Essential fatty acid deficiency and the small intestine
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CHAPTER 4

EFFECTS OF ESSENTIAL FATTY ACID DEFICIENCY ON ENTEROHEPATIC CIRCULATION OF BILE SALTS IN MICE

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ABSTRACT

Essential fatty acid (EFA) deficiency in mice has been associated with increased bile production, which is mainly determined by the enterohepatic circulation (EHC) of bile salts. To establish the mechanism underlying the increased bile production, we characterized in detail the EHC of bile salts in EFA-deficient mice using stable isotope technique, without interrupting the normal EHC. Farnesoid X receptor (FXR) has been proposed as an important regulator of bile salt synthesis and homeostasis. In Fxr−/− mice we additionally investigated to what extent alterations in bile production during EFA deficiency were FXR-dependent. Furthermore, we tested in differentiating Caco-2 cells the effects of EFA-deficiency on expression of FXR-target genes relevant for feedback regulation of bile salt synthesis.

EFA deficiency enhanced bile flow and biliary bile salt secretion were associated with elevated bile salt pool size and synthesis rate (+146% and +42%, respectively, p<0.05), despite increased ileal bile salt reabsorption (+228%, p<0.05). Cyp7a1 mRNA expression was unaffected in EFA-deficient mice. However, ileal mRNA expression of Fgf15 (inhibitor of bile salt synthesis) was significantly reduced, in agreement with absent inhibition of the hepatic bile salt synthesis. Bile flow and biliary secretion were enhanced to the same extent in EFA-deficient wild type and Fxr−/− mice, indicating contribution of other factors besides FXR in regulation of EHC during EFA deficiency. In vitro experiments show reduced induction of mRNA expression of relevant genes upon chenodeoxycholic acid (CDCA) and GW4064 stimulation in EFA-deficient Caco-2 cells.

In conclusion, our data indicate that EFA deficiency is associated with interrupted negative feedback of bile salt synthesis, possibly due to reduced ileal Fgf15 expression.
INTRODUCTION

Essential fatty acid (EFA) deficiency is a frequent condition in patients with cholestasis or cystic fibrosis\textsuperscript{1,2,3} and has various effects on bile production and absorption of dietary fat.\textsuperscript{4,5,6,7} Bile salts are essential for bile production, secretory processes and efficient intestinal absorption of dietary fat. In our lab we developed a mouse model to study the effects of EFA deficiency \textit{in vivo}.\textsuperscript{7,8} Previous studies in this mouse model have shown that EFA deficiency-associated fat malabsorption is not caused by impaired bile formation, like it has been implied earlier in a rat model for EFA deficiency.\textsuperscript{6} In EFA-deficient mice an increase in bile flow and biliary secretion was observed.\textsuperscript{7} The physiological importance and the underlying mechanism of elevated bile flow and biliary secretion of bile salts during EFA deficiency in mice remains unclear. Bile flow and biliary secretion of bile salts are mainly influenced by the circulation of bile salts from the liver to the intestine, and their reabsorption back to the liver via the portal circulation. This enterohepatic circulation (EHC) of bile salts involves many hepatic and intestinal transporters responsible for the uptake and excretion of bile salts and results in efficient preservation of bile salts within the body.\textsuperscript{9} Under physiological conditions, per enterohepatic cycle less than 5 percent of the total amount of bile salts present in the body, i.e. the bile salt pool, is lost via the feces. Under steady state conditions this fraction of bile salts lost is compensated by hepatic bile salt synthesis.\textsuperscript{9,10} Although increased bile flow and biliary secretion have been reported in EFA-deficient mice, it remained unclear how EFA deficiency in mice affects the EHC of bile salts. To address this question, in the current study we measured different steps of the EHC \textit{in vivo} in EFA-deficient mice by stable isotope dilution technique. We compared the outcomes of the measured bile salt synthesis and pool size by the stable isotope dilution technique with the classical determination of these parameters, namely by determining the expression of the gene encoding cholesterol 7α-hydroxylase (\textit{Cyp7a1}), which is the rate limiting enzyme in bile salt synthesis.\textsuperscript{11,12} Shortly, by stable isotope dilution technique, different parameters of the EHC (synthesis, pool size, fractional turnover rate, ileal reabsorption and cycling time) were determined \textit{in vivo} by means of the intravenous injection of a stably labeled bile salt and subsequent determination of plasma enrichment of the label. This method, known as the stable isotope dilution technique, has been developed and validated previously in our lab to measure bile salt kinetics \textit{in vivo} without interrupting the normal EHC.\textsuperscript{9,10} We have chosen to inject the stably labeled cholate (\textsuperscript{2}H\textsubscript{4}-cholate), as this is the primary and most abundant bile salt in humans and rodents; total bile acid pool consists of 30% to 50% and 50% to 80% of cholate (CA) in humans and rodents, respectively.\textsuperscript{10} To confirm our \textit{in vivo} findings, we additionally measured expression of relevant intestinal genes in the EHC of bile salt synthesis in small intestinal model for EFA deficiency (post confluent Caco-2 cells) upon stimulation with chenodeoxycholic acid (CDCA) and GW4064 compound, both potent agonists of FXR. Elucidating the mechanism behind the elevated bile flow during EFA deficiency, might help to understanding and treat fat malabsorption during EFA deficiency. Our study demonstrates that increased secretion of bile salts during EFA deficiency in mice is associated with enhanced bile salts synthesis, despite increased reabsorption of bile salts in the intestine. Our results clearly show that increased bile production during EFA
deficiency cannot be contributed exclusively to FXR, but that other factors must contribute as well. *In vivo* and *in vitro* data show impaired transcriptional regulation of genes involved in intestinal regulation of bile salt homeostasis under EFA-deficient conditions. This suggests an impaired intestinal feedback mechanism of bile salt synthesis during EFA deficiency.

**MATERIAL AND METHODS**

**Mice and housing**

Male wild type mice (~25 g) on a free virus breed (FVB) background were obtained from Harlan (Horst, the Netherlands) and were housed in a light- and temperature-controlled facility. Tap water and food were allowed *ad libitum*. In a separate experiment, where we aimed to determine whether the effects of EFA deficiency on the EHC of bile salts were FXR-dependent, we used male homozygous (*Fxr*−/−) and wild type (*Fxr*+/+) mice on mixed (C57BL/6J×129/OlaHsd) background of 25-40 g. These mice were generated previously by Deltagen, Inc. (Redwood City, CA) and bred at the animal facility of the University of Groningen. Food intake and fecal excretion were monitored during a 72h period at the end of the experiment. For clarity reasons, mice fed the EFA-deficient or control diet (on FVB background) will be mentioned as *EFA-deficient* or *control mice*. *Fxr*+/+ and *Fxr*−/− mice fed the EFA-deficient or control diet will also be entitled as *EFA-deficient* or *control*, respectively, with the genotype indicated in addition. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen, Netherlands.

**Experimental diets**

As in our previous studies, we used high-fat (humanized) EFA-deficient (#4141.08) and EFA-sufficient (control, #4141.07) diets (16 wt% and 34 energy% fat), which were custom synthesized by Arie Blok BV (Woerden, the Netherlands). Essential, unsaturated fatty acids in EFA-sufficient diet were replaced by saturated fatty acid in EFA-deficient diet; EFA-deficient diet was particularly reduced in linoleic acid (essential fatty acid) concentration. In detail, EFA-deficient diet contained 64 mol% palmitic acid (C16:0), 18 mol% stearic acid (C18:0), 13 mol% oleic acid (C18:1ω-9) and 5 mol% linoleic acid (C18:2ω-6). Isocaloric EFA-sufficient diet contained 36 mol% C16:0, 5 mol% C18:0, 31 mol% C18:1ω-9 and 29 mol% C18:2ω-6. Fatty acid contents of the diets were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids. Subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography as described previously.

**Material for stable isotope dilution technique and cell culture experiments**

Consistent with previous studies with stable isotope dilution technique, we administered 2H4-cholate ([2,2,4,4,2H]-cholate) of 98% isotopic purity, which was purchased from Isotec (Miamisburg, OH). Cholyglycine hydrolase from *Clostridium perfringens* was obtained from Sigma Chemicals (St. Louis, MO) and pentafluorobenzylbromide (PFB) was purchased from Fluka Chemie (Buchs, Neu-Ulm, Switzerland). For the *in vitro*...
studies chenodeoxycholic acid was purchased from Calbiochem (CDCA; San Diego, CA, USA) and GW4064 from Tocris Bioscience (Ellisville, MO, USA).

Methods

Induction of EFA deficiency
Mice were fed the EFA-deficient or control diet for 8 weeks, consistent with previous studies in EFA-deficient mice. After 8 weeks of EFA-deficient or control diet, mice underwent a 72h fat balance test, bile cannulation and stable isotope dilution test (details see below). Afterwards, the mice were anesthetized and sacrificed through cardiac puncture. The marker of EFA deficiency, triene/tetraene ratio (C20:3ω-9/C20:4ω-6), was determined in erythrocyte lipids as described previously. Protocol for induction of EFA deficiency in Caco-2 cells is described in section “Stimulation of differentiating EFA-deficient and control Caco-2 cells to CDCA and GW4064”.

Fat absorption
Absorption of major dietary fatty acids was assessed by measuring food intake and collecting feces for 72h. Net amount of fat absorbed was calculated by subtracting the fecal excretion of the major fatty acids (stearate, palmitate, oleate and linoleic acid) from the amount of fatty acids ingested, determined by gas chromatography.

Stable isotope dilution
The stable isotope dilution test was performed as previously described, slightly modified. Three days prior to the end of the experiment, after the fat absorption test was completed, 400 μg of 2H4-cholate in a solution of 0.5% NaHCO3 in PBS was slowly injected intravenously under isoflurane anesthesia. At different time points (12, 24, 36, 48 and 60h) blood samples of 75 μl were collected by orbital puncture under isoflurane anesthesia to determine the isotope enrichment in plasma. Blood, collected in microhematocrit tubes containing heparin, was centrifuged (4,000 rpm for 10 min) and plasma was stored at -20°C until further analysis. Samples used for baseline isotope abundance measurements were obtained by orbital puncture from a separate group of mice.

Bile collection
After the mice were anesthetized with Hypnorm/Diazepam mixture, the bile duct was cannulated during 30 minutes and bile flow was determined gravimetrically (1 g/ml). During the cannulation, body temperature was maintained by placing the mice in a humidified incubator (37°C).

Sample collection
The small intestine was excised, flushed with ice-cold PBS and the last part (terminal ileum) was harvested for gene (mRNA) expression. The livers were excised and weighed. Subsequently, small pieces were cut out for mRNA and biochemical analysis. Prior to storage at -80°C, tissues were snap-frozen in liquid nitrogen.
Cell culture
The human colon carcinoma cell line Caco-2 was obtained from the American Type Tissue Culture Collection (Manassas, VA, USA). Before the experiment, the cells were maintained in DMEM (Gibco BRL, USA) supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1% nonessential amino acids and 0.25% human transferrin in a humidified 5% CO₂ atmosphere under standard conditions.

Stimulation of differentiating EFA-deficient and control Caco-2 cells to CDCA and GW4064
For the experiment, cells (between passage 21 and 40) were seeded at 0.5 × 10⁷ cells/well. Cells were made EFA-deficient according to the adapted protocol of Spalinger et al.¹⁵ Shortly, medium was replaced by DMEM supplemented with dialyzed FBS (control cells) or with delipidated FBS (EFA-deficient cells) one day after seeding. Delipidation of FBS was performed by means of di-isopropylether and 80 ml butan-1-ol extraction. Seven days after complete confluence, the cells were exposed to serum-free DMEM containing chenodeoxycholic acid (CDCA; 50 or 250 µM), GW4064 (1 µM) or vehicle for 24 hours. Afterwards, cells were harvested for fatty acid profile determination and quantitative PCR. All experiments were performed at least in triplicate.

Analytical methods
Biliary bile salts and lipids
Bile salt concentration in bile was determined by an enzymatic fluorimetric assay.¹⁶ Biliary phospholipids and cholesterol were determined as described by Kuipers et al.¹⁷

Gas chromatography
Fatty acids in erythrocytes, food and feces of the mice, and fatty acids in Caco-2 cells were analyzed by extracting, hydrolyzing and methylating total dietary fatty acids as described by Muskiet et al.¹³ Subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography.⁷ Bile salt composition of bile samples was determined by capillary gas chromatography.⁹

Preparation of plasma samples for isotope analysis and gas-liquid chromatography-electron capture negative chemical ionization mass spectrometry (GLC-MS)
Plasma and bile samples were prepared for GLC-MS analysis on a Finnigan SSQ7000 Quadrupole GC-MS machine as described previously by Stellaard et al.¹⁸ Isotope dilution technique has been described in detail by Hulzebos et al.¹⁰ Shortly, enrichment of ²H₄-cholate in plasma was determined as the increase of the M₄/M₀-cholate relative to baseline measurements and is expressed as the natural logarithm of atom percent excess (ln APE). From the decay curve of ln APE (calculated by linear regression analysis), daily fractional turnover rate (FTR; equals the slope of the regression line) and pool size ([(administered amount of label x isotopic purity x 100) / e⁰ of the y-axis of the ln APE curve] of cholate were calculated. Subsequently, cholate synthesis rate was calculated by multiplying pool size and FTR. In addition, the amount of cholate reabsorbed per day, the cycling time and biliary secretion rate of cholate were calculated as described previously.⁹,¹⁰ Cholate was the most abundant bile acid in the bile salt pool of both EFA-
Table 1 Primer and probe sequences used for the quantitative PCR. Genes indicated in capital font are of human origin used for Q-PCR analysis in Caco-2 cells. Remaining primers and probes were used for quantitative PCR analysis in mouse tissues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Forward Primer</th>
<th>Reversed Primer</th>
<th>TaqMan® Probe</th>
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<td>18s</td>
<td>X03205</td>
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<td>Fgfr4</td>
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</tbody>
</table>

EFA DEFICIENCY AND BILE SALT METABOLISM
deficient (~58%) and control mice (~58%). Therefore, we assumed that the parameters calculated for cholate were representative for the complete bile salt pool.

**Measurement of mRNA expression by quantitative PCR (Taqman)**

mRNA expression of hepatic genes involved in bile salt synthesis, hepatic transporter genes (for bile salts, cholesterol and phospholipids), ileal genes implicated in enterohepatic circulation of bile salts and FXR-target genes in Caco-2 cells were determined by quantitative PCR as described previously. PCR results in the liver were normalized to the RNA expression of the housekeeping gene 18s, in ileum to the housekeeping gene β-actin and in Caco-2 cells to GAPDH. The sequences of the primers and probes are listed in Table 1.

**Heuman index**

The Heuman index is a numeric representation of hydrophilic-hydrophobic balance, corresponding with the ability of bile acids to solubilize dietary fats. For determination of the hydrophobicity of the bile salt pool, we calculated the Heuman index after quantification of major biliary bile salts by gas chromatography.

**Statistical analysis**

Using SPSS version 12.0.2 statistical software (Chicago, IL, USA), we calculated significance of differences between EFA-deficient and control (FVB) mice with the Mann-Whitney U-test.

For the experiment with the Fxr−/− mice and their wild type littermates on either EFA-deficient or control diet, statistical analysis was assessed by One-Way ANOVA test followed by a post hoc Bonferroni correction.

For in vitro experiments, data were statistically analyzed using Student’s two-tailed t test. For all experiments, p-values below 0.05 were considered statistically significant.

**Table 2** Animal characteristics of FVB mice fed EFA-deficient or control diet for 8 weeks. Values are represented as means ± SD (n=6 mice per group). *p<0.05 is the significant difference between EFA-deficient and control FVB mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>EFA-deficient</th>
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<tbody>
<tr>
<td></td>
<td>Body weight (g)</td>
<td>28.9 ± 2.2</td>
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<tr>
<td></td>
<td>Liver weight (g)</td>
<td>1.2 ± 0.1</td>
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<tr>
<td></td>
<td>Daily food intake (g)</td>
<td>4.3 ± 1.0</td>
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<tr>
<td></td>
<td>Triene/tetraene ratio</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Fat absorption (% of total fat)</td>
<td>99.4 ± 0.2</td>
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</tbody>
</table>

**RESULTS**

Body weight and food intake were not different between EFA-deficient and control FVB mice (Table 2). Consistent to our previous findings in EFA-deficient mice, liver weight was higher in EFA-deficient mice compared with controls (Table 2), most probably due to
fat accumulation as indicated by a trend towards an increased concentration of triglycerides (117.4±57.4 nmol.mg⁻¹ protein in control mice versus 176.5±57.7 nmol.mg⁻¹ protein in EFA-deficient mice, NS) and significantly increased cholesterol (57.6±10.1 nmol.mg⁻¹ protein in control mice versus 83.1±8.6 nmol.mg⁻¹ protein in EFA-deficient mice, p<0.05) in the livers of EFA-deficient mice (data not shown). After 8 weeks of EFA-deficient diet-feeding, the triene/tetraene ratio (biochemical marker of EFA deficiency) was increased in erythrocytes of EFA-deficient mice (0.23±0.06 versus 0.01±0.00 in control mice, p<0.05) (Table 2). The induction of EFA deficiency in mice decreased fat absorption by approximately 20% (Table 2).

In our second experiment we assessed the possible contribution of FXR to EFA-deficient phenotype of the EHC in Fxr⁻/⁻ mice and their wild type littermates with or without EFA deficiency. During the last decade, the nuclear farnesoid X receptor (FXR) has been identified as an important regulator of the bile salt metabolism.²¹,²²

<p>| Strain C57BL/6Jx129/OlaHsd background on EFA-deficient or control diet for 8 weeks. *p&lt;0.05 EFA-deficient mice versus control with the same genotype, #p&lt;0.05 Fxr⁻/⁻ versus Fxr⁺/⁺ on the same diet. Values are represented as means ± SD (n=5-7 mice per group). |</p>
<table>
<thead>
<tr>
<th>Fxr⁻/⁻</th>
<th>Control</th>
<th>EFA-deficient</th>
<th>Control</th>
<th>EFA-deficient</th>
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<tr>
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<td>Daily food intake (g)</td>
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<td>Fat absorption (% of total fat)</td>
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</tbody>
</table>

Bile salts are the natural ligands of FXR and can activate the FXR in the ileum. In ileum, FXR activated by the bile salts induces release of fibroblast growth factor 15 (Fgf15; homologue to human FGF19), which is transported to the liver to inhibit the bile salts synthesis.²³ Fgf15 has been characterized as important component of the gut-liver signaling pathway that regulates the bile salt synthesis. Similar to EFA-deficient and control FVB mice, after 8 weeks of diet there was no difference in body weight or food intake between EFA-deficient Fxr⁻/⁻ or Fxr⁺/⁺ mice compared with mice of the same genotype on control diet (Table 3). Liver weight was higher in EFA-deficient Fxr⁻/⁻ mice compared with the same mice on control diet (Table 3). In Fxr⁺/⁺ mice this difference did not reach the significant value (Table 3). Upon EFA-deficient diet-feeding, both Fxr⁻/⁻ and Fxr⁺/⁺ mice became EFA-deficient compared with mice of the same genotype on control diet, as indicated by increased triene/tetraene ratio (0.15±0.07 in EFA-deficient Fxr⁺/⁺ mice and 0.17±0.08 in EFA-deficient Fxr⁻/⁻ mice versus 0.01±0.00 in control mice of both genotypes, p<0.05) and by fat malabsorption (Table 3). Interestingly, fat malabsorption was less profound in EFA-deficient Fxr⁻/⁻ mice than in their EFA-deficient wild type littermates (-22% in EFA-deficient Fxr⁻/⁻ mice compared with Fxr⁻/⁻ mice on control diet, and -29% in EFA-deficient Fxr⁺/⁺ mice compared with Fxr⁺/⁺ mice on control diet, p<0.05).
**EFA deficiency is associated with increased bile flow, but has no effect on biliary bile salt composition**

We determined bile flow and biliary secretion rates by bile cannulations. In accordance with previous findings EFA deficiency significantly increased bile flow (+78% in EFA-deficient versus control mice, p<0.05) (Table 4).  

Table 4 Bile flow and biliary secretion rates in FVB mice fed EFA-deficient or control diet for 8 weeks. Values are represented as means ± SD (n=6 mice per group). *p<0.05 is the significant difference between EFA-deficient and control FVB mice.

<table>
<thead>
<tr>
<th>Strain FVB</th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (µl.min⁻¹.100g⁻¹ BW)</td>
<td>4.3 ± 1.3</td>
<td>7.6 ± 2.6*</td>
</tr>
<tr>
<td>Bile salts (µl.min⁻¹.100g⁻¹ BW)</td>
<td>121 ± 30</td>
<td>376 ± 196*</td>
</tr>
<tr>
<td>Phospholipids (µl.min⁻¹.100g⁻¹ BW)</td>
<td>12 ± 6</td>
<td>22 ± 6*</td>
</tr>
<tr>
<td>Cholesterol (µl.min⁻¹.100g⁻¹ BW)</td>
<td>1 ± 0</td>
<td>2 ± 0*</td>
</tr>
</tbody>
</table>

Biliary secretion rates of bile salts and phospholipids were two- to threefold higher in EFA-deficient compared with the control FVB mice (each p<0.05) (Table 4). To determine if EFA deficiency results in altered composition of bile salt pool, we determined biliary bile salt composition and calculated its hydrophobicity, using the Heuman index number. EFA deficiency did not result in major changes in bile salt composition (Figure 1) or in the Heuman index (-0.20 ± -0.06 in EFA-deficient versus -0.21 ± -0.03 in control mice, NS).

In order to investigate if enhanced bile flow in EFA-deficient mice was dependent of biliary bile salt secretion, we plotted the bile flow (y-axis) against the biliary bile salt output (x-axis) (data not shown).

Figure 1 Biliary bile salt composition in EFA-deficient (white bars) and control (black bars) mice. More than 90% of all bile salts are represented and values are expressed as percentage of total bile salts. Data represent means of 5-6 mice per group. Data are means ± SD of n=6 mice per group. *p<0.05 is the significant difference between the two groups.
Figure 2 (a) Decay curve of the intravenously injected $^2$H$_4$-cholate in EFA-deficient and control mice. Enrichment of the administrated $^2$H$_4$-cholate was determined in plasma during 60 hours after the administration of the label. From the curve (b) pool size (y-intercept), (c) synthesis, (d) FTR (slope), (e) reabsorption and (f) cycling time of cholate were determined for individual mice. Data are means ± SD of 6 mice per group. *p<0.05 is the significant difference between EFA-deficient (white bars) and control (black bars) mice.
There is a classical linear relationship between these two parameters, indicating that EFA deficiency in mice does not affect the normal bile formation. Increased bile flow during EFA deficiency is most likely caused by the increased bile salt output.

*Increased bile salt secretion during EFA deficiency corresponds with enhanced synthesis, pool size and reabsorption of bile salts*

To evaluate the effects of EFA deficiency on different parameters of bile salt homeostasis without interrupting the normal EHC, we performed the stable isotope dilution technique by intravenous injection of $^{2}$H$_{4}$-cholate. Decay curve of plasma enrichment of $^{2}$H$_{4}$-cholate over time indicates different kinetics of cholate in EFA-deficient mice compared with control mice (Figure 2a). EFA deficiency was associated with higher pool size (Figure 2b: 40 ± 1 μmol.100g$^{-1}$ in EFA-deficient mice versus 16 ± 7 μmol.100g$^{-1}$ in control mice, p<0.05), higher synthesis rate (Figure 2c: 11 ± 2 μmol.100g$^{-1}$day$^{-1}$ in EFA-deficient mice versus 8 ± 2 μmol.100g$^{-1}$day$^{-1}$ in control mice, p<0.05) and lower fractional turnover rate (Figure 2d: 0.6 ± 0.2 per day versus 0.3 ± 0.01 per day in control mice, p<0.05) of cholate. Reabsorption of cholate in ileum was enhanced in EFA-deficient mice (Figure 2e: 512 ± 275 μmol.100g$^{-1}$day$^{-1}$ versus 156 ± 29 μmol.100g$^{-1}$day$^{-1}$ in control mice, p<0.05), while the cycling time of cholate was not affected by EFA deficiency (Figure 2f: 2.3 ± 1.1 hours in EFA-deficient versus 2.4 ± 1.1 hours in control mice, NS).

*mRNA expression of genes involved in EHC in EFA-deficient mice*

By means of quantitative PCR we determined the mRNA expression of hepatic and ileal genes implicated to be important in bile salt homeostasis or bile flow (Figure 3). EFA deficiency did not have a major effect on the mRNA expression of the gene encoding the rate-limiting enzyme in bile salt synthesis, namely Cyp7a1 (0.62 ± 0.31 versus 1.24 ± 0.59 in control mice, NS) (Figure 3a). The mRNA expression of Cyp8b1, which encodes the gene of the enzyme catalyzing the cholic acid synthesis in the liver and is feedback-inhibited by bile salts, was significantly increased in EFA-deficient mice (2.23 ± 0.8 versus 1.2 ± 0.3 in control mice, p<0.05) (Figure 3a). However, EFA deficiency did not have a major effect on the mRNA expression of other genes involved in hepatic bile salt synthesis (Fxr, Shp, Fgfr4 and Cyp27a1) (Figure 3a). When activated by bile salts in the intestine, FXR activates the mRNA expression and release of Fgf15, which subsequently travels to the liver via the portal circulation in order to inhibit the hepatic bile salt synthesis. We observed a decrease in mRNA expression of Fgf15 gene in the terminal ileum of EFA-deficient (FVB) mice, despite an increased bile salt synthesis and secretion (Figure 3b). These data are confirmed by measurement of Fgf15 in Fxr$^{-/-}$ mice on mixed background (C57BL/6Jx129/OlaHsd); in EFA-deficient Fxr$^{-/-}$ mice, Fgf15 mRNA expression was significantly lower than in Fxr$^{-/-}$ mice on control diet (Figure 4b). These data indicate an effect of the EFA deficiency on Fgf15 mRNA expression, independent of the genetic background of the mice. mRNA of Fgf15 was almost absent in Fxr$^{-/-}$ mice on both EFA-deficient and control diet (Figure 4b), demonstrating that Fgf15 mRNA expression is regulated by the FXR, as reported previously. The mRNA expression of FXR itself and genes encoding the ileal bile salt-transporters (Osta, Ostβ, Ibabp and Asbt) was not significantly affected by EFA deficiency in mice (Figure 3b).
In order to evaluate whether the expression of hepatic transporters of bile salts, phospholipids and cholesterol correlates with the observed increase in bile flow in EFA-deficient mice, we measured the mRNA expression of genes encoding the hepatic transporters (Figure 3c). However, EFA deficiency did not affect the mRNA expression of the majority of the genes, except for a decrease in Mrp3 (basolateral organic anion transporter) (Figure 3c).24

Figure 3 mRNA expression of genes involved in (a) hepatic bile salt synthesis, (b) ileal bile salt transport and (c) hepatic transport of bile salts (BS), phospholipids (PL) and cholesterol (CL). Data represented means ± SD of n=6 mice per group. *p<0.05 is the significant difference between EFA-deficient (white bars) and control (black bars) mice.

EFA deficiency associated increase in bile flow is FXR-independent
To study to what extent alterations in bile production upon EFA deficiency were FXR-dependent, we determined the bile flow by bile cannulation in Fxr−/− mice and their wild
type littermates on EFA-deficient and control diet. Bile flow was enhanced by EFA deficiency in Fxr+/+ and Fxr/-/ mice (+97% and +112%, respectively, compared with mice of the same genotype on control diet, p<0.05) (Figure 4a; Table 5). This was accompanied by an increase in biliary secretion of bile salts in EFA-deficient Fxr+/+ and Fxr-/- mice (+97% and +112%, respectively, compared with mice of the same genotype on control diet, p<0.05) (Figure 4a; Table 5). This was accompanied by an increase in biliary secretion of bile salts in EFA-deficient Fxr+/+ and Fxr-/- mice (+36% in EFA-deficient Fxr+/+ mice compared with control Fxr+/+ mice, NS, and +105% in EFA-deficient Fxr-/- mice compared with control Fxr-/- mice, p=0.054). Although the differences did not reach significant values, there is a trend towards a higher biliary secretion of bile salts in EFA-deficient mice with both genotypes. Secretion of phospholipids showed a trend towards an increased secretion in the EFA-deficient mice, regardless of the genotype (+91% in EFA-deficient Fxr+/+ mice compared with control Fxr+/+ mice, NS, and +179% in EFA-deficient Fxr-/- mice compared with control Fxr-/- mice, p<0.05). These data indicate that the increase in biliary secretion of bile salts and phospholipids is independent of FXR.

**CDCA and GW4064 stimulation of EFA-deficient Caco-2 cells**

To study direct effects of EFA deficiency on FXR activation, in vitro experiments were performed in post confluent Caco-2 cells treated with CDCA and GW4064, both very potent (and the latter one highly specific) FXR agonists. Upon confluence Caco-2 cells spontaneously differentiate and develop small intestinal features, as indicated by the expression of enterocyte markers lactase and sucrose-isomaltase. As expected, after 8-10 days in EFA-deficient medium Caco-2 cells showed clear signs of EFA deficiency as indicated by significantly lower levels of linoleic acid (Figure 5a) and ω-6 family of fatty acids (Figure 5b) in EFA-deficient compared with control Caco-2 cells. Triene/tetraene ratio, biochemical marker of EFA-deficiency, was clearly increased in EFA-deficient Caco-2 cells (0.23±0.06 in EFA-deficient and 0.08±0.05 in control cells; p<0.05; data not shown). This clearly shows that EFA deficiency in Caco-2 cells resembles the situation in humans and mice during EFA deficiency and shows that this is a valid intestinal in vitro model of EFA deficiency. Cellular mRNA expression of intestinal differentiation marker lactase was significantly lower in EFA-deficient cells, compared with control Caco-2 cells, without affected morphology of the EFA-deficient cells.
EFA DEFICIENCY AND BILE SALT METABOLISM

(unpublished data). These data are in agreement with our previous in vivo observations in EFA-deficient mice having reduced mRNA expression and enzyme activity of lactase in mid intestine of EFA-deficient mice. Upon treatment with a physiological concentration of CDCA (50 µM) FGF19 mRNA expression was slightly increased in control, and to a lesser extent in EFA-deficient Caco-2 cells, although the values did not reach the significant difference (Figure 5c). FGF19 mRNA expression was further increased in control Caco-2 cells upon stimulation with higher CDCA concentration of 250 µM (resembling the concentrations bile salts reabsorbed during EFA deficiency in mice) (Figure 5c). This effect was also seen in EFA-deficient cells after treatment with 250 µM CDCA (Figure 5c). Treatment with GW4064 (1 µM) did not increase mRNA expression of FGF19 in either control or EFA-deficient Caco-2 cells (Figure 5c). Although there was no significant difference in FGF19 mRNA expression between EFA-deficient and control cells, EFA-deficient cells seemed to have slightly lower mRNA expression of FGF19 compared to control cells in all conditions.

Table 5 Bile flow and biliary secretion rates in Fxr+/- mice and their wild type littermates (C57BL/6Jx129/OlaHsd background) on EFA-deficient or control diet for 8 weeks. Values are represented as means ± SD (n=5-7 mice per group).

<table>
<thead>
<tr>
<th>Strain C57BL/6Jx129/OlaHsd</th>
<th>Fxr+/+</th>
<th>Fxr-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EFA-deficient</td>
</tr>
<tr>
<td>Bile flow (µl.min⁻¹.100g⁻¹.BW)</td>
<td>3.3 ± 0.3</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>Bile salts (µl.min⁻¹.100g⁻¹.BW)</td>
<td>176 ± 71</td>
<td>239 ± 117</td>
</tr>
<tr>
<td>Phospholipids (µl.min⁻¹.100g⁻¹.BW)</td>
<td>17 ± 2</td>
<td>33 ± 9</td>
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Both CDCA (lower and higher concentrations) and GW4064 (1 µM) significantly induced the mRNA expression of FXR target-gene IBABP in control Caco-2 cells (Figure 5d). This effect was almost completely absent in EFA-deficient Caco-2 cells (Figure 5c), as indicated by absent induction IBABP expression upon 250 µM CDCA and GW4064 treatment in EFA-deficient cells and significantly lower expression of IBABP in EFA-deficient compared with control cells. Only upon a concentration of 50 µM of CDCA there was a slight increase in IBABP mRNA expression in EFA-deficient cells compared with the expression in unstimulated EFA-deficient cells. mRNA concentration of IBABP was significantly lower in EFA-deficient cells compared with control cells in all conditions.

DISCUSSION

We determined the effects of EFA deficiency on bile flow and kinetic parameters of the EHC of bile salts in mice, since we previously observed an increased bile flow and biliary bile salt secretion in combination with fat malabsorption. The mechanism of altered bile production during EFA deficiency in mice had remained unclear. Insight into this mechanism will hopefully allow us to design new strategies to interpret and treat fat malabsorption, specifically in EFA deficiency. Our data show that EFA deficiency in mice...
increases bile salt synthesis and intestinal reabsorption, resulting in a profoundly increased bile salt pool size, biliary bile salt secretion and bile flow. In mice in which FXR was genetically inactivated, EFA deficiency resulted in similar changes as in wild type mice, regarding biliary secretion of bile salts and bile flow. This finding indicates that the changes in bile production during EFA deficiency were essentially FXR-independent. Finally, our data suggest that the increased bile salt synthesis may be related to down-regulation of ileal Fgf15 gene expression, interfering with the negative feedback regulation of hepatic bile salt synthesis.

Consistent with our previous findings, after 8 weeks of EFA-deficient diet, mice were clearly EFA-deficient. Although previous studies in EFA-deficient mice reported slightly increased cholate and slightly decreased β-muricholate fraction in bile, in present study EFA deficiency in mice was not associated with major changes in biliary bile salt composition. These data underscore our previous findings that neither a decrease in biliary flow, nor major alterations in bile composition are the cause of fat malabsorption in EFA-deficient mice. Theoretically, increased biliary bile salt secretion during EFA deficiency could act as a compensatory mechanism for reduced absorption of fat. This is, however, conflicting with studies in rats where fat absorption is similar to that in mice (80-84%), despite a decreased biliary secretion.6,26 We cannot exclude that the proposed compensatory mechanism during EFA deficiency is differentially regulated among the different species.

Enhanced bile flow was associated with increased biliary output of bile salts and phospholipids. The ratio between bile salts and lipids was similar between EFA-deficient and control mice (data not shown), suggesting unaltered coupling of bile salts to lipids in bile upon EFA deficiency. Since the mRNA expression of the hepatic genes encoding transporters for cholesterol (Abcg5/Abcg8) and phospholipids (Mdr2) was not significantly changed upon EFA deficiency in mice, it is tempting to assume that the increased output of lipids in EFA deficiency was entirely based on the increased bile flow. Increased bile salt secretion was not associated with altered expression of several genes encoding hepatic bile salts transporters (Bsep, Ntcp, Mrp2 and Oatp1), possibly due to the fact that the expression of the transporters is not rate limiting factor for the increased secretion rate of bile salts.27 Moreover, bile salt transporters are localized along the hepatic acinus, while the bile salt transport is localized mainly at the periportal zone.28 This implies that the number of hepatocytes, rather than the gene expression of transporters, is the rate limiting factor during the altered bile salt secretion rate in mice.

Whole body kinetics of cholate,10 clearly demonstrated that the increased biliary secretion of bile salts is associated with increased bile salt synthesis. The mRNA expression of Cyp7a1 gene was not affected by EFA deficiency. However, studies on bile salt formation under different conditions have shown that altered synthesis of bile salts is not always correlated to changes in Cyp7a1 mRNA expression.29,30 Previous studies in EFA-deficient mice revealed unaltered mRNA expression of Cyp7a1.7 Since Cyp7a1 has been shown to have a remarkable circadian mRNA expression, with highest levels during the night, we cannot exclude that the differences in expression between EFA-deficient and control mice would have been different during the night.31 Increased bile salt synthesis was accompanied by the increased mRNA expression of Cyp8b1 gene, encoding the enzyme responsible for the synthesis of cholic acid and control over
Figure 5 The effects of EFA deficiency on mRNA expression of FXR-target genes in Caco-2 cells after CDCA and GW4064 stimulation. (a) Linoleic acid concentrations (mol%) in control (black bars) and EFA-deficient (white bars) Caco-2 cells 7 days after confluence. (b) Fatty acid families of essential fatty acids (ω-3 and ω-6) and nonessential fatty acids (ω-7 and ω-8). Control and EFA-deficient cells were treated with vehicle, 50 or 250 µM CDCA or 1 µM GW4064 for 24 hours. Subsequently, cells were harvested for RNA isolation and quantitative PCR analysis of relative mRNA expression of FXR-target genes (c) FGF19 and (d) IBABP was performed. Data of fatty acid determination represent ±SEM of four independent experiments. Quantitative PCR data represent ±SEM of at least three cell experiments. *p<0.05 is the significant difference between the EFA-deficient versus control Caco-2 cells with the same treatment status. #p<0.05 is the significant difference between treated and non-treated Caco-2 cells of the same phenotype.
the ratio of cholic acid over chenodeoxycholic acid in the bile. However, as stated previously, our data on biliary composition in EFA-deficient mice do not show major differences in the concentrations cholic acid and chenodeoxycholic acid in bile compared with control animals. This suggests that the increased Cyp8b1 mRNA gene expression does not lead to major physiological changes in EFA-deficient mice. Unlike Cyp7a1, Cyp8b1 was shown to have the highest mRNA expression during the day; this could be an explanation for the detected difference in Cyp8b1, but not in Cyp7a1, mRNA expression between EFA-deficient and control mice. Our findings on mRNA gene expression measurements of Cyp7a1 and Cyp8b1 underscore the importance of physiological measurements, along with mRNA expression of genes in order to properly study the EHC of bile salts in vivo. The expression of other relevant genes involved in bile salt synthesis (Shp, Cyp27a1) remained similar in EFA-deficient compared with control mice, while the bile salt synthesis was increased.

Stable isotope dilution study revealed an enlargement of the bile salt pool in EFA-deficient mice, suggesting an impaired feedback inhibition of the hepatic bile salt synthesis. The FTR, representing the fraction of the pool renewed each day, was decreased in EFA-deficient mice, while the reabsorption of the bile salts in the intestine was increased. Normally, the enhanced bile salt reabsorption is expected to activate the FXR in the ileum and thereby induce the release of Fgf15 into the portal circulation, which in turn eventually inhibits the bile salts synthesis in the liver. To our surprise, we found a decreased mRNA expression of Fgf15 gene in EFA-deficient mice, indicating a disruption in the intestinal feedback regulation of bile salt synthesis. We realize that the experimental setting we performed in this study does not allow for direct evidence demonstrating that the increased bile salts synthesis in EFA-deficient mice is directly related to, or the result of, the lower plasma concentration of Fgf15. So far, it has not been possible to determine the concentration of Fgf15 in the (portal) plasma of EFA-deficient mice and all of the studies performed so far on the role of Fgf15 in bile salt metabolism in mice are based on the ileal mRNA expression of this gene. For this reason, we performed in vitro experiments in EFA-deficient Caco-2 cells. Although there might be a trend in lower FGF19 mRNA expression in EFA-deficient compared with control Caco-2 cells, we do not see a significant difference after stimulation with CDCA. Unfortunately, we were not able to measure reliable concentrations of FGF19 secreted in medium of stimulated EFA-deficient and control cells. Song et al. recently reported successful measurements of secreted FGF19 in media of cultured human hepatocytes after CDCA treatment for 24 hours. However, we cannot exclude differential regulation of FGF19 secretion in hepatic and intestinal cell lines after CDCA stimulation. In EFA-deficient mice the mRNA expression of Asbt gene and the gene encoding intestinal heteromeric basolateral transporter Ostα/β did not correlate with increased bile salt reabsorption. This suggests a limited role for these transporters in enhanced reabsorption during EFA deficiency in mice and is in agreement with several previous findings. mRNA expression of the cytosolic protein IBABP was not significantly decreased in EFA-deficient mice. However, our in vitro experiments revealed impaired induction of IBABP mRNA gene expression in EFA-deficient Caco-2 cells after CDCA and GW4064 stimulation. Discrepancy between in vivo and in vitro data requires further research. Taken together, in vivo and in vitro data
indicate that besides FXR other factors contribute to the altered EHC of bile salts during EFA deficiency.

In order to determine to what extent the effects of EFA deficiency on bile production were FXR-dependent, we repeated key experiments in mice lacking FXR and their wild type littermates. The rationale behind this was based on two findings; first, during the past decade FXR has been shown to play a crucial role in controlling bile acid homeostasis,\textsuperscript{41,42} and second, our data on bile salt kinetics during EFA deficiency in mice corresponded to a certain extent with changes in bile salt kinetics upon FXR-inactivation observed by Kok et al.\textsuperscript{9} Similar to the situation in \textit{Fxr}\textsuperscript{-/-} mice, EFA-deficient mice showed enhanced pool size, increased synthesis and enhanced bile salt reabsorption of bile salts and similar cycling time compared with control mice. Despite the similarities in the separate effects of EFA deficiency and FXR deficiency in mice on bile salt kinetics, we showed that when combined, the effects of EFA deficiency with additional FXR-inactivation on bile flow and biliary secretion are similar to the effects of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Proposed mechanism of altered bile salt homeostasis during EFA deficiency in mice. Despite an increased secretion of bile salts from the liver to the intestine, bile salt synthesis and pool are increased, while the circulating time of bile salts is unaltered in EFA deficient mice. We suggest that the reduced \textit{Fgf15} mRNA expression might be responsible for the lack of the inhibition of hepatic synthesis of bile salts.}
\end{figure}

EFA deficiency alone. This indicates that when combined, the effects of EFA deficiency on bile flow and biliary secretion rate of bile salts are independent of FXR. Kok et al. proposed that the defective negative feedback inhibition of hepatic cholate synthesis was the consequence of the absence of FXR \textit{in vivo}. The underlying mechanism, however, remained unclear.\textsuperscript{9} In our study we show that the defective negative feedback inhibition of bile salt synthesis in EFA-deficient mice is probably due to reduced \textit{Fgf15} expression. In Figure 6, we propose the mechanism responsible for altered bile salt kinetics during EFA deficiency in mice. The increased bile salt secretion is consistent
with increased synthesis, larger pool size and enhanced ileal reabsorption, without affecting the cycling time of bile salts. We suggest that the preserved bile salt synthesis is due to an intestinal (intracellular) defect leading to a decreased, instead of increased, expression of \textit{Fgf15}. However, the exact intracellular effects of EFA deficiency on bile salt activation of FXR and subsequent regulation of the \textit{Fgf15} gene expression and its secretion remain to be elucidated. EFA deficiency can possibly lead to increased cellular permeability and reduced membrane integrity in the enterocytes, resulting in impaired uptake of bile salts in the ileal enterocytes. To our knowledge, studies on cellular permeability in the enterocytes during EFA deficiency have not been performed yet. Further studies of the effects of EFA deficiency on cellular function will hopefully help us understand how this correlates to the intestinal regulation of the negative feedback synthesis of bile salts.

In conclusion, our study demonstrates that EFA deficiency in mice clearly affects bile salt metabolism at several steps during the EHC of bile salts. We show, indirectly, that reduced intestinal function is at least partly involved in EFA deficiency associated alterations in the gut-liver signaling during the bile salt homeostasis. Further studies are required to determine if adaptations in bile homeostasis can allow for improved fat absorption during EFA deficiency.

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REFERENCES

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Song KH, Li T, Owsley E, Strom S, and Chiang JY. *Hepatology* 2009; 49(1): 297-305.


