Synchronized Activity of Organic Cation Transporter 3 (Oct3/Slc22a3) and Multidrug and Toxin Extrusion 1 (Mate1/Slc47a1) Transporter in Transplacental Passage of MPP⁺ in Rat

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The aim of the present study was to investigate the expression, localization, and function of organic cation transporter 3 (Oct3, Slc22a3) and multidrug and toxin extrusion protein 1 (Mate1, Slc47a1) in the rat placenta. Using qRT-PCR and Western blotting techniques, we demonstrated abundant Oct3 and Mate1 mRNA and protein expression achieving significantly higher levels than those in the maternal kidney (positive control). Immunohistochemical visualization revealed preferential localization of Oct3 on the basolateral, i.e., fetus facing side of the placenta, whereas Mate1 positivity was located in the labyrinth area predominantly on the apical, i.e., maternal side of the placenta. To investigate the role of these transporters in the transplacental pharmacokinetics, the in situ method of dually perfused rat term placenta was employed in open- and closed-circuit arrangements; 1-methyl-4-phenylpyridinium (MPP⁺) was used as a model substrate of both Oct3 and Mate1. We provide evidence that Oct3 and Mate1 cause considerable asymmetry between maternal-to-fetal and fetal-to-maternal transport of MPP⁺ in favor of fetomaternal direction. Using closed-circuit experimental setup, we further describe the capacity of Oct3 and Mate1 to transport their substrate from fetus to mother even against a concentration gradient. We conclude that Oct3, in a concentration-dependent manner, takes up MPP⁺ from the fetal circulation into the placenta, whereas Mate1, on the other side of the barrier, is responsible for MPP⁺ efflux from placenta to the maternal circulation. These two transport proteins, thus, form an efficient transplacental eliminatory pathway and play an important role in fetal protection and detoxication.

Key Words: organic cation transporter 3; multidrug and toxin extrusion transporter 1; placenta; pregnancy; pharmacokinetics; MPP⁺.

The placenta is a crucial organ for proper fetus development, enabling communication between the maternal and fetal circulations. Equipped with various transport and biotransformation proteins, placenta, on one hand, facilitates transport of nutrients to the developing fetus and, on the other hand, ensures its protection against harmful compounds from the maternal circulation. It has been widely accepted that placenta is not only a passive barrier but it can also actively defend the fetus against maternal toxins. This active protective role of the placenta has mainly been attributed to ATP-binding cassette (ABC) drug efflux transporters that are functionally expressed on the apical, maternal-facing membrane, such as P-glycoprotein (Cecкова-Novotna et al., 2006) and breast cancer resistance protein (Hahnova-Cygalova et al., 2011). To date, much less attention has been paid to the role of the solute carrier (SLC) family of transporters in transplacental pharmacokinetics. In this study, we focus on placental expression, localization, and function of organic cation transporter 3 (Oct3; in the text, transporter symbols with all letters in uppercase (OCT/MATE) are used for human genes and proteins, whereas symbols with only the first letter in uppercase (OCT/Mate) are used for genes/proteins in other mammal species). This protein was first cloned from the rat placenta (Kekuda et al., 1998), and its expression was consequently reported in a variety of tissues, including the brain, lung, intestine, heart, spleen, skeletal muscle, blood vessels, kidney, and liver (Lee et al., 2009). Of all organic cation transporters, OCT3 is the one most abundantly expressed in the placenta (Ganapathy and Prasad, 2005; Kekuda et al., 1998) recognizing many endogenous compounds such as the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺), tetraethylammonium, agmantine, cimetidine, prazosin, metformin, dopamine, and norepinephrine (Nies et al., 2011). Abundant placental expression and wide substrate specificity indicate the importance of OCT3 on the maternofetal interface; however, its role in transplacental pharmacokinetics is still not fully understood.

Several researchers have employed various in vitro, in situ, and in vivo models to investigate the expression and function...
of OCT3 in the placenta; these include JAR human placental choriocarcinoma cell line (Martel and Keating, 2003), human placental basal membrane vesicles (Sata et al., 2005), in situ perfused human term placenta (Terti et al., 2010), and Oct3 knockout mice (Zwart et al., 2001). However, this research has provided inconsistent and often contradictory outcomes. In their original work, Kekuda et al. (1998) suggested that OCT3 may be responsible for placental uptake of cationic xenobiotics from the fetal circulation and “may hence be a key player in the barrier function of the placenta to protect the developing fetus from possible deleterious effects of xenobiotics that may be present in the maternal circulation.” Contradictory views of OCT3 role in the placenta were later presented by other authors suggesting that “OCT3 may transfer MPP+ from placenta to fetus” (Ganapathy and Prasad 2005) and “OCT3 constitutes a leak pathway for fetal exposure” (Lee et al. 2009).

To understand the role of OCT3 in drug transport across biological membranes, it must be remembered that OCT3 cannot mediate transcellular passage (i.e., transport across both basolateral and apical membranes) of organic cations on its own. In excretory organs, such as kidney and liver, OCTs have been shown to facilitate the first step of cation excretion, i.e., uptake of organic cations across the basolateral membrane into the cell. The second step, active efflux of the cationic compounds from the cell across the apical membrane, is accomplished by other transport protein(s) such as P-glycoprotein (MDR1, ABCB1) or multidrug and toxin extrusion protein (MATE1, SLC47A1) (Giacomini et al., 2010; Koepsell et al., 2007; Nies et al., 2011; Yonezawa and Inui, 2011). In the placenta, such a vectorial transfer of cationic endo- and xenobiotics has not been systematically explored to date.

The aim of this study was to investigate the expression and localization and clarify the function of Oct3 in the rat placenta. We also aimed to search for a “collaborating” transporter on the localization and clarify the function of Oct3 in the rat placenta. The second step, active efflux of the cationic compounds from the cell across the apical membrane, is accomplished by other transport protein(s) such as P-glycoprotein (MDR1, ABCB1) or multidrug and toxin extrusion protein (MATE1, SLC47A1) (Giacomini et al., 2010; Koepsell et al., 2007; Nies et al., 2011; Yonezawa and Inui, 2011). In the placenta, such a vectorial transfer of cationic endo- and xenobiotics has not been systematically explored to date.

The aim of this study was to investigate the expression and localization and clarify the function of Oct3 in the rat placenta. We also aimed to search for a “collaborating” transporter on the apical membrane of the placenta that would efflux organic cations from the trophoblast cell into the maternal circulation. We used MPP+ as a model toxin and prototypical organic cation substrate of OCT3 (Masuda et al., 2006; Sata et al., 2005; Wu et al., 2000), MATE1 (Tanhara et al., 2007), and P-glycoprotein (Bleasby et al., 2000; Martel et al., 1996). Using the technique of in situ dually perfused rat term placenta, we show that Oct3, in a concentration-dependent manner, takes up MPP+ from the fetal circulation into the placenta, whereas Mate1 is responsible for MPP+ efflux to the maternal circulation. Our results suggest that these two transport proteins control the transplacental pharmacokinetics of organic cations and play an important role in fetal protection and detoxication.

MATERIALS AND METHODS

Reagents and chemicals. 1-methyl-4-phenylpyridinium iodide (MPP+), corticosterone, metformin hydrochloride, and cimetidine were obtained from Sigma-Aldrich (St Louis, MO). The radiolabeled ['H]MPP+ was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Rabbit polyclonal antibodies anti-Oct3, directed to the Oct3 (catalog no. BS5339) (70 kDa) and anti-Mate1 directed to Mate1 (catalog no. sc-138983) (65 kDa) were obtained from BioWorld Technology, Inc. (MN) and Santa Cruz Biotechnology, Inc. (CA), respectively. The loading control for Western blot, rabbit polyclonal anti-β-actin antibody (42–45 kDa) was purchased from Sigma-Aldrich. Horseradish peroxidase–linked donkey anti-rabbit immunoglobulin G, F(ab')2 fragment was purchased from GE Healthcare (Prague, Czech Republic). GF120918, dual P-glycoprotein and BCRP inhibitor, was from GlaxoSmithKline (Greenford, UK). All other chemicals were of analytical grade.

Cell cultures. HRP-1 rat trophoblast cells, a generous gift from Dr Michael Soares (University of Kansas City, KS) (Soares et al., 1987), were used in our studies as a potential in vitro model of rat placental barrier (Staud et al., 2006). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100μg/ml streptomycin, 1mM sodium pyruvate, and 50μg/ml β-mercaptoethanol. Cells from passage 17 were used for qRT-PCR and Western blot analysis.

Animals. Pregnant Wistar rats were purchased from Biotest Ltd (Konárové, Czech Republic) and were maintained in 12:12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on 21st gestation day. Fasted rats were anesthetized with 40 mg/kg of pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL) administered into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Královy (Charles University in Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986).

RNA isolation and reverse transcription-polymerase chain reaction analysis. Placentas and maternal kidneys, as a positive control, from four different animals of 21st gestation day were collected. The organs were frozen in liquid nitrogen immediately after dissection and stored at −70°C until analysis. Expression of Oct1, Oct2, Oct3, Mate1, and Mate2 mRNA was analyzed using qRT-PCR on 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) as described previously (Bacakova et al., 2009). Total RNA was isolated from the term placenta, maternal kidney, and HRP-1 rat trophoblast cells using Qiagen RNeasy Mini Kit (Bio-Consult Laboratories spol. s r.o., Czech Republic) and converted into cDNA via High Capacity cDNA reverse transcription kit (Life Technologies, Foster City, CA). Reaction mixture contained 30 ng of analyzed cDNA. The amplification of each sample was performed in triplicate using TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay for Oct1 (Slc22a1, Rn00562250_m1), Oct2 (Slc22a2, Rn00580893_m1), Oct3 (Slc22a3, Rn00570264_m1), Mate1 (Slc47a1, Rn01460731_m1), and Mate2 (Slc47a2, Rn02061013_m1) provided by Life Technologies. The time-temperature profile used in the “fast” mode was 95°C for 3 min; 40 cycles: 95°C for 7 s, 60°C for 25 s. For greater precision of the mRNA quantification results, two housekeeping genes were selected by the geNorm algorithm (Vandesompele et al., 2002): Ywhaz (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) (Ywhaz_Q1, NM_013011, exon5/exon6; GENERI BIOTECH Ltd, Hradec Královy, Czech Republic) and was used because of its stable expression in the placenta (Meller et al., 2005), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (4352338E; Life Technologies) was used because of its stable expression in the kidney (Soedel et al., 2006). Expression values of each sample were obtained as described previously (Raadilova et al., 2009; Vandesompele et al. 2002). The expression data were normalized by the geometric mean of GAPDH and Ywhaz expressions; the relative expression between reference (maternal kidney) and term placenta and HRP-1 rat trophoblast cells was determined by normalization of data.

Membrane preparation. A modified method of Chen et al. (2005) was used for membrane preparation. Briefly, maternal kidney, rat term placenta, and HRP-1 rat trophoblast cells were minced in ice-cold RIPA buffer (Sigma-Aldrich), containing 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 2 μg/ml aprotinin, and 50 μg/ml benzamidine, and then maternal kidney and term placenta were homogenized using a Magna Lyser Instrument (Roche Diagnostics, Prague,
Czech Republic) at 6500 rpm for 2 × 30 s; HRP-1 rat trophoblast cells were homogenized using Ultrasonic processor UP100H (Hielscher-Ultrasonic Technology, Teltow, Germany). The supernatants were obtained after a 3000 × g centrifugation at 4 °C for 10 min. The protein concentration was determined with the BCA assay (Pierce, Rockford, IL), and the samples were stored at −80 °C.

**Western blot analysis.** Crude membrane–containing homogenates of rat term placenta, maternal kidney, and HRP-1 cells proteins (20 μg) were incubated with sample buffer at room temperature for 30 min and separated by SDS-PAGE on 7.5 and 10% polyacrylamide gel for Oct3 and Mate1, respectively. After the proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA), the membrane was blocked for 1 h with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membranes were then incubated with primary antibodies for 1 h at concentrations of 1:1000 and 1:100 for Oct3 and Mate1, respectively. Then the membranes were washed four times with TBST and incubated for 1 h with a horseradish peroxidase–linked donkey anti-rabbit IgG antibody at concentrations 1:2000 and 1:200 for Oct3 and Mate1, respectively. Then the membranes were washed with TBST buffer, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare) and subjected to autolumino-grammetry for 1–5 min. The immunoreactive bands on the exposed films were quantified as described previously (Brcakova et al., 2009). Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

**Immunohistochemistry.** Preparations of the rat term placental tissue were performed as described previously (Pavek et al., 2003). Specimens of the placenta were fixed in 4% paraformaldehyde and then paraffin embedded (et al., 2006). Specimens of the placenta (thickness, 7 μm) were rehydrated through a series from xylene to ethanol solutions. The antigen Oct3 for immunohistochemistry. Sections of the placenta (thickness, 7 μm) were blocked with 3% H2O2 in PBS solution for 15 min. Subsequently, the slides were developed with a secondary antibody, goat anti-rabbit IgG antibody at concentrations 1:2000 and 1:200 for Oct3 and Mate1, respectively. After washing with TBST buffer, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare) and subjected to autolumi-nography for 1–5 min. The immunoreactive bands on the exposed films were quantified as described previously (Breakova et al., 2009). Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

**Dual perfusion of the rat placenta.** The method of dually perfused rat term placenta was used in our study, as described previously (Staud et al., 2006). In brief, one uterine horn was excised and submerged in heated Ringer’s saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Krebs perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring fetuses by ligatures. The umbilical artery was catheterized by use of a 24-gauge catheter connected to the fetal reservoir and perfused at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner, and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighed glass vial to check for a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments; pH values in the perfusion reservoirs were maintained by controlled oxygenation with carbon dioxide mixture (5% CO2/95% O2) as described previously (Pavek et al., 2001). At the end of experiment, placenta was perfused with radioactive-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences), and its radioactivity was measured to detect tissue-bound MPP+ (Tri-Carb 2900TR; PerkinElmer).

Two types of perfusion experiments were used in this study, i.e., open-circuit and closed-circuit (recirculation) perfusion systems:

**Open-circuit perfusion system.** Open-circuit perfusion system was employed to study maternal-to-fetal and fetal-to-maternal clearances of MPP+ at various concentrations. In this experimental setup, MPP+ was added to either maternal (maternal-to-fetal studies) or fetal (fetal-to-maternal studies) reservoir immediately after successful surgery. After 5 min stabilization period, the sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5 min interval, radioactivity was measured, and transplacental clearance was calculated.

**Closed-circuit (recirculation) perfusion system.** Closed-circuit (recirculation) perfusion system was employed to investigate the potential of Oct3/Mate1 to remove MPP+ from the fetal circulation. In this experimental setup, both maternal and fetal sides of the placenta were infused with equal concentrations of MPP+ and after 5 min stabilization, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were collected every 10 min from the maternal and fetal reservoirs, and [3H]MPP+ concentration was measured. This experimental setup ensures steady MPP+ concentra-tion on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio at equilibrium; any net transfer of the substance implies transport against a concentration gradient and is evidence for active transport.

**Effect of substrate concentration on transplacental clearance.** Open-circuit perfusion system was employed to investigate the effect of various MPP+ concentrations on maternal-to-fetal and fetal-to-maternal clearances. MPP+ with a trace amount of [3H]MPP+ was added to the maternal or fetal reservoir in the following concentrations: 0.001, 0.01, 0.1, 1, 10, 100, or 1000 μM. The inflowing MPP+ concentration was maintained constant for the duration of the experiment; transplacental clearances of MPP+ were calculated as described below.

**Effect of inhibitors on transplacental passage of MPP+.** To study the ef-fect of inhibitors on fetomaternal MPP+ concentration ratio at equilibrium, both maternal and fetal sides of the rat term placenta were perfused with low, non-saturating concentration (0.001 μM) of [3H]MPP+ in closed-circuit perfusion system. Effect of several compounds was investigated; MPP+ (1000 μM), corticosterone (100 μM), metformin (100 μM), or GF20918 (2 μM) was added to both maternal and fetal reservoirs to inhibit the transporters and to demonstrate their effect on fetomaternal equilibrium of MPP+. The inhibitor concentrations were based on previous studies: corticosterone (Wu et al., 1998), GF20918 (Cygalova et al., 2009), metformin (Ito et al., 2011), and metformin (Tsuda et al., 2009).

**Effect of pH on transplacental passage of MPP+.** To study the effect of pH on fetomaternal MPP+ concentration ratio at equilibrium, both maternal and fetal sides of the placenta were perfused with low, non-saturating concentration (0.001 μM) of [3H]MPP+ in closed-circuit perfusion system. pH in the maternal reservoir was adjusted to 6.5, 7.3, or 8.5. In the fetal reservoir, pH 7.3 was used in all experiments.

**Pharmacokinetic analysis of transport activity in the placenta.** Organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of MPP+ in open-circuit perfusion system (Staud et al., 2006). Average data from the intervals of 25 to 35 min of placenta perfusion were used for the following calculations. Maternal-to-fetal transplacental clearance (Clma) was calculated according to Equation 1.

\[
Cl_{ma} = \frac{C_{i} \cdot Q_{i}}{C_{ma} \cdot W_{p}}
\]

(1)

where \(C_{i}\) is MPP+ concentration in the umbilical vein effluent, \(Q_{i}\) is the umbilical flow rate, \(C_{ma}\) is MPP+ concentration in the maternal reservoir, and \(W_{p}\) is the wet weight of the placenta. Fetal-to-maternal transplacental clearance (Clma) was calculated according to Equation 2.

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\[ \text{Cl}_{\text{lm}} = \frac{(C_{fa} - C_{fp})Q_a}{C_{fa}W_p} \]  

(2),

where \(C_{fp}\) is MPP:\(\) concentration in the fetal reservoir entering the perfused placenta via the umbilical artery.

**Statistical analysis.** For each group of placental perfusion experiments, the number of animals was \(n \geq 3\). For the qRT-PCR and Western blot analysis, the number of samples was \(n \geq 4\). Data are presented as means ± SD. Statistical significance was examined by unpaired Student’s t-test or one-way ANOVA followed by Bonferroni test using Graphpad Prism 5.0 software (Graphpad Software, Inc., San Diego, CA); the same software was used for curve fitting. A difference of \(p < 0.05\) was considered statistically significant.

**RESULTS**

**Real-time PCR Analysis**

The mRNA levels of Oct and Mate isoforms in the rat term placenta, maternal kidney, and HRP-1 rat trophoblast cells as evaluated by qRT-PCR are shown in Fig. 1. Of all Oct isoforms tested, only Oct3 mRNA was detected in the placenta in significant amount (345-fold expression compared with that in maternal kidney), whereas mRNA expressions of Oct1 and Oct2 isoforms achieved less than 3 and 1% of those in maternal kidney, respectively (Fig. 1A). Similarly, we observed abundant expression of Mate1 mRNA in the placenta (13 times higher when compared with maternal kidney), whereas Mate2 mRNA expression was approximately 6 times higher compared with kidney (Fig. 1B). In the HRP-1 rat trophoblast cells, only Oct1 mRNA expression was detected (Figs. 1A and B).

**Western Blot Analysis**

Protein quantification of Oct3 and Mate1 was performed in homogenates of the placenta, maternal kidney, and HRP-1 rat trophoblast cells. The relative amount of Oct3 and Mate1 protein was expressed as a percentage of the maternal kidney values. The Oct3 and Mate1 protein expressions in placenta were 1.6- and 1.4-fold higher compared with that in maternal kidney, respectively (Fig. 2). The HRP-1 rat trophoblast cells showed very low protein expression of Oct3 and Mate1 compared with maternal kidney (Fig. 2).
**Immunohistochemical Localization of Oct3 and Mate1 in the Rat Term Placenta**

The antigen retrieval immunohistochemistry at the light microscopy level was carried out for localization of Oct3 and Mate1 in the rat term placenta using polyclonal primary antibody Rabbit anti-rat OCT3 and primary antibody Rabbit anti-rat MATE1, respectively. The positivity of the Oct3 in the rat term placenta is almost exclusively located in the syncytiotrophoblast of the labyrinth area, namely in layers II and III of the trophoblast cells (Fig. 3A). No positivity was detected in fetal capillaries, labyrinth zone, or spongiotrophoblast. These data support the hypothesis that Oct3 is present in the basolateral membrane of the syncytiotrophoblast layer of the rat term placenta. On the other hand, Mate1 positivity was located in syncytiotrophoblast of labyrinth area (predominantly on maternal side); no staining positivity of Mate1 was detected in fetal capillaries, in labyrinth zone, or in spongiotrophoblast (Fig. 3B). However, more precise visualization techniques will be required to specify the exact localization of the transporters in the placental tissue.

**Open-Circuit Perfusion Experiments: Effect of Substrate Inflow Concentrations on Transplacental Clearance in Maternal-to-Fetal and Fetal-to-Maternal Direction**

The maternal or fetal side of the placenta was infused with various concentration of MPP+ (0.001, 0.01, 0.1, 1, 10, 100, or 1000 μM) with trace amount of [3H]MPP+. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in substrate concentration resulted in significant changes in transplacental clearance, confirming involvement of a capacity-
limited transport mechanism (Figs. 4A and B). Less than 1% of the total MPP⁺ dose was detected in placentas after perfusion experiments, suggesting limited tissue binding and negligible effect on clearance calculations.

Comparing fetal-to-maternal and maternal-to-fetal clearances, we observed significant asymmetry in favor of fetal-to-maternal direction (Figs. 5A and B), suggesting an involvement of effective antiport system mediating transplacental permeation of cationic substrates from fetal to maternal circulation. This asymmetry was most pronounced at low MPP⁺ concentration (0.001μM), where fetal-to-maternal clearance was 123 times higher than that in the opposite direction (Fig. 5A). On the other hand, at high MPP⁺ concentration (1000μM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical values, and the asymmetry was almost annulled (Fig. 5B), confirming saturation of the transport proteins and limited role of their transport activities.

FIG. 5. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions at (A) low, nonsaturating and (B) high, saturating MPP⁺ concentrations. MPP⁺ with [3H]MPP⁺ tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by Equations 1 and 2 (see Materials and Methods section). At low substrate concentration (0.001μM), where fetal-to-maternal clearance was 123 times higher than that in the opposite direction (Fig. 5A). On the other hand, at high MPP⁺ concentration (1000μM), fetal-to-maternal and maternal-to-fetal clearances reached almost identical values, and the asymmetry was almost annulled (Fig. 5B), confirming saturation of the transport proteins and limited role of their transport activities.

Closed-Circuit Perfusion Experiments: Effect of Inhibitors on Transplacental Passage of MPP⁺

To investigate the potential of Oct3/Mate1 to remove their substrates from fetal circulation, MPP⁺ was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.001μM in closed-circuit experiment setup. A steady decrease in the MPP⁺ concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion (Fig. 6, inset), confirming the ability of the compound to cross the placenta in fetal-to-maternal direction even against the concentration gradient. This decline was fully blocked by coinfusion of MPP⁺ (1000μM) or partially blocked by corticosterone (100μM), metformin (100μM), or cimetidine (100μM). No changes were observed when GF120918 (2μM), a P-glycoprotein inhibitor, was coinfused (Fig. 6).

Closed-Circuit Perfusion Experiments: Effect of pH on Transplacental Passage of MPP⁺

To investigate the effect of pH on fetal-to-maternal transport of cations, MPP⁺ was added to both maternal and fetal reservoirs at nonsaturating concentration of 0.001μM in closed-circuit experiment setup. pH in the maternal reservoir was set to 6.5, 7.3, or 8.5, whereas pH 7.3 was used in fetal reservoir. At pH 7.3 in both maternal and fetal reservoirs, a steady decrease in the MPP⁺ concentration in the fetal reservoir was observed followed by concentration equilibration after approximately 40 min of perfusion. This decline was notably blocked by adjusting maternal pH to 8.5 resulting in significantly higher ratio between fetal and maternal MPP⁺ concentrations, thus confirming proton-dependent transport of cations across the placenta (Fig. 7).

DISCUSSION

In this study, we describe the expression and localization of Oct3 and Mate1 transport proteins in the rat term placenta and investigate their role in transplacental pharmacokinetics.

Available knowledge on the placental expression of OCT3 suggests that it is expressed in the fetal-facing basolateral membrane of the placenta (Kekuda et al., 1998; Sata et al., 2005), where it can function as an influx transporter of cationic drugs. However, contradictory views on the role of OCT3 in the transplacental pharmacokinetics can be found in the current literature (Ganapathy and Prasad, 2005; Kekuda et al., 1998; Lee et al., 2009). Considering that organic cations cannot cross biological membranes by passive diffusion, it is obvious that after OCT3-mediated influx of a cationic compound into the trophoblast cells, another membrane transporter is responsible for efflux of the molecule from the placenta. As the
transport of organic cations from the trophoblast has not been systematically investigated to date, we searched for a clue in other excretory organs such as the kidney and liver where efflux of organic cations across the apical membrane is thought to occur by a separate transporter-mediated process, either by an ABC drug efflux transporter (e.g., P-glycoprotein) (Bleasby et al., 2000; Martel et al., 1996) or by a cation-H\(^+\) exchange mechanism (e.g., OCTNs or recently discovered MATEs) (Giacomini et al., 2010; Otsuka et al., 2005; Yonezawa and Inui, 2011).

We hypothesized that a similar scenario might occur in the placenta and, therefore, investigated Oct3-mediated influx and P-glycoprotein– and/or Mate1-mediated efflux of MPP\(^+\) at the maternofetal interface.

First, we explored the expression and localization of Oct3 in the rat term placenta. Mate1 is a very recently identified transporter (Otsuka et al., 2005), and only limited information regarding its placental expression is available in the current literature. No Mate1 mRNA was detected in the murine (Aleksunes et al., 2008) and human placenta (Otsuka et al., 2005). On the other hand, Terada et al. (2006) found abundant Mate1 mRNA expression in the rat placenta. Here, we observed prominent placental expression of Mate1 at both mRNA and protein levels, significantly exceeding those of maternal kidney. Similar results were obtained for Oct3 mRNA and protein expression. We also planned to characterize the rat trophoblast HRP-1 cells as an alternative model for in vitro studies of transport of organic cations from the trophoblast.

FIG. 6. Effect of inhibitors on elimination of MPP\(^+\) from the fetal circulation. In the closed-circuit perfusion setup, [\(^3\)H]MPP\(^+\) was simultaneously infused to both the maternal and the fetal sides of the placenta at equal concentrations of 0.001\(\mu\)M, and fetal perfusate was recirculated for 60 min; at the end of the perfusion, fetal and maternal MPP\(^+\) concentrations were compared. Fetal [\(^3\)H]MPP\(^+\) concentrations decreased from 0.001\(\mu\)M down to 0.00015\(\mu\)M and stabilized after 40 min of perfusion (see inset). This decrease was significantly inhibited by cold MPP\(^+\) (1000\(\mu\)M), corticosterone (100\(\mu\)M), metformin (100\(\mu\)M), and cimetidine (100\(\mu\)M). GF120918 (2\(\mu\)M), on the other hand, had no effect on MPP\(^+\) elimination from the fetal reservoir. Data are presented as means \(\pm\) SD of at least three experiments. \(*p < 0.05, **p < 0.01, ***p < 0.001; \) statistically significant difference from MPP\(^+\) 0.001\(\mu\)M (ANOVA followed by Bonferroni test).

FIG. 7. Effect of maternal pH on elimination of MPP\(^+\) from the fetal circulation. In the closed-circuit perfusion setup, [\(^3\)H]MPP\(^+\) was simultaneously infused to both the maternal and the fetal sides of the placenta at equal concentrations of 0.001\(\mu\)M, and the fetal perfusate was recirculated for 60 min. Fetal pH was set to 7.3, and the pH values in the maternal reservoir were set to 6.5, 7.3, or 8.5. At the end of the experiment, fetal and maternal MPP\(^+\) concentrations were compared. It is evident that higher concentration of protons in the maternal circulation results in higher fetal-to-maternal transport of MPP\(^+\), indicating the role of a proton-cation antiporter system on the apical side of the placenta. Data represent means \(\pm\) SD of at least three experiments. \(**p < 0.01, ***p < 0.001 (ANOVA followed by Bonferroni test).\)
placental transport of organic cations; however, negligible expression of Oct3 and Mate1 at both mRNA and protein levels were detected, and this model was, therefore, excluded from the following functional studies. Immunohistochemical visualization revealed preferential localization of Oct3 on the fetal side of the rat placenta, which is a finding consistent with the current literature (Sata et al., 2005). On the other hand, Mate1 appears to be localized preferentially toward the maternal circulation, which is in agreement with its apical localization in the kidney and liver (Otsuka et al., 2005), supporting our hypothesis of its role in trophoblast-to-mother efflux of substrates.

To investigate the function of Oct3 and Mate1 in the rat placenta and to quantify their role in transplacental pharmacokinetics, the in situ method of dually perfused rat term placenta was used in our study as a well-established model to investigate placental physiology (Kertschanska et al., 2000; Stulc et al., 1995), pathophysiology (Jakoubek et al., 2008), and pharmacology (Cygalova et al., 2009; Pavek et al., 2003; Staud et al., 2006). MPP⁺ was chosen as a model toxin and prototypical organic cation substrate. MPP⁺ is the active metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine that has been shown to cause symptoms that mimic Parkinson disease (Wang et al. 2011). It is an ideal candidate model for our studies as it is a well-established substrate of both OCT3 (Sata et al., 2005; Wu et al., 2000) and MATE1 (Terada and Inui, 2008). In addition, it is not subjected to metabolic degradation (Sayre, 1989), and little is known regarding its transplacental passage.

In the initial studies, a range of MPP⁺ concentrations from 0.001 to 1000μM was tested in open-circuit perfusion to evaluate the effect of influx concentration on transplacental clearance. In the case of linear pharmacokinetics, clearance is independent of concentration; however, in both fetal-to-maternal and maternal-to-fetal directions, we observed strong dependence of transplacental clearance on MPP⁺ concentration, which indicates nonlinear pharmacokinetics and involvement of a saturable transport system. When comparing fetal-to-maternal and maternal-to-fetal clearances at low, nonsaturating concentrations, we can quantify a measure of fetal protection by a transport system in the placenta. In the case of MPP⁺, fetal-to-maternal clearance was 123 times faster than maternal-to-fetal one; we believe this huge asymmetry in transplacental clearances is caused by a concerted action of Oct3 and Mate1. In Figure 8, we compare asymmetries in transplacental clearances among several compounds that are substrates of other placental efflux transporters that we investigated previously under identical condition (Cygalova et al., 2009; Staud et al., 2006). This comparison shows that Oct3/Mate1 combo offers surprisingly high level of fetal protection; nevertheless, the role of other cationic transporters (such as Octn1, Octn2, and NET [Ganapathy and Prasad, 2005; Koepsell et al., 2007]) and lipid solubility of the compounds (Cygalova et al., 2009) may affect this clearance ratio comparison.

To investigate the ability of Oct3/Mate1 to remove MPP⁺ already present in the fetal compartment, closed-circuit perfusion was used in which both maternal and fetal sides of the placenta were perfused with nonsaturating concentration of MPP⁺ (0.001μM) and the fetal perfusate was recirculated. In the case of linear pharmacokinetics, both concentrations remain unchanged for the duration of the experiment as...
demonstrated with antipyrine (Cygalova et al., 2009). Here we observed considerable decrease in fetal MPP+ concentrations, demonstrating the ability of MPP+ to cross the placenta from fetus to mother even against its concentration gradient, thus confirming the involvement of an active transport mechanism. Once inside the trophoblast cells, MPP+ can be eliminated into the maternal circulation across the apical membrane either by P-glycoprotein (Bleasby et al., 2000; Martel et al., 1996) or by organic cation-H+ antiporter system (Ganapathy et al., 1988).

In closed-circuit perfusion setup, we first tested possible effect of P-glycoprotein on the transplacental passage of MPP+ by employing GF120918 as an inhibitor. Although in our previous studies GF120918 effectively inhibited P-glycoprotein in the rat placenta (Cygalova et al., 2009; Pavek et al., 2003), here we did not record any change in transplacental passage of MPP+, suggesting P-glycoprotein does not have a substantial role in elimination of the organic cation from the fetus (Fig. 6). We, therefore, further focused on the activity of placental Mate1 and evaluated the effect of proton concentration in the maternal circulation on MPP+ placental transport. Changing pH values from 6.0 to 8.5. Terada et al. (2006) reported Mate1 transport activity to be pH-dependent in vitro in HEK293 cells transiently expressing MATE1. In our in situ study, we employed a similar range of pH on the maternal side of the placenta, showing that the oppositely directed H+-gradient can drive the secretion of organic cations from the placenta to mother. These data indicate that Mate1 on the apical membrane is the collaborating partner of Oct3 in fetal-to-maternal excretion of cations (Fig. 9). We speculate that the H+-gradient in the syncytiotrophoblast is regulated by an ATP-driven H+ pump (Simon et al., 1992) and/or by Na+/H+ exchangers (Sibley et al., 2002) that have been localized in the placenta of several species as important mechanisms for syncytiotrophoblast homeostasis.

Apart from MPP+, a model toxin used in this study, many other molecules have been recognized as substrates/inhibitors of OCT3 and/or MATE1 transporters, including endogenous compounds (e.g., corticosterone, estradiol, progesterone, dopamine, epinephrine, histamine, and serotonin), clinically used drugs (e.g., acyclovir, metformin, procainamide, imipramine, diltiazem, quinine, amantadine, cimetidine, topotecan, and tenofovir), and other toxins and environmental pollutants (e.g., cocaine, paraquat, ethidium, and nicotine) (Nies et al., 2011). It is, therefore, very likely that drug-drug interactions or disruption of the Oct3/Mate1-mediated eliminatory pathway may affect transplacental pharmacokinetics of these substrates and limit the detoxication capacity of the placenta. We addressed this issue in our study by employing various inhibitors of Oct3 and/or Mate1 to investigate their role in the fetal-to-maternal elimination of MPP+. In particular, we used metformin, a frequently prescribed oral antidiabetic and an Oct3 and Mate1 substrate/inhibitor (Tsuda et al., 2009), cimetidine, an H2 antagonist and an inhibitor of Oct3 (Sata et al., 2005) and Mate1 (Ito et al., 2011), and corticosterone as an endogenous compound and inhibitor of Oct3 (Wu et al., 1998). All of these compounds were capable of, at least partially, decreasing the fetal-to-maternal transplacental elimination of MPP+. We, therefore, suggest that the Oct3/Mate1 excretory pathway of the placenta may be compromised by endogenous and exogenous compounds, which may eventually result in unpredictable outcome of medication during pregnancy or even toxicity to the sensitive fetoplacental unit.

In conclusion, we describe the expression and localization of Oct3 on the basolateral, fetus-facing side and of Mate1 on the apical, mother-facing side of the rat placenta. Furthermore, using in situ method of dually perfused rat term placenta, we provide evidence that these two transporters, in a
concerted action, remove their substrates from the fetal circulation and pump them to the maternal one, even against concentration gradient. Based on these results, we propose that Oct3 and MATE1 form an efficient transplacental eliminatory pathway and play an important role in fetal protection and detoxication.

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