Essential fatty acid deficiency in mice is associated with hepatic steatosis and secretion of large VLDL particles

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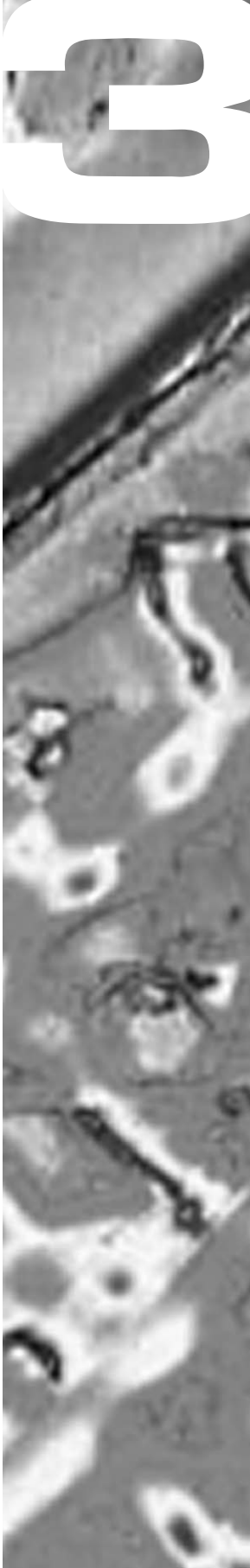

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ABSTRACT

Background: Essential fatty acid (EFA) deficiency in mice decreases plasma triglyceride (TG) concentrations and increases hepatic TG content. We evaluated *in vivo* and *in vitro* whether decreased hepatic secretion of TG-rich VLDL contributes to this consequence of EFA deficiency.

Methods: EFA deficiency was induced in mice by feeding an EFA-deficient (EFAD) diet for eight weeks. Hepatic VLDL secretion was quantified in fasted EFAD and EFA-sufficient (EFAS) mice using the Triton WR-1339 method. In cultured hepatocytes from EFAD and EFAS mice, VLDL secretion into medium was measured by quantifying [^{3}H]-glycerol incorporation into TG and phospholipids (PL). Hepatic expression of genes involved in VLDL synthesis and clearance was measured, as were plasma activities of lipolytic enzymes.

Results: TG secretion rates were quantitatively similar in EFAD and EFAS mice *in vivo* and in primary hepatocytes from EFAD and EFAS mice *in vitro*. However, EFA deficiency increased the size of secreted VLDL particles, as determined by calculation of particle diameter, particle sizing by light scattering and evaluation of TG-to-apoB ratio. EFA deficiency did not inhibit hepatic lipase and lipoprotein lipase activities in plasma, but increased hepatic mRNA levels of apoAV and apoC-II, both involved in control of lipolytic degradation of TG-rich lipoproteins.

Conclusions: EFA deficiency does not affect hepatic TG secretion rate in mice, but increases the size of secreted VLDL particles. Present data suggest that hypotriglyceridemia during EFA deficiency is related to enhanced clearance of altered VLDL particles.

INTRODUCTION

Development of hepatic steatosis is a well-established manifestation of essential fatty acid (EFA) deficiency in animal models. It was first described in 1958 in rats by Alfin-Slater *et al.*[1] and in 1970 by Fukazawa and Sinclair *et al*.[2; 3]. The excess lipid deposited in the liver during EFA deficiency can theoretically result from increased uptake of circulating lipids, enhanced *de novo* lipogenesis, decreased fatty acid oxidation, decreased hepatic lipoprotein secretion, or from a combination of these. Increased hepatic lipogenesis and decreased fatty acid oxidation could indeed contribute, since polyunsaturated fatty acids are physiological suppressors of fatty acid synthesis through down-regulation of SREBP1c[4-8], and inducers of hepatic fatty acid oxidation through activation of PPAR-alpha (PPARa), respectively. The quantitative contribution of increased lipogenesis and decreased oxidation to EFA deficiency-induced hepatic steatosis has not been established. While the effects of EFA deficiency on induction of hepatic steatosis are fairly consistent in the literature, the consequences for hepatic lipoprotein secretion and plasma lipid profiles are less clear. Fukazawa *et al*.[3] reported decreased triglyceride (TG) and phospholipid (PL) secretion from perfused livers of EFAD rats. However, EFA deficiency has also been associated with enhanced hepatic TG secretion rates in rats[9-12]. Similarly, data on lipoprotein clearance during EFA deficiency are equivocal. Activities of plasma lipoprotein lipase (LPL) and hepatic lipase (HL) were reported to be increased in EFAD rats by Nilsson *et al*.[2; 13-14], whereas Levy and colleagues described decreased plasma LPL activity in EFAD rats[15; 16].

Recently, we characterized a mouse model for EFA deficiency in which hepatic TG levels are increased and plasma TG concentrations are decreased[17]. To preclude the confusion from isolated studies on EFA deficiency in different species and models, we have chosen to characterize this mouse model in detail. Previously, we reported characteristics of EFAD mice with respect to growth, intestinal fat absorption, bile formation and fatty acid composition in specific organs[17-19]. In the present study, we investigated whether EFA deficiency affects hepatic VLDL secretion in mice *in vivo* and in isolated mouse hepatocytes *in vitro*. Our data indicate that EFA deficiency in mice does not quantitatively affect hepatic VLDL-TG secretion but increases VLDL particle size. We hypothesize that clearance rate of these large lipoproteins is increased to yield low plasma TG levels.

MATERIALS AND METHODS

Materials

Triton WR-1339, Triton X-100, fatty acid-free bovine serum albumin (BSA), oleic acid and heptadecanoic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [^{3}H]-glycerol was purchased from New England Nuclear (Boston, MA, USA), glycerol tri-[9,10(n)-[^{3}H]-oleate from Amersham Biosciences (Buckinghamshire, UK) and glycerol trioleate from Fluka Chemie, Sigma-Aldrich (Zwijndrecht, the Netherlands). 4-15% SDS ready gels were from Biorad (Hercules, CA, USA), heparin was obtained from Leo Pharma BV (Weesp, the Netherlands) and all cell culture materials were from Costar (Cambridge, MA, USA).

Animals

Male wildtype mice with a free virus breed (FVB) background were obtained from Harlan (Horst, the Netherlands). When starting the experimental diets, mice were approximately eight weeks old and were housed in a light-controlled (lights on 6 AM-6 PM) and temperature-controlled (21°C) facility with free access to tap water and standard laboratory chow (RMH-B, Arie Blok BV, Woerden, the Netherlands). The experimental protocols were 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% protein, 46 energy% carbohydrate and 34 energy% fat, respectively, and had the following fatty acid composition: 41.4 mol% palmitic acid (C16:0), 47.9 mol% stearic acid (C18:0), 7.7 mol% oleic acid (C18:1n-9) and 3 mol% linoleic acid (C18:2n-6). An isocaloric EFA-sufficient (EFAS) diet was used as control diet, containing 20 energy% protein, 43 energy% carbohydrate and 37 energy% fat with 32.1 mol% C16:0, 5.5% C18:0, 32.2 mol% C18:1n-9 and 30.2% C18:2n-6 (custom synthesis, diet numbers 4141.08 (EFAD) and 4141.07 (EFAS), respectively; Arie Blok BV, Woerden, the Netherlands).

Experimental procedures

Induction of EFA deficiency in mice

Mice were fed standard laboratory chow containing 6 weight% fat from weaning, and switched to EFAD or EFAS diet at 8 weeks of age. After 8 weeks on EFAD or EFAS diet, 6 mice of each dietary group were anesthetized by halothane/NO_2 and a large blood sample was obtained by cardiac puncture for determination of plasma lipid levels, lipoprotein profile and plasma and erythrocyte fatty acid composition.

Blood was collected in heparinized tubes and plasma and erythrocytes were separated by centrifugation at 2400 rpm for 10 min (Eppendorf Centrifuge, Eppendorf, Germany). Fresh erythrocyte samples were hydrolyzed and methylated[20] for gas-chromatographic analysis of fatty acid profiles. After liver excision, tissue aliquots (30 mg) were immediately stored in liquid nitrogen for mRNA isolation. The remaining liver tissue was stored at -80°C until further analysis.

Fast Protein Liquid Chromatography (FPLC)

For plasma lipoprotein size fractionation, 200 μl of pooled plasma from EFAD- and EFAS-diet fed mice (n=6 per group) was separated by FPLC on a Superose 6 HR10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Triglyceride, phospholipid and cholesterol concentrations in the obtained fractions (0.5 ml) were measured as described below.

In vivo VLDL secretion in EFAD and EFAS mice

In mice fed EFAD or EFAS diet for 8 weeks (n=6 per group), plasma lipolysis was blocked by retro-orbital injection of Triton WR-1339 (12.5 mg/100 μl phosphate-buffered saline) after an overnight fast. Blood samples (75 μl) were obtained from the retro-orbital plexus under halothane anesthesia, before and at 60 min intervals after Triton injection for 4 hours. Blood was collected in micro-hematocrit tubes containing heparin, and was centrifuged at 2400 rpm for 10 min (Eppendorf Centrifuge, Eppendorf, Germany) for isolation of plasma and blood cells. At the end of the experiment, a large blood sample was obtained by cardiac puncture, after which the liver was removed and stored at -80°C until further analysis. From the last blood sample, the plasma VLDL fraction (d 0.93-1.006 g/ml) was isolated by ultra-centrifugation. For this purpose, 800 μl of NaCl solution with a density of 1.006 g/ml, containing 0.02% NaN_3, was added to 200 μl plasma, followed by centrifugation for 100 min at 120 000 rpm at 4°C in an Optima TM LX table top centrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA). The top layer containing the VLDL fraction was isolated by tube slicing, and the volume was recorded by weight. A 30 μl portion was used for particle size determination using dynamic light scattering (details see below) and the remaining VLDL fraction was stored at -80°C until further analysis.

Post-heparin HL and LPL activity in plasma of EFAD and EFAS mice

Separate groups of EFAD and EFAS mice (n=6 per group) were fasted for 4 hours, after which a baseline blood sample (150 μl) was obtained by orbital bleeding under halothane anesthesia, for determination of baseline plasma lipase activity. Subsequently, an intravenous bolus of 0.1 U of heparin per gram bodyweight was

injected, and 10 min later a post-heparin blood sample (150 μl) was obtained by orbital bleeding. Blood was collected in heparinized micro-hematocrit tubes, immediately centrifuged at 2400 rpm for 10 min (Eppendorf Centrifuge, Eppendorf, Germany) and isolated plasma was frozen in 10% glycerol in liquid nitrogen and stored at -80°C until *in vitro* analysis of LPL and HL activities[21].

For the LPL and HL assay, 10 μl of plasma was incubated with 200 μl of ultrasonified substrate containing 1 ml Triton X-100 (1%), 1 ml Tris-HCl (1M), 2 ml of heat-inactivated human serum, 2 ml of fat-free BSA (10%), 42 mg triolein and 5 μl glycerol-tri-(9,10(n)-[^{3}H])-oleate (5mCi/ml), with or without addition of 50 μl NaCl (5M) to block LPL activity. After 30 min incubation at 37°C, lipolysis was stopped by adding 3.25 ml of heptane / methanol / chloroform (100/128/137, v/v/v) and 1 ml of 0.1M K_2CO_3. After centrifugation for 15 min at 3600 rpm at room temperature, extracted hydrolyzed fatty acids were quantified by scintillation counting. Lipase activities were calculated according to the formula: [dps sample - dps blank] / dps 200 μl LPL-substrate x factor, in which the factor = (2.45 (volume aqueous phase) x 4.74 (total added FFA in mol) / [0.76 (extraction efficiency) x 0.5 (reaction time in h) x 0.01 (plasma volume in ml)]). Post-heparin LPL activity was calculated by subtracting post-heparin hepatic lipase activity (i.e., lipase activity inhibited by 1M NaCl) from the total post-heparin lipase activity.

In vitro VLDL secretion from cultured EFAD and EFAS hepatocytes

Isolation of hepatocytes from mice fed EFAD or EFAS diet for 8 weeks was performed as described previously[22; 23]. Hepatocytes were plated in 35 mm 6-well plastic dishes pre-coated with collagen (Serva Feinbiochemica, Heidelberg, Germany) at a density of $1.0x10^6$ cells per well, suspended in 2 ml of William's E medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 0.20 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, μ50 g/ml gentamycin and 50 nM dexamethasone. Cells were maintained in a humidified incubator at 37°C and 5% CO_2. After a 5 hour attachment period the medium was refreshed. Cells were cultured overnight, medium was removed and hepatocytes were washed and incubated for 4 hours with hormone-free and FCS-free (HF-SF) William's E medium supplemented with 1.7% fat-free albumin, insulin, penicillin / streptomycin and gentamycin. Medium was replaced by 1 ml HF-SF William's E medium per well containing 22 μM [^{3}H]-glycerol (4.4 μCi per well), 3 μM glycerol, 0.75 mM oleic acid (C18:1) complexed with fatty acid-free BSA. After 24 hours incubation, medium was collected and centrifuged for 2 min at 13000 rpm to remove debris, and stored at -80°C until further analysis. Hepatocytes were washed with ice-cold Hank's balanced salt solution (HBSS) and scraped into 2 ml of HBSS for lipid extraction.

Analytical techniques

Plasma lipids were measured using commercially available assay kits from Roche (Mannheim, Germany) for triglyceride and total cholesterol, and from WAKO chemicals GmbH (Neuss, Germany) for phospholipids. ApoB protein levels were determined by Western blotting. Proteins from plasma VLDL fractions (10 μl VLDL/lane) were separated on 4-15% ready gels and blotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) by tank-blotting. Membranes were blocked overnight in a 4% skimmed milk power solution in Tris-buffered saline containing 0.1% Tween-20 (TTBS) and subsequently incubated with the primary antibody (human polyclonal anti apoB, cross-reactive with mouse, Roche, Mannheim Germany, 726494) diluted 1:100000 in TTBS for 2 hours at room temperature. After washing, anti-sheep IgG linked to horseradish peroxidase (Calbiochem, San Diego, CA, 402100), diluted 1:10000 in TTBS was added for 1 hour. Detection was carried out using ECL, according to manufacturer's instructions (Amersham, Roosendaal, the Netherlands) and bands of apoB were quantified using Image Masters VDS system (Amersham Pharmacia Biotech, Uppsala, Sweden).

VLDL size and volume distribution profiles were analyzed by dynamic light scattering, using a Nicomp model 370 submicron particle analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA, USA). Particle diameters were calculated from the volume distribution patterns provided by the analyzer. TG-rich lipoprotein diameters were also estimated using the equation according to Fraser and Harris *et al*.[24; 25]: diameter (nm) = 60 x ([0.211xTG/PL] + 0.27).

Essential fatty acid status was analyzed by hydrolyzing, methylating and extracting plasma and erythrocyte lipids as described previously[20]. For fatty acid analysis of cultured hepatocytes, lipids were extracted from aliquots of mechanically homogenized cell suspensions[26], followed by methylation procedures as described above. Butylated hydroxytoluene was added as antioxidant. Heptadecanoic acid (C17:0) was added to all samples as internal standard prior to extraction. Fatty acid methyl esters were separated and quantified by gas liquid chromatography on a Hewlett Packard gas chromatograph model 6890, equipped with a 50mx0.2mm Ultra 1 capillary column (Hewlett Packard, Palo Alto, CA) and a FID detector, using program conditions as described previously[17]. Individual fatty acid methyl esters were quantified by relating areas of their chromatogram peaks to that of the internal standard C17:0. Relative concentrations (mol%) of erythrocyte and hepatocyte fatty acids were calculated by summation of fatty acid peak areas and subsequent expression of the area of each individual fatty acid as a percentage of this amount.

Lipids secreted into medium by EFAD and EFAS mouse hepatocytes and cellular lipids were subjected to the lipid extraction procedure mentioned above. [^{3}H]-TG and [^{3}H]-PL fractions were isolated from lipid extracts using thin-layer chromatography (TLC) (20x20 cm, Silica gel 60 F254, Merck), with hexane/diethyl-ether/acetic acid (80:20:1, v/v/v) as solvent. After iodine staining, the [^{3}H]-containing TG- and PL-spots were delineated and scraped into vials and assayed for radioactivity by scintillation counting. A portion of the extracted lipids was dissolved in chloroform containing 2% Triton X-100. After chloroform evaporation and resuspension in H_2O, total cellular TG concentration was determined using the TG assay kit mentioned previously.

Protein concentrations in isolated mouse hepatocytes were determined according to Lowry *et al*.[27], using Pierce bovine serum albumin as standard. Secreted apoB in medium of EFAD and EFAS mouse hepatocytes was concentrated with fumed silica and delipidated as described by Vance *et al*.[28] ApoB protein was separated by SDS-PAGE using 4-15% gradient gels at 100V for 30 min followed by 150V for 90 min. Subsequently, gels were subjected to the silver staining procedure as described by Curtin *et al*.[29]. The relative intensities of apoB100 and apoB48 bands were determined using a CCD camera of Image Masters VDS system (Amersham Pharmacia Biotech, Uppsala, Sweden).

For measurement of mRNA expression levels by real-time PCR, total RNA from EFAD and EFAS liver tissue aliquots was isolated using TRI Reagent (Sigma T9424) according to the manufacturer's instructions. Isolated total RNA was converted to single-stranded cDNA with M-MLV reverse transcriptase by the reverse transcription procedure from the manufacturer's protocol (Sigma). Real-time quantitative PCR was performed by using the ABI prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Primers were obtained from Invitrogen and a template-specific 3'-TAMRA, 5'-6-FAM labeled Double Dye Oligonucleotide probe was obtained from Eurogentec, Seraing, Belgium. Primers and probes used in these studies for Acc1, ApoB, Fas, Mttp, Srebp1a, Srebp1c, 18S, ß-actin, hmgCoAS-m have been described previously[30-32]. Acc2 forward primer: CAT ACA CAG AGC TGG TGT TGG ACT, reverse primer: CAC CAT GCC CAC CTC GTT AC, probe: CAG GAA GCC GGT TCA TCT CCA CCA G, GenBank accession NM_133904, ApoAV forward primer: GAC TAC TTC AGC CAA AAC AGT TGG A, reverse primer: AAG CTG CCT TTC AGG TTC TCC T, probe: CTT CTG TGG CTG GCC CAT CAC GC, GenBank accession NM_080434, ApoC-I forward primer: GGG CAG CCA TTG AAC ATA TCA, reverse primer: TTG CCA AAT GCC TCT GAG AAC, probe: CCC GGG TCT TGG TCA AAA TTT CCT TC, GenBank accession NM_007469, ApoC-II forward primer: TTA CTG GAC CTC TGC

CAA GGA, reverse primer: CCC TGA GTT TCT CAT CCA TGC, probe: CCA AAG ACC TGT ACC AGA AGA CAT ACC CGA, GenBank accession NM_009695, ApoC-III forward primer: CCA AGA CGG TCC AGG ATG C, reverse primer: ACT TGC TCC AGT AGC CTT TCA GG, probe: CCA TCC AGC CCC TGG CCA CC, GenBank accession NM_023114, Cpt1a forward primer: CTC AGT GGG AGC GAC TCT TCA, reverse primer: GGC CTC TGT GGT ACA CGA CAA, probe: CCT GGG GAG GAG ACA GAC ACC ATC CAA C, GenBank accession NM_013495, Cpt1b forward primer: CCC ATG TGC TCC TAC CAG ATG, reverse primer: CAC GTG CCT GCT CTC TGA GA, probe: CCC AGG CAA AGA GAC AGA CTT GCT ACA GC, GenBank accession NM_009948. All expression data were subsequently standardized for ß-actin, which was analyzed in separate runs.

Calculations and statistics

All results are presented as means ± S.D. for the number of animals indicated. Data were statistically analyzed using Student's t-test or, in absence of normal distribution, Mann-Whitney-U test. Level of significance was set at $p<0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

RESULTS

In previous studies[17; 18], we developed and characterized a murine model for diet-induced EFA deficiency by feeding mice an EFAD diet for eight weeks, which resulted in pronounced biochemical hallmarks of EFA deficiency such as increased triene/tetraene ratios[33; 34]. After eight weeks of EFAD diet feeding, body weights of mice were significantly lower compared to EFA-fed counterparts (29.5±1.7 g vs. 32.6±3.3 g, $p<0.001$), which is likely related to impaired dietary fat absorption during EFA deficiency, as described previously[17; 35; 36]. No other clinical characteristics of EFA deficiency, such as alopecia or tail necrosis, were observed in EFAD mice. Figure 1 shows that the EFAD diet decreased concentrations of EFA and LCPUFA and increased levels of non-essential fatty acids in plasma VLDL and in erythrocytes.

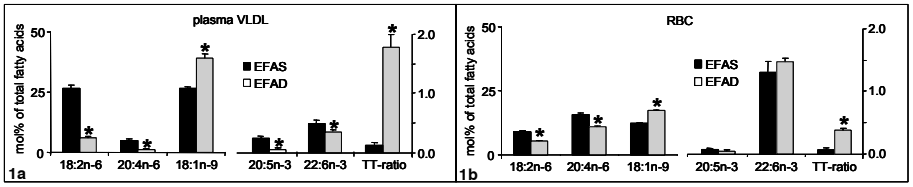


Figure 1: Linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6), oleic acid (C18:1n-9), eicosapentaenoic acid (C20:5n-3), docosahexaenoic acid (C22:6n-3) and the TT-ratio in plasma VLDL and in RBC of EFAD and EFAS mice. Fatty acid concentrations are in mol% of total fatty acids.Data represent means±SD of 6 mice per group. *$p<0.05$ for EFAD vs. EFAS.

Effects were more pronounced in VLDL than in erythrocytes. Similar to previous studies, plasma triglyceride concentration was decreased in EFAD mice (EFAD 0.4±0.1 mM, EFAS 0.8±0.6 mM, $p<0.05$), which was predominantly due to a decrease in the VLDL-sized lipoprotein fraction (Figure 2). Cholesterol concentrations were higher in fractions 15-20 of the FPLC profile of EFAD mice, which may indicate the presence of large HDL particles, or increased amounts of IDL/LDL-sized particles.

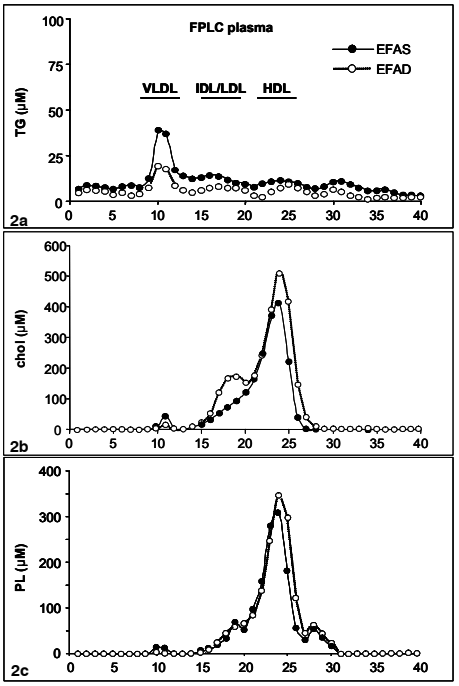


Figure 2: Plasma triglyceride (TG), cholesterol (chol) and phospholipid (PL) concentrations in FPLC fractions of EFA-deficient (EFAD) and EFA-sufficient (EFAS) mice. Data represent lipid concentrations in pooled plasma samples of 6 mice per group.

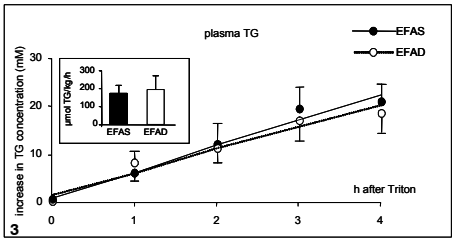
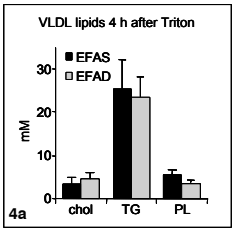
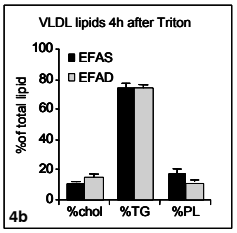


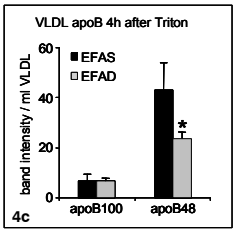
Figure 3: Increase in plasma triglyceride (TG) concentration in mice fed EFA-deficient (EFAD) or EFA-sufficient (EFAS) diet for 8 weeks, before and at 60 min intervals after Triton WR-1339 injection. Plasma accumulation of TG was similar in EFAD and EFAS mice, indicating equal VLDL-TG production rates (193±78 vs. 173±46 μmol TG/kg/h for EFAD and EFAS mice, respectively; NS). Data represent means ± SD of 6 mice per group.

Figure 3 shows that, upon lipolysis blockage by Triton WR-1339, plasma accumulation of TG was similar in EFAD and EFAS mice, indicating equal VLDL-TG production rates (193±78 vs. 173±46 μmol TG/kg/h for EFAD and EFAS mice, respectively; NS). Figure 4 shows that TG and PL levels in the VLDL fraction isolated 4 hours after Triton administration were similar in EFAD and EFAS mice.

ApoB100 concentrations in this fraction were also similar in EFAD and EFAS mice, yet, apoB48 was significantly lower in VLDL of EFAD mice (Figure 4c, $p<0.05$). The decreased apoB48 concentration but the unaffected TG secretion rate suggests an increased size of secreted VLDL particles during EFA deficiency.

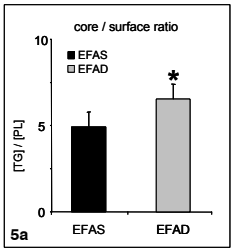


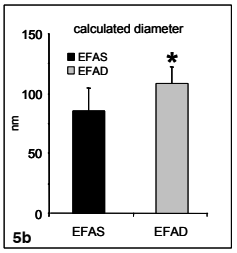


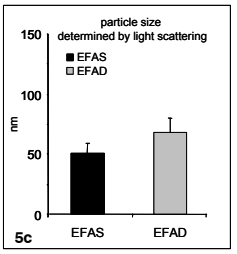


Triglyceride (TG), phospholipid (PL) and cholesterol in plasma VLDL**(4a)**, relative TG, PL and cholesterol in plasma VLDL, expressed as % of total lipid.**(4b),** ApoB100 and apoB48 in plasma VLDL of EFAD and EFAS mice **(4c)**. Plasma VLDL was isolated 4h after Triton administration. Data represent means±SD of 6 mice / group, *$p<0.05$ for apoB48 of EFAD vs. EFAS mice.

Lipoprotein particle size in plasma of EFAD mice was estimated by three methods. The core-to-surface ratio in the isolated TG-rich lipoprotein fraction (4 hours after Triton) was significantly higher in EFAD than in EFAS mice (6.6±0.9 vs. 4.9±1.2, $p<0.05$, Figure 5a). Upon calculation of lipoprotein diameters according to Fraser and Harris *et al*.[24; 25], TG-rich lipoproteins from EFAD mice similarly appeared larger than from EFAS mice (Figure 5b, $p<0.05$). Finally, determination of TG-rich lipoprotein size using dynamic light scattering also indicated that plasma lipoproteins from EFAD mice were larger than from EFAS mice; however, this difference did not reach statistical significance ($p=0.37$, Figure 5c).







Core-to-surface ratio in the isolated VLDL fraction (4h after Triton) of EFAD and EFAS mice, estimated by the ratio of TG (mM) and PL (mM). Data represent means ± SD of 5-6 mice per group, *$p<0.05$ for EFAD vs. EFAS mice. **(5a);** Lipoprotein diameter (nm) calculated as: diameter (nm) = 60 x ([0.211 x TG/PL] +0.27). TG-rich lipoproteins from EFAD mice were significantly larger than those from EFAS mice **(5b).** Data represent means ± SD of 6 mice / group, *$p<0.05$ for EFAD vs. EFAS mice. TG-rich lipoprotein size (nm) measured by dynamic light scattering **(5c).** Data represent means ± SD of 6 mice per group, $p=0.37$ for EFA-deficient vs. EFA-sufficient mice.

In addition to quantifying VLDL-TG production in mice *in vivo*, VLDL-TG production was determined in cultured hepatocytes from EFAD and EFAS mice *in vitro*, to exclude a possible influence of confounding metabolic effects of the systemic circulation on VLDL production.

Figure 6 shows that primary hepatocytes from EFAD mice cultured for 48 hours displayed the classical biochemical markers of EFA deficiency, including decreased levels of LA, ALA and their long-chain metabolites AA and DHA and increased concentrations of non-essential fatty acids of the n-7 and n-9 family. Biochemical indications for EFA deficiency were more pronounced in TG than in PL of hepatocytes, as described previously[19].

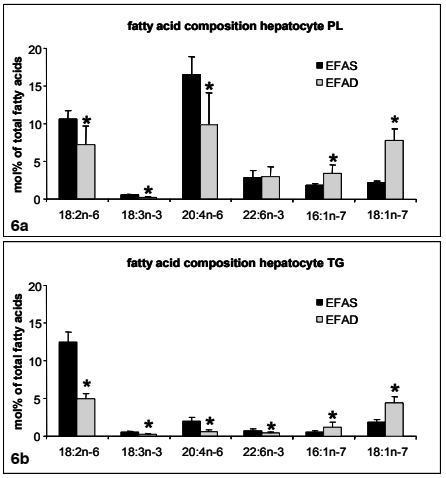


Figure 6: Fatty acid composition of phospholipids (PL) and triglycerides (TG) of cultured hepatocytes from EFA-deficient (EFAD) and EFA-sufficient (EFAS) mice. Concentrations of linoleic acid (LA, C18:2n-6), alpha-linolenic acid (ALA, C18:3n-3), arachidonic acid (AA, C20:4n-6), docosahexaenoic acid (DHA, C22:6n-3), palmitoleic acid (PA, C16:1n-7) and oleic acid (OA, C18:1n-9), are expressed as mol% of total fatty acids. Data represent means ± SD of 6 mice per group.*$p<0.05$ for differences between EFAD and EFAS hepatocytes.

After 24 hours of incubation with [^{3}H]-glycerol and oleic acid, EFAD hepatocytes had incorporated significantly more label into TG and PL than EFAS hepatocytes, compatible with higher rates of TG and PL synthesis (Figure 7a). Total intracellular TG mass was approximately two-fold higher in hepatocytes from EFAD mice, compared with those from EFAS mice (figure 7b). Specific cellular TG activity was similar in EFAD and EFAS cells (Figure 7c). EFAD hepatocytes secreted similar amounts of [^{3}H]-labeled TG into the medium, but significantly less phospholipids compared to EFAS mice (Figure 7d). The apoB content was lower in medium from EFAD than from EFAS cells (Figure 7e), indicating secretion of a decreased number of particles.

Since hepatic TG production was not affected by EFA deficiency in mice, hypotriglyceridemia is likely attributable to accelerated clearance of TG-rich lipoproteins. Determination of mRNA levels of genes involved in hepatic lipogenesis and VLDL assembly, using real-time quantitative PCR, showed a significantly increased expression in EFAD mice of Acc1, which produces malonyl-CoA for fatty acid synthesis (Figure 8).

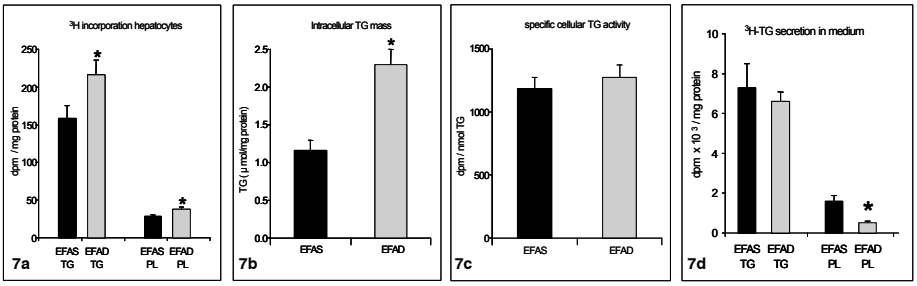


Figure 7a: [^{3}H]-incorporation into TG and PL by EFAD and EFAS hepatocytes after 24h of incubation with [^{3}H]-glycerol and oleic acid. Data represent mean values ± SD measured in hepatocytes from 2 individual mice per group, n=6 separate measurements per mouse. *p<0.001 for differences between EFAD and EFAS hepatocytes.**Figure 7b:** Total intracellular TG mass (µmol/mg protein) in hepatocytes from EFAD and EFAS mice. Data represent mean values±SD measured in hepatocytes from 2 mice per group, n=6 separate measurements per mouse. *p<0.001 for differences between EFAD and EFAS hepatocytes. **Figure 7c:** Specific cellular TG activity (dpm/nmol TG) in cultured hepatocytes from EFAD and EFAS mice (n=2 mice per group). Data represent means ± SD from 6 separate measurements per mouse, p=0.08 for EFAD vs. EFAS hepatocytes. **Figure 7d:** [^{3}H]-TG and [^{3}H]-PL secretion into medium (dpm x 103 / mg protein) by hepatocytes from EFAD and EFAS mice (n=2 mice per group). Data represent means ± SD from 6 measurements per mouse. *p<0.001 for differences between [^{3}H]-PL secretion by EFAD and EFAS hepatocytes. [^{3}H]-TG levels were not significantly different between groups.

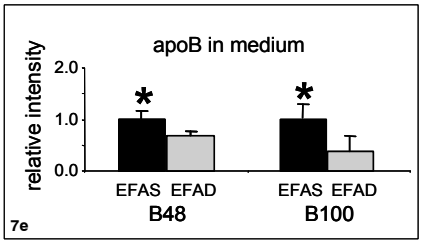


Figure 7e: ApoB48 and apoB100 in medium from EFAD and EFAS hepatocytes (n=2 mice per group). Data represent means ± SD from 6 separate measurements per mouse. *p<0.01 for differences in apoB content in medium of EFAD and EFAS hepatocytes.

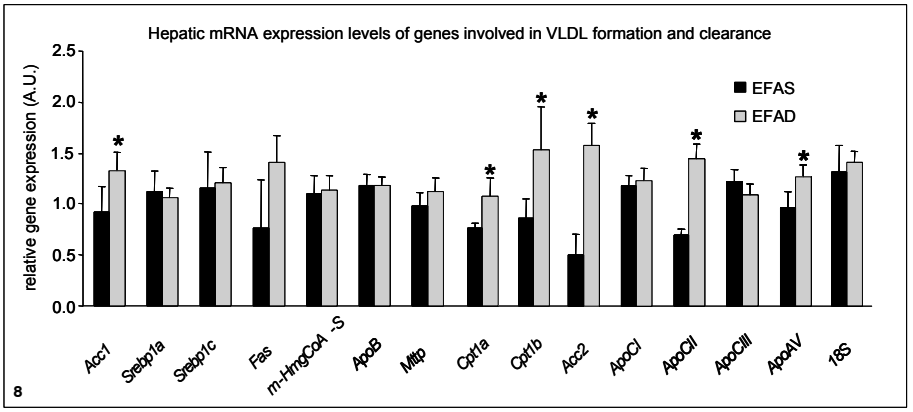


Figure 8: Hepatic mRNA expression levels of genes involved in VLDL formation and clearance, normalized to ß-actin. Data represent mean values ± SD of 6 mice per group, p<0.05 for differences in mRNA levels in EFAD vs. EFAS mice.

Expression of Fas, a critical gene for lipogenesis, tended to be higher in EFAD livers, but the difference did not reach statistical significance. Hepatic expression of Acc2, Cpt1a and Cpt1b was significantly increased in EFAD mice, compatible with increased hepatic fatty acid oxidation. mRNA levels of ApoB and Mttp, key regulators

of VLDL formation, were similar in EFAD and EFAS livers. Expression of ApoC-III, a lipoprotein lipase (LPL) inhibitor, was similar in EFAD and EFAS mice but mRNA levels of ApoC-II and apoA-V, involved in modulation of lipoprotein lipase activity, were significantly higher in livers of EFAD mice compared to controls. Figure 9 shows the *in vitro* activities of post-heparin plasma hepatic lipase (HL) and lipoprotein lipase (LPL) in EFAD and EFAS mice. No significant differences in either HL or LPL activities were detected between the two groups.

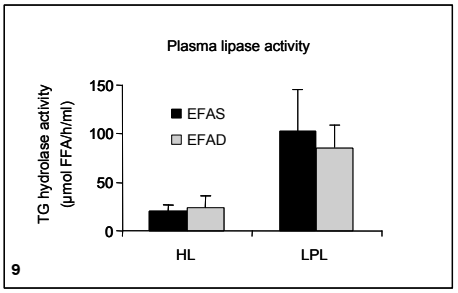


Figure 9: Post-heparin hepatic lipase (HL) and lipoprotein lipase (LPL) activities expressed as triglyceride hydrolase activity (µmol FFA/h/ml) measured in plasma of EFAD and EFAS mice (n=5-6 mice per group). No significant differences were detected between the two groups.

DISCUSSION

We previously demonstrated that EFA deficiency in mice is associated with increased hepatic and decreased plasma TG concentrations[17]. In the present study, we investigated whether decreased hepatic VLDL secretion contributes to these metabolic consequences of EFA deficiency. Our *in vivo* and *in vitro* data indicate that EFA deficiency does not affect quantitative hepatic TG secretion, but alters VLDL size and composition. We speculate that large VLDL particles, perhaps in combination with increased apoCII expression, may be subject to increased clearance rates, resulting in hypotriglyceridemia.
To test this hypothesis experimentally, clearance rates and plasma lipid levels could be measured in EFAD and EFAS mice after infusing a defined lipoprotein emulsion of labeled particles, fractionated into homogenous size populations as described by Rensen *et al.*[37]. Alternatively, although technically more challenging, VLDL particles could be isolated from EFAD and EFAS mice, labeled *ex vivo*, and then clearance rates of EFAD-derived VLDL and of EFAS-derived VLDL could be determined, each in EFAD and EFAS mice.

Fatty acid profile analyses of erythrocytes, plasma VLDL and isolated hepatocytes confirmed the presence of EFA deficiency in our mouse model. As previously demonstrated[17], plasma TG levels were decreased in EFAD mice, particularly in the

VLDL fraction as determined by FPLC. Hepatic VLDL-TG secretion, however, was not decreased in EFAD mice *in vivo* (determined by the Triton method), or in EFAD hepatocytes (determined by [^{3}H]-glycerol incorporation) *in vitro*. Decreased hepatic TG and PL secretion has been reported in studies with perfused livers of EFAD rats[3]. However, the isolated perfused liver model has its limitations regarding physiological lipoprotein secretion, due to lack of hormonal and metabolic feedback from the circulation, with the perfusate usually only containing erythrocytes and fatty acids. The discrepancy regarding *in vivo* studies on lipoprotein clearance in EFAD rats[13; 15] and our EFAD mouse model may be explained by species specificity. Previous studies have demonstrated that EFA deficiency has different effects on bile formation in rats and in mice[17; 36; 38].

Although no quantitative differences were detected in hepatic TG secretion rates, secreted VLDL particles were significantly larger under EFAD conditions. The production of larger VLDL particles could be deduced from several independent observations. During EFA deficiency, the concentration of PL in the VLDL fraction was more profoundly decreased than that of TG (-80% vs. -50%, respectively; Figure 2), indicating production of particles with increased core-surface ratio. The plasma VLDL fraction isolated by ultracentrifugation (after Triton) contained less apoB48 in EFAD than in EFAS mice. Since a single apoB48 molecule is present per VLDL particle, this indicates secretion of a reduced number of VLDL particles *in vivo*. In line with these observations, estimations of particle size by various means also indicated that VLDL particles in EFAD mice were larger than in controls. *In vitro*, EFAD hepatocytes similarly secreted equal amounts of labeled TG but lower amounts of PL and apoB into the medium. An increase in VLDL particle size has previously been reported in EFAD rats[15] and EFAD guinea pigs[39; 40]. We hypothesize that during EFA deficiency, the increased concentration of saturated acyl chains of hepatic PL or the decreased concentration of unsaturated acyl chains (that is, insufficient hepatic EFA-rich PL availability) affect the surface coating of nascent lipoproteins, resulting in relative TG-oversaturation of secreted VLDL. Thus, hepatic VLDL-TG secretion rates apparently are not quantitatively affected during EFA deficiency in mice. By inference, the decreased plasma TG concentration must be due to increased VLDL clearance.

Differences in VLDL clearance during EFA deficiency in mice could result from increased activities of lipolytic enzymes such as hepatic lipase and lipoprotein lipase. However, we found no indications that EFA deficiency affects the *in vitro* activities of hepatic or lipoprotein lipase in EFAD mice. Alternatively, VLDL clearance could be enhanced secondary to alterations of VLDL particles, as was also suggested by

Sinclair *et al*. [2] for enhanced TG clearance from plasma of EFAD rats. In EFAD mice, intravascular lipoprotein metabolism could be influenced by altered interactions with apoC-II, with the recently identified apoAV, or with LPL or phospholipid transfer protein (PLTP)[41; 42]. The decreased EFA content of VLDL surface- and core-lipid acyl chains, as well as the decreased PL/TG ratio, affects the physical structure of VLDL particles during EFAD, which could increase the affinity or binding sites for apoC-II or apoA-V. In addition, it could be hypothesized that a more saturated surface layer in EFAD VLDL can accommodate slightly better the appearance of TG from the core of the lipoprotein at the interface, where TG serves as substrate for lipases. Hamilton and Small[43-45] demonstrated that lipoprotein TG is not completely segregated into the core oil phase, but is also present in small proportions (±3%) intercalated in the PL surface layer. Although the exact mechanism by which LPL gains access to VLDL-TG is not known, the surface TG, with carbonyl groups arranged at the aqueous interface, provides the main pool for interaction with lipolytic enzymes. The decreased PUFA content of lipoprotein-TG and -PL in EFAD mice may enhance incorporation of TG in the PL monolayer at the aqueous interface, thus increasing accessibility to lipases. During lipoprotein TG hydrolysis, the transfer of excess surface PL to HDL is mediated PLTP. Rao *et al*.[46] reported that small VLDL have less affinity for PLTP than large. The EFAD VLDL size and particle surface packing may thus affect the binding affinity for PLTP and thereby its efficiency as a PL carrier, and VLDL metabolism.
Interestingly, both apoA-V and apoC-II mRNA levels were significantly increased during EFA deficiency in mice, which may be compatible with enhanced VLDL catabolism. The increased VLDL particle size could also account for increased clearance from the plasma. In chylomicron studies, Quarfordt and Goodman[47] and Chajek-Shaul *et al*.[48] demonstrated that large particles are cleared more rapidly from plasma than small particles. Production of VLDL with a larger size implies that fewer particles are being secreted to account for the similar TG production rates. Martins *et al*.[49] postulated that particle number strongly affects lipoprotein clearance rate, with small numbers of particles being cleared more rapidly than large numbers, possibly due to a receptor-saturable process involving the availability of apoE.

The relation between murine EFA deficiency and VLDL particle size may be related to the availability of PL for lipoprotein assembly. Under conditions of reduced PL availability for VLDL assembly, e.g., during choline deficiency in rats, VLDL particles with an increased core-to-surface ratio are produced[50; 51]. We speculate that a similar situation may apply in murine EFA deficiency. Previously, we demonstrated that EFA deficiency in mice profoundly increases the amount of PL secreted into bile[17]. Biliary PL are predominantly composed of phosphatidylcholine (PC), similar to PL used for

VLDL assembly. The increased biliary secretion of PC into the intestine may limit the availability of PC for hepatic lipoprotein assembly, thus leading to production of VLDL particles of increased size.
In addition to decreasing plasma TG levels, EFA deficiency in mice increased hepatic TG content, both *in vivo* and *in vitro*. Interestingly, genes involved in fatty acid oxidation (Cpt1a, Cpt1b, Acc2) were upregulated in livers of EFAD mice, compatible with activation of transcription factor PPARa[52]. It is well-known that EFA and LCPUFA are natural ligands for PPARa and it was unexpected that PPARa-regulated genes were upregulated during EFA deficiency. Possibly, increased levels of non-essential LCPUFA (n-9 and n-7 family) in EFAD livers can also activate PPARa. Increased *de novo* synthesis of n-9, n-7 and saturated fatty acids from acetyl-CoA may engender increased rates of hepatic TG synthesis during EFA deficiency. Present data suggest that unimpaired VLDL-TG secretion rates, in combination with increased hepatic TG synthesis, causes hepatic TG accumulation in EFAD mice.

For speculations on the potential clinical implications of our current observations, we attempted to relate our findings on the effects of EFA deficiency on lipoprotein metabolism in mice to reports on cystic fibrosis (CF) patients, in whom EFA deficiency is frequently observed. Unfortunately, no human CF data are available in which information is simultaneously provided on presence of steatosis, plasma TG concentrations, and VLDL particle size and clearance. Yet, indirect indications offer some support for extrapolation of our present findings to the human condition, although caution is warranted. Levy and Lepage[53] reported on the combination of hypertriglyceridemia and diminished plasma PL concentrations in EFAD CF patients compared to non-CF siblings. Interestingly, plasma VLDL of EFAD CF patients was relatively TG-enriched compared to non-CF sibs, suggestive of increased particle size of these lipoproteins in CF. However, a similar finding was reported for non-EFAD CF patients, and no data on steatosis were provided. In 1999, Lindblad *et al*.[54] reported that 35% of CF patients had steatosis, and that the level of the EFA linoleic acid in plasma PL negatively correlated with the degree of steatosis.

We conclude that the steatosis and hypotriglyceridemia during EFA deficiency in mice is a combined result of unimpaired hepatic TG secretion, increased hepatic synthesis of non-essential fatty acids and secretion of large VLDL particles which may be subject to rapid clearance rates.

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