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Functional expression of breast cancer resistance protein and cholesterol effect in human erythrocyte membranes

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In human erythrocyte membranes, various influx and efflux transporters are functionally expressed. However, their transport characteristics and modulation under disease states are not fully understood. In this study, we first examined the expression and detailed transport characteristics of breast cancer resistance protein (BCRP), an efflux ABC transporter, using inside-out membrane vesicles (IOVs) prepared from human erythrocytes, and then studied the effect of membrane cholesterol on BCRP function. The expression of BCRP was confirmed by western blotting; most of them being homodimers. The uptake of lucifer yellow (LY), a fluorescent BCRP substrate, into IOVs was time-, temperature-, and ATP-dependent, and the concentration of ATP which induced half-maximal stimulation of LY uptake was calculated to be 0.39 mM. The uptake of LY by IOVs was saturable with a Km value of 166 μM, and was inhibited by various BCRP inhibitors and substrates, such as fumitremorgin C and mitoxantrone. When membrane cholesterol content was increased by treating IOVs with cholesteryl hemisuccinate, LY uptake decreased with increasing cholesterol content. These results suggest that transport activity of BCRP in human erythrocyte membranes may be suppressed under disease states, such as hypercholesterolemia, that increase membrane cholesterol content.

1. Introduction

Breast cancer resistance protein (BCRP; ABCG2), an efflux transporter, is a member of the ATP-binding cassette (ABC) transporter family (Murakami and Takano 2008). In contrast to other ABC transporter families, such as P-glycoprotein (MDR1; ABCB1) and multidrug resistance-associated proteins (MRPs; ABCC), which have two ATP-binding domains, however, BCRP is a half-ABC transporter with one ATP-binding domain. It is suggested that BCRP functions as a homodimer in the plasma membrane, though the mechanism of homodimer formation is not clearly understood (Mao and Unadkat 2015). BCRP is expressed in a variety of tumor cells as well as in many normal human tissues including the small intestine and liver, and pumps out relatively hydrophilic drugs and endogenous compounds from the cells (Takano et al. 2006).

In mammalian erythrocyte membranes, various ABC efflux transporters including BCRP have been detected (Yumoto et al. 2009; Zhou et al. 2005). Jonker et al. (2002) reported that mice lacking Bcrp1 became extremely sensitive to the dietary chlorophyll-breakdown product pheophorbide a, resulting in severe phototoxic lesions on light-exposed skin. Also, in the erythrocytes of Bcrp1$^{-/-}$ mice, levels of the heme precursor and phototoxin protoporphyrin IX (PPIX) were markedly increased. In addition, Zhou et al. (2005) suggested that increased expression of Bcrp during erythroid maturation might play a role in decreasing cellular PPIX level in mice. Therefore, BCRP in erythrocyte membranes may have a role in protecting erythrocytes from excessive accumulation of PPIX, possibly by pumping out this compound from the cells. However, the transport function of BCRP in human erythrocytes is not fully understood, especially at a membrane level. De Wolf et al. (2007) studied cGMP transport using vesicles isolated from mouse and human erythrocytes. They found that cGMP was transported by Mrp4 and Bcrp1 in mouse erythrocyte membranes, whereas BCRP had a negligible role in cGMP transport in human erythrocyte membranes. Therefore, further studies are needed to characterize BCRP-mediated transport in human erythrocyte membranes.

Cholesterol is a lipid that is essential as a component of animal cell membranes, which maintains membrane structural integrity and fluidity. It also serves as a precursor for the biosynthesis of various important endogenous compounds like steroid hormones. In addition, cholesterol is known to interact with and modulate certain membrane transporters such as P-glycoprotein (Clay et al. 2015). However, the effect of cholesterol on BCRP function in erythrocyte membranes is not known.

In the present study, we first examined the expression and transport function of BCRP in human erythrocyte membranes. For this purpose, inside-out membrane vesicles were prepared from human erythrocytes, and used for expression and transport studies. We then examined the effect of membrane cholesterol, which is known to be increased in hypercholesterolemia, on BCRP function in human erythrocytes.

2. Investigations and results

2.1. Expression of BCRP protein in human erythrocyte membranes

First, the expression of BCRP protein in human inside-out membrane vesicles (IOVs) prepared was confirmed by western blot analysis under non-reducing and reducing conditions. Under non-reducing conditions (without 2-mercaptoethanol), the band density of the 140 kDa protein was much stronger than that of the 70 kDa protein (Fig. 1, left). On the other hand, under reducing conditions, the band density of the 70 kDa protein became relatively stronger than that of the 140 kDa protein (Fig. 1, right). These results may indicate that BCRP in human erythrocyte membranes predominantly exists as a homodimer.

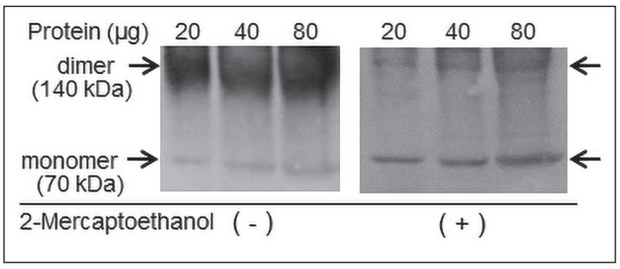

Fig. 1: Western blot analysis of BCRP protein expression in human erythrocyte membranes. IOVs were treated with SDS buffer with or without 2-mercaptoethanol for 5 min at 95 °C, and applied to SDS-PAGE (20, 40, and 80 μg protein/lane). The protein was blotted on a PVDF membrane and detected with a mouse monoclonal antibody for BCRP.

2.2. Characteristics of BCRP-mediated transport of lucifer yellow (LY) in human erythrocyte IOVs

Characteristics of LY uptake were examined in IOVs prepared from human erythrocytes. The uptake of LY in the presence of ATP was time-dependent and increased linearly with time for up to 40 min at 37 °C, whereas the uptake in the absence of ATP (in the presence of AMP) was negligible (Fig. 2A). The uptake of LY also showed marked temperature-dependence, and at 4 °C, no substantial uptake was observed (Fig. 2B). In addition, the uptake of LY was highly dependent on ATP, and increased with the increase in ATP, but became saturated at higher concentrations (Fig. 2C). The ATP concentration which induced half-maximal stimulation of LY uptake was calculated to be 0.39 mM, using a Michaelis-Menten type equation.

2.3. Concentration-dependence of LY uptake in human erythrocyte IOVs

The uptake of LY was measured at various concentrations of LY (25–1000 μM). ATP-dependent uptake of LY was saturable (Fig. 3), and the Michaelis constant (Km) and maximum uptake rate (Vmax) estimated from the Eadie-Hofstee plot were 166 μM and 4.3 nmol/mg protein/30 min, respectively.

2.4. Effect of BCRP inhibitors and substrates on LY uptake in human erythrocyte IOVs

The concentration-dependent effect of BCRP inhibitors, fumitremorgin C (FTC) and Ko143, on LY uptake was examined in human erythrocyte IOVs. FTC inhibited LY uptake in a concentration-dependent manner, and its IC_{50} value was calculated to be 3.4 μM (Fig. 4A). Similarly, a concentration-dependent inhibitory effect of Ko143 on LY uptake was observed with the IC_{50} value being 1.5 μM (Fig. 4B). We next examined the effect of BCRP substrates, mitoxantrone and sulfasalazine, on LY uptake. The uptake of LY by IOVs was inhibited by these compounds in a concentration-dependent manner (Fig. 4C, D). The IC_{50} values of mitoxantrone and sulfasalazine on LY uptake were 34.7 μM and 1.6 μM, respectively.

2.5. Effect of membrane cholesterol on LY uptake in human erythrocyte IOVs

Human erythrocyte IOVs were pretreated with various concentrations of cholesteryl hemisuccinate (CHS), and after washing the IOVs, cholesterol content in the IOVs was measured. As shown in Fig. 5A, membrane cholesterol content increased with increasing concentrations of CHS used for the pretreatment. There was a good inverse relationship (R^2 = 0.998) between membrane cholesterol content and BCRP-mediated LY transport activity, and corresponding to the increase in the membrane cholesterol of IOVs, LY uptake decreased (Fig. 5B). The effect of cholesterol on LY uptake was further examined in IOVs prepared from washed human erythrocytes pretreated with CHS. Similar to the results shown in Fig. 5, increased membrane cholesterol content and decreased LY uptake were also observed in IOVs prepared by this method (Fig. 6).

3. Discussion

BCRP was initially identified in a doxorubicin-resistant MCF-7/AdrVp breast cancer cell line as an efflux transporter, which conferred resistance of the cells to anthracycline anticancer drugs

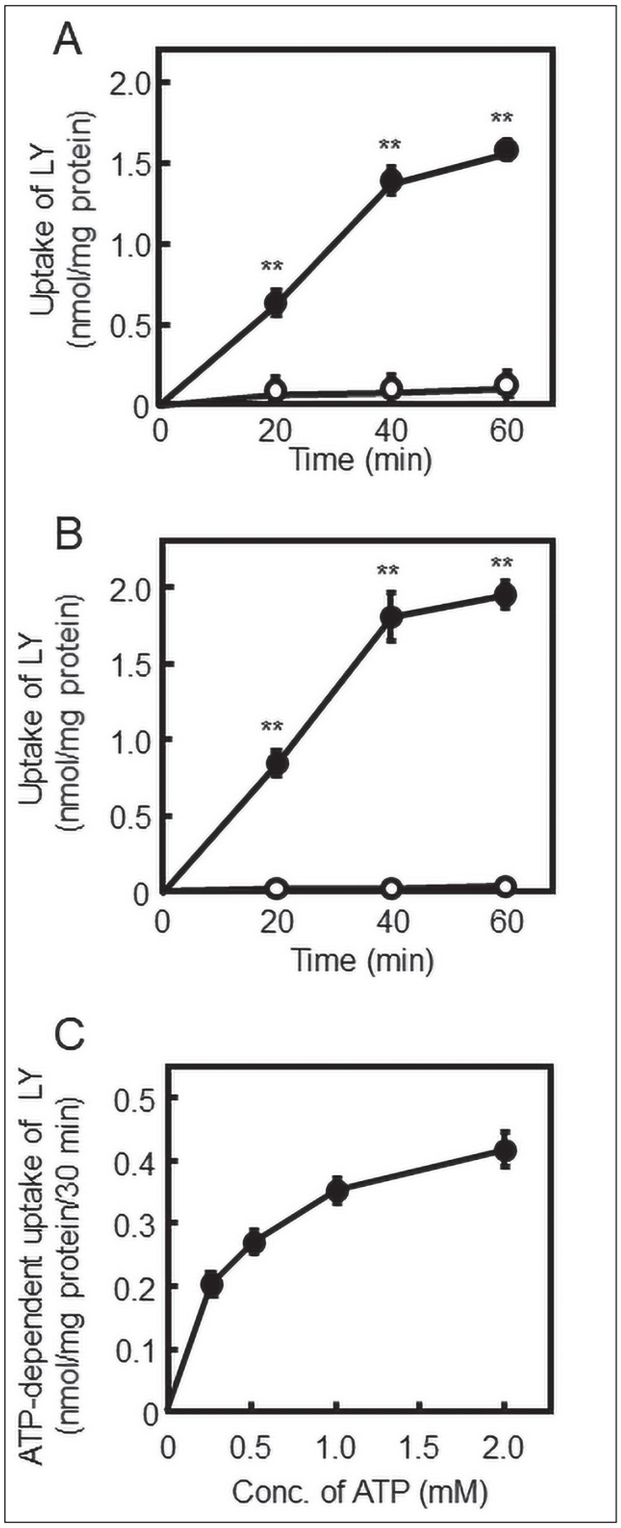

Fig. 2: Time-, temperature-, and ATP-dependence of LY uptake in human IOVs. IOVs were incubated with LY (50 μM); (A) in the presence of 1 mM ATP (closed circles) or 1 mM AMP (open circles) at 37 ºC, (B) at 37 ºC (closed circles) or 4 ºC (open circles) in the presence of 1 mM ATP, and (C) for 30 min at 37 ºC in the presence of various concentrations of ATP (0–2 mM). Each value represents the mean ± S.E. (n=3). **p<0.01, significantly different from the uptake value with 1mM AMP (A) and from the uptake value at 4 ºC (B) at each time point.

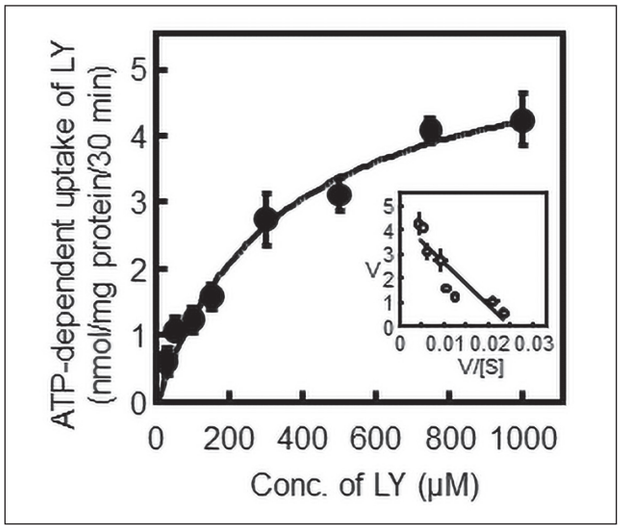

Fig. 3: Concentration-dependence of LY uptake in human IOVs. IOVs were incubated with various concentrations (25 μM–1 mM) of LY for 30 min at 37 ºC in the presence of 1 mM ATP or 1 mM AMP. The ATP-dependent uptake was calculated by subtracting the uptake with AMP from that with ATP at each LY concentration. The inset shows Eadie-Hofstee plot of ATP-dependent uptake. Each value represents the mean ± S.E. (n=3)

(Doyle et al. 1998). Later, it was found that BCRP was expressed in various normal human tissues, including the small intestinal epithelial cells, the liver hepatocytes, and the endothelial cells of brain microvessels. BCRP transports many drugs such as anthracenes, camptothecin derivatives, and nucleoside analogs as substrates. Also, many drugs such as tyrosine kinase inhibitors, HIV protease inhibitors, and calcium channel blockers inhibit the transport function of BCRP. As a result, BCRP is now recognized as one of the key drug transporters involved in clinically relevant drug disposition (International Transporter Consortium 2010; Mao and Unadkat 2015).

The expression of BCRP in erythrocyte membranes is found in various animal species including humans (Shi et al. 2018). In this study, we confirmed BCRP protein expression in human erythrocyte membranes. In addition, based on the western blot analysis under non-reducing and reducing conditions, it was suggested that BCRP in human erythrocyte membranes predominantly exists as a homodimer (Fig. 1), as reported by Leimanis and Georges (2007). There were several reports showing the role of cysteine residues in human BCRP, and Cys-603 in the extracellular domain was suggested to be important for homodimer formation by intermolecular disulfide bridging (Henriksen et al. 2005; Kage et al., 2005; Wakabayashi et al. 2006). On the other hand, the disulfide bridge formed by Cys-603 residues may not be essential for BCRP protein expression and function. The mechanism underlying BCRP homodimer formation and its relationship with membrane localization and transport function are still ambiguous.

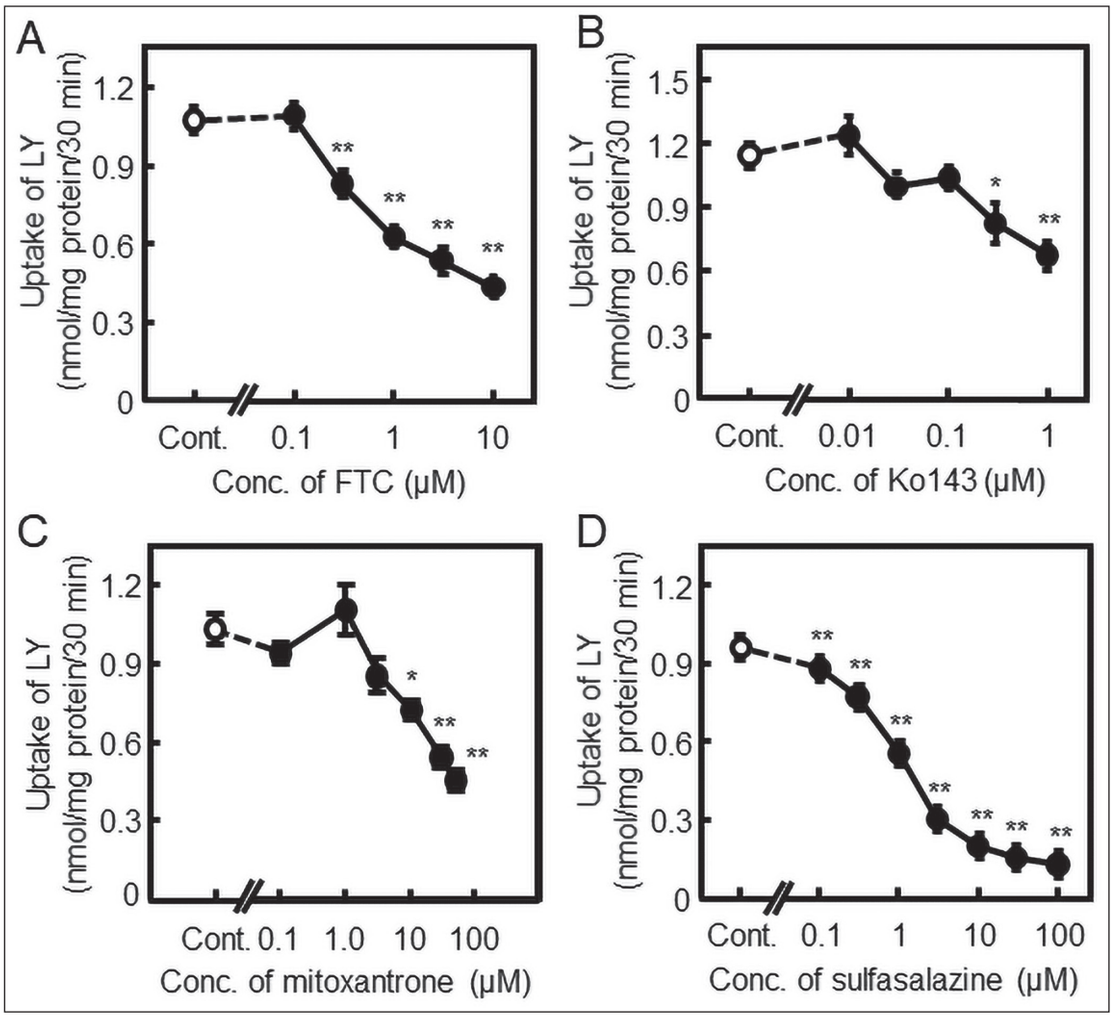

Fig. 4: Effect of various inhibitors and substrates of BCRP on LY uptake in human IOVs. IOVs were incubated with 50 μM LY at 37 ºC for 30 min in the absence (open circles) or presence (closed circles) of various concentrations of FTC (A), Ko143 (B), mitoxantrone (C), and sulfasalazine (D). Each value represents the mean ± S.E. (n=3) *p<0.05, **p<0.01, significantly different from each control.

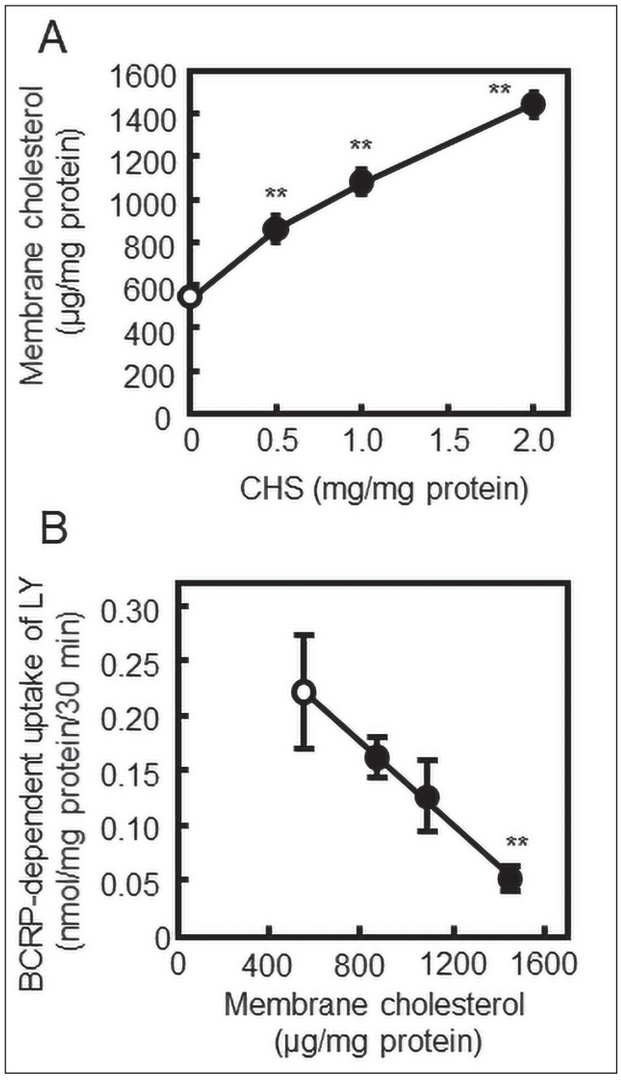

Fig. 5: Effect of CHS treatment of human IOVs on membrane cholesterol content (A) and the relationship between membrane cholesterol content and BCRP function (B). IOVs were incubated with various concentrations of CHS (0.5–2.0 mg/mg protein) at 37 ºC for 1 h, and membrane cholesterol content and BCRP-dependent uptake of LY were measured in CHS-treated IOVs. Each value represents the mean ± S.E. (n=3) **p<0.01, significantly different from each control.

In this study, LY was used as a substrate of BCRP for transport experiments using human erythrocyte IOVs. LY uptake in IOVs was highly dependent on the presence of ATP, and the half-maximal stimulatory concentration of ATP on LY uptake was 0.39 mM (Fig. 2). Using human erythrocyte IOVs, we previously reported that the concentration of ATP which induced half-maximal stimulation of 5-(and-6)-carboxy-2', 7' dichlorofluorescein (CDCF) uptake was 0.54 mM (Yumoto et al. 2009), a value similar to that for LY uptake. In human erythrocyte membranes, CDCF was predominantly transported by multidrug resistance-associated protein (MRP) 5. Therefore, ABC efflux transporters expressed in human erythrocytes such as BCRP and MRP5 may have similar sensitivity to ATP. Assuming that ATP concentration in human erythrocytes is 1 mM (Board 1981), these ABC transporters would efficiently pump out their substrates from the erythrocytes.

Deng et al. (2016) used LY as a substrate to study the effect of albumin on BCRP-mediated transport using membrane vesicles prepared from Sf9 insect cells expressing BCRP. They showed that LY uptake by the vesicles was saturable, and the Km value without albumin was 31.2 µM. The value was smaller than the value obtained in this study (166 µM), which may be due to the different composition of the membranes (insect cell membranes vs. human erythrocyte membranes). In fact, Sjöstedt et al. (2017) showed that the Km value (78.1 µM) of LY uptake in membrane vesicles obtained from BCRP-expressing HEK293 cells (human derived cell line) was higher than that (45.1 µM) in membrane vesicles obtained from BCRP-expressing Sf9 cells, though the Km value in this study was still somewhat higher than that observed in BCRP-expressing HEK293 cell membranes.

FTC and Ko143 are well recognized as selective BCRP inhibitors, and Ko143 is a FTC analogue having a more potent inhibitory effect than FTC (Mao and Unadkat 2015). We observed concentration-dependent inhibitory effects of FTC and Ko143 on LY uptake (Fig. 4), confirming that LY was taken up by BCRP in human erythrocyte IOVs. The uptake of LY in IOVs was also inhibited by mitoxantrone, a typical BCRP substrate. Sulfasalazine was also reported to be a BCRP substrate and was suggested to be useful as an in vivo BCRP probe (Urquhart et al. 2008; Yamasaki et al. 2008). Though there might be some inconsistent results, sulfasalazine inhibited LY uptake in erythrocyte IOVs in this study.

Cholesterol is a major sterol lipid of animal cell membranes, and maintains membrane structural integrity and fluidity. In the membranes, cholesterol is unevenly distributed with a preference for ordered microdomains like lipid rafts. Recently, study results suggest the important role of cholesterol in regulating membrane

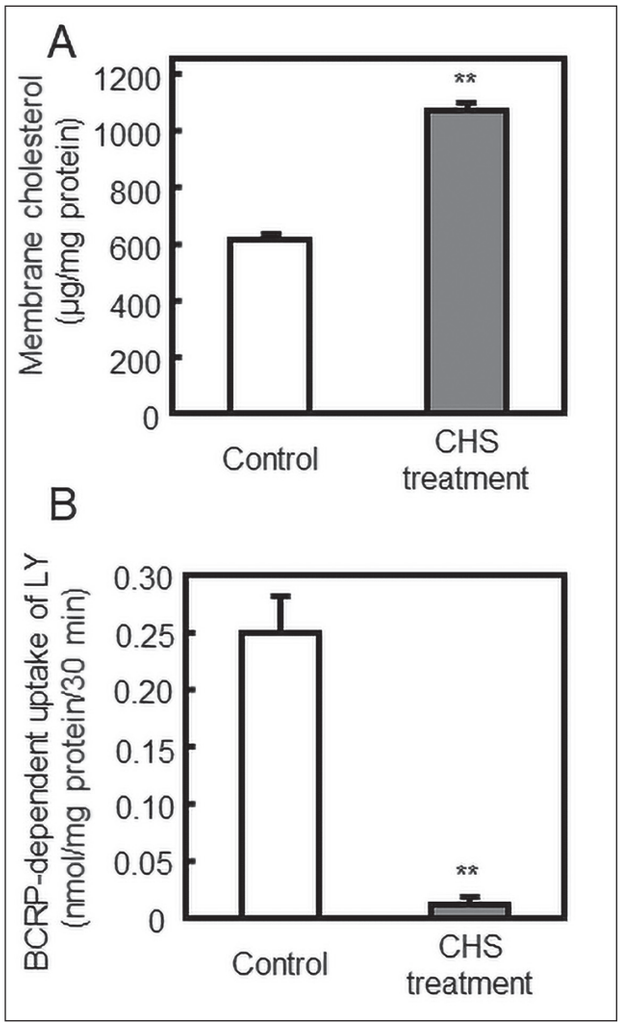

Fig. 6: Effect of CHS treatment of human erythrocytes on membrane cholesterol content (A) and BCRP function (B) in IOVs prepared from CHS-treated erythrocytes. Washed human erythrocytes were incubated with CHS (0.25 mg/mL) at 37ºC for 2 h, and membrane cholesterol content and BCRP-dependent uptake of LY were measured in IOVs prepared from CHS-treated erythrocytes. Each value represents the mean ± S.E. (n=3) **p<0.01, significantly different from each control.

protein structure, function, and dynamics (Grouleff et al. 2015; Song et al. 2014). Cholesterol may modulate proteins directly by binding to the protein, or indirectly through changes in membrane properties, such as the fluidity and membrane thickness.

Storch et al. (2007) examined the membrane localization of BCRP in the canine kidney epithelial cell line MDCKII-BCRP transfected with the cDNA encoding human BCRP, and found that BCRP was located in cholesterol-enriched lipid rafts/caveolae domain of the plasma membrane. They also examined the effect of cholesterol on BCRP activity, and showed that cholesterol depletion provoked a decrease in BCRP activity. In addition, potentiation of BCRP activity by cholesterol was observed in BCRP-expressing Sf9 cell membranes (Pál et al. 2007). In contrast, in this study, BCRP activity decreased in human erythrocyte IOVs pretreated with CHS as well as in IOVs prepared from erythrocytes pretreated with CHS, suggesting that cholesterol enrichment may suppress BCRP activity in human erythrocytes. The reason for this apparent discrepancy is not known at present. Cholesterol content in human erythrocyte membranes under normal physiological conditions may be sufficient for BCRP to fully exert its function, and further enrichment of cholesterol may result in the suppression of BCRP activity.

In conclusion, the transport properties of BCRP in human erythrocytes were characterized at a membrane level using IOVs, in which BCRP existed as a homodimer, and enrichment of membrane cholesterol suppressed the transport activity of BCRP. Cholesterol content in erythrocyte membranes is increased in patients suffering from various diseases such as familial hypercholesterolemia and acute coronary syndrome (Martínez et al. 1996; Tziakas et al. 2007). Therefore, under these disease states, efflux of endogenous BCRP substrates such as PPIX from the erythrocytes, as well as distribution of BCRP substrate drugs into the erythrocytes, may be changed.

4. Experimental

4.1. Chemicals and reagents

LY CH potassium salt and sulfasalazine were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). CHS tris salt and Ko143 were purchased from Nacalai Tesque (Kyoto, Japan). FTC and mitoxantrone dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibody for BCRP, BXP-21 (sc-58222), was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the secondary antibody (peroxidase-labeled affinity purified antibody to mouse IgG (H+L)) was purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD, USA). All other chemicals used for the experiments were of the highest grade commercially available.

4.2. Preparation of inside-out human erythrocyte membrane vesicles (IOVs)

Fresh blood was collected in tubes containing EDTA from healthy volunteers after obtaining written informed consent. IOVs were prepared from human erythrocytes by the method described previously (Takano et al. 2010). Briefly, the washed erythrocytes from fresh blood (5 mL) were suspended in one volume of 51 mM sodium phosphate buffer (pH 8.0) followed by rapid mixing with 8.5 volumes of ice-cold lysis buffer (5.1 mM sodium phosphate buffer, pH 8.0), and stirred for 15 min on ice. The unsealed ghosts were pelleted by centrifugation (32,000 g for 15 min at 4 °C), and washed three times with ice-cold lysis buffer (60 mL) to remove all residual hemoglobin. To initiate vesiculation, the pelleted ghosts were mixed with two volumes of 0.51 mM sodium phosphate buffer (pH 9.7) and incubated for 90 min on ice followed by 30 min at 37 °C. The vesicles formed were pelleted by centrifugation (32,000 g for 15 min at 4 °C), and washed twice with 60 mL of buffer A (100 mM D-mannitol, 10 mM HEPES/Tris, pH 7.4). The final pellet containing IOVs was suspended in buffer A and stored at -80 °C until use.

4.3. Detection of BCRP protein in human IOVs

The expression of BCRP protein in human IOVs was evaluated by western blot analysis after SDS-polyacrylamide gel electrophoresis (PAGE), as described previously (Klokouzas et al. 2003; Leimanis and Georges 2007). Briefly, the IOVs were mixed with a loading buffer and separated by a 7.5% SDS-polyacrylamide gel using a total protein amount of 20, 40, or 80 μg, after denaturation at 95 °C for 5 min with or without 2-mercaptoethanol. The protein was blotted on a PVDF membrane at 4 °C, and detected by a mouse monoclonal antibody for BCRP (1:200 dilution) as the primary antibody and horseradish peroxidase-labeled affinity-purified antibody for mouse IgG (1:5000 dilution) as the secondary antibody at room temperature (20–25 °C). The antibody complexes were visualized with Piece™ ECL Western Blotting Substrate reagents (Thermo Fisher Scientific K.K., Tokyo, Japan).

4.4. Study of LY uptake by IOVs

The uptake of LY, a specific substrate of BCRP, by IOVs was measured by a rapid filtration technique, as described previously (Takano et al. 2010; Yumoto et al. 2010). Briefly, IOVs (3 mg protein/mL) were suspended in buffer B (100 mM NaCl, 100 mM D-mannitol, 10 mM HEPES/Tris, pH 7.4) on ice. The substrate mixture containing LY (50 μM) was prepared in buffer C (80 mM KCl, 100 mM D-mannitol, 10 mM $MgCl_2$, 10 mM phosphocreatine disodium salt, 100 μg/mL creatine phosphokinase, 1 mM ATP or AMP, 10 mM HEPES/Tris, pH 7.4) on ice before the experiments. After preincubation for 10 min at 37 or 4 °C, the reaction was initiated by adding the substrate mixture (80 μL) to the IOVs (20 μL) at each temperature. At the stated times, the incubation was stopped by diluting the reaction mixture with 1 mL of ice-cold stop solution (buffer D; 100 mM KCl, 100 mM D-mannitol, 10 mM HEPES/Tris, pH 7.4). The contents of the tube were immediately poured onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter), and then the filters were washed once with 5 mL of the ice-cold stop solution.

In the inhibition studies, the half-maximal inhibitory concentration (IC_{50}) value was determined with the following Hill equation:

$V = 100/[1+([I]/IC_{50})^{\gamma}]$

where [I] is the concentration of an inhibitor, V is the transport rate in the presence of the inhibitor (% of control), and γ is the Hill coefficient. Graphical representation of the data was performed by the nonlinear least-squares function included in R software, and the IC_{50} value was determined through curve fitting to the above equation.

4.5. Modulation of cholesterol content in human IOVs

Modulation of membrane cholesterol content in IOVs was achieved by incorporation of CHS, a hydrophilic cholesterol ester, by a method similar to that described previously (Nabekura et al. 1996). Briefly, IOVs (300 μL, 6 mg protein/mL) and 150 μL, 300 μL, or 600 μL of CHS (6 mg/mL) in buffer A were suspended in enrichment buffer (3.5% polyvinylpyrrolidone and 1% bovine serum albumin in buffer A, final volume 9.45 mL) and were incubated at 37 °C for 1 h. After CHS treatment, IOVs were washed twice with buffer A (20 mL) by centrifugation (40,000 g for 15 min at 4 °C). We also prepared IOVs from washed human erythrocytes pretreated with CHS. For this purpose, washed human erythrocytes were incubated with 9 volumes of CHS (0.25 mg/mL) at 37 ºC for 2 h, and after incubation, IOVs were prepared as described above. Cholesterol content in IOVs was measured using LabAssay™ cholesterol (Wako Pure Chemical Industries, Osaka, Japan).

4.6. Analytical methods for LY and protein

After the uptake experiments, the filters were incubated with distilled water (1.5 mL) for 1 h at room temperature (20–25 °C) with periodic vortex mixing, and samples were collected to determine the concentration of LY with a Hitachi fluorescence spectrophotometer F-2700 (Tokyo, Japan) (Ex 420 nm, Em 530 nm). Protein concentration was measured using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

4.7. Statistical analysis

The data was expressed as the mean±S.E. Statistical analysis was performed using the Student's t-test, or by one-way analysis of variance followed by Tukey's test for multiple comparisons. The level of significance was set at $*p < 0.05$ or $**p < 0.01$.

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Conflicts of interest: None reported.

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