Role of organic anion-transporting polypeptides, OATP-A, OATP-C and OATP-8, in the human placenta–maternal liver tandem excretory pathway for foetal bilirubin

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Recent functional studies have suggested that, in addition to simple diffusion, carrier-mediated transport may play an important role in foetal unconjugated bilirubin (UCB) uptake by the placenta. We have investigated the role of organic anion-transporting polypeptides (OATPs) in UCB transport by the placenta–maternal liver tandem. RNA was obtained from human liver (hL), human placenta (hPl) at term, and purified (> 80%) cytochrome-7-positive mononucleated human trophoblast cells (hTCs). By analytical reverse transcription (RT)-PCR, agarose gel electrophoresis separation and sequencing, the mRNA of OATP-A (*SLC21A3*) and OATP-8 (*SLC21A8*) was identified in hL, hPl and hTCs, whereas that of OATP-C (*SLC21A6*) was detectable only in hL. Real-time quantitative RT-PCR revealed that in hL the abundance of mRNA was OATP-8 > OATP-C >> OATP-A, whereas in hPl and hTCs this was OATP-8 >> OATP-A >> OATP-C. Expression levels for these OATPs were hL >> hTCs > hPl. Injection of mRNA of OATP-A, OATP-C or OATP-8 or RNA from

hL, hPl or hTCs into *Xenopus laevis* oocytes conferred on them the ability to take up [³H]17 β -D-glucuronosyl oestradiol ([³H]E₂17 β G) and [³H]UCB, although in the case of OATP-A mRNA, the induced uptake of [³H]UCB was very low. *Cis*-inhibition of [³H]E₂17 β G and [³H]UCB uptake by both unlabelled E₂17 β G and UCB was found in all cases. The affinity and efficiency of [³H]UCB transport was OATP-C > OATP-8. Kinetic parameters for [³H]UCB uptake induced by RNA from hTCs resembled most closely those of OATP-8. In conclusion, our results suggest that OATP-8 may play a major role in the carrier-mediated uptake of foetal UCB by the placental trophoblast, whereas both OATP-8 and OATP-C may substantially contribute to UCB uptake by adult hepatocytes.

Key words: bilirubin, liver, placenta, pregnancy, transport, trophoblast.

INTRODUCTION

In most mammals, bilirubin is produced from biliverdin as the end product of haem catabolism. Unconjugated bilirubin (UCB) is bound to albumin in the systemic circulation. In the adult, UCB is taken up from blood by the liver. In this organ, UCB is biotransformed to more water-soluble glucuronic-acid-conjugated derivatives, and is subsequently actively secreted into the bile by an ATP-dependent mechanism mediated by multidrug resistance-associated protein 2 (MRP2) [1].

During intrauterine life, both the very high foetal production of bilirubin, together with the very low expression of uridine diphosphate glucuronyltransferase 1A1 in the foetal liver [2], probably contribute to the fact that UCB concentrations are higher in foetal than in maternal serum [3,4]. Although UCB could play a physiological role in the protection against oxidative stress [5], this pigment can also cause irreversible damage or even death when it becomes accumulated and reaches the brain at toxic levels [6,7], the effects being manifested in the kernicterus syndrome [8]. Owing to the immaturity of foetal hepatobiliary function [9], elimination across the placenta is the major route for the excretion of the majority of foetal UCB [10].

Owing to the lipophilicity of UCB and the existence of a bilirubin concentration gradient across the placenta in the foetus-to-mother direction [3,4], the mechanism responsible for UCB elimination from the foetal blood has been traditionally

accepted as being one of simple diffusion across the placenta [11]. However, using plasma membrane vesicles from human placental trophoblast [12] and the *in vivo* experimental model of *in situ* perfused rat placenta at term [13], functional evidence for an important role of carrier-mediated transport of UCB across the placenta has been found.

The first step in the transplacental transfer of foetal UCB is the uptake from the foetal blood by the trophoblast. Results from functional studies [12] have suggested that one or several members of the human organic anion-transporting polypeptide (OATP, *SLC21A*) family (symbols from the Human Gene Nomenclature Committee Data Base are shown in *italics*) may be candidate carriers for the transport of UCB across this pole of the trophoblast plasma membrane. This resembles what happens at the basolateral plasma membrane of hepatocytes. It has been suggested that the uptake of UCB by liver cells occurs in part by simple diffusion [14] and in part by high-affinity carrier-mediated processes [15], which may be accounted for by OATP-C (*SLC21A6*) [16]. However, this is a matter of controversy, because contradictory results have been obtained by separate groups using different expression systems [16,17]. The role of OATP-8 (*SLC21A8*) in UCB uptake by the liver is not clear. The available studies indicate that when expressed in HEK293 cells, this carrier is able to transport at least monoglucuronosyl bilirubin [18].

The aim of the present work was to gain information on the molecular bases of carrier-mediated transport of foetal UCB by

Abbreviations used: Bf, concentration of unbound [³H]UCB; C_t, threshold cycle; E₂17 β G, 17 β -D-glucuronosyl oestradiol; HSA, fatty acid-free human serum albumin; hL, human liver; hPl, human placenta; hTCs, human trophoblast cells; K_d, binding affinity constant; OATP, multispecific organic anion-transporting polypeptides; RT, reverse transcription; TE buffer, 1 mM EDTA/10 mM Tris, pH 8.0; UCB, unconjugated bilirubin.

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the placenta–maternal liver tandem, by investigating the potential involvement of three members of the SLC21A family of carriers for cholephilic organic anions, i.e. OATP-A (*SLC21A3*), OATP-C and OATP-8 in the uptake of UCB by the trophoblast from the foetal blood and by the maternal hepatocytes from maternal blood. Although in the present work it has been assumed that OATPs are located at the basal pole of trophocyte and hepatocyte plasma membranes, their existence in the apical domain and in intracellular membranes cannot be ruled out.

MATERIAL AND METHODS

Chemicals

Unlabelled UCB, 17 β -D-glucuronosyl oestradiol (E₂17 β G) sodium salt, fatty acid-free human serum albumin (HSA), Percoll and culture media were purchased from Sigma-Aldrich (Madrid, Spain). 17 β -D-Glucuronosyl [6,7-³H]oestradiol ([³H]E₂17 β G; specific activity: 40.5 Ci/mmol) was from PerkinElmer Life Sciences (Madrid, Spain). [³H]UCB (specific activity: 11 Ci/mol) was produced by biosynthetic labelling from the precursor 3,5-[³H] δ -aminolevulinic acid (specific activity: 2 Ci/mmol, PerkinElmer Life Sciences), given intravenously to bile-fistula dogs [19], with isolation and crystallization of the product as reported previously [20]. To minimize its photodegradation, the dissolution and incubation of UCB were performed using aluminium foil-wrapped glassware in a room under a safe red light. The [³H]UCB was dissolved in warm chloroform (< 55 °C) stabilized with 0.5 % (v/v) ethanol, distributed into vials, dried under a nitrogen stream, and the vials were sealed under an argon atmosphere for storage at –20 °C in the dark. The UCB thus obtained contained less than 0.5 % impurities, as determined by mathematical modelling of serial ultrafiltrations of [³H]UCB in a solution of 10 μ M HSA [21].

Isolation of trophoblast cells from human placenta (hPI)

Samples from human liver (hL) biopsies, from cases of negative diagnoses of liver disease and term placentas from uncomplicated pregnancies, were donated by the Department of Gastroenterology and the Department of Obstetrics and Gynecology respectively, at the University Hospital of Salamanca, Spain, in accordance with the protocols and consent forms approved by the Human Subjects Committee of this hospital.

A combination of previously described methods [22–24] was used to isolate mononucleated human trophocytes (hTCs). A portion of approx. 60 g of placental tissue was thoroughly washed with 500 ml of culture medium 199. This was minced with scissors and washed three times with 100 ml of sterile ice-cold PBS, each time passing the ground tissue through a sterile stainless steel grid. Tissue was enzymatically disaggregated using enzymes

from Roche (Barcelona, Spain). The disaggregating cocktail contained trypsin (137.5 units/ml), dispase II (1.2 units/ml) and DNase (108.3 units/ml). The tissue was incubated with this cocktail at 37 °C for 15 min. Released cells were separated from the residual tissue by filtration through a sieve and centrifugation (300 *g* for 7 min). Cells were resuspended in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum. The disaggregation procedure and cell collection were repeated three more times with the remaining tissue and all cells were pooled together. The hTCs were obtained from the middle band (density ranging from 1.051 to 1.065 g/l) of a discontinuous Percoll gradient from 70 % to 10 % (v/v) centrifuged at 1200 *g* for 20 min. This step was carried out twice. Cells collected from the second Percoll gradient were washed and resuspended with a small volume of PBS.

Total cell numbers were counted using a haemocytometer. Viability was found to be higher than 97 % according to the Trypan Blue exclusion test. To count cytokeratin-7-positive cells collected at different steps of the purification procedure, after fixation on glass slips, the cells were stained immunohistochemically, using monoclonal mouse anti-human cytokeratin-7 antibody (Novocastra, Atom, Barcelona, Spain) and VECTASTAIN® ABC peroxidase kit (Vector Laboratories, Peterborough, U.K.). To obtain the pictures shown in Figure 1, fixed cells were first stained immunohistochemically as above, except that the secondary antibody was AlexaFluor® 594 goat anti-mouse (Molecular Probes, Leiden, The Netherlands), and then stained with haematoxylin/eosin (Sigma-Aldrich).

Quantitation of gene expression by real-time reverse transcription (RT)-PCR

Total RNA was isolated from freshly obtained total hPI tissue, hTCs and hL biopsies using RNAeasy spin columns from Qiagen (Izasa, Barcelona, Spain). After being treated with RNase-free DNase I (Roche), RNA was quantified fluorimetrically with the RiboGreen RNA-Quantitation kit (Molecular Probes). DNA was synthesized from 2 μ g of total RNA using random nonamers and avian myeloblastosis virus RT (Enhanced Avian RT-PCR kit; Sigma-Genosys, Cambridge, U.K.) according to the instructions supplied by the vendor.

Primer oligonucleotides obtained from Sigma-Genosys (Table 1) were designed with the assistance of Primer Express software (PerkinElmer Applied Biosystems, Madrid, Spain) for DNA fragments in the described sequences, and their specificity was checked using BLAST software. The presence of expression for the selected transporter genes was investigated by the detection and subsequent sequencing of mid-size fragments of specific cDNA amplified by 40 cycles of hot-start PCR, using AmpliTaq Gold Polymerase and the primers shown in Table 1.

Table 1 Oligonucleotide primer sequences used for analytical (A) and real-time quantitative (Q) PCR

Target	PCR Type	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size (bp)	Position (5' → 3')	Accession number
OATP-A	A	GTCAAACAAGCTGCCACAT	TATCCAGGTATGGCAGCCAAA	446	1871–2316	NM_134431
OATP-A	Q	AAGACCAACGCAGGATCCAT	GAGTTTCACCCATTCCACGTACA	101	1141–1241	NM_134431
OATP-C	A	TGCTTTGCATGTGCTGGAAA	TTGCCACTTGAAGATTGCAAC	604	1015–1618	NM_006446
OATP-C	Q	GAATGCCCCAAGAGATGATGCTT	AACCCAGTGCAAGTGATTCAAT	154	1701–1854	NM_006446
OATP-8	A	CTGCAATGGATTCAAGATGTTCTT	TGCAAGGCCAATGACTGGAC	607	113–719	NM_019844
OATP-8	Q	GTCCAGTCATTGGCTTTGCA	CAACCCAACGAGAGTCTTAGG	111	700–810	NM_019844

Real-time quantitative PCR was then performed using AmpliTaq Gold polymerase (PerkinElmer Applied Biosystems) in an ABI Prism 5700 Sequence Detection System (PerkinElmer Applied Biosystems). The thermal cycling conditions were as follows: a single cycle at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 60 s. Detection of amplification products was carried out using SYBR Green I (Molecular Probes). Non-specific products of PCR, as detected by 2.5 % agarose gel electrophoresis or melting temperature curves (Figure 2), were not found in any case. Measurement of target gene expression was carried out using standard curves generated by fitting the $\log_{10}$ of the amount of DNA template against the threshold cycle (C_t) values. The DNA used to build standard curves was obtained previously by RT-PCR from commercial hL total RNA (Invitrogen, Madrid, Spain), purified by 2.5 % agarose gel electrophoresis, followed by extraction and the amount determined by the PicoGreen test (Molecular Probes). 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 (PerkinElmer Applied Biosystems).

Uptake studies in *Xenopus laevis* oocytes

Mature female frogs (*Xenopus laevis*), purchased from Regine Olig (Hamburg, Germany), were used. Animals received humane care as outlined in the National Institutes for Health guidelines for the care and use of laboratory animals. Experimental protocols were approved by the Ethical Committee for Laboratory Animals of the University of Salamanca. Harvesting and preparation of oocytes were carried out as described elsewhere [25].

Synthesis of mRNA for injection into oocytes was performed using recombinant plasmids containing the open reading frame DNA of human OATP-A, OATP-C or OATP-8, all kindly supplied by Dr. Peter J. Meier (Department of Clinical Pharmacology, Zurich University Hospital, Switzerland). These plasmids were isolated from *Escherichia coli* using the Qiagen Plasmid Mini Kit (IZASA, Barcelona, Spain) and further linearized with restriction enzymes (Roche). mRNAs were synthesized using the T7 mMessage mMachine kit (Ambion; bioNova Científica, Madrid, Spain). *X. laevis* oocytes were microinjected with TE buffer (1 mM EDTA/10 mM Tris, pH 8.0) either alone, which were used to determine non-specific uptake/binding of the substrates, or containing the following amounts of RNA: 25 ng of total RNA from hL, hPI or hTCs, obtained as described above, or 21 ng of mRNA of each carrier. Oocytes were used 2 days after RNA injection, when, on the basis of preliminary experiments on the time-course of functional expression for these carriers, the uptake rate was highest (results not shown).

Uptake studies were carried out using groups of 12 oocytes per data point. Experiments were repeated using three different frogs. Oocytes were washed with substrate-free uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 and 10 mM Hepes/Tris, pH 7.0) and incubated with 100 μl of uptake medium containing the desired amount of the substrate and inhibitor to be tested at 25 °C for the indicated time. Uptake was stopped by the addition of 4 ml of ice-cold uptake medium. The oocytes were washed three times before being collected and placed, in groups of three to increase the radioactivity signal, in vials for dissolution in 200 μl of 10 % (w/v) SDS.

To determine [^3H]UCB uptake by oocytes, the incubation medium was prepared in a similar way to those used previously in studies aimed at measuring [^3H]UCB transport across plasma membrane vesicles [12,15]. Thus [^3H]UCB was dissolved in

DMSO, and then sonicated and filtered (0.22 μm pore size, Millipore, Bedford, MA, U.S.A.) and uptake medium containing 10 μM HSA was added. [^3H]UCB concentrations were determined by measuring the radioactivity in a liquid-scintillation counter (LS-6500; Beckman, Madrid, Spain), before more uptake medium was added to reach the final desired concentrations of the compounds and to reduce the proportion of DMSO in the final solution to less than 1 % (v/v). The approximate concentration of unbound [^3H]UCB (B_f) was calculated using parameters obtained by other workers [15]. Since it has been emphasized that in order to calculate exactly the value of B_f it is necessary to determine the binding affinity constant (K'_i) under the same circumstances as those used in transport studies, we used experimental conditions as close as possible to those used to determine K'_i [15]. These included pH, temperature and the use of HSA from the same source and at equal concentration.

Statistical methods

Unless otherwise stated, results are expressed as means $\pm$ S.E.M. For kinetic analyses, the values were fitted to a Michaelis–Menten equation $\{V = V_{\text{max}} \cdot [S]/(K_m + [S])\}$. Estimations made by linear and non-linear regression analyses were obtained using the UltraFit-v2.1 software provided by Biosoft (Cambridge, U.K.). 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

Expression of OATP-A, OATP-C and OATP-8 in hTCs

Since previous functional studies [12] had suggested the involvement of members of OATP family of carriers in foetal UCB uptake by the placenta, in the present work further efforts to elucidate the presence of OATPs able to transport UCB in trophoblast cells were undertaken. As a first step in this direction, an enriched fraction of hTCs (as indicated by the immunohistochemically localized presence of the specific marker cytokeratin-7) was obtained by two serial centrifugations in discontinuous Percoll gradients of enzymatically disaggregated placental cells (Figure 1).

Figure 1 Isolation of trophoblastic cells

(A) Enrichment in cytokeratin-7-positive cells, presumably trophocytes, from suspensions of disaggregated placental cells at different steps of the isolation procedure. (B, C). Representative pictures of cytokeratin-7-positive cells stained with haematoxylin/eosin and immunostained with mouse anti-human-cytokeratin-7 antibody plus AlexaFluor® 594 goat anti-mouse antibody as observed without (B) and with (C) UV light excitation.

Figure 2 Specificity and linearity of PCR conditions

First negative derivative curves calculated from plots of SYBR Green I-DNA fluorescence of amplified PCR products corresponding to OATP-A (A), OATP-C (B) and OATP-8 (C) gene fragments versus changes in temperature ($-dF/dT$). Resulting peaks correspond to the DNA melting temperature. Insets show the amplified PCR products visualized on 2.5 % agarose gel after electrophoresis. Lane 1, standard DNA ladder; lane 2, negative control without DNA template; lane 3, PCR product using cDNA obtained by RT of commercial hL RNA as a template. (D) Standard curves generated by fitting the $\log_{10}$ of the amount of DNA template (number of copies) added against the C_t values. Linear regression resulted in the following curve equations: OATP-A, $y = -3.517x + 37.82$, correlation coefficient (R) = 0.9991; OATP-C, $y = -3.594x + 38.81$, $R = 0.9996$; OATP-8, $y = -3.338x + 36.25$, $R = 0.9996$; and 18S rRNA, $y = -3.796x + 42.32$, $R = 0.9976$. In all cases, $P < 0.001$. Values for each data point are means $\pm$ S.D. PCR was performed in triplicate for each sample.

Conventional RT-PCR plus bi-directional sequencing of mid-size fragments of OATP-A, OATP-C and OATP-8 purified by agarose gel electrophoresis resulted in amplicons of the expected size. The identity in the central region of the fragments with a length of > 350 bp, in absence of uncertainties reported by the sequencer, was 100 % for OATP-A and OATP-8. Using conventional RT-PCR up to 45 cycles, mRNA of OATP-C was not detectable in hPI or hTCs.

To validate the measurement of RNA abundance by real-time quantitative RT-PCR using SYBR Green I as the detection system, it is necessary to distinguish between desired and undesired amplification products. With this aim, melting curves were acquired for each sample and correlated to the agarose gel electrophoresis results (Figures 2A–2C). The negative first derivatives of the melting curves showed single large peaks, at 78 °C for OATP-A and OATP-C and 77 °C for OATP-8, suggesting the amplification of a single product in each reaction. This was confirmed by agarose gel electrophoresis, which revealed distinct single bands for the amplified products of the expected size: 101 bp for OATP-A, 154 bp for OATP-C and 111 bp for OATP-8. Melting curves and electrophoresis gels for the negative control obtained in reactions without adding

DNA template showed no discernible peaks or bands respectively (Figures 2A–2C).

Standard curves generated by fitting the C_t value versus the $\log_{10}$ of the amount of DNA template (number of copies) previously obtained as described above and added to the reactions, showed the linearity of the fluorescence signal, at least in the $200 - 2 \times 10^6$ copy range, for all targets used in this study (Figure 2D). Differences in the relative efficiencies of the PCR amplifications, as calculated from the slope of the curves, did not exceed 0.4 units among curves obtained with the SYBR Green I detection system, and 0.5 units with curves for 18S rRNA obtained using the TaqMan® probe (Figure 2D). These results indicated that neither non-specific products nor primer-dimers appeared in any case, and that the measurements of the amount of DNA obtained by RT were accurate for all three carriers. Moreover, differences in the efficiency among RT reactions were corrected by expressing the results as the amount of 18S rRNA in each sample.

Although the mRNA of OATP-C was not detected in hTCs by analytical RT-PCR, real-time quantitative RT-PCR using specific shorter target sequences did permit its detection, although to a very limited extent, in both hPI (approx. 14 copies/ 10^9 copies of 18S rRNA) and in hTCs (approx. 64 copies/ 10^9 copies of 18S

Figure 3 Levels of mRNA for OATP-A, OATP-C and OATP-8

Values are means $\pm$ S.E.M. from determinations carried out by real-time quantitative RT-PCR using total RNA obtained from hL, hPl or isolated human trophocytes. At least four different preparations for each tissue were obtained and RT-PCR was performed in triplicate for each sample. Expression levels were normalized on the basis of the content of 18S rRNA measured in the same sample. * $P < 0.05$ as compared with liver; † $P < 0.05$, as compared with placenta; ‡ $P < 0.05$ on comparing OATP-A and OATP-C with OATP-8 in trophocytes by the Bonferroni method for multiple-range testing.

rRNA). The abundance of mRNA of OATP-A in hPl was much higher than that of OATP-C but much lower than that of OATP-8. As compared with hPl, the mRNA of these latter two carriers was highly enriched in hTCs (Figure 3). In the hL, mRNA of OATP-8 was also the most abundant, whilst that of OATP-C was less than half the amount found for OATP-8. In contrast, the amount of mRNA of OATP-A was approx. 200-fold lower than that of OATP-8. In all three cases, the mRNA was markedly higher in hL than in hTCs (Figure 3).

Transport studies

Before studying the ability of OATP-A, OATP-C and OATP-8 mRNAs to induce the uptake of [³H]UCB by *X. laevis* oocytes, the uptake of [³H]E₂17βG, a prototypic substrate of OATP-A [26], OATP-C [27] and OATP-8 [18,27], was investigated. As expected, mRNA of all three carriers was able to induce a marked uptake of [³H]E₂17βG (Figure 4A). Moreover, this was inhibited by UCB and unlabelled E₂17βG.

To validate the model, total RNA from hL, hPl and hTCs was injected in *X. laevis* oocytes and their ability to take up [³H]E₂17βG was measured. A similar marked enhancement in [³H]E₂17βG uptake was observed in oocytes injected with RNA from hL or hTCs (Figure 4B). Although to a lower extent, RNA from hPl also conferred the ability for [³H]E₂17βG uptake. When included in the incubation medium, unlabelled UCB and E₂17βG were able to significantly *cis*-inhibit [³H]E₂17βG uptake by oocytes injected with RNA from hPl, hTCs or hL (Figure 4B).

In similar experiments in which OATP-A, OATP-C or OATP-8 expression in oocytes was induced by injection of the corresponding mRNA, a marked ability to carry out [³H]UCB uptake was conferred by OATP-C and, to a lower extent, by OATP-8. OATP-A induced a very low increase in [³H]UCB uptake as compared with that observed in oocytes injected with TE buffer alone (Figure 5A). Also in these experiments, the presence of either unlabelled UCB or E₂17βG was found to reduce carrier-induced enhancement in [³H]UCB uptake (Figure 5A).

When total RNA from hL, hPl and hTCs was injected in *X. laevis* oocytes, a marked enhancement in their ability

Figure 4 Uptake of [³H]-E₂17βG by *Xenopus laevis* oocytes injected with TE buffer alone (TE) or containing (A) 21 ng mRNA of OATP-A, OATP-8 or OATP-C, or (B) 25 ng total RNA from whole hPl tissue, isolated human trophocytes, or hL

Oocytes (36 per data point counted in groups of three) from three different frogs were incubated with 10 μ M of the indicated substrate at 25 °C for 60 min. Self- and cross-inhibition by unlabelled E₂17βG and UCB respectively, were determined by including 100 μ M of one of these compounds in the incubation medium. Values are means $\pm$ S.E.M. * $P < 0.05$ as compared with uptake in the absence of unlabelled compounds; † $P < 0.05$, on comparing [³H]E₂17βG uptake in the absence of inhibitors with uptake by oocytes injected with TE buffer alone, by the paired *t*-test.

Figure 5 Uptake of [³H]UCB by *Xenopus laevis* oocytes injected with TE buffer alone (TE) or containing (A) 21 ng mRNA of OATP-A, OATP-8 or OATP-C or (B) 25 ng total RNA from whole hPI tissue, isolated human trophocytes or hL

Oocytes (36 per data point counted in groups of three) from three different frogs were incubated with 10 μ M of the indicated substrate at 25 °C for 60 min. Self- and cross-inhibition by unlabelled E₂17βG and UCB respectively, were determined by including 100 μ M of one of these compounds in the incubation medium. Values are means $\pm$ S.E.M. **P* < 0.05 as compared with uptake in the absence of unlabelled compounds; †*P* < 0.05, on comparing [³H]UCB uptake in the absence of inhibitors with uptake by oocytes injected with TE buffer alone, by paired *t*-test.

to take up [³H]UCB was found. The strongest effect was seen in oocytes injected with RNA from hL (Figure 5B). Although to a much lower extent, RNA from hPI also conferred the ability to take up [³H]E₂17βG. This capability was higher for RNA from hTCs, but without reaching the level of RNA from hL. In all three cases, co-incubation of oocytes with [³H]UCB and either unlabelled UCB or E₂17βG resulted in a significant reduction in [³H]UCB uptake (Figure 5B). It must be stated that, in order to maximize the radioactivity taken up by the oocytes, the incubations in experiments shown in Figures 4 and 5 were carried out for 60 min, which, as will be discussed below, were not initial velocity conditions.

Owing to the difficulty in distinguishing between the effects of unlabelled inhibitors on the carrier and on [³H]UCB binding to HSA, self-inhibition was confirmed in studies using a range of [³H]UCB concentrations. This also permitted kinetic analysis. Thus in order to perform these studies under initial velocity conditions, the time-course of [³H]UCB uptake by oocytes expressing OATP-C, OATP-8 or injected with RNA from hTCs was investigated. The results shown in Figure 6(A) indicate that

Figure 6 Uptake of [³H]UCB by *Xenopus laevis* oocytes injected with TE buffer alone (TE) or containing 21 ng mRNA of human OATP-C or OATP-8 or 25 ng of total RNA from isolated human trophocytes

After 2 days in culture, incubations with [³H]UCB were carried out. [³H]UCB uptake was calculated by subtracting the amount of radioactivity retained in oocytes injected with TE buffer alone to that retained in oocytes injected with RNA. Experiments were carried out with oocytes (36 per data point counted in groups of three) obtained from three different frogs. Values are means $\pm$ S.E.M. (A) Time-course of [³H]UCB uptake in incubations with a calculated unbound [³H]UCB concentration (Bf) of 130 nM. (B) Saturation kinetics of [³H]UCB uptake in incubations with a theoretical Bf between 0 and 260 nM for 10 min.

10 min is an appropriate time to obtain a marked uptake signal still within the linear period of the uptake process. Using this time, further incubations with [³H]UCB were carried out in the 0–260 nM range of calculated Bf. The findings suggested the presence of a non-saturable process; probably diffusion plus non-specific binding, which was measured in oocytes injected with TE buffer alone (Figure 6B). Although it is not possible to distinguish the contributions of each of the two components mentioned above, the slope of this curve can be assumed as an estimation of the maximal limit for the value of the diffusion constant (*K_D*) (Table 2).

When [³H]UCB uptake by oocytes injected with RNA was calculated by subtracting the radioactivity retained in oocytes injected only with TE and incubated with the same amount of total radioactivity, clear saturation curves were generated by plotting [³H]UCB uptake by oocytes injected with mRNA of OATP-C, OATP-8 or total RNA of hTCs (Figure 6B). Under

Table 2 Kinetic parameters for [³H]UCB uptake by *Xenopus laevis* oocytes

Apparent affinity constant (K_m), maximal velocity of transport (V_{max}) and efficiency of transport (E_t) of [³H]UCB uptake by *Xenopus laevis* oocytes in which mRNA of OATP-C or OATP-8 or RNA from human trophocytes (hTCs) was injected. The diffusion constant (K_D) was calculated in oocytes injected with TE buffer alone. Calculations were carried out by fitting the data shown in Figure 6(B) either to a Michaelis-Menten equation (hTCs, OATP-C and OATP-8) or to a linear function (TE). Values are means $\pm$ S.D.

	hTCs	OATP-C	OATP-8	TE
K_m (nM)	25.6 $\pm$ 8.1	7.6 $\pm$ 1.2	39.1 $\pm$ 6.4	
V_{max} (pmol/10 min/oocyte)	4.8 $\pm$ 0.8	6.5 $\pm$ 0.3	4.3 $\pm$ 0.4	
E_t (V_{max}/K_m)	0.19	0.86	0.11	
K_D (mL/10 min/oocyte)				2.14 $\pm$ 0.13

the experimental circumstances used in the present study, the solubility of unbound [³H]UCB is expected to be approx. 65 nM [28], thus higher values of Bf, calculated from total content in [³H]UCB, were merely theoretical. Accordingly, the values of [³H]UCB uptake against unbound [³H]UCB concentrations were fitted to a Michaelis-Menten equation only for Bf values within the solubility range (Table 2). Nevertheless, the kinetic constants did not differ significantly when the whole theoretical range of Bf was included in the calculation (results not shown), because saturation of the transport process was almost reached at a Bf value below the solubility limit.

Comparison of the values found for the apparent affinity constant (K_m) indicated that the carrier affinity for [³H]UCB and transport efficiency, at least in this model, were higher for OATP-C than for OATP-8. The kinetic parameters for [³H]UCB uptake by oocytes injected with the RNA from hTCs resemble more closely those of oocytes expressing OATP-8 than those of cells expressing OATP-C (Table 2), which is consistent with the abundance of mRNA of both carriers detected in hTCs by real-time quantitative RT-PCR (Figure 3).

DISCUSSION

Although the mechanism for placental transfer of foetal bilirubin has traditionally been accepted as being due to simple diffusion, evidence for the existence of a carrier-mediated transport of UCB across the placenta has been recently obtained *in vitro* using plasma membrane vesicles from hTCs [12] and cultured human choriocarcinoma BeWo cells [29], and also *in vivo*, using *in situ* perfused rat placenta [13]. The results of the present study contribute to the identification of a role for OATP isoforms in carrier-mediated UCB uptake by both components of the placenta-maternal liver tandem excretory pathway for foetal bilirubin.

The first step in the transfer of UCB across the placenta is its uptake from the foetal blood by the trophoblast. In previous studies using plasma membrane vesicles, we have shown that for other cholephilic organic anions, such as bile acids, this process probably involves an anion-exchanger [30]. Using the same experimental model, we have recently found functional evidence for a role of one or several members of the human OATP family in the transport of UCB across this pole of the trophoblast plasma membrane [12], which is probably similar to what happens at the basolateral plasma membrane of hepatocytes [15,16,18].

Before discussing results based on kinetic studies, it is necessary to consider that, on one hand, the calculated kinetic parameters are approximate for three reasons: (i) the similarity

in functionality of carriers between the *in vivo* situation and that occurring when these proteins are expressed in *X. laevis* oocytes is not known; (ii) K'_t was not determined using strictly the same experimental circumstances as those used in transport studies; and finally (iii) α -fetoprotein, which binds UCB with less affinity than albumin, but which is produced in large quantities by the foetal liver and yolk sac, may play a role in the transport of UCB in the foetal serum affecting uptake of this pigment by the placenta [31]. On the other hand, there is no reason to expect that in the *in vivo* situation there are either dramatically different values for kinetic parameters or changes in their relative magnitude among the different carriers studied here.

At the placental step of foetal UCB excretion, our results clearly point to two important facts: (i) hTCs contain the machinery required to perform carrier-mediated UCB transport, and (ii) OATP-8 is expressed in the placenta, the abundance of its mRNA being particularly enriched in hTCs. Moreover, although OATP-C seems to be a more efficient carrier in transporting UCB than OATP-8, OATP-C is also expressed at a much weaker level in the placenta than OATP-8, which together with the fact that OATP-A is both sparingly abundant in trophoblast cells and is a poor carrier for UCB, leads to the conclusion that OATP-8 probably plays a major role in the uptake of foetal UCB by the placenta. This hypothesis is consistent with the K_m value for UCB uptake found in oocytes injected with RNA from hTCs. This and the K_m for OATP-8 were of the same order, but slightly higher than that previously found in experiments using plasma membrane vesicles from the foetal pole of the trophoblast [12]. Small quantitative differences between kinetic parameters found in both studies are probably related to model-associated characteristics, such as contamination of the membrane preparations with membranes of other subcellular origins. Moreover, the contribution of additional unidentified carriers to placental UCB uptake by membrane vesicles absent in the oocyte model cannot be ruled out.

Regarding the uptake of UCB by the adult hL, the following comments on the role of the isoforms of OATPs included in the present study do not rule out the involvement of additional carriers in this process. Although transport of substrates other than UCB by OATP-A was initially found to be sensitive to inhibition by UCB [32], and we have found that mRNA of this carrier induced a low ability to take up this pigment by *X. laevis* oocytes, the expected contribution of OATP-A is small, owing to both the low efficiency of this carrier in transporting UCB and the relatively low expression in this organ. Indeed, although OATP-A was initially identified in the liver [32], further screening of its expression in several tissues suggested that it cannot be considered as an important liver carrier for organic anions [33]. In fact, the expression of this carrier is high in some non-hepatic tissues, particularly in different regions of the human brain [34].

With regard to the role of OATP-C and OATP-8 in bilirubin uptake by the liver, the results of the present study support previous reports indicating the involvement of both carriers in this process [16,18]. Although OATP-C has been found to confer the ability to take up UCB to HEK293 transfected cells [16], other authors have failed to observe enhanced UCB uptake by this carrier using HeLa cells as the expression system [17]. In our study, the injection of OATP-C mRNA into *X. laevis* oocytes conferred on these cells the ability to carry out high affinity UCB uptake.

Regarding the contribution by OATP-8, this carrier has been previously found to be able to transport monoconjugated bilirubin when expressed in HEK293 [18]. The present study indicates that OATP-8 is also able to transport UCB. If extrapolation of the kinetic parameters found for carriers expressed in oocytes to the *in vivo* situation is assumed (with caution due to the reason given above), an approximate calculation of the OATP-C/OATP-8

ratio for the efficiency of transport, corrected for the level of expression in hL, results in a value for OATP-C that is approx. 3-fold higher than the contribution of OATP-8 in UCB uptake by the liver. However, it must be borne in mind that at normally low serum concentrations of unbound UCB, the predominant relevance of OATP-C in this function could be increased.

Serum concentrations of unbound UCB are higher during intrauterine life, as suggested by the higher concentration of unbound UCB in umbilical cord serum as compared with normal adult serum [35,36], which is in agreement with the diminished bilirubin binding capability of serum collected from premature infants [37] and the higher total bilirubin concentration in foetal serum. This situation is consistent with an important role in placental UCB uptake for the carrier with the highest K_m value, i.e., OATP-8. In contrast, in the adult, the presence of both OATP-C and OATP-8 with different affinities for the substrate, can collaborate to extract UCB, their contribution being dependent on the serum concentration of unbound UCB.

In conclusion, our results suggest that OATP-8, but not OATP-C, may play a major role in the carrier-mediated uptake of foetal UCB by the placental trophoblast, whereas both OATP-8 and OATP-C may contribute substantially to UCB uptake by adult hepatocytes, OATP-A playing a minor role in both steps of the placenta-maternal liver tandem excretory pathway for foetal bilirubin.

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