether 24-OHC induces expression of ABCA1 and ABCG1 in SH-SY5Y cells. ABCA1 and ABCG1 protein were barely detectable in the absence of 24-OHC, but significantly increased in a concentration-dependent manner upon addition of 24-OHC. The effect of 24-OHC at 10  $\mu$ M was comparable to that of the synthetic ligands TO901317 (TO), a ligand of LXR, and retinoic acid (RA), a ligand of RXR (Fig.1).

However, their expression levels were much lower than those in differentiated human macrophage cell line THP-1 (data not shown). Therefore, the author next examined their expression in differentiated SH-SY5Y cells.

SH-SY5Y cells were differentiated with all-*trans* retinoic acid for three days, whereupon 24-OHC was added to the medium for 16 h. Because dendrite elongation of SH-SY5Y cells was observed, differentiation into neuron like cells was confirmed (Fig.2). ABCA1 and ABCG1 protein were faintly but significantly expressed in differentiated SH-SY5Y even in the absence of 24-OHC, and strongly increased in a concentration-dependent manner upon addition of 24-OHC, and the effect of 24-OHC at 10  $\mu$ M was comparable to that of TO+RA (Fig.1). These results suggest that ABCA1 and ABCG1 genes in SH-SY5Y cells well respond to LXR ligands after differentiation, and that exogenously added 24-OHC induces LXR-dependent genes as efficiently as the synthetic ligands.

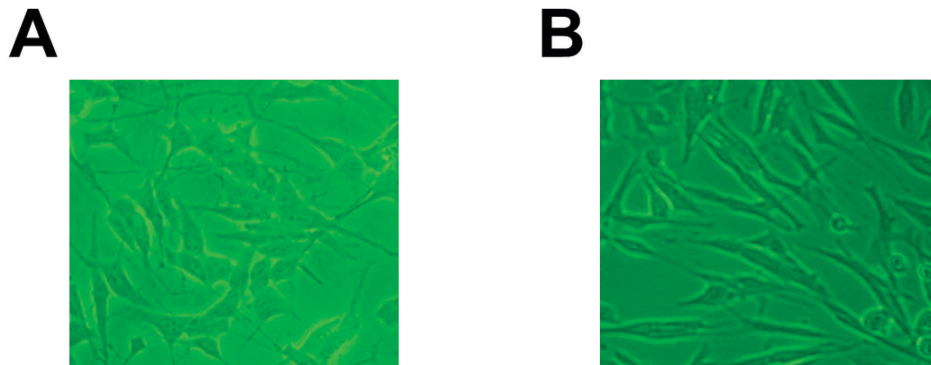
#### ***HDL-dependent efflux of 24-OHC from differentiated SH-SY5Y cells***

It has been reported that 25-hydroxycholesterol is transported from J774 mouse macrophage cells in the presence of apoA-I or HDL (22). The author examined whether 24-OHC was also transported from differentiated SH-SY5Y cells in the presence of apoA-I or HDL. SH-SY5Y cells were first differentiated, and expression of ABCA1 and ABCG1 was induced with TO+RA. In the absence of TO+RA treatment, ~5% of 24-OHC emerged into the medium within 4 h; no significant apoA-I-dependent 24-OHC efflux was observed (Fig. 3A). After treatment with TO+RA, a slight apoA-I-dependent 24-OHC efflux was observed, while no significant increase was observed by the TO+RA treatment.



**Figure 1. Effects of 24-OHC, the synthetic LXR ligand TO901317 (TO), and the RXR ligand 9-cis retinoic acid (RA) on the expression of ABCA1 and ABCG1 in differentiated SH-SY5Y cells.**

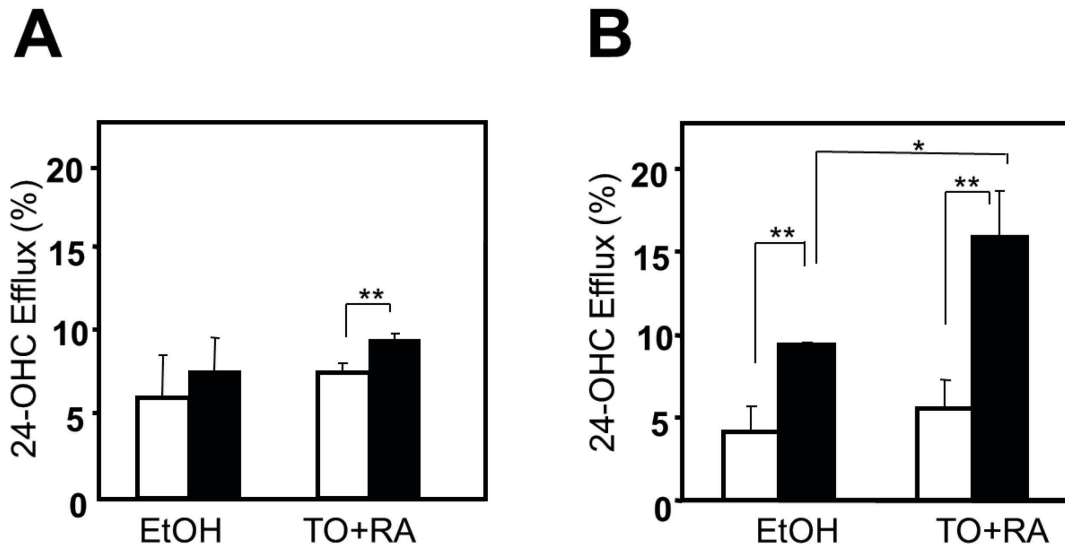
$\beta$ -actin was analyzed as a control. Western blot analysis was performed using whole-cell lysates from undifferentiated and differentiated SH-SY5Y cells treated with the indicated concentrations of 24-OHC or TO (5  $\mu$ M) + RA (5  $\mu$ M) for 16 h.



**Figure 2. Morphology of undifferentiated (A) or differentiated (B) SH-SY5Y cells.**

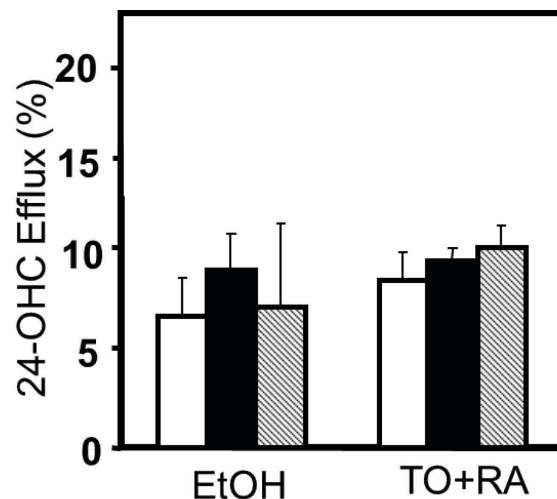
Ten  $\mu\text{M}$  all-trans retinoic acid was added to the culture medium a day after seeding and cells were cultured for 5 days for differentiation of SH-SY5Y cells.

The author also examined 24-OHC efflux in the presence of apoE3 and the isoform apoE4 (Fig. 4). ABCA1 mediates cholesterol efflux in the presence of apoE as efficiently as apoA-I (23). ApoE is the major apolipoprotein in the brain and apoE isoforms and levels strongly influence Alzheimer's disease pathology and risk (24). However, we observed no significant 24-OHC efflux in the presence of either the apoE3 or apoE4 variant (Fig. 4). Because 25-OHC efflux from J774 cells has been observed in the presence of HDL as well as apoA-I (22), the author examined efflux in the presence of HDL; the author observed significant 24-OHC efflux even without TO+RA treatment, and this efflux was further stimulated by treatment (Fig. 3B).



**Figure 3. Effects of LXR/RXR ligands on apoA-I- or HDL-dependent 24-OHC efflux from differentiated SH-SY5Y cells.**

Differentiated SH-SY5Y cells were cultured on collagen-coated 24 well plates and labeled with [<sup>3</sup>H] 24-OHC (0.1  $\mu$ Ci/200  $\mu$ L, 24 h). Labeled cells were incubated with 0.1% ethanol (control) or 5  $\mu$ M TO901317 and 5  $\mu$ M RA (TO+RA) for 16 h. Next, cells were incubated in the absence (empty bars) or in the presence (filled bars) of either 10  $\mu$ g/mL apoA-I (A) or 50  $\mu$ g/mL HDL (B) for 4 h at 37°C. 24-OHC efflux was calculated from the [<sup>3</sup>H] counts in the medium as a percentage of the total counts (medium + lysate). Each bar represents mean  $\pm$  S.D. (n=3). \*,  $p$ <0.05. \*\*,  $p$ <0.01.



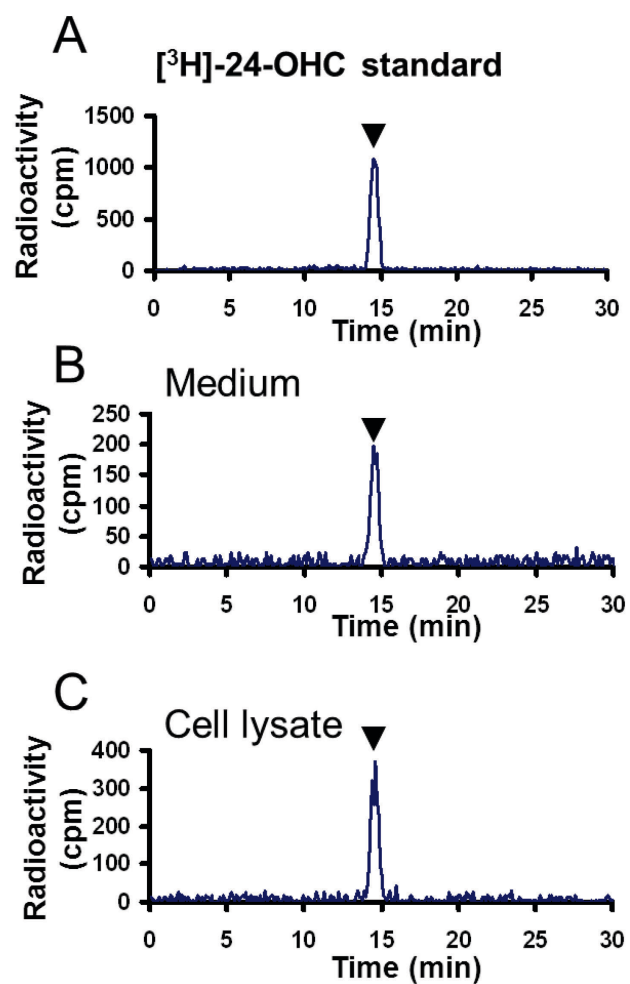
**Figure 4. Effects of LXR/RXR ligands on apoE-dependent 24-OHC efflux from differentiated SH-SY5Y cells.**

Differentiated SH-SY5Y cells were treated as described for Fig. 3, and then incubated in the absence (empty bars) or in the presence of 10  $\mu\text{g/mL}$  apoE3 (filled bars) or apoE4 (hatched bars) for 4 h at 37°C. 24-OHC efflux was calculated as described for Fig. 3.

Each bar represents mean  $\pm$  S.D. (n=3).

***Efflux of 24-OHC, but not its metabolites, from differentiated SH-SY5Y cells***

The majority of 24-OHC in the brain is present in non-ester form (25), but the sulfate ester can also be detected (26). To determine whether 24-OHC is metabolized in SH-SY5Y cells, and which form of 24-OHC is transported from cells, we performed Radio-HPLC analysis. The main radioactive peak in medium and in cell lysate after the efflux assay was detected at the same position (14.8 min) as the standard [ $^3\text{H}$ ]-24-OHC. 24-OHC sulfate, whose retention time is shorter than that of 24-OHC



**Figure 5. HPLC analysis of 24-OHC metabolites.**

[<sup>3</sup>H] compounds obtained from medium (B) and cell lysate (C) after 24-OHC efflux in the presence of HDL were analyzed using HPLC with a reversed-phase column.

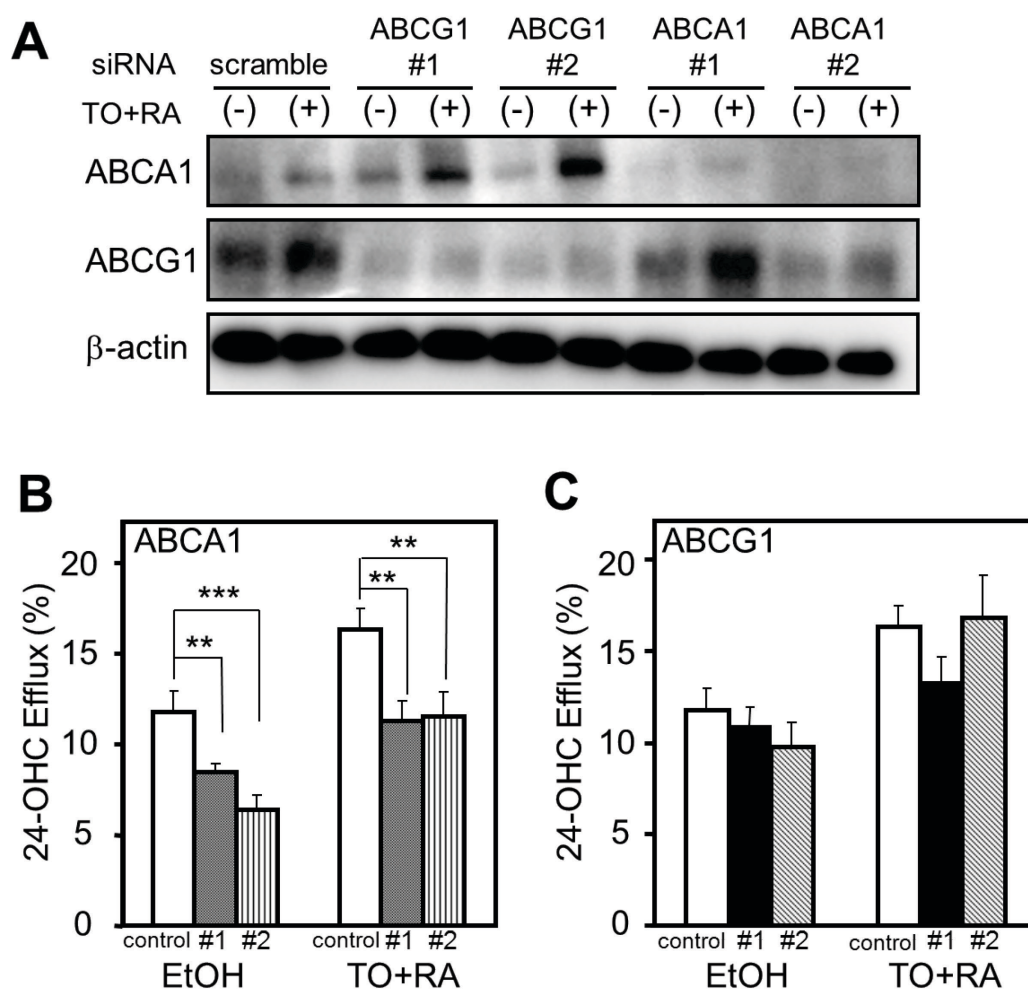
Analysis of the 24-OHC standard compound is presented in (A).

(27), was not detected (Fig.5). These results suggest that 24-OHC itself is transported from differentiated SH-SY5Y cells in the presence of HDL.

#### ***Involvement of ABCA1 in HDL-dependent 24-OHC efflux from SH-SY5Y cells***

Cholesterol efflux by ABCA1 is apoA-I-dependent (5-6), whereas efflux by ABCG1 is HDL-dependent (7, 28-29). In order to determine whether 24-OHC efflux from differentiated SH-SY5Y cells in the presence of HDL is mediated by ABCG1, we suppressed expression of ABCG1 and ABCA1 using siRNAs. siRNAs against ABCG1 and ABCA1 efficiently suppressed the corresponding gene's expression in the differentiated SH-SY5Y cells (Fig. 6A). siRNAs against ABCA1 significantly decreased 24-OHC efflux from differentiated SH-SY5Y cells in the presence of HDL, relative to scrambled siRNA (Fig.6B). In contrast, no significant decrease in 24-OHC efflux was observed after treatment with ABCG1 siRNAs. Figure 6A shows that ABCA1 expression was stimulated when ABCG1 expression was suppressed by siRNAs. Therefore, it was possible that increased ABCA1 compensated for the lost function of ABCG1. siRNA#1 against ABCA1 did not affect ABCG1 expression, whereas siRNA#2 slightly suppressed it; furthermore, the level of ABCG1 expression did not correlate with HDL-dependent 24-OHC efflux (Fig. 6C). These results suggest that ABCA1 is involved in HDL-dependent 24-OHC efflux from differentiated SH-SY5Y cells, but the involvement of ABCG1 remains unclear. Simultaneous treatment with siRNAs against ABCA1 and ABCG1 negatively affected survival of differentiated SH-SY5Y cells especially in the presence of 24-OHC (data not shown), leading the author to take an alternative approach to use HEK293 cells expressing ABCG1.





**Figure 6. Effects of ABCA1 and ABCG1 siRNAs on HDL-dependent 24-OHC efflux from differentiated SH-SY5Y cells.**

SH-SY5Y cells plated in 24-well plates were transfected with siRNA (10 nM) against ABCA1 (A) or ABCG1 (B) and allowed to differentiate for 3 days. Cells were loaded with [<sup>3</sup>H] 24-OHC for 24 h, and treated with or without 5  $\mu$ M TO901317 and 5  $\mu$ M RA (TO+RA) for 16 h. Next, cells were incubated with 50  $\mu$ g/mL HDL for 4 h at 37°C. 24-OHC efflux was calculated as described for Fig. 3. Each bar represents mean  $\pm$  S.D. (n=4). \*\*,  $p < 0.01$ . \*\*\*,  $p < 0.001$ .

## DISCUSSION

24-OHC is the major cholesterol metabolite in the brain (1); high cholesterol turnover catalyzed by cholesterol 24-hydroxylase is essential for neural functions, especially learning (30). The author hypothesized that 24-OHC produced in neuronal cells is actively eliminated via transporters.

The author predicted that 24-OHC would be eliminated by the transporter(s) whose expression is induced by intracellular accumulation of 24-OHC itself. 24-OHC is an endogenous ligand of a nuclear receptor LXR; 24-OHC therefore induces transcription of ABCA1 and ABCG1 genes in macrophages by activating the LXR/RXR heterodimer (19, 31). However, it was reported that 24-OHC is not produced in SH-SY5Y cells (32), and indeed, the author could not detect 24-OHC production under our experimental conditions (data not shown). Therefore, the author added 24-OHC exogenously and see if 24-OHC induces expression of ABCA1 and ABCG1. ABCA1 and ABCG1 genes in differentiated SH-SY5Y cells well responded to exogenously added 24-OHC and synthetic ligands and 24-OHC (10  $\mu$ M) induced them as efficiently as the synthetic ligands (Fig. 1). This concentration is physiologically relevant, as free 24-OHC in the brain was estimated at up to 30  $\mu$ M (25, 33).

24-OHC efflux was clearly observed in the presence of HDL even without TO+RA treatment, and this efflux was further stimulated by the treatment (Fig. 2C). HDL in the brain contains apoE, produced by glial cells, instead of apoA-I. Brain HDL supplies cholesterol to neuronal cells (31). Because it is hard to prepare enough amounts of purified brain HDL for this study and brain HDL is not commercially available, the author used peripheral HDL instead of brain HDL in this study. 24-OHC efflux in the presence of apoA-I was quite low compared to efflux in the presence of HDL. Because cholesterol efflux by ABCA1 is dependent on lipid-free apolipoproteins, and because efflux by ABCG1 is HDL-dependent, it was possible that ABCG1 is responsible for 24-OHC efflux from differentiated SH-SY5Y cells.

To examine if 24-OHC efflux to HDL was mediated by ABCG1, the author suppressed the expression of ABCG1 and ABCA1 using siRNAs. Surprisingly, siRNA against ABCG1 had no significant effect, whereas siRNA against ABCA1 decreased 24-OHC efflux from differentiated SH-SY5Y cells in the presence of HDL. Because ABCA1 expression was induced when ABCG1 expression was suppressed, the author speculated that increased levels of ABCA1 compensated for the lower levels of ABCG1 (Fig. 4A). However, when ABCA1 expression was suppressed, the 24-OHC efflux did not correlate with ABCG1 expression. Therefore, the involvement of ABCG1 in 24-OHC efflux from differentiated SH-SY5Y cells remains unclear. From these results, the author took an alternative approach to reveal the involvement of ABCG1 in 24-OHC efflux from neuronal cells in chapter 2.

ABCG4 was not analyzed in this study, since no antibody, which recognizes endogenously expressed ABCG4, was available. Because ABCG4 is expressed in brain and is required for sterol transport (34), it is possible that ABCG4 is also involved in 24-OHC.

In conclusion, the author demonstrated that efflux of 24-OHC from neuron-like cells is HDL dependent and ABCA1, at least, is involved in 24-OHC efflux from neuron-like cells.

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## **CHAPTER 2**

### **Efflux of 24-OHC from HEK293 cells expressing ABCA1 and ABCG1**



## CHAPTER 2

### 24-OHC efflux from HEK293 cells expressing ABCA1 and ABCG1

ATP-binding cassette protein (ABC) A1 and G1 play major roles in cholesterol homeostasis. ABCA1 promotes cholesterol and phospholipid efflux from cells to lipid-poor apolipoprotein (apo)A-I but not mature high-density lipoprotein (HDL) particles (1-2), while ABCG1 promotes cholesterol efflux to HDL and other lipoprotein particles but not lipid-poor apoA-I (3), suggesting that the two transporters could have complementary activities *in vivo*.

ABCG1 and HDL promote efflux of 7-ketocholesterol and 7- $\beta$ -hydroxycholesterol in transfected HEK293 cells and macrophages, while ABCA1 and apoA-I do not stimulate efflux of these oxysterols (4). Efflux of other oxysterols such as 25-hydroxycholesterol (25-OHC) occurs apoA-I- and HDL- dependent manner in macrophages (5). These results suggest that 25-OHC can be exported by both ABCA1 and ABCG1 pathways.

(24S)-Hydroxycholesterol (24-OHC), which is a brain specific cholesterol metabolite generated by 24-hydroxylase in neuron. Interestingly, there is no report whether ABCA1 and ABCG1 are involved in 24-OHC efflux from cells.

In chapter 1, the author demonstrated that efflux of 24-OHC is HDL-dependent and that ABCA1 is involved in the HDL-dependent 24-OHC efflux from neuron-like cells. Here, the author raised two questions about 24-OHC efflux from cells to HDL. First question is whether ABCA1 can transport 24-OHC to HDL as a lipid acceptor and the second question is whether ABCG1 transports 24-OHC.

In this chapter, the author demonstrated that ABCA1 transports 24-OHC to HDL as a lipid acceptor and ABCG1 is also involved in 24-OHC efflux to HDL using HEK293 cells stably expressing ABCA1 or ABCG1.

## **MATERIALS AND METHODS**

### ***Materials***

24-OHC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). Cholesterol was purchased from Wako Pure Chemical Industries (Osaka, Japan). [22, 23-<sup>3</sup>H] 24-OHC (specific activity: 50-60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). [1, 2, 6, 7-<sup>3</sup>H] Cholesterol (specific activity: 50-60 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). HDL from human plasma was purchased from Athens Research & Technology (Athens, GA, USA). ApoA-I from human plasma was purchased from Calbiochem (San Diego, CA, USA).

### ***Cell culture***

Human embryonic kidney (HEK)293 cells, HEK293 cells stably expressing ABCA1 and HEK293 cells stably expressing ABCG1 were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL benzylpenicillin potassium, 100 µg/mL streptomycin sulfate, and 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### ***[<sup>3</sup>H] 24-OHC and cholesterol efflux from HEK293 cells, stably expressing ABCA1 or ABCG1***

HEK293 cells, stably expressing ABCA1, ABCA1 MM (K939M and K1952M) mutant (6), ABCG1, or ABCG1 KM (K120M) mutant (7) were plated in poly-D-lysine coated 24-well plates at  $1 \times 10^5$  cells/well. Cells were loaded with [<sup>3</sup>H] 24-OHC (0.5 µCi/mL) and unlabeled 24-OHC (10 µM), or [<sup>3</sup>H]cholesterol (1 µCi/mL) and unlabeled cholesterol (10 µM), in DMEM containing 10% FBS for 24 h at 37°C. Next, cells were incubated with DMEM containing 0.1% BSA for 1 h, cells washed

once with PBS containing 0.1% BSA, and once with PBS alone. HEK/ABCA1 cells were incubated for 24 h at 37°C with DMEM containing 0.02% BSA with HDL (50 µg/mL) or apoA-I (10 µg/mL). HEK/ABCG1 cells were incubated for 4 h at 37°C with DMEM containing 0.02% BSA with or without HDL (50 µg/mL). Medium was collected and centrifuged at  $3,000 \times g$  for 3 min to remove cell debris. The cells remaining on the dish were washed twice with ice-cold PBS containing 0.1% BSA, then with PBS, and lysed in 3:2 (v/v) hexane:isopropanol. Total radioactive counts in medium and cell lysate were measured using a liquid scintillation counter.

### ***Data analysis***

Percent efflux was calculated by dividing the radioactivity in the medium by the sum of the radioactivity in the medium and cell lysate. All data are presented as the mean  $\pm$  SD. Experiments were done at least twice independently. The statistical significance of differences between mean values was analyzed using the non-paired t-test. Multiple comparisons were performed using the Dunnet test following ANOVA. A value of  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### ***HDL-dependent efflux of 24-OHC by ABCA1***

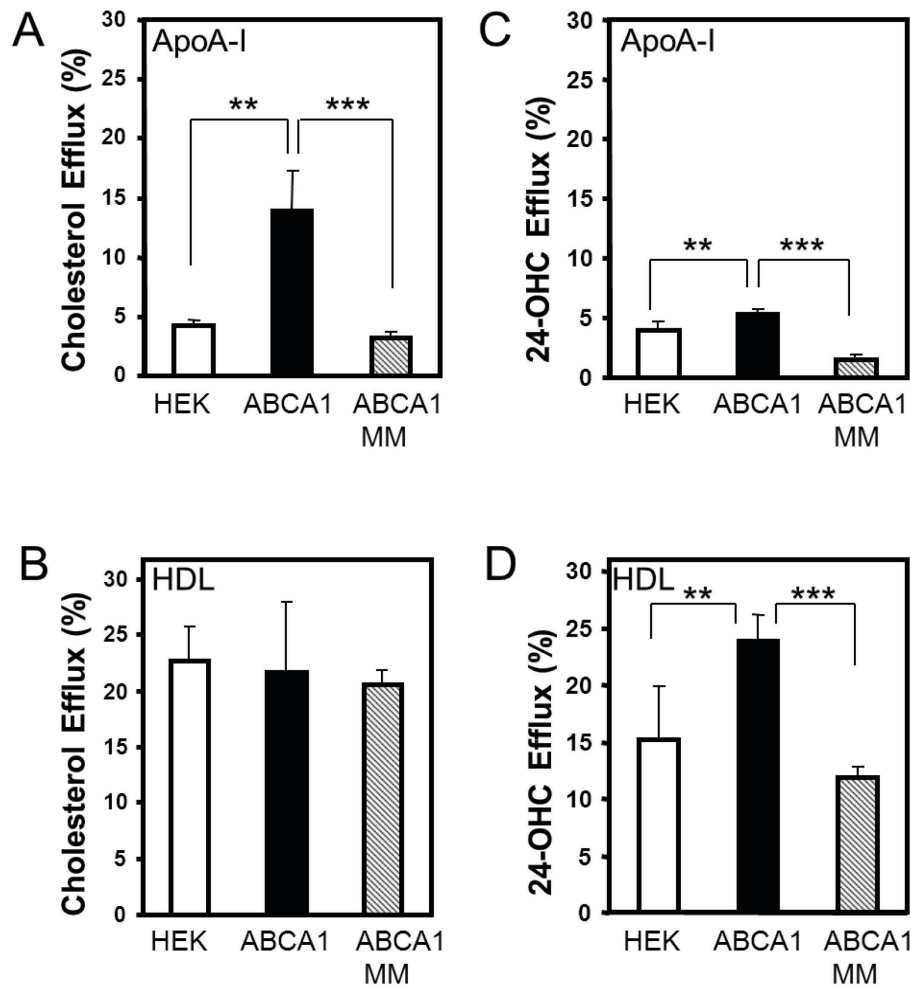
To determine the involvement of ABCA1 in 24-OHC efflux, the author studied HEK293 cells stably expressing ABCA1. At first, the author examined whether cholesterol efflux in the presence of apoA-I, but not HDL, when functional ABCA1 was expressed in HEK293 cells (HEK/ABCA1). As a result of this study, the author observed apoA-I-dependent cholesterol efflux from HEK/ABCA1, but not HDL (Fig.1-A, B) as reported (1-2). On the other hand, apoA-I-dependent cholesterol efflux was observed in neither HEK293 cells nor HEK293 cells stably expressing

ABCA1-MM (HEK/ABCA1-MM), in which the lysine residues critical for ATP hydrolysis are replaced with methionine. These results suggested that apoA-I-dependent cholesterol efflux is mediated by ABCA1 in this system as reported.

Next, the author examined lipid acceptor dependency for 24-OHC efflux using this system. 24-OHC efflux from HEK/ABCA1 was clearly observed in the presence of HDL (Fig. 1D), whereas efflux in the presence of apoA-I was marginal (Fig. 1C). HDL-dependent 24-OHC efflux was observed from neither HEK293 cells nor HEK/ABCA1-MM cells. These results suggested that HDL-dependent 24-OHC efflux is mediated by ABCA1.

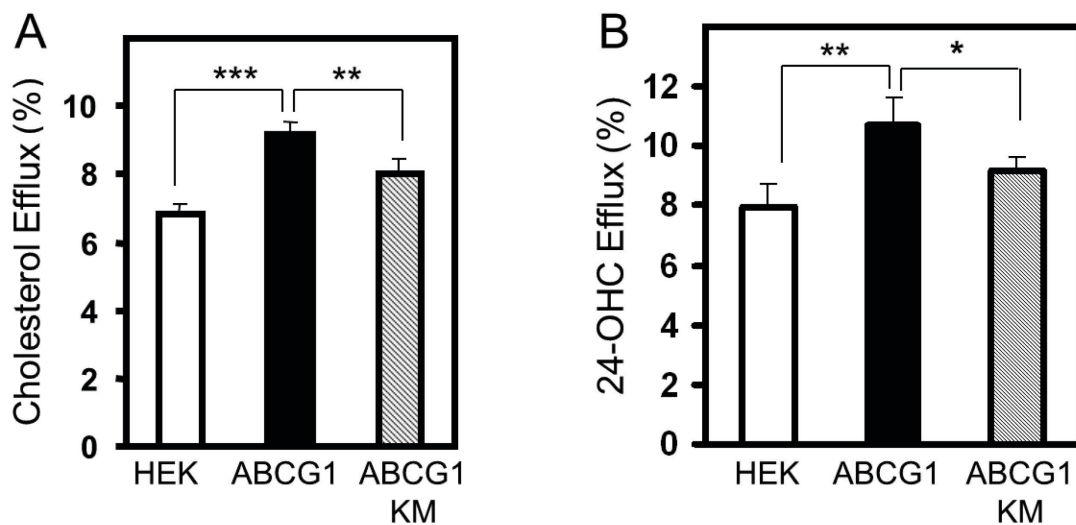
#### ***ABCG1 is also involved in HDL-dependent 24-OHC efflux***

To demonstrate whether ABCG1 also transports 24-OHC, the author studied HEK293 cells stably expressing ABCG1. When functional ABCG1 was expressed in HEK293 cells, the author observed cholesterol efflux in the presence of HDL as reported (3, 6-7) (Fig. 2A); 24-OHC efflux from HEK/ABCG1 was also clearly observed in the presence of HDL (Fig. 2B). Non-functional ABCG1, in which the lysine residue critical for ATP hydrolysis is replaced with methionine, exhibited neither cholesterol nor 24-OHC efflux activity. These results suggest that ABCG1 transports 24-OHC from cells to HDL.



**Figure 1. Cholesterol and 24-OHC efflux from HEK/ABCA1 cells in the presence of apoA-I or HDL.**

HEK293 cells expressing ABCA1 or a mutant ABCA1MM were loaded with 1  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ]cholesterol (A, B) or 0.5  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ] 24-OHC (C, D), and then incubated with apoA-I (A, C) or HDL (B, D) for 24 h. 24-OHC efflux was calculated as described for Fig. 2. Each bar represents mean  $\pm$  S.D. (n=4). \*\*,  $p < 0.01$ . \*\*\*,  $p < 0.001$ .



**Figure 2. Cholesterol and 24-OHC efflux from HEK/ABCG1 in the presence of HDL.**

HEK293 cells expressing ABCG1 or the ABCG1KM mutant were loaded with 1  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ] cholesterol (A) or 0.5  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ] 24-OHC (B) for 24 h. Next, cells were incubated with HDL for 4 h. 24-OHC efflux was calculated as described for Fig. 2. Each bar represents mean  $\pm$  S.D. (n=4). \*,  $p < 0.05$ . \*\*,  $p < 0.01$ . \*\*\*,  $p < 0.001$ .

## DISCUSSION

ABCA1 promotes cholesterol and phospholipid efflux from cells to lipid-poor apoA-I but not mature HDL particles, while ABCG1 promotes cholesterol efflux to



HDL and other lipoprotein particles but not lipid-poor apoA-I (1-2, 7). However, it remained unclear whether ABCA1 and ABCG1 are involved in HDL-dependent 24-OHC efflux. Here, the author showed HDL-dependent 24-OHC efflux is mediated by both ABCA1 and ABCG1.

To determine the involvement of ABCA1 and ABCG1 in 24-OHC efflux, the author studied HEK293 cells stably expressing ABCA1 or ABCG1 (Figs. 1 and 2). 24-OHC efflux from HEK/ABCA1 and HEK/ABCG1 was clearly observed in the presence of HDL, whereas efflux in the presence of apoA-I was marginal. Taken together, these results suggest that both ABCA1 and ABCG1 mediate 24-OHC efflux to HDL.

Because cholesterol efflux by ABCA1 is apoA-I-dependent, it was surprising to observe 24-OHC efflux by ABCA1 in the presence of HDL, but not apoA-I (1-2). However, it was previously reported (9) that the function of ABCA1 does not depend on apoA-I, and that ABCA1 can transport cholesterol in the presence of bile salts in the medium. Therefore, it has also been proposed that lipid accumulation within the extracellular domain via ATP hydrolysis-dependent lipid transport causes conformational changes that generate apoA-I-binding site(s) on the surface of the extracellular domain of ABCA1, and that apoA-I bound to these site(s) is directly loaded with lipids by ABCA1 (9). The amphipathic molecule 24-OHC may not be reserved in the extracellular domain of ABCA1, but may escape to the medium and bind to HDL as a lipid acceptor in the medium.

In this study, the author showed HDL-dependent 24-OHC efflux is mediated by both ABCA1 and ABCG1. The author also demonstrated that 24-OHC is eliminated from neuronal cells to HDL in chapter 1. These results suggest that ABCA1 and ABCG1 are involved in 24-OHC efflux from neuronal cells to HDL.

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## **CHAPTER 3**

### **Neuronal cell protection by ABCA1 and ABCG1 from 24-OHC induced cell death**



## CHAPTER 3

### Neuronal cell protection by ABCA1 and ABCG1 from 24-OHC induced cell death

(24*S*)-Hydroxycholesterol (24-OHC) is a brain specific cholesterol metabolite and generated by cholesterol 24-hydroxylase (CYP46) in neurons (1). This oxysterol is one of the natural ligands of liver X receptor (LXR), a central player in the regulation of cholesterol metabolism (2-3). LXR is also expressed in the central nervous system and is involved in the regulation of brain cholesterol homeostasis. The synthetic LXR ligand TO901317 (TO) was reported to induce the expression of ATP binding cassette transporter (ABC) A1 and ABCG1 in murine astrocytes (4).

In chapter 1, the author demonstrated that expression of ABCA1 and ABCG1 is induced by 24-OHC and TO in neuron-like cells. Moreover, the author revealed that ABCA1 and ABCG1 transport 24-OHC from cells to HDL as a lipid acceptor in chapters 1 and 2. These results suggested that ABCA1 and ABCG1 controlled by LXR regulate 24-OHC concentration in neuronal cells. It has been reported that 24-OHC is present at high concentrations up to 30  $\mu$ M in the brain. High concentration of 24-OHC is toxic to neuron and induces apoptosis (5-7). The author expected that ABCA1 and ABCG1 would protect neurons against the toxicity of 24-OHC, if they are involved in 24-OHC efflux from neuronal cells.

In this chapter, the author examined whether ABCA1 and ABCG1 prevent 24-OHC-induced neuronal cell death using rat primary neurons.

## **MATERIALS AND METHODS**

### ***Materials***

24-OHC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). TO901317 was purchased from ALEXIS Biochemicals (San Diego, CA, USA). 9-cis retinoic acid (RA) was purchased from Sigma. Rat cerebral neuron was purchased from DS PHARMA MEDICAL (Osaka, Japan). LDH assay kit was purchased from Promega (Madison, WI, USA). TaqMan Fast Universal PCR Master Mix was purchased from Life Technologies Corporation (Carlsbad, CA USA). ABCA1, ABCG1 specific primers were purchased from Life Technologies Corporation (Carlsbad, CA USA).

### ***Isolation of rat cerebral neurons***

Primary cerebral neurons were isolated from rat by using neuron isolation kit (DS PHARM BIOMEDICAL) and then plated on Poly-D-Lysine coated plate at  $2 \times 10^5$  cells/mL. The cells were cultured in Neurobasal (Invitrogen, Carlsbad, CA, USA) supplemented with 200 mM L-glutamine and 2% B27 supplement (Invitrogen). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. After cultured at least for 6 days, rat primary neurons were used.

### ***Quantitative real-time PCR analysis***

Total RNA was extracted from rat primary neurons using RNeasy kit according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed using 7500 Fast Real-Time PCR System (PE Applied Biosystems, Forster City, CA, USA) with TaqMan Fast Universal PCR Master Mix. PCR was performed through 40 cycles of 95°C for 3 sec and 60°C for 30 sec after preincubation at 95°C for 20 sec. The Primer of ABCA1 (Assay ID: Rn00710172\_m1), ABCG1 (Assay ID: Rn00585262\_m1) and GAPDH were purchased from Applied Biosystems.



### ***24-OHC induced neuronal cell death***

Rat primary neurons were treated with or without TO901317 (5  $\mu$ M) and RA (5  $\mu$ M) for 16 h. The cells were treated with indicated concentrations of 24-OHC for 24 h with or without TO901317 (TO) and RA. For the determination of cell viability, LDH release was measured by using LDH assay kit (Promega).

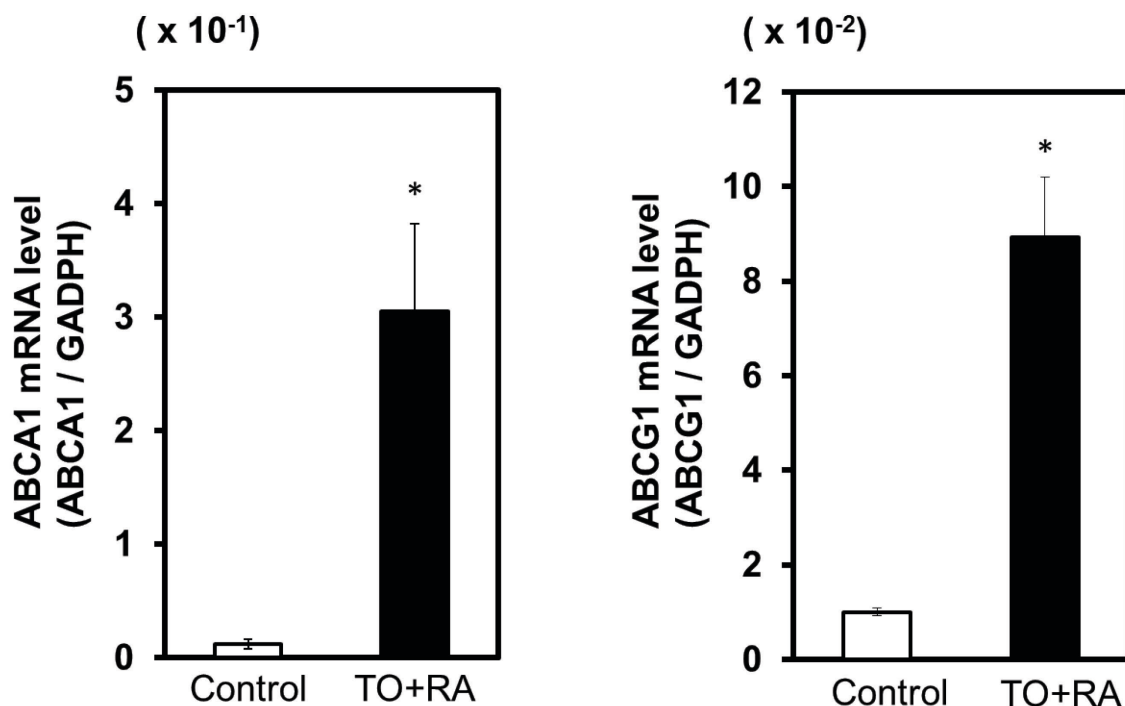
### ***Data analysis***

All data are presented as the mean  $\pm$  S.D. The statistical significance of differences between mean values was analyzed using the non-paired t-test. A value of  $p < 0.01$  was considered statistically significant.

## **RESULTS**

### ***Induction of ABCA1 and ABCG1 expression in rat primary neuron by LXR/RXR ligands***

It has been reported that the synthetic LXR ligand TO does not induce the expression of ABCA1 and ABCG1 efficiently in murine primary neurons while it induces their expression well in murine primary astrocytes (4). Therefore, the author first examined whether LXR/RXR ligand induces expression of ABCA1 and ABCG1 in rat primary neurons. By the treatment with TO and RA for 16 h, the mRNA expression of ABCA1 and ABCG1 in rat primary neurons was significantly increased by 25.9-fold and 8.91-fold, respectively (Fig.1-A,B). This result suggested that expression of ABCA1 and ABCG1 is efficiently induced by LXR/RXR ligands in rat primary neurons.



**Figure 1. Effect of LXR/RXR ligands on the expression of ABCA1 and ABCG1 in rat primary neurons.**

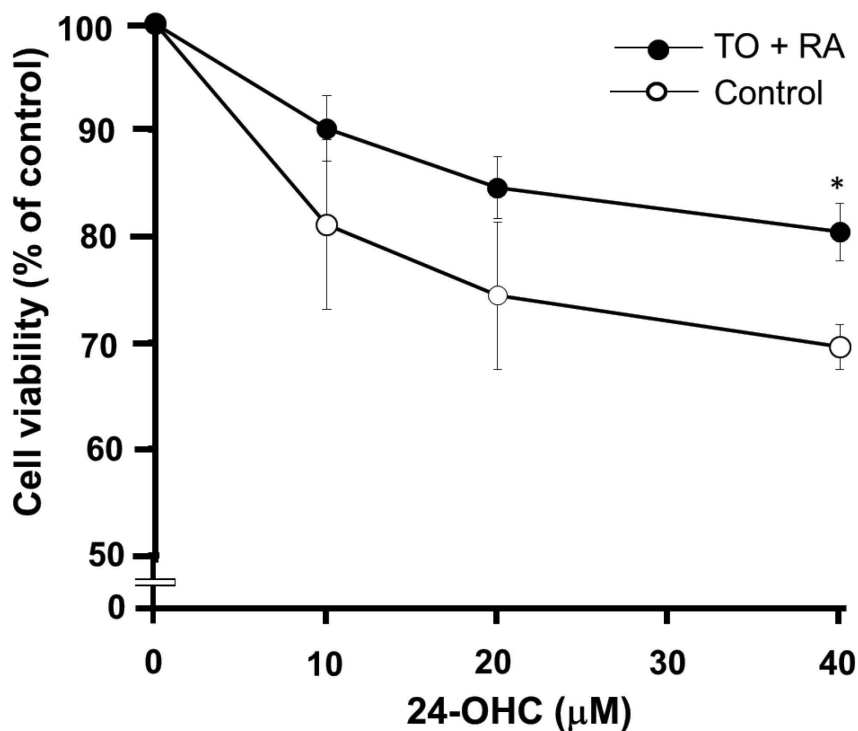
Rat primary neurons were treated with 0.1% ethanol alone (control) or 5  $\mu$ M TO901317 and 5  $\mu$ M 9-cis retinoic acid (TO + RA) for 16 h. Relative ABCA1 (A) and ABCG1 (B) mRNA levels were determined by quantitative real-time PCR analysis. mRNA level was normalized with GAPDH mRNA. Each bar represents the mean  $\pm$  S.D. (n =4).

\* $p < 0.01$ , significantly different from the control.

#### ***Involvement of ABCA1 and ABCG1 in the protection of neurons against 24-OHC***

24-OHC is toxic to neurons and induces apoptosis (7). The author expected that the expression of ABCA1 and ABCG1 would protect neurons against the toxicity of 24-OHC, if they are involved in 24-OHC efflux. The author examined this possibility by using rat primary cerebral neurons (Fig. 2). 24-OHC induced cell death in a concentration-dependent manner as revealed by LDH release assay. When primary

neurons were pretreated with TO and RA for 16 h to induce the expression of ABCA1 and ABCG1 beforehand, the neurons became significantly more resistant to 24-OHC. This result suggests that ABCA1 and ABCG1 protect neurons against toxicity of 24-OHC by exporting it from cells.



**Figure 2. Effects of LXR/RXR ligands on 24-OHC-induced cell death in rat primary neuron.**

Rat primary neurons were cultured on poly-D-lysine-coated 96 well plates for 6 days. Cells were incubated with 5 µM TO901317 and 5 µM RA (TO+RA) or 0.1% ethanol (control) for 16 h. Cells were then incubated with indicated concentrations of 24-OHC with or without TO+RA for 24 h and LDH release to the culture medium was analyzed. Each bar represents mean  $\pm$  S.D. (n=3).

\* $p < 0.01$ , significantly different from the control.

## **DISCUSSION**

The conversion of cholesterol to 24-OHC by CYP46 in neurons plays an important role for the brain function, especially learning. At the same time, 24-OHC possesses potent cytotoxicity, and it induces apoptosis of neuronal cells by generating free radicals (5-6). Therefore, 24-OHC must be eliminated from cells as soon as possible after its production.

In chapters 1 and 2, the author suggested that ABCA1 and ABCG1 export 24-OHC from neuronal cells. Therefore, the author expected that ABCA1 and ABCG1 would protect neurons against the toxicity of 24-OHC. In this study, the author proposed that ABCA1 and ABCG1 prevent 24-OHC-induced neuronal cell death.

There was no report that expression of ABCA1 and ABCG1 is induced by LXR/RXR ligand in rat primary neurons. The author wanted to examine whether the protein expression of ABCA1 and ABCG1 is increased by LXR/RXR ligand. However, no antibodies, which recognize endogenously expressed rat ABCA1 or ABCG1, were available. Therefore, the author examined whether mRNA level of ABCA1 and ABCG1 is induced by LXR/RXR ligand in rat primary neurons. As a result, the author succeeded in demonstrating that the expression of ABCA1 and ABCG1 is induced by TO and RA in rat primary neurons.

Next, the author examined whether ABCA1 and ABCG1 prevent 24-OHC-induced apoptosis. Neuronal cell death was observed in about 30% of rat primary neurons, when they were treated with 40  $\mu$ M 24-OHC for 24 h. In contrast, when they were pre-treated with LXR ligands and the expression of ABCA1 and ABCG1 was induced, neuronal cell death was observed only in about 20% of rat primary neurons. These results suggest that ABCA1 and ABCG1 prevent 24-OHC-induced neuronal cell death.

In this chapter, the author showed that the induction of expression of ABC proteins protects primary cerebral neurons against the toxicity of 24-OHC.

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## CONCLUSIONS

The purpose of the present study in this thesis was to clarify the involvement of ABCA1 and ABCG1 in 24-OHC efflux from neuronal cells. The author revealed that 24-OHC efflux from neuronal cells to HDL is mediated by ABCA1 and ABCG1. In addition, ABCA1 and ABCG1 prevent 24-OHC-induced neuronal cell death. The results described in each chapter can be summarized as follows.

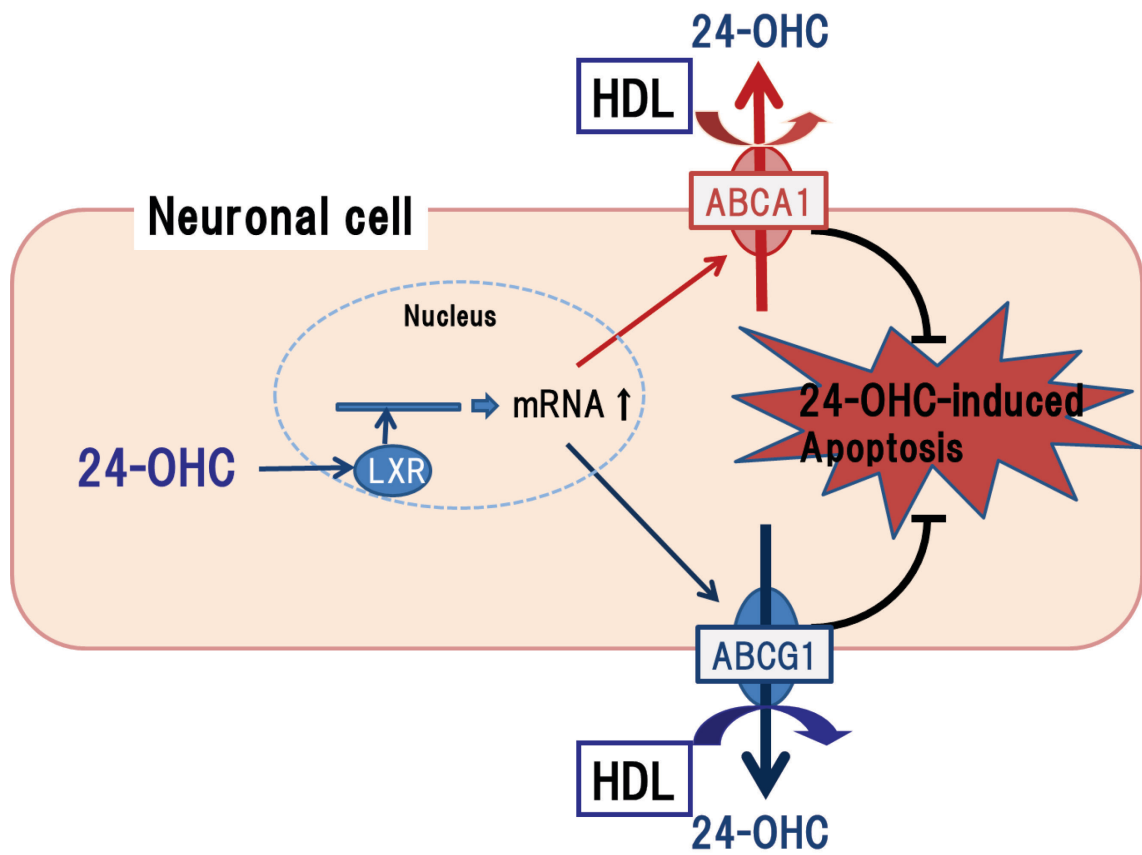
In chapter 1, the author revealed that the expression of ABCA1 and ABCG1 is induced by 24-OHC in differentiated SH-SY5Y cells and that the induction is 24-OHC concentration dependent. Next, the author demonstrated that efflux of 24-OHC from differentiated SH-SY5Y cells is HDL dependent but not apoA-I dependent and that the efflux is stimulated by the treatment with LXR/RXR ligands. Moreover, the 24-OHC efflux is suppressed after the treatment with ABCA1 siRNA. These results suggest that ABCA1 is involved in HDL-dependent 24-OHC efflux from differentiated SH-SY5Y cells. On the other hand, because expression of ABCA1 was induced after the treatment with ABCG1 siRNA, the involvement of ABCG1 in 24-OHC efflux remained unclear.

In chapter 2, the author demonstrated that ABCA1 and ABCG1 transport 24-OHC from cells stably expressing ABCA1 or ABCG1. Cholesterol efflux from HEK293 cells stably expressing ABCA1 was apoA-I dependent but not HDL dependent as previously reported. In contrast, 24-OHC efflux by ABCA1 was HDL dependent but not apoA-I dependent. Cholesterol and 24-OHC efflux from HEK293 cells stably expressing ABCG1 was HDL dependent. These results suggest that HDL-dependent 24-OHC efflux is mediated by both ABCA1 and ABCG1.

In chapter 3, the author examined whether ABCA1 and ABCG1 prevent the 24-OHC-induced neuronal cell death using rat primary neuron. Neuronal cell death was observed in about 30% of rat primary neurons, when they were treated with 40  $\mu$ M 24-OHC for 24 h. In contrast, when they were pre-treated with LXR ligands and the expression of ABCA1 and ABCG1 was induced, neuronal cell death was observed only in about 20% of rat primary neurons. These results suggest that ABCA1 and ABCG1 prevent 24-OHC-induced neuronal cell death.

High cholesterol turnover is essential for brain functions and 24-OHC is the major cholesterol metabolite in the brain. However, 24-OHC is toxic to neuronal cells. From the findings demonstrated in this study, the author proposes that two ABC proteins, ABCA1 and ABCG1, are involved in active elimination of 24-OHC in the presence of HDL as a lipid acceptor and protect neuronal cells from apoptosis (Figure 1).





**Figure 1. ABCA1 and ABCG1 actively eliminate 24-OHC in the presence of HDL and protect neuronal cells from apoptosis.**



## LIST OF PUBLICATIONS

A Akihiro Matsuda, Kohjiro Nagao, Michinori Matsuo, Noriyuki Kioka, and Kazumitsu Ueda

**24(S)-hydroxycholesterol is actively eliminated from neuronal cells by ABCA1**

*Journal of Neurochemistry* 2013, 126, 93–101

### Related Publications

B Sumio Ohtsuki, Shingo Ito, Akihiro Matsuda, Satoko Hori, Takaaki Abe and Tetsuya Terasaki

**Brain-to-blood elimination of 24S-hydroxycholesterol from rat brain is mediated by organic anion transporting polypeptide 2 (oatp2) at the blood–brain barrier**

*Journal of Neurochemistry*, 2007, 103, 1430–1438



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