Effects of bile salts on hepatic lipoprotein production

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CHAPTER 5: Differential effects of farnesoid x receptor–deficiency on hepatic and intestinal TG–rich lipoprotein formation in mice

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ABSTRACT

The bile salt-activated Farnesoid X receptor (FXR) may provide a link between bile salt and triglyceride (TG) metabolism: disruption of FXR leads to hypertriglyceridemia in mice. To provide a mechanistic basis for the latter finding we have investigated the role of FXR in the formation of TG-rich lipoproteins by liver and intestine. FXR-deficient (Fxr<sup>-/-</sup>) mice showed a 1.8-fold increase of basal plasma TG concentration compared to control mice, due to an increased TG content in the Very Low Density Lipoprotein (VLDL)-sized fractions. However, similar VLDL-TG production rates were measured in control and Fxr<sup>-/-</sup> mice. The diameter of nascent VLDL particles (d<1.006 g/ml) of Fxr<sup>-/-</sup> mice was increased compared to controls (+49%, p < 0.05) while their apoB content was reduced (apoB100: -25%, apoB48: -60%), indicating that larger, TG-enriched VLDL particles were produced by Fxr<sup>-/-</sup> mice. Hepatic mRNA levels of key genes involved in lipogenesis (Acc1, Fas, Dgat1) and VLDL assembly (Apob, Apobec, Mttp, Apoe, Pltp) were similar in both groups. In contrast, hepatic mRNA levels of genes apoc2 and lpl, involved in lipolysis of TG-rich lipoproteins, were down-regulated by 51% and 74%, respectively in Fxr<sup>-/-</sup> mice. However, total plasma lipase activity was not different between the groups. Surprisingly, production of TG-rich chylomicrons by the intestine was impaired in Fxr<sup>-/-</sup> mice (-50%, p<0.05) and chylomicron particle size was reduced (-50%, p<0.05). The data presented in this study show that disruption of FXR differentially affects formation of TG-rich lipoproteins in liver and intestine. Hypertriglyceridemia in Fxr<sup>-/-</sup> mice probably results from impaired processing of differently structured TG-rich lipoproteins.
INTRODUCTION

Conversion of cholesterol into bile salts is the major metabolic pathway for elimination of cholesterol from the body. Bile salts are secreted via the bile into the small intestine, where they act to facilitate uptake of dietary fat by the enterocytes. The conversion of cholesterol into bile salts is regulated through feed-forward activation by metabolites of cholesterol, the oxysterols, and feedback repression by bile salts [1]. Oxysterols bind and activate the nuclear liver X receptor (LXR, Nr1h2/Nr1h3) [2], which induces expression of the rate-limiting enzyme in hepatic biosynthesis of bile salts, cholesterol-7α hydroxylase (Cyp7a1) [3,4]. Bile salts repress their own synthesis by inhibition of Cyp7a1 gene transcription via activation of the Farnesoid X receptor [5]. Parks et al [6] and Makishima et al [5] have demonstrated that bile salts are natural ligands for the Farnesoid X-activated receptor (FXR, Nr1H4) [7,8]. FXR is a member of the nuclear hormone receptor superfamily and is primarily expressed in liver, kidney and intestine [9]. Upon activation, FXR heterodimerizes with the Retinoid X receptor (RXR) [10] and binds to a FXR response element (FXRE, also known as bile acid response element or BARE), consisting of two nucleotide inverted repeats separated by one nucleotide (IR-1), to induce gene transcription [7,11,12]. Bile salt-activated FXR has been shown to down-regulate the expression of Cyp7A1 [5,6,13] via activation of a second nuclear receptor, Shp (short heterodimer partner, Nr0b2). Induced expression of Shp inhibits the actions of Lrh-1 (Nr5a2), a nuclear receptor required for basal Cyp7A1 expression [9,14,15].

Bile salt-activated FXR has been shown to regulate not only bile salt biosynthesis, but also the expression of proteins involved in bile salt transport and secretion. These include Ntcp (Slc10a1) [16], the hepatic basolateral sodium taurocholate co-transporting peptide, the hepatic canalicular transport protein bile salt export pump (Bsep or Abcb4) [17,18] and the intestinal ileal bile acid binding protein (I-Babp) [6,19,20]. These findings have been interpreted to indicate a central role for FXR in the maintenance of bile salt homeostasis. Recently, FXR has also been implicated in regulation of genes involved in lipoprotein metabolism. Expression of genes encoding apolipoprotein C-II, apolipoprotein E and the phospholipid transfer protein (PLTP) [21-23] were shown to be increased upon FXR activation and the apolipoprotein AI gene (Apoa1) was reported to be down-regulated upon FXR activation [24].

Generation of mice lacking FXR (FXR null mice) reported by Sinal et al. [16] provided further evidence for a link between bile salts and lipoprotein metabolism. On a
control diet, FXR null mice showed significantly elevated plasma levels of triglycerides, total cholesterol and phospholipids, compared to control mice. In addition, FPLC analysis of plasma from FXR null mice showed an increased apolipoprotein B100 and apolipoprotein B48 content in the Very Low Density Lipoprotein (VLDL) fraction. Since each VLDL particle contains a single apoB molecule and, therefore, secretion of apoB molecules corresponds with the number of VLDL particles produced [25], these data indicated that the increased triglyceride concentration in plasma could very well be attributed to increased production of VLDL. However, the cause of hypertriglyceridemia associated with FXR deficiency has remained elusive so far.

In this study, FXR null mice generated by homologous recombination (Fxr<sup>-/-</sup>) were used to examine the effects of FXR deficiency on hepatic and intestinal secretion of TG-rich lipoproteins. The results presented in this study demonstrate that disruption of FXR differentially affected composition and size of both liver-derived nascent VLDL and intestinal chylomicron particles. Alterations in composition and size of hepatic and intestinal TG-rich lipoproteins may affect processing of the particles, resulting in elevated plasma TG concentrations. Our data provide further evidence for a role for FXR in control of hepatic and intestinal lipoprotein metabolism.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male wild type (Fxrf<sup>+/+</sup>), Fxrf<sup>+/−</sup> and Fxrf<sup>−/−</sup> mice on a mixed C57BL/6J/ SV129 background (Tularik Inc, San Francisco, USA) were housed in a light- and temperature controlled facility. The animals were fed a commercially available lab chow (RMH-B, Hope Farms BV, Woerden, The Netherlands, containing 51.3% carbohydrate, 6.2% fat and approximately 0.01% cholesterol (w/w)) and tap water, *ad libitum*. The mice received humane care and experimental procedures were in accordance with local guidelines for use of experimental animals.

**Plasma, liver and intestinal tissue sampling**

In the morning (after a 9-h fast), a large blood sample was collected by cardiac puncture and centrifuged. Plasma thus obtained was stored at -20°C until analyzed. The liver was
quickly removed, weighed and frozen in separate portions for RNA isolation and lipid analysis. Parts of the liver were frozen in isopentane and used for microscopic examination. Small intestine was removed and flushed with PBS containing 5mM taurocholate. Proximal, middle and distal parts (3 portions each) were frozen in separate portions for RNA isolation.

**In vivo VLDL–triglyceride production rate**

After a fasting period of 9 hours, wild type, \( Fxr^{(+/-)} \) and \( Fxr^{(-/-)} \) mice (n=6 per group) received a retro-orbital injection of 12.5 mg Triton WR-1339 in 100 µl PBS. Tail blood samples were taken under light isoflurane anesthesia before and 1, 2 and 3 hours after the injection of Triton WR-1339. At 4 hours after Triton WR-1339 injection a large blood sample was collected by cardiac puncture. The large blood sample was used for isolation of VLDL. The collected blood samples were used for triglyceride measurements. VLDL triglyceride production rate was calculated from the slope of the triglyceride concentration vs. time curve. The slopes thus obtained, reflecting individual TG production rates, were normalized to body weight and used to determine mean TG production rates per experimental group.

**Intestinal production of TG–rich lipoproteins**

After an overnight fast, male wild type and \( Fxr^{(-/-)} \) mice (n=6 per group) were intravenously injected with Triton WR-1339 (12.5 mg/100 µl PBS). Immediately thereafter, mice were intragastrically administered a lipid bolus containing 200 µl olive oil, in which 10 µCi \(^{3}\)H-triolein and 2 µCi \(^{14}\)C-oleic acid was dispersed. Blood samples (75 µl) were collected before (t=0h) and at one, two and three hours after label administration via retro-orbital puncture. After 4 h, a large blood sample was drawn via cardiac puncture. The content of \(^{3}\)H and \(^{14}\)C in plasma (20 µl) was measured by liquid scintillation counting. The chylomicron fraction was immediately isolated from 300 µl plasma by ultracentrifugation (Optima TLX table top centrifuge, Beckman Instruments, Palo Alto, CA, USA) as described by Kendrick *et al.* [26].

\[ \text{FXR-deficiency in mice affects hepatic and intestinal lipoprotein formation} \]
Hepatic RNA isolation and measurement of mRNA levels by Real Time–PCR (Taqman)

Total RNA was isolated from ~30 mg of liver tissue with the Trizol method (GIBCO, Paisley, United Kingdom). RNA was converted to single stranded cDNA by a reverse transcription procedure with AMV-RT (Promega, Madison, WI, USA) according to manufacturer’s protocol using random primers. cDNA levels were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

For the PCR amplification studies, an amount of cDNA corresponding to 20 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to manufacturer’s protocol and optimized for amplification of the particular gene using the appropriate forward and reverse primers (GIBCO) and a template specific 3’-TAMRA, 5’-FAM labeled Double Dye Oligonucleotide probe (Eurogentec). In the same experiments, calibration curves were run on serial dilutions of pooled 8x concentrated cDNA solution as used in the assay, resulting in series containing 4x, 2x, 1x, 0.5x, 0.25x, 0.125x, 0.062x and 0.031x of the cDNA present in the assay incubation. The data obtained were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, USA). All quantified expression levels were within the linear part of the calibration curves and were calculated by using these curves. PCR results were normalized to \( \beta\)-Actin mRNA levels. The sequences of the primers and probes used in this study are listed in Table I.

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<th>Sequences (5’ to 3’)</th>
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Table 1. RT-PCR primers

Ref. [58] (rat)

Ref. [58]

AF374267
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After a 4-h fast, 6 male wild type and $Fxr^{-/-}$ mice were injected with heparin i.v. (0.1 U in PBS/g body weight). Blood samples were drawn (150 µl) via retro-orbital puncture before and 10 minutes after injection with heparin. Lipoprotein lipase and hepatic lipase activity in plasma were determined exactly as described earlier [27].

**Isolation and culture of mouse hepatocytes**

Isolation and culture of mouse hepatocytes was performed as described previously [28] using wild type (n=2) and $Fxr^{-/-}$ mice (n=2). Hepatocytes were plated in 35 mm 6-well plastic dishes (Costar Corp., Cambridge, MA, USA), precoated with collagen (Serva Feinbiochemica, Heidelberg, Germany) at a density of $1.0 \times 10^6$ cells/well in 2 ml of William’s E medium containing insulin, fetal calf serum (FCS), dexamethasone and penicillin/streptomycin. After overnight incubation the medium was removed and hepatocytes were washed with hormone-free and FCS-free (HF-SF) William’s E medium and subsequently incubated with HF-SF William’s E medium for 3 hours. After 3 hours the medium was removed and replaced by 1 ml HF-SF William’s E medium per well containing 25 µM $[^{3}H]$-glycerol (4.4 µCi/well), 0.75 mM oleate (C18:1) complexed with FFA-free (free fatty acid free) bovine serum albumin. After 24 hours incubation the medium was collected and centrifuged to remove debris. Hepatocytes were washed with
ice-cold Hank’s balanced salt solution (HBSS) and scraped into 2 ml of HBSS for lipid extraction.

**Bile salt uptake in hepatocytes**

Steady state intracellular bile salt concentrations were determined after a 24-h incubation with either 100 µM or 200 µM taurocholate (TC). At the start of the experiment, the William’s E complete medium was removed from the cells and replaced by 1 ml HF-SF William’s E medium containing 0.75 mM oleate (C18:1) complexed with bovine serum albumin, pen/strep, gentamicin, 0.5 µCi [³H]-TC (3.47 Ci/mmol) supplemented with unlabeled TC (final TC concentration 100 µM or 200 µM). Cells were incubated in 5% CO₂ at 37°C for 24 h. The assay was terminated by aspirating the medium and washing the cells three times with cold HBSS containing unlabeled TC (TC concentration 1 mM ). Cells were scraped into 1 ml HBSS and lysed by pushing the cell suspension several times through a syringe. An aliquot of the cell suspension was used for total cellular protein measurement (according to Lowry [29]) and another aliquot of the cell suspension (250 µl) was used for determination of radioactivity by liquid scintillation counting. Intracellular TC accumulation was expressed as nanomol TC per milligram cellular protein.

**mRNA expression levels in cultured primary mouse hepatocytes**

Total RNA of wild type and Fxr⁻/⁻ mouse hepatocytes was isolated by using the SV Total RNA Isolation System (Promega RNA, Madison, WI, USA) according to the manufacturer’s instructions. RNA was processed for real-time PCR as described in the section “Hepatic RNA isolation and measurement of mRNA levels by Real Time-PCR (Taqman)”.

**Hepatic lipid and protein analyses**

Livers were homogenized and hepatic concentrations of triglycerides, total cholesterol (Roche Diagnostics), phospholipids and free cholesterol (Wako Chemicals, Neuss, Germany) were measured using commercial kits and after lipid extraction according to Bligh and Dyer [30] and redissolving the lipids in 2% Triton X-100 in water. Protein concentrations in livers were determined according to Lowry [29] using bovine serum albumin as standard (Pierce).
Histology
Liver histology was examined on frozen liver sections after Oil-Red-O (ORO) staining for neutral lipids by standard procedures.

Plasma lipid analyses
Plasma triglycerides, phospholipids, HDL cholesterol, free cholesterol and total cholesterol were determined using commercially available kits (Roche Diagnostics and Wako Chemicals). Lipoproteins of wild type, \( Fxr^{+/+} \) and \( Fxr^{-/-} \) mice were separated using FPLC (fast protein liquid chromatography) on a Pharmacia Superose 6B 10/30 column. The contents of ApoB100, ApoB48 and ApoA-I in the fractions were visualized by Western blotting. Proteins in the fractions were separated by SDS-PAGE using 4-15% gradient gels (Ready Gels, BioRad, Hercules CA, USA) and transferred to nitrocellulose membranes (Hyperbound, Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The membranes were incubated with sheep-anti-human apoB (Roche Diagnostics) or rabbit-anti-human apoA-I (Calbiochem, San Diego, CA, USA), followed by incubation with donkey-anti-sheep IgG HRP or donkey-anti-rabbit IgG HRP and visualized by ECL detection (all three Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions.

VLDL isolation and analyses
VLDL (d < 1.006 g/ml) was isolated by density gradient ultracentrifugation. Hereeto, 300 µl plasma was adjusted to 800 µl with a NaCl/KBr solution of density 1.006 g/ml, containing 1 mM EDTA and NaN\(_3\), and centrifuged (627.000 g, 100 minutes) in a Optima TM LX table top ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA). VLDL was isolated by tube slicing and the volume was recorded by weight. ApoB100 and ApoB48 were determined by Western blotting, using antibodies against human ApoB raised in sheep. Triglyceride and cholesterol content were determined as described for plasma. Phospholipids were determined using a commercial available kit (Wako Chemicals).

Lipoprotein size determination
VLDL or chylomicron size and volume distribution profiles were analyzed by dynamic scattering using a Nicomp model 370 submicron particle analyzer (Nicomp Particle Sizing
FXR-deficiency in mice affects hepatic and intestinal lipoprotein formation

Systems, Santa Barbara, CA, USA). Particle diameters were calculated from the volume distribution patterns provided by the analyzer.

Statistics

All values represent mean ± standard deviation for the number of animals, wells or experiments indicated. Statistical analysis of two groups was assessed by Mann-Whitney U test. Level of significance was set at p<0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, USA).

RESULTS

Plasma and hepatic lipid parameters in FXR-deficient mice

As shown in Figure 1, disruption of Fxr resulted in a significant increase of basal plasma lipid concentrations in chow-fed mice.

Plasma TG concentrations were 1.8-fold increased and total cholesterol concentrations were 1.6-fold increased in Fxr<sup>−/−</sup> mice, compared to wild type mice. Sinal et al. and Lambert et al. [16, 37] reported similar changes in plasma lipids in their strain of Fxr<sup>−/−</sup> mice, generated by a Cre-Lox procedure. Fxr<sup>+/−</sup> mice showed plasma lipid levels that were intermediate between those of controls and Fxr<sup>−/−</sup> mice.
FPLC analysis of plasma lipoprotein profiles confirmed that the increase in plasma TG was mainly caused by an increased TG content in Very Low Density Lipoproteins-sized fraction (VLDL) (Figure 2). Western blot analysis of apoB’s in the fractions 15-20 showed an increased content of apoB100 and apoB48.
**Figure 3** shows the cholesterol distribution: the elevated plasma cholesterol concentrations in $Fxr^{(-/-)}$ mice were mainly due to an increased concentration in the HDL-sized fractions (fractions 30-32). Western blot analysis of apoA-I contents in the fractions 27-33 revealed that the HDL fraction of plasma from $Fxr^{(-/-)}$ mice contained more of these apolipoproteins. The hepatic lipid contents are summarized in **Table 2** and were similar in the three groups. This is in contrast to data reported by Sinal *et al* [16], showing a 2-fold increased hepatic TG content in $Fxr^{(-/-)}$ mice.

**Table 2. Liver lipid parameters**

<table>
<thead>
<tr>
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<th>WT</th>
<th>$Fxr^{(+/-)}$</th>
<th>$Fxr^{(-/-)}$</th>
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<tbody>
<tr>
<td>Triglycerides (nmol/mg liver)</td>
<td>7.22 ± 3.63</td>
<td>9.51 ± 4.15</td>
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<tr>
<td>Phospholipids (nmol/mg liver)</td>
<td>32.8 ± 1.7</td>
<td>32.8 ± 1.4</td>
<td>34.3 ± 0.6</td>
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<td>Free cholesterol (nmol/mgliver)</td>
<td>4.68 ± 0.15</td>
<td>4.09 ± 0.18</td>
<td>4.44 ± 0.27</td>
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<tr>
<td>Cholesterylester (nmol/mgliver)</td>
<td>0.40 ± 0.21</td>
<td>0.37 ± 0.15</td>
<td>0.33 ± 0.23</td>
</tr>
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</table>

Livers of non-fasted male wild type, $Fxr^{(+/-)}$ and $Fxr^{(-/-)}$ mice were homogenized and concentrations of lipids were measured as described under “Experimental Procedures”. Each value represents the mean ± S.D.; n=6 per group.

**Disruption of Fxr results in production of large VLDL particles**

To determine whether elevated plasma TG levels in $Fxr^{(-/-)}$ mice were due to increased hepatic production of VLDL particles, fasted wild type mice, $Fxr^{(+/-)}$ mice and $Fxr^{(-/-)}$ mice were injected with Triton WR-1339 to calculate VLDL-TG production rates. Similar VLDL-TG production rates were calculated from the plasma TG vs. time curves for the three groups (**Figure 4**).
The concentrations of TG, phospholipids, free cholesterol and cholesteryl esters in nascent VLDL particles isolated from plasma obtained at 4 h after Triton WR-1339 injection from wild type and Fxr$^{(-/-)}$ mice are summarized in Table 3.

### Table 3. VLDL lipid composition

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<tr>
<td>Triglycerides (mM)</td>
<td>11.5 ± 6.9</td>
<td>17.8 ± 2.8*</td>
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<tr>
<td>Phospholipids (mM)</td>
<td>2.0 ± 0.8</td>
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<tr>
<td>Free cholesterol (mM)</td>
<td>2.0 ± 0.5</td>
<td>2.5 ± 0.4</td>
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<tr>
<td>Cholesteryl ester (mM)</td>
<td>0.2 ± 0.3</td>
<td>0.04 ± 0.05*</td>
</tr>
<tr>
<td>Total lipids (mM)</td>
<td>15.9 ± 7.0</td>
<td>23.7 ± 2.9*</td>
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<tr>
<td>Core: Surface lipids</td>
<td>2.6 ± 1.2</td>
<td>3.7 ± 1.7</td>
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<tr>
<td>Diameter (nm)</td>
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</tr>
<tr>
<td>Volume ($10^4$ nm$^3$)</td>
<td>14.2 ± 2.0</td>
<td>26.4 ± 5.7*</td>
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</table>

Male wild type and Fxr$^{(-/-)}$ mice were injected with Triton WR-1339. At 4 hours after injection, VLDL was isolated from plasma by density gradient ultracentrifugation. Lipid concentrations and particle diameter were measured. Particle volume was calculated using the equation:

$$\text{Volume} = \frac{4}{3} \pi \left(\frac{1}{2}\text{diameter}\right)^3$$

Each value represents the mean ± S.D. n=6 (wild type); n=6 (Fxr$^{(-/-)}$); *, p < 0.05.
Concentrations of TG, PL and free cholesterol in nascent VLDL particles of $Fxr^{(-/-)}$ mice were increased compared to VLDL from wild type mice. In addition, the mol% of surface lipids (PL and free cholesterol) was reduced relative to that of core lipids (TG and cholesteryl esters), leading to a mean increase of the core:surface ratio by 41.9%. Accordingly, direct measurement of VLDL particle size revealed that the diameter of nascent VLDL particles isolated from $Fxr^{(-/-)}$ mice was significantly larger than those obtained from control mice.

The increase in calculated particle volume (factor 1.86) was of similar magnitude as the increase in VLDL-TG content (factor 1.55), indicating that larger VLDL particles containing more TG were produced. Since VLDL-TG production rates were similar for control and $Fxr^{(-/-)}$ mice (Figure 4), these findings indicate a decrease in number of VLDL particles produced. This was confirmed by Western blot analysis of the apoB content of the nascent VLDL fractions from control and $Fxr^{(-/-)}$ mice (Figure 5). A 43% reduction in VLDL-apoB100 and a 66% reduction in VLDL-apoB48 was observed in $Fxr^{(-/-)}$ mice compared to control (p<0.05).

Figure 5. Apolipoprotein B contents in nascent VLDL particles of wild type, $Fxr^{(+/-)}$ mice and $Fxr^{(-/-)}$ mice. Plasma was obtained by cardiac puncture at 4 hours after injection of Triton WR1339. VLDL was isolated by density ultracentrifugation and was applied to SDS-polyacrylamide gel electrophoresis followed by Western blotting. Each lane represents VLDL-apoB from an individual mouse. Left panel, wild type control mice. Right panel, $Fxr^{(-/-)}$ mice. The intensities of ApoB100 and apoB48 from VLDL of $Fxr^{(+/-)}$ mice were compared with the intensity of the VLDL-apoB bands from wild type mice.

Although the TG concentration and apoB concentration in VLDL fractions of $Fxr^{(-/-)}$ mice was increased compared to wild type mice, the hepatic basal mRNA expression levels of key genes involved in lipogenesis ($Acc1$, $Fas$, $Dgat1$), cholesterol biosynthesis ($Hmgr$, $Hmgs$) and VLDL assembly ($Apob$, $Apobec$, $Mttp$, $Apoe$, $Plt$) were similar in the three groups (Figure 6a). In spite of the strongly reduced apoB48 content of nascent VLDL, expression of the apoB-editing enzyme ($Apobec$) was not affected. Although $Apoe$ mRNA expression was statistically significantly decreased in $Fxr^{(-/-)}$ mice, the decrease in $Apoe$ expression was small (-14%, compared to control, p<0.05). The hepatic mRNA levels of
the transcription factors Srebp-1c and Srebp-2, involved in control of de novo lipogenesis and cholesterol synthesis, respectively, were both down regulated in Fxr<sup>-/-</sup> mice (Figure 6b), but this was not reflected in expression of their target genes. In contrast, the genes encoding apoC-II and lipoprotein lipase (LPL), involved in lipolysis of VLDL, were severely down-regulated (Figure 6c, Apoc2: -51%, Lpl: -74% in Fxr<sup>-/-</sup> mice compared to control, p<0.05). Expression of apoC-I, apoC-III and hepatic lipase, on the other hand, remained unaffected.

![Figure 6. Changes in relative hepatic gene expression in wild type, Fxr<sup>+/−</sup> and Fxr<sup>-/-</sup> mice, determined by Real Time PCR.](image)

**Plasma LPL activity in control and Fxr<sup>-/-</sup> mice**

The activity of lipoprotein lipase (LPL) and hepatic lipase (HL) in plasma was determined in control and Fxr<sup>-/-</sup> mice, since decreased LPL activity in plasma might explain the higher VLDL-TG concentrations observed in Fxr<sup>-/-</sup> mice under basal conditions. However, Figure 7 shows that basal LPL and HL activity was similar in plasma of control and Fxr<sup>-/-</sup> mice, indicating that disruption of FXR does not result in decreased activity of the lipolytic enzymes LPL and HL.
Previously, we showed that bile salts inhibit VLDL-TG production in primary human, rat and mouse hepatocytes in vitro [31-33]. To determine whether this bile salt-mediated effect was FXR-dependent and could contribute to the effects observed in vivo, primary hepatocytes isolated from control and Fxr(-/-) mice labelled with 3H-glycerol were incubated without or with 100 µM or 200 µM taurocholate (TC).

Because FXR has been reported to regulate expression of hepatic bile salt transporters [17,34-36], mRNA expression of these transporters in cultured primary hepatocytes of control and Fxr(-/-) cells was measured and steady state intracellular taurocholate concentrations were determined after a 24-h incubation with 100 µM or 200 µM TC.
Figure 8a shows that the mRNA expression levels of basolateral bile salt transporters Ntcp and Oatp were upregulated 2.2-fold and 1.7-fold, respectively. In contrast, the bile salt export pump Bsep was down regulated by 56% in \( \text{Fxr}^{-/-} \) hepatocytes. After a 24-h incubation with 100 or 200 µM TC, intracellular steady state TC concentrations in \( \text{Fxr}^{-/-} \) hepatocytes were 1.93-fold or 1.52-fold higher than observed in control hepatocytes under the same experimental conditions (Figure 8b, \( P<0.05 \)).

Under control conditions, hepatocytes lacking FXR secreted 29% less newly synthesized \(^3\text{H}-\text{TG} \) than control hepatocytes did (Figure 9c, \( P<0.05 \)), while intracellular TG synthesis and TG mass were similar in both cell types (Figure 9a and 9b). Incubation with 100 µM TC resulted in a significant decrease in \(^3\text{H}-\text{TG} \) secretion in both control cells and \( \text{Fxr}^{-/-} \) cells (-60% and -85%, respectively, \( P<0.05 \)). Incubation with 200 µM TC further decreased \(^3\text{H}-\text{TG} \) secretion by control and \( \text{Fxr}^{-/-} \) cells (-72% and -90%, respectively, \( p<0.05 \) compared to control) (Figure 9c). In both cell types, TC incubation resulted in a slightly increased TG synthesis, although TG mass was not affected by TC.
incubation (Figures 9a and 9b). Because a linear relationship exists between intracellular BS concentration and inhibition of VLDL-TG secretion [31,32], the higher intracellular TC concentrations might explain the more pronounced inhibitory effect of TC on VLDL-TG secretion in \( Fxr^{-/-} \) hepatocytes (Figure 9c), which was apparently FXR-independent.

![Figure 9. Triglyceride synthesis and VLDL-triglyceride secretion in cultured wild type and \( Fxr^{-/-} \) hepatocytes after 24 h TC incubation.](image)

To determine whether absence of FXR had similar effects on expression of genes involved in lipoprotein metabolism in vitro, mRNA levels of genes in VLDL assembly, lipolysis and clearance were determined. Figure 10 shows that the mRNA expression levels of apoB, apoE and MTP were significantly increased in \( Fxr^{-/-} \) hepatocytes (+18%, +29% and +96%, respectively, compared to control, \( P<0.05 \)), in contrast to the data obtained in vivo in the liver of \( Fxr^{-/-} \) mice (Figure 6b). Apoc2 expression was also increased 1.6-fold in hepatocytes of \( Fxr^{-/-} \) mice, but down-regulation of \( Lpl \) expression was also observed in cultured primary hepatocytes of \( Fxr^{-/-} \) mice (Figure 10).
Recently, Lambert et al. [37] reported a significantly increased production of TG-rich lipoproteins upon Triton administration in non-fasted Fxr null mice. We did not find an increased hepatic production of VLDL after a 9-hour fasting period. The increased production of TG-rich lipoproteins reported by Lambert et al. might thus be caused by an increased production of chylomicrons by the intestine. Therefore, we measured total TG production rate (endogenous VLDL-TG + chylomicron TG production) after intravenous administration of Triton WR-1339 and an intragastric bolus of olive oil containing $^3$H-triolein and $^{14}$C-oleic acid in wild type and $Fxr^{-/-}$ mice (Figure 11). The endogenous VLDL TG production rate (determined as in Figure 4) was subtracted from total TG production rate to calculate chylomicron TG production rate in wild type and $Fxr^{-/-}$ mice. As shown in the insert of Figure 11, the calculated chylomicron production rate appeared to be impaired in $Fxr^{-/-}$ mice compared to control mice (86 ± 66 vs. 211 ± 89 µmol TG/h/kg, p<0.05).

**Disruption of Fxr results in altered formation of chylomicrons in the intestine**

Figure 10. Changes in relative hepatic gene expression, determined by Real Time PCR in cultured wild type and $Fxr^{-/-}$ hepatocytes. RNA was isolated from cultured wild type and $Fxr^{-/-}$ hepatocytes after 24 h incubation in control medium and converted to cDNA. Levels of cDNA were measured by Real Time PCR. Results were normalized to $\beta$-Actin mRNA levels. Open bars: wild type hepatocytes. Closed bars: $Fxr^{-/-}$ hepatocytes.
The concentrations of triglycerides, phospholipids, free cholesterol and cholesteryl ester in nascent chylomicron particles isolated from plasma 4 h after Triton WR-1339 injection are summarized in Table 4. Although the concentrations of triglycerides, phospholipids and free cholesterol in nascent chylomicrons from Fxr\(^{(-/-)}\) mice were decreased compared to controls, none of these differences reached statistical significance. However, direct measurement of chylomicron particle size revealed that 4h after administration of the lipid bolus, the mean diameter of the chylomicron particles of Fxr\(^{(-/-)}\) mice was decreased by 38% compared to controls (160 ± 42 vs. 257 ± 15 nm, p<0.005, Table 4).

![Image of calculated chylomicron TG accumulation in plasma from wild type and Fxr\(^{(-/-)}\) mice after injection of Triton WR-1339 and a subsequent intragastric bolus of olive oil.](image_url)

**Figure 11.** Calculated chylomicron TG accumulation in plasma from wild type and Fxr\(^{(-/-)}\) mice after injection of Triton WR-1339 and a subsequent intragastric bolus of olive oil. Male wild type (n=6) and Fxr\(^{(-/-)}\) mice (n=6) received at t=0 injection of Triton WR-1339 followed by an intragastric bolus of olive oil. Chylomicron TG accumulation in time was calculated by subtracting mean endogenous VLDL-TG accumulation (figure 4) from the mean total TG accumulation per group at each time point. (○) wild type mice. (●) Fxr\(^{(-/-)}\) mice. The inset shows the calculated total TG and chylomicron TG production rate in µmol TG/kg/hr, from the mean plasma TG vs. time curve. (○) Wild type controls. (●)Fxr\(^{(-/-)}\) mice. Values represent the mean ± S.D.

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Male wild type and Fxr\(^{(-/-)}\) mice were injected with Triton WR-1339 and a subsequent intra-gastric bolus of olive oil. At 4 hours after injection, nascent chylomicrons were isolated from plasma by density gradient ultracentrifugation. Particle volume was calculated using the equation Volume = 4/3 x π x (½diameter\(^2\)). Each value represents the mean ± S.D.; n=6 (wild type); n=6 (Fxr\(^{(-/-)}\)); *, p < 0.05.
The chylomicron particle volume was decreased by 72% (P<0.001) in $Fxr^{-/-}$ mice compared to controls. Surprisingly, the chylomicron apoB48 protein content was 2.2-fold increased in $Fxr^{-/-}$ mice (Figure 12), indicating that more particles were produced by the intestinal cells, that should contain a smaller amount of TG.

Lipolysis of dietary TG in the intestine and subsequent uptake of fatty acids by the enterocytes may significantly affect intestinal chylomicron formation and secretion. Therefore, intestinal fat absorption was measured in controls and $Fxr^{-/-}$ mice by applying a intragastric bolus of $^3$H-triolein and $^{14}$C-oleic acid before intravenous administration of Triton WR-1339. Figures 13a and 13b show the time course of plasma appearance of radioactivity for $^3$H-triolein and $^{14}$C-oleic acid, respectively. $Fxr^{-/-}$ mice showed a clearly decreased accumulation of both radioactive labels in plasma in the time frame studied (P<0.05). This confirms the results in Figure 11 that secretion of lipids packed into chylomicrons is impaired in $Fxr^{-/-}$ mice. The ratio between $[^3]$H and $[^{14}]$C in plasma did not show significant differences between control and $Fxr^{-/-}$ mice (Figure 13c), indicating that intestinal lipolysis of TG was unaffected in $Fxr^{-/-}$ mice.
FXR-deficiency in mice affects hepatic and intestinal lipoprotein formation

**DISCUSSION**

Excess cholesterol is to a large extent removed from the body via conversion into bile salts and their subsequent loss in feces. Control of bile salt metabolism is therefore of vital importance for maintaining cholesterol homeostasis. The farnesoid X receptor (FXR) has been shown to control expression of multiple genes involved in bile salt metabolism [5,6,13] and is therefore implicated to play a central role in control of bile salt and cholesterol metabolism in the body. Disruption of FXR in mice has been demonstrated to not only affect bile salt metabolism but also provoke significant changes in triglyceride metabolism [16]. The data presented in this study demonstrate that FXR deficiency in mice differentially affects the formation of TG-rich lipoproteins in liver and intestine. Basal plasma triglyceride (TG) concentrations were increased nearly 2-fold in Fxr-deficient mice, but hepatic VLDL-TG production rates were similar in controls and Fxr<sup>−/−</sup> mice. However, larger TG-enriched VLDL particles were produced by Fxr<sup>−/−</sup> mice. In contrast, intestinal chylomicron production was impaired in Fxr<sup>−/−</sup> mice and chylomicron particle size was reduced. Although LPL and HL activities in the circulation were not affected in Fxr<sup>−/−</sup> mice, the presence of differently structured TG-rich lipoproteins may hamper lipolytic processing of the particles leading to the hypertriglyceridemic profile observed in Fxr<sup>−/−</sup> mice.

The observed hyperlipidemia in Fxr<sup>−/−</sup> mice (Figure 1) was not accompanied by hepatic steatosis, as was the case in Fxr-null mice generated by Cre-lox technology.
reported by Sinal et al [16]. The different observations in hepatic fat content may be related to strain specificity or differences in diet composition.

The lipoprotein profile obtained by FPLC separation clearly showed that the elevated plasma TG concentration in Fxr<sup>(/-)</sup> mice was almost entirely in the VLDL-sized fractions (Figure 2). Yet, in vivo studies, similar hepatic TG production rates were measured in wild type, heterozygous and homozygous Fxr-deficient mice. This is in contrast to observations reported by Lambert et al [37] who reported a more than 2-fold increase in plasma TG accumulation at 3h after Triton administration in Fxr-null mice compared to wild type controls. However, it should be noted that plasma TG accumulation in Fxr<sup>(/-)</sup> mice in our study was similar to that in Fxr-null mice studied by Lambert et al (985 mg/dl vs. ~1300 mg/dl). In contrast, plasma TG accumulation in their control mice was 1.5-2 fold lower than reported by us and others for wild type C57Bl6 mice (600 mg/dl vs. 903-1327 mg/dl) [40-44]. Our values for wild type mice were slightly lower than reported in other studies, but this may be related to species differences. The Fxr<sup>(/-)</sup> mice have a mixed SV129/C57BL6 background in contrast to the studies mentioned in which wild type C57BL6 mice were used.

We also examined the composition and diameter of the nascent VLDL particles that were present in the blood 4 h after Triton-WR1339 injection. Although TG production rates were similar in all groups, Fxr<sup>(/-)</sup> mice were shown to produce larger particles that were enriched in TG. The increase in TG content (1.6 fold) and particle volume (1.8 fold) was balanced by a decreased production of VLDL particles, represented by a ~60% decrease in apoB protein content. Each VLDL particle contains a single apoB molecule and, therefore, secretion of apoB molecules corresponds with the number of VLDL particles produced [25]. These results demonstrate that FXR-deficiency leads to production of differently structured VLDL particles, which may reflect a disturbed intracellular VLDL formation. Although expression of hepatic genes involved in lipogenesis and VLDL assembly was not affected in Fxr<sup>(/-)</sup> mice (Figure 6b), control of VLDL assembly has often been demonstrated to occur predominantly at the post-translational level [45-48]. How FXR-deficiency interacts at this level remains to be established.

Secretion of newly synthesized VLDL-TG in vitro in Fxr-deficient hepatocytes was decreased by 29% compared to VLDL-TG production by control hepatocytes (Figure 9c), despite increased mRNA expression levels of the microsomal triglyceride transfer protein (MTP), a well-known stimulator of VLDL production. Incubation with the bile salt taurocholate (TC) inhibited VLDL-TG secretion both in Fxr-deficient and wild type
FXR-deficiency in mice affects hepatic and intestinal lipoprotein formation

hepatocytes, indicating that bile salt-mediated inhibition of VLDL-TG and apoB secretion demonstrated in our earlier studies [31,32,49] is not controlled by FXR. Upon TC incubation, Fxr-deficient hepatocytes accumulated more TC compared to controls (2-fold), which coincided with a 2-fold induced mRNA expression of the basolateral bile salt uptake transporters Ntcp (Slc10a1) and Oatp (Slc21a1) and a 50% reduced mRNA expression of the canalicular bile salt export pump Bsep (Abcb4). In vitro and in vivo experiments [50] showed that Ntcp is down-regulated and Bsep expression is upregulated via the bile salt-activated nuclear hormone receptors FXR and Shp [17,34-36]. This coordinate regulation of bile salt transporters appears to be absent in Fxr-deficient hepatocytes.

Our in vivo and in vitro data demonstrate that the hyperlipidemic plasma profile observed in Fxr-deficient mice was not caused by an increased VLDL-TG production by the liver. We hypothesized that an increased chylomicron production in Fxr(-/-) mice might contribute to the hypertriglyceridemic plasma profile. Therefore, we examined intestinal fat absorption and chylomicron production in wild type controls and Fxr(-/-) mice. In contrast to our hypothesis, chylomicron TG production was decreased by 60% in Fxr(-/-) mice. Measurement of particle size of the secreted nascent chylomicrons revealed that Fxr(-/-) mice produced smaller chylomicrons compared to wild type mice (diameter 160 nm vs. 257 nm, respectively, p<0.005) containing less TG. Surprisingly, the chylomicrons produced by Fxr(-/-) mice contained more apoB48, indicating that more particles were produced. It is not very likely that a disturbed activity of the microsomal triglyceride transfer protein (MTP) plays an important role in decreasing the chylomicron size, since impaired MTP lipid transfer activity was shown to decrease secretion of chylomicron particles similar to the inhibitory effect on VLDL secretion [51]. Intracellular accumulation of ceramide (hydrolytic product of the phospholipid sphyngomyelin) may play a role in control of intestinal chylomicron secretion. Recently, Kirby and co-workers demonstrated that CEL-deficient mice showed similar alterations in chylomicron composition and secretion as described for Fxr(-/-) mice in this study [52]. CEL (pancreatic bile salt-stimulated carboxyl ester lipase or cholesterol esterase) deficiency was associated with a decreased capacity to break down ceramide, which has been reported to reduce secretion of intestinal lipoproteins [53]. The underlying mechanism is still unknown.

Labelled free fatty acids and triacylglycerols appeared in plasma TG of both controls and Fxr(-/-) mice in exactly the same ratio as administered in the intragastric lipid bolus (Figure 13c), which indicates that intestinal lipolysis of TG and subsequent uptake of fatty acids was not impaired in Fxr(-/-) mice. Instead, intestinal uptake of fatty acids appeared to
be increased, since significantly less unabsorbed labelled fatty acids and TG could be recovered from the intestine of \( Fxr^{(-)} \) mice (data not shown). The 60% decrease in chylomicron production rate may be caused by impaired assembly or secretion of chylomicrons by the enterocytes, resulting in accumulation of intracellular lipids. However, microscopical examination of the intestines of \( Fxr^{(-)} \) mice under non-fasted conditions did not show accumulation of intracellular lipids (data not shown).

From the data presented in this study, hypertriglyceridemia in \( Fxr^{(-)} \) mice could not be explained by increased hepatic or intestinal TG production rates or by impaired activity of lipoprotein lipase and hepatic lipase in the circulation (Figure 7). However, disruption of FXR led to secretion of larger, TG-enriched VLDL particles by the liver and secretion of smaller chylomicrons by the intestinal cells. From studies in diabetic models, large TG-rich VLDL particles (VLDL1) were demonstrated to have a longer retention time in plasma than smaller VLDL particles (VLDL2) [54-56]. In addition, processing of VLDL1 particles was shown to result in formation of small dense LDL particles (pattern B LDL), which is associated with increased risk of development of cardiovascular disease [57]. Small postprandial VLDL-like chylomicrons are also associated with increased plasma TG levels; small chylomicrons were suggested to compete with VLDL particles for lipolysis, thereby increasing the retention of VLDL in the circulation [56]. Hepatic mRNA expression of ApoC-II, which normally functions as a stimulatory co-factor for LPL, was significantly reduced in \( Fxr^{(-)} \) mice compared to controls. This could result in reduced ApoCII presence on VLDL and chylomicron particles and therefore less susceptibility to LPL-mediated lipolysis. In addition, the differently structured TG-rich lipoproteins may bind to remnant receptors (LDL receptor, VLDL receptor, LRP) with less affinity, which would also increase their retention time in plasma.

We conclude that FXR deficiency differentially affects the composition and size of TG-rich lipoproteins in liver and intestine in mice. A disturbed processing of the differently structured TG-rich apoB-containing lipoproteins may be responsible for the hypertriglyceridemia in Fxr-deficient mice.

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