Treatment of EFA deficiency with dietary triglycerides or phospholipids in a murine model of extrahepatic cholestasis

Anniek Werner, Rick Havinga, Folkert Kuipers, and Henkjan J. Verkade

Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, Academic Hospital Groningen, 9700 RB Groningen, The Netherlands

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Werner, Anniek, Rick Havinga, Folkert Kuipers, and Henkjan J. Verkade. Treatment of EFA deficiency with dietary triglycerides or phospholipids in a murine model of extrahepatic cholestasis. Am J Physiol Gastrointest Liver Physiol 286: G822–G832, 2004. First published December 11, 2003; 10.1152/ajpgi.00425.2003.—Essential fatty acid (EFA) deficiency during cholestasis is mainly due to malabsorption of dietary EFA (23). Theoretically, dietary phospholipids (PL) may have a higher bioavailability than dietary triglycerides (TG) during cholestasis. We developed murine models for EFA deficiency (EFAD) with and without extrahepatic cholestasis and compared the efficacy of oral supplementation of EFA as PL or as TG. EFAD was induced in mice by feeding a high-fat EFAD diet. After 3 wk on this diet, bile duct ligation was performed in a subgroup of mice to establish extrahepatic cholestasis. Cholestatic and noncholestatic EFAD mice continued on the EFAD diet (controls) or were supplemented for 3 wk with EFA-rich TG or EFA-rich PL. Fatty acid composition was determined in plasma, erythrocytes, liver, and brain. After 4 wk of EFAD diet, induction of EFAD was confirmed by a sixfold increased triene-to-tetraene ratio (T/T ratio) in erythrocytes of noncholestatic and cholestatic mice (P < 0.001). EFA-rich TG and EFA-rich PL were equally effective in preventing further increase of the erythrocyte T/T ratio, which was observed in cholestatic and noncholestatic nonsupplemented mice (12- and 16-fold the initial value, respectively). In cholestatic mice, EFA-rich TG was superior to EFA-rich TG in decreasing T/T ratios of liver TG and PL (each P < 0.05) and in increasing brain PL concentrations of the long-chain polyunsaturated fatty acids (LCPUFA) docosahexaenoic acid and arachidonic acid (each P < 0.05). We conclude that oral EFA supplementation in the form of PL is more effective than in the form of TG in increasing LCPUFA concentrations in liver and brain of cholestatic EFAD mice.

oral supplementation; long-chain polyunsaturated fatty acids; brain; liver; triglycerides; phospholipids; essential fatty acid deficiency

ESSENTIAL FATTY ACIDS (EFA) and their long-chain polyunsaturated metabolites have been recognized to play a role in growth, development of the central nervous system, eicosanoid production, and control of lipid homeostasis. Because of the inability of mammalian cells to synthesize EFA de novo, adequate EFA levels depend entirely on sufficient dietary intake and absorption. Conditions leading to fat malabsorption, such as cholestasis, have been associated with a high incidence of essential fatty acid deficiency (EFAD; see Refs. 10, 33, 38). In a rat model of cholestasis, we recently demonstrated that impaired intestinal absorption of EFA is the main contributor to EFAD in cholestatic conditions, rather than altered postabsorptive EFA metabolism (23). So far, no adequate oral strategies for prevention or treatment of EFAD during cholestasis have been developed. In regular diets, 90% of EFA are present as acyl esters in triglycerides (TG), and only 10% are present as acyl esters in phospholipids (PL) and cholesterol esters (41). Under physiological conditions, however, the intestine receives significant amounts of EFA as biliary PL, which contain up to 40 mol/mol EFA [mostly linoleic acid (LA)], esterified at the sn-2 position. TG and PL have different intestinal absorption mechanisms. Because of their hydrophobic nature, TG are insoluble in the aqueous intestinal lumen, and products of TG lipolysis, i.e., free fatty acids and monoglycerides, highly depend on bile components for solubilization in mixed micelles. After absorption by the enterocyte, fatty acids and monoglycerides are reesterified into TG and secreted in lymph as the major core components of chylomicrons. PL, on the other hand, are relatively independent of bile components for intestinal absorption. PL have a higher tendency than TG to interact with water and can associate into liquid crystals (bilayers; see Ref. 42), which have been suggested to play a role in luminal lipid solubilization under bile-deficient conditions (27). PL can be absorbed intact or after partial digestion to lysoglycerolipid and free fatty acids (7, 21, 36). PL of luminal origin are predominantly used by enterocytes for assembly of the surface coat of chylomicrons secreted into lymph. In conditions of cholestasis, i.e., during decreased or absent bile formation, TG absorption is severely impaired. PL can facilitate dietary lipid absorption under bile-deficient conditions by enhancing luminal lipid solubilization and by providing surface components for lipoprotein assembly (13, 42). Additionally, PL have been postulated to have greater postabsorptive bioavailability (8, 12, 19, 37, 44). On the basis of these characteristics, we hypothesized that PL could be a better vehicle for oral EFA supplementation during cholestasis than TG. In this study, we compared the efficacy of EFA-rich PL and of EFA-rich TG for treatment of EFAD under cholestatic conditions in mice. For this purpose, we developed a model for both EFAD and cholestasis by feeding mice an EFAD diet followed by bile duct ligation (BDL). Subsequent oral supplementation with EFA either as TG or as PL demonstrated that the latter resulted in higher concentrations of EFA-derived long-chain polyunsaturated fatty acids (LCPUFA) in target organs as brain and liver.

MATERIALS AND METHODS

Animals

Wild-type mice with a free virus breed background were obtained from the breeding colony at the Central Animal Facility, Academic Medical Center (Amsterdam, the Netherlands). Male mice (body weight 25–35 g) were housed in a light-controlled (lights on 6:00 AM–8:00 PM) facility. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
AM-6:00 PM) and temperature-controlled (21°C) facility and were 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 Diets**

The EFAD diet contained 20% energy as protein, 46% energy as carbohydrate, and 34% energy as fat, respectively, and had the following fatty acid composition: 41.4 mol/mol palmitic acid (C16:0), 47.9 mol/mol stearic acid (C18:0), 7.7 mol/mol oleic acid (C18:1n-9) and 3 mol/mol LA (C18:2n-6). An isocaloric EFAS-sufficient (EFAS) diet was used as a control diet, containing 20% energy as protein, 43% energy as carbohydrate, and 37% energy as fat with 32.1 mol/mol C16:0, 5.5% C18:0, 32.2 mol/mol C18:1n-9, and 30.2% C18:2n-6 [custom synthesis, diet numbers 4141.08 (EFAD) and 4141.07 (EFAS); Hope Farms]. For EFA supplementation, EFAD chow pellets were finely pulverized and homogeneously mixed with either TG oil or PL oil (lecithin), dissolved in water with ethanol. Both TG and PL (lecithin) were dissolved in water with ethanol. Both TG and PL oils were purified from crude soybean oil and had the following fatty acid profile (wt/wt): 53.5% C18:2, 7.6% C18:3, 22.8% C18:1, 10.7% C16:0, 4.0% C18:0, and <0.5% of C14:0, C16:1, C20:0, C20:1, C22:0, and C24:0 each (TG); and 56.1% C18:2, 8.2% 18:3, 13.2% C18:1, 16.1% C16:0, 4.5% C18:0, and <0.5% of C14:0, C16:1, C20:0, C20:1, C22:0, and C24:0 each (PL). We aimed to supplement the EFAD mice with 2.5 mg LA/day. For the TG-oil, the percentage of fatty acid mass relative to the glycerol backbone mass is 72.3%, of which 56.1% is LA. To obtain equimolar amounts of LA in the two EFA-supplemented diets, we added 5.6 g TG (= 0.006 mol TG/kg chow) and 5.8 g PL (= 0.007 mol PL), resulting in 0.003 mol LA/kg chow for both oils. On the basis of average daily chow intake of 3 g (measured in pilot experiments with EFAD mice), this resulted in 2.5 mg LA supplementation/day. The PL and TG soybean oils were a generous gift from Unimills (Zwijndrecht, The Netherlands).

**Experimental Procedures**

**Induction of EFAD in mice and oral administration of EFA-rich TG or PL** Mice were fed standard laboratory chow containing 6 wt/wt fat (RMH-B; Hope Farms) from weaning. Before starting the EFAD diet, a blood sample was obtained by tail bleeding under halothane anesthesia for determination of baseline EFA status. Blood was centrifuged, and erythrocyte samples were separated by centrifugation at 2,400 rpm for 10 min (Eppendorf Centrifuge, Eppendorf, Germany). Plasma and erythrocyte samples were hydrolyzed and methylated the same day (26) for gas-chromatographic analysis of fatty acid profiles. All mice were then fed a high-fat (16% weight as fat), EFAD diet for 4 wk. Subsequently, mice were randomly assigned to an EFAD diet supplemented with either EFA-rich TG or EFA-rich PL, or continued on the EFAD diet for 3 wk (n = 5/group). At weekly intervals, mice were weighed, chow containers were weighed to monitor food intake, and blood samples were taken by tail bleeding for determination of EFA status.

**BDL in EFAD mice and oral administration of EFA-rich PL or TG** A separate group of mice was fed the EFAD diet for 3 wk, after which their bile ducts were ligated by placing three sutures proximal to the gallbladder under halothane/NO2 anesthesia. Animals were allowed to recover from surgery for 1 wk during which the EFAD diet was continued. Subsequently, i.e., after 4 wk on the EFAD diet, mice were randomly assigned to an EFAD diet enriched with either EFA-rich TG or EFA-rich PL, or continued on the EFAD diet for 3 wk. A separate control group of non-bile duct-ligated control mice received an EFAS diet for 7 wk (n = 6 for each dietary group). During the entire experiment, body weight, chow ingestion, plasma liver enzymes, and EFA status were determined at weekly intervals. After 3 wk of supplementation, mice were terminated under anesthesia by heart puncture, by which a large blood sample (0.6–1.0 ml) was obtained, and liver and brain were removed and stored at –80°C for fatty acid analysis. A schematic overview of the experimental design is depicted in Fig. 1.

**Analytical techniques.** Fatty acid status was analyzed by hydrolyzing, methylation, and extracting total plasma lipids and erythrocyte membrane lipids as described by Musket et al. (26). For fatty acid analysis of brain and liver, tissue samples were mechanically homogenized in 0.9% NaCl and lipids were extracted from aliquots of tissue homogenate as described by Bligh and Dyer (6). Lipid extracts were fractionated into PL, cholesterol esters, triacylglycerols, diacylglycerols, monoaoylglycerols, and free fatty acids using TLC (20 × 20 cm, Silica gel 60 F254; Merck), with hexane-diethyl ether-acetic acid (80:20:1, vol/vol/vol) as solvent. TLC plates were dried and colored by iodine, and PL and TG spots were scraped off and methylated (26). To account for losses during lipid extraction, heptadecanoic acid (C17:0; Sigma, St. Louis, MO) was added to all samples as internal standard before extraction. Butylated hydroxytoluene was added as antioxidant. Chow pellets were freeze-dried and mechanically homogenized, and from aliquots of each diet, lipids were hydrolyzed, methylated, and extracted as described above. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph model 6890, equipped with a 50 m × 0.2 mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA).
CA) and an FID detector, using program conditions as described previously (43). Individual fatty acid methyl esters were quantified by relating areas of their chromatogram peaks to that of the internal standard C17:0. Plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and cholesterol concentrations were determined using routine clinical procedures. Plasma total bile salt levels were determined according to Mashige et al. (22). Liver and brain histology was examined on frozen tissue sections after Oil Red O (ORO) staining, which colors neutral lipids (mainly TG) with an orange-red tint, and on parafomaldehyde-fixed, paraffin-embedded tissue sections after hematoxylin-eosin (HE) staining by standard procedures.

Calculations and Statistics

Relative concentrations (mol/mol) of plasma, erythrocyte, liver, and brain fatty acids were calculated by summation of all fatty acid peak areas and subsequent expression of the area of each individual fatty acid as a percentage of this amount. EFA status was evaluated by comparing molar percentages of individual EFA and LCPUFA and by calculating markers for EFAD such as the triene-to-tetraene ratio (T/T ratio; 20:3n-9/20:4n-6) in different body compartments (16).

All results are presented as means ± SD for the number of animals indicated. Data were statistically analyzed using Student’s $t$-test or, for comparison of more than two groups, ANOVA test with post hoc Bonferroni correction. The level of significance was set at $P < 0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS

EFAD in Noncholestatic Mice

In a previous study (43), we developed a murine model for diet-induced EFAD by feeding mice an EFAD diet for up to 8 wk, which resulted in a pronounced dietary fat malabsorption. In the present study, feeding the EFAD diet for 4 wk did not decrease body weight, but mice stopped growing [baseline: 32.4 ± 2.9 g; at 4 wk: 34.1 ± 3.3 g, not significant (NS)]. After 3 wk of EFA supplementation, no differences in body weight were found between mice fed EFA-rich TG (33.6 ± 3.9 g) or EFA-rich PL (33.1 ± 1.9 g, NS). Average chow intake was 3.5 ± 0.5 g/day in both dietary groups throughout the experimental period, indicating an average daily LA intake of 3 mg. Because EFAD has been reported to have species-specific effects on bile formation (20, 43), we measured the effects of dietary EFAD and EFA supplementation on plasma bile salt levels. Development of EFAD was associated with increasing plasma bile salt concentrations (21 ± 4 μM at baseline to 40 ± 7 μM after 4 wk of EFAD diet). Three weeks of supplementation with TG or PL tended to reverse the increased plasma bile salt levels (32 ± 10 and 38 ± 6 μM, respectively), but differences were not statistically significant.

Figure 2 shows the molar percentages of relevant polyunsaturated fatty acids (PUFA) in erythrocyte membrane lipids of noncholestatic mice during EFAD diet feeding (control), EFA-rich PL supplementation, or EFA-rich TG supplementation. The classic biochemical parameter describing EFA status is the T/T ratio, i.e., the molar ratio between the nonessential fatty acid eicosatrienoic acid (20:3n-9) and the LCPUFA arachidonic acid (AA; 20:4n-6). Development of EFAD is associated with an elevated T/T ratio. Compared with baseline (0.02 ± 0.01), the T/T ratio in erythrocytes of noncholestatic mice increased more than sixfold ($P < 0.001$) after 4 wk and 16-fold after another 3 wk of EFAD diet ($P < 0.001$, Fig. 2A). EFA supplementation, either with EFA-rich PL (0.09 ± 0.02) or EFA-rich TG (0.09 ± 0.03), completely prevented this further increase in the T/T ratio ($P < 0.001$ for either PL or TG vs. EFAD) and tended to decrease the ratio compared with 4-wk values. Molar percentages of LA (Fig. 2B) and of its corresponding long-chain polyunsaturated metabolite AA (Fig. 2C)
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Fig. 3. Body weight in bile duct-ligated mice fed EFAD diet or EFAD diet supplemented with PL or TG and of noncholestatic EFAS control mice. Data represent means ± SD of 5 mice/group. *P < 0.01 for TG, EFAD, and EFAS in week 0 vs. week 7. #P < 0.05 for PL vs. TG, EFAD, and EFAS in week 7. For PL-fed mice, body weight was not significantly different in week 7 compared with baseline (week 0).

were decreased after 4 wk on the EFAD diet (both P < 0.001). After another 3 wk on this diet, LA levels had further decreased (P < 0.001), whereas AA levels remained at a stable low level. Supplementation with either EFA-rich TG or PL reversed the decreased LA and AA concentrations with similar efficacy (P < 0.005; Fig. 2, B and C). In control mice fed the high-fat EFAS diet for 7 wk, LA and AA levels remained stable (LA: 8.9 ± 0.6, AA: 15.7 ± 0.6; data not shown). Molar percentages of the n-3 EFA α-LA (ALA) were low in all dietary groups and did not change significantly during 4 or 7 wk of EFAD diet, nor during 3 wk of EFA supplementation (data not shown).

EFAD in Cholestatic Mice

Under physiological (noncholestatic) conditions, mice fed a high-fat EFAS diet for 7 wk gradually increased in body weight from 25.8 ± 0.6 to 40.2 ± 2.2 g (Fig. 3). Development of EFAD and cholestasis interrupted physiological growth in mice. Nonsupplemented and TG-supplemented EFAD mice significantly lost weight during the 7-wk experiment (from 25.8 ± 1.5 g at baseline to 22.7 ± 1.2 and 23.2 ± 1.5 g, respectively; each P < 0.01). Supplementation of EFA with PL, however, prevented this weight loss completely, resulting in a stable body weight throughout the experiment (PL: 27.0 ± 2.5 g, P < 0.05 for PL vs. TG and EFA).

Plasma bile salt concentrations significantly increased from 21 ± 4 μM at baseline to 46 ± 11 μM after 3 wk on the EFAD diet. In the 4th wk of BDL, plasma bile salt concentrations had strongly increased in all groups. However, EFA supplementation in the form of either PL or TG profoundly mitigated the increase in plasma bile salt concentration compared with nonsupplemented mice (PL: 1.242 ± 163 μM; TG: 1.104 ± 252 μM; EFAD: 1.983 ± 510 μM; P < 0.05 for either TG or PL vs. EFAD in week 7; P < 0.005 for all groups in week 3 vs. week 7).

As expected, ALT, AST, and AP activity in plasma strongly increased after induction of extrahepatic cholestasis (Table 1). No profound differences between the three experimental groups were noted in these parameters. Plasma cholesterol concentrations had remained fairly constant after 4 wk of EFAD diet/1 wk of BDL. After another 3 wk of cholestasis, however, plasma cholesterol levels were significantly higher in PL- and TG-supplemented mice compared with nonsupplemented mice.

The T/T ratio (20:3n-9/20:4n-6) in total plasma lipid increased during development of EFAD and cholestasis from 0.01 ± 0.00 at baseline to 0.51 ± 0.28 in week 4 (P < 0.001; Fig. 4). Upon supplementation with PL or TG, the T/T ratio decreased to 0.32 ± 0.17 and 0.36 ± 0.03, respectively, whereas in nonsupplemented mice the T/T ratio further increased to 0.61 ± 0.20. Plasma molar percentages of LA and AA were significantly lower in week 4 compared with baseline values (P < 0.001). A further decrease in LA levels was partially prevented only after supplementation with PL and not with TG (P < 0.05 for PL vs. EFAD at week 7; TG vs. EFAD NS).

Because total plasma lipid fatty acid composition in nonfasted animals is strongly determined by postprandial dietary TG, we also analyzed fatty acid profiles of the isolated plasma PL fraction of the terminal blood sample in week 7 (Fig. 5). The T/T ratio tended to be lower and LA, AA, and docosahexaenoic acid (DHA) concentrations higher in TG- and PL-fed mice compared with nonsupplemented mice, but differences were not statistically significant. Fatty acid concentrations were not significantly different between the two supplementation groups.

The fatty acid composition of the plasma compartment is largely determined by the plasma TG fraction, which reflects

Table 1. Plasma AST, ALT, AP, and cholesterol

<table>
<thead>
<tr>
<th>Week 0</th>
<th>Week 4 (1 wk after BDL)</th>
<th>Week 7 (4 wk after BDL)</th>
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<tbody>
<tr>
<td></td>
<td>PL</td>
<td>TG</td>
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<tr>
<td>AST, U/I</td>
<td>48±30</td>
<td>659±323*</td>
</tr>
<tr>
<td>ALT, U/I</td>
<td>34±14</td>
<td>354±199*</td>
</tr>
<tr>
<td>AP, U/I</td>
<td>81±31</td>
<td>895±228*</td>
</tr>
<tr>
<td>Cholesterol, mM</td>
<td>3.2±1.2</td>
<td>3.3±1.7</td>
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Data represent means ± SD of 4–6 mice/group. EFAD, essential fatty acid deficiency. Plasma aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), and cholesterol were measured at baseline (week 0), after 1 wk of bile duct ligation (BDL); week 4), and after 4 wk of BDL (week 7) in triglyceride (TG)-supplemented, phospholipid (PL)-supplemented, or nonsupplemented mice. *P < 0.001 for AST, ALT, and AP concentrations in week 4 compared with baseline. †P < 0.001 for TG- and PL-supplemented mice in week 4 vs. week 7. ‡P < 0.01 for cholesterol levels in TG- and PL-supplemented mice in week 7 vs. week 4. §P < 0.005 for cholesterol levels in EFAD vs. TG- and PL-supplemented mice in week 7. www.ajpgi.org
dietary fatty acid composition to a certain extent. Fatty acid profiles in erythrocyte membranes are assumed to be a more stable reflection of overall EFA status. The T/T ratio in erythrocytes was 0.02 ± 0.01 at baseline and increased to 0.12 ± 0.06 in week 4 (i.e., 1 wk after BDL, *P < 0.001; Fig. 6). Continuation of the EFAD diet in cholestatic mice induced a further increase of the erythrocyte T/T ratio, which was prevented by supplementation with either PL or TG (Fig. 6). LA decreased from 10.5 ± 0.5 mol/mol at baseline to 6.4 ± 1.7 mol/mol in week 4 (Fig. 6B). Supplementation with PL for 3 wk resulted in higher LA concentrations compared with the EFAD group (5.3 ± 1.5 vs. 3.5 ± 1.5 mol/mol, respectively; *P < 0.005). TG-supplemented mice had intermediate LA levels at 7 wk (4.3 ± 0.8; TG vs. EFAD, NS). AA molar percentages in cholestatic mice remained remarkably stable during the entire experiment in all dietary groups as follows: 15.3 ± 2.9 at baseline; 15.7 ± 1.0 in week 4 (Fig. 6C) and 15.3 ± 4.0 (PL), 13.3 ± 4.3 (TG), and 13.2 ± 1.8 (EFAD) in week 7 of the study period (NS). Surprisingly, when comparing AA values of cholestatic with those of noncholestatic EFAD mice (Fig. 2C), we observed that BDL completely prevented the decline in AA during EFAD diet feeding. AA levels in cholestatic EFAD mice remained similar to those in noncholestatic EFAD mice (15.4 ± 0.5, NS).

Because the liver is the principal site of desaturation and elongation of EFA into their long-chain polyunsaturated metabolites, we analyzed the fatty acid composition of liver TG and liver PL fractions. In liver PL (Fig. 7), the T/T ratio was significantly lower in PL-fed mice than in TG-fed and EFAD mice after 4 wk of cholestasis (*P < 0.05, Fig. 7A). LA, AA, and DHA concentrations were not significantly different between PL-, TG-, and nonsupplemented cholestatic mice. Yet, LA and AA levels of liver PL were significantly lower in cholestatic animals than in noncholestatic EFAS mice (*P < 0.001).

Differential effects of TG and PL supplementation were also observed in the liver TG fraction (Fig. 8). Although LA levels were similar in all three cholestatic groups, the T/T ratio of PL-supplemented mice was comparable to that of noncholestatic EFAS mice (Fig. 8A), whereas T/T ratios of TG-supplemented and EFAD mice were significantly higher (*P < 0.05). AA was significantly higher in PL-supplemented mice compared with TG- or nonsupplemented mice (PL: 1.4 ± 0.4 mol/mol; TG: 0.7 ± 0.2 mol/mol; EFAD: 0.7 ± 0.2 mol/mol, *P < 0.05). Similarly, DHA levels were higher in the PL group compared with the TG and EFAD groups, and even higher than in EFAS control mice (Fig. 8D).

ORO staining for neutral lipids on frozen liver sections showed lipid accumulation in periportal (zone 1) but not in perivenous (zone 3) hepatocytes of EFAD noncholestatic mice. EFAS noncholestatic mice did not show hepatic lipid accumulation. In paraffin-embedded HE-stained liver sections of cholestatic mice, extensive bile duct proliferation was observed in all three dietary groups, with extensive hepatocyte damage resulting from toxic bile accumulation. No accumulation or zonal distribution of lipid was observed in livers of cholestatic mice. There were no overt histological differences between the three cholestatic groups (data not shown).

Because the central nervous system is a well-known target organ for LCPUFA and contains particularly high levels of DHA, we analyzed fatty acid profiles of brain tissue samples. PL-supplemented mice had significantly higher AA and DHA concentrations than both TG-supplemented and EFAD mice. This suggests a differential impact of dietary fatty acid composition on brain lipid status in cholestatic mice. A similar pattern was observed in liver lipids, which might be explained by the central role of the liver as the primary source of fatty acids for the brain.
Fig. 5. T/T ratio (A), LA (B), AA (C), and docosahexaenoic acid (DHA; D) in the plasma PL fraction of cholestatic mice, after 7 wk of experimental diet feeding. Individual fatty acid concentrations are expressed as molar percentages of total fatty acids. Data represent means \pm SD of 4–6 mice/group. *P < 0.001 for the T/T ratio and LA in PL- and TG-supplemented and nonsupplemented mice compared with noncholestatic EFAS mice. **P < 0.05 for AA in PL, TG, and nonsupplemented mice compared with noncholestatic EFAS controls. #P < 0.05 for DHA in EFAD vs. EFAS mice in week 7; DHA in TG-fed mice and PL-fed mice was not significantly different from noncholestatic EFAS mice.

Fig. 6. T/T ratio (A), LA (B), and AA (C) in RBC of cholestatic mice during the 7-wk period of experimental diet feeding. Individual fatty acid concentrations are expressed as molar percentages of total fatty acids. Data represent means \pm SD of 4–6 mice/group. *P < 0.001 for the T/T ratio and for LA in week 0 vs. week 4. **P < 0.05 for T/T ratios of EFAD mice compared with PL- and TG-supplemented mice in week 7. #P < 0.005 for LA in PL-supplemented vs. nonsupplemented EFAD mice in week 7. LA concentrations in TG-fed mice were not significantly different from those of EFAD mice. AA levels were not significantly different between noncholestatic EFAS mice and any of the cholestatic EFAD mice throughout the experimental period.
concentrations in PL isolated from brain tissue than TG-fed and EFAD mice (each \( P < 0.05 \) for differences between PL vs. TG and EFAD; Fig. 9), and even slightly higher DHA concentrations than noncholestatic EFAS mice, although the latter difference was not significant. The third major brain fatty acid, 22:4 n-6, was similarly lower in TG-fed and EFAD mice compared with noncholestatic controls \[ 1.69 \pm 0.13 \text{ mol/mol (TG)}, 1.63 \pm 0.12 \text{ mol/mol (EFAD)}, \text{ and } 2.09 \pm 0.20 \text{ mol/mol (EFAS)}, P < 0.01 \], whereas PL-supplemented mice had 22:4 n-6 concentrations comparable to noncholestatic EFAS mice \[ 1.83 \pm 0.13 \text{ mol/mol, NS; data not shown} \].

**DISCUSSION**

In the present study, we compared the efficacy of EFA-rich TG and EFA-rich PL for correcting EFAD under cholestatic conditions in mice. We hypothesized that PL would be more effective than TG for oral treatment of EFAD in cholestatic liver disease, since dietary TG are profoundly malabsorbed during cholestasis and PL are less dependent on bile for intestinal absorption. The present results indicate that, indeed, oral supplementation with PL is superior to oral TG in increasing EFA-derived LCPUFA concentrations in brain and liver.

We used and adapted a murine model for diet-induced EFAD that we developed and characterized previously (43). Ligation of the common bile duct in mice fed an EFAD diet for 3 wk resulted in acute extrahepatic cholestasis, combined with a diet-induced EFAD. Both our cholestatic and noncholestatic mouse models for EFAD developed the characteristic biochemical hallmarks of EFAD in plasma and erythrocyte fatty acid profiles (1, 16, 46). Plasma and erythrocyte EFA and LCPUFA concentrations strongly decreased, and levels of nonessential fatty acids, such as mead acid (20:3 n-9) and oleic acid, concomitantly increased. The cessation of growth that we observed in EFAD mice is likely related to impaired dietary fat absorption during EFAD, which has been described previously (3, 4, 20). Superimposing extrahepatic cholestasis on EFAD in mice subsequently resulted in weight loss. Bile-diverted rats compensate for bile deficiency-induced fat malabsorption by increasing their chow ingestion (24), in contrast to rats with BDL (23). In our bile duct-ligated EFAD mice, chow intake similarly remained constant, suggesting that two causes for fat malabsorption are present in our mouse model (bile deficiency and EFAD), the combination of which presumably induced weight loss. BDL profoundly elevated plasma bile salt concentrations, which were already slightly elevated by prior EFAD diet feeding. Liver histology revealed extensive bile duct proliferation and parenchymal damage in cholestatic mice, accompanied by jaundice within days after bile duct closure. The rapid onset of these biochemical and morphological parameters of EFAD and cholestasis in our mice provided us with an effective model for studying EFAD under acute cholestatic conditions.

In cholestatic EFAD mice, oral administration of EFA-rich PL completely restored DHA and AA concentrations in the
target organs brain and liver, in contrast to EFA-rich TG, which did not improve these parameters relative to continuation of the EFAD diet.

It is well known that the high levels of DHA and AA in the excitable retinal and synaptosomal membranes of the central nervous system are crucial for adequate membrane reactivity and function of receptor proteins (2, 11, 34). The molecular mechanism underlying the high concentrations of DHA and AA in the brain is unknown. Scott and Bazan (35) demonstrated that the majority of brain DHA originates from hepatic elongation and desaturation of n-3 fatty acids and subsequent redistribution to the brain. Additionally, astrocytes and brain endothelial cells are capable of synthesizing LCPUFA from EFA, which may constitute a minor but still relevant source of DHA and AA for the central nervous system (15, 25, 28, 45). The plasma compartment appears to be the main source of brain DHA and AA. LCPUFA are partly present in plasma as lipoprotein components, but Purdon et al. (29) and Rapoport et al. (31) demonstrated in rats that LCPUFA esterified within circulating lipoproteins do not enter the brain to a measurable extent, and that only the unesterified form is incorporated. Unesterified LCPUFA bound to albumin are a well-recognized plasma source of PUFA for the brain (14), but concentrations of particularly n-3 fatty acids are low, and it is questionable whether the free fatty acid compartment accounts for the majority of LCPUFA supply to the brain (39). LCPUFA are also present in plasma as lyso-phosphatidylcholine (PC), bound to albumin (30). Thies et al. (40) and Bernoud et al. (5) reported data suggesting that the brain preferentially absorbs DHA as sn-2 lyso-PC compared with unesterified DHA. Because dietary PL are partly absorbed as lyso-PL and partly as intact PL molecules, the highly efficient increase in brain LCPUFA concentration after PL supplementation in our study could be in line with these observations, supporting the presumed high bioavailability of dietary PL (8, 12, 19, 37). Surprisingly, PL supplementation seemed to increase brain DHA to an even slightly higher level than in noncholestatic mice fed the EFAS diet. However, because both EFAD and EFAS diets contain very low amounts of ALA, prolonged EFAS diet feeding may result in marginal DHA levels. The TG- and PL-enriched diets equally provided supplementary ALA, which has the capacity to increase DHA levels, even compared with EFAS mice. However, comparison between the TG- and the PL-fed groups indicated that only PL supplementation increased brain DHA.

We did not find indications that EFAD and cholestasis affected the activity of hepatic desaturation and elongation enzymes required for AA and DHA synthesis from their respective precursors. The ratios between 20:3n-6 and 20:4n-6, as a marker for Δ5 desaturase activity, and between 22:5n-3 and 22:6n-3, as a marker for Δ6 desaturase activity, were not different between supplemented and nonsupplemented mice, nor between cholestatic and noncholestatic EFAD mice. The present observations are in agreement with previous results in EFAS bile duct-ligated rats, which showed no indications
for altered postabsorptive EFA metabolism during cholestasis (23).

Oral PL more efficiently improved LCPUFA concentrations in brain and in liver than oral TG. Interestingly, PL and TG reversed parameters of EFAD in plasma and erythrocytes with equal efficacy. Demarne et al. (13) and Minich et al. (23) demonstrated in cholestatic rats that absorption of unsaturated fatty acids, and of EFA in particular, is relatively preserved compared with that of saturated species. Quantitative absorption studies would be required to fully exclude differences in net enteral uptake of TG-EFA and PL-EFA. In addition, the products of TG and PL lipolysis (2-monoacylglycerol and 1-lyso-PL) follow qualitatively different routes within the enterocyte, possibly resulting in different postabsorptive metabolic pathways of the attached EFA.

Although we could not demonstrate differential effects of the TG and PL supplements in the plasma PL fraction, fatty acid profiles of plasma PL closely corresponded with fatty acid profiles as measured in liver PL. If PL on the surface of chylomicron particles were specifically targeted for EFA delivery to the brain, then plasma PL fatty acid analysis is likely to miss differential effects of oral PL over TG if blood samples are not taken during or immediately after fat absorption, due to the rapidity of chylomicron clearance (17).

Our present data are in contradiction with the paradigm that the erythrocyte membrane fatty acid composition is a tentative index for overall body EFA status. Rather, our data are in line with the recent questioning by several authors of the validity of extrapolating erythrocyte membrane fatty acid profiles to their status in target organs for EFA. Korotkova and Strandvik (18) demonstrated that EFAD in rats differentially affects fatty acid profiles of erythrocyte, serum, jejunum, ileum, and colon PL. Rioux et al. (32) reported that, in piglets, plasma PL are good indicators for liver and bile PL fatty acids, but not for brain fatty acid composition. Our results in mice support these observations, since, indeed, fatty acid profiles of the isolated plasma PL fraction closely corresponded with those of liver PL but not of brain PL. Data suggest that specific channeling of PUFA to target organs as the central nervous system occurs at the expense of less critical tissues such as erythrocytes.

Supplementation of EFA as PL not only improved biochemical parameters of EFAD in cholestatic mice, but oral PL also prevented weight loss during EFAD and cholestasis, whereas oral TG did not. Nishioka et al. (27) recently demonstrated that enteral infusion of PL-cholesterol liposomes partially corrects lipid malabsorption in bile-diverted rats, compatible with a facilitating effect of enteral PL supplementation on fat uptake during bile deficiency. Although no information on fat balance is available from the present studies, it is tempting to speculate that EFA-rich PL supplementation partially corrects fat malabsorption in EFAD cholestatic mice.

A surprising observation was the remarkably stable AA concentration in erythrocytes of cholestatic EFAD mice compared with noncholestatic EFAD mice. Although in the latter AA levels decreased after starting the EFAD diet, BDL per se appeared to maintain AA concentrations at a

Fig. 9. LA (A), AA (B), and DHA (C) in brain PL of cholestatic mice after 7 wk of experimental diet feeding and of noncholestatic EFAS controls (dotted line). Individual fatty acid concentrations are expressed as molar percentages of total fatty acids. Data represent means ± SD of 4–6 mice/group. **P < 0.05 for LA concentrations of EFAD mice compared with PL-fed and EFAS mice. AA and DHA were significantly higher in brain PL of PL-fed mice compared with TG-fed and EFAD mice (#P < 0.01).
normal level. Tso (41) reported that human bile PL provide up to 1.7 g AA to the intestine per day. An average Western adult diet supplies 1.8 g AA daily (9), indicating that biliary PL secretion in the intestine provides a significant portion of enteral AA. Our results support the concept that biliary secretion of AA in the form of PL quantitatively affects overall body AA homeostasis.

EFAD in pediatric cholestatic patients has been proven difficult to correct by merely increasing EFA ingestion. It should be realized that, in patients, in contrast to the presented model, cholestasis usually develops gradually, and EFAD is not the result of a lack of dietary EFA content. Yet, our present experiments suggest that oral administration of EFA-rich PL might be an effective treatment strategy for reversing EFAD in patients with cholestatic liver disease. The efficacy of oral PL supplementation for treatment of EFAD in pediatric patients with cholestatic is currently under investigation.

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