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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.**

***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].

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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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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