Fat malabsorption in essential fatty acid-deficient mice is not due to impaired bile formation

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Werner, Anniek, Deanna M. Minich, Rick Havinga, Vincent Bloks, Harry Van Goor, Folkert Kuipers, and Henkjan J. Verkade. Fat malabsorption in essential fatty acid-deficient mice is not due to impaired bile formation. Am J Physiol Gastrointest Liver Physiol 283: G900–G908, 2002; 10.1152/ajpgi.00094.2002.—Essential fatty acid (EFA) deficiency induces fat malabsorption, but the pathophysiological mechanism is unknown. Bile salts (BS) and EFA-rich biliary phospholipids affect dietary fat solubilization and chylomicron formation, respectively. We investigated whether altered biliary BS and/or phospholipid secretion mediate EFA deficiency-induced fat malabsorption in mice. Free virus breed (FVB) mice received EFA-containing (EFA+) or EFA-deficient (EFA−) chow for 8 wk. Subsequently, fat absorption, bile flow, and bile composition were determined. Identical dietary experiments were performed in multidrug resistance gene-2-deficient [Mdr2−/−] mice, secreting phospholipid-free bile. After 8 wk, EFA−-fed wild-type [Mdr2+/+ and Mdr2−/−] mice were markedly EFA deficient [plasma triene (20:3n-9)-to-tetraene (20:4n-6) ratio > 0.2]. Fat absorption decreased (70.1 ± 4.2 vs. 99.1 ± 0.3%, P < 0.001), but bile flow and biliary BS secretion increased in EFA− mice compared with EFA+ controls (4.87 ± 0.36 vs. 2.87 ± 0.29 μl·min−1·100 g body wt−1, P < 0.001, and 252 ± 30 vs. 145 ± 20 nmol·min−1·1·100 g body wt−1, P < 0.001, respectively). BS composition was similar in EFA− and EFA+ fed mice. Similar to EFA− Mdr2−/− mice, EFA− Mdr2−/− mice developed fat malabsorption associated with twofold increase in bile flow and BS secretion. Fat malabsorption in EFA− mice is not due to impaired biliary BS or phospholipid secretion. We hypothesize that EFA deficiency affects intracellular processing of dietary fat by enterocytes.

Fat absorption; multidrug resistance gene-2; ATP-binding cassette; polyunsaturated fatty acids; phospholipid; bile salt

The absorption process have been investigated in control and EFA− rats, i.e., lipolysis of dietary triglycerides by lipases, solubilization of lipolytic products by bile components, uptake by the enterocyte and intracellular reesterification to triglycerides and, finally, chylomicron formation and secretion into lymph (2, 21). Available data from rat models of EFA deficiency have indicated that neither fat digestion nor fatty acid uptake by the enterocyte is responsible for the fat malabsorption encountered in EFA deficiency (2, 21). In rats, EFA deficiency resulted in significantly decreased bile flow and biliary secretion of bile salts, phospholipids, and cholesterol (20, 21). Acyl chain analysis of biliary phosphatidylcholine (PC) from EFA− rats revealed that biliary PC contents of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) were decreased to 10 and 26% of control values, respectively, and were replaced by oleic acid (18:1n-9) (2). In addition to alterations in bile, intracellular events, such as fatty acid reesterification and chylomicron production, were severely impaired (2, 21). It thus seems that EFA deficiency in rats affects bile formation and/or intracellular processing of fat by the enterocyte, resulting in fat malabsorption. In a previous study (32), we demonstrated the role of biliary PC in intestinal chylomicron formation, using multidrug resistance gene-2 Mdr2−/− mice [gene symbol: ATP-binding cassette-B4 (ABC-B4)] that are unable to secrete phospholipids into bile (26). We reasoned that this animal model could be important to further delineate the pathophysiological mechanism of EFA− induced fat malabsorption.

If altered biliary phospholipid secretion were involved in EFA−-associated fat malabsorption, one would expect fat absorption in Mdr2−/− mice to be relatively unaffected by changes in the EFA status. If, however, the pathophysiological mechanism would not involve alterations in biliary phospholipid secretion, EFA deficiency would be expected to equally affect the process in Mdr2−/− and in wild-type [Mdr2+/+] mice. In the present study, we investigated the role of bile formation in general and of biliary phospholipid secretion in particular in the pathophysiology of EFA−...
associated fat malabsorption. We first developed and characterized a murine model for EFA deficiency with respect to fat absorption and bile formation. We then compared fat absorption and bile formation in EFA and EFA-sufficient (EFA\(^+\)) \textit{Mdr2}\(^{−/−}\) mice.

**MATERIALS AND METHODS**

**Animals**

Mice homozygous for disruption of the \textit{Mdr2} \(^{−/−}\) and \textit{Mdr2} \(^{+/+}\) with a free virus breed (FVB) background were obtained from the breeding colony at the Central Animal Facility (Academic Medical Center, Amsterdam, The Netherlands) (13). All mice were 2–4 mo old and weighed 25–30 g. Mice were housed in a light-controlled (lights on 6 AM–6 PM) and temperature-controlled (21°C) facility and allowed tap water and chow (Hope Farms, Woerden, The Netherlands) ad libitum. The experimental protocol was approved by the Ethics Committee for Animal Experiments, Faculty of Medical Sciences (University of Groningen, The Netherlands).

**Experimental Procedures**

Fat absorption and bile secretion in EFA\(^−\) mice. FVB mice (\(n = 14\)) were fed standard low-fat chow (6 weight\% fat, 14 energy\% fat) for standardization for 1 mo after which they were anesthetized with halothane and a baseline blood sample was obtained by tail bleeding for determination of EFA status. Blood was collected in microhematocrit tubes containing heparin, and plasma was separated with an Eppendorf centrifuge at 9,000 rpm for 10 min and stored at \(−20°C\) until analysis. Subsequently, mice were randomly assigned to either an EFA\(^−\) or an EFA\(^+\) diet. The EFA\(^−\) and EFA\(^+\) diets were isocaloric and contained 16 weight\% fat. The EFA\(^−\) chow contained 20, 34, and 46 energy\% from protein, fat, and carbohydrate, respectively, and had the following fatty acid profile: 32 mol\% palmitic acid (16:0), 6 mol\% stearic acid (18:0), 32 mol\% oleic acid (18:1n-9), and 30 mol\% linoleic acid (18:2n-6) (custom synthesis; Hope Farms). The EFA\(^−\) diet had identical energy percentages derived from protein, fat, and carbohydrate, and had the following fatty acid composition: 41 mol\% 16:0, 48 mol\% 18:0, 8 mol\% 18:1n-9, and 3 mol\% 18:2n-6 (custom synthesis; Hope Farms). At intervals of 2 wk, blood samples were taken in the manner described above. After 4 and 8 wk of experimental diet, a 72-h fecal fat balance was performed involving quantitative feces collection and determination of chow intake. At \(8 wk\), mice were anesthetized by intraperitoneal injection of Hypnorm (fentanyl/ fluanisone) and diazepam, and gallbladders were cannulated for collection of bile for 1 h as described previously (31). At the end of bile collection, a large blood sample (0.6–1.0 ml) was obtained by heart puncture.

Hepatic expression of \textit{Cyp7A} and \textit{Cyp27}. A separate group of male FVB mice was fed EFA\(^−\) or EFA\(^+\) chow for 8 wk (\(n = 6\) mice per dietary group). After \(8 wk\), animals were anesthetized with halothane, and blood was collected by cardiac puncture for lipid analysis. Livers were removed, and liver samples were immediately frozen in liquid nitrogen and stored at \(−80°C\) for determination of mRNA levels of two key enzymes in bile salt biosynthesis, cholesterol 7α-hydroxylase (\textit{Cyp7A}) and sterol 27-hydroxylase (\textit{Cyp27}).

Plasma accumulation of oral \({\textsuperscript{1}H}\text{[triolein and \({\textsuperscript{14}C}\text{]oleic acid after Triton WR-1339 injection}}. In a separate experiment, male FVB mice fed EFA\(^−\) or EFA\(^+\) chow for 8 wk (\(n = 5\) per group) were intravenously injected with 12.5 mg Triton WR-1339 (12.5 mg/100 ml PBS) to block lipolysis of circulating lipoproteins in the blood. Subsequently, an intragastric fat bolus was administered containing 200 \(µl\) olive oil, in which 10 \(µCi\) glycerol tri-[9,10\(\alpha\)-\textsuperscript{3}H]oleate ([\textsuperscript{3}H]triolein) (Amersham, Arlington Heights, IL) and 2 \(µCi\) \[^{14}\text{C}\]oleic acid (NEN Laboratories, Boston, MA) were dispersed. Before (\(t = 0\)) and at 1, 2, 3, and 4 h after label administration, blood samples (75 \(µl\)) were taken by tail bleeding as described above. The content of \[^{3}H\] ([\textsuperscript{3}H]) and \[^{14}C\] ([\textsuperscript{14}C]) in plasma (25 \(µl\)) was measured by scintillation counting (Packard Instruments, Downers Grove, IL).

Plasma appearance of retinyl palmitate after retinol administration. In addition to the absorption of fatty acids during EFA deficiency, the plasma appearance of the less polar lipid molecule retinol (vitamin A) was investigated. Retinol (5,000 IU) in an olive oil bolus (100 \(µl\) per mouse) was administered intragastrically to EFA\(^−\) mice and control mice (\(n = 7\) per group), and blood samples were taken at 0, 2, and 4 h after bolus administration.

Fat absorption and bile secretion in EFA\(^−\) \textit{Mdr2} \(^{−/−}\) mice. Male \textit{Mdr2} \(^{−/−}\) mice (\(n = 14\)) were fed low-fat chow (6 weight\% fat; 14 energy\% fat) for standardization for 1 mo. Baseline blood samples were taken for determination of plasma EFA status, after which mice were randomly assigned to either the EFA\(^−\) or the EFA\(^+\) diet. Diets were identical to those described above. After 8 wk of feeding the respective diets, blood samples were obtained by tail bleeding under halothane anesthesia. As in the \textit{Mdr2} \(^{+/+}\) mice, fat absorption was measured by means of a 72-h fecal fat balance. Retinol in a bolus of olive oil was administrated intragastrically to EFA\(^−\) \textit{Mdr2} \(^{−/−}\) mice (\(n = 7\)) and their EFA\(^+\) controls (\(n = 7\)). Blood samples were taken at 0, 2, and 4 h after bolus administration. Gallbladders were cannulated, and bile was collected for 1 h as described above (31). At the end of bile collection, mice were killed after obtaining a large blood sample (0.6–1.0 ml) by heart puncture.

**Analytical Techniques**

Fatty acid status was analyzed by extracting, hydrolyzing, and methylating total plasma lipids, liver homogenates, and biliary lipids according to the method described by Lepage and Roy (19). To account for losses during lipid extraction, heptadecenoic acid (C17:0) was added to all samples as an internal standard before extraction and methylation procedures, and butylylated hydroxytoluene was added as an antioxidant. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard model 6890 gas chromatograph, equipped with a 50 m \(×\) 0.2 mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a flame ionization detector. The injector and detector were set at 260 and 250°C, respectively. The oven temperature was programmed from an initial temperature of 160°C to a final temperature of 290°C in three temperature steps (160°C, held 2 min; 160–240°C, ramp 2°C/min, held 1 min; 240–290°C, ramp 10°C/min, held 10 min). Helium was used as a carrier gas with a constant flow rate of 0.5 ml/min. Individual fatty acid methyl esters were quantified by relating the areas of their chromatogram peaks to that of the internal standard heptadecanoic acid (C17:0).

Plasma lipid levels (cholesterol, HDL-cholesterol, triglycerides, free fatty acids, and phospholipids) were measured using commercially available kits (Roche Diagnostics, Mannheim, WAKO Chemicals, Neuss, Germany) according to the instructions provided. Cholesterol, cholesterol ester, and triglycerides in liver tissue were determined after Bligh and Dyer (4) lipid extraction, and biliary bile salt composition was
measured as described previously. Total protein concentrations of liver homogenates were determined according to the method described by Lowry et al. (23).

Total RNA was isolated from liver tissue using TRIzol reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer’s instructions. Single-stranded cDNA was synthesized from 4.5-μg RNA and subsequently subjected to quantitative real-time detection RT-PCR (12, 14). The following primers and probes were used: for 

\[ \text{Cyp7A1 (GenBank accession no. L23754): 5'} - \text{CAG GAT GCT CTG TGT} \]
\[ \text{TCA-3'} \] (forward primer), 5’-AGG CAT ACA TCC CTG CCG
tGA CAC CTC C-3’ (reverse primer), 5’-TGC AAA ACC ATG AAT

CTG TCA TGA GAC CTC C-3’ (probe).

For Cyp27 (M73231, M62401): 5’-GCC TTG GTG ACT-3’ (forward primer), 5’-CCG GAG TCC ATC ACA ATG-3’ (forward primer), 5’-TCC GCC TGT GCT CGT GTC GTA CCA C-3’ (reverse primer), 5’-GCC TTC GGG AAG

CAG CAC-3’ (probe); and for β-actin (M12481): 5’-AGC CAT GGA GTG ACT CCA-3’ (forward primer), 5’-TCT CCG GAG TCC ATC ATG ACA ATG-3’ (reverse primer), 5’-TGT

CCG TGT GCT GTC GTA CCA CCA C-3’ (probe). For each real-time PCR, 4-μl cDNA was used in a final volume of 20 μl, containing (in mmol/l) 500 of forward and reverse primers and 200 of probe, 250 MgCl₂, 10 deoxyribonucleoside triphosphate mix, and 5 μl real-time PCR buffer (10×), and 1.25 U Hot GoldStar (Eurogentec). Real-time detection PCR was performed on the ABI PRISM 7700 (PE Applied Biosystems) initialized by 10 min at 95°C to denature the complementary DNA followed by 40 PCR cycles each at 95°C for 15 s and 60°C for 1 min.

Feces and chow pellets were freeze-dried and then mechanically homogenized. From aliquots of each, lipids were extracted, hydrolyzed, and methylated (19). Resulting fatty acid methyl esters were analyzed by gas chromatography for the fatty acid content as described above. Fatty acids were quantified using heptadecaenoic acid (17:0) as the internal standard.

Retinyl palmitate concentrations in plasma were determined after two extractions with hexane (5). Retinyl acetate was added to plasma samples as an internal standard before lipid extraction. Samples were resuspended in ethanol and analyzed by reverse-phase HPLC using a 150 × 4.6 mm Symmetry RP18 column (Waters, Milford, MA) (36). The peak area of retinyl palmitate was normalized to that of retinyl acetate. At each time point, concentrations were expressed as micromoles of retinyl palmitate per liter of plasma.

Calculations

**Fatty acid status in plasma and in bile.** Relative concentrations (mol%) of plasma, liver, and biliary phospholipid fatty acids were calculated using the summed areas of major fatty acid peaks (palmitic, stearic, oleic, linoleic acids) and then expressing the area of each individual fatty acid as a percentage of the total area of the fatty acid peaks. Linoleic acid was measured by subtracting the amount of fatty acid excreted in feces in 72 h from the amount of this fatty acid ingested in 72 h (net fat absorption). This quantity was subsequently expressed as a percentage of the amount of fatty acid ingested in 72 h (coefﬁcient of fat absorption; 100-fold the ratio between (amount ingested and the amount recovered in feces) and the amount ingested). Net amount of fat absorption was calculated by subtracting the fecal loss of the four major fatty acids from the amount ingested.

**Statistics**

All results are presented as means ± SD for the number of animals indicated. Data were statistically analyzed using Student’s two-tailed t-test. The level of significance for all statistical analyses was set at P < 0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

**RESULTS**

**Body Weight and Chow Ingestion in EFA⁻ and EFA⁺ Mdr2⁻/-⁻ Mice.**

Body weight was monitored at the start of experimental feeding and then every 2 wk for 8 wk. No differences in basal or final weight were found for Mdr2⁻/-⁻ mice fed EFA⁺ or EFA⁻ chow (basal: 24.4 ± 2.0 and 24.6 ± 1.3 g; final: 23.3 ± 1.0 and 21.9 ± 2.0 g, respectively). Chow intake measured after 4 and 8 wk of experimental diet feeding was similar in both dietary groups (data not shown).

**Essential Fatty Acid Status**

**Triene-to-tetraene ratio in plasma and liver.** The classic biochemical parameter describing EFA status, the triene-to-tetraene ratio (20:3n-9/20:4n-6), was measured in plasma at baseline and every 2 wk for 8 wk and in liver after 8 wk of feeding EFA⁻ chow. The baseline plasma triene-to-tetraene ratio was 0.02 ± 0.00, which is well below the cutoff value for EFA deficiency, i.e., 0.19 ± 0.00, for plasma and 0.05 ± 0.00, which is well below the cutoff value for EFA deficiency, i.e., 0.19 ± 0.00, for liver, in contrast to control mice (0.01 ± 0.00 for controls). After 8 wk on EFA⁻ chow, mice had a pronounced EFA deficiency with triene-to-tetraene ratios of 0.66 ± 0.05 for plasma and 0.56 ± 0.09 for liver, in contrast to control mice (0.01 ± 0.00 for plasma; P < 0.001 and 0.02 ± 0.00 for liver; P < 0.001) (data not shown).

**Characterization of EFA Deficiency in Mice**

Because EFA deficiency has not been characterized before in a murine model, plasma and liver lipids were measured. No differences were found in cholesterol, HDL-cholesterol, free fatty acid or phospholipid concentrations in plasma between EFA⁺ and EFA⁻ mice (Table 1). Plasma triglyceride concentrations

Table 1. Plasma lipid concentrations in mice fed EFA⁺ or EFA⁻ chow for 8 wk

<table>
<thead>
<tr>
<th>Lipid</th>
<th>EFA⁺</th>
<th>EFA⁻</th>
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<tr>
<td>Cholesterol</td>
<td>2.04 ± 0.23</td>
<td>2.21 ± 0.12</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.44 ± 0.16</td>
<td>1.41 ± 0.08</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>0.48 ± 0.07</td>
<td>0.27 ± 0.05*</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.58 ± 0.10</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>2.84 ± 0.44</td>
<td>3.11 ± 0.17</td>
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</table>

Concentrations are given in millimoles per liter. No. = 7 mice per group. EFA⁺, essential fatty acid-containing; EFA⁻, EFA-deficient. *P < 0.001.
significant fat accumulation (triglyceride, unesterified fatty acids) during 72 h from their dietary intake in 72 h and then absorption was calculated by subtracting fecal excretion of these fatty acids during 72 h from their dietary intake in 72 h. Absorption was calculated by subtracting fecal excretion of these fatty acids during 72 h from their dietary intake in 72 h and then multiplying the result by 100 to obtain a coefficient (as detailed in MATERIALS AND METHODS). Data represent means ± SD of 7 mice per group, **P < 0.001.

Fat Absorption

Fecal fatty acid balance. The fecal balance revealed a decreased absorption of total dietary fat in EFA− mice compared with their EFA+−fed controls (P < 0.01) (Fig. 1). The absorption coefficient for EFA− mice was 70.1 ± 1.6% compared with absorption coefficients above 95% for mice fed EFA+ chow. Individual fatty acid balances for palmitic, stearic, oleic, and linoleic acids were calculated (Fig. 1). In EFA− mice, absorption of saturated fatty acids (palmitic and stearic) was more affected than absorption of unsaturated fatty acids (oleic and linoleic).

Bile Secretion and Composition

Table 3 shows that bile flow and biliary secretion of bile salt, cholesterol, and phospholipids during a 1-h period immediately after interruption of the enterohepatic circulation were higher in EFA− mice compared with controls (for each parameter, P < 0.001). Theoretically, alterations in bile salt hydrophobicity could contribute to fat malabsorption in EFA deficiency. However, bile salt composition appeared to be similar between both dietary groups (Table 4). EFA deficiency-associated changes in acyl chain composition of biliary PC were analyzed by gas chromatography. Relative concentrations (mol%) of Δ16:1n-7, Δ18:1n-9, and Δ18:1n-7 were higher (P < 0.001), and concentrations of 16:0, 18:2n-6, 18:0, and 20:4n-6 were lower (P < 0.05) in bile of EFA− mice compared with mice fed EFA+ chow (data not shown).

Cyp7A and Cyp27 mRNA Levels

To determine whether the increased bile salt secretion in EFA− mice might be due to increased hepatic bile salt synthesis, hepatic mRNA levels of Cyp7A and Cyp27 were measured by quantitative real-time RT-PCR, using β-actin mRNA as a housekeeping signal. No significant differences in hepatic mRNA levels of Cyp7A and Cyp27 were observed between mice fed EFA+ and EFA− chow (1.00 ± 0.64 vs. 0.63 ± 0.29 for Cyp7A and 1.00 ± 0.22 vs. 1.14 ± 0.19 for Cyp27) (Fig. 2). Values represent the ratio of specific hepatic mRNA levels of Cyp7A and Cyp27 to the hepatic mRNA level of β-actin, normalized to the EFA+ control group.

Plasma Accumulation of [3H]Triolein and [14C]Oleic Acid After Triton WR-1339 Administration

Figure 3 shows the time course of plasma [3H] and [14C] radioactivity after intragastric administration of [3H]triolein and [14C]oleic acid. EFA− mice had a slightly increased plasma concentration of both radioactive labels during the studied time frame, reaching a significant difference at 3 h after bolus administration. If EFA deficiency would differentially affect lipolysis and fatty acid uptake, an altered [3H]-to-[14C] ratio in

Values are expressed as a percentage of the total amount. Greater than 90% of all bile salts are represented. Minor metabolites (<1% of total area) have been excluded. Data represent means ± SD. No. = 5–7 mice per group, **P < 0.01.
plasma would be expected. However, when expressed as the ratio of plasma $[^3\text{H}]-[^{14}\text{C}]$ (Fig. 3C), no significant difference was found between EFA$^{-}$ and EFA$^{+}$ mice. Both for EFA$^{-}$ and EFA$^{+}$-fed mice, thin-layer chromatography revealed that $[^3\text{H}]$ as well as $[^{14}\text{C}]$ radioactivity was predominantly (>95%) present in the triglyceride fraction.

**Plasma Appearance Of Retinyl-Palmitate After Retinol Administration**

The absorption of dietary oleic acid, a polar lipid (class III, soluble amphiphile) (30) was only mildly impaired in EFA$^{-}$ mice (Fig. 1). In a separate experiment, we investigated plasma appearance of retinol (vitamin A), a less polar lipid (class I, insoluble nonswelling amphiphile), after its intragastric administration. Plasma concentrations of retinyl palmitate were significantly lower in EFA$^{-}$ mice compared with controls at 2 and 4 h after retinol administration (Fig. 4).

**Body Weight and Chow Ingestion in EFA$^{-}$ and EFA$^{+}$ Mdr2$^{(-/-)}$ Mice**

Basal body weights of Mdr2$^{(-/-)}$ mice entering the EFA$^{+}$ and EFA$^{-}$ group were similar (26.1 ± 1.6 vs. 25.4 ± 1.5, respectively). However, body weights of Mdr2$^{(-/-)}$ mice fed EFA$^{-}$ chow gradually decreased compared with Mdr2$^{(-/-)}$ mice fed EFA$^{+}$ chow, and by 8 wk this resulted in a significantly lower body weight for EFA$^{-}$-compared with EFA$^{+}$-fed Mdr2$^{(-/-)}$ mice (20.2 ± 1.0 vs. 25.3 ± 1.1 g, respectively; $P < 0.01$). No significant difference in chow intake was observed between the two dietary groups after 4 or 8 wk on either diet (data not shown).

**EFA Status**

Triene-to-tetraene ratio in plasma and liver. The baseline triene-to-tetraene ratios (20:3n-9/20:4n-6) in plasma and liver were significantly higher in Mdr2$^{(-/-)}$ compared with Mdr2$^{(+/-)}$ mice (0.035 ± 0.003 vs. 0.018 ± 0.001; $P < 0.001$) but were still well below the cutoff value for EFA deficiency (0.2). Mdr2$^{(-/-)}$ mice fed EFA$^{-}$ chow for 8 wk developed EFA deficiency according to triene-to-tetraene ratios in plasma (0.46 ± 0.03) and in liver (0.38 ± 0.04) in contrast to the EFA$^{+}$-fed Mdr2$^{(-/-)}$ controls (0.01 ± 0.01 for plasma, $P < 0.001$; and 0.02 ± 0.00 for liver, $P < 0.01$).

![Graph showing relative mRNA levels of Cyp7A and Cyp27 in EFA$^{+}$ and EFA$^{-}$ mice](image)

**Fig. 2.** Hepatic mRNA levels of Cyp7A and Cyp27 in mice fed EFA$^{+}$ or EFA$^{-}$ chow for 8 wk determined by quantitative real-time RT-PCR. Values represent the ratio of specific hepatic mRNA levels of Cyp7A and Cyp27 to that of β-actin, normalized to the EFA$^{+}$ control group. Data represent means ± SD of 6 mice per group. No significant differences were found between groups.

![Graph showing plasma appearance of [3H]triolein and [14C]oleic acid](image)

**Fig. 3.** Plasma appearance of [3H]triolein (A) and [14C]oleic acid (B) in mice after an intragastric load of olive oil containing these fats and after intravenous injection of Triton WR-1339 at time 0. Mice were fed EFA$^{+}$ or EFA$^{-}$ chow for 8 wk. Blood samples were taken hourly for 4 h, and fats were extracted from plasma after extraction. Thin-layer chromatography was performed to isolate the triglyceride fraction for determination of radioactivity by scintillation counting. Scanning of the thin-layer plates indicated that essentially all radioactivity (95%) was in the triglyceride fraction. Data represent means ± SD of 5 mice per group (disintegrations·s$^{-1}$·μl plasma$^{-1}$).

**Fig. 4.** Time course of triene-to-tetraene ratios in plasma (C) and liver (D) in EFA$^{+}$ and EFA$^{-}$ mice after intragastric administration of 200 μl of 20:3n-9 or 20:4n-6 oleic acid at 8 wk. The ratio of [3H]-to-[14C] in the administered bolus was 4.43 ± 0.05. Data represent means ± SD of 5 mice per group. No significant differences were found between groups at any time point.

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Characterization of EFA deficiency in Mdr2$^{-/-}$ mice. Plasma concentrations of cholesterol and phospholipid were increased, whereas the plasma triglyceride level was decreased in EFA$^-$/Mdr2$^{-/-}$ mice compared with their EFA$^+$-fed controls ($P < 0.05$) (Table 5). Similar to the situation in Mdr2$^{+/-}$ mice, a pronounced hepatic fat accumulation characterized by increased levels of triglyceride, unesterified and esterified cholesterol, was observed in EFA$^-$/Mdr2$^{-/-}$ mice (Table 6).

The fecal fat balance revealed a decreased dietary fat absorption in EFA$^-$/Mdr2$^{-/-}$ mice compared with EFA$^+$ controls ($P < 0.01$; Fig. 5). Absorption coefficients for saturated fatty acids (palmitic and stearic) were lower than absorption coefficients of unsaturated fatty acids (oleic and linoleic) in EFA$^-$/Mdr2$^{-/-}$ mice. Plasma concentrations of retinyl palmitate after intragastric administration of retinol were significantly lower in EFA$^-$/Mdr2$^{-/-}$ mice compared with EFA$^+$ controls ($P < 0.01$; Fig. 6).

Bile Secretion and Composition

As in EFA$^-$/Mdr2$^{+/-}$ mice (Table 3), bile flow was increased in EFA$^-$/Mdr2$^{-/-}$ mice compared with their EFA$^+$-fed counterparts ($P < 0.05$). Bile flow and bile salt secretion were higher in Mdr2$^{-/-}$ mice than in Mdr2$^{+/-}$ mice ($P < 0.01$) (Table 7). Bile salt composition was virtually identical between EFA$^+$ and EFA$^-$-fed Mdr2$^{-/-}$ mice (Table 8).

Table 5. Plasma lipid concentrations in Mdr2$^{-/-}$ mice fed EFA$^+$ or EFA$^-$ chow for 8 wk

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>EFA$^+$</th>
<th>EFA$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>$1.39 \pm 0.14$</td>
<td>$1.82 \pm 0.21^*$</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>$1.04 \pm 0.12$</td>
<td>$1.30 \pm 0.19^*$</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>$0.46 \pm 0.09$</td>
<td>$0.34 \pm 0.12^*$</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>$0.61 \pm 0.08$</td>
<td>$0.66 \pm 0.11$</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>$2.18 \pm 0.18$</td>
<td>$2.82 \pm 0.24^*$</td>
</tr>
</tbody>
</table>

Concentrations are given in millimoles per liter. No. = 7 mice per group. *$P < 0.05$.

DISCUSSION

We investigated the mechanism of fat malabsorption in EFA deficiency in mice with particular emphasis on the possible role of altered bile formation as proposed by Levy et al. (20, 21) based on studies in rats. To specify the contribution of biliary phospholipid secretion, studies were performed in EFA$^-$ and EFA$^+$ Mdr2$^{+/-}$ and Mdr2$^{-/-}$ mice; the latter are unable to secrete phospholipids into their bile. Yet, we first had to develop and characterize a murine model for EFA deficiency. Our data indicate that dietary fat absorption is reduced in EFA$^-$/Mdr2$^{-/-}$ mice compared with EFA$^+$ controls. The mechanism underlying this EFA deficiency-associated fat malabsorption does not likely involve alterations in bile formation (including bile flow, bile salt secretion rate, bile salt composition, or phospholipid secretion rate) nor changes in fat digestion (lipolysis). Rather, our data strongly indicate that EFA deficiency in mice affects intracellular events of fat absorption that occur in the enterocyte.

Biochemical EFA deficiency conventionally defined by a molar ratio of eicosatrienoic acid (20:3n-9)-to-arachidonic acid (20:4n-6) above 0.20 in plasma (16) was already reached after only 2 wk of EFA$^-$ diet feeding to mice. This rapidity of onset makes the mouse an attractive and versatile model for studying EFA deficiency.

Table 6. Hepatic lipid concentrations in Mdr2$^{-/-}$ mice fed EFA$^+$ or EFA$^-$ chow for 8 wk

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>EFA$^+$</th>
<th>EFA$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>$101.0 \pm 22.2$</td>
<td>$225.8 \pm 95.9^*$</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>$45.8 \pm 2.1$</td>
<td>$81.4 \pm 11.8^*$</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>$11.6 \pm 1.5$</td>
<td>$41.7 \pm 7.6^*$</td>
</tr>
</tbody>
</table>

Concentrations are given in nanomoles per milligram of protein. No. = 7 mice per group. *$P < 0.05$. 

Fig. 4. Time course of retinyl palmitate concentration in plasma of mice fed EFA$^+$ or EFA$^-$ chow for 8 wk after intragastric administration of 5,000 IU retinol at time 0. Data represent means ± SD of 7 mice per group. $P < 0.05$ at 2 and 4 h after bolus administration.

Fig. 5. Fat absorption coefficients of total dietary fat and separately of major dietary fatty acids (16:0 + 18:0 + 18:1n-9 + 18:2n-6) in Mdr2$^{-/-}$ mice fed EFA$^+$ or EFA$^-$ chow for 8 wk. Feces were collected after a 72-h period in which chow intake was monitored by weighing the chow container. Absorption was calculated by subtracting fecal excretion of these fatty acids during 72 h from their dietary intake in 72 h and then multiplying the result by 100 to obtain a coefficient (as detailed in MATERIALS AND METHODS). Data represent means ± SD of 7 mice per group. $P < 0.01$ for all lipid classes.
similar to EFA– rats (3, 34), EFA– mice experienced changes in plasma and liver fat contents. Specifically, EFA– mice have decreased plasma triglyceride and increased hepatic triglyceride and cholesterol levels. In EFA– rats, Wanon et al. (33) observed alterations in HDL cholesterol concentration, with defective translocation of HDL cholesterol into bile and concurrently increased hepatic very low-density lipoprotein (VLDL)-cholesterol secretion. Lipoprotein abnormalities were also found by Levy et al. (22) in EFA– cystic fibrosis (CF) patients. Compared with their EFA+ counterparts, EFA– CF patients had increased plasma triglyceride levels (specifically in the VLDL, LDL, HDL2, and HDL3 fractions) and decreased plasma HDL- and LDL-cholesterol. Also, lipoprotein size was altered in these EFA– patients, with larger VLDL, LDL, and HDL2 particles and smaller HDL3 particles. It could be speculated that EFAs, as constituents of triglycerides, phospholipids, and cholesterol esters, may be essential for regulation of lipoprotein metabolism. Although the effects of EFA deficiency are species specific, it appears that an adequate EFA status is required for efficient intestinal and hepatic processing of lipoproteins.

In addition to the changes in plasma and liver lipids, EFA deficiency in mice was associated with fat malabsorption. The coefficients of fat absorption in EFA– mice (60–70%) were somewhat lower than corresponding values in EFA+ rats (80–90%) (2, 15, 28). In all of these studies, EFA deficiency was induced by feeding the animals high-fat EFA– diets almost entirely composed of saturated fatty acids. Apart from species specificity, the difference in coefficients of fat absorption could be related to the amount and type of fat in the diet. In the present study, mice were fed high-fat chow (16 weight%), whereas Hjelte et al. (15) used chow diets containing only 7 weight% fat. Levy et al. (21) reported that lipolytic activity in EFA– rats was unchanged compared with control rats. Our present results in EFA– mice are compatible with this observation. The appearance of [3H]triolein in plasma after its intragastric administration was similar in EFA– mice and control mice. If anything, the [3H] label was recovered from plasma of EFA–-fed mice even at higher concentrations compared with EFA+–fed controls. The explanation of this phenomenon may involve the choice of the lipid oleic acid. The absorption of dietary oleic acid was only mildly impaired during EFA deficiency (Fig. 1). In addition, a tracer effect could not be excluded. When we investigated the absorption of a less polar lipid (retinol) after its intragastric administration, both EFA+ Mdr2+/+ and EFA– Mdr2–/– mice showed decreased plasma concentrations compared with their EFA+ controls, which underlines the occurrence of lipid malabsorption during EFA deficiency.

Rather than differences in fat digestion (lipolysis), it could have been expected that alterations in bile formation contributed to fat malabsorption in EFA– mice, in analogy to the situation in EFA– rats (20). The pathophysiology of EFA deficiency-associated fat malabsorption in mice could be due to increased biliary bile salt secretion rates, analogous to previous data in rats. However, bile flow and biliary bile salt and phospholipid secretion rates were increased during EFA deficiency. Robins and Fasulo (27) reported that EFA– hamsters have increased hepatic bile flow and biliary bile salt and cholesterol secretion compared with controls. It is not known, however, whether EFA– hamsters have a decreased coefficient of fat absorption. Our observation in EFA– mice, together with the available data on EFA– rats and hamsters, indicate that the effects of EFA deficiency on bile formation are species specific. Present data exclude the fact that EFA– fat malabsorption is due to decreased rates of biliary bile salt secretion in mice, in contrast to the situation in rats (20, 21). Theoretically, an increase in the contribution of hydrophilic bile salts (to total bile salts) could

Table 7. Bile flow and biliary secretion rates in Mdr2–/– mice fed EFA+ or EFA– chow for 8 wk

<table>
<thead>
<tr>
<th></th>
<th>EFA+</th>
<th>EFA–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow</td>
<td>6.57 ± 1.57</td>
<td>9.27 ± 2.17*</td>
</tr>
<tr>
<td>Bile salts</td>
<td>260 ± 53</td>
<td>540 ± 182*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.97 ± 0.28</td>
<td>1.31 ± 0.35</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.10 ± 0.26</td>
<td>0.07 ± 0.16</td>
</tr>
</tbody>
</table>

Biliary output rates are given in 1 microliters·min⁻¹·100 g body weight⁻¹ or 2 nanomoles·min⁻¹·100 g body weight⁻¹. No. = 5–7 mice per group. *P < 0.05.

Table 8. Biliary bile salt composition in Mdr2–/– mice fed EFA+ or EFA– chow for 8 wk

<table>
<thead>
<tr>
<th>Bile Salt</th>
<th>EFA+</th>
<th>EFA–</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate</td>
<td>63.7 ± 7.3</td>
<td>56.2 ± 3.4</td>
</tr>
<tr>
<td>β-Muricholate</td>
<td>28.0 ± 8.0</td>
<td>35.9 ± 4.1</td>
</tr>
<tr>
<td>α-Muricholate</td>
<td>4.8 ± 1.7</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>1.0 ± 0.6</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>1.6 ± 0.9</td>
<td>0.8 ± 0.4</td>
</tr>
</tbody>
</table>

Values expressed are a percentage of total amount. Greater than 90% of all bile salts are represented. Minor metabolites (<1% of total area) have been excluded. Data represent means ± SD of 7 mice per group.
contribute to impaired solubilization of dietary fats. Yet, biliary bile salt composition was virtually unchanged in EFA− mice compared with controls.

The increased biliary secretion rate of bile salts, immediately after interruption of the enterohepatic circulation, strongly suggests an expansion of the bile salt pool size in EFA deficiency.

Bile salts negatively affect their own biosynthesis by repressing the expression of Cyp7A via the FXR-SHP1-LRH1-Cyp7A pathway with Cyp7α encoding the enzyme cholesterol 7α-hydroxylase that catalyzes the first step of the neutral pathway in bile salt synthesis (6–8, 24, 25). In our experiments, however, Cyp7A and Cyp27 mRNA levels were similar in livers from EFA− and EFA−/fed mice. We speculate that EFA deficiency in mice impairs the capacity of bile salts to exert negative feedback inhibition on their own hepatic biosynthesis, but the mechanism hereof remains unclear.

Not only biliary bile salts but also biliary phospholipids play a role in dietary fat absorption, e.g., in supplying surface components for the assembly of chylomicron particles in enterocytes (17, 29). Under physiological conditions, overall fat absorption in Mdr2−/− mice is only slightly decreased compared with control mice (95 vs. 98%), based on 72-h fecal fat balance measurements. On the other hand, kinetics of chylomicron formation are clearly delayed in Mdr2−/− mice (32). Therefore, a quantitative alteration in biliary phospholipid secretion was not likely to contribute to EFA deficiency-associated fat malabsorption. Yet, fat malabsorption during EFA deficiency could still be due to qualitative changes in biliary phospholipid composition. Replacement of polyunsaturated acyl chains (linoleoyl, arachidonoyl) by saturated or monounsaturatedated species could theoretically be responsible for impaired chylomicron assembly and secretion. In accordance with findings by Bennett Clark et al. (2) in EFA− rats, the acyl chain composition of biliary PC in EFA− mice showed less essential (i.e., 18:2n-6, 20:4n-6) and more non-EFAs (i.e., 18:1n-9, 18:1n-7, 16:1n-7). If acyl chain composition of bile phospholipids were important for fat malabsorption during EFA deficiency, one would expect that EFA deficiency would not or to a much lesser extent affect fat absorption in Mdr2−/− mice. Present data, however, clearly indicate that fat absorption in EFA− Mdr2−/− mice was affected similarly as in EFA− Mdr2−/− mice. In EFA− Mdr2−/− mice, as in EFA− Mdr2−/− controls, bile flow and biliary bile salt secretion were increased. On the basis of the similar fat absorption coefficients we found in EFA− mice with and without biliary phospholipid secretion, we conclude that the effect of biliary PC acyl chain composition is not of pathophysiological relevance for EFA deficiency-associated fat malabsorption.

Rather than due to alterations in bile secretion, fat malabsorption during EFA deficiency may be caused by other steps involved in fat absorption. Intestinal mucosal phospholipids normally contain large amounts of 18:2n-6 and 20:4n-6, and during EFA deficiency, the levels of these fatty acids are markedly decreased (9, 10, 35). The resultant structural changes in membranes, and the increased cellular turnover rate in the intestinal mucosa reported in EFA− rats (28) could be responsible for decreased dietary fat absorption. Based on our study and previous studies (2, 21) in EFA− rats, the intraluminal events involved in fat absorption (i.e., reesterification, chylomicro assembly, and/or chylomicon secretion) are involved in EFA deficiency-associated fat malabsorption.

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REFERENCES

ESSENTIAL FATTY ACID DEFICIENCY IN MICE