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Mechanisms for the transport of unconjugated bilirubin in human trophoblastic BeWo cells

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Abstract To evaluate mechanisms that mediate passage of unconjugated bilirubin (UCB) across placenta, the transport of [^{3}H]UCB was studied in the human trophoblastic, BeWo cell line. When plotted against the unbound UCB concentration [B_f], uptake exhibited saturative kinetics with a similar apparent K_m (~30 nM) for BeWo cells grown either in polarized (Transwell) or non-polarized fashion (dish). UCB release from cells, but not uptake, was inhibited by sulfobromophthalein but not by taurocholate, and almost abolished by MK571, a specific inhibitor of the activity of multidrug resistance-associated proteins (MRPs). *MRP1* and *MRP5* were both present in BeWo cells and the expression of *MRP1*, but not *MRP5*, was markedly higher in polarized cells. These data indicate that UCB is taken up from the fetal circulation by a still undefined, saturative process not shared by other organic anions and is then excreted to maternal circulation by proteins of the MRP family.

Key words: Placenta; Expression; Fetus; Multidrug resistance-associated protein; Bilirubin; Transport

1. Introduction

Although bilirubin transport and metabolism have been largely studied in adults, little information is available on these processes during fetal life. Fetal production of unconjugated bilirubin (UCB) is higher than in adult life, and there is a physiological, sudden increase in hemoglobin catabolism perinatally [1]. Theoretically, the fetus is at risk for accumulation of UCB, due to the almost complete absence in the fetal liver of the only enzyme that conjugates UCB, uridine-diphosphate glucoronosyltransferase-1A1 [2]. The fetus is also more susceptible to neurotoxicity from retained UCB, because α-fetoprotein has a lower ability to bind UCB than does adult serum albumin [3].

Transplacental passage of UCB has been assumed to occur by passive diffusion [4], but it has not been investigated whether there exist specific systems for the transport of UCB across the placenta, akin to those demonstrated in hepatocytes for UCB [5,6], and in plasma membrane vesicles of trophoblasts for other organic anions, such as bile acids. For bile acids, the mechanisms of basolateral uptake are both passive and carrier-mediated [7]. Export at the apical pole, however, is ATP-dependent, suggesting the involvement of an ABC protein [8], most likely the bile salt export pump (BSEP), which is also present in the liver [9]. Other ABC proteins, such as MDR1 and CFTR [10,11], as well as multidrug resistance-associated proteins MRP1 and MRP5 [12], are present in both human placenta and hepatocytes.

The mechanism(s) by which UCB is exported across the apical (maternal-facing) domain of the trophoblast have not been studied as yet. We, therefore, have investigated the mechanisms involved in the transfer of UCB across a cell line (BeWo) derived from human choriocarcinoma [13], which has been used often as a model to study the mechanisms of transplacental passage of nutrients and metabolites [14–16]. The results suggest that, in placental cells, UCB is taken up by carrier-mediated diffusion and secreted by an active mechanism mediated by one or more MRPs.

2. Materials and methods

2.1. Chemicals

[^{3}H]Bilirubin (specific activity 40×10^3 dpm/μg) was labeled biosynthetically in bile-fistula rats by intravenous infusion of 3,5-[^{3}H] δ-aminolevulinic acid and highly purified as described previously [17]. MK571 was purchased from Alexis (San Diego, CA, USA). All other reagents, including fatty acid free HSA (lot. no. 93H9345), were of analytical grade and were obtained from Sigma (Milan, Italy).

2.2. Cell cultures

BeWo cells were cultured under standard conditions in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum and 1% antibiotics (10 000 U/ml penicillin and 10 mg/ml streptomycin). The cells were maintained in 75-cm^2 Falcon flasks at pH 7.4 and 37°C, under a humidified atmosphere of 5% CO_2. After 3 days in culture, the cells were harvested by exposure (1–2 min) to 0.25% trypsin and 0.02% EDTA, and transferred onto either 35-mm Petri dishes or onto Transwell-clear polyester filters (12- or 24-mm diameter) (Costar, New York, USA). Cells were seeded in dishes at a density of 0.5×10^6 cells/dish [18]. The medium was replaced with fresh medium after 24 h and transport studies were performed the following day. In Transwell filters, cells were seeded at a density of $0.1–0.2\times10^6$cells/cm^2, and the medium was replaced every other day. As confirmed by achievement of a maximum transepithelial electrical resistance of 140 Ω/cm^2 (Millicell-ERS epithelial voltohmmeter and electrodes, Millipore Corp., Bedford, MA, USA) and by phase contrast microscopy [19], the cells formed polarized, confluent monolayers in 5 days, and were used for transport experiments.

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2.3. Measurement of cellular uptake of UCB

All the transport measurements were carried out at room temperature (22–24°C) and under dim light. Cells grown on dishes were washed once with 1.5 ml of transport buffer (25 mM HEPES/Tris pH 7.4, 40 mM NaCl, 100 mM sucrose, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5 mM glucose). [^{3}H]UCB was dissolved in 30 μl DMSO, and diluted 100-fold with transport buffer containing 30 μM HSA. Measurement of uptake was initiated by the addition of 1.0 ml of this medium containing a free (unbound) UCB concentration [B_f] ranging from of 5 to 100 nM [20]. After incubation for 60 s, uptake was arrested by removing the medium and then immediately washing the cells three times with 1.5 ml of ice-cold transport buffer. The cells were then dissolved in 1 ml of 2% sodium dodecyl sulfate/0.2 N NaOH, and 500 μl of the cell lysate was added to 10 ml of scintillation liquid (Filtercount, Packard, Groningen, The Netherlands), and radioactivity measured. Protein content of the cell lysate was measured with the bicinchonic acid reagent [21].

Cells grown on Transwell polyester filters were handled identically, except that the uptake medium was added only to the apical (maternal), upper compartment, while the lower, basolateral (fetal) chamber was loaded with transport buffer without UCB or HSA. Procedures otherwise were the same as for cells grown in dishes. Preliminary experiments showed that, under these conditions, less than 1% of the UCB was transferred from the apical to the basal side over 60 min.

2.4. Efflux experiments

BeWo cells grown on dishes were preincubated for 60 min at 37°C with [^{3}H]UCB and 30 μM HSA at [B_f] ranging from 10 to 40 nM [20]. The medium was then removed and cells were rinsed quickly with 1.5 ml of ice-cold transport buffer which contained neither UCB nor HSA. A volume of 1 ml of transport buffer containing 30 μM HSA, but not UCB, was then added to the cells, and efflux of UCB followed at room temperature for intervals of 0, 5, 10 and 20 min by radioassay of [^{3}H]UCB retained by the cells as described for the uptake experiments. The residual UCB at each time interval was expressed both as pmol UCB/mg protein, and as a percentage of the initial UCB content following UCB preloading (t_0). To assess the inhibitory effect of other organic anions on UCB efflux, BSP, taurocholate and MK571 were added to the transport buffer containing 30 μM HSA but not UCB, and retention of UCB by the preloaded cells measured as above.

Cells grown to confluence on Transwell were preloaded, by incubation for 60 min at 37°C with HSA-bound [^{3}H]UCB placed in the apical chamber, and then washed as described for the dish-grown cells. After loading, 1.5 ml of the transport buffer without HSA or UCB was placed in the lower chamber and the apical portion of the cells exposed for 0–20 min to 1.0 ml of transport buffer containing HSA but not UCB. The retained UCB and protein were analyzed as for dish-grown cells. The effects on UCB efflux of different organic anions and MK571 were tested by inclusion in the buffered HSA solutions placed in the apical chamber.

2.5. Isolation of total RNA and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared from 3-days-old dish-grown or 5-days-old filter-grown BeWo cells by a single-step guanidinium isothiocyanate–phenol–chloroform extraction procedure [22]. The same procedure was used to isolate total RNA from normal human placenta obtained both at term and from the first trimester (12th week of gestation).

RT-PCR, to amplify and detect *MRP1* and *MRP5*, was performed using a Gene Amp PCR system 2400 (Perkin-Elmer, Norwalk, CT, USA). The sequences of the primers for amplification of the human *MRP1* gene [23] (accession number L05628) are: sense primer (CTGTTTTGTTTTCGGGTTCC); antisense primer (GATGGTGGACTGGATGAGGT). Sequences for the amplification of the human *MRP5* gene [12] (accession number U83661) are: sense primer (CCTGGAGAGGACACACATGA); antisense primer (GGTGTCAAACTCCACCACCT). The amplification of cDNA was run for 30 cycles for *MRP1* and 40 cycles for *MRP5* at 95°C for 5 min, 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. Final elongation was run at 72°C for 5 min. For each RT-PCR reaction β-actin was amplified also (β-actin Control Reagents, PE Biosystems, Foster City, CA, USA). The PCR products were separated by electrophoresis in 2.5% agarose gels and visualized by UV light in the presence of ethidium bromide.

2.6. Statistical analysis

For each experiment, UCB transport was measured in duplicate in at least three separate cell preparations. Results are expressed as mean ± S.D. of at least three experiments and P values < 0.05 obtained by two-tailed Student's t test were considered significant. K_m and V_{max} were determined from a computer-assisted program (SigmaPlot, Landel Scientific, San Rafael, CA, USA) according to the Michaelis–Menten equation.

3. Results

3.1. UCB uptake by BeWo cells

Fig. 1 shows the uptake kinetics of [^{3}H]UCB by non-polarized (Fig. 1A) and polarized (Fig. 1B) cells. In both types of cells, [^{3}H]UCB uptake exhibited a saturative function when plotted against the unbound concentration of UCB, [B_f], indicating a carrier-mediated mechanism. In non-polarized cells (Fig. 1A), the kinetic constants gave an apparent K_m of 37.4 ± 4.9 nM, and a V_{max} of 23.9 ± 1.3 pmol UCB/mg protein per min. When UCB uptake was measured in polarized cells (Fig. 1B) a comparable apparent K_m of 27.2 ± 6.0 nM was observed, whereas the V_{max} was 5.9 ± 0.6 pmol UCB/mg protein per min, or about one fourth the V_{max} in non-polarized cells ($P < 0.001$).

As shown in Table 1, UCB uptake in BeWo cells, at [B_f] = 15 nM, was not inhibited by the addition of 100–150 μM BSP, 20 μM indomethacin, or 50 μM taurocholate, indicating that the uptake mechanism(s) for UCB were not

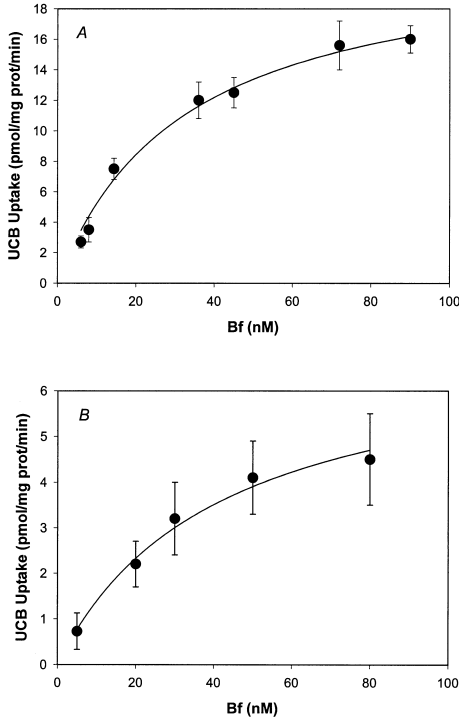

Fig. 1. Uptake of [^{3}H]UCB into BeWo cells. Initial [^{3}H]UCB uptake was measured at room temperature and in dim light by incubating the cells for 60 s with UCB–HSA solutions yielding 0–90 nM unbound (free) UCB [B_f]. Uptake was expressed as pmol UCB/mg protein per min. A: UCB uptake by non-polarized cells, grown in a Petri dish; B: UCB uptake by polarized cells, grown in a Transwell, from a buffered UCB–HSA solution in the apical-facing, upper chamber. In both cases, uptake was saturative with similar K_m values but with a V_{max} value almost four times greater for non-polarized cells.

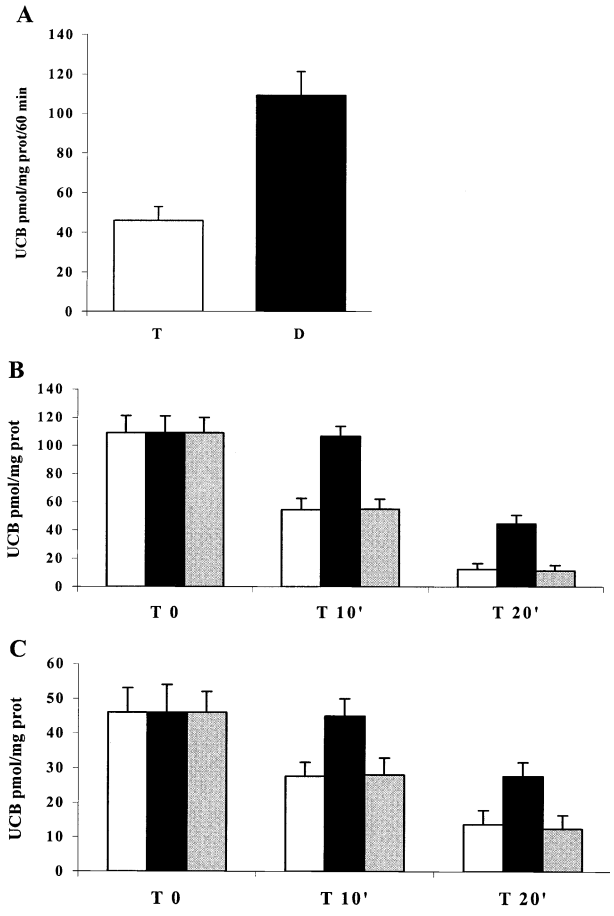

Fig. 2. Cellular uptake and efflux of [^{3}H]UCB. Cells were preloaded by exposure to 5 μM [^{3}H]UCB and 30 μM HSA ([B_f] = 15 nM) for 60 min at 37°C in the dark. Cells were then washed and exposed to a medium containing 30 μM HSA without UCB, and residual cellular content of [^{3}H]UCB determined by radioassay at different intervals. A: Cellular content after 60 min of preloading from a UCB–HSA solution in Transwell (white bar) and dishes (black bar). B: UCB content of non-polarized, dish-grown cells at different time intervals. UCB content decreased over time (white bars). When 150 μM BSP was added (black bars) UCB efflux was significantly impaired both after 10 and at 20 min. The addition of 50 μM taurocholate (gray bars) did not affect cellular release of UCB. C: UCB content of polarized, Transwell-grown cells after different time intervals. As with non-polarized cells, BSP (black bars) but not taurocholate (gray bars) significantly inhibited UCB cellular extrusion.

shared by these other organic anions. Conversely, both BSP and indomethacin enhanced UCB uptake and this effect was vastly greater when BeWo cells were grown in a polarized fashion ($P < 0.01$), suggesting that these two organic anions may interfere with the cellular efflux of UCB. Such enhancement was not seen when taurocholate was added.

3.2. UCB efflux from preloaded BeWo cells

UCB efflux from preloaded BeWo cells was measured in the absence and presence of BSP and taurocholate. Fig. 2A shows that, after incubation for 60 min at [B_f] of 15 nM, non-polarized cells grown in dishes took up over twice as much UCB compared to polarized cells grown in Transwells (109 ± 12 vs. 46 ± 7 pmol UCB/mg protein, $P < 0.001$). Fig. 2B shows that, when preloaded non-polarized cells were exposed to a medium without UCB, but containing 30 μM HSA to trap UCB (control), the intracellular UCB content decreased progressively to 25 ± 5% at 20 min ($P < 0.001$ vs. time 0). Addition of 150 μM BSP decreased UCB efflux significantly, with virtually all UCB retained at 10 min ($P < 0.01$ vs. control) and 41 ± 5% retained at 20 min ($P < 0.01$ vs. control). With preloaded polarized cells grown on Transwells (Fig. 2C), addition of 150 μM BSP likewise inhibited UCB efflux markedly; virtually all the initial UCB was retained at 10 min and 62 ± 6% after 20 min ($P < 0.002$ vs. non-polarized cells, $P < 0.01$ vs. polarized controls). In both polarized and non-polarized cells, 50 μM taurocholate and 20 μM indomethacin did not affect cellular release of UCB.

To test the possible involvement of MRP family member(s) in UCB efflux, BeWo cells preloaded with UCB were exposed to 100 μM of MK571, a specific inhibitor of the function of MRP proteins [23,24]. As shown in Fig. 3, UCB efflux from polarized BeWo cells was nearly abolished by MK571 at 10 min and, after 20 min, 81 ± 7% of the initial UCB content was still retained (time 0). A significant but lower inhibitory effect of MK571 was observed in non-polarized BeWo cells ($P < 0.01$ vs. control, and $P < 0.05$ vs. polarized cells).

3.3. MRP expression in BeWo cells and human placenta by RT-PCR analysis

The expression of the genes encoding MRP proteins was studied by RT-PCR analysis of total RNA extracted from BeWo cells grown for 3 days in dishes or for 5 days on Transwell, and compared to that observed in total human placenta. A band of the expected size (287 bp) (Fig. 4, upper panel) was obtained from RNA prepared from placenta harvested during the first (lane 3) and the third (lane 4) trimester of pregnancy. A band of the same size was also observed in RNA extracted

Table 1
Effect of other organic anions on UCB uptake in BeWo cells

	Non-polarized BeWo cells[a]		Polarized BeWo cells[b]	
	UCB transport (pmol/mg protein per min)	Change vs. control (%)	UCB transport (pmol/mg protein per min)	Change vs. control (%)
Control (UCB+HSA)	8.3 ± 0.8		2.1 ± 0.4	
+BSP 100 μM	10.5 ± 0.7	+26 ± 2¶	6.3 ± 0.8*	+200 ± 38*
+BSP 150 μM	11.4 ± 0.8	+38 ± 3¶	10.1 ± 1.1*	+380 ± 52*
+Indomethacin 20 μM	9.2 ± 0.6	+7 ± 0.5	4.5 ± 0.6*	+114 ± 28*
+Taurocholate 50 μM	8.1 ± 0.9	−3 ± 1	2.3 ± 0.4	+10 ± 9

Initial uptake was measured for 60 s at 22–24°C at concentrations of 5 μM [^{3}H]UCB and 30 μM HSA ([B_f] = 15 nM), in the presence or absence of possible competitors. ¶$P < 0.02$ and *$P < 0.001$ vs. control.
[a]Grown in Petri dishes.
[b]Grown on Transwell filters.

from BeWo cells, but the intensity was greater in RNA derived from polarized cells (lane 2) than from non-polarized cells (lane 1), suggesting that polarization is associated with an increased expression of *MRP1*. As shown in the middle panel of Fig. 4, expression of *MRP5* was observed also, both in human placenta at the two different gestational ages as well as in polarized and non-polarized BeWo cells. In the case of *MRP5*, an evident amplification was obtained only after 40 cycles, suggesting a lower level of expression of this ABC gene as compared to *MRP1* both in human placenta and in BeWo cell line.

4. Discussion

These data indicate that BeWo cells take up UCB by a saturative mechanism not shared by BSP, taurocholate or indomethacin and that, after entry into cell, UCB is secreted via a process which is inhibited by BSP and by the specific inhibitor of MRP proteins, MK571. These results, showing that BeWo choriocarcinoma cells are able to transport UCB at unbound concentrations below the maximum aqueous solubility of 70 nM [25], suggest that the trophoblasts of the human placenta may utilize carrier-mediated processes to efficiently transfer UCB from the fetus to the mother [26]. This is in contrast to the long-held belief that this process is simply due to passive diffusion [4].

The uptake process in BeWo cells was saturative. The saturative kinetics and the apparent affinity constant (K_m) value we observed in BeWo cells are similar to those we found previously in rat liver basolateral plasma membrane vesicles [20] and freshly isolated rat hepatocytes [17], indicating that this phenomenon may act at physiological conditions. While the apparent K_m value was comparable in polarized and non-polarized BeWo cells, the V_{max} value was almost three times greater in the non-polarized cells. This is likely due to (a) a different expression of carrier molecules in polarized and non-polarized cells, and/or (b) a greater countervailing cellular efflux of UCB in polarized cells.

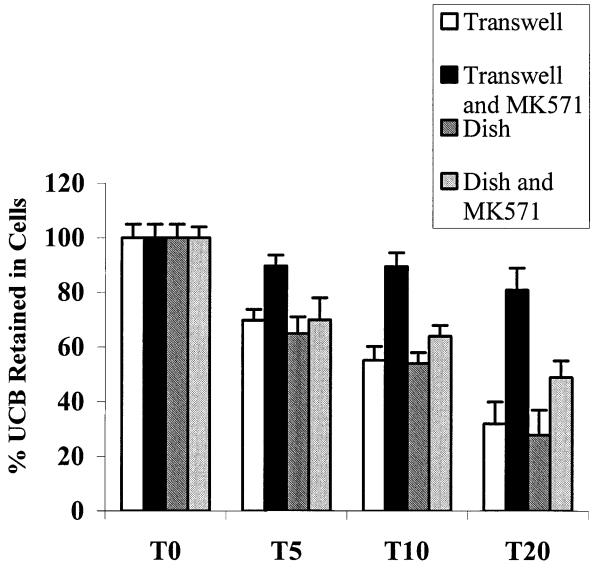

Fig. 3. Inhibition of cellular efflux of [^{3}H]UCB by MK571. Cells were preloaded with [^{3}H]UCB as in Fig. 2, and UCB efflux followed at various time intervals in the absence or presence of 100 μM MK571. Data are expressed as % of the initial content (time 0) which was retained. Inhibition of UCB efflux by MK571 was greater in polarized cells (black bars) as compared to that observed in non-polarized cells (gray bars). The percentage of [^{3}H]UCB released over the time by non-polarized (stripped bars) and polarized (white bars) cells was similar, in spite of the greater initial content of [^{3}H]UCB after preloading in non-polarized cells.

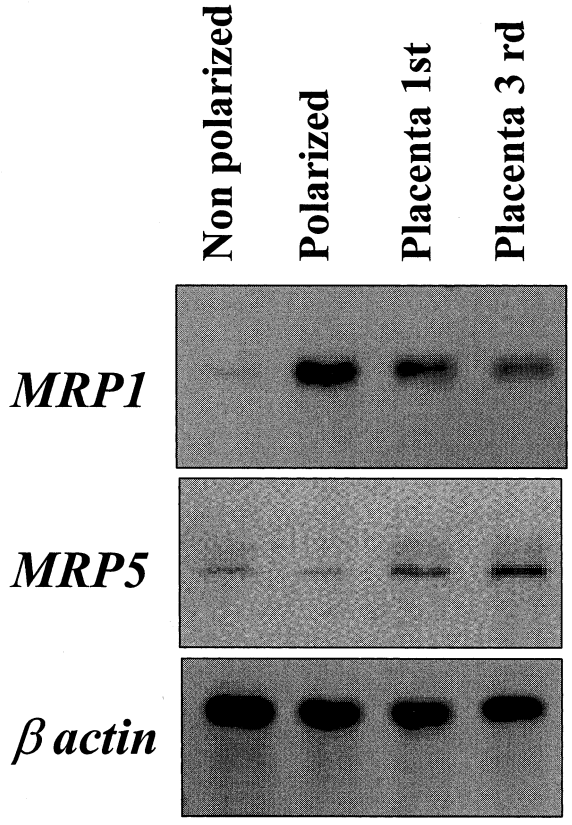

Fig. 4. *MRP1* and *MRP5* expression in human placental tissue and BeWo cells assessed by RT-PCR. RNA was extracted from human placenta at the first and third trimester and from BeWo cells grown on dishes or Transwells. PCR products stained with ethidium bromide were visualized under UV light. *MRP1* and *MRP5* were detected in all samples. The amplification of *MRP1* after 30 cycles resulted in a product of 287 bp, which was much more highly expressed in polarized than in non-polarized BeWo cells. *MRP1* expression was rather comparable in human placenta at the two different gestational ages. Expression of *MRP5*, as detected after 40 cycles of amplification (325 bp), was not significantly different between polarized vs. non-polarized cells, or in the placenta from the third vs. the first trimester. In both tissues, *MRP1* was more highly expressed than *MRP5*.

BSP, an organic anion which reportedly shares some of the uptake mechanisms of UCB in the liver [20,27], did not inhibit, but actually enhanced UCB uptake in non-polarized BeWo cells, especially when added to the apical compartment of polarized cells on the Transwell (Table 1). This is evidence that mechanisms for UCB uptake across the membrane of BeWo cells and, by inference, normal placental trophoblasts differ from those in hepatocytes. It suggests further that UCB uptake does not involve the OATP family, which is expressed in the human placenta [28], but does not appear to be involved in the transport of UCB [29] while it is actively involved in BSP uptake [30]. The lack of inhibition of UCB uptake by taurocholate further supports this conclusion, since taurocholate is also transported by OATP proteins.

The data shown in Fig. 2, together with the stimulatory effect on UCB uptake elicited by BSP and indomethacin,

but not taurocholate (Table 1), suggest that, in BeWo cells, particularly when polarized, UCB may be exported by a transport mechanism which is also shared by BSP and indomethacin; their inhibition of efflux of the pigment from the cell increases the 'net' uptake of UCB. This concept is supported by the finding (Fig. 2) that addition of BSP to the external medium strikingly decreased efflux of UCB from preloaded BeWo cells in a time-dependent fashion, both in non-polarized and polarized cells. The partial escape from this inhibition at 20 min as compared with 10 min is likely due to export of BSP from the cell after initial loading, and suggests that BSP *cis*-inhibited the extrusion of UCB, particularly at the apical level, as indicated by the greater inhibition in polarized cells. As with uptake, taurocholate did not influence cellular efflux of UCB, indicating that the export protein for UCB at the apical pole of the BeWo cells apparently does not transport taurocholate, thus excluding the involvement of the BSEP [31].

The nearly complete abolition of the apical UCB efflux from BeWo cells by MK571, a specific inhibitor of MRP proteins [24], and considerable inhibition by BSP and indomethacin, known to be substrates for MRP proteins at concentrations similar to those used in the present study [32], suggest the involvement of an MRP protein in the export of UCB from these cells. This was also shown by our recent data indicating that UCB is a substrate for both *YCF1* and *YLL015* gene products in yeast [33]. Of the six *MRP* genes in *Saccharomyces cerevisiae*, *YCF1* and *YLL015* show the greatest homology for human *MRP1* and *MRP2* [34,35]. The involvement of MRP2 in ATP-dependent export of UCB from mammalian cells has previously been excluded, since liver plasma membrane vesicles derived from TR^- rats, genetically deficient in mrp2, retain unimpaired ATP-dependent transport of UCB [36]. As in human placenta, PCR analysis revealed the presence of both *MRP1* and *MRP5* in BeWo cells, whether non-polarized or polarized (Fig. 4). The association of higher UCB efflux in polarized BeWo cells with a higher expression of *MRP1*, without a parallel increase of *MRP5* expression, supports a major role of MRP1 in UCB transport. The additional involvement of other MRPs in the whole placenta cannot be excluded, however, since the pups of *mrp1*-knockout mice do not evidence excessive hyperbilirubinemia or UCB toxicity at birth [37]. The low and unchanged level of *MRP5* mRNA before and after polarization, together with the recent report that MRP5 is involved in the transport of nucleotide analogues [38], renders it unlikely that this carrier plays an active role in UCB transport

Collectively, our data point to the existence of specific transporters of UCB at both the basal (fetal-facing) and apical (maternal-facing) membranes of the trophoblast, that mediate the passage of UCB from fetal to maternal blood. The marked inhibition by MK571 of UCB export from polarized BeWo cells strongly suggests that the extrusion of UCB is accounted for by an MRP protein. Although the molecular mechanisms of placental uptake of UCB from the fetus remain undefined, our studies suggest that MRP1 may mediate UCB transfer from the placental trophoblasts to the maternal circulation, and thus protect the fetus from UCB toxicity.

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