CHAPTER 4

CHOLIC ACID INDUCES A CFTR DEPENDENT BILIARY SECRETION AND LIVER GROWTH RESPONSE IN MICE

Submitted

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Abstract

Introduction
Cystic fibrosis (CF) mouse models do not display cirrhotic CF liver disease. We hypothesized that a cirrhotic CF liver disease equivalent may be provoked in CF mice by hydrophobic bile salt exposure.

Methods
We studied the effect of acute (intravenous) and chronic (dietary) cholate administration on bile formation, bile composition and histology in a CF mouse model.

Results
Basal bile production, i.e. without bile salt administration, was similar in Cftr−/− mice and controls. Intravenous taurocholate stimulated bile salt secretion and bile flow similarly in both genotypes. Cholate administration for 3 weeks also increased bile salt secretion similarly in Cftr−/− mice and controls. However, cholate administration for 3 weeks increased bile flow in Cftr−/− mice to a lower extend, resulting in a significantly higher biliary bile salt concentration, compared with controls. In controls, but not in Cftr−/− mice, chronic cholate increased both the biliary phospholipid-to-bile salt (molar) ratio and bile hydrophobicity. Cholate administration for 3 weeks did not induce liver histology analogous to cirrhotic cystic fibrosis liver disease in Cftr−/− mice. However, chronic cholate administration induced a liver growth (+52%, p<0.001) and parenchymal proliferation response (Ki67) in controls that was lacking in CF mice.

Conclusion
We conclude that chronic CA administration induces a Cftr dependent biliary secretion and liver growth and proliferation response in mice. Chronic CA exposure did not induce cirrhotic cystic fibrosis liver disease in CF mice. This response includes Cftr dependent cytoprotective changes in the bile composition. These findings point to a, potentially protective, Cftr dependent, hepatic response to prolonged hydrophobic bile salt exposure.
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INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the CFTR gene (1,2). Cystic fibrosis liver disease (CFLD) develops in 5-10% of cystic fibrosis patients (3). It is a serious complication of CF (4,5). CFLD is characterized by cirrhosis and patients often present with splenomegaly, hypersplenism and complications of portal hypertension, including variceal bleeding and ascites (6,7). Although the synthesis and excretory liver function are usually spared, in some CFLD patients liver transplantation can be indicated (8,9). Despite major advances in basic knowledge about cystic fibrosis, the pathogenesis of CFLD is still unexplained.

In the liver, CFTR is expressed exclusively at the apical membrane of the cholangiocytes lining the bile ducts (10,11). CFTR in cholangiocytes mediates water secretion, and controls the pH of bile (12,13). Consequently, loss of CFTR function may increase the concentration of potentially cytotoxic bile salts in bile and increase its viscosity, leading to occlusion of small bile ducts and, ultimately, obstructive biliary cirrhosis (14-16).

CF mice may show minor gallbladder and liver abnormalities that do not lead to loss of liver function (17-20). In general, mice produce bile that is relatively hydrophilic (21). Hydrophilic bile salts are considered less cytotoxic than hydrophobic ones (22,23). The hydrophilic profile of murine bile could prevent or mitigate liver disease in CF mice.

To test this hypothesis we sought to change the composition of murine bile to one containing more hydrophobic bile salts. To this end, we either acutely or chronically challenged CF mice with cholic acid (CA), a bile salt with strong detergent action or monitored effects on bile production, composition, serum alanine transaminase (ALT), liver morphology, and histology (23).

MATERIALS AND METHODS

Animals. We used C57Bl/6;129 Cfrtm1CAM mice and Cfrtm1CAM littermate controls. All mice were bred and accommodated at the Animal Experimental Center of the Erasmus Medical Center in Rotterdam, The Netherlands (17). Mice were housed in a light-controlled (lights on 6 AM to 6 PM) and temperature-controlled (21°C) facility, and had free access to tap water and a semi-synthetic diet (SRM-A; Hope Farms BV Woerden, The Netherlands) from the time of weaning. All experiments were performed with mice of 10-20 weeks of age. Group size varied per experiment from 5-7 mice per genotype. Experimental protocols were approved by the Ethical Committee for Animal Experiments of Erasmus MC. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Experimental procedures. To test our hypothesis we used a mouse gall bladder cannulation model (24,25). We first applied an acute bile salt infusion, as previously described (25). We infused taurine-conjugated cholate (TCA) (43 mM dissolved in phosphate-buffered saline, pH
7.4) via the jugular vein using an infusion pump. The bile salt dosage was increased every 30 minutes in a stepwise manner (dosage steps 0, 50, 300, 450 and 600 nmol.min⁻¹). During bile salt infusion, bile was collected in 15 minutes fractions.

Different groups of Cftr⁻/⁻ and control mice were either fed a control diet consisting of standard chow or the same diet enriched with cholate (CA 0.5% wt/wt) for 3 weeks. Additional experimental groups of Cftr⁻/⁻ mice and controls were fed the diet for 3 months. After gallbladder cannulation as described above, genuine bile production was determined by bile collection for 30 minutes. The bile was collected for analysis. Mice were then sacrificed by cardiac puncture, blood was drawn by cardiac puncture and livers samples were immersed in neutral buffered formalin.

Analytical techniques. Biliary bile salt concentrations were determined by an enzymatic fluorometric assay (26). Lipids were extracted from the bile (27). The phospholipid concentrations were determined using a spectrophotometric assay (28). Biliary bile salt composition was determined by capillary gas chromatography (29). The hydrophobicity of bile salts in bile was calculated according to the Heuman index based on the fractional contribution of the mayor bile salt species (23). ALT was determined in plasma samples.

For histological analysis of the liver samples, hematoxylin/eosin staining was applied on paraffin embedded sections. To quantify mitotic active cells we used Ki-67 immunostaining using a rabbit anti Ki-67p monoclonal antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) (30).

An experienced hepato-pathologist (A.S.H.G.) assessed the liver samples in a blinded fashion. Histology of the liver parenchyma and portal tracts were evaluated separately for inflammation, fibrosis and Ki-67 mitotic activity. The score was performed in a semi-quantitative approach on a scale from 0 to 3 (0: absent, 1: sporadic, 2: regular, 3: frequent) (31).

Statistical analysis. Analyses were performed using SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL). All nominal results are reported as means ± SEM. The ordinal histology results are reported as median and range. Differences between study groups were evaluated using the Mann-Whitney U test. The level of significance was set at a P value of less than 0.05.

**RESULTS**

Bile production and bile salt secretion after interruption of the enterohepatic circulation. Bile production, assessed over 30 minutes immediately after interruption of the enterohepatic circulation, did not differ significantly between Cftr⁻/⁻ mice and controls (mean of 2 time points in first 30 minutes: 6.3±0.7 vs. 5.8±0.5 μl.min⁻¹.100 g⁻¹, respectively; Figure 1A). Bile salt
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concentrations and bile salt secretion rates were also similar between $\text{Cftr}^{-/-}$ mice and controls (Figure 1B and C).

**Bile production and bile salt secretion during acute IV taurocholic acid (TCA) administration.** Administration of TCA (iv) dose-dependently increased bile production (Figure 1A). The dose-response relationship was similar for $\text{Cftr}^{-/-}$ mice and controls, indicating that bile production was not impaired in CF mice. A concomitant dose-dependent increase in bile salt concentrations and bile salt secretion rates was observed upon TCA infusion, in both $\text{Cftr}^{-/-}$ mice and controls (Figure 1B and C). A linear correlation was observed between the bile salt secretion rate and bile flow (Figure 1D). The slopes derived after linear regression analysis, were similar for $\text{Cftr}^{-/-}$ mice and controls, indicating similar levels of bile salt dependent bile flow. These observations strongly suggest that the bile flow in mice, both under basal conditions and after acute IV TCA administration, is (predominantly) generated via CFTR independent mechanisms.

![Figure 1](image-url)

**Figure 1.** Biliary parameters during intravenous taurocholic acid (TCA) administration. Biliary bile flow (A), bile salt concentration (B), bile salt secretion rate (C) and relationship between bile salt secretion rate and bile flow (D) in Cftr knockout mice ($\text{Cftr}^{-/-}$) and control littermates ($\text{Cftr}^{+/+}$) during intravenous infusion with TCA in stepwise increasing dosage of. The grey symbols in (D) represent baseline values before the start of TCA infusion. Data are presented as means ± SEM of $N=5-7$ mice per group. There was no significant difference between $\text{Cftr}^{-/-}$ and $\text{Cftr}^{+/+}$ mice, at any of the individual time points, for bile flow, bile salt concentration and bile salt secretion rate.
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Bile production and bile salt composition during chronic cholic acid feeding. Feeding a CA containing diet for 3 weeks increased bile flow, both in Cfr<sup>-/-</sup> mice and controls. However, the increase was larger in controls than in Cfr<sup>-/-</sup> mice (Figure 2A). The CA diet increased the biliary bile salt concentration in Cfr<sup>-/-</sup> animals but, not in controls (Figure 2B). Chronic CA feeding increased the bile salt secretion rate in both Cfr<sup>-/-</sup> mice and controls (Figure 2C).

To assess cytotoxicity, we measured the biliary phospholipid concentration and calculated the phospholipid-to-bile salt ratio. During regular chow feeding, biliary phospholipid concentrations were similar in Cfr<sup>-/-</sup> mice and controls (Figure 2D), as were the phospholipid-to-bile salt ratios (Figure 2E). CA feeding appeared to increase phospholipid concentrations to a similar magnitude in Cfr<sup>-/-</sup> and controls although the apparent differences between means did not reach statistical significance (Figure 2D). CA administration increased the phospholipid-to-bile salt ratio in controls. Because CA feeding increased the biliary bile salt concentration in Cfr<sup>-/-</sup> mice (but not in controls; see above) the phospholipid-to-bile salt ratio, under these conditions, was significantly lower in Cfr<sup>-/-</sup> mice than in controls (Figure 2E). We found no significant difference in serum ALT in Cfr<sup>-/-</sup> mice after CA diet compared to normal diet (Figure 2F). The CA diet, however, significantly increased ALT, compared to normal in controls and not in Cfr<sup>-/-</sup> mice.

CA feeding markedly increased the fractional contribution of CA to the total bile salt pool of the bile. In control mice, it rose to 90%, whereas in Cfr<sup>-/-</sup> mice it reached near 100%. In the control animals, the remaining 10% was attributed to deoxycholic acid (DCA), which is formed from CA by intestinal bacteria (Figure 2G). Based on this result, after CA diet, the Heuman hydrophobicity index of the bile was higher in controls than in Cfr<sup>-/-</sup> mice (0.061 vs. 0.003, respectively, P<0.01, Figure 2H).

On regular chow, serum ALT levels were similar in Cfr<sup>-/-</sup> mice and controls (Figure 2F). CA feeding increased serum ALT had in controls, suggesting that, despite the concomitant increase in the phospholipid-to-bile salt ratio, the bile produced was more cytotoxic (Figure 2F). In Cfr<sup>-/-</sup> mice, the CA diet induced a similar mean increase in serum ALT levels, despite a markedly lower hydrophobicity index. However, the variation between individual animals was larger than for controls, suggesting that a subset of Cfr<sup>-/-</sup> animals may be more prone to develop lesions of the biliary tract.
Figure 2. Biliary parameters and bile composition before and after chronic dietary cholic acid (CA) administration. Biliary bile flow (A), bile salt concentration (B), bile salt secretion rate (C), phospholipid concentration (D), phospholipid-to-bile salt ratio (E) Serum ALT levels (F), percent contribution of the bile salts cholate, deoxycholate and others (chenodeoxycholate, deoxycholate, ursodeoxcholate, α-muricholate and β-muricholate) in bile (G) and Heuman index of total bile salts in bile representing the hydrophobicity of bile salts (H) in Cftr knockout mice (Cftr−/−) and control littermates (Cftr+/+) after a regular or 0.5% CA (wt/wt) chow diet for 3 weeks. Data are presented as means ± SEM or percentage if appropriate of N=6-7 mice per group. *P-value<0.05.
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*Cftr* dependent effects of chronic CA exposure on liver weight. Similar to previous observations in other CF mouse models, our *Cftr*−/− mice tended to be smaller than littermate controls (Figure 3A) (32). CA feeding did not markedly affect body weight. On regular chow, *Cftr*−/− mice and controls had similar absolute and relative liver weights (Figure 3C and 3E). Chronic CA administration increased the absolute and relative liver weight in controls, but not in *Cftr*−/− mice (+52%, p<0.001), indicating that chronic CA administration induces a hepatic, *Cftr*-dependent adaptive mechanism to stimulate liver enlargement (Figure 3C and 3E). The CA-induced liver growth response (+33%, p<0.01) was also observed in control mice with a genetic background (FVB;129Sv) different from the one of the *Cftr*−/− littermates (C57Bl6;129). The liver growth response to CA-diet was lost in mice expressing mutant *Cftr* (homozygous F508del *Cftr* mice; *Cftr*tm1EUR), indicating that it is largely independent of subtle differences between mouse strains and requires functional CFTR, Figure 3 B, 3D and 3F) (33).

*Cftr* dependent effects of chronic CA exposure on liver histology. On a regular chow diet, parenchymal cell proliferation was enhanced in *Cftr*−/− mice, relative to controls (Figure 4C), but no signs of parenchymal inflammation were evident. Chronic CA administration increased parenchymal mitotic activity in controls to a level similar to that found in *Cftr*−/− mice on either regular chow or after chronic CA exposure. Concomitantly, 3 week CA feeding significantly increased the parenchymal inflammation score in controls (Figure 4D). Prolonged (3 month) CA exposure exceeds the 3 weeks findings, showing increased parenchymal mitotic activity and parenchymal inflammation, particularly observed in controls. CA feeding did not generate pathological changes in the portal tract areas of the *Cftr*−/− mice, or controls (Figure 4 A and B).

**DISCUSSION**

In mice, both acute and chronic hydrophilic bile salt administration markedly increased biliary bile salt production and flow rates. *Cftr*−/− mice showed a reduced capacity to adapt to chronic bile salt administration. Although biliary bile salt production was similar the increase in bile flow was less pronounced in *Cftr*−/− mice compared controls. This reduced capacity, of *Cftr*−/− mice, to increase bile flow resulted in significantly higher biliary bile salt concentrations. No such defect was observed upon acute intravenous bile salt loading, suggesting that only long-term adaptations to bile salt loading, such as hepatic cell proliferation (discussed below), is perturbed in CF. These results imply that in mice, in contrast to humans, either *Cftr* does not significantly contribute to canalicular or ductular fluid secretion or that, in the absence of *Cftr* is substituted by other anion channels.
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Figure 3. Body and liver weight before and after chronic dietary cholic acid (CA) or UDCA administration. Body weight (A/B), absolute liver weight (C/D) and relative liver weight reported as a percentage of the body weight (E/F) in Cftr knockout mice (Cftr-/-) or ΔF508 Cftr mice (Cftrtm1EUR) and their respective control littermates (Cftr+/+) after a regular or 0.5%-CA (wt/wt) chow diet for 3 weeks. Data are presented as means ± SEM of N=5-7 mice per group. *P-value<0.05.
We had expected that in CF conditions prolonged CA feeding would increase in biliary bile salt concentration together with increased level of secondary hydrophobic bile salts. We anticipated that this combination of hydrophilic bile salts would result in more cytotoxic bile. However, we found that under these conditions, bile of Cftr<sup>-/-</sup> mice contained little of the secondary bile salt deoxycholate (DCA), whereas in controls its level in bile was markedly enhanced by CA feeding. Since DCA is produced exclusively by intestinal bacteria, these results suggest that alterations in the composition of this micro flora, which are typical of CF, reduce DCA production (34-36). Alternatively, absorption of DCA may be reduced in CF. This low availability of DCA may reduce the cytotoxicity of bile. Therefore, this hitherto unknown mechanism may protect CF mice against bile salt-induced biliary disease.

A previous study reported the development of spontaneous liver disease in CF mice (18). The mice used in these studies had a genetic background (C57BL/6J) different from the animals used by us. This difference between strains indicates that genetic modifiers might affect the
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hepatobiliary phenotype. Considering the potential effect of the intestinal micro flora on bile composition, environmental factors may also play a role (36). Peptamen, a liquid diet formulation used to prevent intestinal obstruction (used in ref. 14, but not by us), has been shown to promote small intestinal bacterial overgrowth in CF mice (37). This may lead to enhanced production of secondary bile salts, in turn leading to the production of a more cytotoxic bile and, consequently, liver disease.

We found that CA feeding significantly increased liver mass in control mice. Similar findings have been reported previously (38). This earlier study showed that CA feeding also enhanced liver regeneration after partial hepalectomy. It was proposed that this response is mediated through the Farnesoid X receptor (FXR) nuclear receptor that is directly activated by specific bile salt, including CA and DCA. In accordance, we have found earlier that UDCA, which is a limited FXR ligand, does not resemble the effect of CA on liver growth (39). In this study, oral feeding of UDCA (0.5% wt/wt) to Cftr-WT mice for 3 weeks, did not result in a significant increase in relative liver weight (0.048 ±0.003 vs. 0.044±0.001, respectively; unpublished data).

Presently, we found no increase in liver mass in CA-fed Cftr<sup>−/−</sup> and Cftr<sup>ΔF508/ΔF508</sup> mice. This indicates that FXR signaling may be perturbed in CF mice, a notion, which is reinforced by a previous study showing that FXR-controlled genes are down-regulated in the ileum of CF mice, possibly, because of reduced bile salt uptake in ileocytes (40).

In summary, we show that, in mice, CFTR plays a role in the hepatic adaptive response to high bile salt exposure. The CF condition is characterized by a reduction in bile salt-induced hepatic cell proliferation, increase in biliary bile salt levels and difference in secondary bile salt composition. These changes suggest that the CF liver may be more prone to develop hepatobiliary disease upon (chronic) exposure to hepatotoxic substances.

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