The molecular mechanism of Atp8b1-deficiency
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Chapter 8

Summary and Perspectives
Summary

Progressive familial intrahepatic cholestasis (PFIC) is a severe form of cholestasis found in young children. Three types of PFIC are known, PFIC1-3, which are caused by mutations in ATP8B1, ABCB11, and ABCB4, respectively. All these genes code for transporter proteins necessary for proper bile formation. Impairment of either of these proteins results in reduced hepatobiliary bile salt transport and thus cholestasis. These diseases are progressive and eventually may lead to liver failure if the patients are not transplanted. This thesis focuses on the mechanism of ATP8B1-associated disease.

PFIC1 and BRIC1 (a mild form of PFIC1) are caused by mutations in the gene ATP8B1. The purpose of the research presented in this thesis was to study the pathophysiological mechanisms that underlie ATP8B1-associated disease. To study this, we have used a mouse model for PFIC1 disease, the Atp8b1<sup>G308V/G308V</sup> mouse.

An overview of bile salt synthesis, the mechanism of bile formation and proteins involved is given in Chapter One. In addition, a general review about inherited forms of intrahepatic cholestatic liver diseases is given.

Chapter Two describes the characterization of the Atp8b1<sup>G308V/G308V</sup> mice (129/SvImJ strain). The mice are homozygous for the most common PFIC1 mutation found in the Amish kindred, a point mutation which replaces the glycine at amino acid 308 with a valine. Patients homozygous for this mutation have decreased bile salt secretion and suffer from severe cholestasis and progressive liver disease. Although the animals did show increased serum bile salt levels, these mice have no impaired hepatobiliary bile salt excretion. Instead, these mice displayed enhanced biliary bile salt secretion compared to wildtype animals. Whereas wildtype mice developed cholestasis upon infusion of the hydrophobic bile salt taurodeoxycholate (TDC), Atp8b1 deficient mice maintained high bile salt output. In these experiments we observed enhanced hydroxylation of TDC to taurocholate in Atp8b1 deficient mice, which explains the protection of these mice against TDC-induced cholestasis. Feeding of a cholate supplemented diet led to a dramatic, but variable, increase in the serum bile salt concentration of mutant mice, whereas there was no effect in wild type mice. The conclusion at that time was that Atp8b1 deficiency affects bile salt homeostasis without impairment of bile secretion, and that the phenotype probably might be caused by higher uptake of bile salts in the intestine. This aspect was subsequently studied in more detail. In Chapter Three intestinal bile salt absorption was studied in the context of coprophagy. Coprophagy means that animals eat their feces. Wildtype and Atp8b1<sup>G308V/G308V</sup> mice were housed in metabolic cages (which precludes coprophagy) and were...
fed control or cholate-supplemented diets. Bile salt species were analyzed in portal and systemic blood. In case of a normal diet, coprophagy had little effect on the peripheral bile salt composition. In case of a cholate-supplemented diet, the amount of secondary bile salts in the peripheral and portal bile salt composition was substantially increased by coprophagy. So, coprophagy is an important factor in studies involving bile salt feeding. No distinct differences in portal or peripheral bile salt concentrations were observed between Atp8b1<sup>G308V/G308V</sup> and wildtype animals. These data suggested that there is no enhanced intestinal bile salt reabsorption in Atp8b1<sup>G308V/G308V</sup> mice.

Chapter Four presents data which demonstrate that the enhanced serum bile salt levels in Atp8b1<sup>G308V/G308V</sup> mice are not caused by enhanced intestinal reabsorption of bile salts. We studied intestinal bile salt transport by means of Ussing chamber experiments and intestinal bile salt perfusions in situ. We did not find an altered intestinal bile salt uptake in Atp8b1<sup>G308V/G308V</sup> mice compared to wildtype mice. Bile salt feeding resulted in enhanced serum bile salt levels in Atp8b1<sup>G308V/G308V</sup> mice, although intestinal bile salt uptake was decreased rather than increased. This study shows that Atp8b1<sup>G308V/G308V</sup> mice have a similar intestinal bile salt handling as wildtype mice.

The main conclusion of Chapter Five is that absence of Atp8b1 leads to an instable canalicular membrane of the hepatocyte. In Atp8b1<sup>G308V/G308V</sup> mice, infusion of taurocholate resulted in enhanced biliary output of cholesterol (two-fold) and canalicular ectoenzymes, compared to wildtype mice. Importantly, bile of Atp8b1<sup>G308V/G308V</sup> mice contained phosphatidylserine, an aminophospholipid that is normally confined to the cytoplasmic leaflet of the canalicular membrane. This phenotype depended on bile salt hydrophobicity since infusion of tauroursodeoxycholate caused none of these effects. Moreover, single-pass perfusion of the isolated liver with TDC resulted in a significantly reduced biliary output and a concomitant increase in hepatic retention of TDC. From these observations we hypothesized that Atp8b1 is important to reduce the outer leaflet content of phosphatidylserine to increase the relative sphingolipid content, which together with cholesterol forms a rigid, liquid-ordered membrane that is resistant towards detergent bile salts; Atp8b1-deficiency thus leads to loss of the normal phospholipid asymmetry of the canalicular membrane. As a result, the canalicular membrane becomes more sensitive to extraction of cholesterol by hydrophobic bile salts. We hypothesize that this causes impaired activity of ABCB11 and, therefore, cholestasis.

In Chapter Six we demonstrate that the increased biliary cholesterol output observed in Atp8b1<sup>G308V/G308V</sup> mice is not depending on the presence of the sterol transporter Abcg5/g8. To demonstrate this we generated double transgenic Atp8b1<sup>G308V/G308V</sup>/Abcg8<sup>−/−</sup> (GF) mice. Infusion
of TC in these GF mice resulted in the same phenotype as in Atp8b1\(^{G308V/G308V}\) mice, indicating that enhanced biliary cholesterol output was due to nonspecific extraction rather than enhanced Abcg5/g8 activity.\(^1\) These data support our theory that absence of Atp8b1 brings the canalicular membrane in a more liquid-disordered state which allows nonspecific extraction of cholesterol.

Mice lacking both Abcb4 and Atp8b1 were studied in Chapter Seven. The presence of Atp8b1 is not of great importance when Abcb4 is lacking as well, because the phenotype of the double mutant (MF) mice is less severe than that of Atp8b1\(^{G308V/G308V}\) mice: extraction of ectoenzymes is decreased in the MF animals compared to Atp8b1\(^{G308V/G308V}\) mice, as well as the fibrosis compared to Abcb4\(^{-/-}\) mice.\(^2\) From these data we hypothesize that the outer leaflet of the canalicular membrane is rather bile salt resistant despite the absence of Atp8b1. We propose a model in which PC flopping by Abcb4 brings the canalicular membrane in a more liquid-disordered state. Under normal conditions, this is counteracted by PS flipping by Atp8b1. In the absence of PC flopping, the action of Atp8b1 is less crucial.

**Perspectives**

Our studies show that the absence of Atp8b1 has dramatic effects on the proper function of the canalicular membrane. There is uncontrolled lipid and ectoenzyme extraction from the membrane by hydrophobic bile salts and biliary bile salt excretion is impaired. Studies in mice, however, have a major drawback because there is a substantial difference between mice and man at the level of bile salt homeostasis. This difference can be explained by the ability of mice to rehydroxylate hydrophobic bile salts. In this way, they alter the toxic bile salts into more hydrophilic ones, which are less strong detergents and cause less damage. Thus, our PFIC1 mouse model (the Atp8b1\(^{G308V/G308V}\) mouse) as well as our PFIC3 model (the Abcb4\(^{-/-}\) mouse) have much less severe phenotypes than their corresponding human patients. A way to circumvent this is to create a more “humanized” mouse, which lacks the ability of rehydroxylation. The enzymes responsible for this very efficient detoxification belong to the family of the cytochrome P450 system. However, the cytochrome P450 enzymes that mediate these hydroxylations are not properly identified and most likely include several P450 enzymes. Mice lacking the liver NADPH-cytochrome P450 reductase (Por) have a disrupted P450 system because of the absence of the obligatory electron donation by this enzyme to all P450 proteins.\(^3\) In mice this leads to a reduction in bile salt production of ~90%, because conversion of cholesterol to bile salts involves several P450 enzymes (e.g. Cyp7a1). The mice show a

\(^1\) Because Abcg8\(^{-/-}\) mice are on a C57Bl/6J background, all these experiments were done with mice on this background. The C57Bl/6J background gives rise to different parameters in bile formation (see Perspectives).

\(^2\) Because Abcb4\(^{-/-}\) mice are on a FVB background, all these experiments were done with mice on this background. The FVB background gives rise to different parameters in bile formation (see Perspectives).
decreased bile production. It would be interesting to study the phenotype of double mutant mice, harboring both the liver-specific Por disruption and the Atp8b1 mutation. Depending on the damage seen in the liver, it could be of interest as well to feed these animals a diet containing “human” bile salts. Thus, feeding hydrophobic bile salts will replace the murine bile salt pool for a more human bile salt pool and hopefully will lead to liver disease that resembles the pathology in PFIC1 patients.

Other mouse models may also be relevant for these studies. In 2007, van Herwaarden et al. published the first results with the Cyp3a mouse. All Cyp3a genes are knocked out in this mouse, leaving them with impaired detoxification capacity upon challenge with a chemotherapeutic agent. The composition of the bile salt pool in the Cyp3a mice should be studied and in case of reduced hydroxylation this suggests a role for one or more of the Cyp3a genes in (re)hydroxylation of bile salts. If this is the case, a double transgenic mouse for all Cyp3a genes and Atp8b1 could provide a powerful model for PFIC1.

Genetic background is an issue which should be addressed in this context. Most of the research was done with mice from the 129Sv/ImJ strain (chapter 2, 3, 4 and 5). For the purpose of producing double mutant mice we had to switch to C57Bl/6J (chapter 6) and FVB (chapter 7) mice. Although the Atp8b1 mutation remained the same, the physiology of the different backgrounds appeared to be different, as can be seen in table 1. Wildtype FVB mice show a low biliary bile salt output compared to the wildtype 129Sv/ImJ and C57Bl/6J mice. Since the biliary bile salt output is the driving force for bile formation, it is not surprising that the biliary cholesterol and phospholipid output are also low in wildtype FVB mice compared to the wildtype 129Sv/ImJ and C57Bl/6J mice. Notwithstanding these differences in bile formation, the effect of Atp8b1 deficiency is qualitatively the same in all strains.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>129Sv</th>
<th>C57Bl/6</th>
<th>FVB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type Atp8b1G308V/G308V</td>
<td>Wild-type Atp8b1G308V/G308V</td>
<td>Wild-type Atp8b1G308V/G308V</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>92 ± 8.0</td>
<td>225 ± 179</td>
<td>194 ± 165</td>
</tr>
<tr>
<td>Bile salt (total µmol/L)</td>
<td>2.0 ± 0.9</td>
<td>18.5 ± 29.8</td>
<td>2.16 ± 0.57</td>
</tr>
<tr>
<td><strong>Bile</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow (µL/min·100 g)</td>
<td>8.0 ± 1.6</td>
<td>12.4 ± 3.6</td>
<td>9.55 ± 2.60</td>
</tr>
<tr>
<td>Bile salt (nmol/min·100 g)</td>
<td>498 ± 278</td>
<td>868 ± 320</td>
<td>502 ± 254</td>
</tr>
<tr>
<td>Phospholipids (nmol/min·100 g)</td>
<td>58.5 ± 17.4</td>
<td>66.4 ± 27</td>
<td>56.9 ± 24.8</td>
</tr>
<tr>
<td>Cholesterol (µL/min·100 g)</td>
<td>4.2 ± 1.0</td>
<td>5.9 ± 3.7</td>
<td>2.2 ± 2.6</td>
</tr>
<tr>
<td>Liver weight (% of body weight)</td>
<td>3.8 ± 0.4</td>
<td>4.5 ± 0.5</td>
<td>4.6 ± 0.5</td>
</tr>
</tbody>
</table>

*Table 1. Comparison of plasma and bile parameters in wildtype and Atp8b1G308V/G308V mice of different strains. Bile parameters are measured in the first ten minutes of the depletion phase.*
Upon bile salt infusion, wildtype FVB animals have a higher biliary cholesterol output than wildtype C57Bl/6J animals (figure 1). This suggests that upon bile salt infusion, Abcg5/g8 secretes more cholesterol in the FVB animals than in the other backgrounds. This could explain the observation that, in contrast to Atp8b1<sup>G308V/G308V</sup> animals on the C57Bl/6J or 129Sv/ImJ background, Atp8b1<sup>G308V/G308V</sup> animals on an FVB background do not show enhanced biliary cholesterol excretion compared to wildtype mice upon bile salt infusion. We hypothesize that when the Abcg5/g8-dependent biliary cholesterol output is high, the aspecific extraction from the canalicular membrane is low and vice versa. To study this, protein expression levels of Abcg5 and Abcg8 should be determined in canalicular membranes from animals against the different backgrounds.

From the studies presented in this thesis it is clear that the Atp8b1<sup>G3038V/G308V</sup> mice have provided us with much information about the canalicular membrane. We have shown that proteins like Atp8b1, Abcb4 and Abcg5/g8 are of great importance in keeping the canalicular membrane intact and functioning properly. By use of the different mouse models, we were able to investigate the role of these proteins <i>in vivo</i>, leading to more insight in the pathophysiology of intrahepatic cholestasis. However, the mouse models did not show a phenotype as severe as the

![Figure 1. Ratio between biliary cholesterol output and bile salt output of wildtype FVB (grey squares), wildtype 129Sv/ImJ (white squares) and wildtype C57Bl/6J (black squares) mice upon TC infusion.](image-url)
ones seen in the patients. To test our theories, better mouse models are needed which resemble the human physiology.

In conclusion, the data described in this thesis have provided us with insights that are of great importance in understanding the function of the canalicular membrane and the pathophysiology of inherited and acquired forms of cholestasis.

Reference List