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Journal of Hepatology 44 (2006) 768–775

Journal of Hepatology

www.elsevier.com/locate/jhep

Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides

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***Background/Aim*: Multidrug Resistance Protein 3 (MRP3) transports bile salts and glucuronide conjugates in vitro and is postulated to protect the liver in cholestasis. Whether the absence of Mrp3 affects these processes in vivo is tested.**

***Methods*: Mrp3-deficient mice were generated and the contribution of Mrp3 to bile salt and glucuronide conjugate transport was tested in (1): an Ussing-chamber set-up with ileal explants (2), the liver during bile-duct ligation (3), liver perfusion experiments, and (4) in vitro vesicular uptake experiments.**

***Results*: The *Mrp3*$^{(-/-)}$ mice show no overt phenotype. No differences between WT and Mrp3-deficient mice were found in the trans-ileal transport of taurocholate. After bile-duct ligation, there were no differences in histological liver damage and serum bile salt levels between *Mrp3*$^{(-/-)}$ and WT mice, but Mrp3-deficient mice had lower serum bilirubin glucuronide concentrations. Glucuronide conjugates of hyocholate and hyodeoxycholate are substrates of MRP3 in vitro and in livers that lack Mrp3, there is reduced sinusoidal secretion of hyodeoxycholate-glucuronide after perfusion with hyodeoxycholate.**

***Conclusions*: Mrp3 does not have a major role in bile salt physiology, but is involved in the transport of glucuronidated compounds, which could include glucuronidated bile salts in humans.**
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***Keywords*: Cholestasis; Liver; Bilirubin; Enterohepatic circulation; Multidrug resistance**

1. Introduction

Members of the ATP-binding cassette (ABC) family of transporters are large membrane proteins that couple the energy released from ATP hydrolysis to active transport of a substrate across the membrane [1]. The ABCC subfamily contains nine transporters, the Multidrug Resistance Proteins 1–9 (MRP1–9) [2–4]. MRPs transport organic anions with broad substrate specificity but their physiological roles are still not fully defined. An example is MRP3, which in humans localizes to the basolateral membranes of epithelial cells from the gut, liver, pancreas, kidney, and the adrenal gland [5–7].

Initial studies on MRP3 focused on the liver. The basal hepatic levels of Mrp3 in rats are very low, but high levels are found in the naturally occurring Mrp2-deficient rat strains and in livers of Dubin–Johnson patients [7–10].

Received 13 May 2005; received in revised form 7 July 2005; accepted 11 July 2005; available online 9 August 2005

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Abbreviations ABC, ATP-binding cassette; Asbt, apical sodium-dependent bile salt transporter; BDL, bile-duct ligation; HC, hyocholate; HDC, hyodeoxycholate; K_m, Michaelis constant; MRP, multidrug resistance protein; PXR, pregnane X receptor; V_{max}, maximal velocity.

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doi:10.1016/j.jhep.2005.07.022

Moreover, treatments that induce cholestasis (e.g. common bile-duct ligation; BDL) also result in a substantial induction of hepatic Mrp3 in rats [9–12], and to some induction in mice as well [13].

A possible explanation for these findings comes from transport assays with rat Mrp3 [14], which showed that Mrp3 transports glycocholate, taurocholate, and taurolithocholate-3-sulfate, the latter two with high affinity. This has led to the suggestion that Mrp3 may be important in the absorption of bile salts in the terminal ileum [15] and in protecting the liver under conditions of impaired bile flow, by transporting toxic organic anions (e.g. bile salts) across the sinusoidal membrane into the circulation for subsequent excretion in the urine [12,16]. It is doubtful whether this would also apply to humans, however, as human MRP3 transports glycocholate and taurocholate with low affinity [16–18]. Mice with a homozygous disruption of *Mrp3* were generated by embryonic stem cell technology to study the role of Mrp3 in bile salt physiology. The characterization of these *Mrp3*$^{(-/-)}$ mice is reported here, extending a preliminary report on these mice [19].

2. Methods

2.1. Animals

The generation of *Mrp3*$^{(-/-)}$ mice is described in online Supplementary material. Mouse stocks were maintained as a cross of FVB/129Ola (50%/50%). Male mice were used in the experiments described. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

2.2. Generation and characterization of antibodies against mouse Mrp3

A fragment of mouse Mrp3 corresponding to amino acids 818–952 was cloned by RT-PCR from liver RNA into the pMalC expression plasmid. Fusion proteins were isolated as previously reported [20]. Serum of a rabbit injected with the fusion protein was collected after four boosts and called A66. The polyclonal serum recognizes mouse and rat Mrp3, but not human MRP3 (not shown).

2.3. Immunoblotting and Immunohistochemistry

Preparation of tissue lysates, size-fractionation on SDS-polyacrylamide gel and blotting were done as described [21]. Mrp3 was detected with rabbit polyclonal serum A66 (1:250) followed by a goat anti-rabbit horseradish-peroxidase conjugate (1:10,000) and visualized with chemiluminescence (ECL, Amersham Pharmacia Biotech). Mrp2 and α-tubulin were detected as described previously [22,23].

Cryosections (4 μm) were air dried overnight and fixed for 7 min in acetone at room temperature for immunohistochemistry. Small intestine was incubated with A66 antiserum (1:1000) followed by incubation with biotin-labeled swine anti-rabbit serum (Dako, Copenhagen, Denmark; 1: 300) and streptavidin-HRP (Dako, Copenhagen, Denmark; 1:500). Immunohistochemistry of other tissues (colon, liver, lung, pancreas) was performed by incubation with A66 antiserum (1:200) followed by HRP-labeled swine-anti-rabbit antiserum (1:200). In control stainings, the primary antibodies were replaced by appropriate normal sera.

2.4. Trans-ileal taurocholate transport experiments and determination of total fecal bile salt excretion

Terminal ileum sections were mounted in an Ussing chamber as described [24]. Experiments were initiated by adding taurocholate (1 mM) to the mucosal or serosal compartment. Samples from both compartments were taken over time, taurocholate concentrations measured spectrophotometrically with 3α-hydroxysteroid dehydrogenase [25] and the mean taurocholate transport rate calculated.

Mrp3$^{(-/-)}$ and WT mice were housed individually in metabolic cages and the total feces produced during 24 h was collected to determine total fecal bile salt excretion. Fecal bile salt content was analyzed as described previously [26].

2.5. BDL experiments

Animals were anesthesized with Hypnorm (1.75 ml/kg)/Dormicum (1.75 ml/kg); the common bile duct was ligated and the gall bladder removed. After 3 or 7 days, mice were anesthesized with methoxyflurane and sacrificed by heart puncture collection of blood. Tissues were collected and frozen in liquid nitrogen or fixed in ethanol/acetic acid/formaldehyde in 0.9% NaCl (40:5:10:45) for further immunoblot and immunolocalization studies. Serum levels of liver enzymes and bilirubin were determined on a Hitachi 911 analyzer. Serum levels and species of bile salts were determined by mass spectrometry as described [27].

2.6. Preparation of [^{3}H]-labeled hyocholate- and hyodeoxycholate glucuronides (HC/HDC-GlcA)

[^{3}H]-HC-GlcA and [^{3}H]-HDC-GlcA were enzymatically synthesized with specific activities of 0.31 μCi/μmol in a modification of the synthesis of [^{14}C]-labeled bile salt glucuronides [28]. In short, a reaction mixture containing 2 mg of microsomal protein from human organ donor liver [29], 0.3 mM HC or HDC, 1 mM UDP-GlcA, 10 μCi UDP glucuronic acid [1-^{3}H] (15 Ci/mmol, Bio Nuclear AB, Bromma, Sweden), 5 mM $MgCl_2$ and 0.1 M imidazole–HCl, pH 6.8, in a total volume of 10 ml was incubated for 60 min at 37 °C. Bile salt glucuronides were extracted from reaction mixtures with ODS cartridges, purified by anion exchange chromatography on Lipidex-DEAP, and characterized by electrospray-mass spectrometry and gas chromatography–mass spectrometry as described before [29]. Purities were 98–99%, yields were 7.7% ([^{3}H]-HC-GlcA) and 10.6% ([^{3}H]-HDC-GlcA). Conjugates with specific activities of 15 mCi/μmol were obtained by omitting unlabeled UDP-GlcA in the reaction mixture.

2.7. Preparation of membrane vesicles and vesicular-transport assays

Control and human MRP3-containing Sf9 membrane vesicles were prepared as described previously [16]. ATP-dependent transport of [^{3}H]-HC-GlcA and [^{3}H]-HDC-GlcA into control and MRP3-containing inside out membrane vesicles was measured using the rapid filtration technique as described previously [21].

2.8. In situ mouse liver perfusion with HDC

Mouse surgery and liver perfusion were performed as described previously [30,31]. HDC (dissolved in Krebs/bicarbonate buffer) was infused at a rate of 225 nmol/min into the inflowing catheter. Bile and perfusate were collected in fractions of 10 and 5 min, respectively, for a time period of 90 min and frozen at −20 °C until analysis. Samples were concentrated by solid phase extraction on an OASIS HLB cartridge (Waters, Milford, MA). Concentrations of taurine-conjugated HDC (THDC) and HDC-GlcA were determined by HPLC on an Omnispher column (Varian, Sint-Katelijne-Waver, Belgium) with Gynkotek pumps (Germering, Germany) using a flow of 0.7 ml/min. Gradient elution was performed using a mixture of 20 mM ammonium formate pH 3.5 and 100% acetonitrile: the %acetonitrile was increased from 28 to 48% during the total run time of 26 min. Peaks were quantified with

evaporative light scattering detection (Alltech Associates, Inc., Deerfield, IL) and identified by Mass Spectrometry. Calibration curves of taurocholate and taurochenodeoxycholate were used to quantify THDC and HDC-GlcA, respectively, because no standards for THDC and HDC-GlcA were available.

2.9. Etoposide sensitivity

Sensitivity of mice to etoposide was determined as previously described [32].

2.10. Statistical evaluation

Results are presented as means ± SD. Differences between groups were evaluated with a Student's *t*-test and a *P*-value <0.05 was considered significant.

3. Results

3.1. Generation of $Mrp3^{(-/-)}$ mice

Two independent properly targeted embryonic stem cell clones were obtained (see Supplementary Methods) and injected into mouse blastocysts. This resulted in chimeric mice that transmitted the $Mrp3^{-}$ allele through the germ line to F1 offspring (Supplementary Fig. 1b). Offspring from $Mrp3^{(+/-)}$ intercrosses were born at the expected Mendelian ratio indicating that there is no selection against the mutant allele during development. Absence of intact Mrp3 in $Mrp3^{(-/-)}$ mice was verified by immunoblotting with two independent rabbit polyclonal sera (Supplementary Fig. 1c and not shown).

The homozygous null mice were healthy, fertile and displayed no overt phenotype. Histological analysis of 3- and 12-month-old male and female mice revealed no differences between WT and $Mrp3^{(-/-)}$ mice (data not shown). In addition, analysis of blood clinical chemistry did not reveal alterations in the $Mrp3^{(-/-)}$ mice (see Supplementary Table 1).

Cells that overproduce MRP3 are resistant to the anticancer drug etoposide. However, no increased sensitivity of $Mrp3^{(-/-)}$ mice for this drug was found at concentrations up to 120 mg/kg, a dose which gave sub-lethal toxicity (e.g. weight loss) in WT mice (not shown). In contrast, 120 mg/kg etoposide is lethal to $Mrp1(-/-)$ mice [32]. Several other MRPs as well as MDR1 P-gp and BCRP transport etoposide and the lack of hypersensitivity in the $Mrp3^{(-/-)}$ mice probably reflects an overlap with these transporters in tissues where *Mrp3* is expressed.

3.2. Tissue distribution of Mrp3

In tissue samples, Mrp3 was detected throughout the gastrointestinal tract, liver, lung, pancreas, bladder, gall bladder and at low levels in the adrenal gland (Fig. 1A). The highest levels of Mrp3 were found in stomach, colon and liver. Mrp3 was not detected in the kidney, brain and testis (Fig. 1A). In the tissues where Mrp3 was present, its absence in the $Mrp3^{(-/-)}$ mice was confirmed (not shown). Additionally, localization of Mrp3 in several of these tissues was observed (Fig. 1B). In pancreas, Mrp3 is localized to pancreatic ductal cells, as was seen for human

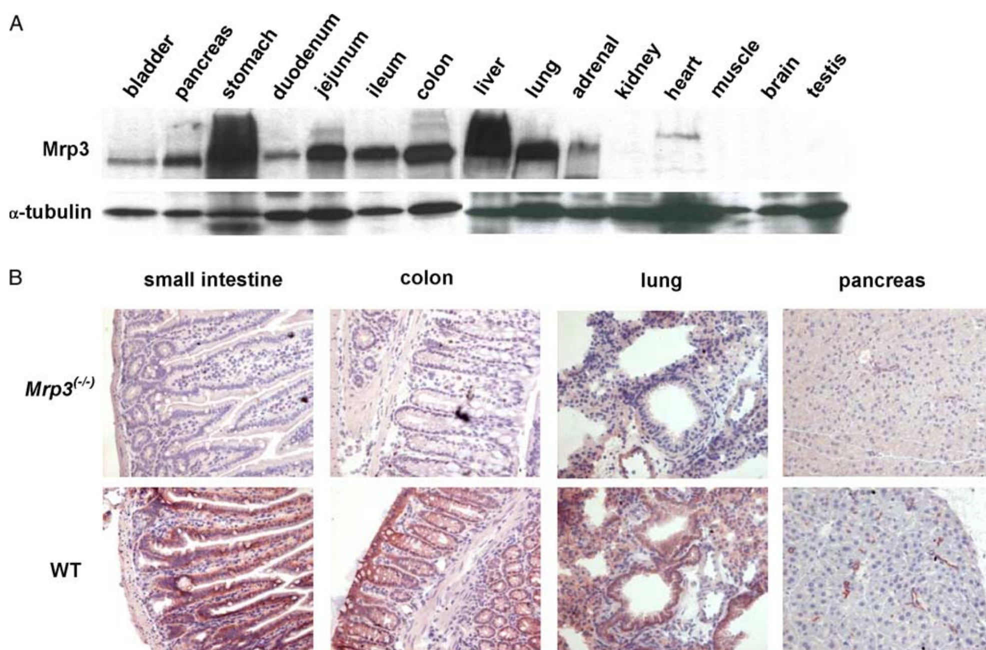

Fig. 1. Tissue distribution of Mrp3 in mice. (A) Detection of Mrp3 in total tissue lysates (30 μg protein per lane) by immunoblot analysis. α-Tubulin was used as loading control. (B) Immunolocalization of Mrp3 in tissue sections of WT and $Mrp3^{(-/-)}$ mice.

MRP3 [7]. In mouse lung Mrp3 was readily detected, even though Scheffer et al. [7] did not find MRP3 in human lung. In the small intestine and colon, murine Mrp3 is localized to the basolateral membrane of enterocytes (Fig. 1B), as recently also demonstrated for rat Mrp3 [15]. Hepatic expression of rat Mrp3 is low, but is highly induced in several models of cholestatic liver disease [9–12]. In contrast to this, basal hepatic expression of Mrp3 in our mice, a cross of FVB and 129Ola (50%/50%), is high.

3.3. Trans-ileal transport of taurocholate and fecal bile salt excretion

Bile salts are taken up into enterocytes from the gut, predominantly at the terminal ileum via the apical sodium-dependent bile salt transporter (Asbt) [33,34]. As Mrp3 is localized to the basolateral membrane of enterocytes (Fig. 1B) and is able to transport bile salts, the possibility that Mrp3 represents the basolateral transporter using an Ussing chamber set-up with terminal ileum sections was tested. No difference in the trans-ileal rate of taurocholate (1 mM) transport from the luminal to serosal compartment was seen between WT and *Mrp3*$^{(-/-)}$ mice (Supplementary Fig. 2A). Transport of taurocholate in the opposite direction was minimal, i.e. <10% of the mucosal to serosal transport (not shown), demonstrating that the ileum sections were intact. Unexpectedly, a minor decrease in fecal bile salt excretion in Mrp3$^{(-/-)}$ mice was found (Supplementary Fig. 2B). However, this decrease is consistent with the absence of an essential role for Mrp3 in the uptake of bile salts from the gut.

3.4. BDL experiments with *Mrp3*$^{(-/-)}$ mice

A BDL model of obstructive cholestasis was used to investigate whether Mrp3 mediates bile salt efflux from the liver under conditions where normal bile flow is impaired. Histological inspection of 3- and 7-day BDL livers revealed a similar pattern of necrotic foci and hyperplasia of bile-duct epithelium in livers of *Mrp3*$^{(-/-)}$ and control mice (not shown). Moreover, there was no difference in the level of hepatic marker enzymes in serum of 3- and 7-day BDL *Mrp3*$^{(-/-)}$ and WT mice (Supplementary Table 2). Levels of hepatic Mrp2 in untreated mice were not different between WT and *Mrp3*$^{(-/-)}$ mice (not shown). Three days of BDL resulted in a 50% reduction in hepatic Mrp2 levels, but no substantial increase in hepatic Mrp3 level (Fig. 2). No substantial induction of Mrp3 was seen in immunoblot analysis after 7 days of BDL either (not shown).

BDL resulted in the expected large increase in serum levels of bile salts in our mice, but no difference between the *Mrp3*$^{(-/-)}$ and control mice was seen (Fig. 3A). Additionally, no differences in bile salt species in the serum of 3-day BDL mutant and WT mice were observed (not shown). However, a difference in serum levels of conjugated bilirubin after 3 days of BDL was observed. The levels of conjugated bilirubin in the mutant mice were only ~60% of those in the control group (Fig. 3B), suggesting that part of the bilirubin formed in hepatocytes is transported to the blood by Mrp3.

3.5. Vesicular uptake of glucuronidated bile salts

Our finding that serum levels of bilirubin glucuronide are lower in the knockout mice prompted us to test whether glucuronidated compounds that are formed during cholestasis are Mrp3 substrates. In humans, cholestasis results

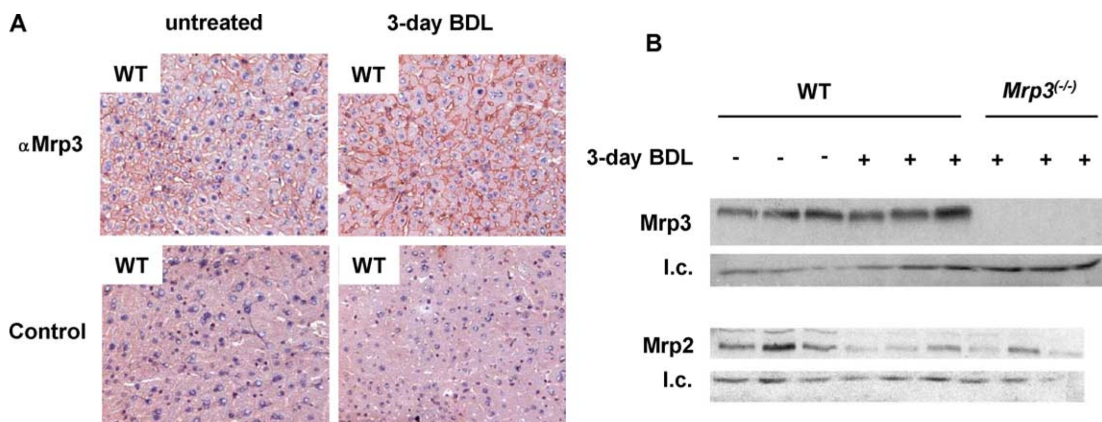

Fig. 2. Detection of Mrp3 and Mrp2 in BDL livers. (A) Mrp3 was analyzed in liver sections from untreated or 3-day BDL WT mice as indicated in the figure. Control stainings in which the αMrp3 antiserum was replaced by normal rabbit serum are shown in the lower panels. (B) Total liver lysates from untreated WT, 3-day BDL WT, and 3-day BDL *Mrp3*$^{(-/-)}$ ($n=3$ mice per group) were prepared and 40 μg protein loaded per lane. Mrp3 and Mrp2 were detected as described in methods. L.c, Gel loading was controlled by ponceau staining of blotted membranes (not shown) and with a non-specific cross-reacting band.

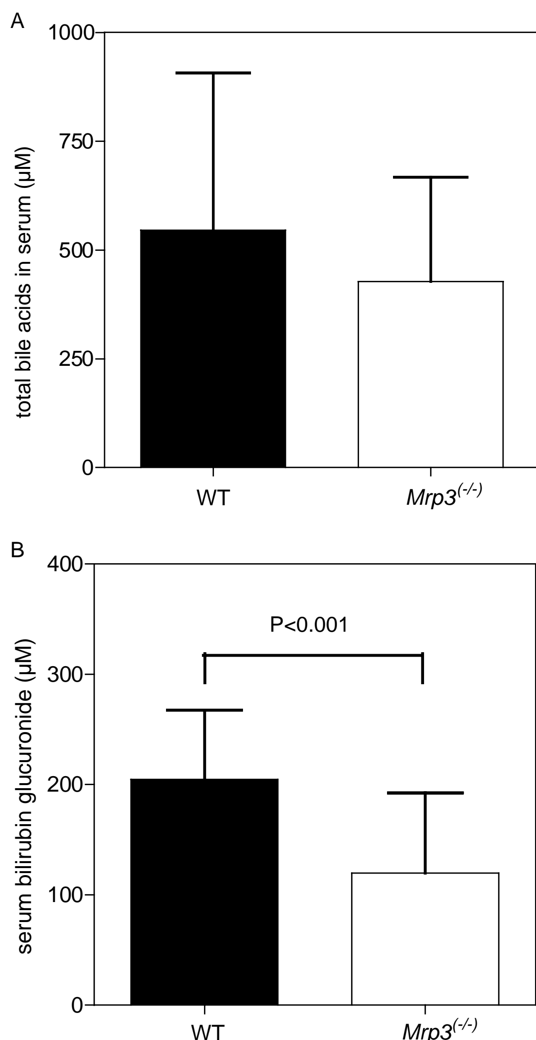

Fig. 3. Serum levels of total bile salts and bilirubin glucuronide after 3 days of BDL in WT and *Mrp3*$^{(-/-)}$ mice. Serum from BDL mice was collected after 3 days. (A) Total serum bile salts ($n=10$ mice per group). The serum bile salt levels of untreated WT and *Mrp3*$^{(-/-)}$ were 1.6 ± 0.9 and 1.9 ± 1.2 μM, respectively ($n=5$, $P=0.65$). (B) Serum concentrations of bilirubin glucuronide. Each bar and error represent the mean ± SD of the total serum bilirubin glucuronide concentration ($n=$ 15 mice per group). The serum bilirubin levels of untreated WT and *Mrp3*$^{(-/-)}$ are not different (see Supplementary Table 1).

in increased bile salt glucuronidation [35]. Using membrane vesicles prepared from Sf9 insect cells, it is found that human MRP3 transports the glucuronidated (secondary) bile salts HDC-GlcA and HC-GlcA in a time-, concentration- and ATP-dependent manner (Fig. 4). ATP-dependent transport followed simple Michaelis–Menten kinetics and the compounds were transported by MRP3 with K_m and V_{max} values of approximately 0.7 ± 0.1 μM and 0.4 ± 0.02 nmol/mg per min (HDC-GlcA) and 0.2 ± 0.04 μM and 0.2 ± 0.02 nmol/mg per min (HC-GlcA). Both the affinity and the catalytic efficiency (V_{max}/K_m) for the two glucuronidated bile salts are the highest reported for any MRP3 substrate to date. We may even have underestimated the V_{max}, as the rate of uptake diminishes very rapidly with time (Fig. 4A and B), making it difficult to determine initial transport rates.

3.6. HDC liver perfusion experiments

In contrast to humans, mice do not form HDC and HDC-GlcA in serum of BDL mice was not detected. To test whether mouse Mrp3 also transports HDCA-GlcA, mouse livers were perfused with HDC. This resulted in the conjugation of HDC to a taurine (94%) or glucuronate (6%) group, and the appearance of both conjugates in bile and perfusate. The absence of Mrp3 only affected the sinusoidal (perfusate) excretion of HDC-GlcA, whereas the canalicular (biliary) excretion of THDC and HDC-GlcA, and the sinusoidal excretion of THDC were not affected (Fig. 5A and B), consistent with the basolateral localization of Mrp3. Moreover, these results show that hepatic Mrp3 also transports glucuronidated bile salts in vivo, thereby influencing their plasma levels.

4. Discussion

Our results with the *Mrp3*$^{(-/-)}$ mouse indicate that Mrp3 does not contribute substantially to bile salt physiology in agreement with the preliminary analysis of our mice by Zelcer [19] and a very recent report on an independently generated *Mrp3*$^{(-/-)}$ mouse by Belinsky et al. [36]. The absence of a role for Mrp3 in intestinal bile salt transport is in line with a recent report that Ostα/Ostβ is a basolateral bile salt carrier in transfected epithelial cells and is coexpressed in ileal enterocytes with the apical bile salt transporter, Asbt [37].

The 3-day BDL treatment of our mice results in a substantial increase in serum levels of bile salts and hepatic marker enzymes that is similar in the *Mrp3*$^{(-/-)}$ and control mice (Fig. 3A and Supplementary Table 2). The urinary excretion of bile salts after BDL also was not different between *Mrp3*$^{(-/-)}$ and control mice (not shown). It is concluded from these results that in our cholestatic mice Mrp3 does not contribute to sinusoidal bile salt efflux. Obviously, this does not exclude a role for MRP3/Mrp3 in the sinusoidal efflux of bile salts in other species, as there are species-specific differences in the affinity of MRP3/Mrp3 for bile salts [14,16] as well as species-specific differences in the composition of the bile salt pool [38].

Whereas a high basal level of Mrp3 in mouse liver and no induction of Mrp3 during BDL were found (Fig. 2), Bohan et al. [13] report that a 2-week BDL treatment in mice resulted in a 6-fold increase in hepatic Mrp3 levels that proceeded through a TNFα-dependent upregulation of Liver Receptor Homologue 1 (Lrh1). Mrp3 levels correlated with serum bile salt levels and from this the authors concluded that Mrp3 has a major role in the basolateral bile salt clearance after BDL. However, repression of Lrh1-mediated transcription in BDL is likely to induce numerous hepatic

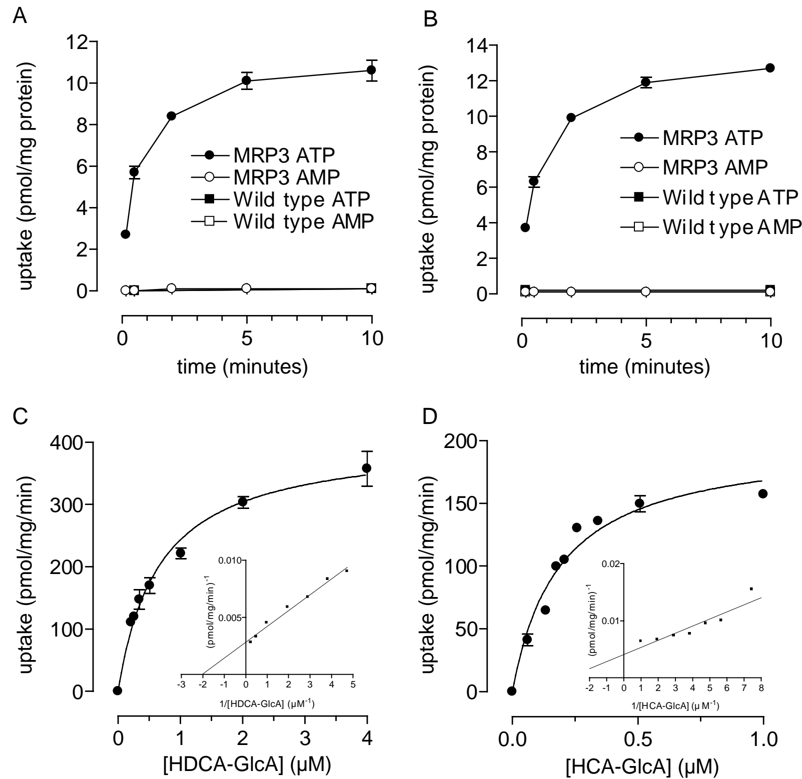

Fig. 4. Time-, concentration- and ATP-dependent transport of HDC-GlcA and HC-GlcA by MRP3. Membrane vesicles containing MRP3 (circles) or control membranes (squares) from Sf9 insect cells were incubated at 37 °C in the presence of 4 mM ATP (closed symbols) or 4 mM AMP (open symbols). (A, B) Time-dependent transport of HDC-GlcA and HC-GlcA, respectively. (C, D) Concentration-dependent transport of HDC-GlcA and HDC-GlcA, respectively. Note that lines representing transport in WT vesicles and in MRP3-containing vesicles in the presence of AMP overlap and are therefore not visible separately. Insets represent Lineweaver–Burk transformations of the presented data. Values are presented as means ± SD.

gene alterations as Lrh1 is a key hepatic nuclear receptor. The discrepancy in Mrp3 upregulation after BDL may be due to differences in experimental set-up between both studies. Bohan et al. [13] used a BDL treatment of 14 days whereas our studies did not extend beyond 7 days. Moreover, mice of a mixed FVB/129 Ola (50/50%) genetic background were used whereas Bohan et al. [13] used C57BLJ mice, which might influence the endogenous hepatic Mrp3 levels. Possibly, the FVB/129 Ola mice have a high basal expression of Mrp3, preventing it from being further induced.

Whereas in mice no substantial amounts of bile salt glucuronides were detected in urine under conditions of BDL (data not shown), in humans cholestasis results in the increased formation and urinary excretion of glucuronidated bile salts [35,39]. This pathway is almost specific for 6α-hydroxylated bile salts such as HC and HDC [28,35,40,41], which are formed in the liver from hepatotoxic lithocholate and chenodeoxycholate [42,43] by PXR-dependent activation of CYP3A [44,45]. In humans, administration of rifampicin, the prototype inducer of Cyp3A4 resulted in significantly enhanced excretion of HC- and HDC-6β-glucuronides in urine [46]. Our vesicular uptake studies demonstrated that MRP3 can transport HDC-GlcA and HC-GlcA, both with high affinity. The transport of HDC-GlcA by MRP3/Mrp3 was further confirmed by the HDC liver perfusion experiments, in which the absence of Mrp3 resulted in lowered sinusoidal excretion of HDC-GlcA. The percentage of HDC that was conjugated to glucuronic acid in mouse liver was low, possibly due to species-specific bile salt metabolism [38]. The detoxification pathway initiated by 6α-hydroxylation that is followed by glucuronidation and subsequent excretion is important for protection of the human liver from hepatotoxic bile salts (e.g. lithocholate) [40,42]. We speculate that MRP3/Mrp3 directs these glucuronidated bile salts to the bloodstream for subsequent urinary excretion.

Although our results with the *Mrp3*$^{(-/-)}$ mice indicate that Mrp3 does not play a role in the transport of major bile salts, Mrp3 does contribute to the sinusoidal excretion of glucuronidated compounds. In humans it may protect the liver during cholestasis from the toxic accumulation of hepatotoxic bile salts. Glucuronidation is also a major route for the detoxification of drugs in the liver and one would expect the absence of Mrp3 to affect excretion of such glucuronosyl derivatives via the urine. Indeed, in recent experiments it is found that *Mrp3*$^{(-/-)}$ mice are unable to secrete morphine-3-glucuronide from the liver resulting in a major alteration in morphine pharmacokinetics [47].

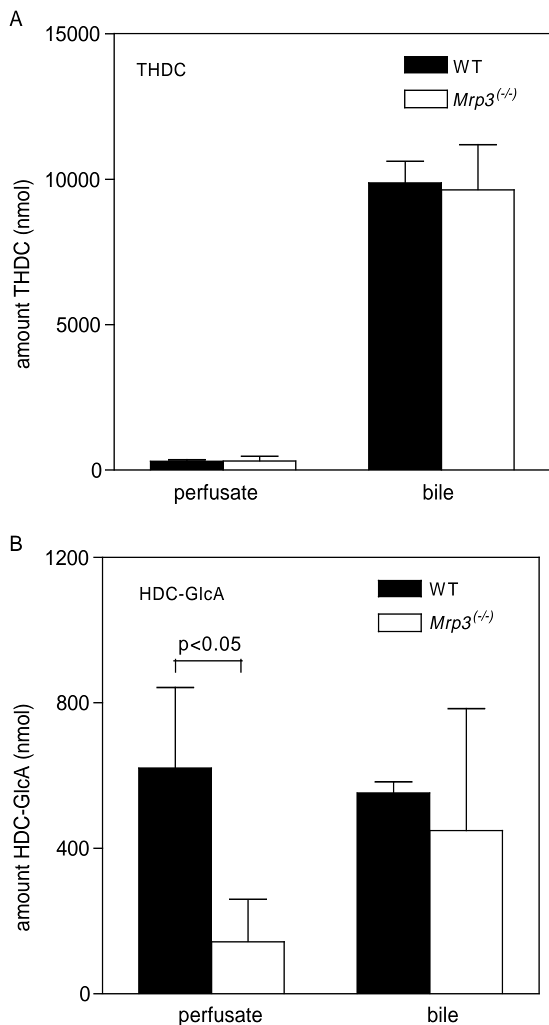

Fig. 5. Cumulative excretion of HDC-GlcA and THDC in perfusate and bile after perfusion of mouse liver with HDC. Livers of WT and *Mrp3*$^{(-/-)}$ mice (three mice per group) were perfused with Krebs-bicarbonate buffer supplemented with 225 nmol HDC per min and the cumulative excretion of THDC (A) and HDC-GlcA (B) in perfusate and bile was determined as described in Section 2.

Acknowledgements

We thank members of the Borst lab for advice and assistance throughout this study and Dr Wim Nooijen of the clinical chemistry lab for analyzing mouse serum. We are grateful for the excellent biotechnical assistance of A.J. Schrauwers. This study was supported in part by grants of the Netherlands Organization for Scientific Research (NWO program 912-02-073) and of the Dutch Cancer Society (NKI 2001-2474).

Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2005.07.022

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