Interplay between glucose, fatty acid and bile salt metabolism in mouse models of fatty liver
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Herrema, H. J. (2010). Interplay between glucose, fatty acid and bile salt metabolism in mouse models of fatty liver. Groningen: s.n.
Bile salt sequestration induces hepatic de novo lipogenesis via FXR and LXRα-controlled metabolic pathways in mice

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Hepatology, 2009
ABSTRACT

Diabetes is characterized by high blood glucose levels and dyslipidemia. Bile salt sequestration has been found to improve both plasma glycaemic control and cholesterol profiles in diabetic patients. Yet, bile salt sequestration is also known to affect triglyceride metabolism, possibly via signaling pathways involving FXR and LXRα. We quantitatively assessed kinetic parameters of bile salt metabolism in lean C57Bl/6J and in obese, diabetic db/db mice upon bile salt sequestration using colesevelam HCl (2% wt/wt in diet) and related these to quantitative changes in hepatic lipid metabolism. As expected, bile salt sequestration reduced intestinal bile salt reabsorption. Importantly, bile salt pool size and biliary bile salt secretion remained unchanged upon sequestrant-treatment due to compensation by de novo bile salt synthesis in both models. Nevertheless, lean and db/db mice showed increased, mainly periportally confined, hepatic triglyceride contents, increased expression of lipogenic genes and increased fractional contributions of newly synthesized fatty acids. Lipogenic gene expression was not induced in sequestrant-treated Fxr−/− and Lxrα−/− mice compared to wild-type littermates, in line with reports indicating a regulatory role of FXR and LXRα in bile salt-mediated regulation of hepatic lipid metabolism. Conclusion: Bile salt sequestration by colesevelam induces the lipogenic pathway in an FXR- and LXRα-dependent manner without affecting the total pool size of bile salts in mice. We speculate that a shift from intestinal reabsorption to de novo synthesis as source of bile salts upon bile salt sequestration affects zonation of metabolic processes within the liver acinus.
INTRODUCTION

Diabetes is a multifactorial disease characterized by increased fasting blood glucose levels and dyslipidemia i.e., high plasma triglyceride and low-density-lipoprotein cholesterol (LDL-C) levels. Controlling blood glucose and cholesterol levels in diabetic patients is critical for delaying the progression of clinical complications e.g., neuropathy and cardiovascular diseases. An efficient way to reduce plasma cholesterol levels is to induce cholesterol secretion in bile, either as bile salt or as free cholesterol. Bile is secreted into the ileum to facilitate absorption of lipids and lipid soluble vitamins. About 95% of secreted bile salts are reabsorbed in the terminal ileum and transported back to the liver via the portal vein (enterohepatic circulation). Besides their function in absorption of dietary fats, bile salts are signaling molecules and exert an important role in regulation of lipid metabolism 1. Interestingly, bile salt metabolism is affected in diabetes which might contribute to the altered lipid profile observed in these patients 2. Knowledge of potential disturbances in bile salt metabolism in type 2 diabetic humans and animal models, however, is still very limited 3.

Increasing fecal bile salt loss by preventing their intestinal reabsorption (sequestration) increases bile salt synthesis and, hence, hepatic cholesterol turnover. As a consequence, LDL-C levels are reduced in hyperlipidemic subjects 4,5. Interestingly, bile salt sequestration also improves glucose levels in type 2 diabetic patients 6-8. Yet, use of bile salt sequestrants has been associated with elevated plasma triglyceride levels 9,10. Bile salt feeding on the other hand, has been shown to improve plasma lipid profiles in these patients 11,12. The regulation of the interrelationship between bile salt and lipid metabolism is still only partly understood. At a molecular level, a key regulatory role is assigned to the bile salt-activated nuclear receptor FXR (NR1H4) 13. Pharmacological activation of FXR has been shown to improve hypertriglyceridemia in mouse models of insulin resistance 14,15 whereas Fxr−/− mice have increased serum triglyceride levels 16. Moreover, administration of the natural FXR-ligand cholate improved plasma triglyceride levels of high fat diet-fed mice via SHP-dependent modulation of the lipogenic gene Srebp1c 17. In the same study, it was shown that the nuclear oxysterol receptor LXRα (NR1H3) is involved in the regulation of lipogenic gene expression upon bile salt feeding. At a physiological level, bile salt-activated signaling pathways are regulated by bile salt concentrations in the liver. We hypothesized that an altered flux of bile salts returning to the liver underlies, at least in part, the consequences on hepatic metabolism observed upon bile salt sequestration. We quantitatively assessed the kinetics of bile salt and hepatic fatty acid metabolism in lean C57Bl/6J mice and in obese and diabetic db/db mice treated with the bile salt sequestrant colesevelam HCl 18. Additionally, we studied the contribution of FXR and LXRα to sequestrant-induced changes in lipogenic gene expression.
Bile salt sequestration reduced intestinal reabsorption of bile salts by 30%. Nevertheless, the bile salt pool size remained unchanged in both models due to a compensatory increase in de novo synthesis of bile salts. Remarkably, sequestrant-treatment significantly increased hepatic triglyceride contents which primarily accumulated in periportal areas. Expression levels of lipogenic genes as well as the fractional contribution of de novo synthesized fatty acids were increased. This lipogenic response appeared to be FXR- and LXRα-dependent. We speculate that a shift from reabsorption to de novo synthesis as the source of biliary bile salts underlies the lipogenic phenotype observed upon bile salt sequestration.

MATERIALS AND METHODS

Animals
Male lean C57Bl6/J and obese, diabetic db/db mice on a C57Bl6/J background (B6. Cg-m +/- Lepr^db/J) were purchased from Charles River Laboratories (L’Arbresle, France and Brussels, Belgium, respectively). Fxr^−/− and Lxrα^−/− mice were generated as described. All animals were housed individually in a temperature- and light-controlled facility. Mice were fed commercially available laboratory chow (RMH-B; Arie Blok, Woerden, The Netherlands), when indicated supplemented with 2% (w/w) colesevelam HCl (Daiichi Sankyo, Inc., Parsippany, NJ, USA), for 2 weeks. Mice were used for experimental procedures at 12 weeks of age. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Experimental procedures
Postprandial blood glucose levels were measured at the start of the experiment and subsequently after 1 week and after 2 weeks of treatment. Additionally, body weight and food intake were determined at these time points. [1-13C]-acetate (2% w/v in drinking water) was provided for 24 h (7AM-7AM), starting at day 13 of the experiment. Blood spots were collected from the tail on filter paper (Schleicher and Schuell No2992, ’s Hertogenbosch, The Netherlands) before and after administration of the label. Blood spots were air-dried and stored at room temperature until analysis. After two weeks on the diets, mice were sacrificed by heart puncture under isoflurane anesthesia. Plasma was stored at -20°C until analyzed. The liver was removed, weighed and snap-frozen in liquid nitrogen. The intestine was excised, flushed with cold (4°C) PBS and subsequently snap-frozen in liquid nitrogen. Both liver and intestine were stored at -80°C until biochemical analysis and RNA isolation.

In a separate experiment, 400 μg [3H4]-cholate (in 0.5% NaHCO in PBS, pH = 7.4) was intravenously administered at day ten of the two-week periods. Subsequently, retro-orbital blood samples (75 μl) were obtained at 12, 24, 36, 48 and 60 h after injection of [3H4]-cholate in chow-fed lean and db/db mice. A pilot study in cole-
Bile salt sequestration and hepatic lipid metabolism

Sevelam-treated animals indicated that, as expected, turnover of [3H4]-cholate was markedly increased. Therefore, blood samples were obtained at 12, 18, 24, 30 and 36 h after administration of [3H4]-cholate in colesevelam-treated lean and db/db mice. Plasma was stored at -20°C until analyzed. Feces were collected over the 60 h experimental period and, after air-drying, kept at room temperature until analysis. After 60 h, mice were anesthetized by intraperitoneal injection of Hypnorm (1 ml/kg) and Diazepam (10 mg/kg) and subjected to gallbladder cannulation for 30 min. During bile collection, body temperature was stabilized using a humidified incubator. Bile was stored at -20°C until analyzed. Animals were sacrificed by cervical dislocation.

Analytical procedures

Blood glucose concentrations were measured using EuroFlashTM test strips (LifeScan Benelux, Beerse, Belgium). Hepatic lipids were extracted according to Bligh & Dyer. Plasma and liver triglyceride and cholesterol contents were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma free fatty acids were determined using a NEFA-C kit (Waco Chemicals, Neuss, Germany). Pooled plasma samples from each group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6 column using an Akta Purifier (GE Healthcare, Diegem, Belgium). Triglycerides in each fraction were determined. Total bile salts in bile and feces were determined by an enzymatic fluorimetric assay.

Liver morphology was assessed by Masson’s trichrome staining of parafin embedded material.

GC/MS analysis and mass isotopomer distribution analysis (MIDA)

Biliary and fecal bile salts were determined by gas chromatography as described. The isotope dilution technique as well as the preparation of plasma samples for analysis of bile salts by gas chromatography-mass spectrometry (GC-MS) were described in detail by Hulzebos et al. Fecal neutral sterols were analyzed as described. Labeling of acetyl-CoA pools with orally provided [1-13C]-acetate was described by Jung et al. Cholesterol was extracted from blood spots and prepared for GC-MS analysis as described. Lipids in liver homogenates were hydrolyzed in HCl/acetonitril. Fatty acids were extracted in hexane and converted to their pentafluorobenzyl (PFB) derivatives. The fatty acid-PFB isotopomer patterns (mass fragments C16:0 m/z 255-259, C18:0 m/z 283-287, C18:1 m/z 281-285) were analyzed using a Agilent 5975 series GC-MS (Agilent Technologies, Santa Clara, CA). GC-MS measurements of fatty acids and MIDA analyses were performed essentially as described.

Calculation of de novo synthesis and chain elongation of fatty acids

Incorporation of [1-13C]-acetate into C16:0 was assumed to be solely the result of de novo lipogenesis via the malonyl-CoA-Fas pathway. The M1 and M3 mass isoto-
pomers of C16:0 were used to calculate the acetyl CoA precursor pool and fractional synthesis of C16:0 as described \(^{28}\).

C18:0 is synthesized from chain elongation of pre-existing (chain elongation) and \textit{de novo} synthesized C16:0 (\textit{de novo} lipogenesis). The M1 mass isotopomer of C18:0 represents the sum of these two processes; the M3 mass isotopomer results only from chain elongation of \textit{de novo} synthesized C16:0. We assumed that the acetate precursor pool enrichment calculated for C16:0 equaled the precursor pool of acetate used to elongate C16:0. C18:0 generated from \textit{de novo} synthesized C16:0 was considered as nonamer of acetate. Therefore, MIDA algorithms using M3 of C18:0 and the acetate precursor pool enrichment calculated for C16:0 were used to calculate the fractional C18:0 synthesis. Subsequently, total M1 mass isotopomer of C18:0 was corrected for the contribution of C18:0 derived from \textit{de novo} synthesized C16:0. Using this methodology, we could calculate the contribution of chain elongation of pre-existing C16:0 to the total fractional C18:0 synthesis.

C18:1 is synthesized by desaturation of C18:0 by Scd1. Using similar methodology to that of C18:0, we could calculate the contribution of chain elongation of pre-existing C16:0 and \textit{de novo} synthesized C16:0 to total fractional synthesis of C18:1.

Throughout the manuscript, chain elongation is defined as synthesis of C18:0 or C18:1 from pre-existing C16:0; \textit{de novo} lipogenesis is defined as the synthesis of C18:0 or C18:1 from \textit{de novo} synthesized C16:0.

**Calculation of CDCA- and CA-derived bile salts**

The murine bile salt species we quantified in bile and feces using GC-MS include cholate, deoxycholate, chenodeoxycholate, \(\alpha\)-muricholate, \(\beta\)-muricholate, \(\omega\)-muricholate, hyodeoxycholate and ursodeoxycholate. We consider cholate and deoxycholate as CA-derived and the others as CDCA-derived bile salts. The contribution of deoxycholate to CA-derived bile salts is minor in bile (~5%), because the majority of DCA that returns to the liver is reconverted to CA in mice. Therefore, the pool size and synthesis rate of cholate as determined \textit{in vivo} using \(^{2}\text{H}_4\)-cholate were used as values for CA-derived bile salt pool size and synthesis. The composition and synthesis of CDCA-derived bile salts were calculated from the ratio of CDCA-derived/CA-derived bile salts and the CA-derived pool and synthesis as determined by calculations of \(^{2}\text{H}_4\)-cholate. The product of the CDCA/CA ratio in feces and CA synthesis represents the synthesis of CDCA-derived bile salts. The product of the CDCA/CA ratio in bile and CA pool size represents the contribution of CDCA-derived bile salts to the total bile salt pool size.

**RNA isolation**

Total RNA was isolated from liver and intestine using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers’ protocol. cDNA was produced as described.
Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to 18S (liver) and β-actin (intestine).

**Statistics**

All values are represented as mean ± standard deviation. Statistical analysis was assessed using the Mann-Whitney-U-test (SPSS 12.0.1 for Windows). P-values were corrected for multiple comparison errors.

Table 1. Basal, plasma and liver parameters of lean and db/db mice (control and colesevelam-treated). Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.
RESULTS

Effects of colesevelam-treatment on food intake, body weight and plasma metabolites in lean and \(db/db\) mice

Lean and \(db/db\) mice were treated with the bile salt sequestrant colesevelam for two weeks. Food intake was increased in colesevelam-treated lean and \(db/db\) mice during treatment compared to untreated controls (Table 1). Body weight gain was unaffected in colesevelam-treated lean mice but decreased in colesevelam-treated \(db/db\) mice. Blood glucose levels increased in control \(db/db\) mice during the two-week intervention period but remained stable in colesevelam-treated \(db/db\) mice. Blood glucose levels remained unchanged upon treatment in lean mice. Fasting insulin levels (not shown) were unchanged and decreased, respectively, in colesevelam-treated lean and \(db/db\) mice. Non-esterified fatty acid (NEFA) and VLDL-triglyceride levels (Supplemental Figure 1) were significantly reduced in colesevelam-treated \(db/db\) mice compared to untreated controls but remained unchanged in lean mice.

![Supplemental Figure 1](image)

Supplemental Figure 1. Plasma lipoprotein profile of control and colesevelam-treated lean and \(db/db\) mice. VLDL-TG was reduced in diabetic mice upon treatment. No changes in VLDL-TG levels were observed in lean mice. Plasma from 6 animals per group was pooled and separated by fast protein liquid chromatography (FPLC) on a Superose 6 column.

Biliary bile salt flux and cholate pool size remain unchanged in colesevelam-treated lean and \(db/db\) mice

Control \(db/db\) mice showed increased feces production and a higher fecal bile salt output, representing hepatic bile salt synthesis, compared to lean controls (Figure 1A and B, resp.). As expected, colesevelam-treatment led to massive increases in fecal bile salt output (Figure 1B). Untreated lean and \(db/db\) mice had similar bile
Bile salt sequestration and hepatic lipid metabolism

Direct end-products of de novo bile salt synthesis are the primary bile salts cholate and chenodeoxycholate. Modifications of these bile salts in liver and intestine give rise to differentially structured primary and secondary bile salts, respectively. Supplemental Table 1 provides details on biliary and fecal bile salt compositions. In short, sequestrant-treatment resulted in a strongly increased relative content of fecal deoxycholate in both groups. Cholate remained the major biliary bile salt species in both models upon sequestrant-treatment. Next, we determined relevant kinetic parameters of cholate, the major primary bile salt species in mice. Untreated db/db mice displayed a larger pool size and a higher synthesis rate of cholate compared to untreated lean mice (Figure 2). Importantly, cholate pool size remained unchanged upon colesevelam-treatment in both models. Synthesis rates of cholate were massively increased upon sequestrant-treatment (+375% and +172%, lean and db/db mice, respectively) and completely compensated for the increased fecal bile

**Figure 1.** Effects of colesevelam on fecal and biliary bile salts. Fecal output (A) was unchanged in both lean and db/db mice upon colesevelam-treatment. As expected, fecal bile salt output (B) of lean and db/db mice was increased upon colesevelam-treatment, indicative for higher hepatic bile salt synthesis. Importantly, bile flow (C) and biliary bile salt output (D) were unchanged upon colesevelam treatment. Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.
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Supplemental Table 1. Fecal and biliary bile salt composition as percentage of total bile salts. Secondary bile salts are marked *. Values are presented as mean ± SD. N=6 animals per group. +; colesevelam-treated

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<th>UDC *</th>
<th>β-M</th>
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Salt loss induced by colesevelam. The calculated amount of cholate reabsorbed from intestines of colesevelam-treated lean and db/db mice was reduced by about 30% compared to untreated controls (Figure 2D). Decreased plasma bile salt levels further reflect a reduced flux of bile salts returning to the liver (Figure 2E).

To gain insight into colesevelam-induced changes in total bile salt pool composition and synthesis of bile salts derived from the primary bile salt species cholate and chenodeoxycholate, we calculated the amount of cholate- and chenodeoxycholate-derived bile salts in the pool as well as their synthesis rates. Upon sequestrant-treatment, the total pools of bile salts remained unchanged in both models (Figure 3A). Nevertheless, the pool size of chenodeoxycholate-derived bile salts was decreased. The synthesis of cholate-derived bile salts was massively increased, whereas synthesis of chenodeoxycholate-derived bile salts remained unchanged in sequestrant-treated mice compared to untreated controls (Figure 3B). Corresponding with increased cholate synthesis, hepatic expression of Cyp7a1, encoding the first and rate-limiting enzyme in bile salt synthesis, and Cyp8b1, encoding the specific enzyme required for cholate synthesis, were massively increased. Cyp27a1 directs bile salt synthesis towards CDCA. Although expression of Cyp27a1 was increased in colesevelam-treated mice, this was not reflected in increased CDCA synthesis (Figure 3C). Despite the fact that bile salt reabsorption was not completely abolished, expression levels of the Fxr-target gene Fgf15 were undetectable in distal ilea of colesevelam-treated lean and db/db mice (Figure 3D).
Cholesterol synthesis is massively increased in colesevelam-treated lean and \textit{db/db} mice colesevelam-treatment increased fecal cholesterol excretion (Figure 4A). Together with a strongly increased synthesis of bile salts this translates into an increased turnover of cholesterol. However, this did not result in reduced plasma concentrations or hepatic contents of cholesterol (Figure 4B and C, respectively). Increased hepatic expression of \textit{HmgCoAr}, encoding the rate-controlling enzyme in cholesterol synthesis, and of \textit{Ldlr} (Figure 4D) indicated the anticipated hepatic com-

![Figure 2.](image)

**Figure 2.** The pool size of cholate is unchanged in lean and \textit{db/db} mice after a two-week colesevelam-treatment. The kinetics of cholate were calculated from the decay of [\textsuperscript{3}H\textsubscript{4}]-cholate from plasma (18). Despite substantial fecal bile salt loss (Figure 1B) in colesevelam-treated lean and \textit{db/db} mice, cholate pool size (A) was unchanged in both groups. Hence, the fractional turnover rate (FTR) (B) and synthesis rate (C) of cholate were massively increased. Both lean and \textit{db/db} mice had reduced absorption of cholate (D) upon treatment with colesevelam. Additionally, total plasma bile salts (E) were reduced in colesevelam-treated mice. Reabsorbed cholate is represented as percent of cholate secreted in bile. Values are presented as mean ± SD. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.
Figure 3. Decreased chenodeoxycholate (CDCA)-derived bile salt pool size and synthesis in colesevelam-treated mice. The composition and synthesis of the total bile salt pool were calculated from the ratio of CDCA-derived/choolate (CA)-derived bile salts and the pool size and synthesis of cholate as determined in vivo using $[^3H]$-cholate. The product of CDCA-derived/CA-derived bile salt ratio in feces and CA synthesis represents the synthesis of CDCA-derived bile salts. The product of the CDCA-derived/CA-derived bile salt ratio in bile and CA pool size represents the contribution of CDCA-derived bile salts to the total bile salt pool size. The total bile salt pool size (A) was unchanged in colesevelam-treated lean and db/db mice. Synthesis (B) of CA-derived bile salts was massively increased whereas synthesis of CDCA-derived bile salts was unaffected. Hepatic expression (C) of Cyp7a1, Cyp8b1 and Cyp27a1 were massively increased. Interestingly, intestinal expression (D) of the FXR-target gene Fgf15 was undetectable in colesevelam-treated mice whereas expression of Ibabp, another FXR target gene was unchanged.

Legend continues on next page
Values are presented as mean ± SD. In panel A-B, * and # indicate significant differences in total (CA + CDCA-derived) pool and synthesis. Gene expression levels are presented as relative expression normalized to 36B4 (liver) and 18S (intestine). N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively. +; colesevelam-treated. CA-derived; cholate + deoxycholate. CDCA-derived; chenodeoxycholate, α-muricholate, β-muricholate, ω-muricholate, hyodeoxycholate and ursodeoxycholate.

**Figure 4.** A massive increase in *de novo* cholesterol synthesis completely compensates for the loss of cholesterol via increased conversion into bile salts and increased fecal loss after colesevelam-treatment. Fecal cholesterol output (A) was increased in lean and db/db mice upon colesevelam-treatment. Yet, plasma concentrations (B) and hepatic contents (C) of cholesterol remained unchanged. Increased hepatic expression levels (D) of *Ldlr* and *HmgCoAr*, key genes involved in hepatic cholesterol metabolism, strongly pointed towards compensatory cholesterol synthesis. To assess this, the fraction of newly synthesized cholesterol was calculated by means of [1-13C]-acetate incorporation using Mass Isotopomer Distribution Analyses (MIDA). The fraction of newly synthesized cholesterol was indeed massively increased upon colesevelam-treatment in lean and db/db mice (E). Values are presented as mean ± SD. Gene expression levels are presented as relative expression normalized to 36B4. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.
Figure 5. Increased fatty acid synthesis and chain elongation underlie the increased hepatic triglyceride content after colesevelam-treatment. Colesevelam-treatment lead to increased hepatic fat contents (Table 1) which was mainly periportally defined (A) vs (B), lean control vs lean colesevelam respectively. Increased expression of the key lipogenic genes Srebp1c, Acc1, Fas and Scd1 (C) were highly suggestive of enhanced lipogenesis in colesevelam-treated mice. The fraction of newly synthesized C16:0 (D) was significantly increased in colesevelam-treated lean mice only. The fractions of newly synthesized C18:0 and C18:1 were significantly increased in lean and db/db mice. Additionally, chain elongation (defined as C16:0 + C:2) and desaturation were increased in Colesevelam-treated lean and db/db mice. Values are presented as mean ± SD. Gene expression levels are presented as relative expression normalized to 36B4. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively. In panel D-F, * and # indicate significant differences in total (de novo + chain elongated) fraction of newly synthesized fatty acids. +; colesevelam-treated.
pensatory response in cholesterol metabolism after colesevelam-treatment. To quantify this, the fraction of newly synthesized cholesterol was determined by analysis of the incorporation of [1-\(^{13}\)C]-acetate into plasma cholesterol. Fractional cholesterol synthesis was indeed robustly increased in colesevelam-treated mice (Figure 4E).

**Hepatic triglyceride content is increased due to enhanced contribution of lipogenesis in colesevelam-treated mice**

Both colesevelam-treated lean and \( db/db \) mice had modestly increased (lean +50%, \( db/db \) +23%) hepatic triglyceride contents compared to untreated controls (Table 1).Remarkably, fat accumulated primarily in periportal areas upon bile salt sequestration (Figure 5A and B). Increased hepatic expression of key lipogenic genes (Srebp1c, Acc1, Fas and Scd1) (Figure 5C) was highly suggestive of enhanced synthesis of fatty acids. Indeed, the total fractions of newly synthesized C16:0, C18:0 and C18:1, as determined by incorporation of [1-\(^{13}\)C]-acetate followed by MIDA analysis, confirmed that synthesis of these major hepatic fatty acid species was increased. Additionally, we calculated the contribution of *de novo* synthesis and chain elongation to the total fractional C18:0 and C18:1 synthesis.\(^{28}\) The increased total fraction of newly synthesized fatty acids was mainly attributable to increased chain elongation in colesevelam-treated lean and \( db/db \) mice (Figure 5D).

**Supplemental Figure 3.** Hepatic expression of \( Fxr \) and \( Fxr \)-target genes. Hepatic expression of \( Fxr \) and of the well-defined \( Fxr \)-target genes \( Shp \), \( Ntcp \) and \( Bsep \) were unaffected in colesevelam-treated lean mice compared to controls. In \( db/db \) mice, however, hepatic expression of \( Shp \) and \( Ntcp \) were reduced and increased, respectively, which is indicative of reduced FXR-signaling. Expression of \( Bsep \) was unchanged in colesevelam HCl-treated \( db/db \) mice compared to controls. Gene expression levels are presented as relative expression normalized to 18S. N=6 animals per group. Significant differences within or between groups are indicated # and *, respectively.
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Lipogenic gene expression upon sequestrant-treatment is Fxr and Lxrα dependent

Bile salt-mediated changes in expression of one of the major regulators of lipogenesis, Srebp1c, have been reported to be regulated by both FXR- and LXRα-regulated pathways. Surprisingly, expression levels of well-defined FXR- and LXRα-target genes were differentially or not at all affected in colesevelam treated lean and db/db mice (Supplemental Figure 3 and 4, resp.). To further address the role of hepatic FXR and LXRα in the lipogenic response to bile salt sequestration, Fxr-/- and Lxrα-/- mice and wild-type littermates were treated with colesevelam for two weeks. The key lipogenic genes Srebp1c, Acc1, Fas and Scd1 were significantly increased in livers of sequestrant-treated wild-type mice compared to untreated controls (Figure 6). Lipogenic genes, however, were barely affected in sequestrant-treated Fxr-/- and Lxrα-/- mice. These results support earlier observations on the regulatory roles of these nuclear receptors in the response to bile salt-mediated changes in lipid metabolism.

DISCUSSION

This work provides novel insights in the interrelationship between bile salt and lipid metabolism in lean and diabetic db/db mice treated with the bile salt sequestrant
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colesevelam. To the best of our knowledge, this is the first report which quantitatively shows that, despite massively induced fecal bile salt loss upon sequestrant-treatment, bile salt pool sizes and biliary bile salt secretion rates remain unaffected. Additionally, we show that bile salt sequestration induces hepatic fatty acid synthesis and elongation. An altered hepatic bile salt gradient due to decreased reabsorption but increased de novo synthesis of bile salts likely affects specific aspects of hepatic bile salt signaling. The lipogenic response appears to be dependent on FXR- and LXRα-signaling as was evident from studies in the respective knockout mice.

Knowledge of possible disturbances in bile salt metabolism in type 2 diabetic humans and animal models is very limited 3. For the first time, to our knowledge, we report data on kinetic alterations of bile salt metabolism in diabetic db/db mice and show that db/db mice have an increased pool size and synthesis rate of bile salts compared to lean controls. As suggested for db/db mice 15 and liver-specific insulin receptor knockout (LIRKO) mice 30, disturbed hepatic insulin signaling may directly contribute to changes in bile salt synthesis. Indeed, insulin was shown to reduce plasma bile salts in type 1 diabetic rats 31 possibly via FOXO1-mediated regulation of Cyp7a1 32. Further studies beyond the scope of this work, are needed to further unravel underlying mechanisms of disturbed bile salt metabolism in type 2 diabetes.

Db/db mice responded favorably to sequestrant-treatment: blood glucose levels stabilized while NEFA and VLDL-TG levels decreased. These parameters were unchanged in lean mice. Importantly, the pool size of the primary bile salt species cholate as well as the total pool size of bile salts remained unchanged in sequestrant-treated lean and db/db mice. Remarkably, only the synthesis of cholate was massively increased: synthesis of chenodeoxycholate-derived bile salts was not affected at all. In humans, an increased cholate-to-chenodeoxycholate ratio would result in a more hydrophilic bile salt pool which has been associated with decreased susceptibility for gallstone disease 33. Colesevelam-treatment might therefore be beneficial for prevention of gallstone formation in type 2 diabetic humans who have an increased prevalence of gallstones 34. Bile salt reabsorption was reduced by 30% in both models. Although bile salt reabsorption was not fully impaired, Fgf15 expression levels were not detectable in ilea of sequestrant-treated wild-type mice. Possibly, bile salt sequestration decreases the cellular content of bile salts below a certain threshold value necessary to activate FXR in enterocytes as observed in an in vivo study in rabbits 35.

Interestingly, hepatic triglyceride contents of colesevelam-treated lean and db/db mice were enhanced which appeared to be mediated by an increased de novo synthesis of hepatic fatty acids and chain elongation. In contrast to our data, other studies addressing the effects of bile salt sequestration on lipid metabolism showed that bile salt sequestration prevented triglyceride accumulation in the liver 36,37. It should be realized that those studies were performed in high fat diet-fed mice in which the beneficial effects of bile salt sequestration are likely partly attributable to
sequestrant-induced malabsorption of lipids. In addition, strain-specific responses to sequestrant-treatment cannot be ruled out.

At a molecular level, the interrelationship between bile salt and lipid metabolism is generally accepted to be mediated by FXR. Nevertheless, data to explain the exact mechanisms of this relationship are still very inconsistent. Pharmacological activation of FXR has been shown to reduce FFA levels in insulin-resistant rodents. Absence of FXR signaling in Fxr−/− mice leads to increased VLDL-TG levels in plasma of these mice suggestive of a role for Fxr in control of VLDL assembly.

Colesvelam-treatment induced hepatic expression levels of the lipogenic gene Srebp1c in lean and db/db mice. Hepatic expression levels of the lipogenic gene Srebp1c were reduced in Fxr−/− mice compared to controls. Conversely, FXR activation was also shown to repress the expression of Srebp1c in a pathway involving SHP. Expression levels of the FXR-target gene Shp were unaffected and decreased in colesvelam-treated lean and db/db mice, respectively. These results are suggestive of SHP-independent regulation of Srebp1c upon sequestrant-treatment. Supportive of SHP-independent regulation of lipogenic gene expression by FXR was the observation that FXR regulates the transcription of the lipogenic gene Fas through direct binding to the Fas promoter. Since expression levels of well-known FXR-target genes were differentially affected in lean and db/db mice, we studied the role of FXR in the lipogenic response of sequestrant-treatment in Fxr−/− mice and found that in contrast to wild-type mice littermates, lipogenic gene expression levels were barely affected. Srebp1c, is strongly regulated by the oxysterol receptor LXRa. Increased synthesis of cholesterol, as occurs in sequestrant-treated mice, could possibly lead to increased hepatic levels of oxysterols and, hence, activation of LXRa. Expression levels of established LXRa-target genes, however, were unaffected in sequestrant-treated lean and db/db mice, suggestive of unchanged LXRa signaling. Yet, investigation in sequestrant-treated Lxra−/− mice revealed that lipogenic gene expression was not increased in these mice compared to untreated wild-type littermates. Our results from colesvelam treated Fxr−/− and Lxra−/− mice confirm earlier findings that FXR and LXRa are both involved in regulation of bile salt-mediated changes in lipogenic pathways. The exact molecular mechanisms via which these nuclear receptors signal regulate the lipogenic response to bile salt sequestration exceed the scope of this report. At a physiological level, bile salt-mediated signaling pathways are dependent on the concentration of bile salts in the liver acinus. We speculate that the concept of metabolic zonation might add to the understanding of the observed hepatic effects upon bile salt sequestration. Hepatocytes localized around the portal vein display different metabolic activities than those lining the central vein, e.g., bile salt and fat synthesis are pericentrally localized processes whereas cholesterol synthesis is mainly carried out by portal hepatocytes. As we show in the current report, the amount of bile salt molecules reabsorbed in ilea of colesvelam-treated mice was decreased by ~30% with a subsequent reduction in plasma bile salt levels and,
hence, reduced bile salt signaling in periportal hepatocytes. Newly synthesized bile salts, which accommodate a much larger fraction of the bile salt pool of colesevelam-treated mice compared to controls, are primarily secreted by pericentrially localized cells and possibly exert differential signaling functions. Selective periportal fat accumulation and differentially affected expression levels of hepatic Fxr-target genes support this hypothesis. Additionally, it was shown that Cyp7a1, which is exclusively expressed in pericentral hepatocytes, translocates to a larger area of the liver lobulus with more involvement of periportally localized cells in sequestrant-treated rats. Our working model is summarized in Chapter 8, Figure 1. It should be stressed that this hypothesis requires dedicated investigation.

In conclusion, we show that colesevelam-treatment increases lipogenesis and chain elongation in mice which, at least at the level of gene expression, is dependent on FXR and LXRα. A shift from reabsorption to de novo synthesis as the source of biliary bile salts affects the sinusoidal gradient of bile salts. This shift modifies the regulation of genes and proteins involved in bile salt synthesis and bile salt-mediated regulation of metabolism and possibly underlies the phenotypical response to colesevelam-treatment in mice.

ACKNOWLEDGEMENTS

We are indebted to Rick Havinga for excellent contributions to the mouse studies performed. Additionally, we are grateful to Theo Boer and Elles Jonkers for their excellent technical assistance with GC/MS analyses.
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