Effects of essential fatty acid deficiency on enterohepatic circulation of bile salts in mice

S. Lukovac, E. L. Los, F. Stellaard, E. H. H. M. Rings, and H. J. Verkade

Pediatric Gastroenterology, Department of Pediatrics, Beatrix Children’s Hospital, Groningen University Institute for Drug Exploration (GUIDE), Center for Liver, Digestive and Metabolic Diseases, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Submitted 10 March 2009; accepted in final form 13 July 2009

Lukovac S, Los EL, Stellaard F, Rings EH, Verkade HJ. Effects of essential fatty acid deficiency on enterohepatic circulation of bile salts in mice. Am J Physiol Gastrointest Liver Physiol 297: G520–G531, 2009. First published July 16, 2009; doi:10.1152/ajpgi.00091.2009.—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 and a selective FXR agonist 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 because of reduced ileal Fgf15 expression.

fgf15; FGF19; small intestine; stable isotope dilution

ESSENTIAL FATTY ACID (EFA) deficiency is a frequent condition in patients with cholestasis or cystic fibrosis (3, 43, 47) and has various effects on bile production and absorption of dietary fat (27–29, 49). Bile salts are essential for bile production, secretory processes, and efficient intestinal absorption of dietary fat. In our laboratory we developed a mouse model to study the effects of EFA deficiency in vivo (31, 49). Previous studies in this mouse model have shown that EFA deficiency-associated fat malabsorption is not caused by impaired bile formation, as has been implied earlier in a rat model for EFA deficiency (28).

In EFA-deficient mice an increase in bile flow and biliary secretion was observed (49). 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 (25). Under physiological conditions, per enterohepatic cycle less than 5% 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 (17, 25). 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 present study we measured different steps of the EHC 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 (Cyp7a1), which is the rate-limiting enzyme in bile salt synthesis (2, 9). Shortly, by stable isotope dilution technique, different parameters of the EHC (synthesis, pool size, fractional turnover rate, ileal reabsorption, and cycling time) were determined 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 laboratory to measure bile salt kinetics in vivo without interrupting the normal EHC (17, 25). We have chosen to inject the stably labeled cholate (2H4-cholate), since this is the primary and most abundant bile salt in humans and rodents; the total bile acid pool consists of 30–50% and 50–80% of cholate in humans and rodents, respectively (17). To confirm our 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 (postconfluent Caco-2 cells) upon stimulation with chenodeoxycholic acid (CDCA) and GW4064 compound, both potent agonists of farnesoid X receptor (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 in which we aimed to determine whether the effects of EFA deficiency on the EHC mechanism of bile salts were FXR dependent, we used male homozygous (FXr/--) and wild-type (FXr+/+) mice on mixed (C57BL/6Jx129/OlaHsd) background of 25–40 g. These mice were generated previously by Deltagen (Redwood City, CA) and bred at the animal facility of the University of Groningen (25). Food intake and fecal excretion were monitored during a 72-h period at the end of the experiment. For clarity reasons, mice fed the EFA-deficient or control diet (on FVB background) will be referred to as “EFA-deficient” or “control” mice. FXr+/+ and FXr/-- mice fed the EFA-deficient or control diet will also be called 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 (no. 4141.08) and EFA-sufficient (control, no. 4141.07) diets (16 wt% and 34 energy% fat), which were custom synthesized by Arie Bloks (Woerden, the Netherlands) (31, 49). Essentially, unsaturated fatty acids in EFA-sufficient diet were replaced by saturated fatty acid in an EFA-deficient diet; the EFA-deficient diet was particularly reduced in linoleic acid (essential fatty acid) concentration. In detail, the EFA-deficient diet contained 64 mol% palmitic acid (C16:0), 18 mol% stearic acid (C18:0), 13 mol% oleic acid (C18:2n-9), and 5 mol% linoleic acid (C18:2n-6). The isocaloric EFA-sufficient diet contained 36 mol% C16:0, 5 mol% C18:0, 31 mol% C18:1n-9, and 29 mol% C18:2n-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 (34, 49).

Materials for Stable Isotope Dilution Technique and Cell Culture Experiments

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

Methods

Induction of EFA deficiency. Mice were fed the EFA-deficient or control diet for 8 wk, consistent with previous studies in EFA-deficient mice (31, 48, 49). After 8 wk of EFA-deficient or control diet, mice underwent a 72-h fat balance test, bile cannulation, and stable isotope dilution test. Afterward, the mice were anesthetized and euthanized through cardiac puncture. The marker of EFA deficiency, triene-to-tetraene ratio (C20:3n-9/C20:4n-6), was determined in erythrocyte lipids as described previously (31, 49). Protocol for induction of EFA deficiency in Caco-2 cells is described in Stimulatation 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 72 h. Net amount of fat absorbed was calculated by subtracting the fecal excretion of the major fatty acids (stearate, palmitate, oleate, and linoleate) from the amount of fatty acids ingested, determined by gas chromatography (49).

Stable isotope dilution. The stable isotope dilution test was performed as previously described, slightly modified (17). 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 60 h) 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 min, 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) (25).

Sample collection. The small intestine was excised and 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). Before the experiment, the cells were maintained in DMEM (GIBCO-BRL) supplemented with 10% FBS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1% nonessential amino acids, and 0.25% glutamine. Culture medium was composed of DMEM containing 4.5 g/l glucose, 1% nonessential amino acids, 1% vitamins, and 1% sodium pyruvate. Cells were grown at 37°C in a humidified incubator (37°C) (25).

Stimulation of differentiating EFA-deficient and control Caco-2 cells with CDCA and GW4064. For the experiment, cells (between passages 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. (45). Shortly, medium was replaced by DMEM supplemented with dialyzed FBS (control cells) or with delipidated FBS (EFA-deficient cells) 1 day after seeding. Delipidation of FBS was performed by means of di-iso-propylether and 80 ml butan-1-ol extraction. Seven days after complete confluence, the cells were exposed to serum-free DMEM containing CDCA (50 or 250 μM), GW4064 (1 μM), or vehicle for 24 h. Afterward, 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 fluorometric assay (33). Biliary phospholipids and cholesterol were determined as described by Kuipers et al. (26).

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. (34). Subsequent separation and quantification of fatty acid methyl esters was performed by gas chromatography (49). Bile salt composition of bile samples was determined by capillary gas chromatography (25).

Preparation of plasma samples for isotope analysis and GLC-MS. Plasma and bile samples were prepared for gas-liquid chromatography-electron capture negative chemical ionization mass spectrometry (GLC-MS) analysis on a Finnigan SSQ7000 Quadrupole GC-MS machine as described previously by Stellard et al. (46). Isotope dilution technique has been described in detail by Hulzebos et al. (17).

Shortly, enrichment of $H_4$-cholate in plasma was determined as the increase of the $M_4/M_0$-cholate ratio relative to baseline measurements and is expressed as the natural logarithm of atom percent excess (In 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 / isotopic purity × 100)/intercept 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 the biliary secretion rate of cholate were calculated as described previously (17, 25). Cholate was the most abundant bile acid in the bile salt pool of both EFA-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. 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-transporter genes (for bile salts, cholesterol, and phospholipids), ileal expression of hepatic genes involved in bile salt synthesis, hepatic mRNA expression by quantitative PCR.

Statistical Analysis

Using SPSS version 12.0.2 statistical software (Chicago, IL), 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 by Student’s two-tailed t-test.

For all experiments, $P$ values below 0.05 were considered statistically significant.

RESULTS

Body weight and food intake were not different between EFA-deficient and control FVB mice (Table 2). Consistent with our previous findings in EFA-deficient mice, liver weight was higher in EFA-deficient mice compared with controls (Table 2), most probably owing to fat accumulation as indicated by a trend toward an increased concentration of triglycerides [117.4 ± 57.4 nmol/mg protein in control mice vs. 176.5 ± 57.7 nmol/mg protein in EFA-deficient mice (NS)] and significantly increased cholesterol (57.6 ± 10.1 nmol/mg protein in control mice vs. 83.1 ± 8.6 nmol/mg protein in EFA-deficient mice, $P < 0.05$) in the livers of EFA-deficient mice (data not shown) (49). After 8 wk 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 vs. 0.01 ± 0.00 in control mice, $P < 0.05$) (Table 2). The induction of EFA deficiency in mice decreased fat absorption by ~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 FXR has been identified as an important regulator of the bile salt metabolism (10, 32). 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 (20). Fgf15 has been characterized as an important component of the gut-liver signaling pathway that regulates the bile salt synthesis. Similar to EFA-deficient and control FVB mice, after 8 wk 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 vs. 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 vs. control mice, $P < 0.05$) (Table 4) (49). 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 whether 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 (14). EFA deficiency did not result in major changes in bile salt composition (Fig. 1) or in the Heuman index (~0.20 ± ~0.06 in EFA-deficient vs. ~0.21 ± ~0.03 in control mice, NS).

To investigate whether 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). 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
<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Forward Primer</th>
<th>Reversed Primer</th>
<th>TaqMan probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>NM_007393</td>
<td>AGC CAT GTA ACC ATC CA</td>
<td>TCT CCG GAG ATC ACA ATG</td>
<td>TGT CCG TGT ATG CCT CTC TCT GTA CCA C</td>
</tr>
<tr>
<td>18s</td>
<td>X03205/X0086/M11188</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Fxr</td>
<td>U09416</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Fxrα</td>
<td>U09417</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Fxrβ</td>
<td>AK002513</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Fgfr4</td>
<td>BC100260.1</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>NM_007824.2/M_012942.1</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Cyp27a1</td>
<td>AK004977/M38566</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Cyp8b1</td>
<td>NM_010012</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Bsep</td>
<td>NM_021022</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Ntcp</td>
<td>AB003303</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Mdr2</td>
<td>NM_022090.1</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Abcg5</td>
<td>NM_031884/NM_053754</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Abcg8</td>
<td>NM_022437/NM_026180</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>IBABP</td>
<td>NM_002508</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Bsep</td>
<td>NM_000145</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Bsep</td>
<td>NM_031884/NM_053754</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
<tr>
<td>Bsep</td>
<td>NM_145932.3/M_221376.4</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
<td>CCA AGG GCT CTC TGT G</td>
</tr>
</tbody>
</table>

Primers and probes indicated in upper case are of human origin used for quantitative PCR analysis in Caco-2 cells. Remaining primers and probes were used for quantitative PCR analysis in mouse tissues.
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. The 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 (Fig. 2A). EFA deficiency was associated with higher pool size (Fig. 2B: 40 ± 1 $\mu$mol/100 g in EFA-deficient mice vs. 16 ± 7 $\mu$mol/100 g of in control mice, P < 0.05), higher synthesis rate (Fig. 2C: 11 ± 2 $\mu$mol·100 g$^{-1}$·day$^{-1}$ in EFA-deficient mice vs. 8 ± 2 $\mu$mol·100 g$^{-1}$·day$^{-1}$ in control mice, P < 0.05), and lower fractional turnover rate (Fig. 2D: 0.6 ± 0.2 per day vs. 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 (Fig. 2E: 512 ± 275 $\mu$mol·100 g$^{-1}$·day$^{-1}$ vs. 156 ± 29 $\mu$mol·100 g$^{-1}$·day$^{-1}$ in control mice, P < 0.05), whereas the cycling time of cholate was not affected by EFA deficiency (Fig. 2F: 2.3 ± 1.1 h in EFA-deficient vs. 2.4 ± 1.1 h 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 (Fig. 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 vs. 1.24 ± 0.59 in control mice, NS) (Fig. 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 vs. 1.2 ± 0.3 in control mice, P < 0.05) (Fig. 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, Fgf14, and Cyp27a1) (Fig. 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 to inhibit the hepatic bile salt synthesis (20). We observed a decrease in mRNA expression of Fgf15 gene in the terminal ileum of EFA-deficient (FVB) mice, (Fig. 3B). These data are confirmed by measurement of Fgf15 in Fxr$^{++}$/+ mice on a mixed background (C57BL/6Jx129/OlaHsd); in EFA-deficient Fxr$^{++}$/+ mice, Fgf15 mRNA expression was significantly lower than in Fxr$^{++}$/+ mice on control diet (Fig. 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 (Fig. 4B), demonstrating that Fgf15 mRNA expression is regulated by the FXR, as reported previously (20). The mRNA expression of FXR itself and genes encoding the ileal bile salt-transporters (Ostra, Ostβ, 

---

**Table 2. Animal characteristics of FVB mice fed EFA-deficient or control diet for 8 wk**

<table>
<thead>
<tr>
<th>Strain</th>
<th>FVB</th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.9±2.2</td>
<td>27.1±1.9</td>
<td></td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.2±0.1</td>
<td>1.4±0.1*</td>
<td></td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>4.3±1.0</td>
<td>4.9±1.6</td>
<td></td>
</tr>
<tr>
<td>Triene-to-tetraene ratio</td>
<td>0.0±0.0</td>
<td>0.2±0.1*</td>
<td></td>
</tr>
<tr>
<td>Fat absorption, % of total fat</td>
<td>99.4±0.2</td>
<td>80.9±2.9*</td>
<td></td>
</tr>
</tbody>
</table>

*Values are represented as means ± SD (n = 6 mice per group). *P < 0.05 is the significant difference between essential fatty acid (EFA)-deficient and control free virus breed (FVB) mice.

---

**Table 4. Bile flow and biliary secretion rates in FVB mice fed EFA-deficient or control diet for 8 wk**

<table>
<thead>
<tr>
<th>Strain</th>
<th>FVB</th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow, µl·min$^{-1}$·100 g$^{-1}$·body wt</td>
<td>4.3±1.3</td>
<td>7.6±2.6*</td>
<td></td>
</tr>
<tr>
<td>Bile salts, µl·min$^{-1}$·100 g$^{-1}$·body wt</td>
<td>121±30</td>
<td>376±196*</td>
<td></td>
</tr>
<tr>
<td>Phospholipids, µl·min$^{-1}$·100 g$^{-1}$·body wt</td>
<td>12±6</td>
<td>22±6*</td>
<td></td>
</tr>
<tr>
<td>Cholesterol, µl·min$^{-1}$·100 g$^{-1}$·body wt</td>
<td>1±0</td>
<td>2±0*</td>
<td></td>
</tr>
</tbody>
</table>

*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.

---

**Fig. 1. Biliary bile salt composition in essential fatty acid (EFA)-deficient and control 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 2 groups. α-M, α-muricholate; DC, deoxycholate; C, cholate; CDC, chenodeoxycholate; HDC, hydoxycholate; UDC, ursodeoxycholate; β-M, β-muricholate; ω-M, ω-muricholate.
Ibabp, and Asbt) was not significantly affected by EFA deficiency in mice (Fig. 3B).

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 (Fig. 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) (Fig. 3C) (15).

**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 both \(Fxr^{+/+}\) and \(Fxr^{-/-}\) mice (+97% and +112%, respectively, compared with mice of the same genotype on control diet, \(P < 0.05\)) (Fig. 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 toward a higher biliary secretion of bile salts and phospholipids in EFA-deficient mice with both genotypes. Secretion of phospholipids showed a trend toward 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 postconfluent Caco-2 cells treated with CDCA and GW4064, both very potent FXR agonists (the latter one highly specific). Upon confluence Caco-2 cells spontaneously differentiate and develop small intestinal features, as indicated by the expression of enterocyte markers lactase and sucrase-isomaltase (6). As expected, after 8–10 days in EFA-deficient medium Caco-2 cells showed clear signs of EFA deficiency as indicated by significantly lower...
lactase expression in mid intestine of EFA-deficient mice (31).

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 (Fig. 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) (Fig. 5C). This effect was also seen in EFA-deficient cells after treatment with 250 μM CDCA (Fig. 5C). Treatment with GW4064 (1 μM) did not increase mRNA expression of FGF19 in either control or EFA-deficient Caco-2 cells (Fig. 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 with control cells in all conditions.

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 (Fig. 5D). This effect was almost completely absent in EFA-deficient Caco-2 cells (Fig. 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

![Bile flow](image)

**Table 5. Bile flow and biliary secretion rates in Fxr<sup>−/−</sup> mice and their wild-type littermates (C57BL/6Jx129/OlaHsd background) on EFA-deficient or control diet for 8 wk**

<table>
<thead>
<tr>
<th>Strain C57BL/6Jx129/OlaHsd</th>
<th>Fxr&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Fxr&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EFA-deficient</td>
</tr>
<tr>
<td>Bile flow, μL·min&lt;sup&gt;−1&lt;/sup&gt;·100 g&lt;sup&gt;−1&lt;/sup&gt; body wt</td>
<td>3.3 ± 0.3</td>
<td>6.5 ± 1.5</td>
</tr>
<tr>
<td>Bile salts, μL·min&lt;sup&gt;−1&lt;/sup&gt;·100 g&lt;sup&gt;−1&lt;/sup&gt; body wt</td>
<td>176 ± 71</td>
<td>239 ± 117</td>
</tr>
<tr>
<td>Phospholipids, μL·min&lt;sup&gt;−1&lt;/sup&gt;·100 g&lt;sup&gt;−1&lt;/sup&gt; body wt</td>
<td>17 ± 2</td>
<td>33 ± 9</td>
</tr>
</tbody>
</table>

Values are represented as means ± SD (n = 5–7 mice per group).
data suggest that the increased bile salt synthesis may be related to downregulation of ileal Fgf15 gene expression, interfering with the negative feedback regulation of hepatic bile salt synthesis.

Consistent with our previous findings, after 8 wk 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 the present study EFA deficiency in mice was not associated with major changes in biliary bile salt composition (49). 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 in which fat absorption is similar to that in mice (80–84%), despite a decreased biliary secretion (28, 35). 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 owing to the fact that the expression of the transporters is not a rate-limiting factor for the increased secretion rate of bile salts (50). Moreover, bile salt transporters are localized along the hepatic acinus, whereas the bile salt transport is localized mainly at the perportal zone (12). 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 (17) 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 (18, 30). Previous studies in EFA-deficient mice revealed unaltered mRNA expression of Cyp7a1 (49). 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 (36). 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 the ratio of cholic acid over CDCA in the bile (51). However, as stated previously, our data on biliary composition in EFA-deficient mice do not show major differences in the concentrations of cholic acid and
CDCA 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 (21). Our findings on mRNA gene expression measurements of Cyp7a1 and Cyp8b1 underscore the importance of physiological measurements, along with mRNA expression of genes 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, whereas 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, whereas 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 (20, 22). 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 a 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 are based on the ileal mRNA expression of this gene (1, 13, 20, 22, 24, 40). 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. (44) recently reported successful measurements of secreted FGF19 in media of cultured human hepatocytes after CDCA treatment for 24 h. 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 (18, 25, 41). mRNA expression of the cytosolic protein Ibabp was not significantly decreased in EFA-deficient mice. However, our in vitro experiments revealed impaired induction of IBAAP mRNA gene expression in EFA-deficient Caco-2 cells after CDCA and GW4064 stimulation. The 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.

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 (8, 23), 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. (25). Similar to the situation in Fxr−/− 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 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. (25) proposed that the defective negative feedback inhibition of hepatic cholate synthesis was the consequence of the absence of FXR in vivo. The underlying mechanism,
however, remained unclear. In our study we show that the defective negative feedback inhibition of bile salt synthesis in EFA-deficient mice is probably due to reduced Fgf15 expression. In Fig. 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 Fgf15. However, the exact intracellular effects of EFA deficiency on bile salt activation of FXR and subsequent regulation of the 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 whether adaptations in bile homeostasis can allow for improved fat absorption during EFA deficiency.

ACKNOWLEDGMENTS
The authors thank Rick Havinga, Ingrid Martini, Juul Baller, Renze Boerhof, and Theo Boer for excellent technical assistance.

GRANTS
This study was supported by the Dutch Digestive Foundation (MLDS).

REFERENCES


