Lack of biliary lipid excretion in the little skate, Raja erinacea, indicates the absence of functional Mdr2, Abcg5, and Abcg8 transporters

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Oude Elferink, Ronald P. J., Roelof Ottenhoff, Gert Fricker, David J. Seward, Nazzareno Ballatori, and James Boyer. Lack of biliary lipid excretion in the little skate, Raja erinacea, indicates the absence of functional Mdr2, Abcg5, and Abcg8 transporters. Am J Physiol Gastrointest Liver Physiol 286: G762–G768, 2004. First published December 30, 2003; 10.1152/ajpgi.00424.2003.—The ABC transporters bile salt export pump (BSEP; encoded by the ABCB11 gene), MDR3 P-glycoprotein (ABCB4), and sterolin 1 and 2 (ABCG5 and ABCG8) are crucial for the excretion of bile salt, phospholipid, and cholesterol, respectively, into the bile of mammals. The current paradigm is that phospholipid excretion mainly serves to protect membranes of the biliary tree against bile salt micelles. Bile salt composition and cytotoxicity, however, differ greatly between species. We investigated whether biliary phospholipid and cholesterol excretion occurs in a primitive species, the little skate, which almost exclusively excretes the sulphated bile alcohol scymnolsulphate. We observed no phospholipid and very little cholesterol excretion into bile of these animals. Conversely, when scymnolsulphate was added to the perfusate of isolated mouse liver perfusions, it was very well capable of driving biliary phospholipid and cholesterol excretion. Furthermore, in an erythrocyte cytosis assay, scymnolsulphate was found to be at least as cytotoxic as taurocholate. These results demonstrate that the little skate does not have a system for the excretion of phospholipid and cholesterol and that both the MDR3 and the two half-transporter genes, ABCG5 and ABCG8, have evolved relatively late in evolution to mediate biliary lipid excretion. Little skate plasma membranes may be protected against bile salt micelles mainly by their high sphingomyelin content.

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Abcg5

ing excretion of both lipids in mouse livers that express

we studied the effect of scymnolsulphate on phospholipid

malian bile, we investigated whether perfusing skate liver with

hydroxyl groups. Because this bile salt is not found in mam-

same nucleus as cholate but bears a longer side chain with three

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little skate secretes scymnolsulphate into bile, almost exclu-

Bile formation in this animal has been well studied in the past

200 million years ago. Various types of bile salts and bile alcohols

exist in the animal kingdom, some of which are much less

cytotoxic than the hydrophobic mammalian bile salts (12). The

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MATERIALS AND METHODS

Skate liver perfusion. Male little skates (Raja erinacea, 0.7–1.2 kg

body wt) were collected by net from Frenchman’s Bay in Maine and

maintained for up to 4 days in tanks equipped with flowing sea water

(15°C) at the Mount Desert Island Biological Laboratory (Salsbury

Cove, ME). Livers were removed from the skates and perfused in an

erythrocyte-free, recirculating perfusion system at 15°C as previously

described (20, 22). The perfusion medium consisted of well-oxygen-

ated, heparinized elasmobranch Ringer solution containing 5 mM

urea and 5 mM HEPES/TRIS (pH 7.5). The bile duct was cannu-

lated with a 17-cm segment of polyethylene tubing (PE-90). Because

the proximal gallbladder and cystic duct are intrahepatic and cannot

be ligated, the cystic duct was excluded by inserting a plug at the neck

of the gallbladder through an incision at the gallbladder apex. The

plug, which consisted of a plastic cap of an 18-G hypodermic needle

covered with two layers of Parafilm, was secured into the gallbladder

with sutures. Next, the collateral tributaries of the portal vein were

ligated, and the portal vein was cannulated with a 2- to 3-cm segment

of polyethylene tubing (PE-205) attached to an equal length of latex

tubing. After the portal vein was cannulated, the liver was flushed

with 40–50 ml of heparinized elasmobranch Ringer solution. The liver

was then excised and perfused at a rate of 30 ml/min, which produced

a perfusion pressure of ~2–4 cmH2O, which is optimal for bile

production and O2 consumption in the isolated perfused skate liver

(20); Liver weights of the animals were 26.0 ± 8.1 g (n = 25). The

first 150 ml perfusate were discarded after a single passage; subse-

sequently, a recirculating perfusion was performed with a reservoir

containing 100 ml of perfusion medium. The medium was continu-

ously filtered and aerated with humidified air. The filter upstream from

the perfusate reservoir consisted of a 200-μm silk-screen mesh

stretched over a small funnel, whereas the downstream filter was a

Millipore filter holder containing a prefiber (AP25–042–00) and a

1.2-μm filter (RAWP–047–00). Bile was collected in 30-min intervals

while a 0.5-ml sample was also taken from the perfusate. The

perfusion was carried out for 7 h; after 2 h, 5 μmol of the indicated

bile salts were added to the recirculating medium every 30 min (10

μmol/h). Bile volume was measured gravimetrically, assuming a

density of one. ICG or dibromosulphotalein (DBSP) were added after

1 h of perfusion at an initial concentration of 10 μM (1 μmol/liver).

Bile and perfusate samples were collected every hour for 8 h. ICG

concentrations were measured spectrophotometrically at 805 nm, after
dilution of the samples in elasmobranch Ringer containing 0.25%

bovine serum albumin. DBSP was measured spectrophotometrically

585 nm, after dilution with 0.1 M sodium pyrophosphate buffer (pH

8.2).

cDNA library construction and screening. RNA was isolated from

skate liver (R. erinacea) as described (6) and used for construction of

cDNA library in a ZAP expression vector (Stratagene). The library

was screened under low stringency with two probes that corresponded to

the killifish ABC region of the bile salt export pump (Bsep) as

previously described (6). PCR was used to identify DNA fragments of

appropriate size that were subsequently sequenced for identification of

ABC transporter orthologues.

Mouse liver perfusion. Male mice of FVB/N genetic background

were bred in our own colony (Academic Medical Center, The

Netherlands). Isolated liver perfusions were carried out in a recirculat-
ing fashion (exactly as described in Ref. 11). Bile samples were collected

in 10-min intervals. Ten minutes after the start of the perfusion, the

bile salt infusions were started and continuously infused in the

perfusion medium at a rate of 600 nmol-min−1·100 g body wt−1. Bile

salt, phospholipid, and cholesterol were determined using fluores-

ten enzymatic assays (as described in Ref. 17). In all experiments, bile

flow was measured by weighing the bile samples, assuming a specific

density of 1 g/ml.

Purification and analysis of scymnolsulphate. Scymnolsulphate

(Fig. 1) was purified from pooled gallbladder bile from spiny dogfish

(Squalus acanthias; basically following the procedure described in

Ref. 15). Briefly, lyophilized bile was dissolved in chloroform/meth-

anol/acetic acid (33:15:1) and subjected to consecutive silica gel 60

chromatographies. The final purification was performed by reverse-

phase adsorption chromatography using Serdolit PAD 1-resins (Serva

Electrophoresis, Heidelberg, Germany). Desorption was achieved by

washing the resins with methanol. By this procedure, 10 g lyophi-

lized dogfish bile yielded ~500 mg scymnolsulphate. The bile alcohol

was characterized by negative ion first atom bombardment mass spectrom-

etry (JEOL JMS-700, JEOL, Eching, Germany) and 13C-nuclear

magnetic resonance (360, MHz-spectrometer Aspect 3000; Bruker,

Karlsruhe, Germany). All bile salts were purchased from Sigma

Aldrich (St. Louis, MO). Bile salt-mediated cytolysis was performed

Fig. 1. Structural formulas of taurocholate (TC; A) and scymnolsulphate (B).
Table 1. Composition of skate bile compared with that of mouse bile

<table>
<thead>
<tr>
<th></th>
<th>Skate</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>Bile flow, µmol/h·g liver⁻¹</td>
<td>2.9±0.5</td>
<td>93±8.3</td>
</tr>
<tr>
<td>[Bile salt], mM</td>
<td>35.0±6.0</td>
<td>26.9±2.4</td>
</tr>
<tr>
<td>[Phospholipid], µM</td>
<td>11.5±4.2</td>
<td>1540±210</td>
</tr>
<tr>
<td>[Cholesterol], µM</td>
<td>2.3±1.6</td>
<td>220±30</td>
</tr>
</tbody>
</table>

Values are means ± SE. The lipid concentrations were determined in the first bile sample after cannulation. In the skate, this represents a 30-min sample, and in the mouse this is a 15-min sample. Bile salt concentration ([Bile salt]) is defined here as reaction equivalents in the enzymatic assay using 3-OH steroid dehydrogenase. [Phospholipid], phospholipid concentration; [cholesterol], cholesterol concentration.

exactly as described by Velardi et al. (26) using washed human erythrocytes. All our experiments on animals complied with guidelines of the institutional care and use committee. Statistical differences were analyzed by two-tailed Student’s t-test with unequal variance.

RESULTS

Endogenous bile secretion parameters in the little skate. Analysis of bile samples from the little skate (Table 1) revealed that these animals do not secrete significant amounts of phospholipid or cholesterol into bile. For comparison, the composition of mouse bile is given as well. Because bile flow rates in the skate are extremely low compared with the mouse, the values are given as biliary concentrations rather than as secretion rates. Using standards of known concentration (as determined by HPLC-mass spectrometry), we could show that sphingomyelin is quantitatively measured in our enzymatic assay with 3-OH steroid dehydrogenase. Although bile salt concentrations are comparable in bile from skate and mouse, the amounts of cholesterol and phospholipid are several orders of magnitude lower in skate bile. Analysis of skate gallbladder bile by thin-layer chromatography confirmed the absence of phosphatidylycholine and aminophospholipids, phosphotidylserine, and phosphatidylethanolamine. A small quantity of sphingomyelin was observed. The question therefore arose whether the skate expresses a functional phospholipid secretion system.

Skate liver perfusion with TC and TCDC. To investigate this, isolated skate livers were perfused with TC and TCDC, two different mammalian bile salts (Fig. 2). These bile salts were added to the perfusate at 2 h after the start of the perfusion at a rate of 10 µmol/h. As a control, perfusion was carried out without addition of bile salt. TC was efficiently secreted by perfused skate livers and maintained an increase in bile flow compared with livers that were not perfused with bile salt (Fig. 2A). At the highest secretion level, ~60% of the administered TC was secreted into bile (Fig. 2B). Note that there is a 2- to 3-h lag before the administered bile salts appear from the end of the biliary cannula because of the slow bile flow rates. TCDC was much less well secreted and led to a reduction in bile flow toward the end of the experiment. Neither of the two mammalian bile salts stimulated significant phospholipid secretion (Fig. 2C), which did not exceed 200 pmol·h⁻¹·g liver⁻¹. Hence the phospholipid-to-bile salt ratio in the little skate is >100-fold lower than in rodent liver. Cholesterol secretion was barely detectable in the control perfusions of skate livers (Fig. 2D). On perfusion with either TC or TCDC, the cholesterol concentration in skate bile tended to rise, but the values were variable and not significantly different from the control perfusion. In Fig. 3, the relationship between bile flow and biliary bile salt output is given for the skate (during TC perfusion) and for mice (during endogenous bile salt secretion). Please note that for skate, the data are expressed per hour and gram of liver, whereas for the mouse, the data are expressed per minute and 100 g body wt. When expressed as such, the relationships are quite similar. Because mice are ~25 g and have a liver of ~1 g, the bile salt output in mice is ~15 times higher than in the skate. Irrespective of the different rates, the water volume generated per micromole of bile salt is virtually identical (12 and 16 µl/µmol in skate and mouse, respectively, see equations in Fig. 3, A and B). When phospholipid output is plotted against bile salt output for both skate and mice (Fig. 3C), it becomes clear that there is no bile salt-driven phospholipid secretion in the skate. When the same is done for cholesterol (Fig. 3D), some cholesterol secretion is observed, but this is only slightly dependent on bile salt secretion. These data suggest that the skate does not have a
functional homolog of the mammalian Mdr2/MDR3 P-glycoprotein nor ABCG5/ABCG8, which is essential for biliary excretion of phospholipid and cholesterol, respectively.

This possibility was confirmed by RT-PCR of skate mRNA, which identified orthologs of Mdr1, Bsep, and Mrp2 (5, 6) but not for Mdr2, Abcg5, and Abcg8.

Mouse liver perfusion with scymnolsulphate. To assess whether scymnolsulphate is capable of driving phospholipid secretion in the presence of Mdr2 Pgp, we purified scymnolsulphate from gallbladder bile of the spiny dogfish and perfused mouse livers with the purified bile alcohol. In the perfused mouse liver, this bile alcohol was readily secreted (Fig. 4B), indicating that the mouse ABC transporters, Bsep and possibly Mrp2, recognize and transport this substrate. Secretion of scymnolsulphate caused substantial phospholipid secretion (Fig. 4C), demonstrating that in the presence of the canalicular phospholipid translocator Mdr2, scymnolsulphate is entirely capable of stimulating phospholipid secretion. In addition, scymnolsulphate elicited significant cholesterol secretion (Fig. 4D). In Fig. 5, the relationship between bile salt...
and phospholipid secretion was compared for scymnolsulphate and tauroursodeoxycholate in mice.

Cytoxicity of scymnolsulphate. We subsequently analyzed the cytoxicity of scymnolsulphate by incubation of human erythrocytes with increasing concentrations of the conjugated bile alcohol and measured the extent of cell lysis. Several known mammalian bile salts were analyzed in parallel, so that a direct comparison of cytotoxicity could be made. Washed erythrocytes were incubated with the indicated concentrations of scymnolsulphate, TC, tauroursodeoxycholate, and taurodeoxycholate (TDC), and cell lysis was assessed by centrifugation and measurement of the hemoglobin concentration in the supernatant (Fig. 6). Quite surprisingly, it was found that scymnolsulphate induced erythrocyte lysis at lower concentrations than TC. Hence, scymnolsulphate is more cytolytic than TC. Because the little skate lives at considerably lower temperatures than 37°C, we also repeated the experiment at 15°C. At this lower temperature, the cytotoxicity was considerably lower, but the relative cytotoxicity of the various bile salts remained the same (data not shown).

Biliary excretion of DBSP and ICG by perfused skate liver. Because Mdr2 is also thought to contribute to biliary excretion of hydrophobic organic compounds that bind to or partition into biliary vesicles or micelles [such as ICG (13)], additional studies measured biliary ICG excretion in the perfused skate liver and compared its excretion with that of another anionic dye, DBSP, which is a relatively hydrophilic substrate for the canalicular Mrp2 transport protein. ICG and DBSP were added to perfusate at the 1-h time interval, and 1 h later, <4% of the DBSP remained in the perfusate (<0.4 μM), whereas ~10–15% of the ICG (1.0–1.5 μM) remained, indicating a slightly faster clearance of DBSP. Given the slow bile flow rate in the skate, these dyes did not appear in collected bile until the third collection interval (from 1–2 h after administering the dyes; Fig. 7). DBSP was excreted into bile more efficiently than ICG such that over this collection interval the amount of DBSP in bile was four times that of ICG (Fig. 7). However, both of these compounds were concentrated in bile, indicating active hepatobiliary transport. DBSP reached its maximum biliary concentration at the seventh collection interval and started declining by the eighth hour, whereas ICG concentration continued to rise until the end of the experiment (Fig. 7).

DISCUSSION

The present study analyzed the ability of skate liver to secrete phospholipid and cholesterol. We observed that under basal conditions, the little skate excretes hardly any phospholipid and cholesterol into bile. The skate virtually excretes only lipid and cholesterol into bile. We therefore administered mammalian bile salts, which drive lipid excretion in mammals, to the perfusate of perfused skate livers. TC was readily secreted by skate livers, as we have shown previously for 3H-TC. (4).

More recently, the skate homolog of the BSEP was cloned and characterized; this transporter has similar affinities for TC and TCDC as the rat Bsep (6). Interestingly, TCDC, when
administered to the perfusate, was very poorly excreted; the amount of bile salt in bile was not significantly different from that in livers without bile salt. In addition, bile flow in TCDC-treated liver tended to be lower. This may indicate that the hydrophobic TCDC either is not easily transported by skate liver or that it could induce cholestasis. The first possibility is not very likely because TCDC was found to compete with TC for transport by skate liver plasma membrane vesicles (1). Hence, it seems likely that TCDC is also transported. The excretion of TC was not accompanied by lipid excretion, suggesting that the skate does not have a phospholipid translocating system, similar to the rodent Mdr2 or the human MDR3 P-glycoprotein. The possibility exists that, due to their physical properties, conjugated bile alcohols such as scymnosulphate are not able to extract phospholipid or cholesterol from the canalicular membrane. We therefore also investigated whether scymnosulphate could drive biliary lipid secretion in mice. Infusion of scymnosulphate in normal mice led to a strong excretion of the conjugated bile alcohol into bile. This again is not surprising, because it was recently shown that rat Bsep has a similar affinity for scymnosulphate as skate Bsep (6). In mice, scymnosulphate elicited lipid secretion of similar magnitude as TC. The scymnosulphate-driven phospholipid and cholesterol secretion in perfused mouse livers demonstrate that this conjugated bile alcohol is quite capable of extracting lipids from the membranes of mice liver. This adds evidence to the suggestion that the skate does not have the capability of translocating phospholipid. The surprising finding was that scymnosulphate was at least as cytotytic as TC. Mice have a bile salt pool that consists of 30–70% of TC, the remainder being taumuricholate. The latter bile salt is much less cytolytic than TC and comparable with tauroursodeoxycholate. This creates the rather paradoxical situation that mice, which have a less cytolytic bile salt pool than skate, are susceptible to bile salt-induced liver damage if they do not secrete lipids (as is the case in the Mdr2−/− model), whereas the skate does not seem to have this problem. Similar to the Mdr2−/− mouse, the little skate does not excrete significant amounts of lipid. Several possible explanations exist. The skate might excrete other, as yet unidentified, compounds that interact with micelles of scymnosulphate so as to reduce the phospholipid solubilizing capacity. We have not made an extensive search for phospholipids other than PC, PS, PE, and SM, by thin-layer chromatography. None of these phospholipids that are normally found were present in significant concentrations in skate bile, and only a minor quantity of sphingomyelin was detected. Alternatively, the outer leaflet of the skate canalicular membrane might be adapted in such a way that it can withstand the detergent effects of scymnosulphate. It has been reported (24) that skate liver membranes have an exceptionally high anisotropy, compared with rat membranes, when each is measured at their respective body temperatures. This indicates that skate liver membranes are exceptionally rigid, which could result from a relatively high sphingomyelin content. If this analysis of liver plasma membranes also pertains to the canalicular membrane, it would confer a stronger resistance of this membrane toward the bile alcohol.

We also found that cholesterol excretion into bile is very low in the skate, even on administration of TC or TCDC to the perfusate. Although it was thought for a long time that the excretion of these two lipids is highly coupled, several lines of evidence now indicate that this is not the case, at least in terms of their transport mechanisms. First, although cholesterol excretion in the Mdr2−/− mouse is nearly absent, this can be increased by infusion of more hydrophobic bile salts such as TC or taurodeoxycholate or by feeding the animals a diet containing cholate so as to increase the percentage of hydrophobic bile salt (18). In this situation, cholesterol excretion is induced, but phospholipid excretion remains absent and the excretion of the two lipids can be uncoupled. Second, it has recently been discovered that cholesterol excretion into bile is critically dependent on the expression of the two half-transporter genes *Abcg5* and *Abcg8* (27). Knockout mice for these two genes have a 10-fold reduced cholesterol excretion, whereas phospholipid excretion is not affected. Conversely, mice that overexpress *Abcg5* and *Abcg8* in the liver have a marked increase in biliary cholesterol excretion, again without a big change in phospholipid excretion (28). Hence, although the excretion of both phospholipid and cholesterol is exquisitely dependent on bile salt excretion, the translocation/extrusion mechanisms seem to be mediated by separate transporter proteins. The skate does not excrete cholesterol to any significant extent, even under conditions that drive phospholipid-independent cholesterol excretion in the mouse. This suggests that the skate, an evolutionarily ancient vertebrate, may not only lack an Mdr2 homolog but also the functional homologs of *Abcg5* and *Abcg8*. These important mammalian lipid transporters presumably developed later in evolution.

Evidence has been presented in the past to suggest that hydrophobic drugs such as ICG are also transported via Mdr2 Pgp, because biliary ICG excretion is impaired in *Mdr2−/−* mice. This phenomenon can, however, also be a secondary consequence of the absence of lipid in the bile of these animals, which acts as a sink for this hydrophobic drug. The presence of high concentrations of ICG in bile of the little skate, lacking an Mdr2 homolog, suggests that biliary elimination, at least for a large part, is achieved by active transporters other than Mdr2 Pgp.

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