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Keywords:

Trophoblast; sulfate conjugate; breast cancer resistance protein (BCRP); multidrug resistance-associated protein (MRP); mitoxantrone; BODIPY FL prazosin.

Abstract:

The breast cancer resistance protein (BCRP) and the multidrug resistance-associated proteins (MRPs) have the ability to eliminate sulfate conjugates but it is not known if this constitutes one of their roles in the placenta. To determine this, the BeWo cell line was used as a model of placental trophoblast cells and we examined the fate of two common sulfotransferase substrates, 4-nitrophenol and acetaminophen. At 0.5–200 μM, acetaminophen sulfate did not alter the accumulation of the BCRP substrates BODIPY FL prazosin or mitoxantrone in BeWo monolayers indicating a lack of interaction of BCRP with acetaminophen sulfate. 4-nitrophenyl sulfate increased the accumulation of BODIPY FL prazosin only at 200 μM, indicating it to be a BCRP inhibitor at high concentrations. Efflux studies and bidirectional transport studies examining the effect of BCRP/MRP inhibitors on the efflux of intracellularly generated 4-nitrophenyl sulfate and acetaminophen sulfate, indicated that one or more of the MRP isoforms played a major role in the elimination of 4-nitrophenyl sulfate and acetaminophen sulfate across the basolateral (fetal-facing) and apical (maternal-facing) trophoblast membranes respectively. BCRP played only a minor role in the elimination of these two sulfate conjugates across the apical membrane. Our study shows that a yet undetermined role of trophoblast efflux transporters is the elimination of sulfate conjugates

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Text of paper:

MRP isoforms and BCRP mediate sulfate conjugate efflux out of BeWo cells.

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1. Introduction

The placenta has the capacity of promoting elimination of drugs and natural substances by sulfation (Sodha and Schneider, 1984, Stanley et al., 2001). Since sulfate metabolites bear a negative charge at physiological pH it is likely that passive diffusion would not be the predominant mechanism of their elimination out of the placenta. This indicates transporter-mediated elimination and suggests the involvement of either uptake transporters or efflux transporters.

Among the efflux transporters, the breast cancer resistance protein (BCRP) mediates biliary elimination of sulfoconjugates in rat and mice (Zamek-Gliszczynski et al., 2006c, Adachi et al., 2005). Human BCRP also has the capacity to eliminate sulfate conjugates (Grube et al., 2007, Zamek-Gliszczynski et al., 2006a, Imai et al., 2003). A major part of the work regarding the involvement of multidrug resistance-associated protein (MRP) isoforms in the excretion of sulfate conjugates has been done in rats where MRP2 excreted bile acid conjugates as well as xenobiotic sulfate conjugates (Zamek-Gliszczynski et al., 2006a). MRP3 and MRP4 have been reported to interact with the sulfate conjugates of xenobiotics, steroids and bile acids, while MRP1 has been reported to transport steroid conjugates (Zamek-Gliszczynski et al., 2006a, Deeley and Cole, 2006). Thus literature would suggest that MRP isoforms and BCRP are prime candidates to mediate the transport of sulfated metabolites.

The sulfate-eliminating role of the efflux transporters has been demonstrated in other tissue but it is not yet known whether they play a similar role in the placenta. However, species as well as tissue differences exist with regards to the relative contribution of the efflux transporters responsible for the elimination of sulfate conjugates (Zamek-Gliszczynski et al., 2005, Zamek-Gliszczynski et al., 2006c, Enokizono et al., 2007). In addition, altered sulfate transport has the ability to produce toxicity. For example, impaired hepatic export of troglitazone sulfate, the major metabolite of the antidiabetic drug troglitazone, has the potential to reduce bile acid efflux and produce hepatotoxicity. (Kostrubsky et al., 2001, Funk et al., 2001). Therefore, it is of significance to elucidate the role of sulfate conjugate excretion in tissues capable of sulfation to understand mechanisms controlling exposure to potentially toxic metabolites. In the placenta, excretion of sulfate conjugates is critical for controlling fetal exposure to potentially toxic metabolites and is largely unexplored.

Substances in the maternal circulation have to permeate through the syncytiotrophoblast cells, the fetal connective tissue, and the fetal capillary endothelium in order to reach the fetal circulation. The syncytiotrophoblast layer expresses sulfotransferase enzymes, as well as efflux

transporters capable of eliminating sulfate conjugates. It is likely that these cells play a central role in reducing fetal exposure to substances in the maternal circulation firstly through sulfation, and secondly through sulfate metabolite elimination. Among the efflux transporters, BCRP is abundantly expressed in the syncytiotrophoblast cells (Maliepaard et al., 2001, Ceckova et al., 2006) and is located predominantly in the brush border (maternal-facing) membrane (Grube et al., 2007, Evseenko et al., 2007). MRP2, MRP3 and MRP4 are localized predominantly at the syncytiotrophoblast brush border membrane (St-Pierre et al., 2000, Azzaroli et al., 2007). MRP1 and MRP5 are expressed on both apical and basolateral membranes (St-Pierre et al., 2000, Atkinson et al., 2003, Meyer Zu Schwabedissen et al., 2005).

The objective of the present work was to determine the efflux transporters responsible for the elimination of sulfate conjugates out of trophoblast cells using the BeWo cell line as a model. The BeWo cell line is derived from a malignant choriocarcinoma, and in culture consists predominantly of cytotrophoblast-like cells. The mRNA of BCRP and several MRP isoforms are expressed in BeWo cells, and protein expression has been determined for BCRP, MRP1, MRP2 and MRP5 (Evseenko et al., 2006, Azzaroli et al., 2007, Young, 2005, Pascolo et al., 2003). We have previously determined that the sulfotransferase isoforms SULT1A1 and SULT1A3 are functional in BeWo cells. In this study**,** we examined the fate of two common substrates of sulfation pathways, 4-nitrophenol and acetaminophen**.** Acetaminophen and 4-nitrophenol were chosen because they are both substrates of SULT1A1, and their sulfate conjugates are demonstrated to be/likely to be eliminated by BCRP or MRP isoforms in other species.

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2. Materials and methods

2.1. Materials

The BeWo cell line (clone b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO). BODIPY® FL prazosin, penicillin-streptomycin solution, 200mM L-glutamine, and minimal essential medium nonessential amino acid (MEM-NEAA) solution were purchased from Invitrogen (Carlsbad, CA). MK-571 and leukotriene C4 were obtained from Biomol (Plymouth Meeting, PA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Norcross, GA). [^{3}H] mitoxantrone (4 Ci/mmol) was obtained from Moravek Biochemicals Inc. (Brea, CA). All other materials and cell culture reagents were from Sigma (St. Louis, MO).

2.2. BeWo cell culture

The BeWo cell line was cultured as described previously (Bode et al., 2006). Passages 29 through 45 of the cells were used in this study. Briefly, cells were grown at 37ºC in an atmosphere of 5% CO_2 and saturated relative humidity. They were maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% heat-inactivated FBS and 1% each of 10,000 U/ml penicillin with 10,000 µg/mL streptomycin solution, 200 mM L-glutamine solution, and 10 mM MEM-NEAA. At 70-90% confluence, cells were detached with 0.5% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA) diluted in phosphate buffered saline (PBS) at a 1:10 ratio. Cells were then subcultured at a ratio of 1:10.

2.2. Accumulation studies

For accumulation studies cells were seeded onto poly-d-lysine and fibronectin coated plates at a cell density of 12,500 cells/cm^{2} as previously published (Bode et al., 2006). BeWo monolayers were used for the uptake studies once they reached 90-100% confluency. All utilized solutions were pre-warmed to 37ºC. The cells were washed twice in HBSS containing 25mM glucose (HBSS-Glc). They were then allowed to equilibrate in HBSS-Glc at 37ºC on a shaker (~ 50 r.p.m.) for 30 minutes. Cells were then treated with HBSS-Glc solutions containing mitoxantrone in the presence of either known BCRP inhibitors fumitremorgin C (5 µM) or chrysin (100 µM), or 4-nitrophenyl sulfate (0.5-200 µM) or acetaminophen sulfate (0.5-200 µM). For the time-dependent studies, 20nM [^{3}H] mitoxantrone (4 Ci/mmol) was used. For the others 10 µM unlabeled mitoxantrone containing 20 nM [^{3}H] mitoxantrone (final specific activity 0.02 Ci/mmol) was used. Cells were incubated for 150 minutes with shaking (50 r.p.m). Following this, the dosing solutions were aspirated off and the cells were washed thrice with ice-cold HBSS-Glc. The cells were then lysed for 2 hours at 37ºC with a lysing solution containing 0.5% Triton X-100 in 0.2 N NaOH. The cell lysates were analyzed by liquid scintillation counting and their protein

content determined by the BCA assay kit (Pierce Chemical, Rockford, IL). Accumulation studies of 500 nM BODIPY® FL prazosin (Invitrogen, Carlsbad, CA) was performed in a similar manner with the following exceptions: the dosing solutions were prepared in HBSS-Glc containing 2% BSA and the lysing solution contained 2% Triton X-100. Fluorescence was detected using a Bio-Tek FL600 Microplate Fluorescence Reader (excitation: 485 nm, emission: 535 nm).

2.3. Efflux studies

For efflux studies, cells were seeded onto poly-d-lysine and fibronectin coated plates at a cell density of 12,500 cells/cm^2 as mentioned previously (Bode et al., 2006) and used at confluency. All solutions were pre-warmed to 37°C. Cells were washed thrice in HBSS and incubated for 30 minutes in HBSS containing inhibitor. Inhibitors used were 5 µM and 25 µM MK-571; 10 and 100 µM indomethacin; 5 µM fumitremorgin C; 200 µM chrysin; and 0.1 µM and 0.2 µM leukotriene C4. Following this, cells were freshly incubated with either 1 µM 4-nitrophenol or 250 µM acetaminophen, in the presence of inhibitors, for 2 hours and 4 hours respectively. The incubation medium was collected, centrifuged at 2,500 RCF for 15 minutes, and analyzed by LC-MS/MS.

2.4. Bidirectional transport studies

For the bidirectional transport studies BeWo cells were seeded onto 0.4 µM pore size Transwell® plates (Costar Corporation, Acton, MA) at a density of 45,000 cells/cm^2. Transwells were coated with human placental collagen prior to seeding as per the method of Bode et al (Bode et al., 2006). All experiments were conducted at confluency. Stock solution of 4-nitrophenol was prepared in HBSS. All utilized solutions were pre-warmed to 37°C. The volume of solutions added to the apical chamber was 0.5 mL while that added to the basolateral chamber was 1.5 mL. Cell monolayers were incubated in HBSS at 37ºC with shaking (50 r.p.m.) for 20 minutes with or without inhibitors of ABC transporters. Inhibitors used were 25 µM MK-571, 0.2 µM leukotriene C4, or 5 µM fumitremorgin C. The preincubation solution was then aspirated off and the cells were incubated with 50 µM 4-nitrophenol solution containing inhibitors. Control cells contained vehicle instead of the inhibitors. Portions of the receiver chamber were withdrawn at predetermined time points (final time point was 45 minutes) and replaced with fresh HBSS containing inhibitor. At the end of the experimental period, the donor solution was withdrawn. All samples were centrifuged at 2,500 x g for 15 minutes and a portion of the supernatant analyzed by LC-MS/MS. The bidirectional transport of 0.5 µM fluorescein served as a marker of monolayer integrity.

2.5. LC-MS/MS analysis of 4-nitrophenyl sulfate and acetaminophen sulfate

Samples from transport studies were analyzed by LC-MS/MS (LC coupled to Quattro Triple Quadrupole mass spectrometer). Analytes were separated on a Phenomenex Luna C18 column (2.0 x 50 mm, 5 µM pore size). The injection volume was 20 µL. 4-nitrophenol (PNP), 4-nitrophenyl sulfate (PNPS) and the internal standard 3-methyl-4-nitrophenol (MPNP) were separated using a mobile phase gradient at a flow rate of 0.3 mL/min. Solvent A was water containing 0.1% formic acid, while solvent B was acetonitrile containing 0.1% formic acid. The solvents were held at 95:5 A:B for 2 minutes, ramped to 30:70 A:B from 2 to 4.5 minutes, maintained at 30:70 A:B from 4.5 to 9.5 minutes, changed to 95:5 A:B from 9.5 to 10 minutes and then held at this composition until 15 minutes. Analytes were detected in negative ion mode using multiple reaction monitoring: PNP (138 → 108), PNPS (217.95 → 138.05), and MPNP (152 → 122) (Fig. 1a). To reduce exposure of the mass spectrometer to possible salts and cellular contaminants in the sample, the HPLC eluent was diverted to waste for the first 4 minutes.

Acetaminophen, acetaminophen sulfate (AS), and the internal standard cimetidine were separated using a mobile phase gradient at a flow rate of 0.3 mL/min. Solvent A was water containing 0.1% formic acid, while solvent B was acetonitrile containing 0.1% formic acid. The solvents were held at 100:0 A:B for 1 minute, ramped to 50:50 A:B from 1 to 2.5 minutes, maintained at 50:50 A:B from 2.5 to 4.5 minutes, changed to 100:0 A:B from 4.5 to 5.5 minutes, and then held at this composition for another 3.5 minutes. AS and cimetidine were detected in negative ion mode: AS (230.1→ 149.8), cimetidine (251.2 → 156.9) (Fig. 1b). The HPLC eluent was diverted to waste for the first 3 minutes.

Analytes were quantified from standard curves with the standards prepared in HBSS. The calibration curve of 4-ntrophenyl sulfate was linear between 3-260 ng/mL (R^2 = 0.9923), while that of acetaminophen sulfate was linear between 1.3-270ng/mL (R^2 = 0.9993). The lower limit of detection of acetaminophen sulfate was 1.4 ng/mL. At the lowest concentration, the S/N ratio of 4-nitrophenyl sulfate was 100.

2.6. Determination of sulfotransferase enzyme activities

BeWo cell lysate was prepared and sulfotransferase activities measured according to the procedure of Hu et al. (Hu et al., 2003) with slight modifications. BeWo cells at confluence were scraped up in PBS. The cells were centrifuged, PBS was aspirated away, and the cells were then suspended in 50 mM potassium phosphate buffer (pH 7.4) and homogenized on ice for 30 seconds with a Polytron

homogenizer. The homogenate was spun down at 14,000 rpm for 15 minutes, the supernatant withdrawn and spun down at 14,000 rpm for 15 minutes again. The resultant supernatant was stored at -80ºC for further use. Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL). Sulfotransferase activities were measured as follows: Cell lysate at a final concentration of 0.9 mg protein/mL was added to 100 µM 3'-phosphoadenosine-5'-phosphosulfate (PAPS, Sigma, St. Louis, MO) and 1 µM 4-nitrophenol/250 µM acetaminophen in a total reaction volume of 100 µL. The mixture was incubated at 37ºC for 2 hours in the case of 4-nitrophenol and 4 hours in the case of acetaminophen. The reaction was stopped by the addition of 25 µL of 94% acetonitrile/6% glacial acetic acid containing the internal standards 3-methyl-4-nitrophenol or cimetidine. The samples were then centrifuged at 14,000 rpm for 15 minutes and the supernatant analyzed by LC-MS/MS. The analytical methods were the same as used in section 2.5.

2.7. Permeability and excretion rate calculations

The following equation was used to calculate permeability in either the apical to basolateral direction ($P_{app,A\ to\ B}$) or basolateral to apical directions ($P_{app,B\ to\ A}$) (Young et al., 2006) :

$$P_{app} = \frac{dC}{dt} \times \frac{V_r}{A} \times C_d$$

Where dC/dt is the change in concentration in the receiver compartment with respect to time, V_r is the volume of the receiver compartment, A is the growth surface area, and C_d is the initial concentration of the compound added to the donor compartment. The permeability directional ratio (PDR) was calculated as follows:

$$PDR = P_{app,\ B\ to\ A} / P_{app,\ A\ to\ B}$$

Metabolite excretion rates (V_{mt}) were calculated as reported before (Jeong et al., 2004, Chen et al., 2005) . Metabolite excretion rates were obtained by monitoring the change of metabolite concentration (C_m) in the receiver chamber as a function of time.

$$V_{mt} = \frac{dC_m}{dt} \times V_r$$

2.8. Statistical analyses

Statistical significance was determined using One-way analysis of variance (ANOVA) followed by Dunnett's post-comparison test (GraphPad Prism software) or the student's t-test as deemed appropriate. A p-value of less than 0.05 was considered to be statistically significant.

3. Results and discussion

3.1 Effect of acetaminophen sulfate and 4-nitrophenyl sulfate on the accumulation of mitoxantrone and BODIPY® FL prazosin

BCRP, MRP1, MRP2, and MRP5 proteins are expressed in BeWo cells (Pascolo et al., 2003, Young, 2005, Evseenko et al., 2006), while MRP2 protein expression is weak (Young, 2005). In BeWo cells, BCRP mRNA levels were reported to be much higher than those of MRP1 or MRP2 (Serrano et al., 2007). In syncytiotrophoblast BCRP and MRP2 are localized to the apical membrane, while MRP1 and MRP5 are detected on both membranes. Assuming similar transporter localization in syncytiotrophoblast and BeWo cells, the initial hypothesis was that BCRP would predominantly mediate sulfate metabolite elimination across the apical trophoblast membrane. To examine this, the effects of acetaminophen sulfate and 4-nitrophenyl sulfate on the accumulation of the BCRP substrates BODIPY® FL prazosin and mitoxantrone were determined. If the sulfates altered accumulation of the BCRP substrates, it would indicate an interaction of BCRP with the sulfates, and would imply a potential role of BCRP in sulfate elimination.

BODIPY® FL prazosin and mitoxantrone were used at concentrations that are typically used for BCRP (Cerveny et al., 2006, Lee et al., 2007). The initial experiments were performed to optimize the accumulation time so as to produce a maximum difference in accumulation in the presence/absence of a BCRP inhibitor. Thus, the accumulation studies were performed in the presence/absence of a BCRP-specific inhibitor fumitremorgin C (FTC). On an average, fumitremorgin C increased the accumulation of BODIPY® FL prazosin by 1.5-2 fold and that of mitoxantrone by 1.3-1.5 fold. Although, accumulation of both mitoxantrone and BODIPY® FL prazosin reached steady state after the first hour of incubation (Fig. 2), accumulation of both substrates in the presence/absence of FTC was on an average most significantly different at 150 minutes. Hence an accumulation period of 150 minutes was utilized for all subsequent experiments.

Acetaminophen sulfate (0.5-200 μM) did not affect the accumulation of either mitoxantrone or BODIPY® FL prazosin suggesting no interaction with BCRP (Figs. 3a and 3b). Under the same conditions, chrysin which is a nonspecific BCRP inhibitor (Gyemant et al., 2005) and fumitremorgin C increased accumulation by 1.5-2 fold. 4-nitrophenyl sulfate (0.5-200 μM) did not alter the accumulation of mitoxantrone (Fig. 3b). 4-nitrophenyl sulfate did not affect the accumulation of BODIPY® FL prazosin either except at 200 μM where it produced a statistically significant increase in accumulation suggesting it to be a potential BCRP inhibitor at this concentration (Fig. 3a). The increase in accumulation (1.5-1.8 fold) was comparable to that produced by fumitremorgin C (1.5-2 fold). Hence, 4-nitrophenyl sulfate can be concluded to be an inhibitor of BCRP only at high concentrations. Although not as effective, this is in agreement with previous studies (Suzuki et al., 2003) where 4-nitrophenyl sulfate inhibited BCRP-mediated transport of estrone sulfate in membrane vesicles obtained from mouse lymphoma P388 cells (IC_{50} = 53 μM). Testing higher concentrations of 4-nitrophenyl sulfate were deemed to be irrelevant as 4-nitrophenol sulfation in BeWo cytosolic homogenate is negligible at concentrations greater than 100 μM. As 4-nitrophenyl sulfate or acetaminophen sulfate did not generally affect accumulation of the BCRP substrates, the results suggested that BCRP does not play a role in the elimination of these specific sulfated substrates in the trophoblast.

The structure of the membrane-bound ATP-binding cassette (ABC) family of transporters reveals that they consist of trans-membrane domains and cytosolic domains. The substrate-binding sites of these transporters are usually located on the trans-membrane domains and substrates gain access to the binding site from the membrane-cytosolic interface (Sharom, 2006). It can be contended that in these experiments the charged sulfates would not be able to permeate the cells and thus would not gain access to the transporter-binding site. BeWo cells, however, have been reported to take up organic anions via temperature-sensitive mechanisms indicating carrier-mediated transport (Serrano et al., 2007). Although 4-nitrophenyl sulfate or acetaminophen sulfate uptake has not been demonstrated in BeWo cells, both sulfates are taken up by carrier-mediated processes in isolated hepatocytes (Sakuma-Sawada et al., 1997b, Sakuma-Sawada et al., 1997a). BeWo cells have the ability to produce 4-nitrophenyl sulfate intracellularly upon incubation with 4-nitrophenol. To assess if intracellularly-generated 4-nitrophenyl sulfate affected the accumulation of mitoxantrone in a manner different from that mentioned above, BeWo cell monolayers were incubated with 4-nitrophenol (0.5-200 μM). We found that under these conditions the accumulation of mitoxantrone decreased by 10-30%. As this was not dose dependent and not statistically significant at all concentrations, it indicated that intracellularly generated 4-nitrophenyl sulfate did not interact with BCRP and reaffirmed the previous conclusion that BCRP plays only a minor role in sulfate elimination.

3.2. Efflux of the sulfate metabolites across the apical membrane

To identify the efflux transporters mediating sulfate elimination, BeWo monolayers grown on multiwells were incubated with 4-nitrophenol or acetaminophen. The incubation medium was analyzed for 4-nitrophenyl sulfate or acetaminophen sulfate. Since the transport medium was monitored for the sulfate metabolite, the experiments produced information on sulfate elimination across the apical trophoblast membrane. The amounts of 4-nitrophenyl sulfate and acetaminophen sulfate detected in the transport medium increased linearly as a function of time (data not shown). Incubation periods of 2 hours and 4 hours were selected for future experiments with 4-nitrophenol (1 µM) and acetaminophen (250 µM) respectively.

Efflux of acetaminophen sulfate across the apical membrane underwent a dose-dependent decrease in the presence of the MRP inhibitors MK-571 and indomethacin (Fig. 4). Another MRP inhibitor leukotriene C4 (LTC4) did not produce any effect. The BCRP-specific inhibitor FTC at 5 µM also produced a significant decrease in efflux; the amount of acetaminophen sulfate effluxed being ~77% of vehicle-treated controls. Compared to fumitremorgin C, much lesser amounts were eliminated at the highest concentrations of MK-571 (~26%) and indomethacin (~58%). Similar results were obtained with the apical efflux of 4-nitrophenyl sulfate (Fig. 5). The percentage of 4-nitrophenyl sulfate detected in the transport medium was ~20% in the presence of 25 µM MK-571, ~70% in the presence of 100 µM indomethacin, ~85% in the presence of 5 µM FTC and undetectable in the presence of chrysin. Leukotriene C4 did not produce any effect.

Inhibition by MK-571, indomethacin, and FTC indicated that the apical efflux of 4-nitrophenyl sulfate and acetaminophen sulfate are mediated by the MRP isoforms as well as by BCRP. The MRP inhibitors utilized were not specific to a particular isoform and protein of all the MRP isoforms identified in BeWo cells so far (i.e., MRP 1, 2, and 5), are expressed on the apical syncytiotrophoblast membrane to some extent at least (St-Pierre et al., 2000, Azzaroli et al., 2007, Meyer Zu Schwabedissen et al., 2005). This along with weak/negligible expression of MRP2 in BeWo (Pascolo et al., 2003, Evseenko et al., 2006, Young, 2005) led to the conclusion that the MRP isoforms mediating apical sulfate efflux are likely MRP1 and/or MRP5.

3.3. Elimination of 4-nitrophenyl sulfate across the basolateral membrane

To examine sulfate metabolite elimination across the basolateral membrane, and also to determine if 4-nitrophenyl sulfate undergoes preferential efflux across any of the membranes, BeWo monolayers were grown on Transwells. Monolayers were incubated with 4-nitrophenol in either the apical or the basolateral chamber. The receiver chambers were analyzed for 4-nitrophenol and 4-nitrophenyl sulfate. Control experiments indicated that 4-nitrophenol (0.5-50 µM) did not compromise monolayer integrity as $P_{app,\ A\text{-}B}$ and $P_{app,\ B\text{-}A}$ of the paracellular marker fluorescein in the presence of 4-nitrophenol were

similar to the values obtained when fluorescein was run alone (data not shown). Excretion rates of 4-nitrophenyl sulfate were similar across the apical and basolateral membranes.

To investigate the basolateral elimination of 4-nitrophenyl sulfate, the apical to basolateral transport of 50 µM 4-nitrophenol was determined in the presence of various inhibitors. Both MRP inhibitors MK-571 (25 µM) and LTC4 (0.2 µM) inhibited the excretion rate of 4-nitrophenyl sulfate across the basolateral membrane by approximately 50% (Fig. 6). The results suggest that one or more of the MRP isoforms (likely MRP1 or MRP5) mediate the basolateral elimination of 4-nitrophenyl sulfate. Although FTC did not produce a statistically significant change in 4-nitrophenyl sulfate elimination across the basolateral membrane, it reduced the excretion rate to almost the same extent as the MRP inhibitors. BCRP is predominantly localized to syncytiotrophoblast apical membrane (Grube et al., 2007, Evseenko et al., 2007). It is likely that BCRP inhibition drives 4-nitrophenyl sulfate excretion across a basolateral membrane transporter as has been reported with hesperitin sulfate elimination in Caco-2 (Brand et al., 2008).

In our hands BeWo monolayer integrity was compromised (i.e. the monolayers lifted off the sides of the transwell membranes) at incubation times longer than 60 minutes. Acetaminophen sulfate was not detectable in the transport medium within this period of time, and hence we were unable to determine the excretion pattern of acetaminophen sulfate across the apical and basolateral membranes.

3.4. Effect of inhibitors on sulfate metabolite formation in BeWo cell lysate

To determine if the inhibitors used in the preceding section selectively affected transport, their effect on sulfate formation in BeWo homogenate was determined. Only inhibitor concentrations that had produced a statistically significant change in the efflux or transport studies (sections 3.2 and 3.3) were used. For 1 µM 4-nitrophenol, in the presence of 5 µM MK-571, 25 µM MK-571, 100 µM indomethacin, 200 µM chrysin, and 5µM FTC, the amount of 4-nitrophenyl sulfate produced was 36%, 11%, 1%, 74%, and 131% of the vehicle-treated controls respectively (Fig. 7). The changes in all these cases were statistically significant. For 50 µM 4-nitrophenol, compared to the vehicle-treated controls, in the presence of 25 µM MK-571, 54% of 4-nitrophenyl sulfate was produced. Leukotriene C4 did not produce any change in the amount of 4-nitrophenyl sulfate produced.

Comparing the effect of the transporter inhibitors on the formation and efflux of 4-nitrophenyl sulfate, we can see that 100 µM indomethacin inhibited metabolism and apical efflux to approximately the same extents. In the presence of 5 µM and 25 µM MK-571 respectively, only 36% and 11% of 4-nitrophenyl

sulfate was formed with respect to the vehicle-treated controls, while the amount effluxed were 47% and 19% of the controls respectively. Thus no conclusion could be reached on the role of MRP isoforms in the apical efflux of 4-nitrophenyl sulfate. On the other hand, although leukotriene C4 significantly decreased the basolateral elimination of 4-nitrophenyl sulfate, it did not inhibit the formation of 4-nitrophenyl sulfate. Since out of the MRP isoforms detected on the basolateral syncytiotrophoblast membrane, MRP1 and MRP5 protein are expressed in BeWo cells, it would be reasonable to propose that either MRP1 or MRP5 mediate 4-nitrophenyl sulfate excretion across the basolateral trophoblast membrane. Chrysin, which was used as a BCRP inhibitor, almost completely inhibited both the formation and efflux of 4-nitrohenyl sulfate. Thus, the chrysin results could not be used to reach any conclusion about the role of BCRP in the efflux of 4-nitrophenyl sulfate. Fumitremorgin C did not inhibit the formation of 4-nitrophenyl sulfate but decreased its efflux (~14%). Thus it can be concluded that BCRP mediates a minor portion of the apical efflux of 4-nitrophenyl sulfate.

None of the inhibitors changed the formation of acetaminophen sulfate (Fig. 8). Therefore based on the efflux studies it can be concluded that either MRP1 or MRP5 mediate the apical elimination of acetaminophen sulfate with a minor contribution from BCRP.

4. Conclusions

Efflux transporters in the syncytiotrophoblast, located on the maternal-facing membrane, have the ability to reduce fetal exposure by pumping substances back into the maternal circulation. This may be one of their primary roles in the placenta. MRP isoforms and BCRP mediate sulfate elimination in other human tissues (Brand et al., 2008, Hu et al., 2003, Chen et al., 2005, Jeong et al., 2004), but it has not yet been examined whether they play a similar role in the placenta. Our results, show for the first time, that another role of trophoblast MRP and BCRP is sulfate conjugate elimination, although BCRP appears to play only a minor role in sulfate efflux in BeWo cells. In mice and rat, BCRP and MRP 2-4 mediate the hepatic elimination of acetaminophen sulfate (Zamek-Gliszczynski et al., 2005, Zamek-Gliszczynski et al., 2006c, Zamek-Gliszczynski et al., 2006b). Compared to acetaminophen sulfate, much less is known about the transporters responsible for 4-nitrophenyl sulfate efflux in other tissues or even species. However, based upon the excretion pattern and (Higaki et al., 2003) and the hepatic expression of efflux transporters (Zamek-Gliszczynski et al., 2006a) it is likely that at least a portion of 4-nitrophenyl sulfate elimination is via MRP 2-4 and BCRP. Our results show that BCRP is responsible for a minor portion of the efflux of 4-nitrophenyl sulfate from the apical membrane of BeWo cells, while either MRP1 or MRP5 mediate its basolateral efflux. Apical elimination of acetaminophen sulfate is mediated most likely, primarily by MRP1/MRP5, with a minor contribution from BCRP. Thus similar to their rodent counterparts, trophoblast efflux transporters have the ability to eliminate sulfate metabolites of small molecules.

Our studies also revealed that the transporter inhibitors affected not only efflux, but also sulfate conjugation. In several cases MK-571 at similar concentrations (as used in this study) or higher has been shown to inhibit sulfate efflux out of whole cells (Jeong et al., 2004, Hu et al., 2003, Zhang et al., 2007, Walle et al., 1999). In many of these cases the effect of the inhibitor on metabolism was not examined. While it has been shown that MK-571 did not affect apigenin sulfation, in this study the metabolism experiment was performed for 30 minutes whereas the intact cell efflux experiments were performed for 2 hours (Hu et al., 2003). Our work shows for the first time that MK-571 and indomethacin affected both formation and efflux of 4-nitrophenyl sulfate. This underscores the importance of performing metabolic studies along with transport studies to tease out the effect that the inhibitors have on efflux, from that they have on sulfate formation.

The placenta synthesizes increasing amounts of estrogens with advancing pregnancy. The syncytiotrophoblast cells as well as cultured trophoblast cells express several sulfotransferase isoforms that can sulfate estrogens. Although their exact role in placental steroid metabolism has not yet been demonstrated, it is likely that they play a part in placental estrogen metabolism, at least when local estrogen concentrations are high. Common classes of drugs consumed during pregnancy include antidiabetics, antiasthmatics, antiepileptics, medicines for psychotrpic disorders, non-steroidal anti inflammatory drugs, antihistamines, and antacids. Unless the objective is to treat the fetus (e.g. to prevent maternal to fetal transmission of HIV), the overall goal of drug delivery during pregnancy is reduction of fetal exposure to drugs. Several of these drugs are also substrates of the sulfotransferase enzymes. The sulfate eliminating capability of trophoblast efflux transporters indicates that together with the sulfotransferase enzymes, they have the ability to maintain hormone homeostasis, as well as reduce fetal exposure by the process of sulfation.

Compared to other tissues, the placenta expresses high quantities of the steroid sulfatase enzyme (STS) whose physiological function in the placenta is the deconjugation of dehydroepiandrosterone sulfate (DHEAS) and 16-hydroxyl DHEAS taken up from the fetus for the *de novo* synthesis of estrogen in the syncytiotrophoblast. It was proposed that OATP2B1 on the basolateral membrane and BCRP on the apical membrane function together to eliminate dehydroepiandrosterone sulfate taken up from the fetal circulation. In a subsequent study, it was mentioned that the high sulfatase concentrations in the syncytiotrophoblast makes this dual transport system an unlikely *in vivo* process (Ugele et al., 2008). The same reasoning can be extended against the role of trophoblast efflux transporters in the elimination of sulfate metabolites. However, compared to physiological substrates such as estrone sulfate and dehydroepiandrosterone sulfate, the steroid sulfatase enzyme exhibits a lower affinity for small molecules such as 4-nitrophenylsulfate (K_m = 400 µM for arylsulfatase C), whereas SULT1A1 sulfates small molecules such as 4-nitrophenol at much lower concentrations (i.e., reported K_m values of SULT1A1 for 4-nitrophenol ~ 1 µM). The differential affinity indicates that in spite of the high expression

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of steroid sulfatase in the placenta, it is likely that the sulfotransferase enzymes and the efflux transporters act in concert to reduce unwarranted fetal exposure to at least small molecules.

None of the inhibitors used in this study completely inhibited the efflux of either acetaminophen sulfate or 4-nitrophenyl sulfate. This suggests the involvement of other transporters such as the organic anion transporters (OATs) or the organic anion transporting polypeptides (OATPs) both of which are known to transport sulfates (Jeong et al., 2004, Hu et al., 2003, Konig et al., 2006). In placenta OATP-E/4A1 and OATP-B/2B1 are localized on apical and basolateral trophoblast membranes respectively, while OAT4 is localized on the basolateral trophoblast membrane (Young, 2005, Ugele et al., 2008). Future studies should examine if additional roles of placental organic anion transporters and organic anion transporting polypeptides include sulfate metabolite elimination. Further we also saw that chrysin inhibited of the formation of 4-nitrophenyl sulfate. Chrysin and other dietary flavonoids are known to inhibit sulfotransferase enzymes. It would be interesting to observe if other dietary polyphenols exert a similar effect on placental sulfotransferase enzymes.

Acknowledgement

The authors would like to thank Deborah Galinis, Ph.D., Cephalon Inc., for her critical comments and suggestions on several experiments performed in this study.

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