Handling of Biliverdin by the Rat Placenta

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Abbreviations used:
Bilirubin (BR); Biliverdin (BV); Biliverdin IXα reductase (BVRα); 5-Carboxy-X-rhodamine triethylammonium salt (ROX); Estradiol 17β-D-Glucuronide, E₂17βG; Heme oxigenase (HO); Organic anion-transporting polypeptide (Oatp); Taurocholic acid (TC).

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ABSTRACT

Fetal liver immaturity is accompanied by active heme catabolism. Thus fetal biliary pigments must be excreted toward the mother by the placenta. To investigate biliverdin handling by the placenta-maternal liver tandem, biliverdin-IXα was administered to 21-day pregnant rats through the jugular vein or the umbilical artery of an “in situ” perfused placenta. Jugular administration resulted in the secretion into maternal bile of both bilirubin and biliverdin (3:1). However, when biliverdin was administered to the placenta, most of it was transformed into bilirubin before being transferred to the maternal blood. Injecting *Xenopus laevis* oocytes with mRNA from rat liver or placenta enhanced their ability to take up biliverdin, which was inhibited by estradiol 17β-D-glucuronide. The expression of three Oatp isoforms in this system revealed that they have different ability to transport biliverdin (Oatp1/1a1>Oatp2/1a4>Oatp4/1b2). The abundance of their mRNA in rat trophoblast was Oatp1/1a1>>Oatp4/1b2>Oatp2/1a4. The expression of biliverdin-IXα reductase in rat placenta was detected by RT-PCR/sequencing and Western blot. The relative abundance of biliverdin-IXα reductase mRNA (determined by real-time quantitative RT-PCR) was fetal liver>placenta>maternal liver. Common bile duct ligation in the last week of pregnancy induced an up-regulation of biliverdin-IXα reductase in maternal liver, but had no effect on fetal liver and placenta. In conclusion, several members of the Oatp family may contribute to the uptake of fetal biliverdin by the rat placenta. Prior to being transferred to the mother, biliverdin is extensively converted into bilirubin by biliverdin-IXα reductase, whose expression is maintained even though bilirubin excretion into maternal bile is impaired.

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INTRODUCTION

The first products of heme catabolism are Fe, CO and the blue-green pigment biliverdin (BV) (32). Although the main product is BV-IXα, small amounts of the other three isomers (BV-IXβ, BV-IXγ and BV-IXδ depending on the protoporphyrin IX bridge cleaved), in particular BV-IXβ, are also produced in humans and other mammals (40). BV-IXα is further biotransformed to bilirubin (BR) by BV-IXα reductase (BVRα), which is expressed in many tissues (21), including the placenta (25). BR is conjugated in the liver with glucuronic acid by BR uridine diphosphate-glucuronosyl transferase-1A1 (3) prior secretion into bile.

Owing to the immaturity of the fetal liver, physiologically relevant hepatobiliary elimination of BR does not occur. Only a small amount of BR-IXβ is secreted into fetal bile, where it is the major heme catabolite (40). Unlike BR-IXα, the IXβ isomer is relatively polar and may not require glucuronidation for its biliary excretion (22). The concentrations of unconjugated BR are higher in fetal than in maternal serum (15,27). Two facts contribute to this difference: a very active heme catabolism, and hence BR production in the fetus, together with very low BR uridine diphosphate-glucuronosyl transferase activity in the fetal liver (14). Thus, during intrauterine life, the placenta is the major route for the excretion of fetal biliary pigments (for a review, see 19).

The physiological advantages of the transformation of BV into BR have only recently been understood. Thus generation of endogenous CO, a potent vasodilator, may play a role in the control of local fetal-placental vasculature tone (26). Moreover, in the presence of reactive oxygen species, BR can be oxidized to BV and then recycled by BVRα back to BR (1). However, beneficial antioxidant properties are limited to low concentrations because BR can also cause irreversible damage or even death when it becomes accumulated and reaches the brain at high levels (9). It has also been suggested that the reduction of BV to BR could have the evolutionary advantage of facilitating the placental excretion of bile pigments by simple diffusion (33). However, in vitro (35) and in vivo (5) studies have suggested that under physiological circumstances the major pathway for BR transfer across the placenta involves carrier-mediated transport across both poles of the plasma membrane of the
human trophoblast (35). Moreover, BR does not undergo any major biotransformation during its residence in the rat placenta (5).

Using “in situ” perfused rat placenta we determined that antipyrine, but not BR, is readily able to cross the rat placenta in the mother-to-fetus direction (5). Vectorial properties for transplacental BR transfer are consistent with the moderate increase in serum BR concentrations observed in the fetuses of pregnant rats with hyperbilirubinemia due to common bile duct ligation (17).

How fetal biliary pigments are taken up by the placenta is not well understood. Proteins of the organic anion transporting polypeptide (OATPs) family (10), in particular human OATP-C/1B1 and OATP8/1B3, have been reported to confer the ability to take up unconjugated BR when expressed in *Xenopus laevis* oocytes (6). However, the mRNA of OATP-C/1B1 is almost absent in isolated human trophoblast cells, whereas OATP-8/1B3 is clearly expressed in this epithelium (6).

Regarding BV, although this biliary pigment is able to inhibit BR transfer in rat placenta when co-administered through the umbilical artery of “in situ” perfused rat placentas (5), it has been shown that BV itself is poorly transferred, without prior reduction to BR, across the guinea pig placenta (25).

The aim of the present study was to gain information on the handling of fetal BV by elucidating whether several Oatp isoforms involved in the liver uptake of organic anions may play a role in the carrier-mediated uptake of fetal BV by the rat placenta and liver, and by measuring the levels of expression of BVRα in the three elements of the fetal-liver-placenta-maternal liver trio involved in the handling of fetal BV in native form or biotransformed into BR.
MATERIAL AND METHODS

Chemicals and animals

Biliverdin (BV) IXα hydrochloride (more than 95% pure, as confirmed by HPLC, see below) was obtained from Frontier Scientific (Logan, UT, USA), which is considered a reliable source of this compound, with sufficient purity for most biomedical investigations (23). 5-carboxy-X-rhodamine triethylammonium salt (ROX) was from Molecular Probes (Leiden, The Netherlands). Estradiol 17-β-D-glucuronide (E₂17βG) and unconjugated bilirubin (BR) was from Sigma-Aldrich (Madrid, Spain). [³H]-Taurocholic acid (TC, 5 Ci/mmol) was obtained from PerkinElmer Life Sciences (Pacisa&Giralt, Madrid, Spain).

Pregnant rats on day 21 of gestation (~370 g) from the University of Salamanca Animal House (Salamanca, Spain) and mature female frogs (Xenopus laevis), purchased from Regine Olig (Hamburg, Germany), were used. The animals received humane care as outlined in the National Institutes of Health guidelines for the care and use of laboratory animals (Institutional Animal Care and Use Committee Guidebook, 2nd ed, 2002). Experimental protocols were approved by the Ethical Committee for Laboratory Animals of the University of Salamanca. Harvesting and preparation of oocytes from Xenopus laevis were performed as previously described (6).

Excretion of biliverdin by the rat placenta-maternal liver tandem

To carry out these experiments, the rats were anesthetized with sodium pentobarbital (Nembutal N.R.; Abbot, Madrid, Spain) by intraperitoneal injection (50 mg/kg body weight). When needed, polyethylene catheters were inserted into the maternal left jugular vein (for infusion) and the left carotid artery (for sampling). Bile flowing through a polyethylene catheter implanted into the common bile duct was collected in pre-weighed vials placed on ice and protected from light to prevent the degradation of biliary pigments. Immediately after surgical preparation, the animals were left for an equilibration period of 20 min before starting sample collection.

In situ single-pass perfusion of rat placenta was carried out as previously described in detail (4). Briefly, one of the placentas was exposed and perfused through the
umbilical artery using a heparinized saline solution (0.05% heparin, 150 mM NaCl) at 37°C at a constant inflow of 500 µl/min with a peristaltic pump. An incision in the umbilical vein was performed to permit free outflow. Perfusion pressure was measured as an indicator of placental perfusion resistance and was considered to be appropriate when it remained relatively constant and lower than 20 cm H₂O throughout the experimental period.

To compare BV handling by the placenta-maternal liver tandem versus the maternal liver alone or the placenta alone, four separate set of experiments were carried out, in which BV in 150 mM NaCl at pH 7.8 was administered: i) 0.1 µmol BV was administered to the mother as a bolus (50 µl/min over 5 min) through the jugular vein. ii) 1.0 µmol BV was single-pass-perfused (500 µl/min over 5 min) through the umbilical artery, followed by continuous perfusion of this placenta with heparinized saline solution throughout the rest of the experimental period. iii) To prevent the biotransformation of the BV by the maternal liver and kidneys once it had been transferred to the mother through the placenta, immediately before administering 1.0 µmol BV through the umbilical artery the maternal liver and kidneys were rapidly isolated from the maternal circulation using previously emplaced ligatures. The viability of these preparations during the experimental period was checked by measuring steady-state concentrations of urea in serum (7) and glucose in blood (Accutrend Sensor kit, Roche Diagnostics, Barcelona, Spain). iv) To investigate the profile of spontaneous accumulation of biliary pigments in serum in this model, similar experiments were carried out except that vascular isolation of the liver and kidneys was not followed by administration of BV through the umbilical artery. Samples of maternal serum and/or bile were collected periodically to carry out analytical measurements.

To investigate the effect of blocking the maternal biliary excretion of biliary pigments on the expression of BVRα in the fetal liver - placenta - maternal liver trio, complete obstruction of the maternal common bile duct was imposed on day 14 of gestation and samples of the above tissues were collected on day 21, as described elsewhere (34).
Measurement of gene expression levels

To determine mRNA levels by real-time RT-PCR, tissue samples were immediately immersed in the RNA stabilization reagent RNAlater (Qiagen, Izasa, Barcelona, Spain) and stored at 4ºC until total RNA was isolated using RNAeasy spin columns from Qiagen (Izasa, Barcelona, Spain). After treatment with RNase-free DNase I (Roche, Barcelona, Spain), RNA was quantified fluorimetrically with the RiboGreen RNA-Quantitation kit (Molecular Probes). cDNA was synthesized from 2 µg of total RNA using random hexamers and avian myeloblastosis virus RT (Enhanced Avian RT-PCR kit; Sigma-Genosys, Cambridge, UK), according to the instructions supplied by the vendor.

Real-time quantitative PCR was then performed using AmpliTaq Gold polymerase (Applied Biosystems, Madrid, Spain) in an ABI Prism 5700 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as follows: a single cycle at 95ºC for 10 min followed by 40 cycles at 95ºC for 15 s and at 60ºC for 60 s. Detection of amplification products was carried out using SYBR Green I. Non-specific products of PCR, as detected by 2.5% agarose gel electrophoresis or melting temperature curves, were not found in any case. The results of mRNA abundance for each target gene in each sample were normalized on the basis of its 18S rRNA content, which was measured with the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems).

The primer oligonucleotides sequences and conditions for measuring the absolute abundances of Oatp1/1a1 (gene symbol Slc21a1/Slco1a1), Oatp2/1a4 (Slc21a5/Sclo1a4) and Oatp4/1b2 (Slc21a10/Sclo1b2) and rat placental lactogen type II, a specific marker for trophoblastic cells, have been described elsewhere (34). The primer oligonucleotide sequences for BVRα were: forward primer (position 138-160, GeneBank Accession Number BC078766) 5’-TTG GAG TGG TAG TGG TTG GTG TT-3’ and reverse primer (position 288-266) 5’-TCC AAA GAA ATC TGC CGT ACT TC-3’. All primers were designed with the assistance of Primer Express software (Applied Biosystems) and their specificity was checked using BLAST. They were obtained from Sigma-Genosys. Sequencing of the amplified fragments of rat BVRα cDNA in maternal liver, fetal liver and placenta resulted in similarity of 100% with previously described sequence (GeneBank Accession Number BC078766).
Western blot analyses on liver and placenta homogenates were carried out in 7.5% SDS-polyacrylamide gels using equal protein loading (150 µg per lane) as confirmed by staining of membranes after transfer with Ponceau Red-S (Sigma-Aldrich). Rabbit polyclonal antibody OSA-400 against BVRa was from Stressgen Bioreagents (bioNova, Madrid). Donkey anti-rabbit IgG horseradish peroxidase-conjugated antibody NA934 and enhanced chemiluminiscence reagents were from Amersham Pharmacia Biotech (Freiburg, Germany).

Uptake studies using *Xenopus laevis* oocytes
Using the PolyAT tract mRNA Isolation System purchased from Promega (Innogenetic, Barcelona, Spain), poly(A) mRNA to be injected into the oocytes was isolated from total RNA, obtained from rat liver or placenta as described above. The synthesis of cRNA of rat Oatp1/1a1, Oatp2/1a4 and Oatp4/1b2 for injection into oocytes was performed using recombinant plasmids containing the open reading frame (ORF) DNA of these transporters, all kindly supplied by Dr. Peter J. Meier, Dr. Bruno Stieger and Dr. Bruno Hagenbuch (Zurich University Hospital, Switzerland). These plasmids were isolated from *E. coli* using the Qiagen Plasmid Mini Kit (Izasa) and further linearized with restriction enzymes. Capped and poly(A) tailed cRNAs were synthesized using the T7 mMessage mMachine Ultra kit (Ambion; bioNova Cientifica, Madrid, Spain).

*Xenopus laevis* oocytes were microinjected with TE buffer (1 mM EDTA, 10 mM Tris, pH 8.0) either alone, for determining non-specific uptake/binding of the substrates, or containing the following amounts of RNA: 25 ng mRNA from rat liver or placenta or 7 ng cRNA of each Oatp. Oocytes were used 2 days after RNA injection, when - on the basis of preliminary experiments on the time-course of the functional expression for these carriers - the uptake rate was highest (data not shown).

Uptake studies were carried out using groups of 12 oocytes per data point. The data represent the results of at least three separate sets of RNA injections. Oocytes were washed with substrate-free uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes/Tris, pH 7.0) and incubated with 100 µl of
uptake medium containing 50 µM [³H]-TC or 100 µM BV at 25°C for 1 h. Uptake was stopped by the addition of 4 ml ice-cold uptake medium. The oocytes were washed three more times before being collected and were placed in vials individually (to measure radioactive substrate by liquid scintillation) or in groups of two oocytes (to increase the absorbance signal in HPLC analysis of BV uptake).

**Analytical and statistical methods**

Bile flow was determined gravimetrically. BR concentrations in bile were determined by a modification of the diazotized sulfanilic acid coupling method (18) and BV by HPLC as described below. To determine BV and BR in serum, both pigments were extracted (2), and subsequently dissolved in methanol. The mixture was treated to transform BR glucuronides into the corresponding methyl esters as described elsewhere (5), evaporated under nitrogen and dissolved in 200 µl lysis/extraction solution (methanol containing 50 pmol ROX as internal standard for HPLC) to be analyzed by HPLC using 1 ml/min flow rate in a solvent gradient system. The initial mobile phase composition was 100% phosphate buffer (2.5 mM, pH 7.2). After 1 min, the solvent was progressively changed to 50% methanol and 50% phosphate over 2 min. This elution system was maintained for 1 min before starting a 5-min linear change to 100% methanol, which was maintained until the end of the runtime (20 min). Measurement of absorbances at 378, 450 or 567 nm wavelength for, BV, BR or ROX, respectively, were used for peak detection.

To measure BV in oocytes, these were placed in vials (in groups of two oocytes) and treated with 150 µl lysis/extraction solution and sonicated for 5 min in an ice-chilled bath. Then, oocyte lysates were centrifuged at 20,000 xg for 5 min and supernatants were used to measured BV by HPLC.

Results are expressed as means±SD. Regression lines were calculated by the least-squares method. To calculate the statistical significance of the differences between groups, the paired t-test or the Bonferroni method for multiple-range testing were used, as appropriate.
RESULTS

To evaluate the overall handling of BV by the placenta-maternal liver tandem 1.0 µmol BV was administered through the umbilical artery of “in situ” perfused rat placentas in a single-pass perfusion system (Figure 1). Only 1.6% of the pigment, almost all of it (99%) biotransformed into BR, was recovered in maternal bile over the following 90 min (15.5±3.2 nmol BR plus 0.13±0.05 nmol BV). Moreover, in a different set of experiments, 1.0 µmol BV was administered through the umbilical artery of “in situ” perfused rat placentas just after vascular isolation of the maternal liver and kidneys (Figure 2). The result was that a peak in serum BV concentrations appeared before the general deterioration, which occurred after 20 min, as monitored by increasing levels of urea and decreasing glucose concentrations in maternal serum. A transient peak of BV concentrations was followed by a delayed (5-10 min), and progressive, increase in unconjugated BR, but not in conjugated BR (data not shown), concentrations in maternal serum (Figure 2). The magnitude of the increase in serum concentrations for BV and BR are consistent with the amount of BR secreted into bile when BV was administered to animals with intact liver and kidney circulation. No detectable spontaneous accumulation of any of these pigments was observed in animals up to 20 min after vascular isolation of the liver and kidneys when BV was not administered through the umbilical artery (BV was not detected and BR concentrations remained below 0.3 µM) (Figure 2).

To measure the proportion of BV converted into BR by the maternal liver after crossing the placenta, a dose of 10% of that administered through the umbilical artery, i.e., 0.1 µmol, was administered directly into the maternal circulation by injection through the jugular vein (Figure 3). Approximately 80% of this amount was eliminated in bile over the following 90 min as a mixture of BR and BV. Most of the secreted pigments (80%, 61.7±3.6 nmol) were found to be BV biotransformed into BR, but a smaller although considerable amount (20%, 15.7±4.0 nmol) was secreted in bile as BV.

To study the ability of several Oatp isoforms to transport BV, these transporters were expressed in Xenopus laevis oocytes and BV uptake was measured in a HPLC system. ROX was used as internal standard, because BV and ROX showed similar
(>98%) recoveries from oocytes undergoing the lysis/extraction protocol (data not shown), similar retention time in HPLC, and low interferences between them in the recording of absorbance chromatograms (Figure 4A). Using this method, the presence of endogenous BV in *Xenopus laevis* oocytes was detected. This is not surprising because this pigment has been found previously in eggs from very different species (22). The technique permitted linear detection of BV over a large concentration range (Figure 4B) and a clear discrimination between non-specific uptake in non-injected oocytes (Figure 4C) and transport-mediated uptake. Indeed, injection of oocytes with mRNA from rat placenta resulted in an enhanced uptake of BV (Figure 4D). When carrier-mediated uptake (uptake in injected oocytes minus that in non-injected oocytes) was calculated, the ability of three members of the OATP family, involved in the uptake of organic anions by the rat liver to induce sodium-independent uptake of TC (Oatp4/1b2 > Oatp1/1a1 > Oatp2/1a4) was observed (Figure 5). This was more marked for BV (Oatp1/1a1 > Oatp2/1a4 > Oatp4/1b2) (Figure 5). A similar ability to enhance overall uptake of TC was induced by injection of mRNA from either rat placenta or maternal liver, and both were similarly inhibited by E\(_2\)17βG (Figure 6), a substrate shared by several OATP isoforms (10). Moreover, mRNA from placenta induced a significantly higher BV uptake than that from maternal liver (Figure 6). E\(_2\)17βG also induced a dramatic inhibition of BV uptake in both cases (Figure 6). It should be noted that in experiments carried out with oocytes injected with total mRNA overall uptake was the result of uptake/efflux balance, since export pumps were probably also expressed.

To investigate the relevance of Oatps as transporters responsible for BV uptake by the rat placenta their expression levels in this organ were determined by measurement of their absolute abundance of mRNA (Figure 7A). In all cases, the expression levels were markedly lower in placenta than in maternal liver. Moreover, the most abundant isoform in the placenta was Oatp1/1a1. When these values were corrected by the amount of trophoblast, using the rat placental lactogen type II mRNA as a marker, the abundance of mRNA for Oatp1/1a1 was 7- to 8-fold higher than that for Oatp2/1a4 and Oatp4/1b2 (Figure 7B).

To study the potential contribution of fetal and maternal livers and the placenta to the biotransformation of fetal BV into BR, the relative abundance of BVR\(\alpha\) in these
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organs was determined by real time quantitative RT-PCR, after confirming that no non-specific products were generated during amplification, as revealed by the appearance of a single peak in the DNA melting curves and 2.5% agarose gel electrophoresis of amplified products (Figure 8A).

To compare the relative expression of this enzyme in different tissues, the abundance of its mRNA and protein were considered per gram of tissue (Figure 8B) and by western blot per fixed amount of total protein (Figure 8B, inset), respectively. The highest relative expression was found in fetal liver, followed by the placenta and the maternal liver. To evaluate the total relevance of each organ in the overall activity of this enzyme in the whole animal, the relative abundance of mRNA was corrected by the weight of each organ (Figure 8C). The highest total amount of mRNA was found in maternal liver, followed by the fetal liver and the placenta.

Cholestasis imposed in pregnant rats during the last week of pregnancy induced a marked increase in maternal serum total BR concentrations from 0.14±0.03 mg/dl in controls (n=6) to 3.02±0.51 mg/dl in the group with cholestasis (n=6; p<0.05), together with an up-regulation of BVRα in maternal liver (Figures 8B and 8C). However, the repercussion on fetal serum BR concentrations was less marked (from 0.31±0.06 in controls (n=12) to 0.58±0.08 mg/dl in the group with cholestasis (n=12; p<0.05), and was not accompanied by any effect on the expression of BVRα in fetal liver (Figures 8B and 8C). The expression of BVRα in the placenta, which is exposed to blood from both compartments, was not effected by maternal cholestasis either (Figures 8B and 8C).
DISCUSSION

Many studies have been conducted recently to shed light on the role of plasma membrane transporters in the rat and human placental handling of endogenous substances and xenobiotics (16,20,29). The present study provides direct evidence that several of the Oatps expressed in rat placenta and maternal liver are able to take up BV from the fetal and maternal circulations. Among them, Oatp1/1a1 and Oatp4/1b2 are abundantly expressed in the maternal liver, and together with the contribution to a lesser extent of Oatp2/1a4 may play an important role in BV uptake from the maternal blood. As regards the placenta, Oatp1/1a1, owing to its ability to transport BV and its high expression as compared to that of Oatp2/1a4 and Oatp4/1b2, is probably the most important of these three transporters contributing to BV uptake by this organ from fetal blood. Nevertheless, other transporters may also participate in this function.

The observation that when mRNA from liver or placenta was injected in *Xenopus laevis* oocytes the uptake by these cells was higher for BV than for TC, is likely due to the fact that Na\(^+\)-independent uptake, which was the type measured in the present study, could be more important for BV than for TC. It should be noted that at the basolateral surface of rat hepatocytes, TC uptake is mainly governed by the very efficient Na\(^+\)-taurocholate cotransporting polypeptide (Ntcp, gene symbol *Slc10a1*) (11). Moreover, the expression of export pumps may differently reduce the net amount of TC and BV found in the cells after 60 min incubation.

In “in vivo” experiments, only a minor part of the amount of BV that was taken up by the rat placenta was rapidly released toward the mother, followed by a delayed release of BR. This suggests that the transport of BV from the trophoblast toward the mother is very poor and/or that placental biotransformation of BV into BR is very efficient. Indeed, our results confirm the expression in rat placenta of BVR\(\alpha\), whose enzymatic activity has previously been reported (25). Moreover, expression levels of this enzyme in the placenta, which were higher than that in maternal liver, are consistent with the observed extensive biotransformation of BV into BR during placental transit. The fact that the expression of BVR\(\alpha\) in fetal liver was even higher than in placenta, suggests that an important part of endogenous BV produced by the
fetus could be transformed into BR by the fetal liver prior to being taken up by the placenta.

Although several human OATPs, namely OATP-C/1B1 and OATP8/1B3, are able to transport BR (6,8) and could play a role in BR uptake by the placenta (6), little is known about the fate of this pigment, or that of BV, during the rest of trans-placental transit (19).

Inside the trophoblast, BR is probably partly bound to lipids and proteins such as glutathione S-transferase (22). Functional studies have suggested that from there BR would be exported across the apical pole of the trophoblast via an ATP-dependent mechanism (35). Whether one or several isoforms of multidrug resistance associated proteins (MRPs), such as MRP1 and MRP2, which are expressed in human (38) and rat placenta (34,37), are involved in this process is not known. Canalicular MRP2 plays a key role in the secretion into bile of mono- and di-glucuronides of BR (12). However, owing to the low UDP-glucuronosyl transferase activity of the fetal liver and the absence of placental biotransformation of unconjugated BR during transplacental transfer when this compound is administered through the umbilical artery of “in situ” perfused rat placentas (5), MRP2 is not expected to play an important role in BR transfer across the placenta. Moreover, the excretion of dicarboxylate organic anions related to BV into rat bile is also mediated by MRP2-independent mechanisms (24), suggesting that this export pump is not responsible for the poor release of BV from the trophoblast into the maternal blood across the apical pole of this epithelium.

Like MRP2, MRP1 is also able to perform ATP-dependent transport of bilirubin glucuronides (12). Although indirect evidence has suggested that MRP1 may also be involved in the efflux of unconjugated BR from human choriocarcinoma BeWo cells (28), its role in BR and BV transport across the placenta remains to be elucidated.

An interesting observation of the present study was that the impairment in maternal bile secretion induced an up-regulation of BVRA in the maternal liver. This probably enhances the ability of this organ to regenerate BR from BV as part of the redox cycle, BR being an important defense mechanism against oxidative stress (1), such as that caused by maternal cholestasis (30). However, probably due to the lower
exposure of fetal liver to compounds accumulated in maternal blood (17), the expression of BVRα in fetal liver was not affected by maternal cholestasis. The absence of changes in BVRα expression in paired placentas is consistent with the concept that hepatobiliary-like excretory mechanism of the placenta are for the most part unidirectional (5), and therefore constitute an efficient barrier that excludes, at least in part, cholephilic compounds from the trophoblast.

Since we have previously reported that several Oatps (Oatp1/1a1, Oatp2/1a4 and Oatp4/1b2) and Mrps (Mrp1, Mrp2 and Mrp3) are indeed up-regulated in rat placenta in response to maternal cholestasis (34), the fact that BVRα expression in maternal liver, but not in placenta, was sensitive to maternal cholestasis suggests that the placenta would be equipped with different mechanisms for regulating gene expression in response to accumulation in the maternal compartment of cholephilic organic anions. Indeed, although several endogenous as well as exogenous substances may activate the nuclear receptors - the pregnane X receptor (PXR) and the constitutive adrostane receptor (CAR) - resulting in changes in the expression of genes such as Slco1a4, Abcc2 (for Mrp2) and Abcc3 (for Mrp3) in the rat liver (13,36), these receptors are poorly expressed in rat placenta (37). Nevertheless, the expression of several Oatps and Mrps can be induced in rat placenta by maternal cholestasis (34), which in addition to these events also causes enhanced oxidative stress and apoptosis (31). This results in an overall impairment of the ability of this organ to transfer cholephilic organic anions, such as bile acids, from the fetus to the mother (34).

In conclusion, our results suggest that among transporters involved in fetal BV uptake by the rat placenta, several Oatps, in particular Oatp1/1a1, may be involved. Once in the placenta, and prior to transfer to the mother, BV is extensively converted into BR by BVRα, which is highly expressed in this organ. The small amount of BV that reaches the maternal blood is efficiently taken up, probably in part by Oatp1/1a1, Oatp2/1a4 and Oatp4/1b2, and biotransformed into BR, which joins fetal BR transferred as such by the placenta, to be eliminated mainly through secretion into bile by the maternal liver.
REFERENCES


FIGURE LEGENDS

Figure 1. Time course of the increase in biliverdin (BV, closed circles) and bilirubin (BR, open circles) bile output after the administration of 1 µmol BV over 5 min through the umbilical artery of the in situ perfused rat placenta-maternal liver. Values are means±SD from 5 experiments. The inset depicts the schematic representation of the experimental model.

Figure 2. (A) Changes in serum glucose (solid line) and urea (dashed line) concentrations following rapid isolation from the circulation of the liver and kidneys of pregnant rats at min 0 using ligatures previously emplaced around the portal vein, the hepatic artery, and the renal arteries and veins. (B) Time course of biliverdin (BV, closed circles) and bilirubin (BR, open circles) concentrations in maternal serum samples collected for 0 to 20 min. Immediately after vascular isolation of maternal liver and kidneys, 1 µmol BV was (circles, n=5) or was not (squares, n=4) administered over 5 min through the umbilical artery of the in situ perfused rat placenta on day 21 of pregnancy. Values are means±SD.

Figure 3. Time course of the increase in biliverdin (BV, closed circles) and bilirubin (BR, open circles) bile output after the administration of 0.1 µmol BV as a 5-min bolus through the jugular vein of 21-day pregnant rats. Values are mean±SD from 5 experiments. The inset depicts the schematic representation of the experimental model.

Figure 4. Validation of the method to measure biliverdin (BV) uptake by *Xenopus laevis* oocytes using HPLC. (A). Representative HPLC chromatogram obtained by continuous recording of absorbance at wavelengths of 378 nm and 567 nm after injection into the chromatograph of 100-µl samples of 50 pmol 5-carboxy-rhodamine (ROX) used as internal standard (grey line) and 100 pmol BV-IXα (solid line). (B). Linearity of the signal (peak height) versus the amount of BV dissolved in 100 µl methanol and subsequently injected into the chromatograph with (closed circles) or without (open circles) undergoing procedure of mixing-lysis-extraction with two oocytes. Values are means±SD from three determinations per data point. (C and D) Representative chromatograms from BV measurements in the supernatant of
suspensions prepared by an incubation-lysis-extraction procedure carried out with two oocytes that had been injected with TE buffer (C) or mRNA of rat placenta (D) two days before carrying out incubation with 100 µM BV-IXα at 25°C for 60 min.

**Figure 5.** Sodium-independent uptake of [³H]-taurocholic acid ([³H]-TC) and biliverdin-IXα (BV) by *Xenopus laevis* oocytes injected with 7 ng cRNA of Oatp1/1a1, Oatp2/1a4 or Oatp4/1b2. Oocytes were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes/Tris, pH 7.0) containing 50 µM [³H]-TC or 100 µM BV-IXα at 25°C for 60 min. BV uptake was measured by HPLC according to an incubation-lysis-extraction protocol carried out with two oocytes in each measurement. Values are means±SD from at least 15 determinations and represent net uptake, calculated by subtracting the uptake by oocytes injected with TE buffer without cRNA. *, p<0.05, on comparing by the Bonferroni method of multiple range testing with the uptake of the same substrate by oocytes injected with Oatp1/1a1 cRNA.

**Figure 6.** Sodium-independent uptake of [³H]-taurocholic acid ([³H]-TC) and biliverdin-IXα (BV) by *Xenopus laevis* oocytes injected with 25 ng mRNA from rat placenta or liver (B, D). Oocytes were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes/Tris, pH 7.0) containing 50 µM [³H]-TC or 100 µM BV-IXα in the presence or absence of 100 µM estradiol 17β-D-Glucuronide (E₂17βG) at 25°C for 60 min. BV uptake was measured by HPLC according to an incubation-lysis-extraction protocol carried out with two oocytes in each measurement. Values are means±SD from at least 15 determinations and represent net uptake, calculated by subtracting the uptake by oocytes injected with TE buffer without mRNA. *, p<0.05, on comparing uptake in the absence or the presence of E₂17βG by the paired t-test.

**Figure 7.** Absolute abundance of mRNA for Oatp1/1a1, Oatp2/1a4 and Oatp4/1b2 in liver, placenta (A) and trophoblast (B) in 21-day pregnant rats. Values are means±SD from determinations carried out by real-time quantitative RT-PCR using total RNA obtained from these organs. At least 10 different preparations for each tissue were obtained and RT-PCR was performed in triplicate for each sample. Levels of mRNA in each sample were normalized on the basis of the content in 18S
rRNA. Values for trophoblast tissue were calculated by correcting the abundance of mRNA for each carrier by that for rat placental lactogen type II (rPL-II), used as a specific trophoblast marker. *, $p<0.05$, on comparing different carriers in the same tissue by the Bonferroni method of multiple range testing. †, $p<0.05$, on comparing liver and placenta by the paired $t$-test.

**Figure 8.** (A). Validation of conditions to measure biliverdin IX$\alpha$ reductase (BVR$\alpha$) mRNA levels by SYBR Green I-based real-time quantitative RT-PCR. Representative plot of the first negative derivative curves calculated from plots of the SYBR Green I-DNA fluorescence of the amplified PCR products versus changes in temperature ($-dF/dT$). The resulting peak corresponds to the melting temperature of an amplified specific BVR$\alpha$ fragment. The inset shows the amplified PCR product visualized on 2.5% agarose gel after electrophoresis. Lane 1: standard DNA ladder; PCR was carried out without DNA template (lane 2) or with cDNA obtained by RT of rat kidney RNA (calibrator) as template (lane 3). (B) Relative abundance of BVR$\alpha$ mRNA in different organs determined by SYBR Green I-based real-time quantitative RT-PCR. Values represent means±SD from the percentage of expression per gram of tissue. Abundance of BVR$\alpha$ mRNA was compared with the calibrator used in all reactions. Expression levels were normalized on the basis of the content of 18S rRNA measured in the same sample. The inset shows representative Western-blot analysis of liver and placenta homogenates from the same experimental groups. (C) Values of BVR$\alpha$ mRNA abundance as calculated per whole organ. In six pregnant rats (cholestasis group) the common bile duct was ligated on day 14 of pregnancy. Six (maternal liver) or twelve (placenta and fetal liver) different preparations for each tissue in each group were obtained on day 21 of pregnancy and RT-PCR was performed in triplicate for each sample. *, $p<0.05$ on comparing control with maternal cholestasis; †, $p<0.05$ on comparing fetal liver and placenta with maternal liver; ‡, $p<0.05$ on comparing placenta with fetal liver. Comparisons were carried out by the Bonferroni method for multiple-range testing.