Published in final edited form as:

Biochem Pharmacol. 2018 October ; 156: 467–478. doi:10.1016/j.bcp.2018.09.012.

Role of the Efflux Transporters BCRP and MRP1 in Human Placental Bio-disposition of Pravastatin

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Abstract

The expression and activity of human placental transporters during pregnancy could be altered by several factors including pathological changes associated with preeclampsia. The aims of this study were to identify the placental efflux transporters involved in the bio-disposition of pravastatin, determine the protein expression of these transporters and their encoding genes as well as the activity of pravastatin uptake in placentas obtained from patients with preeclampsia. ATP-dependent uptake of [3H]-pravastatin by trophoblast tissue apical and basal membrane vesicles exhibited sigmoidal kinetics. The curved shapes of Eadie–Hofstee plots indicate that more than one placental transporter are involved in the uptake of pravastatin. ATP-dependent uptake of [3H]-pravastatin into vesicles expressing MRP1–5, BCRP, and P-gp, as well as the results of inhibition studies suggest that BCRP and MRP1 are the major placental efflux transporters responsible for the *in vitro* uptake of pravastatin. Compared to placentas from healthy pregnancies, preeclamptic placentas had increased number of syncytial knots with increased expression of BCRP in their apical membrane and increased expression of MRP1 in the cytoplasm of the syncytiotrophoblast and in cytoplasm of syncytial knots. There was a concomitant increase in *ABCC1* but not in *ABCG2* gene expressions in preeclamptic placentas. ATP-dependent uptake of [3H]-pravastatin by vesicles prepared from apical membranes of preeclamptic placentas was similar to the uptake by vesicles prepared from placentas obtained after uncomplicated pregnancies (13.9 ± 6.5 vs 14.1 ± 5.8 pmol·mg protein⁻¹·min⁻¹). The transporter-specific changes in the expression of BCRP and MRP1 in preeclamptic placentas did not affect the efflux activity of transporters localized on the apical membrane of the syncytiotrophoblast.

Graphical Abstract

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Keywords

Pravastatin; Preeclampsia; Pregnancy; Placenta; Efflux Transporters

1. Introduction

Preeclampsia complicates up to 8% of pregnancies worldwide and is a major cause of maternal and neonatal morbidity and mortality. Because preeclampsia and cardiovascular diseases share many pathophysiological pathways, pravastatin—3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor—has been used in preclinical studies to reverse various pathophysiological changes associated with preeclampsia [1–5]. The NICHD network of Obstetrics Fetal Pharmacology Research Centers (OPRC) is conducting a phase I/II pilot study ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01717586) Identifier NCT01717586) to assess the safety and pharmacokinetic properties of pravastatin in women at high-risk for preeclampsia. The study consists of a series of clinical trials randomizing high-risk pregnant women to escalating dose of pravastatin or placebo. Each trial consists of 20 patients (10 randomized to pravastatin and 10 to placebo) with singleton non-anomalous pregnancies and a history of prior severe preeclampsia that required delivery before 34 completed weeks. The preliminary results showed that pravastatin reduced the rates of preeclampsia, preeclampsia with severe features and preterm delivery before 37 weeks compared to placebo [6]. These data suggest the efficacy of pravastatin in prevention and early management of preeclampsia.

Human placenta act as a functional barrier between the maternal and fetal circulations and is composed of apical (brush border, maternal facing) and basal (fetal facing) plasma membranes of the syncytiotrophoblast as well as fetal endothelial cells. The low permeability of pravastatin across biological membranes including human placenta is to be expected because of its hydrophilic properties [7]. However, data obtained in our laboratory revealed that $18 \pm 4\%$ of the drug was transferred from the maternal to the fetal circuit of the dually perfused placental lobule. Furthermore, the transfer of pravastatin in the Fetal to Maternal (F→M) direction was significantly higher than its transfer in the opposite direction (M→F) [8]. Taken together these data suggest the involvement of trophoblast uptake and efflux transporters in the placental bio-disposition of pravastatin. Several efflux and uptake transporters expressed in syncytiotrophoblast and fetal endothelial cells could affect the bidirectional transfer of endogenous metabolites and xenobiotics/medications between the maternal and fetal circulations [9]. The function of efflux transporters localized on the apical membrane of the syncytiotrophoblast is to limit the transfer of compounds across the placenta, therefore decreasing fetal exposure to compounds present in the maternal circulation; while the same transporters localized on the basal membranes will extrude the compounds to the fetal circulation [9].

The major class of transporters identified in the placenta is ATP-Binding Cassette (ABC) superfamily including the P-glycoprotein (P-gp) encoded by the ABCB1 gene, the multidrug resistance proteins MRP1 encoded by the ABCC1 gene, MRP2 encoded by the ABCC2 gene, MRP3 encoded by the ABCC3 gene, MRP4 encoded by the ABCC4 gene, and MRP5 encoded by the ABCC5 gene, as well as the breast cancer resistance protein (BCRP, ABCG2), with expression levels that vary depending on gestational age [10, 11].

During pregnancy, the expression and activities of placental transporters could be affected by several factors including pathological changes associated with preeclampsia. It is well accepted that the activation of the inflammatory cascade that occurs in normal pregnancy is further exacerbated by preeclampsia [12]. There are several reports addressing the impact of inflammation on efflux transporters in human trophoblast tissue [13–15]. An increase in BCRP and P-gp mRNA and protein expression was reported in preterm placentas with inflammation over preterm placentas without inflammation [13]. In primary human trophoblast cells following exposure to tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), expression of BCRP and P-gp at both the gene and protein levels decreased, expression of MRP1 at the gene level increased, while protein expression of MRP1 was not affected [15]. Furthermore, a slight increase in expression of BCRP and P-gp encoding genes was reported in preeclamptic placentas [13]. These studies suggest that alterations in the expression and the activities of efflux transporters in placentas from preeclamptic patients are plausible and that the response could be transporter-specific.

Since placental transporters are the major determinants of pravastatin transplacental transfer, it is imperative to determine the effect of preeclampsia on these transporters, in order to assess the extent of fetal exposure to pravastatin during pregnancy. The aims of this study were to identify placental efflux transporters involved in bio-disposition of pravastatin, determine the gene and protein expression of these transporters as well as the activity of pravastatin uptake in placentas obtained from patients with preeclampsia compared with placentas obtained from term pregnant women without preeclampsia.

2. Materials and methods

2.1. Chemicals

[³H]-pravastatin calcium salt (specific activity, 10 Ci/mmol) and [³H]-dihydroalprenolol (DHA) (specific activity, 80 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Non-radioactive pravastatin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Vesicles prepared from baculovirus infected insect cells (Sf9) expressing human MRP1, MRP2, MRP3, and BCRP transporter proteins were purchased from Corning Gentest (Woburn, MA, USA). Vesicles purified from human (K) cells overexpressing the MDR1/P-gp, MRP4, and MRP5 transporter proteins were purchased from Solvo Biotechnology through Sigma-Aldrich (St. Louis, MO, USA).

For Western blot, mouse monoclonal antibodies against BCRP (B-1, sc-377176), MRP1 (QCRL-1, sc-18835), MRP2 (M2 III-6, sc-59608), MRP3 (M3 II-21, sc-59612), MRP4 (F-6,

sc-376262), MRP5 (E-10, sc-376262), and P-gp (c219, SIG-38710) were purchased from Santa Cruz Biotechnology, Inc (Dallas, TX, USA) and Covance (Princeton, NJ, USA). Rabbit monoclonal anti-cytokeratin 7 (EPR17078, ab181598) antibodies were purchased from Abcam (Cambridge, MA, USA). Goat anti-mouse IgG CY5 (PA45009) and goat anti-rabbit IgG CY5 (PA45011) secondary antibodies were purchased from GE healthcare Bio-Science Corp. (Piscataway, NJ, USA).

For Immunohistochemistry (IHC), mouse monoclonal antibodies against BCRP (ab3380) and MRP1 (ab24102) were purchased from Abcam (Cambridge, MA, USA). Goat anti-mouse IgG secondary antibody conjugated to Horseradish Peroxidase (BA-9200) was purchased from Vector Laboratories (Burlingame, CA, USA).

For *ABCC1* and *ABCG2* gene expression assays using Q-RT-PCR, the Zymo TRI Reagent® and the Direct-zol™ RNA MiniPrep Kit were purchased from Zymo Research Corporation (Irvine, CA), and the High-Capacity RNA-to-cDNA™ Kit and the TaqMan probes and master mix were acquired from Thermo Fisher Scientific (Waltham, MA).

All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

2.2. Clinical material

Placentas from uncomplicated term pregnancies (control) between 37–0/7 and 39–6/7 weeks of gestation and from patients with preeclampsia between 27–3/7 and 40–2/7 weeks of gestation were obtained immediately following vaginal or abdominal deliveries from the Labor and Delivery Ward of the John Sealy Hospital, the teaching hospital of the University of Texas Medical Branch, Galveston, Texas, according to a protocol approved by the Institutional Review Board. Preeclampsia was diagnosed according to the criteria set by the American College of Obstetricians and Gynecologists (ACOG 2002). Placentas from patients with history of maternal infection, systemic diseases, and drug or alcohol abuse during pregnancy were excluded from this investigation.

2.3. Preparation of placental apical (brush border) and basal membrane vesicles

Placental apical and basal membrane vesicles were prepared according to established protocols [16–19]. Briefly, placental tissue was dissected from the maternal side and washed three to four times in ice-cold saline (0.9% NaCl). The minced tissue was transferred to sucrose–Sucrose-HEPES-Tris (SHT) Buffer (250 mM sucrose, 10 mM HEPES-Tris, pH 7.4) containing 1 mM EDTA and stirred for 1 h to disrupt brush border membranes. The tissue suspension was filtered through mesh strainer with pore size of 250 µm, and the tissue residue collected in 50 mM Tris-HCl (pH 7.4). Filtrate was used to prepare apical (brush border) membrane vesicles, and tissue residue was used to prepare basal membrane vesicles.

Apical membranes were isolated through differential centrifugation steps of filtrate carried out at 4 °C (1000 x g for 10 minutes followed by 10,000 x g for 15 minutes, followed by 46,000 x g for 60 min). The pellet was resuspended in SHT buffer using Wheaton 358044 15 ml Potter-Elvehjem Tissue homogenizer, and incubated on ice in SHT buffer containing 10 mM MgCl₂ for 20 min under continuous stirring followed by centrifugation at 3,000 x g for

10 min at 4°C. Supernatant was then centrifuged at 46,000 x g for 60 min at 4°C, and the pellet was resuspended in SHT Buffer using a 26-gauge needle, aliquoted and stored at -80°C.

Tissue residue in 50 mM Tris-HCl containing basal membranes was portioned and sonicated using a ¾ inch high-gain probe for 10 sec at 240 W (Vibra-cell, Sonics) on ice. Sonicated tissue was collected on a 250 µm mesh strainer, and stirred gently in 5 mM Tris-HCl, (pH 7.4) on ice for 1 hour. Tissue was collected again using the 250 µm mesh strainer, and gently stirred in SHT Buffer containing 1mM EDTA on ice for 30 min. Portions were then sonicated twice at 240 W for 20 sec each, and centrifuged at 3000 x g for 10 min at 4°C, followed by supernatant centrifugation at 46,000 x g for 60 min at 4°C. Pellet was resuspended in SHT Buffer, aliquoted and stored at -80°C.

Basal membrane vesicles were further purified before use by discontinuous sucrose gradient of 40% sucrose (w/w) overlaid with 30% sucrose (w/w) which was overlaid with 25% sucrose (w/w), modified from Eaton and Oakey, 1994 [18]. Crude vesicles were layered over sucrose solutions, and the sucrose gradient tubes were centrifuged at 141,000 x g for 4 hours at 4°C using a swinging bucket rotor (SW Ti40), followed by centrifugation of the interface between the 25/30% sucrose layers at 46,000 x g for 60 min at 4°C. Pellet was resuspended in SHT Buffer using a 26-gauge needle, aliquoted and stored at -80°C.

2.3.1 Electron microscopy, purity and sidedness of apical and basal membrane vesicles—Electron microscopy was performed according to the established and previously published protocol from our laboratory [19]. Cross-contamination of apical membranes by basal, and basal membranes by apical, was determined by the binding activity of [³H]-dihydroalprenolol (DHA) [20] and by alkaline phosphatase assay [18], respectively, using AnaSpec Sensolyte pNPP Alkaline Phosphatase Colorimetric Assay Kit (Fremont, CA, USA). Contamination of vesicle preparations by intracellular membranes of endoplasmic reticulum and by mitochondria was assessed by Abcam Cytochrome c Reductase Assay Kit (Cambridge, MA, USA) and Sigma-Aldrich Cytochrome c Oxidase Assay Kit (St. Louis, MO, USA), respectively.

Inside-out configuration of vesicles was determined by acetylcholinesterase assay [21, 22]. Since in inside-out vesicles (IOV) ATP-binding domain and the substrate binding site of the efflux transporter localized on the outside of the membrane bilayer, the ATP-dependent uptake of a substrate by IOV in *vitro* corresponds to its *in vivo* efflux by the transporter protein.

Protein concentrations were measured using Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.

2.4. ATP-dependent uptake of [³H]-pravastatin by placental apical and basal membrane vesicles

Each reaction was carried out in 120 µl of MOPS buffer (50 mM MOPS-Tris, 70 mM KCl, 10 mM MgCl₂ final concentrations, pH 7.4) containing 90 µg apical or 30 µg basal vesicles, in triplicate. The reaction solutions were pre-incubated with pravastatin for 5 minutes at

37°C and the uptake was initiated by the addition of either 5 mM ATP or 5 mM AMP (as control). The reaction was stopped after 1 min for apical membrane vesicles and after 2 mins for basal membrane vesicles by the addition of 3 ml ice cold Wash Buffer (40 mM MOPS-Tris, 70 mM KCl, pH 7.0), and vesicles were isolated by rapid filtration through a Whatman glass fiber filter strip (pore size 0.7 µm; Whatman, Clifton, NJ, USA) using a Brandel Cell Harvester (Brandel, Gaithersburg, MD, USA). The amount of [³H]-pravastatin was determined by Tri-Carb 4910 TR 110V Liquid Scintillation Counter (PerkinElmer, Waltham, MA, USA). ATP-dependent uptake of pravastatin was reported as the difference in its uptake activity in the presence and absence of ATP and expressed as fmol-mg protein⁻¹.min⁻¹. The rate of ATP-dependent uptake of [³H]-pravastatin was used to obtain the saturation curves necessary to calculate the K_t and V_{max} for the reactions. The apparent kinetic parameters K_t and V_{max} were determined by fitting the allosteric sigmoidal equation using the GraphPad Prism 5 software version 5.01.

2.5. ATP-dependent uptake of [³H]-pravastatin by vesicles prepared from baculovirus infected insect cells (Sf9) or purified from human (K) cells overexpressing human transporter proteins

The commercially available vesicles expressing the transporters MRPs1–5, BCRP and P-gp were screened for their interaction with pravastatin. ATP-dependent uptake of 1 µM [³H]-pravastatin was determined using 30 µg of vesicles proteins, in duplicate. The reaction was carried out as above, but with the addition of 2 mM Glutathione to the assay buffers containing MRPs-overexpressing vesicles. The reaction was stopped after 2 mins by the addition of 3 mL ice cold Wash Buffer. Vesicles prepared from insect cells (Sf9) infected with wild type virus were used as a control. ATP-dependent uptake of [³H]-pravastatin was calculated and reported as above. The apparent kinetic parameters K_t and V_{max} were determined by fitting the Michaelis–Menten equation using the GraphPad Prism 5 software (GraphPad Co. Ltd, San Diego, CA, USA).

2.6. Western blot analysis of apical and basal membranes

40 µg of apical and basal membrane vesicles were resolved on a 4–7.5% SDS-polyacrylamide gel electrophoresis (PAGE), and subsequently electrotransferred onto nitrocellulose membranes at 110 V for 3 hours at 4°C. After blocking overnight at 4°C with 5% nonfat dried milk in phosphate-buffered saline, membranes were probed overnight with the following primary antibodies: c219 (1:100) against P-gp, B-1 (1:50) against BCRP, QCRL-1 (1:20) against MRP1, M2 III-6 (1:20) against MRP2, M3 II-21 (1:20) against MRP3, F-6 (1:20) against MRP4, E-10 (1:20) against MRP5, and EPR17078 (1:30) against cytokeratin 7. Immunoreactivity was detected with ECL PLEX goat anti-mouse or goat anti-rabbit Ig CY5 (1:10,000) secondary antibodies using Typhoon FLA 9000 (GE Healthcare, Piscataway, NJ). Vesicles from baculovirus infected insect cells (Sf9) expressing human BCRP, MRP1, MRP2, MRP3, and vesicles purified from human (K) cells overexpressing the MDR1/P-gp, MRP4, and MRP5 were used as positive controls.

2.7. Effect of chemical inhibitors on the ATP-dependent uptake of [³H]-pravastatin

The effect of inhibitors on the ATP-dependent uptake of [³H]-pravastatin was determined as above with the addition of inhibitors to the reaction mixture. The final concentration of [³H]-

pravastatin in the reaction mixture was 1 μM , i.e., approximately equal to its apparent K_t values for transporters localized on apical and basal membranes. To confirm that efflux transporters mediate ATP-dependent uptake of [^3H]-pravastatin into placental IOV, indomethacin and benzbromarone were used at a concentration of 100 μM . After 5 minutes pre-incubation of reaction mixture with inhibitor at 37 C, the pre-incubation was continued for another 10 minutes with [^3H]-pravastatin. The uptake was initiated by the addition of either 5 mM ATP or 5 mM AMP (as control). The reaction was stopped as described above. All stock solutions of the inhibitors were made in methanol. The maximum final concentration of methanol in the reaction mixture was 0.5%. The control reaction included all components except for the inhibitor and in the presence of 0.5% methanol (v/v).

2.8. ATP-dependent uptake of [^3H]-pravastatin by vesicles prepared from baculovirus infected insect cells (Sf9) overexpressing human BCRP and MRP1 proteins

The effect of a range of [^3H]-pravastatin concentrations between 0.25–5 μM on the velocity of ATP-dependent uptake of [^3H]-pravastatin by baculovirus infected Sf9 insect cells transfected with BCRP and MRP1 human transporters was determined as above. The amount of vesicle proteins expressing BCRP and MRP1 in the reaction solution was 40 μg and 30 μg , respectively. The reaction with BCRP was terminated after 2 min and with MRP1 after 3 min as described above. The rates of ATP-dependent uptake of [^3H]-pravastatin were expressed as $\text{fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

2.9. Immunohistochemistry (IHC)

Samples preparation and immunostaining were performed at the Research Histopathology

Core of the University of Texas Medical Branch, Pathology Department. After collection of the placenta from an uncomplicated pregnancy or from a patient with preeclampsia, a small portion of the placenta measuring 3cm x 2cm x 0.4cm was dissected within 5–10 minutes of delivery, placed in 10% Neutral Buffered Formalin, and after fixation and processing embedded in paraffin. Tissue sections were cut at 4 μm using an HM325 Thermo Scientific microtome, de-paraffinized in four changes of xylene for five minutes each, and then rehydrated through a series of graded alcohol solutions with a final rinse in distilled water (dH₂O). Slides were treated in an epitope retrieval citrate buffer, pH 6 for 20 minutes at 92–100°C using a Black and Decker steamer and then cooled down for a period of 20 minutes at room temperature to facilitate antigen-antibody binding. Slides were rinsed with three changes of dH₂O and placed in a solution of 3% H₂O₂ in dH₂O for 10 minutes to quench endogenous peroxidase. Slides were then rinsed with three changes of distilled water and placed on a Dako Automated Staining System stainer. Between each step, slides were washed using Tris buffered Saline with tween 20 (20X) (Thermo Scientific). Prior to loading the auto-stainer, all primary and secondary antibodies were titrated using Dako antibody diluent with background reducing components (Dako Corporation, Carpinteria, CA). Slides were incubated at room temperature with either anti-BCRP (1:100 for 2 hour, ab3380) or anti-MRP1 (1:160 for 1 hour, ab2410) antibodies. Secondary goat anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:250) were applied for 30 minutes at room temperature.

BCRP and MRP1 protein expression in the tertiary villi of control and preeclamptic placentas were examined by a single pathologist who was blinded to the source of the samples. For each placenta, five randomly selected fields were examined at x200 magnification under a Nikon Eclipse Ci microscope, and the images were captured at x400 magnification using NIS-Elements F software. Within the tertiary villi, the staining was assessed in the syncytial knots (STK), in the syncytiotrophoblast cells (ST) and in the fetal capillary endothelial cells (EC). Using a semi-quantitative grading system, the intensity of BCRP and MRP1 staining was examined in 5 randomly selected fields at x200 magnification. The staining was detected in the following specific locations: a) The apical membrane (apm) of the STK (apmSTK); b) The cytoplasm (c) of the of the STK (cSTK); c) The apical membrane of the ST cells (apmST); d) The cytoplasm of the ST cells (cST); e) The fetal capillary endothelial cell cytoplasm (cEC). Staining grading was performed as follows: Negative (-); Mild (+); Moderate (++) and severe (+++). Due to great variability of BCRP and MRP-1 expression in both groups, a sum of positive and negative grades was generated for each location, resulting in summation scores. After a comparison was made between the summation scores, cytoplasmic to apical membrane score ratios (c/apm score ratio) were obtained for STK, ST and EC, to allow comparisons for BCRP and MRP-1 expression in the control and pre-eclamptic groups.

2.10. RNA isolation and Real-Time Quantitative Polymerase Chain Reaction (Q-RTPCR) for the determination of ABCC1 and ABCG2 genes expression

About 100–200 mg of tissue obtained from each placenta was placed in Zymo TRI Reagent® and homogenized using a Qiagen TissueRuptor equipped with disposable probes (Qiagen, Valencia, CA). Total RNA was isolated from the homogenate using the Direct-zol™ RNA MiniPrep Kit according to the manufacturer's instructions. Isolated RNA was quantitated with a NanoDrop ND-1000 (NanoDrop Technologies, DE) and assessed for quality on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, California, USA) in the Molecular Genomics Core at UTMB. A uniform concentration from each isolated RNA was then reverse-transcribed using High-Capacity RNA-to-cDNA™ Kit in a 20 µl reaction mixture. Real time PCR was subsequently performed using equal concentrations of cDNA in 96-well plates with triplicate for each sample using TaqMan probes for *ABCG2* encoding BCRP (Assay ID: Hs01053790_m1) and *ABCC1* encoding MRP1 (Assay ID: Hs01561502_m1) on a Roche LightCycler 96 real-time PCR system (Roche Diagnostics Corporation, Indianapolis, IN). The TaqMan® Gene Expression Assay for *HPRT1* (Assay ID: Hs99999909_m1) was included in each reaction as the internal control. Data were analyzed using the Rel Quant Analysis mode of LightCycler 96 software version 1.1.0.1320.

2.11. Statistical analysis

All data were expressed as means $\pm$ SD. Data were plotted using GraphPad Prism 5.0 software. Statistical analyses for uptake studies were performed with Minitab 18 using independent Student t-test for the comparisons between two group means, and using one way ANOVA followed by Tukey's post hoc test for multiple comparisons. Sample size for IHC analyses was based on the two-sample t-test and statistical significance was determined

using the non-parametric Mann-Whitney test. Results were considered significant if the p value was less than 0.05.

3. Results

3.1. Characterization of placental apical and basal membrane vesicles.

In a previous report, we presented an electron microscopy image that showed heterogeneous populations of bilayer vesicles from apical membranes [19].

The data presented in Table 1 show that DHA binding in basal membranes increased by more than 17-fold over homogenate while in apical membranes it decreased by almost 2-fold, suggesting negligible cross-contamination of apical membranes by basal membranes. On the other hand, 1.5-fold increase in ALP activity in vesicles prepared from basal membranes suggests that some cross-contamination by apical membranes is possible. The contamination of apical and basal membranes by intracellular membranes of mitochondria was $3 \pm 3\%$ and $11 \pm 4\%$, and endoplasmic reticulum was $13 \pm 8\%$ and $0.15 \pm 0.06\%$ (Table 1).

3.2. ATP-dependent uptake of [³H]-pravastatin by placental apical and basal membrane vesicles

The functional activities of efflux transporters localized on the apical and basal membranes of human placenta were investigated using a range of pravastatin concentrations to generate saturation curves and calculate reaction constants K_t and V_{max} (Figure 1A-D).

Preliminary data on the reaction conditions for vesicles prepared from apical membranes showed that uptake of [³H]-pravastatin was ATP-dependent, linear with protein concentrations up to 100 μg and time up to 1 minute. Analysis of the sigmoidal curve (Figure 1B) showed a mean apparent K_t of $0.8 \pm 0.4 \mu\text{M}$ and V_{max} of $1853 \pm 234 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$.

Vesicles prepared from basal membranes showed linear ATP-dependent uptake of [³H]-pravastatin with protein concentrations up to 30 μg and time up to 2 minute. The apparent K_t and V_{max} values calculated from allosteric sigmoidal curve (Figure 1D) were $1.2 \pm 0.3 \mu\text{M}$ and $553 \pm 34 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$, respectively.

Eadie-Hofstee plots of the data (Figure 1B&D insert) exhibit the characteristic of “curved” shape associated with sigmoidal kinetics.

3.3. Uptake of [³H]-pravastatin by overexpressed vesicles.

The ATP-dependent uptake of [³H]-pravastatin by each efflux transporter was determined using commercially available vesicles overexpressing P-gp, BCRP, and the MRPs1–5. The ATP-dependent uptake of [³H]-pravastatin by vesicles expressing BCRP ($802 \pm 117 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$) and MRP1 ($697 \pm 98 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$) was 4–6 fold higher than its uptake by vesicles expressing MRP5 ($191 \pm 29 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$), MRP2 ($152 \pm 25 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$), P-gp ($152 \pm 57 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$), and MRP4 ($126 \pm 38 \text{ fmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$) (Figure 2).

3.4. Western blot analysis

Western blot analysis showed expression of P-gp, BCRP, MRP2 and MRP3 transporters in vesicles prepared from apical membranes and MRP1, MRP4, and MRP5 transporters in vesicles prepared from basal membranes. In addition, weak immunoreactivity signals were detected for BCRP expression in basal and MRP1 expression in apical membranes (Figure 3).

3.5. Inhibition of ATP-dependent uptake of [³H]-pravastatin

To confirm that efflux transporters mediate ATP-dependent uptake of [³H]-pravastatin by placental IOV, indomethacin and benzbromarone were used at concentration of 100 μ M (Figure 4). At this concentration indomethacin, inhibited 90% of [³H]-pravastatin uptake by MRP1 and MRP4 vesicles, 80% by MRP3 and P-gp vesicles, 60% by MRP2 and MRP5 vesicles, and 50% by BCRP vesicles. In vesicles prepared from apical and basal membranes 100 μ M of indomethacin inhibited uptake of [³H]-pravastatin by 40% and 60%, respectively. On the other hand, 100 μ M of benzbromarone inhibited almost 100% of [³H]-pravastatin uptake by MRP1, MRP2, MRP3, MRP4 and BCRP vesicles and 90% by MRP5 and P-gp vesicles. Also 100 μ M of benzbromarone inhibited 100% of [³H]-pravastatin uptake in placental vesicles prepared from apical membranes, and 70% by vesicles prepared from basal membranes. Taken together, these data indicate that ATP-dependent uptake of [³H]-pravastatin by placental IOV is mediated by several efflux transporters.

3.6. Uptake of [³H]-pravastatin by vesicles overexpressing BCRP and MRP1 transporters

The ATP-dependent uptake of [³H]-pravastatin by BCRP overexpressed vesicles was linear up to 50 μ g of protein over 3 minutes. In vesicles overexpressing MRP1 it was linear up to 50 μ g of protein and up to 4 minutes. The rate of ATP-dependent uptake of [³H]-pravastatin by vesicles expressing BCRP and MRP1 was dependent on pravastatin concentrations and exhibited typical Michaelis–Menten saturation kinetics (Figure 5A-D). Analysis of the hyperbolic saturation curves obtained from vesicles expressing BCRP revealed a mean apparent K_t and V_{max} values of 2.8 ± 0.4 μ M and 3204 ± 2466 fmol·mg protein⁻¹·min⁻¹, respectively (Figure 5B), while the apparent K_t and V_{max} values for pravastatin uptake by vesicles expressing MRP1 was 2.5 ± 1.0 μ M and 836 ± 165 fmol·mg protein⁻¹·min⁻¹, respectively (Figure 5D).

Eadie–Hofstee plot of the data (Figure 5B&D, insert) indicated that the uptake of [³H]-pravastatin by vesicles expressing BCRP and MRP1 in the range of pravastatin concentrations tested exhibited monophasic enzyme kinetics.

3.7. Expression of BCRP and MRP1 in placentas obtained from uncomplicated pregnancies.

In the tertiary villi of placentas from uncomplicated pregnancies (control), BCRP and MRP1 expression were detected in the apical plasma membrane and in the apmSTK and cSTK, in the apmST and cST, as well as in the cEC (Figure 6A&B).

The BCRP summation score was the highest in the apmST, followed by cEC. BCRP c/apm score ratios for ST and STK were 0.58 and 7, respectively. BCRP ST-to-STK score ratio in

the apical plasma membrane was 19.7. These data suggest that in term tertiary villi, BCRP is predominantly localized in the apical plasma membrane of ST and in the cytoplasm of the EC of fetal capillaries (Table 2).

The MRP1 summation score was the highest in the apmSTK. However, MRP1 expression was negative in the majority of the investigated fields containing EC and STK. The MRP1 c/apm score ratios in ST was 0.2. (Table 2), suggesting that in term tertiary villi, MRP1 expression is mainly determined in the apm of ST.

3.8. Expression of BCRP and MRP1 proteins in placentas obtained from patients with preeclampsia.

In the tertiary villi of preeclamptic placentas, we observed strong positive BCRP expression in the apmSTK (Figure 7A). The intensity of staining of the apmSTK showed a significant difference between control and preeclamptic placentas ($p < 0.01$). Furthermore, the mean number for STK in preeclamptic placentas was higher than in the control (11.6 ± 4.2 vs 7.3 ± 2.6). When BCRP expression at the apmSTK, cSTK, apmST and cST were compared between control and preeclamptic groups, no significant difference was found (Table 3).

In preeclamptic placentas, MRP1 expression was higher for both cSTK ($p < 0.001$) and cST ($p < 0.01$), as compared to control placentas (Figure 7B). There were no visible differences between preeclamptic and control placentas concerning MRP1 expression in apmST and apmSTK, as well as in EC (Table 3).

3.9. ABCC1 and ABCG2 genes expression

As shown in Figure 8A, there was no change in *ABCG2* gene expression in preeclamptic placentas compared to the levels observed in controls. However, we observed (Figure 8B) a significant increase in *ABCC1* expression in preeclamptic placentas compared to controls ($P < 0.001$).

3.10. ATP-Dependent uptake of [3H]-pravastatin by apical placental membrane vesicles prepared from control and preeclamptic placentas.

Due to predominant expression of BCRP and MRP1 in apical plasma membrane of ST and STK, the functional activity of efflux transporters involved in ATP-dependent uptake of [3H]-pravastatin was determined by inside-out vesicles prepared from apical membranes. ATP-dependent uptake of [3H]-pravastatin by vesicles prepared from apical membranes of preeclamptic placentas (13.9 ± 6.5 pmol.mg protein⁻¹.min⁻¹) was similar to the uptake of [3H]-pravastatin determined in vesicles prepared from placentas obtained from uncomplicated pregnancies (14.1 ± 5.8 pmol.mg protein⁻¹.min⁻¹)(Figure 9).

4. Discussion

In this study, the function of placental efflux transporters was determined *in vitro* using vesicles prepared from apical and basal membranes of trophoblast tissue obtained from term pregnancies. These membrane vesicles preparations consist of approximately 80% of right-side-out (ROV) and 20% of inside-out (IOV) vesicles. In the IOVs the cytoplasmic ATP-

binding domain and the substrate binding site of the efflux transporter are on the outside of the membrane bilayer and consequently the ATP-dependent uptake of a substrate by IOV *in vitro* corresponds to its *in vivo* efflux by the transporter protein [23]. Recently, Gilibili et al., pointed out several limitations including extensive substrate diffusion into membrane vesicles and the lack of an optimal dynamic window of ATP-dependent uptake that makes this *in vitro* vesicle assay unsuitable for many substrates [24]. In our case, pravastatin is a hydrophilic compound and thus diffuses poorly across membranes. The short duration of uptake linearity (up to 1 and 2 minutes in apical and basal membranes, respectively) also did not affect reproducibility of the uptake assay. However, the high proportion of the ROV with uptake transporters transferring pravastatin to the inside of the vesicles resulted in higher uptake of [³H]-pravastatin in the presence of AMP (Figure 1A&B) than expected from its physicochemical properties.

In this study, the ATP-dependent uptake of [³H]-pravastatin by apical and basal membrane vesicles revealed sigmoidal kinetics and “curved” shapes of Eadie–Hofstee plots suggesting that more than one placental efflux transporter is involved in its uptake (Figure 1B&D). Indeed, using commercially available vesicles expressing individual efflux transporters, the ATP-dependent uptake of [³H]-pravastatin was observed by BCRP, MRP1, MRP5, MRP2, P-gp, and MRP4 vesicles (Figure 2). Furthermore, the expression of BCRP, P-gp, MRP2, and to a lesser extent MRP1 in vesicles prepared from apical membranes and the expression of MRP1, MRP4, and MRP5, and to lesser a extent BCRP in basal membranes was demonstrated by Western blot analysis (Figure 3). In addition, the sigmoidal plot of the uptake could also indicate the existence of at least two drug binding sites in one or more transporters (for example MRP2 [25], MRP1 [26], P-gp [27,28]) with a positive cooperative interaction [25].

The data obtained in this investigation show that the rate of the [³H]-pravastatin uptake by vesicles prepared from apical membranes expressing BCRP, P-gp, MRP2, and to lesser extent MRP1 was 3-fold higher than that by basal membranes expressing MRP1, MRP4, and MRP5. Previously MRP2 and BCRP were identified as the major hepatic transporters that transfer pravastatin to the bile—the major route for its elimination [29]. Although in our study the activity of MRP2 was relatively low in the overexpressed system, its expression in apical membranes of syncytiotrophoblast and known self-cooperative effects between modulator and substrate binding sites suggest its contribution to the efflux of pravastatin from placenta. On the other hand, despite the relatively high expression of MRP4 in basal membranes of syncytiotrophoblast its contribution to the efflux of pravastatin most likely is low because net activity of transporters localized on basal membranes was 3 fold lower than on the apical. Nevertheless, these data support the previously observed higher transfer of pravastatin across dually perfused human placental lobule in the F→M than the M→F direction [8]. Also they are in accordance with observations from the pilot clinical trial in which cord blood pravastatin concentrations, from the majority of neonates in the pravastatin group, were below the detection limit of the assay [6].

Vesicle preparations used in this study express several efflux transporters and pravastatin can be transferred by more than one transporter (Figure 2), making the selective inhibition of an individual transporter very challenging. To confirm that the observed ATP-dependent uptake

of [^3H]-pravastatin by apical and basal membranes was mediated by efflux transporters and for identification purposes, we used 100 μM of indomethacin or benzbromarone. The selection of these two inhibitors and the concentrations were based on their ability to inhibit more than one transporter [30–33]. In contrast to complete inhibition by 100 μM of benzbromarone, the effect of 100 μM of indomethacin on individual transporters, especially BCRP, MRP2 and MRP5, was partial. The latter could explain partial inhibition of [^3H]-pravastatin uptake in apical membranes in the presence of indomethacin (Figure 4). On the other hand, more pronounced inhibition of [^3H]-pravastatin uptake by indomethacin in basal vs apical membranes, could be attributed to 90% inhibition of major transporters expressed in vesicles prepared from basal membranes, i.e. MRP1 and MRP4 (Figure 3). The remaining uptake of [^3H]-pravastatin in the presence of benzbromarone could be explained by the activities of other efflux transporters expressed on basal membranes of syncytiotrophoblast. Thus, the results of inhibition experiments with 100 μM of indomethacin and benzbromarone (Figure 4) showed that the uptake of [^3H]-pravastatin determined in placental vesicles prepared from apical and basal membranes is mediated by multiple efflux transporters. Taking into consideration results from western blot analysis and previously reported high mRNA expression of BCRP [34,35] especially in term placentas [11] as well as relative abundance of MRP1 compared to other MRPs [34, 36], the *in vitro* ATP-dependent uptake of [^3H]-pravastatin by membrane vesicles could be primarily attributed to BCRP and MRP1. In the range of pravastatin concentrations tested, uptake of [^3H]-pravastatin by BCRP or MRP1 overexpressed vesicles showed standard saturation curves with relatively high affinity to the transporters (Figure 5B&D).

The second aim of this investigation was to determine whether expression and activity of BCRP and MRP1 transporters could be affected by pathological changes associated with preeclampsia. Using placentas from term uncomplicated pregnancies as control, we showed that in tertiary villi, BCRP and MRP1 were predominantly localized in the apm ST (Figure 6A&B). We also observed high expression of BCRP, but not MRP1, EC. Our data are concordant with the previous immunohistochemical [37, 38] and immunofluorescence [39] studies confirming the localization of BCRP in the apical membranes of ST cells and in the fetal capillary EC of tertiary villi. It should be noted here, that we also observed a high intensity signal for BCRP expression in vesicles prepared from apical membranes (Figure 3). On the other hand, reports regarding localization of MRP1 are controversial. In a study of term human placenta, immunofluorescent staining showed predominant MRP1 expression in the blood vessels [40], while in this study and in another study [37], the immunohistochemical expression of MRP1 in fetal capillary EC was very low or undetectable. The expression of MRP1 in apical and basal membrane of ST was shown by immunohistochemistry in one study [37], only in the basal membrane by immunofluorescence in the a second study [41], and only in apical membrane by immunofluorescence in a third study [40]. Our immunohistochemistry data revealed MRP1 expression in the apical membrane of ST cells, which is in agreement with the report by St-Pierre. However, the intensity of MRP1 signal on Western blot in vesicles prepared from basal membranes was stronger than in vesicles prepared from apical membranes (Figure 3). This discrepancy in the MRP1 localization could be explained in part by the selectivity and sensitivity of anti-MRP1 antibodies used in different studies. In addition, the precipitation of

MRP1 proteins present in the cytoplasm of ST with basal membranes during preparation of vesicles cannot be excluded. Nevertheless, it appears that the net effect of placental efflux transporters will restrict the transfer of pravastatin across the placenta for the following reasons: (i) A relatively higher expression of BCRP and MRP1 in the apical membrane of ST cells observed in this study and by others [37, 40]; (ii) The larger surface area of apical membranes compared to basal membranes; (iii) The higher activity of efflux transporters localized on the apical membranes rather than the basal membranes.

Preeclamptic placentas had increased number of STK with increased mean expression of BCRP in their apical membranes and increased expression of MRP1 in the cytoplasm of ST cells and STK (Figure 7A&B). Normal villous maturation increases the percentage of STK as gestation progresses [42]. In this study, the average gestational age of preeclamptic placentas was lower than that of control placentas (36.4 ± 1.6 vs 38.6 ± 0.4 weeks.days, $p < 0.001$). Thus, based on the gestational age, the percentage of STK in preeclamptic placentas should be similar or lower compared to control placentas [42]. Therefore, the increased number of STK in preeclamptic relative to control placentas is most likely due to pathological changes associated with the disease and is concordant with previous reports [43,44]. An increase in the number of STK in preeclampsia has been associated with placental ischemia and malperfusion [45–47]. Increased syncytial knotting as a result of hypoxia has also been reported in placentas from women whose pregnancies occurred at high altitude [46]. Furthermore, in this study, the immunohistochemical staining intensity of BCRP in apical membranes of STK of preeclamptic placentas was significantly stronger than in control placentas, indicating increased expression of BCRP in STK in preeclamptic placentas.

Reports on the effect of hypoxia on placental BCRP expression are inconsistent. In explants from term human placentas low (3%) oxygen tension had no effect on BCRP protein levels [48], while placentas from first trimester had increased BCRP immunohistochemical staining under hypoxic conditions in cytotrophoblast and the microvillous membrane of the syncytium [49]. This reported discrepancy in BCRP expression could be attributed to the difference in the placental environment and experimental conditions. Thus, compared to placental explants from term placentas experimentally exposed to low oxygen tension for 24 and 48 hours, the development of placentas from the first trimester occurs in hypoxic conditions. However, in a recent study [50], western blot analysis also showed decreased expression of BCRP in microvillous membrane of placentas from women who gave birth at a high altitude, suggesting down-regulation of BCRP under hypoxic conditions. Previously, Krishnamurthy and colleagues showed that hypoxia upregulated expression of BCRP in mouse hematopoietic stem cells and that this upregulation is regulated by the hypoxia-inducible transcription factor complex HIF-1 [51]. Furthermore, using chemical inhibitors of BCRP transport, authors showed that BCRP protects cells from hypoxia [51]. Thus, it appears that increased protein expression of BCRP in STK of preeclamptic placentas may be a compensatory response to the pathological conditions associated with preeclampsia. In preeclamptic placentas we also observed a slight increase in *ABCG2* mRNA expression (Figure 8), however, in contradiction to the significant increase in protein expression. Similarly, a slight increase in *ABCG2* mRNA expression in preeclamptic placentas was reported by Mason et al. [13]. The discrepancy between *ABCG2* gene and BCRP protein

expression was reported previously by a group of investigators who studied the expression of BCRP/*ABCG2* in human placenta throughout gestation [49, 52] and noted a disconnect between gene expression and translation.

In contrast to BCRP, in this study preeclampsia was associated with increased expression of both *ABCC1* gene and MRP1 protein. On the other hand, Mason et al. did not find apparent changes in expression levels of both *ABCC1* mRNA and MRP1 protein in preterm placentas (25.6–33.0 weeks of gestation) in a small number of patients with severe preeclampsia [13]. Furthermore, in our study expression of MRP1 in preeclamptic placentas was increased only in the cytoplasm of ST and STK. The latter changed the cytoplasmic to apical plasma membrane MRP1 distribution ratio, suggesting a decrease in the proportion of active plasma membrane transporters vs inactive cytoplasmic transporters in preeclamptic placentas. However, transporter-specific changes in mRNA and protein expression of BCRP and MRP1 observed in preeclamptic placentas did not affect the uptake of [³H]-pravastatin by IOVs (Figure 9).

In summary, several efflux transporters are involved in placental distribution of pravastatin with BCRP and MRP1 being the major contributors. However, in addition to the efflux transporters, pravastatin is also a substrate of the uptake transporters OAT4 [53], OATP1A2 and OATP2B1 [54]. Recent data from our laboratory determined the expression of OATPs 1A2, 3A1, 4A1, and 5A1 on apical, OATP2B1 on basal, and OAT4 on both apical and basal membranes of the syncytiotrophoblast [55]. It is assumed that uptake transporters localized on the apical and basal membranes will transfer pravastatin from maternal and fetal circulations, respectively to the syncytiotrophoblast, while the efflux transporters will extrude the drug from the placental tissue (Figure 10). The higher activities of efflux transporters localized on the apical membranes of syncytiotrophoblast and the lack of effect of pre-eclampsia on the rate of transporter-mediated efflux of pravastatin suggest that the drug is unlikely to accumulate in syncytiotrophoblast or fetal circulation. However, the effect of pre-eclampsia on the activity of placental uptake transporters on the transfer of pravastatin is yet to be determined.

ACKNOWLEDGEMENT

The authors appreciate the assistance of Dr. Vsevolod Popov with electron microscopy and also the support of the physicians and nurses of the Labor & Delivery Ward of the John Sealy Hospital, the teaching hospital at UTMB, Galveston, Texas, the Perinatal Research Division, and the Publication, Grant, & Media Support division of the Department of Obstetrics & Gynecology.

This study was supported by the Obstetric-Fetal Pharmacology Research Center network of NICHD [Grant U54 HD047891].

ABBREVIATIONS:

ALP	Alkaline Phosphatase
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein

ABCG2	ATP-binding cassette sub-family G member 2
DHA	dihydroalprenolol
IOVs	inside-out vesicles
MRPs	multidrug resistance-associated proteins
PALP	Placental alkaline phosphatase
P-gp	P-glycoprotein 1
ABCB1/MDR1	multidrug resistance protein 1, ATP-binding cassette sub-family B member 1
SHT buffer	Sucrose-HEPES-Tris Buffer
ST	Syncytiotrophoblast cells
STK	Syncytiotrophoblast knots
EC	Fetal endothelial cells
apm	Apical membrane
c	Cytoplasm

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Figure 1.

Uptake of $[^3\text{H}]$ -pravastatin by (A,B) apical and (C,D) basal membrane vesicles. The effect of a range of $[^3\text{H}]$ -pravastatin concentrations between 0.25–3 μM on the rate of ATP-dependent uptake of $[^3\text{H}]$ -pravastatin was determined using pooled membrane vesicles prepared from 14 term placentas obtained after uncomplicated pregnancies. Percentage of inside-out vesicles (IOVs) in the pooled apical and basal membrane vesicles was $19\% \pm 3\%$ and $20\% \pm 3\%$, respectively. ATP-dependent uptake of $[^3\text{H}]$ -pravastatin by IOV reported as the difference between its uptake in the presence of ATP and in the presence of AMP. In both apical and basal membrane vesicles, the difference between ATP and AMP uptake was statistically significant ($P < 0.05$) for each concentration tested. The data shown are the mean $\pm$ SD of duplicates from two experiments.

Figure 2.

ATP-dependent uptake of [3 H]-pravastatin by vesicles prepared from baculovirus infected Sf9 insect cells transfected with human MRP1, MRP2, MRP3, and BCRP transporters and vesicles purified from human (K) cells overexpressing the P-gp, MRP4, and MRP5 transporters. No ATP-dependent uptake was observed in vesicles prepared from mock-transfected cells (control). In vesicles expressing BCRP, MRP1, MRP2, MRP4 and MRP5, the difference between total and nonspecific uptake was statistically significant ($P < 0.05$). The highest pravastatin uptake was observed into vesicles transfected with BCRP and MRP1 transporters. In BCRP and MRP1 vesicles the ratios of pravastatin uptake in the presence of ATP vs its uptake in the presence of AMP were 2.1 ± 0.1 and 5.0 ± 0.6 , respectively. The data are shown as mean $\pm$ SD of duplicates from three experiments.

Figure 3.

Expression of efflux transporter proteins in vesicles prepared from apical and basal membranes of human placenta. Lanes 1–2 - 0.5 and 0.05 µg of positive controls (vesicles prepared from baculovirus infected insect cells (Sf9) expressing human BCRP, MRP1, MRP2, MRP3, and vesicles purified from human (K) cells overexpressing the MDR1/P-gp, MRP4, and MRP5, and HeLa cells). Lanes 3–5 - vesicles prepared from apical membranes of human placentas (# 1,2,3); Lanes 6–8 - vesicles prepared from basal membranes of

human placentas (#1,2,3). Both preparations showed immunoreactivity toward trophoblast marker cytokeratin 7.

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Figure 4.

The effect of 100 μ M of the transporters inhibitors indomethacin and benzbromarone on ATP-dependent uptake of [3 H]-pravastatin was determined using the following vesicle preparations: (i) vesicles prepared from apical and basal membranes of term human placentas, and (ii) purchased vesicle preparations overexpressing MRP1, MRP2, MRP3, MRP4, MRP5, BCRP, and P-gp. The rates of ATP-dependent uptake of [3 H]-pravastatin are expressed as percent of control (absence of inhibitors) and represent the mean $\pm$ SD of two experiments performed in triplicates.

Figure 5. Rates of [^3H]-pravastatin uptake by baculovirus infected Sf9 insect cells transfected with (A,B) BCRP and (C,D) MRP1 human transporters. The ATP-dependent uptake of [^3H]-pravastatin into vesicles reported as the difference between its uptake in the presence of ATP and in the presence of AMP. In vesicles transfected either with BCRP or with MRP1, the difference between ATP and AMP uptake was statistically significant ($P < 0.01$) for each concentration tested. The data are shown as mean $\pm$ SD of duplicates from two experiments.

Figure 6.

BCRP expression in tertiary villi of placentas obtained from uncomplicated pregnancies (A) or from patients with preeclampsia (B) (x400).

Immunohistochemical (IHC) expression of BCRP was determined in 27 control and 25 preeclamptic placentas. STK, syncytial knots; ST, syncytiotrophoblast. In preeclamptic placentas, the intensity of the IHC signal in the apical membranes of STK is stronger than in the apical membrane of STK in control placentas.

Figure 7.

MRP1 expression in tertiary villi of placentas obtained from uncomplicated pregnancies (A) or from patients with preeclampsia (B) (x400).

Immunohistochemical (IHC) expression of MRP1 was determined in 26 control and 25 preeclamptic placentas. In preeclamptic placentas, the intensity of the IHC signal in the cytoplasm of syncytiotrophoblast (ST) and syncytial knots (STK) is stronger than in the apical membrane of ST and STK in control placentas.

Figure 8A.

ABCG2 expression in placentas obtained from uncomplicated pregnancies and patients with preeclampsia.

For mRNA expression of the *ABCG2* gene encoding the BCRP protein, a total of 22 preeclamptic and 26 control placentas were evaluated. Data shown represent mean $\pm$ SD relative expression levels after normalization to the levels of *HPRT* expression (internal control).

Figure 8B.

ABCC1 expression in placentas obtained from uncomplicated and from patients with preeclampsia.

For mRNA expression of the *ABCC1* gene encoding MRP1, a total of 20 preeclamptic and 26 control placentas were evaluated. Data shown represent mean $\pm$ SD relative expression levels after normalization to the levels of *HPRT* expression (internal control). * $P < 0.001$.

Figure 9.

ATP-Dependent uptake of [³H]-pravastatin by apical placental membrane vesicles prepared from placentas obtained either from uncomplicated pregnancies (n=8) or from patients with preeclampsia (n=8). The average percentage of IOV in preparations from placentas obtained after uncomplicated pregnancies (control) and patients with preeclampsia was 12% ± 3% and 12% ± 4%, respectively. The rates of uptake were normalized to the percentage of IOV in each preparation. The values are given as mean of 8 individual uptake experiments performed in triplicate ± S.D.

Figure 10.

Localization of transporters on apical and basal sides of syncytiotrophoblast. Schematic illustration of Western blots presented in Figure 3 and in recent abstract from our laboratory [55]. Pravastatin is a polar compound and its transfer across biological membranes including the placenta is mediated by the transporters. Uptake transporters localized on apical membranes of syncytiotrophoblast transfer pravastatin from the maternal circulation to the syncytiotrophoblast. From syncytiotrophoblast efflux transporters localized on apical and basal membranes extrude the drug to the maternal and fetal circulations, respectively. The higher activity of efflux transporters localized on apical side of membranes will decrease accumulation of pravastatin by placental tissue and thus less drug will be available for efflux to the fetal side. The uptake transporters localized in basal membranes of syncytiotrophoblast facilitate removal of pravastatin from fetal circulation to the placental tissue. Thus, activity of efflux transporters localized on apical membranes and uptake transporters on basal are essential in limiting fetal exposure. Investigations on the role of uptake transporters and the effect of preeclampsia on their expression and activities is ongoing in our laboratory.

Table 1.

Purity of apical and basal membrane vesicles.

	ALP activity, $\mu\text{mol}\cdot\text{mg}$ $\text{protein}^{-1}\cdot\text{min}^{-1}$ [enrichment]	DHA binding, $\text{fmol}\cdot\text{mg}$ protein^{-1} [enrichment]	Cytochrome C Oxidase activity, Units/mL (%)	Cytochrome C Reductase activity, mUnits/mL (%)
Placental fractions [†]	2.0 ± 0.6 ^I	44.5 ± 3.5 ^I	0.145 ± 0.014 ² (100%)	1131 ± 164 ³ (100%)
Apical membrane	26.7 ± 5.7 [14.7 $\pm$ 5.6]	24.5 ± 0.7 [0.6 $\pm$ 0.1]	0.005 ± 0.005 (3 $\pm$ 3%)	174 ± 60 (13 $\pm$ 8%)
Basal membrane	2.9 ± 1.6 [1.5 $\pm$ 0.5]	732 ± 56 [17.5 $\pm$ 2.6]	0.018 ± 0.005 (11 $\pm$ 4%)	1.5 ± 0.7 (0.15 $\pm$ 0.06%)

Data are expressed as means $\pm$ SD.[†]Placental fractions: homogenate¹; crude mitochondrial²; crude microsomal³^IEnrichment in Alkaline Phosphatase (ALP) activity (marker for apical membranes) and enrichment in dihydroalprenolol (DHA) binding (marker for basal membranes) was calculated relative to placental tissue homogenates.²Percent contamination of placental vesicles prepared from apical and basal membranes by mitochondrial membranes was calculated relative to cytochrome c oxidase activity determined in crude mitochondrial preparations from human placentas.³Percent contamination of placental vesicles prepared from apical and basal membranes by membranes of endoplasmic reticulum was calculated relative to cytochrome c reductase activity determined in crude microsomal preparations from human placentas.

ALP, Alkaline Phosphatase; DHA, dihydroalprenolol.

Table 2.

Summation scores. Sum of all positive and negative grades for BCRP and MRP1 expression in uncomplicated (control) and preeclamptic placentas (PE)

		STK summation apmSTK	scores cSTK	EC summation score	ST summation ampST	score cST
BCRP	Control (n=27)	3	21	34	59	34
	PE (n=25)	26	21	43	55	32
MRP1	Control (n=26)	17	-2	-12	40	8
	PE (n=25)	19	16	-12	46	31

Immunohistochemical expression of BCRP and MRP1 in trophoblast and fetal capillary endothelial cells was graded using a semi-quantitative grading system. The intensity of staining was reported as follows: negative (-), mild (+), moderate (++) and intense (+++). Subsequently, a final numerical score reflecting the protein expression in each group was derived from the sum of all positive and negative grades called the summation score. The summation scores were used for comparison and statistical analysis.

STK, Syncytiotrophoblast knots; ST, Syncytiotrophoblast cells; apm, Apical membrane; c, Cytoplasm; EC, Fetal endothelial cells.

Table 3.

Expression of the efflux transporters BCRP and MRP1 in apical membrane and cytoplasm of syncytial knots and syncytiotrophoblast, as well as in endothelial cells of fetal blood capillaries in placentas from uncomplicated pregnancies (control) and patients with preeclampsia (PE).

	Mean BCRP	Expression	Mann-Whitney Test	Mean MRP1	Expression	Mann-Whitney Test
	Control (n=27)	PE (n=25)	P-values adjusted for ties	Control (n=26)	PE (n=25)	P-values adjusted for ties
apmSTK	0.56±0.51	1.24±0.78	0.001	1.04±0.77	0.78±0.72	0.202
cSTK	0.89±0.32	1.02±0.62	0.454	0.46±0.51	0.82±0.38	0.009
apmST	2.22±0.80	2.24±0.79	0.960	1.69±0.88	1.90±0.89	0.416
cST	1.30±0.54	1.30±0.50	0.931	0.65±0.49	1.32±0.61	<0.001
EC	1.39±0.66	1.74±0.89	0.216	0.27±0.41	0.22±0.33	0.850

Data are expressed as means ± SD.

STK, Syncytiotrophoblast knots; ST, Syncytiotrophoblast cells; apm, Apical membrane; c, Cytoplasm; EC, Fetal endothelial cells.