Refined sugar feeding reduces hepatic bile salt synthesis in mice
Relationship with modulation of FXR via GlcNacylation

Hilde Herrema, Marije Boesjes, Jurre Hageman, Johanna H.M. Stroeve, Renze Boverhof, Michael Müller, Frans Stellaard, Albert K. Groen and Folkert Kuipers

In preparation
ABSTRACT

The concerted actions of several enzymes and transporters in liver and intestine maintain an efficient enterohepatic circulation of bile salts. The bile salt-activated nuclear receptor FXR plays a key role in control of this circulation. Interestingly, synthesis rates and pool sizes of bile salts show large interindividual differences in humans. Besides genetic factors, dietary components have been shown to modulate bile salt metabolism. Refined sugar intake has been shown to decrease bile salt synthesis via yet unknown mechanisms in humans and animal models. In this study, we measured kinetic parameters of bile salt metabolism in mice fed a regular laboratory chow diet or an isocaloric, compositionally identical diet in which the carbohydrate fraction consisted solely of the simple carbohydrate dextrose for two weeks. Biliary (-33%) and fecal bile salt (-34%) secretion were decreased in dextrose-fed mice compared to chow-fed mice. Primary bile salt synthesis was massively (-50%) decreased contributing to a 40% smaller pool size observed in dextrose-fed mice. Interestingly, despite anticipated low bile salt levels in the ileum of dextrose-fed mice, ileal expression of the FXR-target genes \textit{Shp} and \textit{Fgf15} were significantly increased, particularly in the fed state. This effect was absent in intestine-specific \textit{Fxr} knockout (iFxr\textsuperscript{-/-}) mice compared to dextrose-fed wild-type littermates. \textit{In vitro} studies indicate that FXR is posttranslationally regulated by GlcNAcylation upon exposure to glucose. In conclusion, our data show that simple carbohydrate feeding suppresses bile salt synthesis, possibly by a counterintuitive upregulation of intestinal \textit{Fgf15} expression due to GlcNAcylation of FXR.
INTRODUCTION

Bile salts are crucial for bile formation and for efficient absorption of dietary fat. Bile salts are maintained within an efficient enterohepatic circulation through the concerted actions of several enzymes and transporters in liver and intestine. Interestingly, the bile salt pool size of healthy humans is highly variable. Although genetic factors likely add to this phenomenon, this variation is, at least in part, attributable to effects of diet composition. In particular, refined sugars have been shown to impact on bile salt metabolism in humans and experimental animal models. Insight in diet-induced changes in bile salt metabolism is of importance in view of the ongoing increase in refined sugar intake in Western societies. Additionally, bile salts are now recognized as important integrators of metabolism.

Bile salts regulate their own synthesis by activating the nuclear receptor FXR. Activated hepatic FXR reduces the expression of Cyp7a1, catalyzing the first and rate-limiting step in bile salt synthesis, in a SHP-dependent manner. Activation of FXR in the distal ileum induces the expression of Fgf15 (murine ortholog of human FGF19) which is subsequently released into portal blood to downregulate hepatic bile salt synthesis via FGFR4 signaling in the liver. Hepatic Fxr expression has been reported to be regulated by glucose. Additionally, at the posttranslational level acetylation and phosphorylation have been shown to impact on Fxr transcriptional activity. It is currently not known whether FXR is posttranslationally regulated by glucose-hexosamine-derived O-linked β-N-acetylglucosamine modification (GlcNAcylation), a process similar to phosphorylation. GlcNAcylation has been shown recently to be an important regulatory mechanism for modulation of other nutrient-responsive transcription factors like LXR and FOXO1. GlcNAcylation is dependent on the cellular concentration of UDP-GlcNAc, which is produced by the hexosamine biosynthetic pathway (HBP), that depends on the amount of glucose entering the cell.

This study aimed to determine the effects of refined carbohydrate feeding on bile salt synthesis and pool size in mice. We determined quantitative parameters of bile salt metabolism using a stable isotope approach in mice fed a regular laboratory chow diet or an isocaloric, compositionally identical diet in which the carbohydrate fraction consisted solely of the simple sugar dextrose. Since genes involved in bile salt metabolism show a large diurnal variation in activity, gene expression patterns were obtained from mice terminated at the end of the light phase (7AM) or at the end of the light phase (7PM).

Mice fed the dextrose diet showed strongly decreased bile salt synthesis and a reduced pool size compared to chow-fed mice. Plasma bile salts were also reduced in dextrose-fed mice. Remarkably, despite presumably low ileal bile salt levels, dextrose-fed mice displayed massively increased ileal expression levels of the FXR-target genes Shp and Fgf15. These FXR-target genes were not affected in intestine-specific
Fxr knockout mice fed the dextrose diet. Preliminary results indicated that glucose dose-dependently increased FXR GlcNAcylation in vitro thereby possibly enhancing FXR transactivation capacity. In conclusion: our results indicate that dextrose feeding inhibits bile salt synthesis in an FXR-dependent manner via massive induction of ileal Fgf15 expression, possibly due to increased GlcNAcylation of this nuclear receptor.

MATERIALS AND METHODS

Animals
Male C57Bl6/J (Charles River Laboratories, L’Arbresle, France) and iFxr−/− mice and their WT littermates were housed individually in a temperature-controlled room (−22ºC) with a 12h light/dark cycle. Mice were fed commercially available laboratory chow (RMH-B; Arie Blok, Woerden, The Netherlands), high (40% w/w) in complex carbohydrates or a eucaloric semi-synthetic diet (Reference diet 4068.22, Arie Blok, Woerden, The Netherlands) in which the carbohydrate fraction (40% w/w) consisted solely of dextrose for 2 weeks. When indicated, diets were supplemented with 2% (w/w) colesevelam HCl (Daiichi Sankyo, Inc., Parsippany, NJ, USA). Mice were used for experimental procedures at 12 weeks of age. Animal experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Animal experiments
After a two-week diet intervention, mice were terminated at 7AM or at 7PM by cardiac puncture under isoflurane anesthesia. Plasma was stored at -20°C until analyzed. The liver was quickly removed, weighed and snap-frozen in liquid nitrogen. The intestine was excised, flushed with ice-cold PBS and subsequently snap-frozen in liquid nitrogen. Both liver and intestine were stored at -80ºC until biochemical analyses and RNA isolation.

In a separate experiment, 400 μg [2H4]-cholate (0.5% NaHCO in PBS, pH = 7.4) was intravenously administered to chow-fed mice at day ten of the two-week diet intervention. Results from a pilot study pointed towards a decreased bile salt pool size in dextrose-fed mice. Hence, the relative dose of [2H4]-cholate would be much higher in dextrose-fed mice. Therefore, 250 μg [2H4]-cholate was administered to dextrose-fed mice. Subsequently, retro-orbital blood samples were obtained 12, 24, 36, 48 and 60 h after injection of [2H4]-cholate. Plasma was stored at -20°C until analyzed. Feces were collected during the 60h kinetic experiment and, after air-drying, kept at RT until analysis. After 60h, 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 terminated by cervical dislocation.
Analytical procedures

Hepatic and biliary lipids were extracted according to Bligh & Dyer. Plasma and liver triglyceride, total cholesterol and free cholesterol content 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). Hepatic and biliary phospholipid content was determined according to Böttcher et al. Cholesterol in bile was measured according to Gamble et al. Total bile salts in bile and feces were determined by an enzymatic fluorimetric assay.

Preparation of plasma samples for GC/MS analysis of bile salts

Plasma samples were prepared for GC/MS analysis essentially as described. In short, 10μl of plasma was diluted with 100μl demi water, 10μl 0.2M sodiumacetate buffer (pH 5.6), 20μl 0.2M sodium-EDTA and 20μl 0.2M mercaptoethanol. Subsequently, bile salt conjugates were hydrolyzed enzymatically using 0.012U Cholylglycine Hydrolase (overnight, 37°C). Samples were cooled down to RT followed by disruption of bile salt-protein interactions using 40μl 1M sodiumhydroxide (30 minutes, 64°C). Samples were cooled down to RT. Upon adding 80μl 1M hydrochloric acid, the free bile salts were extracted two times with 1 ml diethyl ether containing 10% aceton. The combined diethyl ether layers were evaporated and 20μl of triethylamine was added. Samples were treated with 20μl 10% PFB in acetonitril (30 minutes, RT). After addition of 125μl 1M hydrochloric acid, the PFB esters were extracted with 800μl ethylacetate. The evaporated residue was treated with 20μl BSTFA:pyridine:TMCS (ratio 5:5:0.1, 1h, RT). Samples were evaporated and redissolved in 100μl isooctane. Bile salt derivatives were analyzed using an Agilent 7890A GC connected to an Agilent 5975C MS (Agilent Technologies, Santa Clara, CA) on a narrow bore column (J&W scientific). Fecal neutral sterols were analyzed as described.

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, α-muricholate, β-muricholate, ω-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 in vivo using [2H4]-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 [H4]-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.

**Constructs**

The coding sequences of FXR alpha 1, FXR 3, LXR alpha and LXR beta were amplified and cloned into pcDNA5/FRT/TO V5. The presence of the correct genes was sequence verified. FXR alpha 2 and FXR alpha 4 were cloned from the FXR alpha 1 and FXR alpha 3 constructs, respectively. The 4 amino acids insertion in the hinge region of FXR alpha 1 and FXR alpha 3 were deleted via inverse PCR. Protein expression was verified by western blotting.

**Cell cultures and transfections**

HEK 293 embryonic kidney cells were maintained in DMEM +GlutaMax (Invitrogen, 31966) supplemented with 10% fetal bovine serum (Gibco) and 1% Penicillin-Streptomycin (Gibco) and kept at 37°C in a humidified atmosphere containing 5% CO2. HEK293 cells were transfected using Lipofectamine (Invitrogen) as instructed by the manufacturer with the following details: for each transfection 400,000 cells were seeded on poly-l-lysin coated 10 cm² plates (6wells) one day before transfection. Transfection was performed in opti-MEM reduced serum medium (Invitrogen, 11058). Four hours after transfection, media was changed to complete DMEM with different glucose concentrations for 24 hours.

**Preparation of protein extract**

Cells were washed twice in cold PBS and harvested in 500ul lysis buffer containing 150mM NaCl, 0.5% igepal, 1.5mM MgCl2, 20mM Tris-HCl (pH7.4), 3% glycerol, 1mM DTT, complete protease inhibitors (Roche) and 100uM PUGNac (Sigma A7229). The lysates were homogenized by passing through a 26G needle 3 times and centrifuged for 14.000rpm for 15 min at 4°C. The supernatant was centrifuged again to get lost of all the debris. The pellet was taken up in 250uL 2x sample buffer (4% SDS, 10% β-mercapto-ethanol, 20% glycerol, 0.002% bromophenol blue, 0.1M Tris HCl pH6.8) and stored at -20°C as the pellet fraction of the immunoprecipitation. As the input fraction of the immunoprecipitation, 30ul of supernatant is added to 30ul 4x sample buffer. The supernatant, which is the protein extract, was used for further procedures.

**Immunoprecipitation**

Protein A/G Plus agarose beads (Santa Cruz Biotechnology, sc-2003) were coated with O-linked N-Acetylglucosamine (Affinity BioReagents MA1-072, clone RL2) (20ul beads+1ul AB per 6well) for 1 hour at 4°C with slow agitation. Protein extracts
were precleared by incubation with protein A/G beads for 1 hour at 4°C with slow agitation. The precleared supernatant was incubated with the O-Linked N-acetylglucosamine coated beads overnight at 4°C with slow agitation. The beads were washed 3 times in lysis buffer. 30ul sample buffer was added to each bead fraction. All the fractions were boiled for 5 min just before performing the Western blot analysis and stored at -20°C.

Western Blotting
O-GlcNac levels were determined by SDS-PAGE (10% gel loaded 20ul sample) and blotting (nitrocellulose) with anti-v5 HRP (Invitrogen 1:5000) antibodies. Blots were stripped using stripping buffer (Thermo Scientific). Subsequently, GAPDH levels, which served as loading control, were determined (CalBioChem CB 1001 1:50000). Secondary anti-mouse Ig HRP linked antibody (from sheep) was used at 1:1000 (GE Healthcare).

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 28. 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, Nieuwerkerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to 18S or 36B4 (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).

RESULTS

Effect of dextrose feeding on liver and plasma parameters
Mice were fed a chow diet, high in complex sugars, or a diet in which the carbohydrate fraction consisted solely of the refined sugar dextrose for two weeks (Supplemental Table 1). Bile salt metabolism has a strong day/night variation in which intestinal FXR controls hepatic bile salt synthesis via FGF15 mainly during the dark period and 31. Therefore, mice were terminated at the end of the dark phase (7AM) or at the end of the light phase (7PM). Daily food intake was similar in chow- and dextrose-fed mice (Table 1). Body weights or liver weights did not differ between mice fed the different diets. Plasma triglycerides, cholesterol and non-esterified fatty acid levels (NEFA) levels did not differ between groups. Dextrose-fed mice had
higher liver triglyceride contents compared to chow-fed mice. Interestingly, plasma bile salts were markedly reduced in dextrose-fed mice compared to chow-fed mice at both time points.

Supplemental table 1.
Composition of chow and dextrose diets.

<table>
<thead>
<tr>
<th>Component (g/kg)</th>
<th>Chow</th>
<th>Dextrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr.Prot</td>
<td>227</td>
<td>213</td>
</tr>
<tr>
<td>Cr.Fat</td>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>Cr.Fiber</td>
<td>37</td>
<td>51</td>
</tr>
<tr>
<td>Sugar+Starch</td>
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<td>514</td>
</tr>
<tr>
<td>Minerals</td>
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<tr>
<td>Moisture</td>
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<td>104</td>
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<tr>
<td>Dry Matter</td>
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Table 1. Basal parameters of mice fed chow or dextrose diet for 2 weeks. Values are presented as mean ± SD. N=6 animals per group. TG; triglyceride, FCH; free cholesterol, CHE; cholesterolesters, PL; phospholipids. Data are represented as mean ± SD, n=6 mice per group. * significant differences (p<0.05) between chow- and dextrose-fed groups.

<table>
<thead>
<tr>
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<th>7 AM</th>
<th>7 PM</th>
<th>7 AM</th>
<th>7 PM</th>
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<td></td>
<td>chow</td>
<td>dextrose</td>
<td>chow</td>
<td>dextrose</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.9 ± 1.4</td>
<td>25.0 ± 1.7</td>
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<td>Liver weight (g)</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.2</td>
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<td>Relative liver weight (%)</td>
<td>5.3 ± 0.4</td>
<td>4.5 ± 0.6</td>
<td>4.4 ± 0.2</td>
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<td>24h food intake (g)</td>
<td>4.3 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>4.2 ± 0.2</td>
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<td>Plasma</td>
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<td>TG (mmol/l)</td>
<td>1.37 ± 0.42</td>
<td>1.18 ± 0.48</td>
<td>0.62 ± 0.14</td>
<td>0.46 ± 0.19</td>
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<tr>
<td>FCH (mmol/l)</td>
<td>0.33 ± 0.07</td>
<td>0.32 ± 0.17</td>
<td>0.36 ± 0.17</td>
<td>0.27 ± 0.18</td>
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<tr>
<td>CHE (mmol/l)</td>
<td>1.91 ± 0.21</td>
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<td>1.59 ± 0.19</td>
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<td>NEFA (µmol/l)</td>
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<td>657 ± 219</td>
<td>496 ± 160</td>
<td>422 ± 166</td>
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<td>Bile salts (µmol/l)</td>
<td>22.4 ± 3.9</td>
<td>11.1 ± 2.8 *</td>
<td>10.4 ± 5.0</td>
<td>4.8 ± 3.1 *</td>
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<tr>
<td>Liver</td>
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<tr>
<td>TG (µmol/g)</td>
<td>11.5 ± 1.3</td>
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<td>12.6 ± 1.6</td>
<td>15.4 ± 1.7 *</td>
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<tr>
<td>FCH (µmol/g)</td>
<td>5.2 ± 2.2</td>
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<td>7.1 ± 1.6</td>
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<tr>
<td>CHE (µmol/g)</td>
<td>0.35 ± 0.15</td>
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<td>0.26 ± 0.08</td>
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Decreased biliary and fecal bile salt output in dextrose-fed mice

Bile flow was reduced by ~30% in dextrose-fed mice compared to chow-fed controls (Figure 1), likely due to the decreased bile salt secretion (-34%) in these mice. Additionally, biliary cholesterol and phospholipids were decreased to a similar extent although the difference did not reach statistical significance for cholesterol. Hepatocytes are exclusively responsible for the synthesis of bile salts from cholesterol. 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. Dextrose-fed mice had a lower relative amount of cholate in bile compared to chow-fed mice whereas the relative amount of the secondary, cholate-derived bile salt deoxycholate was higher. In feces of dextrose-fed mice, the relative amount of cholate was lower whereas the relative amounts of the secondary bile salt species deoxycholate and ω-muricholate were higher. Although dextrose feeding decreased both the absolute and the relative amount of cholate in bile, it still represented the major bile salt species in bile (Supplemental Table 2).

Daily fecal bile salt output, representing hepatic synthesis, was decreased in dextrose-fed mice (9.62 ± 1.85 vs 6.32 ± 0.51 μmol/24h/100g body weight, chow vs
Supplemental Table 2. Fractional biliary and fecal bile salt composition. The fraction of CA in biliary bile was lower in dextrose-fed mice whereas the fraction of DC was increased. The fraction of CA was also decreased in fecal bile salts. The secondary bile salt ω-MC was massively increased in dextrose fed mice compared to chow-fed mice. Values are presented as mean ± SD. N=6 animals per group. * significant differences (p<0.05) between groups. α-MC; α-muricholate, DC; deoxycholate, CA; cholate, alloCA; allo-cholate, CDC; chenodeoxycholate, HDC; hydoxycholate, UDC; ursodeoxycholate, β-MC; β-muricholate, ω-MC; ω -muricholate

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<tr>
<th></th>
<th>α-M</th>
<th>DC *</th>
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<th>CDC</th>
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<th>UDC*</th>
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<td></td>
<td></td>
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<tr>
<td>Chow</td>
<td>5.5</td>
<td>34.3</td>
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<td>3.7</td>
<td>11.8</td>
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Figure 2. Effect of dextrose feeding on kinetic parameters of cholate metabolism. A stable isotope dilution technique was used to determine the kinetics of the major rodent bile salt species cholate. Dextrose feeding led to a massive reduction of the pool size of cholate (A). The fractional turnover rate (FTR) (B) was increased. Consequently, the synthesis rate was decreased in dextrose-fed mice compared to chow-fed mice. The biliary cholate secretion (D) and the absorption rate per cycle (E) were decreased, but there was no significant difference in the fractional cholate absorption (F) between chow- and dextrose-fed mice. Values are presented as mean ± SD. N=6 animals per group. * significant differences (p<0.05) between groups. CA; cholate
Dextrose feeding and bile salt metabolism

The pool size of cholate, as determined by a stable isotope approach, was \(~40\%\) smaller compared to chow-fed mice (Figure 2). The FTR was slightly but significantly increased. The cholate absorption efficiency per cycle of the enterohepatic circulation was not significantly affected in dextrose-fed mice (94.3 ± 2.3\% vs. 89.9 ± 4.0\%, chow vs dextrose). The synthesis rate of cholate, \(i.e.,\) the product of pool size and FTR, was reduced by \(~50\%\) in dextrose-fed mice compared to chow-fed mice. The contributions of cholate- and chenodeoxycholate-derived bile salt to the total pool were calculated as described (Experimental procedures). Interestingly, dextrose feeding decreased the pool size of both cholate- and chenodeoxycholate-derived bile salts (Figure 3). The relative pool sizes of cholate- and chenodeoxycholate-derived bile salts were unchanged, however, the relative fractional syntheses were decreased and increased, respectively.

Figure 3. Both the contribution of cholate (CA)- and chenodeoxycholate (CDCA)-derived bile salts to the total pool (A) were decreased in dextrose-fed mice compared to chow-fed mice. Nevertheless, only the absolute synthesis (B) of CA-derived bile salts was decreased whereas the synthesis of CDCA-derived bile salts was unchanged upon dextrose feeding. The relative pool sizes (C) of CA- and CDCA-derived bile salts were unchanged, however, their relative fractional syntheses (D) were decreased and increased, respectively. Values are presented as mean ± SD. N=6 animals per group. * significant differences (p<0.05) between groups. CA-derived; cholate + deoxycholate. CDCA-derived; chenodeoxycholate, α-muricholate, β-muricholate, ω-muricholate, hyodeoxycholate and ursodeoxycholate.
Gene expression patterns in liver and intestine

Bile salts exert negative feedback control on hepatic bile salt synthesis via activation of both hepatic and intestinal FXR. Gene expression levels of genes regulated by bile salts were measured using QPCR. Since plasma bile salts of dextrose mice were low, it was very surprising to find that expression of Shp, one of the key players in negative feedback control of bile salt synthesis and established target-gene of FXR, was increased in livers of dextrose-fed mice (Figure 4). Expression of Cyp7a1, encoding the rate-limiting enzyme in bile salt synthesis, was strongly decreased in 7AM-terminated dextrose-fed mice. This coincided with the decreased cholate synthesis rates and high Shp-expression levels. Interestingly, at 7PM hepatic gene expression levels of dextrose-fed mice were similar those of chow-fed mice. Intestinal FXR is activated during enterohepatic cycling mainly during the night, which leads to increased expression and release of FGF15 to inhibit hepatic bile salt synthesis.

Intestinal expression levels of bile salt-regulated genes were assessed in 3 segments of the ileum, i.e., proximal, mid and distal (Figure 5). Strikingly, despite lower bile salt concentrations in the ilea of dextrose-fed mice, expression levels of Fgf15 were massively increased in the distal ileum. Although the function of Shp in the ileum remains largely unknown, its expression was substantially increased in the mid and distal parts of the ileum of dextrose-fed animals. The effect of dextrose-feeding on expression levels of Shp and Fgf15 were most pronounced in mid and distal ilea of 7AM-terminated mice. Nevertheless, expression levels of these genes were still markedly increased in distal ilea of 7PM-terminated mice.

![Graph](image)

**Figure 4.** Hepatic gene expression levels of chow- and dextrose-fed mice. Mice were terminated at the end of the dark phase (7AM) or at the end of the light phase (7AM). Hepatic expression the Fxr-target genes Shp and Cyp7a1 were increased and decreased, respectively, at 7AM. No differences between groups were measured in mice sacrificed at 7PM. Values are presented as mean ± SD. N=6 animals per group. * significant differences (p<0.05) between groups. Gene expression levels were normalized to 36B4.
Reduced bile salt synthesis upon dextrose feeding is FXR- and bile salt dependent

Despite lower plasma bile salt concentrations, lower biliary bile salt secretion, and hence likely, lower bile salt concentrations in ilea of dextrose-fed mice, the FXR-induced expression of Fgf15 appeared to be much stronger. The contribution of bile salts to this effect was assessed by supplementing the chow and dextrose diets with the bile salt sequestrant colesvelam HCl (2% w/w). This bile salt sequestrant has previously been shown by us to reduce bile salt reabsorption by ~30% and to completely abolish Fgf15 expression in chow-fed mice. Interestingly, dextrose feeding
could not prevent the reduction of $Fgf15$ expression in the presence of colesevelam HCl (Figure 6).

To assess whether enhanced expression levels of $Shp$ and $Fgf15$ were indeed dependent on FXR, gene expression levels in livers and distal ilea of chow and dextrose-fed intestine-specific $Fxr$ knockout ($iFxr^{−/−}$) mice and their wild type (WT) littermates were compared. Intestinal expression of $Fgf15$ was not increased upon dextrose feeding of $iFxr^{−/−}$ mice in contrast to their WT littermates (Figure 7). Additionally, hepatic $Cyp7a1$ expression was decreased in WT mice, as expected, but not in $iFxr^{−/−}$ mice upon dextrose feeding. These findings establish the role of intestinal FXR in dextrose-mediated reduction of bile salt synthesis.

**Fxr GlcNAcylated is elevated by glucose in HEK293 cells**

A stronger FXR-mediated induction of intestinal $Fgf15$ in the presence of a smaller bile salt pool and hence, lower flux of FXR ligands through the ileal enterocytes suggests a stronger FXR transactivation. Glucose-induced GlcNAcylation may be involved, as previously demonstrated for LXR$^{18}$ and FOXO1$^{19}$. To determine whether glucose is able to induce GlcNAcylation of FXR, FXR-transfected HEK293 cells were maintained in medium containing 5mM or 25mM glucose. Immunoprecipitated V5-tagged FXR was analyzed by Western blotting using anti V5 antibodies. Glucose concentration-dependent GlcNAcylation of FXR was indeed observed (Figure 8), demonstrating that such a modification may be responsible for dextrose-effects in vivo.
DISCUSSION

Although the bile salt pool appears to be very efficiently maintained, there is a large intra-individual variation in pool size \(^{1-4}\). Part of this variability can likely be accounted for by differences in diet composition. In particular, refined sugar intake has been shown to induce profound changes in bile salt metabolism \(^{6-8}\). Increasing consumption of refined sugars in Western societies might add to variations in bile salt pool sizes and, possibly, to the development of bile salt-related pathologies. Additionally, knowledge of the impact of diet composition on bile salt metabolism is of great importance for proper design of animal studies. Dedicated studies addressing quantitative changes of refined sugar feeding on (kinetic) parameters of bile salt metabolism, however, are very limited. Therefore, we compared kinetic parameters of bile salt metabolism in mice fed a regular laboratory chow diet or a diet identical in composition and caloric content in which the carbohydrate fraction consisted solely of the refined sugar dextrose. Bile salt pool sizes and biliary bile salt output rates in dextrose-fed mice were substantially reduced compared to chow-fed counterparts. These effects were likely attributable to an impaired hepatic bile salt synthesis. To

![Gene expression liver](image1)

**Figure 7.** Increased ileal *Fgf15* expression upon dextrose feeding is FXR dependent. *iFxr* \(^{-/-}\) mice did not show increased expression levels of *Fgf15* in distal ileum upon dextrose feeding. Values are presented as mean ± SD. *N=6* animals per group. * significant differences within groups. # significant differences between groups. N.D.; not detectable. Gene expression levels were normalized to *36B4* (liver) and *β-actin* (intestine).

![Gene expression distal ileum](image2)

**Figure 8.** FXR is modified by GlcNAc in HEK 293 cells. HEK293 cells were transfected with V5-tagged *Fxrα2* and *Fxrα4* and maintained in DMEM containing low and high concentrations of glucose (- and + resp.). *Lxrβ* and *Lxra*, known to be GlcNAcylated, were included as controls. GlcNAcylation of immunoprecipitated LXRs and FXRs was determined by SDS-PAGE followed by blotting anti-v5.

![V5](image3)
evaluate the mechanisms involved, one has to take into account the circadian rhythmicity in the expression of genes involved in bile salt metabolism. Therefore, mice were terminated at the end of the light phase (7 AM) as well as at the end of the dark phase (7 PM) for assessment of gene expression patterns. Surprisingly, expression of hepatic (Shp) and intestinal (Shp, Fgf15) FXR-target genes were increased in mice fed the dextrose diet compared to chow-fed mice despite decreased plasma and intestinal bile salt levels. Hepatic and intestinal gene expression patterns from chow-fed and dextrose-fed mice differed most at 7 AM and were, in part, blunted in mice terminated at 7 PM. This coincided with the reported activity of hepatic and intestinal FXR. Nevertheless, the “temporal” changes in expression levels of bile salt regulatory genes appeared to be sufficient to substantially lower bile salt synthesis in dextrose-fed mice compared to chow-fed mice. Preliminary results from in vitro studies show that FXR is activated by GclNacylation, a novel glucose-mediated posttranscriptional regulatory mechanism. Although the diets were identical with respect to composition and caloric content, the sources of several components in the dextrose diet differed from those of chow. Since the fiber content of the dextrose diet is lower than of chow, this may lead to altered metabolism of secondary bile salt in the colon of dextrose-fed mice. The contribution of the major secondary bile salt deoxycholate was 8% lower in feces collected from dextrose-fed mice compared to that of chow-fed mice. In contrast, the fraction of fecal α-muricholate was 12% higher in dextrose-fed mice. It cannot be excluded that this is due to altered bacterial composition or to an altered composition of bile salts coming from the ileum. The chow diet contains complex carbohydrates that must undergo several conversions before they can be taken up by enterocytes. The carbohydrate fraction in the dextrose diet consists solely of glucose which can be immediately taken up by the enterocytes and released into the blood stream. Intestinal glucose uptake is predominantly facilitated by GLUT2. Glucose intake leads to a very rapid increase in Glut2 expression levels and translocation of the protein to the plasma membrane. Indeed, we found increased Glut2 expression levels in proximal intestines of dextrose-fed mice. Interestingly, expression patterns of genes involved in bile salt synthesis differed most in the distal ileum in which luminal glucose levels are presumably very low or even absent. At a physiological level, intake of simple sugars leads to a sharp rise in insulin levels in order to maintain blood glucose levels. Both glucose and insulin have been suggested as important regulators of bile salt synthesis. Glucose is a strong regulator of gene expression in general via, amongst others, G6P or metabolites of the pentose phosphate pathway. intermediates of the pentose phosphate pathway have been suggested to upregulate Fxr expression in rat hepatocytes. Although we did not find increased Fxr expression levels in livers of dextrose-fed mice, hepatic expression of the FXR-target genes Shp and Cyp7a1 were increased in dextrose-fed mice terminated at 7 AM. Additionally, ileal expression levels of the FXR-target genes Shp and Fgf15 were massively increased despite low bile salt levels, again suggesting activation of FXR upon dextrose-feeding in mice.
We have previously shown that Fgf15 expression was completely abolished upon bile salt sequestrant feeding in mice. Nevertheless, bile salt reabsorption was by no means completely prevented upon sequestrant treatment of chow-fed animals: 65% of the circulating bile salts were still reabsorbed in the presence of the sequestrant. Since the dextrose diet led to massively induced Fgf15 expression levels, we questioned whether these high expression levels could be maintained when the dextrose diet was supplemented with a bile salt sequestration. Fgf15 expression levels, however, were also abolished in mice fed a sequestrant-supplemented dextrose diet. Therefore, the increased expression of FXR-target genes upon dextrose feeding appeared, at least in part, to be dependent on a certain minimal concentration of bile salts. A study in rabbits in which the bile salt pool was completely removed indeed showed that a minimal amount of bile salts is needed for maintaining Fxr expression. A similar explanation might count for the activation of FXR: although bile salt levels are likely low in ilea of dextrose-fed mice, a certain threshold concentration of bile salts might have been present to activate FXR. Bile salt sequestration possibly decreases the bile salt concentration below this threshold in dextrose-fed mice leading to complete lack of FXR-activated expression of Fgf15.

To establish whether the effects of dextrose feeding on gene expression level were indeed mediated by FXR, intestinal Fxr<sup>−/−</sup> (iFxr<sup>−/−</sup>) mice and their WT littermates were also fed the dextrose diet. Interestingly, we did not find increased expression levels of Fgf15 in the distal ileum of dextrose-fed iFxr<sup>−/−</sup> mice compared to chow-fed iFxr<sup>−/−</sup> mice. These results demonstrate that dextrose feeding indeed exerts its effect on Fgf15 expression levels via FXR. Although the WT littermates of iFxr<sup>−/−</sup> mice showed a similar pattern regarding the expression of Fgf15 and Shp upon dextrose feeding as C57Bl/6J mice did, the response was less pronounced in the first. The response to dextrose feeding in mice may therefore be background-dependent.

The in vivo results were suggestive of activation of the FXR-signaling pathway by additional regulators. FXR transactivation capacity has been shown to be regulated by phosphorylation of its DNA-binding domain by protein kinase C (PKC). Additionally, FXR is post-translationally regulated via acetylation. We questioned whether FXR could be posttranslationally regulated by glucose-hexosamine-derived O-linked β-N-acetylg glucosamine modification (GlcNAcylation), a process similar to phosphorylation, recently described for LXR and FOXO1. GlcNAcylation is regulated by GlcNAc transferase (OGT) and GlcNAc case, two antagonistic enzymes, which respectively add and remove a GlcNAc moiety (from UDP-GlcNAc) to serine and/or threonine moieties on proteins. Preliminary results suggest that FXR is indeed GlcNAcylated in response to high glucose which possibly enhances the transactivation capacity of FXR or increased its affinity for ligands such as bile salts. Taken together, a diet high in the refined sugar dextrose reduces bile salt synthesis via activation of the FXR-signaling pathway, possibly by GlcNAcylation of the protein. Activation of the FXR-signaling pathway in both liver and intestine by this diet
should be taken into account when designing animal studies, especially when FXR-signaling pathways are being addressed. Similar modes of action may underly the reported differences in bile salt pool sizes and synthesis rates in human subjects. In view of the recently identified roles of bile salts in control of a variety of metabolic processes \(^ {12}\), these effects may contribute to or even explain diet/induced effects on metabolism.
REFERENCE LIST


Dextrose feeding and bile salt metabolism