OATP8/1B3-MEDIATED COTRANSPORT OF BILE ACIDS AND GLUTATHIONE.

AN EXPORT PATHWAY FOR ORGANIC ANIONS FROM HEPATOCYTES?

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Running Title: OATP8/1B3-Mediated Bile Acid/GSH Cotransport

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In cholestasis, accumulation of organic anions in hepatocytes is reduced by transporters (several MRPs and OStα/OSTβ) able to extrude them across the basolateral membrane. Here we investigated whether OATP isoforms may contribute to this function. Xenopus laevis oocytes expressing human carboxyl-esterase-1 were able to efficiently load cholic acid (CA)-methyl ester, which was cleaved to CA and exported. Expression of OATP8/1B3 enhanced CA efflux, which was trans-activated by taurocholate, but trans-inhibited by reduced (GSH) and oxidized (GSSG) glutathione. Moreover, taurocholate and estradiol 17β-D-glucuronide, but not bicarbonate and glutamate, cis-inhibited OATP8/1B3-mediated bile acid transport, whereas glutathione cis-stimulated this process, which involved the transport of glutathione itself with a stoichiometry of 2:1 (GSH/bile acid). No cis-activation by glutathione of OATP-C/1B1 was found. Using real-time quantitative RT-PCR, the absolute abundance of OATP-A/1A2, OATP-C/1B1 and OATP8/1B3 mRNA in liver biopsies obtained from patients with different cholestatic liver diseases was measured. In healthy liver, expression levels of OATP-C/1B1 were approximately five-fold those of OATP8/1B3 and >100-fold those of OATP-A/1A2. This situation was not substantially modified in several cholestatic conditions studied here. In conclusion, although both OATP-C/1B1 and OATP8/1B3 are highly expressed, even in several cholestatic liver diseases, and are able to transport bile acids, their mechanisms of action are different. OATP-C/1B1 may be involved in uptake processes, whereas OATP8/1B3 may mediate the extrusion of organic anions by symporting with glutathione as a normal route of exporting metabolites produced by hepatocytes or preventing their intracellular accumulation when their vectorial traffic toward the bile is impaired.

INTRODUCTION

The removal from blood of endogenous and xenobiotic cholephilic organic anions, such as bile acids, bilirubin and steroid hormones, is a major function carried out by the liver. The vectorial transport of these compounds by hepatocytes includes uptake across the basolateral plasma membrane and subsequent secretion into bile across the canalicular plasma membrane. Since for most of these substances simple diffusion plays a minor role in both processes, the overall system depends on the polarized expression of transport proteins (1).

Sodium-taurocholate cotransporting polypeptide (NTCP, gene symbol SCL10A1) mediates efficient sodium-dependent bile acid uptake across the basolateral plasma membrane (2). Together with carriers belonging to the SLC22A family, such as organic anion transporters (OATs) (3) and organic cation transporters (OCTs) (4), several members of the family of organic anion transporting polypeptides (OATPs, gene symbol SLCO) account for most of the sodium-independent uptake of cholephilic organic anions (5,6). OATPs exhibit broad substrate specificity, including conjugated and unconjugated bile acids, bilirubin, thyroid hormones, anionic peptides and numerous drugs (5,6).

Three OATP isoforms with the ability to transport bile acids are expressed in human liver, namely, OATP-A/1A2 (gene symbol SLC01A2), OATP-C/1B1 (SLCO1B1) and OATP8/1B3 (SLCO1B3). The role of OATP-A/1A2 in bile acid uptake by
hepatocytes is probably minor as compared with other OATPs owing to its low expression in these cells (7,8). In contrast, based on its broad specificity, high expression level, and localization at the hepatocyte basolateral plasma membrane, OATP-C/1B1 probably plays a major role in the hepatic uptake of organic anions (9). OATP8/1B3 is also expressed at the same domain of the hepatocyte plasma membrane (10) and exhibits similar broad substrate specificity to that of OATP-C/1B1 (11), which suggests that this transporter could contribute to transporting organic anions in the liver as well as in other epithelial cells expressing it (8).

The driving forces accounting for OATP-mediated transport are not yet well known (12). Rat Oatp1/1a1-mediated transport has been reported to occur through pH-dependent and electroneutral anion exchange, in which the uptake of organic anions is coupled to the efflux of bicarbonate (13). However, the ability of reduced glutathione (GSH) to participate in exchange with organic anions across this antiporter has been also suggested (14). The efflux of intracellular GSH or glutathione S-conjugates also seems to be able to account for the driving force involved in rat Oatp2/1a4-mediated substrate uptake (15). It has been demonstrated that the organic substrate transport carried out by Oatp1/1a1 and Oatp2/1a4 can be potentially bidirectional (15,16), which implies that the overall directionality of the net transport is dependent on substrate gradients across the basolateral membrane of hepatocytes.

Studies carried out with human hepatoblastoma HepG2 cells have indicated that the uptake and extrusion of cholephilic organic anions are modulated by intracellular GSH (17), but not by proton gradients (18). In contrast, OATP-B/2B1 is a pH-sensitive transporter that does not transport GSH and is not activated by the outwardly directed GSH (19). Whether other human members of the OATP family may work as anion exchangers or GSH-mediated transporters remains to be elucidated. In preliminary experiments we unexpectedly found evidence that OATP8/1B3 could behave as a GSH-dependent symporter. Accordingly, we wondered whether this could play a role in cis-activating bile acid transport and, if so, what the functional meaning of this mechanism of action would be.

Under physiological circumstances, several ATP-binding-cassette (ABC) proteins export cholephilic organic anions into bile across the canalicular plasma membrane of hepatocytes, whereas other ABC proteins such as the isoforms 1, 3 and 4 of the multidrug resistance-associated protein (MRP), are up-regulated in response to cholestasis and become a route for the extrusion of cholephilic organic anions across the basolateral membrane (for review, see 20). This may limit the accumulation in hepatocytes of potentially toxic compounds. Therefore, another aim of the present work was to investigate whether OATPs, in particular OATP8/1B3, may contribute to this function.

**Experimental Procedures**

**Chemicals**

Radiolabeled substrates were obtained from American Radiolabeled Chemicals (ITISA Biomedica, Madrid, Spain) or Perkin Elmer Life Sciences (Izasa, Barcelona, Spain), except 22,23-3H labeled bile acids - [3H]taurocholenoxycholic acid (TCDCA; 0.37 TBq/mmol), [3H]taurodeoxycholic acid (TDCA; 1.11 TBq/mmol) and [3H]taurosodeoxycholic acid (TUDCA; 0.37 TBq/mmol), which were prepared by reductive tritiation of their corresponding precursors, as reported (21). [3H]cholic acid (CA) methyl ester (CA-ME) was synthesized by reaction of CA dissolved in methanol/chloroform (1/2, vol/vol) with an excess of freshly distilled diazomethane in diethyl ether at room temperature for 12 h (22). Unlabeled sodium salts of bile acids, CA-ME, estradiol 17β-D-glucuronide (E2<17βG), reduced (GSH) and oxidized (GSSG) glutathione, L-glutamic acid and sodium bicarbonate were purchased from Sigma-Aldrich Quimica (Madrid). As indicated by the supplier, the purity of the bile acids, GSH and GSSG was more than 98% as determined by thin-layer chromatography. All other reagents were of analytical grade.

**Uptake studies in Xenopus laevis oocytes**

Harvesting and preparation of oocytes were carried out as described elsewhere (23) from mature female frogs (*Xenopus laevis*), purchased from Regine Olig (Hamburg, Germany) and treated in accordance with the indications of current Spanish and European laws (RD 223/88 and EU Directive 86/609/CEE) and supervised by the Ethical Committee for Laboratory Animals of the University of Salamanca.
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Synthesis of cRNAs was performed as previously described (23) using recombinant plasmids containing the open reading frame cDNA of the following proteins: human OATP8/1B3 and OATP-C/1B1 cloned in pCMV6-XL and rat Bsep and human carboxyl-esterase-1 (CES1) cloned in pSPORT1. Incubation time after injection of the cRNA in oocytes was selected based on preliminary experiments on the time-course of the functional expression for these carriers (results not shown).

Efflux experiments from Xenopus laevis oocytes

Three different sets of experiments were carried out to investigate the glutathione-induced activation of OATP8/1B3-mediated efflux of bile acids from oocytes:

i) Trans-activation experiments. Oocytes were injected with cRNA of either human CES1 and OATP8/1B3 or CES1 and rat Bsep, which were used as a positive control of bile acid efflux (22). After two days, the oocytes were incubated with 50 µM \[^{14}C\]CA-ME at 25°C for 1 h, which, as has been previously reported (22), permitted the cells to be loaded with \[^{14}C\]CA-ME, which was subsequently hydrolyzed to free \[^{14}C\]CA by CES1. Based in previous studies (22), since the magnitude of \[^{14}C\]CA-ME hydrolysis by endogenous esterases is variable, cotransfection with CES1 was carried out to obtain higher and standardized cleavage activity. Bile acid efflux was determined after transferring the oocytes to fresh radioactivity-free medium with or without the activators or inhibitors to be tested, and incubation at 25°C for 2 h.

ii) Cis-activation of the efflux from oocytes loaded by incubation. The oocytes were incubated with 10 µM \[^{14}C\]taurocholic acid (TCA) in the absence of GSH at 25°C for 120 min, or in the presence of 20 mM \[^{3}H\]GSH at 25°C for 60 min. The efflux of both compounds was determined at different time-points after transferring the oocytes to radioactive substrate-free medium at 25°C.

iii) Cis-activation of the efflux from oocytes loaded by microinjection. The oocytes were directly loaded with 50 nl U medium containing 300 µM \[^{14}C\]GCA alone or with 20 mM \[^{3}H\]GSH. The efflux of both compounds was determined at different time-points after immediate transferring the oocytes to radioactive substrate-free U medium at 25°C. Some oocytes were used to carry out the analysis of the intracellular balance between reduced and oxidized radioactive glutathione. Groups of 3 oocytes were treated with 100 µl of lysis/extraction solution (1-butanol, 2-propanol, glacial acetic acid, and methanol), sonicated in an ice-cold bath for 1 min and centrifuged at 20,000 xg at 4°C for 5 min. The supernatant was analyzed by TLC using a mixture of 1-butanol, 2-propanol, glacial acetic acid, and methanol and water (2.5:2:0.5:1:4) as eluent. In this system, Rf values were 0.45 for GSH and 0.28 for GSSG. In a different set of oocytes, the magnitude of the leak of injected compounds through the hole made in the plasma membrane by the microcapillary was evaluated by measuring the efflux of \[^{3}H\]inulin that was similarly loaded by microinjection.

In these sets of experiments the efflux process was stopped as in the uptake experiments, and the oocytes were processed as described elsewhere (22) for the determination of the amount of radiolabeled compound retained.

Measurement of absolute mRNA levels in human liver

Samples from human liver biopsies were obtained at the University Hospital of Salamanca (Spain) in accordance with established protocols and consent forms were reviewed and approved by the Human Subjects Committee of this hospital. Forty-two patients (age 49.6±14.7 SD; 50% female) were included in the study after being diagnosed at the Gastroenterology Division of the Salamanca University Hospital with one of the liver diseases indicated in Table 1, and showed biochemical
signs of cholestasis. These were elevations above normal values of the serum concentrations of one or more of the following: total bilirubin, alkaline phosphatase and gamma-glutamyl transpeptidase.

Samples were collected for diagnostic purposes and only the remaining tissue was used for the present study. This was immediately immersed in the RNA stabilization reagent RNAlater (Qiagen, Izasa) and stored at -80°C until total RNA was isolated and absolute abundance measured by real-time quantitative PCR using AmpliTaq Gold polymerase (Applied Biosystems, Madrid) in an ABI Prism 5700 Sequence Detection System (Applied Biosystems) as previously described (8). Detection of amplification products was carried out using SYBR Green I (Applied Biosystems). No non-specific product of PCR, as detected by 2.5% agarose gel electrophoresis and DNA melting temperature curves, was found in any case. The results of mRNA abundance for the target genes in each sample were normalized on the basis of its 18S rRNA content, which was measured using the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems). The primer oligonucleotides sequences and conditions for measuring the absolute abundances of OATP-A/1A2, OATP-C/1B1 and OATP8/1B3 have been reported elsewhere (8).

**Statistical Methods**

Results are expressed as means ± SD. For kinetic analyses, values were fitted to Michaelis-Menten or Hill equations. Linear regression analyses were carried out by the method of least-squares. 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**

**OATP8/1B3-mediated uptake of bile acids in Xenopus laevis oocytes**

OATP8/1B3 expression markedly enhanced the ability of the oocytes to take up E₂17βG, a prototypic substrate of OATP8/1B3 (10) as compared with non-injected oocytes (Figure 1A). Since in preliminary experiments no significant difference between oocytes injected with TE buffer and non-injected oocytes regarding their ability to take up bile acids or E₂17βG was found (data not shown), in the present study, non-injected oocytes (wild) were used to determine non-specific uptake. Similarly, the uptake of several bile acid species differing in their conjugation states and degree of hydroxylation was enhanced in oocytes injected with the cRNA of OATP8/1B3 (Figure 1A). However, this effect was lower than for E₂17βG (Figure 1B).

**Trans-activation of OATP8/1B3-mediated bile acid efflux**

Since bile acid transport by several OATP isoforms, such as rat Oatp1/1a1 and Oatp2/1a4, has been reported to be potentially bidirectional and trans-stimulated by glutathione (14,15,16), we investigated these characteristics for OATP8/1B3 using a previously described experimental model (22). To measure bile acid efflux, oocytes expressing CES1 were incubated with radiolabeled CA-ME. This permitted efficiently loading these cells with CA-ME. This was subsequently cleaved to free CA, which then slowly effluxed from the oocytes. This process was enhanced when rat Bsep was expressed in these cells (Figure 2), which was used here as positive control of the experiment. Although to a lower extent, the expression of OATP8/1B3 also enhanced CA efflux (Figure 2). On placing TCA in the extracellular medium, a trans-activation of the OATP8/1B3-mediated efflux of CA was observed (Figure 2); this is usually interpreted as an indication of the bidirectional transport ability of the carrier. To test whether OATP8/1B3 could work as an exchanger trans-activated by glutathione, inwardly directed glutathione gradients were imposed. Surprisingly, extracellular GSH and GSSG, which had no effect on the CA efflux from non-injected oocytes, induced a significant inhibition of OATP8/1B3-mediated CA efflux. Indeed, the reduction in CA content was even lower than that observed in oocytes expressing only human CES1 (Figure 2). We speculated then on the possibility that CA molecules that had effluxed from oocytes could be in part taken up again via OATP8/1B3 due to activation by inwardly directed glutathione gradient, resulting in a net reduction of bile acid efflux.

**Cis-activation of OATP8/1B3-mediated bile acid uptake**

The next experiments were carried out to elucidate whether re-uptake of the bile acid was indeed involved in the diminished net CA efflux observed when glutathione was placed in the extracellular medium. Both GSH and GSSG, but
not other anions such as bicarbonate and glutamate, were able to induce cis-stimulation of OATP8/1B3-mediated uptake of E$_2$17βG, TCA, glycocholic acid (GCA) and CA (Figure 3). As expected, unlabeled E$_2$17βG and TCA inhibited the uptake of the radiolabeled substrates.

We then investigated whether OATP-C/1B1-mediated transport was also sensitive to cis-activation by glutathione. In contrast to what was found for OATP8/1B3, the addition of 20 mM GSH or GSSG to the incubation medium significantly reduced OATP-C/1B1-mediated TCA uptake (Figure 4). Similar results were obtained when bicarbonate, TCA, or E$_2$17βG were added to the incubation medium (Figure 4). These results are consistent with the expected behaviour of OATP-C/1B1, similar to other members of the OATP family, as an organic anion exchanger (5).

The net intracellular accumulation of TCA reflects the balance between both uptake and efflux mechanisms. Accordingly, in the presence of glutathione or bicarbonate in the medium the efflux of substrate taken up previously may decrease net TCA accumulation as compared with that occurring in the absence of inwardly directed glutathione or bicarbonate gradients. The reduction in radiolabeled TCA uptake by E$_2$17βG and TCA was consistent with competition between these substrates for OATP-C/1B1-mediated uptake (Figure 4).

In order to perform kinetic analyses under appropriate initial velocity conditions, the time-course of TCA uptake by oocytes expressing OATP8/1B3 was determined (Figure 5A). The results indicated that 15 min was an appropriate time to obtain a marked signal of uptake still within the linear range of the uptake process. The addition of 20 mM GSH to the extracellular medium had little effect on non-specific TCA uptake but markedly increased OATP8/1B3-mediated TCA uptake (Figure 5B). Saturation curves were obtained by fitting the values of net TCA uptake against TCA concentrations. The best fits for OATP8/1B3-mediated TCA uptake were to Michaelis-Menten equations. Kinetic parameters were calculated from double-reciprocal Lineweaver-Burk plots for TCA (Figure 5C) and similarly for other bile acids (Table 2).

These results revealed that the GSH-induced enhancement in the efficiency of OATP8/1B3-mediated bile acid transport (E$_T$) - defined as the ratio between the maximal velocity of transport (V$_{max}$) and the apparent affinity constant (K$_m$) - was mainly due to increased V$_{max}$, whereas K$_m$ was not significantly modified by the presence of GSH.

**OATP8/1B3-mediated co-transport of glutathione and bile acids**

Since kinetic analysis suggested that GSH activates the translocation of the substrate, whether this was due to an interaction of GSH with the protein with or without co-transport of the activator was investigated. Using radiolabeled GSH, it was found that non-specific GSH uptake was much lower than that observed in oocytes expressing OATP8/1B3 (Figures 6 and 7A). Moreover, when the values of net OATP8/1B3-mediated GSH uptake vs. GSH concentrations were plotted, a saturation curve was obtained. The best fit was a Michaelis-Menten equation, whose kinetic parameters (Figure 7B and Table 2), revealed values of the same range as those for bile acids, although both V$_{max}$ and K$_m$ were markedly higher.

When the ability of bile acids to affect OATP8/1B3-mediated GSH transport was investigated, we found that in the presence of extracellular concentrations of GCA much higher than those of GSH the uptake of the latter was stimulated. Nevertheless, even when GCA concentrations in U medium were much lower than those of GSH, GSH uptake was also stimulated (Figure 6). Moreover, the kinetic study of GSH uptake in the presence of GCA (Figure 7B) revealed that this induced a significant increase in E$_T$ mainly due to enhanced V$_{max}$, whereas K$_m$ was not significantly changed (Table 2).

To determine the stoichiometry of the process, the uptake of bile acids (TCA or GCA) at a fixed concentration (10 µM) was measured in the presence of varying concentrations (1-20 mM) of GSH. The results were then fitted to Hill equations for different Hill numbers (n$_H$), i.e., the number of molecules of activator per molecule of substrate co-transported (Figure 8). For both TCA and GCA the best fit was for n$_H$=2, suggesting that the most probable stoichiometry of OATP8/1B3-mediated cotransport of GSH and bile acids was 2:1, respectively.
Since these studies were carried out in uptake experiments, assuming bidirectional properties of the transporter, whereas in the in vivo situation the glutathione gradient in liver cells is expected to be directed outward, we investigated whether GSH was able to activate OATP8/1B3-mediated efflux. To carry out these experiments, oocytes were first loaded with radiolabeled TCA with or without GSH (Figure 9). Because TCA uptake was activated in the presence of GSH, in order to obtain a similar initial intracellular load these cells were loaded for a shorter time (60 min) while when they were incubated in the absence of GSH the incubation period was longer (120 min). The efflux of TCA was markedly faster when the oocytes were also loaded with GSH (Figure 9A), which was also able to efficiently efflux from oocytes expressing OATP8/1B3 (Figure 9B).

Since during uptake/efflux period GSH could be converted to GSSG, an additional set of experiments was carried out by direct microinjection in the oocytes. $^{3}H$Inulin loaded by microinjection effluxed slowly from these cells (Figure 10A), probably in part through the hole made by the microcapillary. GSH was more rapidly effluxed from oocytes expressing OATP8/1B3 (Figure 10A). To determine whether part of the microinjected radioactivity remained as GSH throughout the experiment and hence could be used as a substrate by OATP8/1B3, TLC analysis of cell lysates was carried out at different time points. This revealed that, even though a certain degree of oxidation of GSH to GSSG during the analytical procedure cannot be ruled out, an important proportion of the radioactivity recovered from these cells was found to be GSH (min 0: $93\pm3\%$; min 10: $48\pm28\%$; min 30: $51\pm29\%$; min 60: $54\pm23\%$). These experiments also confirmed that TCA efflux was faster from oocytes expressing OATP8/1B3 and was further stimulated by co-microinjection with GSH (Figure 10B).

Expression of OATP-A/1A2, OATP-C/1B1 and OATP8/1B3 in human liver

In order to find some clue to understand the physiological relevance of these findings, the steady-state expression levels in human liver of the three isoforms of OATPs with the ability to transport bile acids were measured by real-time quantitative RT-PCR. As compared to OATP-C/1B1 and OATP8/1B3, the abundance of OATP-A/1A2 mRNA was very low both in normal liver and in several cholestatic liver diseases studied here (Table 1). In healthy livers, the expression of OATP-C/1B1 was higher than that of OATP8/1B3. No significant reduction in the expression of these isoforms was observed in any of the groups of cholestatic livers studied here. Moreover a significant correlation between the expression of both OATP-C/1B1 and OATP8/1B3 was observed when all samples were plotted together (Figure 11), indicating that the quantitative relationship between mRNA of both isoforms was approximately 5:1 (OATP-C/1B1 vs. OATP8/1B3). No significant correlation between the abundance of mRNA of OATP-C/1B1 or OATP8/1B3 and any of the biochemical markers of cholestasis used here, namely, serum levels of total bilirubin, alkaline phosphatase and gamma-glutamyl transpeptidase, was found (data not shown).

DISCUSSION

By performing simultaneous determinations of OATP-A/1A2, OATP-C/1B1 and OATP8/1B3 expression, the present study complements previous studies in which this has been measured in percutaneous liver biopsies from patients with cholestatic liver diseases (24-26). Our results confirm previous reports (7,8) indicating that in healthy livers the expression of OATP-A/1A2 is too low to play a major role in the overall hepatic clearance of organic anions from blood. Moreover, the present study suggests that no marked up-regulation of this carrier occurs in cholestatic liver diseases that could change the relevance of OATP-A/1A2 in this function. However, an important role in the re-absorption of bile acids from bile toward the periductular capillary plexus cannot be ruled out (27).

In contrast, the high expression of OATP-C/1B1 and OATP8/1B3 suggests that both may play a major role in the transport of organic compounds across the basolateral membrane of hepatocytes. In the cholestatic conditions studied here, the expression of OATP-C/1B1 and OATP8/1B3 was not significantly impaired. However this is probably not a general rule. Thus, in a previous study of four patients with primary sclerosing cholangitis, the amount of OATP-C/1B1 mRNA was found to be reduced by half (24). The expression of both OATP-C/1B1 and OATP8/1B3 has been also found decreased in types 2 and 3 of progressive familial intrahepatic cholestasis (28). Moreover, in patients with primary biliary cirrhosis III, a significant decrease in the
expression of OATP-C/1B1 has been reported (26). In the present study, a tendency toward a decreased expression of OATP-C/1B1, although still not significant, in patients with primary biliary cirrhosis I-II was found.

The functional results of the present study support, although they do not prove, the concept that OATP-C/1B1, like other members of the OATP family, behaves as an anion exchanger (5). Thus, several compounds induced a marked inhibition in overall OATP-C/1B1-mediated TCA uptake, which was consistent with competition with the substrate, as was probably the case of unlabeled TCA and E2,17βG, but also with activation of OATP-C/1B1-mediated efflux of TCA that had been previously taken up by the cells, as was probably the case of extracellular glutathione and bicarbonate.

Regarding the ability of OATP8/1B3 to transport bile acids, our results are apparently controversial with initial studies by König et al. who had found that OATP8/1B3-transfected HEK293 cells were not able to take up bile acids (10). However, an artificial mutation was inadvertently created during the construction of the recombinant plasmid used to transfect these cells, which decreased the ability of the expressed protein to transport some substrates, such as sulfobromophthalein and abolished that of others, including bile acids (29). Moreover, the ability of OATP8/1B3 to transport TCA and GCA, when this protein was expressed in Xenopus laevis oocytes, has been already described by others (11).

Thus although our results indicate that OATP8/1B3 was able to transport all major bile acids, either unconjugated or amidated with taurine or glycine, in absence of glutathione, the magnitude of this process was similar to that carried out by OATP-C/1B1 only when OATP8/1B3 was activated by GSH or GSSG. In contrast, bicarbonate failed to activate OATP8/1B3-mediated bile acid transport. Since glutathione enhanced V_max without affecting K_m, and GSH itself was transported by OATP8/1B3 in the same direction as bile acids, it could be suggested that this carrier may work as a symporter of organic anions and glutathione. The most probable ratio of the symport process is two molecules of GSH per each cholephilic organic anion co-transported. Moreover, our results suggest that this symporter is potentially bidirectional. This is a very interesting characteristic that has permitted kinetic studies by imposing precise extracellular concentrations of substrate and activator in uptake experiments, which is not possible in efflux studies using Xenopus laevis oocytes because these cells have endogenous GSH export mechanisms, which is a major confounding variable in GSH transport measurements (30).

Thus, the results of the kinetic analyses are in agreement with those obtained for other GSH exporters recently reviewed by Ballatori et al. (12). In general, these carriers exhibit low catalytic efficiency, i.e., the K_m values for GSH are relatively high and the transport velocities (V_max values) are only moderate, leading to a low V_max/K_m ratio. The high K_m values are not unexpected, given that GSH is present in high concentrations within cells (1–10 mM), whereas 100-fold lower K_m values for bile acids are consistent with much lower concentrations of these compounds in hepatocytes. The values of K_m for conjugated and unconjugated CA were similar to those previously reported for the transport of these bile acids by OATP-C/1B1 and rat Oatp1/1a1 and Oatp2/1a4, which lie in the range of 10–35 µM and 35–54 µM, respectively (11,31,32).

These findings have important functional implications. Since the glutathione gradient across the hepatocyte basolateral membrane is directed outwardly, it could be postulated that glutathione extrusion across OATP8/1B3 may behave as a pathway for exporting organic anions. The balance between uptake and efflux across the basolateral membrane of the hepatocyte would be determined by the magnitude and direction of the gradient of a given substrate and the contribution to the overall process of uptake transporters, i.e., NTCP and OATP-C/1B1 and export transporter, such as OATP8/1B3 whose expression levels are approximately five-fold lower than those of OATP-C/1B1 both in healthy liver and in at least the cholestasis liver diseases studied here. Recently, two additional mechanisms have been suggested to contribute with OATP8/1B3 to carrier-mediated bile acid export across the basolateral membrane of the hepatocyte. One of them is MRP4, which, as we have described for OATP8/1B3, is also able to cotransport bile acids with reduced glutathione (33). The other is OSβ-OSTβ, which is up-regulated in cholestasis (34).

Under physiological circumstances, the function of OATP8/1B3 may involve a reduction in the
efficiency of the overall uptake process but, in compensation, it may also represent a rapid mechanism to export to the blood the derivatives produced by the biotransformation of cholephilic organic anions, which are subsequently taken up by other tissues or eliminated by the kidney. Moreover, owing to the fact that the export into the extracellular space of GSH and its adducts is an important step in their turnover, a role of OATP8/1B3 in glutathione homeostasis, as has been proposed for rat Oatp1/1a1 (35), cannot be ruled out.

In addition, when the biliary excretory pathway is acutely impaired, the extrusion of organic anions across the basolateral membrane through OATP8/1B3 may constitute an alternative excretory pathway that is useful for protecting hepatocytes when the expression of OSTα-OSTβ and basolateral MRP isoforms is still very low and adaptative changes in transporter expression, aimed at reducing the toxic insult due to retained biliary constituents, have not yet taken place. Moreover, since the expression of OATP8/1B3 is not markedly impaired in several cholestatic liver diseases, it could be suggested that, at least in these conditions, OATP8/1B3 would contribute to exporting cholephilic organic anions toward the blood.

In conclusion, although both OATP-C/1B1 and OATP8/1B3 are highly expressed, even in several cholestatic liver diseases, and are able to transport bile acids, their mechanisms of action are different. OATP-C/1B1 may be involved in uptake processes, whereas OATP8/1B3 may mediate the extrusion of organic anions by symporting with glutathione as a normal route of exporting metabolites produced by hepatocytes or preventing their intracellular accumulation when their vectorial traffic toward the bile is impaired.
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FOOTNOTES

Abbreviations:
CA, cholic acid; CES1, human carboxyl-esterase-1; E217βG, estradiol 17β-D-glucuronide; GCA, glycocholic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; OATP, organic anion transporting polypeptide; TCA, taurocholic acid; TDCA, taurodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TUDCA, tauroursodeoxycholic acid.

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FIGURE LEGENDS

Figure 1. Total uptake (A) and OATP8/1B3-mediated uptake (B) of estradiol 17β-D-glucuronide (E217βG) and bile acids. *Xenopus laevis* oocytes expressing OATP8/1B3 were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM [3H]TAUR and one of the following bile acids: [3H]taurocholic acid (TCA), [3H]glycocholic acid (GCA), [3H]taurocholesolic acid (TCA), [3H]taurochenodeoxycholic acid (TCDA), [3H]taurochenodeoxycholic acid (TCDCA) or [3H]taurocholic acid (TCDCA), at 25°C for 1 h. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs. OATP8/1B3-mediated uptake was calculated by subtracting the amount of radioactivity taken up by non-injected oocytes from that determined in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies. *, P<0.05, on comparing OATP8/1B3-expressing oocytes with non-injected oocytes by paired t-test. †, P<0.05, as compared with the E217βG uptake by the Bonferroni method of multiple range testing.

Figure 2. OATP8/1B3-mediated efflux of cholic acid (CA) from *Xenopus laevis* oocytes. Oocytes were injected with the cRNA of both human carboxyl-esterase-1 (CES1) and OATP8/1B3, CES1 and rat Bsep, or CES1 alone. After 2 days the cells were first incubated with 50 µM [3H]CA methyl ester at 25°C for 1 h. To measure [3H]CA efflux, the oocytes were then transferred to [3H]CA-free medium in the presence of 0.2 mM taurocholic acid (TCA), 20 mM reduced glutathione (GSH), 20 mM oxidized glutathione (GSSG) or none (Control) and incubated at 25°C for 2 h. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs. *, P<0.05, as compared with oocytes injected only with cRNA of CES1. †, P<0.05, as compared with Control by the Bonferroni method of multiple range testing.

Figure 3. Effect of several anions on OATP8/1B3-mediated uptake of estradiol 17β-D-glucuronide (E217βG) (A), taurocholic acid (TCA) (B), glycocholic acid (GCA) (C) and cholic acid (CA) (D). *Xenopus laevis* oocytes expressing OATP8/1B3 were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing the radiolabeled substrate at a concentration of 10 µM at 25°C for 1 h with or without 20 mM reduced glutathione (GSH), oxidized glutathione (GSSG), bicarbonate (HCO3) and glutamate (Glu) or 50 µM E217βG and TCA. Values of net uptake are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs and were calculated by subtracting the amount of radioactivity measured in non-injected oocytes from that found in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies. *, P<0.05, as compared with Control by the Bonferroni method of multiple range testing.

Figure 4. Effect of several anions on OATP-C/1B1-mediated uptake of taurocholic acid (TCA). *Xenopus laevis* oocytes expressing OATP-C/1B1 were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2 and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM [3H]TCA at 25°C for 1 h with or without 20 mM reduced glutathione (GSH), oxidized glutathione (GSSG) and bicarbonate (HCO3) or 50 µM E217βG and TCA. Values of net uptake are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs and were calculated by subtracting the amount of radioactivity measured in non-injected oocytes from that found in oocytes injected with OATP-C/1B1 cRNA 2 days before carrying out transport studies. *, P<0.05, as compared with Control by the Bonferroni method of multiple range testing.

Figure 5. Kinetic analysis of the cis-activation of OATP8/1B3-mediated taurocholic acid (TCA) uptake by reduced glutathione (GSH). (A) Time-course of OATP8/1B3-mediated TCA uptake by *Xenopus laevis* oocytes incubated with uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM...
CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM [³H]TCA at 25°C. (B) TCA uptake by oocytes incubated with varying concentrations of TCA in the presence (closed symbols) or absence (open circles) of 20 mM GSH at 25°C for 15 min. (C) Lineweaver-Burk plot of OATP8/1B3-mediated TCA uptake in the presence (closed circles) or absence (open symbols) of 20 mM GSH. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs and were calculated by subtracting the amount of radioactivity measured in non-injected oocytes from that found in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies.

Figure 6. Effect of glycocholic acid (GCA) on OATP8/1B3-mediated uptake of reduced glutathione (GSH). *P<0.05, as compared with oocytes incubated in the absence of GCA by the Bonferroni method of multiple range testing. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs. OATP8/1B3-mediated uptake was calculated by subtracting the amount of radioactivity measured in the absence of GCA from that found in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies.

Figure 7. Kinetic analysis of OATP8/1B3-mediated uptake of reduced glutathione (GSH). (A) GSH uptake by Xenopus laevis oocytes expressing (circles) or not (open triangles) OATP8/1B3. The cells were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM glycocholic acid (GCA) with varying concentrations of [³H]GSH at 25°C for 15 min. (B) Lineweaver-Burk plot of OATP8/1B3-mediated GSH uptake in the absence (open squares) and in the presence (closed squares) of 10 µM GCA. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs. OATP8/1B3-mediated uptake (closed circles) was calculated by subtracting the amount of radioactivity measured in non-injected oocytes (open triangles) from that found in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies (open circles).

Figure 8. Hill plots of the cis-activation of OATP8/1B3-mediated taurocholic (TCA) (A-C) and glycocholic acid (GCA) (D-F) uptake by reduced glutathione (GSH). Xenopus laevis oocytes expressing OATP8/1B3 were incubated in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM [³H]TCA or [¹⁴C]GCA and varying concentrations (1-20 mM) of GSH at 25°C for 15 min. Data were fitted to the Hill equation by the method of least-squares for different Hill numbers (nH); nH = 1 (A, D), nH = 2 (B, E) and nH = 3 (C, F). Hill plot is V versus V/[GSH]nH, where V is initial rate of bile acid (BA) uptake, [GSH] is activator concentrations and nH is number of molecules of activator per molecule of substrate interacting with the carrier. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs. OATP8/1B3-mediated uptake was calculated by subtracting the amount of radioactivity measured in non-injected oocytes from that found in oocytes injected with OATP8/1B3 cRNA 2 days before carrying out transport studies.

Figure 9. Glutathione (GSH)-induced cis-activation of OATP8/1B3-mediated efflux of taurocholic acid (TCA) from Xenopus laevis oocytes. Non-injected oocytes (wild) or OATP8/1B3-expressing oocytes were previously loaded (dashed lines) with TCA alone (open symbols) by incubation in uptake medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, adjusted to pH 7.0 with Tris) containing 10 µM [¹⁴C]TCA at 25°C for 120 min, or with [¹⁴C]TCA and [³H]GSH (closed symbols) by incubation in uptake medium containing 10 µM [¹⁴C]TCA with 20 mM [³H]GSH at 25°C for 60 min. To determine [¹⁴C]TCA (A) and [³H]GSH (B) efflux, their content in oocytes were measured at different time-points after transferring the cells to substrate-free medium and incubating them at 25°C. Values are means ± SD from at least 24 determinations.
determinations per data point obtained using oocytes from at least three different frogs. *, P<0.05, as compared with OATP8/1B3-expressing oocytes loaded with TCA without GSH.

**Figure 10. Efflux of inulin, taurocholic acid (TCA) and glutathione (GSH) from Xenopus laevis oocytes previously loaded by microinjection.** Oocytes expressing OATP8/1B3 or wild type cells were microinjected with 50 nl solution containing 100 µM [³H]inulin or 300 µM [¹⁴C]TCA alone or with 20 mM [³H]GSH before being transferred to incubation medium (100 mM choline chloride, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, adjusted to pH 7.0 with Tris) at 25°C for the indicated time. Measurement of the radioactivity remaining in the cells was used to determine: (A) Leak of [³H]inulin through hole made in the plasma membrane during microinjection, and non-specific or OATP8/1B3-mediated GSH efflux. (B) Non-specific or OATP8/1B3-mediated TCA efflux in the presence or absence of microinjected GSH. Values are means ± SD from at least 24 determinations per data point obtained using oocytes from at least three different frogs, except for TLC analysis where 4 groups of 3 oocytes each were analyzed per data point. *, P<0.05, as compared OATP8/1B3-expressing cells with wild oocytes. †, P<0.05, as compared OATP8/1B3-expressing oocytes loaded with TCA with and without GSH. For these comparisons, the Bonferroni method of multiple range testing was used.

**Figure 11. Relationship between absolute amount of mRNA for OATP-C/1B1 and OATP8/1B3 in control liver tissue and that of patients with cholestatic liver diseases.** Values are means of determinations carried out in triplicate in each sample by real-time quantitative RT-PCR using total RNA obtained from samples collected from control healthy liver tissue (n=6) or 42 biopsies obtained from patients with hepatocellular cholestasis (n=12), primary biliary cirrhosis I-II (n=7), non-alcoholic steatohepatitis (n=7), non-viral hepatitis (n=8) and hemochromatosis (n=8). Expression levels were normalized on the basis of the content of 18S rRNA measured in the same sample.
Table 1. Absolute steady-state levels of mRNA for OATPs in Control liver tissue and that of patients with cholestatic liver diseases

|                  | OATP-A/1A2 | OATP-C/1B1 | OATP8/1B3   | 1B1/1B3 Ratio |
|------------------|------------|------------|-------------|---------------|
| Control          | 8±2        | 4894±1208  | 923±148     | 5.3           |
| Hepatocellular Cholestasis | 12±4       | 7444±1146  | 1205±132    | 6.2           |
| Primary Biliary Cirrhosis I-II | 15±7       | 4022±657   | 893±68      | 4.5           |
| Non-Alcoholic Steatohepatitis | 7±4        | 5500±1779  | 873±108     | 6.3           |
| Non-Viral Hepatitis | 6±3        | 5982±1954  | 961±273     | 6.2           |
| Hemochromatosis  | 16±4       | 6288±1448  | 1335±358    | 4.7           |

Samples were collected from control healthy liver tissue (n=6) or 42 biopsies obtained from patients with hepatocellular cholestasis (n=12), primary biliary cirrhosis I-II (n=7), non-alcoholic steatohepatitis (n=7), non-viral hepatitis (n=8) and hemochromatosis (n=8). Expression levels were normalized on the basis of the content of 18S rRNA measured in the same sample. Values are means ± SD. Determinations were carried out in triplicate on each sample by real-time quantitative RT-PCR using total RNA obtained from human liver. No significant difference (P<0.05) as compared with Control by the Bonferroni method of multiple-range testing was found.
Table 2. Kinetic parameters of OATP8/1B3-mediated transport of reduced glutathione (GSH) and bile acids

| Substrate | Activator | $K_m$ (µM) | $V_{max}$ (pmol/15 min/oocyte) | $E_T$ ($V_{max}/K_m$) | R |
|-----------|-----------|------------|------------------|-----------------|---|
| GSH       | -         | 4651±690   | 746±133          | 0.16            | 0.9969 |
| GSH       | GCA       | 4507±460   | 1596±54         | 0.35            | 0.9982 |
| TCA       | -         | 42.2±4.2   | 5.9±0.3         | 0.14            | 0.9988 |
| TCA       | GSH       | 43.3±4.3   | 8.6±0.4         | 0.20            | 0.9989 |
| GCA       | -         | 43.4±17.8  | 4.3±0.6         | 0.10            | 0.9722 |
| GCA       | GSH       | 43.5±16.4  | 5.6±0.8         | 0.13            | 0.9766 |
| CA        | -         | 41.8±13.4  | 7.0±1.0         | 0.17            | 0.9884 |
| CA        | GSH       | 41.6±7.8   | 9.1±0.8         | 0.22            | 0.9958 |

Apparent affinity constant ($K_m$), maximal velocity of transport ($V_{max}$) and efficiency of transport ($E_T$) of OATP8/1B3-mediated uptake by *Xenopus laevis* oocytes. Kinetic parameters were calculated by fitting the values of OATP8/1B3-mediated uptake in the presence or absence of 20 mM GSH or 10 µM GCA as activator to a Michaelis-Menten equation. Values are means ± SD. $^a$, $P<0.05$, as compared with uptake in the absence of GSH by the paired t-test.
Figure 1
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A

Wild Oocytes

OATP8/1B3 mRNA-Injected

Total Uptake (pmol/n/oocyte)

E217βG CA GCA TCA TDCA TCDCA TUDCA

* * *

B

OATP8/1B3-Mediated Uptake (pmol/n/oocyte)

E217βG CA GCA TCA TDCA TCDCA TUDCA

† †
Figure 4
Briz et al.
Figure 7
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**UPTAKE**

- Total
- OATP8/1B3-Mediated
- Non-specific

**S = GSH Concentration (mM)**

**V = GSH Uptake (nmol/15 min/oocyte)**

**A**

**B**

Without TCA

With 10 µM TCA
Figure 8
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A

TCA

$V/[GSH]_{pH}$

$n_H = 1$

$r = 0.9487$

B

TCA

$V/[GSH]_{pH}$

$n_H = 2$

$r = 0.9657$

C

TCA

$V/[GSH]_{pH}$

$n_H = 3$

$r = 0.8835$

D

GCA

$V/[GSH]_{pH}$

$n_H = 1$

$r = 0.9121$

E

GCA

$V/[GSH]_{pH}$

$n_H = 2$

$r = 0.9841$

F

GCA

$V/[GSH]_{pH}$

$n_H = 3$

$r = 0.9144$

V = Bile Acid Uptake (pmol/15 min/oocyte)
| Loading | Efflux |
|---------|--------|
| GSH     | +      |
| OATP8/1B3 | Wild |

### Figure 9

**A**

- Graph depicting TCA content (pmol/ococyte) over time (min) for loading and efflux.
- Comparison of GSH and OATP8/1B3 Wild with +GSH effect.
- Data points marked with asterisks (*) indicate significant differences.

**B**

- Graph showing GSH content (nmol/ococyte) over time (min) for loading and efflux.
- Comparison of OATP8/1B3 Wild with +GSH effect.
- Error bars showing variability in data points.
Figure 10
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**A**

GSH (○, [ ) and Inulin (○, [ ) Content (% of injected)

- **Wild**
- **OATP8/1B3**

Time (min)

0 10 20 30 40 50 60

**B**

TCA Content (% of injected)

- **Wild (TCA)**
- **Wild (TCA+GSH)**
- **OATP8/1B3 (TCA)**
- **OATP8/1B3 (TCA+GSH)**

Time (min)

0 10 20 30 40 50 60
\[ y = 4.76x \]

\[ R = 0.8678, n=48; p<0.001 \]
Oatp8/1B3-mediated cotransport of bile acids and glutathione. An export pathway for organic anions from hepatocytes?

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