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Localization of the placental BCRP/*ABCG2* transporter to lipid rafts: Role for cholesterol in mediating efflux activity

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ABSTRACT

Introduction: The breast cancer resistance protein (BCRP/*ABCG2*) is an efflux transporter in the placental barrier. By transporting chemicals from the fetal to the maternal circulation, BCRP limits fetal exposure to a range of drugs, toxicants, and endobiotics such as bile acids and hormones. The purpose of the present studies was to 1) determine whether BCRP localizes to highly-ordered, cholesterol-rich lipid raft microdomains in placenta microvillous membranes, and 2) determine the impact of cholesterol on BCRP-mediated placental transport *in vitro*.
Methods: BCRP expression was analyzed in lipid rafts isolated from placentas from healthy, term pregnancies and BeWo trophoblasts by density gradient ultracentrifugation. BeWo cells were also tested for their ability to efflux BCRP substrates after treatment with the cholesterol sequestrant methyl-β-cyclodextrin (MβCD, 5 mM, 1 h) or the cholesterol synthesis inhibitor pravastatin (200 μM, 48 h).
Results and discussion: BCRP was found to co-localize with lipid raft proteins in detergent-resistant, lipid raft-containing fractions from placental microvillous membranes and BeWo cells. Treatment of BeWo cells with MβCD redistributed BCRP protein into higher density non-lipid raft fractions. Repletion of the cells with cholesterol restored BCRP localization to lipid raft-containing fractions. Treatment of BeWo cells with MβCD or pravastatin increased cellular retention of two BCRP substrates, the fluorescent dye Hoechst 33342 and the mycotoxin zearalenone. Repletion with cholesterol restored BCRP transporter activity. Taken together, these data demonstrate that cholesterol may play a critical role in the post-translational regulation of BCRP in placental lipid rafts.

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1. Introduction

The breast cancer resistance protein (BCRP/*ABCG2*) is highly expressed on the apical membrane of placental syncytiotrophoblasts, serving a fetoprotective function at the interface of the maternal and fetal circulations [1,2]. BCRP transports substrates away from the placenta and prevents the accumulation of potentially harmful xenobiotics in the fetus [3]. Substrates include commonly prescribed drugs such as the hypoglycemic agent glyburide, the chemotherapeutic drug doxorubicin and the antibiotic nitrofurantoin [4–7]. BCRP also transports estrogenic dietary chemicals that affect sexual differentiation of the fetus including the fungal toxin zearalenone [8–11]. Additionally, BCRP critically prevents cytokine-induced apoptosis and facilitates syncytial formation in placental cells [12,13].

Few studies have offered insight into the post-translational regulation of BCRP by cholesterol and its organization in the plasma membrane. Cholesterol in the plasma membrane aggregates in structures called lipid rafts, which are ordered microdomains also rich in sphingolipids and proteins [14]. Lipid raft order creates a phase-separation between its contents and the disordered phospholipid bilayer [14,15]. Lipid rafts are critical in a number of membrane processes including signal transduction, biochemical synthesis and transport [16].

There is some evidence linking lipid raft integrity and membrane cholesterol content to BCRP function. In cells transfected to

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overexpress BCRP, the BCRP protein localized in lipid raft fractions after density gradient ultracentrifugation [17]. Proteomic analysis of lipid rafts in primary human T cells and mouse spermatozoa also point to the detergent resistance of the BCRP protein [18,19]. Further, BCRP efflux activity is dependent on lipid raft integrity and cholesterol content in canine kidney cells and membrane vesicles overexpressing BCRP [17,20,21]. It is known that cholesterol is critical for fetal development; mothers with chronically low cholesterol are at higher risk for premature delivery and low birth weight babies [22,23]. However, it is possible that cholesterol also plays a critical role in regulating placental BCRP and therefore influences drug transfer from the maternal to fetal circulation.

To date, the majority of studies of the BCRP transporter have investigated the localization of the protein in lipid rafts and the ability of cholesterol to regulate its activity using overexpressing cell-based systems, with little attention paid to native human tissues. Therefore, we hypothesized that BCRP localizes to lipid rafts in microvillus membranes from healthy term placentas and cultured placental cells. Further, we expected that disruption of cholesterol content in placental cells would alter BCRP function and enrichment in lipid rafts, which could potentially enhance drug transfer across the placental barrier.

2. Materials and methods

2.1. Chemicals

Unless stated otherwise, all chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

BeWo human choriocarcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in an incubator at 37 °C with 5% CO_2 in air in a DMEM and F12 1:1 mixture (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (Atlantic Biologicals, Miami, FL) and 1% penicillin-streptomycin (Life Technologies). This cell line recapitulates first trimester trophoblasts by secreting placental hormones and expressing transporters such as BCRP [35]. For all experiments, cells were grown to 70–80% confluence before use. For cholesterol modulation studies, cells were cultured in the presence or absence of pravastatin (PRAV, 10–200 μM) for 48 h or methyl-β-cyclodextrin (MβCD, 5 mM) for 1 h and then HBSS in the absence and presence of cholesterol-MβCD (Sigma C4951) for 30 min [16]. Time points and doses used were based on previous literature and preliminary studies indicating a reduction in cholesterol but no effect on cell viability (Supplemental Fig. 1) [16]. Cells were lysed in buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100 and a protease inhibitor cocktail (Sigma P8340).

2.3. Patient selection and sample collection

Placentas (n = 3) were obtained with written informed consent from healthy women meeting all criteria following term delivery by scheduled Cesarean section (Supplemental Table 1). Inclusion criteria included healthy women between the ages of 18–40 years, term gestation (≥36 weeks), and scheduled Cesarean section without labor. Exclusion criteria included chronic medical conditions, pregnancy-induced medical conditions, maternal infection, clinical chorioamnionitis, medication use (with the exception of prenatal vitamins), maternal smoking, alcohol or drug abuse, multiple pregnancies, and known fetal chromosomal abnormalities [24]. The study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (Protocol #0220100258) and Rutgers University (Protocol #E12-024).

Upon collection, placenta samples for lipid raft analysis were snap frozen and stored at −80 °C until use. Samples for immunohistochemistry were stored in PAXgene Tissue Containers containing PAXgene tissue stabilizer (Qiagen, Germantown, MD).

2.4. Assays for cholesterol, protein and cell viability and growth inhibition

All colorimetric and fluorescent assays were performed using a SpectraMax M3 Multimode Microplate Reader and analyzed with SpectraMax SoftMax Pro 6.3 software (Molecular Devices, Sunnyvale, CA). For analysis of cholesterol in cell lysates and lipid raft fractions, an Amplex Red based detection kit was used according the instructions provided by Sigma-Aldrich. Samples were compared to a standard curve generated from human low density lipoprotein prepared in either cell lysis buffer (20 mM tris-HCl, 150 mM NaCl, 5 mM EDTA, pH = 7.4) or lipid raft extraction buffer (see below). Protein content of cell lysates and lipid raft fractions was quantified using a Detergent Compatible analysis kit (BioRad, Hercules, CA) based on the Lowry method [25].

Viability was measured as a function of the ability of the cells to convert resazurin to fluorescent resorufin as previously described [26]. To assess growth, cell number quantified using a Beckman Coulter Z1 Particle Counter (Indianapolis, IN). At all concentrations used, pravastatin had no effect on either cell growth or viability (Supplemental Fig. 1).

2.5. Subcellular fractionation

Ultracentrifugation and density gradient methods were employed to obtain total cell membranes from BeWo cells, brush border membranes from human term placenta, and lipid rafts using a Beckman L7-55 ultra-centrifuge (Beckman Coulter, Brea, CA) [27–29].

2.5.1. Membrane isolation from BeWo cells

Plasma membranes were collected from BeWo cells using a Percoll-based ultra-centrifugation method as previously described [27]. All ultra-centrifugation steps for membrane isolation were performed using a Type 40.1 Ti rotor (Beckman Coulter, Indianapolis, IN).

2.5.2. Placental brush border membrane extraction

Crude brush border MVM extracts were prepared from human term placentas using an $MgCl_2$-based centrifugation method as previously described [28]. Pure MVM were prepared by subjecting the crude MVM to the protocol a second time. All ultra-centrifugation steps for brush border membrane extractions were performed using a Type 60 Ti rotor (Beckman Coulter). Whole homogenates, nuclear fractions, and crude and pure MVM were analyzed for markers of apical and endothelial membranes, cytoplasm, nuclei and mitochondria using techniques in Western blotting.

2.5.3. Lipid raft extraction

Lipid raft fractions were prepared using a method adapted from a prior report [29]. Brush border membrane extracts and BeWo plasma membranes were incubated in TNE buffer (25 mM Tris HCl, 150 mM NaCl, 5 mM EDTA), supplemented with 1% Lubrol or 1% Triton X-100, respectively, on ice for 1 h. The mixtures were then mixed with OptiPrep (60% iodixanol) to a final concentration of 40% iodixanol in a final volume of 3 mL. This was added to the bottom of an open-top ultra-centrifuge tube and overlayed with 6 mL of 30%

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