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Joanne Hoogerland, Yu Lei, Justina Wolters, Jan Freark Boer, Trijnie Bos, et al.. Glucose-6-Phosphate Regulates Hepatic Bile Acid Synthesis in Mice. *Hepatology*, 2019, 10.1002/hep.30778 . inserm-02379171

HAL Id: inserm-02379171

<https://inserm.hal.science/inserm-02379171>

Submitted on 25 Nov 2019

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Glucose-6-Phosphate Regulates Hepatic Bile Acid Synthesis in Mice

Joanne A. Hoogerland,¹ Yu Lei,¹ Justina C. Wolters,¹ Jan Freark de Boer,^{1,2} Trijnie Bos,¹ Aycha Bleeker,¹ Niels L. Mulder,¹ Theo H. van Dijk,² Jan A. Kuivenhoven,¹ Fabienne Rajas,³ Gilles Mithieux,³ Rebecca A. Haeusler,⁴ Henkjan J. Verkade,¹ Vincent W. Bloks,¹ Folkert Kuipers,^{1,2} and Maaïke H. Oosterveer¹

It is well established that, besides facilitating lipid absorption, bile acids act as signaling molecules that modulate glucose and lipid metabolism. Bile acid metabolism, in turn, is controlled by several nutrient-sensitive transcription factors. Altered intrahepatic glucose signaling in type 2 diabetes associates with perturbed bile acid synthesis. We aimed to characterize the regulatory role of the primary intracellular metabolite of glucose, glucose-6-phosphate (G6P), on bile acid metabolism. Hepatic gene expression patterns and bile acid composition were analyzed in mice that accumulate G6P in the liver, that is, liver-specific glucose-6-phosphatase knockout (*L-G6pc*^{-/-}) mice, and mice treated with a pharmacological inhibitor of the G6P transporter. Hepatic G6P accumulation induces sterol 12 α -hydroxylase (*Cyp8b1*) expression, which is mediated by the major glucose-sensitive transcription factor, carbohydrate response element-binding protein (ChREBP). Activation of the G6P-ChREBP-CYP8B1 axis increases the relative abundance of cholic-acid-derived bile acids and induces physiologically relevant shifts in bile composition. The G6P-ChREBP-dependent change in bile acid hydrophobicity associates with elevated plasma campesterol/cholesterol ratio and reduced fecal neutral sterol loss, compatible with enhanced intestinal cholesterol absorption. **Conclusion:** We report that G6P, the primary intracellular metabolite of glucose, controls hepatic bile acid synthesis. Our work identifies hepatic G6P-ChREBP-CYP8B1 signaling as a regulatory axis in control of bile acid and cholesterol metabolism. (HEPATOLOGY 2019;0:1-14).

Bile acids facilitate absorption of dietary lipids and fat-soluble vitamins in the intestine, but also act as signaling molecules that control glucose, lipid, and energy metabolism.⁽¹⁾ Bile acid metabolism is known to be perturbed in conditions of uncontrolled hyperglycemia and insulin resistance (IR).^(2,3) Bile acid synthesis from cholesterol occurs exclusively in the liver and comprises multiple biochemical reactions initiated by cholesterol 7 α -hydroxylase (CYP7A1), the rate-controlling enzyme in the

“classic” pathway of primary bile acid synthesis. Sterol 12 α -hydroxylase (CYP8B1) subsequently generates 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid; CA) as an end product.^(2,4,5) As a consequence, hepatic CYP8B1 activity determines the contribution of CA produced in the classic pathway relative to 3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid (chenodeoxycholic acid; CDCA). CDCA, in contrast to CA, can also be generated by an “alternative” pathway starting with 27-hydroxylation of cholesterol.⁽⁶⁾ CDCA is

Abbreviations: Acc, acetyl-CoA carboxylase; Ac-H3, acetylated histone 3; Ac-H4, acetylated histone 4; Acly, ATP citrate lyase; bp, base pair; C4, 7 α -hydroxy-4-cholesten-3-one; CA, cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid); CDCA, chenodeoxycholic acid (3 α ,7 α -dihydroxy-5 β -cholan-24-oic acid); ChIP, chromatin immunoprecipitation; ChREBP, carbohydrate response element-binding protein (Mlx1p1); Cyp2c70, cytochrome P450, family 2, subfamily c, polypeptide 70; Cyp7a1, cholesterol 7 α -hydroxylase; Cyp7b1, oxysterol 7 α -hydroxylase; Cyp8b1, sterol 12 α -hydroxylase; Cyp27a1, sterol 27-hydroxylase; DCA, deoxycholic acid; FoxO, forkhead box O; FOXO1, forkhead box protein O1; Fxr, farnesoid X receptor; G6P, glucose-6-phosphate; G6PC, glucose-6-phosphatase, catalytic subunit; GC, gas chromatography; GSD I, glycogen storage disease type 1; H3/4, histone 3 and 4; Hnf4 α , hepatocyte nuclear factor 4 alpha; IgG, immunoglobulin G; IHH, immortalized human hepatocyte; IR, insulin resistance; L-PK, L-type pyruvate kinase; Lrh-1, liver receptor homolog 1; Mafg, MAF BZIP transcription factor G; MCA, muricholic acid (3 α ,6 β ,7 α -trihydroxy-5 β -cholanoic acid and 3 α ,6 β ,7 β -trihydroxy-5 β -cholanoic acid); Shp, small heterodimer partner; shRNA, short hairpin RNA; siChREBP, ChREBP small interfering RNAs; WT, wild type.

Received August 27, 2018; accepted May 15, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30778/supinfo.

Supported by an unrestricted research grant from DSM Nutritional Products (Kaiseraugst, Switzerland). M.H.O. is the recipient of a VIDI grant from the Dutch Scientific Organization and holds a Rosalind Franklin Fellowship from the University of Groningen. R.A.H. is supported by R01HL125649 from the National Institutes of Health. F.K. is supported by CardioVasculair Onderzoek Nederland (CVON2012-03).

efficiently converted to hydrophilic C6-hydroxylated muricholic acids (MCAs) in rodents, but not in humans.⁽⁶⁾ Primary bile acid species are secreted into the intestine, where they can be converted by microbial actions to secondary bile acids with distinct physicochemical properties⁽⁶⁾ that determine their efficacy to promote fat and cholesterol absorption as well as their signaling functions.⁽¹⁾

Bile acid synthesis is increased during postprandial periods and reduced upon fasting.⁽⁷⁾ Insulin and glucose have both been reported to induce the expression of *CYP7A1* in cultured hepatocytes.^(8,9) Moreover, insulin suppresses whereas glucose induces the expression of *Cyp8b1*.^(9,10) Insulin-induced suppression of *Cyp8b1* is mediated by the transcription factor, forkhead box protein O1 (FOXO1).⁽⁴⁾ Under insulin-resistant conditions, constitutive FOXO1 activation shifts the composition of the bile acid pool toward an increased contribution of CA and its hydrophobic microbial metabolite, 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (deoxycholic acid; DCA).⁽⁴⁾ Accordingly, we and others have shown that IR is associated with an increase in CA synthesis^(2,4,5) and a more hydrophobic bile acid pool in humans.⁽²⁾ IR is generally associated with hyperglycemic episodes, enhancing intrahepatic glucose metabolism.^(11,12)

Here, we characterized the direct regulatory role of intrahepatic glucose on bile acid synthesis. After being taken up by hepatocytes, glucose is immediately converted into glucose-6-phosphate (G6P), the primary

intracellular metabolite of glucose that acts as a signaling molecule.⁽¹²⁾ Glycogen storage disease type 1 (GSD I) is an inborn error of carbohydrate metabolism caused by mutations in the glucose-6-phosphatase (glucose-6-phosphatase, catalytic subunit; *G6PC*) gene (GSD Ia) or the glucose-6-phosphate transporter, *SLC37A4* (GSD Ib). GSD I is characterized by a strong accumulation of G6P inside hepatocytes and, importantly, low fasting glucose and insulin levels.⁽¹³⁾ We took advantage of this unique feature to evaluate the effects of intracellular glucose versus blood glucose and insulin and hence to selectively establish the effects of intra- versus extrahepatic glucose on bile acid metabolism. Our data show that, in mice, intrahepatic G6P regulates bile acid metabolism by a carbohydrate response element-binding protein (ChREBP; also known as Mlx1pl)-dependent induction of CYP8B1, resulting in an increased hydrophobicity of biliary bile acids and reduced fecal cholesterol loss. On the other hand, hepatic CYP7A1 expression was regulated by extrahepatic (blood) glucose rather than intrahepatic G6P.

Materials and Methods

ANIMALS

Male adult (8–12 weeks) B6.*G6pc*^{lox/lox} and B6.*G6pc*^{lox/lox}.SA^{creERT2/w} mice,⁽¹⁴⁾ male *L-FoxO1,3,4*^{-/-} and *L-FoxO1,3,4*^{+/-} mice (18–20 weeks old),⁽¹⁵⁾ and

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.30778

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the ¹Department of Pediatrics; ²Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands; ³Institut National de la Santé et de la Recherche Médicale, U1213, Université Claude Bernard Lyon, Villeurbanne, France; ⁴Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Maaïke H. Oosterveer, Ph.D.
Department of Pediatrics
University Medical Center Groningen
Internal Zip Code CA84

Hanzeplein 1
9713 GZ Groningen, The Netherlands
E-mail: m.h.oosterveer@umcg.nl
Tel.: +31 (0)50 361 1253

C57BL/6 mice (12–13 weeks old, local breeding) were housed in a light- and temperature-controlled facility and fed a standard laboratory chow diet (RMH-B; AB-diets, Woerden, The Netherlands). Liver-specific *G6pc*-deficient mice (*L-G6pc*^{-/-}) and wild-type (WT) littermates (*L-G6pc*^{+/+}) were generated as described.⁽¹⁴⁾ For tissue collection, mice were sacrificed by cardiac puncture 10 days after the last tamoxifen injection in nonfasted conditions, unless stated otherwise. In separate experiments requiring bile collection, mice were anesthetized by intraperitoneal injection of Hypnorm (10 mL/kg; Janssen Pharmaceuticals, Tilburg, The Netherlands) and diazepam (10 mg/kg; Actavis, Baarn, The Netherlands), the bile duct was ligated, the gallbladder was cannulated, and bile was collected for 30 minutes.

Male *L-FoxO1,3,4*^{-/-}, *L-FoxO1,3,4*^{+/-} mice and C57BL/6 mice were equipped with a permanent catheter in the right jugular vein for infusions and were allowed a recovery period of at least 4 days. Mice were kept in experimental cages during the experiment and the preceding fasting period, allowing frequent collection of tail blood samples. After overnight fasting, mice were infused for 6 hours with S4048 (a generous gift from Sanofi-Aventis, Frankfurt, Germany, 5.5 mg/mL of phosphate-buffered saline (PBS) with 6% dimethyl sulfoxide at 0.135 mL/h) or vehicle. Blood glucose concentrations were measured in tail blood every 30 minutes during the experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

CONSTRUCTION, PRODUCTION, AND *IN VIVO* TRANSDUCTION OF SHORT HAIRPIN RNAs USING SELF-COMPLEMENTARY ADENO-ASSOCIATED VIRUS VECTORS

To construct the self-complementary (scAAV) adeno-associated virus (AAV) 2/8-U6-shChREBP, the scAAV2-LP1-hFIXco backbone vector was restricted with BamHI and BbsI and the 3,493-bp (base pair) fragment was isolated and ligated. Restriction with BamHI and BbsI removed hFIXco and partially deleted the LP1 promoter, and the U6 promoter driving the expression of the construct was cloned into the vector in antisense orientation. Short hairpin RNA (shRNA) construct directed against ChREBP α/β and scramble

construct were ordered as oligonucleotides (shRNA; 5'-aat tcA AAA AAT GTA GTT TGA AGA TGT GGG TCT CGA GAC CCA CAT CTT CAA ACT ACA TC-3' and 3'-ggc caG ATG TAG TTT GAA GAT GTG GGT CTC GAG ACC CAC ATC TTC AAA CTA CAT TTT TT-5', scramble; 5'-aat tcG TTG TAA GTG GAG GTT TAA GTC TCG AGA CTT AAA CCT CCA CTT ACA ACA CCG GT-3' and 3'-ggc caA CCG GTG TTG TAA GTG GAG GTT TAA GTC TCG AGA CTT AAA CCT CCA CTT ACA AC-5') and cloned into the vector using EcoRI and AgeI. Production, purification, and titration of these AAV2/8 viruses encoding the shRNA directed against ChREBP α and ChREBP β and the scrambled control were performed as described.⁽¹⁶⁾ Mice were injected with 5×10^{12} virus particles per mouse and sacrificed 30 days after virus administration.

IMMORTALIZED HUMAN HEPATOCYTE GLUCOSE STIMULATION AND TRANSIENT TRANSFECTION ASSAYS

For glucose stimulation, immortalized human hepatocyte (IHH) cells⁽¹⁷⁾ were glucose-deprived in Dulbecco's modified Eagle's medium (Thermo Scientific, Landsmeer, The Netherlands) without glucose, supplemented with 1% penicillin/streptomycin, 0.1% fatty-acid-free bovine serum albumin, 16 mU/mL of insulin, 2 mM of GlutaMAX (Thermo Scientific) and 1 mM of glucose for 16 hours. Cells were subsequently incubated with low (1 mM) or high (11 mM) glucose concentrations for 24 hours. For transient transfection assays, IHH cells were transfected for 48 hours using Lipofectamine RNAiMAX Reagent (Thermo Scientific), according to the manufacturer's protocol, with 50 nM of ChREBP small interfering RNAs (siChREBP)⁽¹⁸⁾ or control small interfering RNA (siRNA; #12935-100; Thermo Scientific) in Williams E medium containing 2 mM of glutamine and supplemented with 2% fetal calf serum, 20 mU/mL of insulin, and 50 nM of dexamethasone.

ANALYTICAL PROCEDURES

Blood glucose was measured using a One Touch Ultra glucose meter (LifeScan, Inc., Milpitas, CA). Plasma insulin and glucagon were analyzed using commercially available enzyme-linked immunosorbent assays (Chrysal Chem, Downers Grove, IL and Alpco Diagnostics,

Salem, NH, respectively). To quantify plasma plant sterols, plasma lipids were extracted according to Folch lipid extraction,⁽¹⁹⁾ methanolized, silylated, and analyzed with gas chromatography (GC). Commercially available kits were used to analyze plasma levels of triglycerides (Roche, Mannheim, Germany) and plasma levels of total and free cholesterol (Roche and DiaSys, Holzheim, Germany, respectively). Hepatic glycogen and G6P content was determined as described.⁽²⁰⁾ Plasma and biliary bile acid composition were quantified using liquid chromatography-mass spectrometry; fecal bile acid composition was quantified using capillary GC as described.⁽²¹⁾ The hydrophobicity index of biliary bile acids was calculated according to Heuman.⁽²²⁾ Fecal cholesterol and its derivatives were trimethylsilylated with pyridine, N,O-Bis(trimethylsilyl) trifluoroacetamide, and trimethylchlorosilane (ratio 50:50:1) and quantified by GC.

GENE EXPRESSION ANALYSIS

Total RNA was isolated using TRI-Reagent (Sigma-Aldrich Corp., St. Louis, MO). Complementary DNA was obtained by reverse transcription and amplified using primers and probes listed in Supporting Table S6. mRNA levels were calculated based on a dilution curve, expressed relative to *36b4* for liver and *18S* for IHH cells, and normalized to their controls.

TARGETED PROTEOMICS

Targeted proteomics was applied in homogenized liver tissue by the isotopically labeled peptide standards (G6PC; GLGVDLLWTLEK, CYP8B1; VFGYQSVDGDHR, ChREBP; LGFDTLHGLVSTLSAQPSLK, CYP7A1; LSSASLNIR, oxysterol 7 α -hydroxylase [CYP7B1]; YITFVLNPFQYQYVTK, sterol 27-hydroxylase [CYP27A1]; LYPVVPTNSR, cytochrome P450, family 2, subfamily c, polypeptide 70 [CYP2C70]; TDSSLLSR), containing ¹³C-labeled lysine/arginine (PolyQuant GmbH, Bad Abbach, Germany), according to the workflow described.⁽²¹⁾ The following alterations were made: Lipids were extracted from liver homogenates with diethyl ether before the proteomics workflow and the concentrations were related to the total peptide content, which was determined by a colorimetric peptide assay after tryptic digestion and SPE cleanup (Thermo Scientific). Concentrations of endogenous peptides were calculated from the known concentration of the

standard and expressed in fmol/ μ g of total peptide and expressed relative to the values in the control group.

CELL REPORTER ASSAYS

CV1 cells (ATCC) were transiently transfected using FuGENE 6 Transfection Reagent (Promega, Leiden, The Netherlands). pCMVS4/ChREBP α , pCMVS4/ChREBP β , and pCMVS4/Mlx (kind gifts from M. Herman) were shuttled to pcDNA3.1 using cloning PCR. Primers are listed in Supporting Table S6. The human or mouse PGL3/Cyp8b1 promoter luciferase reporter (−623/+364 bp and −1,582/+115 bp respectively, kind gifts from J. Chiang) or minimal promoter PGL3/ChREBP luciferase reporter (−40/+12; kind gift from H. Towle) was cotransfected with pcDNA3.1/ChREBP α , pcDNA3.1/ChREBP β , pcDNA3.1/Mlx, pcDNA3.1/Hnf4 α (hepatocyte nuclear factor 4 α), or a combination for 48 hours. Cell lysis and luciferase assays were performed using a Dual-Luciferase Reporter Assay System (Promega).

CHROMATIN IMMUNOPRECIPITATION/qPCR

Chromatin immunoprecipitation (ChIP) analysis was performed as described,⁽²³⁾ with the following modifications. Before cross-linking with 1% formaldehyde, livers were homogenized in PBS and cross-linked with 0.5 M of di(N-succinimidyl) glutarate for 45 minutes at room temperature. Immunoprecipitation of samples was performed overnight at 4°C with 3 μ g of ChREBP (Novus), acetylated histone 4 (Ac-H4; Millipore), acetylated histone 3 (Ac-H3; Millipore), HNF4A (Santa Cruz), or normal rabbit immunoglobulin G (IgG) antibody (Santa Cruz). DNA was purified using the PCR Clean-up Extraction Kit (Macherey-Nagel), after which qPCR was performed. Primers are listed in Supporting Table S7.

STATISTICAL ANALYSIS

Statistical analysis was performed using BrightStat software. Differences between two or multiple groups were tested by Mann-Whitney U test or Kruskal-Wallis H test followed by post-hoc Conover pairwise comparisons, respectively. *P* values <0.001 (***), 0.001–0.01 (**), and 0.01–0.05 (*) were considered significant. Correlations were analyzed by Spearman's

correlations coefficient using SPSS software (version 24.0 for Windows; SPSS, Chicago, IL).

Results

HEPATIC G6P ACCUMULATION MODIFIES BILE ACID SYNTHESIS

To establish the selective impact of intracellular glucose on hepatic bile acid synthesis, C57BL/6 mice were infused during 6 hours with S4048, a selective inhibitor of the G6P transporter, SLC37A4, thereby acutely inducing GSD Ib in liver.⁽²⁴⁾ S4048 reduced blood glucose concentrations and increased hepatic G6P and glycogen contents, whereas glucagon-to-insulin ratios were increased (Supporting Table S1). Hepatic mRNA levels of genes involved in bile acid synthesis showed a marked increase in *Cyp8b1* expression, whereas *Cyp7a1* and *Cyp27a1* expression were reduced and *Cyp7b1* and *Cyp2c70* expression remained unchanged (Fig. 1A). S4048 infusion did not alter biliary bile acid composition or plasma bile acid levels (Fig. 1B and Supporting Fig. S1A). Presumably, the time frame of S4048 infusion is too short to translate into altered bile acid composition: The cycling time of the murine bile acid pool is approximately 4–5 hours, and only 5% of biliary bile acids is derived from *de novo* synthesis.⁽²⁵⁾

Next, we performed similar analyses in mice with sustained hepatic G6P accumulation, that is, fasted liver-specific *G6pc* knockout (L-*G6pc*^{-/-}) mice,⁽¹⁴⁾ which exhibited increased glucagon-to-insulin ratios (Supporting Table S1). In these animals, hepatic *Cyp8b1* mRNA levels were also strongly elevated whereas expression of *Cyp7a1*, *Cyp27a1*, *Cyp7b1*, and *Cyp2c70* was significantly lower as compared to L-*G6pc*^{+/+} littermates (Fig. 1C). Altered expression of bile acid synthesis genes in L-*G6pc*^{-/-} mice did translate into a relative increase in CA and CA-derived bile acids (Fig. 1D; Table 1). Similar increases in CA and DCA and concomitant decreases in CDCA and CDCA-derived MCAs were observed in plasma and feces from L-*G6pc*^{-/-} mice (Supporting Fig. S1B,C; Supporting Table S2). Biliary bile acid secretion rates and plasma bile acid concentrations were not different between L-*G6pc*^{-/-} and L-*G6pc*^{+/+} mice (Fig. 1E; Table 1).

Interestingly, hepatic CYP7A1 protein levels, but not *Cyp7a1* mRNA levels, were lower in

L-*G6pc*^{-/-} mice as compared to WT littermates (Fig. 1F), and hepatic CYP7A1 protein levels positively correlated with blood glucose levels (Fig. 1G). Similar correlations were observed for plasma 7 α -hydroxy-4-cholesten-3-one (C4) levels, the product of CYP7A1 and a marker of its activity⁽²⁶⁾ (Fig. 1G). C4 levels were significantly lower in fasted L-*G6pc*^{-/-} mice compared to WT littermates (Supporting Fig. S1D). On the other hand, hepatic CYP8B1 mRNA and protein levels were significantly increased in L-*G6pc*^{-/-} mice irrespective of the feeding state (Fig. 1H).

ChREBP MEDIATES THE INDUCTION OF *Cyp8b1* IN RESPONSE TO HEPATIC G6P ACCUMULATION

To elucidate the mechanism of G6P-dependent control of *Cyp8b1*, we performed S4048 infusions in mice lacking forkhead box O (FoxO) 1,3,4 expression in hepatocytes and in mice with reduced hepatic expression of the G6P-sensitive transcription factor, ChREBP, which is activated in GSD Ia and GSD Ib.^(12,24,27) We confirmed that FoxOs control basal *Cyp8b1* expression,⁽⁴⁾ and found that the S4048-mediated induction of *Cyp8b1* was absent in L-*FoxO1,3,4*^{-/-} mice (Fig. 2A). Interestingly, induction of *Cyp8b1* upon S4048 infusion was also abolished in mice with reduced hepatic *Chrebp α* and *Chrebp β* expression (Fig. 2A and Supporting Fig. S2B). Similar effects were observed on *Cyp8b1* mRNA and protein levels upon hepatic ChREBP knockdown in L-*G6pc*^{-/-} mice (Fig. 2B,C). As shown above, S4048 infusion and hepatic *G6pc* deficiency caused reductions in *Cyp7a1* expression. However, these reductions were not reversed by knockout of FoxOs or knockdown of hepatic ChREBP (Supporting Fig. S2A,C,D). Thus, ChREBP mediates the induction of hepatic *Cyp8b1*, but not the repression of *Cyp7a1*, in liver-specific GSD Ia and GSD Ib mice.

We also tested whether established transcriptional regulators of *Cyp8b1* are altered in response to hepatic G6P-ChREBP signaling. Hepatic expression of nuclear receptor subfamily 1 group H member 4 (farnesoid X receptor; *Fxr*), nuclear receptor subfamily 5 group A member 2 (liver receptor homolog 1; *Lrh-1*), *Hnf4a* (*Hnf4 α*), and MAF BZIP transcription factor G (*Mafg*) remained largely unaffected upon hepatic G6P accumulation (Supporting Fig. S2E,F), indicating that these factors cannot explain the

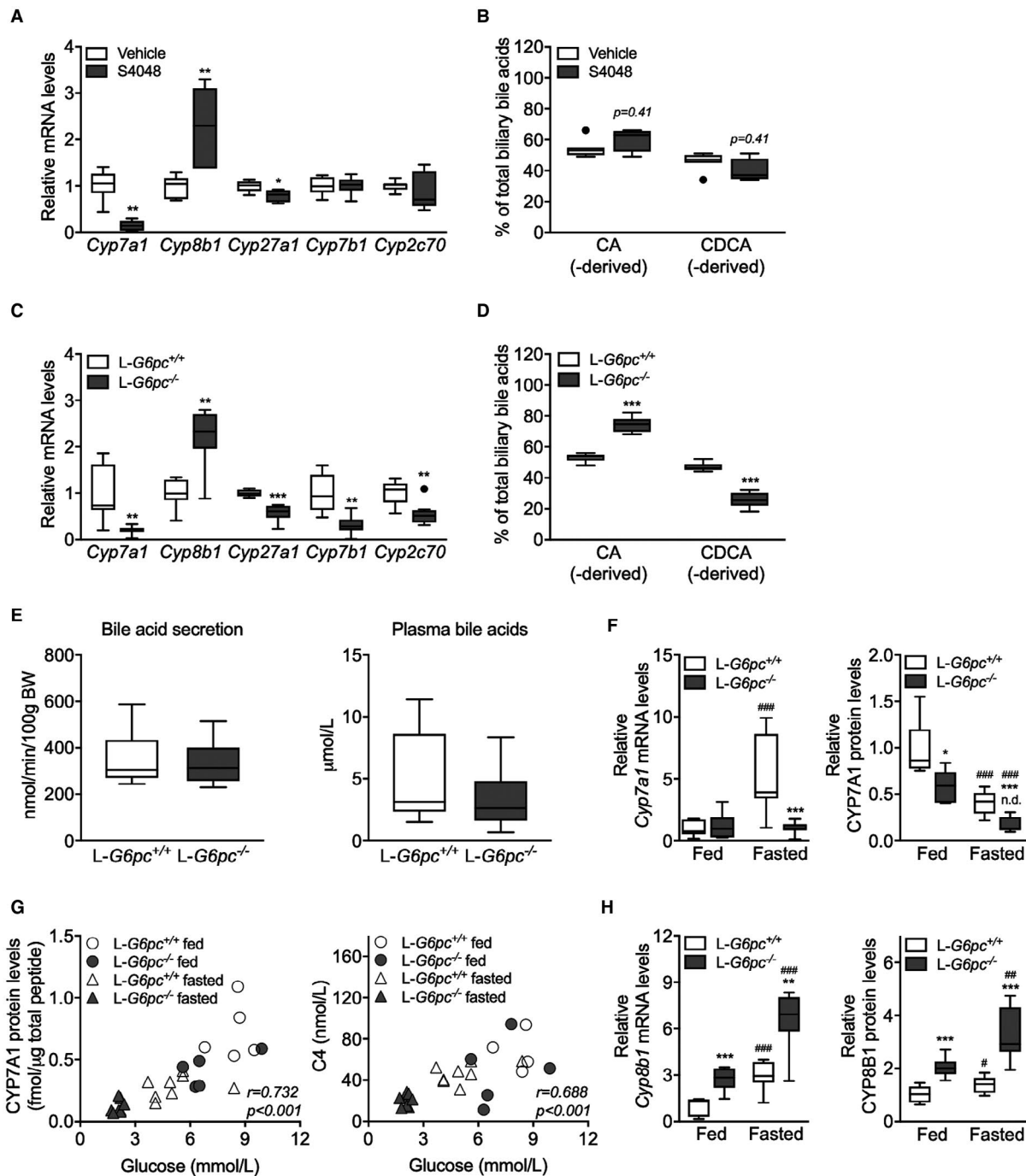


FIG. 1. Hepatic G6P accumulation modifies bile acid synthesis. (A) Hepatic mRNA levels of bile acid synthesis genes and (B) biliary bile acid composition in C57BL/6 mice infused with S4048 or vehicle ($n = 7$). (C) Hepatic mRNA levels of bile acid synthesis genes in overnight fasted *L-G6pc^{-/-}* mice and *L-G6pc^{+/+}* mice ($n = 7-8$). (D) Biliary bile acid composition. (E) Biliary bile acid secretion and plasma bile acid levels in *L-G6pc^{-/-}* and *L-G6pc^{+/+}* mice ($n = 7-8$). (F) Hepatic mRNA and protein levels of CYP7A1 in *L-G6pc^{-/-}* mice and *L-G6pc^{+/+}* mice in either fed state or after an overnight fast ($n = 7-8$). (G) Correlation between blood glucose levels and hepatic CYP7A1 protein levels and correlation between blood glucose levels and plasma C4 levels in *L-G6pc^{-/-}* mice and *L-G6pc^{+/+}* mice in either fed state or after an overnight fast ($n = 7-8$). (H) Hepatic mRNA and protein levels of CYP8B1 in *L-G6pc^{-/-}* mice and *L-G6pc^{+/+}* mice in either fed state or after an overnight fast ($n = 7-8$). Data represent Tukey box plots. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. See also Supporting Fig. S1 and Supporting Tables S1, S2, and S3. Abbreviation: BW, body weight.

TABLE 1. Bile Characteristics in Chow-Fed Male *L-G6pc*^{-/-} Mice and WT Littermates

| | <i>L-G6pc</i> ^{+/+} | <i>L-G6pc</i> ^{-/-} | <i>P</i> Value |
|---|------------------------------|------------------------------|----------------|
| | Median (Range) | Median (Range) | |
| Body weight (g) | 28.4 (21.5-29.9) | 27.5 (25.3-32.5) | 0.645 |
| Bile flow (μL/min/100 g BW) | 12.2 (8.5-15.0) | 14.6 (12.3-18.5) | 0.021 |
| Bile acid secretion (nmol/min/100 g BW) | 305.1 (244.3-587.5) | 313.3 (229.7-514.5) | 0.878 |
| Phospholipid secretion (nmol/min/100 g BW) | 81.1 (75.3-164.9) | 109.7 (93.2-159.4) | 0.038 |
| Cholesterol secretion (nmol/min/100 g BW) | 11.6 (9.5-17.1) | 12.7 (10.7-18.6) | 0.161 |
| Bile acid species secretion (nmol/min/100 g BW) | | | |
| CA | 3.88 (1.18-7.15) | 3.13 (0.86-6.07) | 0.959 |
| GCA | 0.58 (0.21-1.21) | 0.45 (0.31-0.75) | 0.279 |
| TCA | 150.17 (124.91-292.16) | 226.74 (164.34-344.06) | 0.028 |
| TUDCA | 5.11 (3.86-11.26) | 3.92 (2.58-7.33) | 0.105 |
| TCDCA | 2.01 (1.61-4.98) | 2.63 (1.32-5.84) | 0.645 |
| TDCA | 6.59 (3.40-13.85) | 9.18 (2.14-15.74) | 0.442 |
| THDCA | 2.27 (0.56-3.89) | 1.40 (0.79-2.17) | 0.279 |
| α-MCA | 0.40 (0.14-1.48) | 0.31 (0.00-1.18) | 0.279 |
| Tα-MCA | 11.37 (9.18-36.33) | 12.48 (6.90-29.22) | 0.878 |
| β-MCA | 2.65 (0.52-5.13) | 0.42 (0.00-1.17) | 0.007 |
| Tβ-MCA | 117.01 (87.53-212.07) | 52.38 (30.24-117.05) | 0.002 |
| ω-MCA | 2.61 (0.84-7.07) | 0.84 (0.38-1.78) | 0.005 |

Abbreviations: BW, body weight; GCA, glycocholic acid; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THDCA, taurohyodeoxycholic acid; α-MCA, alpha-muricholic acid; Tα-MCA, tauro-alpha-muricholic acid; β-MCA, beta-muricholic acid; Tβ-MCA, tauro-beta-muricholic acid; ω-MCA, omega-muricholic acid. *P* values <0.05 are marked in bold.

induction of *Cyp8b1* in response to G6P accumulation. We noted that the expression of some of these factors was reduced exclusively when ChREBP was knocked down in S4048-treated or *L-G6pc*^{-/-} mice (Supporting Fig. S2E,F), though the biological significance of this is unclear. Hepatic nuclear receptor subfamily 0, group B, member 2 (small heterodimer partner; *Shp*) mRNA levels were lower in S4048-treated and *L-G6pc*^{-/-} mice as compared to their controls, and were further reduced in response to hepatic ChREBP knockdown in *L-G6pc*^{+/+} and *L-G6pc*^{-/-} mice (Supporting Fig. S2E,F). Thus, *Fxr*, *Shp*, *Lrh-1*, *Hnf4α*, and *Mafg* mRNA levels did not consistently follow the pattern of CYP8B1 expression in response to hepatic G6P-ChREBP signaling (Fig. 2A-C and Supporting Fig. S2E,F).

***Cyp8b1* INDUCTION BY G6P-ChREBP IS CELL AUTONOMOUS AND OCCURS IN HUMAN CELLS**

To assess whether this G6P-ChREBP-dependent modulation of CYP8B1 is conserved in human

hepatocytes, we exposed IHH cells, that are glucose responsive,⁽¹⁷⁾ to high- and low-glucose culture media. We also transfected them with siChREBP or scrambled siRNAs under conditions of high-glucose exposure. As expected, high glucose induced *CHREBPα* mRNA levels, as well as the expression of its target genes, *CHREBPβ*, L-type pyruvate kinase (*L-PK*) and apolipoprotein C3 (*APOC3*; Supporting Fig. S2G), whereas siChREBP reduced all of these (Fig. 2D). Combined with the *in vivo* data shown above, these *in vitro* findings demonstrate that ChREBP activity is necessary and sufficient for CYP8B1 induction by intracellular glucose metabolism, in a cell-autonomous manner.

On the other hand, *CYP7A1* expression in IHHs was not similarly regulated. Consistent with published data,⁽⁹⁾ *CYP7A1* mRNA levels were induced upon high-glucose exposure (Supporting Fig. S2G). However, siChREBP did not reverse this effect; in fact, it amplified it (Supporting Fig. S2G). Thus, *CYP7A1* mRNA levels are induced in response to glucose exposure in IHHs, but not through ChREBP. *CYP7B1* was not regulated by glucose or siChREBP

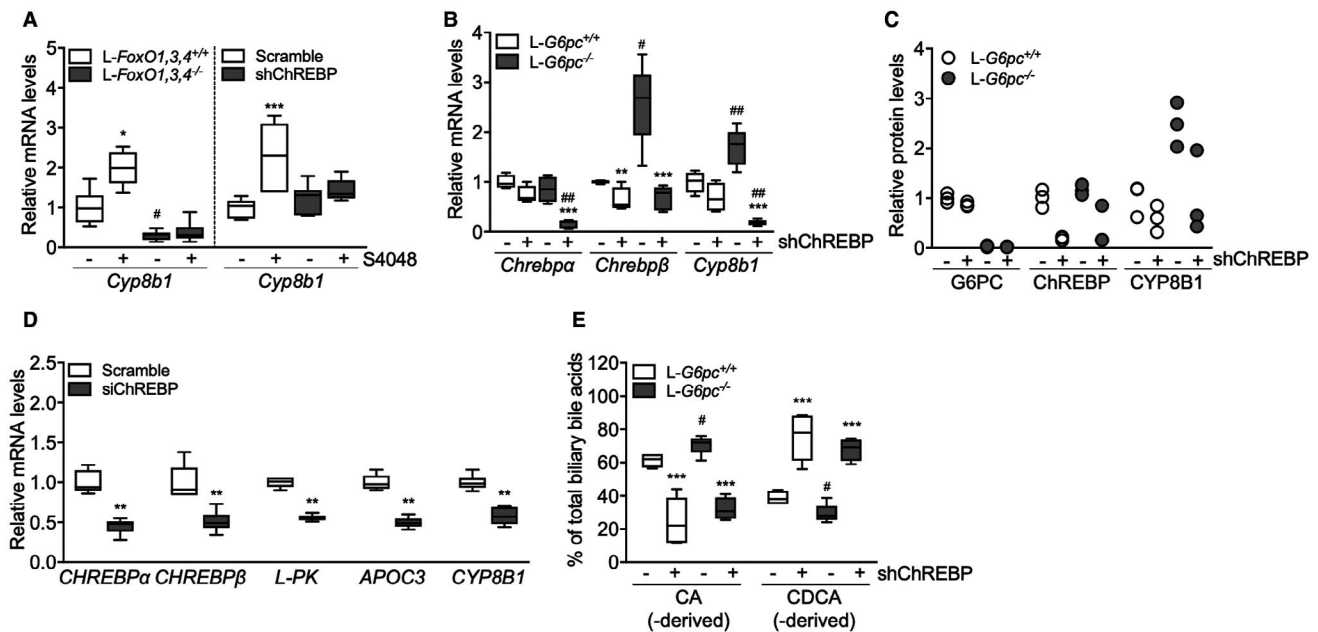


FIG. 2. ChREBP mediates induction of *Cyp8b1* in response to hepatic G6P accumulation. (A) Hepatic mRNA levels of *Cyp8b1* in *L-FoxO1,3,4^{-/-}* and *L-FoxO1,3,4^{+/+}* mice and in C57BL/6 mice treated with either shChREBP or scrambled shRNA, infused with S4048 or vehicle ($n = 7-8$). (B) Hepatic mRNA levels in *L-G6pc^{-/-}* and *L-G6pc^{+/+}* mice, treated with either shChREBP or scrambled shRNA ($n = 4-6$). (C) Hepatic protein levels in *L-G6pc^{-/-}* and *L-G6pc^{+/+}* mice, treated with either shChREBP or scrambled shRNA ($n = 3$). (D) mRNA expression in IHH cells transfected with siChREBP or scramble after high (11 mM) glucose exposure for 24 hours ($n = 6$). (E) Biliary bile acid composition in *L-G6pc^{-/-}* and *L-G6pc^{+/+}* mice treated with either shChREBP or scrambled shRNA ($n = 4-5$). Data represent Tukey box plots. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ indicates significance compared to scrambled shRNA. # $P < 0.05$ indicates significance compared to WT littermates. See also Supporting Fig. S2 and Supporting Tables S4 and S5.

(Supporting Fig. S2G), and *CYP27A1* is not expressed by IHH cells.

HEPATIC G6P-ChREBP SIGNALING REGULATES BILE ACID COMPOSITION

Then, we evaluated whether G6P-ChREBP-dependent induction of *Cyp8b1* translated into qualitative changes in biliary and plasma bile acids. Hepatic G6P accumulation increased the contribution of biliary CA and DCA and increased plasma CA and DCA levels in *L-G6pc^{-/-}* mice whereas administration of shChREBP had the opposite effect (Fig. 2E and Supporting Fig. S2H; Supporting Tables S3, S4, and S5), consistent with the observed decrease in hepatic *Cyp8b1* expression (Fig. 2B,C and Supporting Fig. S2B). Combined, these data indicate that G6P-ChREBP induce qualitative changes in biliary and plasma bile acid composition through induction of hepatic *Cyp8b1* expression.

ChREBP DOES NOT DIRECTLY REGULATE HEPATIC *Cyp8b1* TRANSCRIPTION

We next investigated whether ChREBP directly regulates *Cyp8b1* transcription. Analysis of a hepatic ChREBP ChIP sequencing (ChIP-seq) data set⁽²⁸⁾ indicated potential regulation of *Cyp8b1* by ChREBP. Computational analysis revealed three putative ChREBP response elements similar to the ChREBP consensus sequence (CAYGYGnnnnnCRCRTG) and one element with an alternative sequence (GGGGGYGGGC) in the mouse *Cyp8b1* promoter (Fig. 3A). Cell reporter assays did not show transactivation of the murine or human *Cyp8b1* promoter by ChREBP α or ChREBP β , whereas both *Cyp8b1* reporters used were transactivated by Hnf4 α (Fig. 3B),⁽²⁹⁾ and the minimal acetyl-CoA carboxylase α (acetyl-CoA carboxylase; *Acc*) promoter⁽³⁰⁾ did show ChREBP responsiveness (Fig. 3B). In agreement with these findings, *in vivo* ChIP analysis did

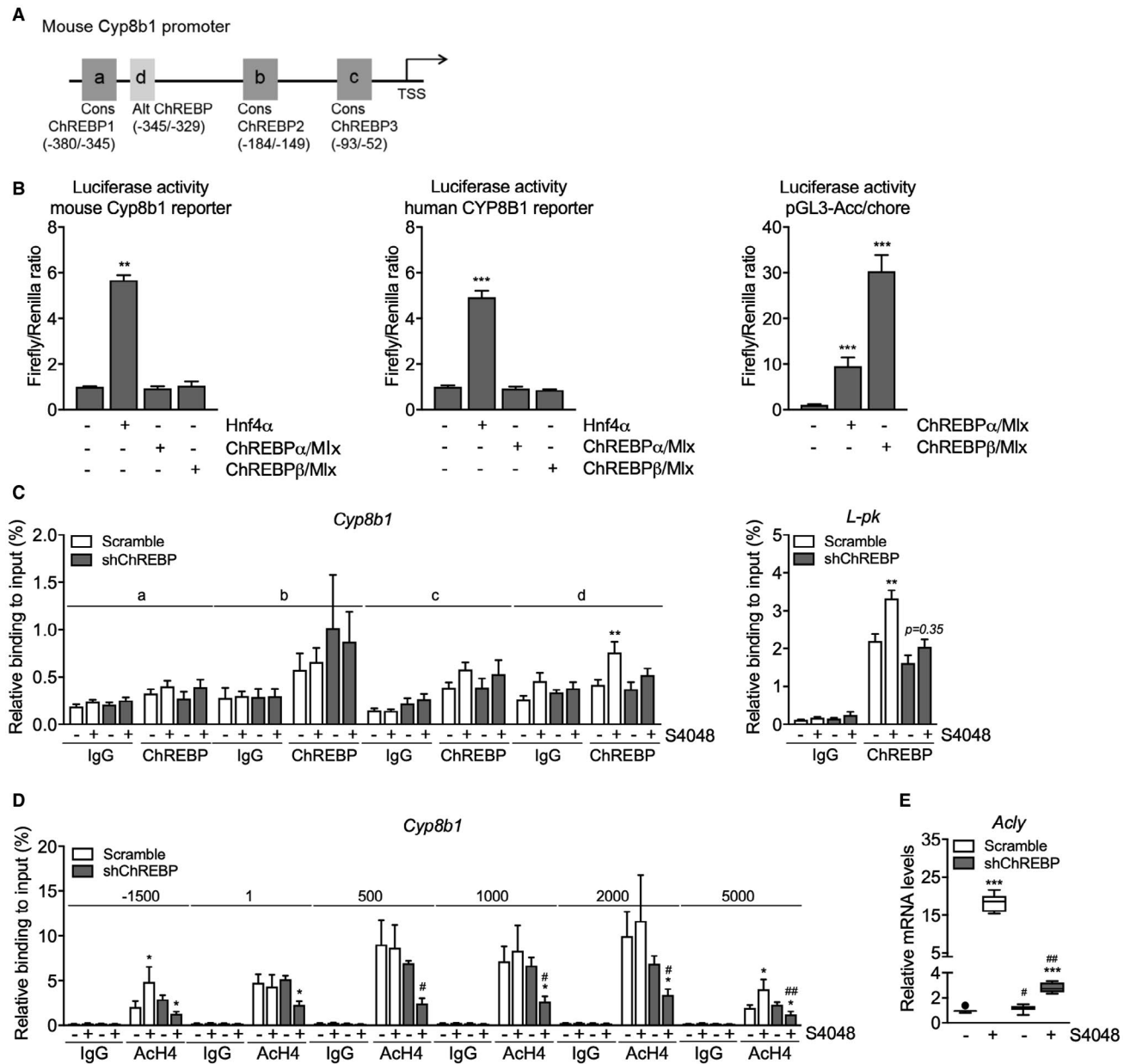


FIG. 3. ChREBP does not directly regulate hepatic *Cyp8b1* transcription. (A) Schematic presentation of putative consensus and alternative ChREBP response elements within the murine *Cyp8b1* promoter. (B) Luciferase activity for the murine and human CYP8B1 promoter reporter and minimal promoter ACC/chore after transfection with Hnf4 α , ChREBP α , and ChREBP β plasmids (n = 5-6). (C) *In vivo* ChIP analysis of the putative ChREBP response elements in the hepatic *Cyp8b1* and *L-pk* gene and (D) of acetylated histone H4 around the hepatic *Cyp8b1* gene in mice treated with either shChREBP or scrambled shRNA and infused with S4048 or vehicle (n = 7). (E) Hepatic mRNA levels of *Acly* in C57BL/6 mice treated with either shChREBP or scrambled shRNA, infused with S4048 or vehicle (n = 7-8). Data are represented as means \pm SEM. *** P < 0.001; ** P < 0.01; * P < 0.05 indicates significance compared to vehicle controls. ## P < 0.01; # P < 0.05 indicates significance compared to controls treated with scrambled shRNA. See also Supporting Fig. S3. Abbreviations: Alt, alternative; chore, carbohydrate response element; Cons, consensus; TSS, transcription start site.

not show a strong interaction of ChREBP with the putative response elements in the mouse *Cyp8b1* promoter whereas S4048 treatment promoted ChREBP

recruitment to the pyruvate kinase L/R (*L-pk*) promoter (Fig. 3A,C).⁽³¹⁾ Moreover, HNF4 α recruitment to the *Cyp8b1* and *L-pk* promoter regions was

not altered upon ChREBP knockdown, indicating that the effect of ChREBP was likely not mediated by increased HNF4 α binding to *Cyp8b1* (Supporting Fig. S3A). We confirmed that acetylated histone 3 and 4 (H3/4) mainly interacted with the transcribed region of the *Cyp8b1* promoter (Supporting Fig. S3B and Fig. 3D).⁽³²⁾ Interestingly, recruitment of Ac-H4 in the promoter region (–1,500 bp) and further downstream (+5,000 bp) in the *Cyp8b1* gene was induced upon hepatic G6P accumulation and reduced upon ChREBP knockdown in S4048-treated mice, whereas we did not observe clear changes in binding of Ac-H3 under conditions of combined hepatic ChREBP knockdown and G6P accumulation (Fig. 3D). Altogether, these findings demonstrate that induction of *Cyp8b1* expression by G6P-ChREBP is associated with increased recruitment of Ac-H4, but not of ChREBP, HNF4 α , or Ac-H3, to the *Cyp8b1* locus. These effects were

paralleled by a ChREBP-dependent induction of ATP citrate lyase (*Acl*) expression (Figs. 3E and 2D), the essential enzyme for glucose-induced histone acetylation *in vitro*.⁽³³⁾

G6P-ChREBP INCREASES BILIARY BILE ACID HYDROPHOBICITY AND REDUCES FECAL STEROL LOSS

A shift in the contribution of CA- versus CDCA-derived bile acids alters the hydrophobicity of the bile acid pool⁽²²⁾ and, in turn, changes the capacity for intestinal lipid solubilization and uptake.^(1,7,34–36) Induction of hepatic *Cyp8b1* expression and relative increase in CA and DCA in L-*G6pc*^{–/–} mice (Fig. 1C,D) increased the hydrophobicity index of the biliary bile acids entering the intestine (Fig. 4A) whereas hepatic ChREBP knockdown reduced this index

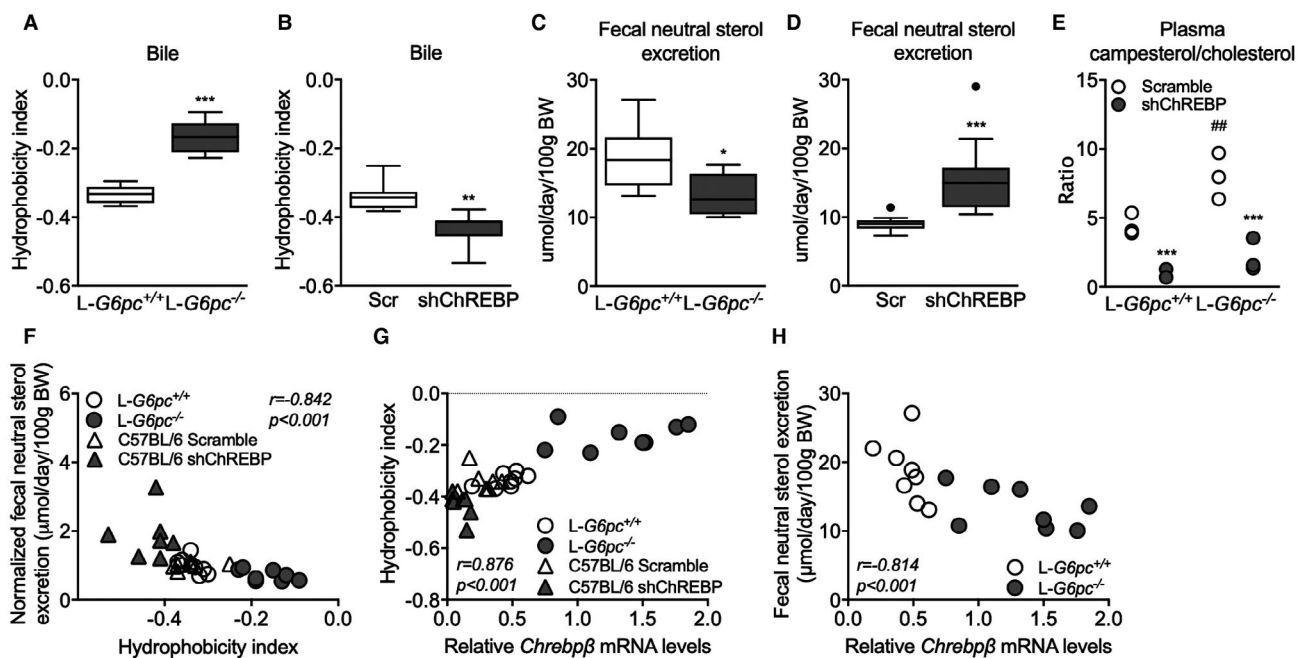


FIG. 4. G6P-ChREBP increases biliary bile hydrophobicity and reduces fecal sterol loss. (A) Bile hydrophobicity index of bile from L-*G6pc*^{–/–} and L-*G6pc*^{+/+} mice and (B) mice treated with either shChREBP or scrambled (Scr) shRNA (n = 7–8). (C) Fecal neutral sterol excretion of L-*G6pc*^{–/–} and L-*G6pc*^{+/+} mice (n = 8) and (D) mice treated with either shChREBP or scrambled shRNA (n = 14). (E) Plasma campesterol/cholesterol ratios in L-*G6pc*^{–/–} and L-*G6pc*^{+/+} mice treated with either shChREBP or scrambled shRNA (n = 3). (F) Correlation between bile hydrophobicity index and normalized fecal neutral sterol excretion and between (G) *Chrebp* mRNA levels and bile hydrophobicity index in L-*G6pc*^{–/–} and L-*G6pc*^{+/+} mice and mice treated with either shChREBP or scrambled shRNA (n = 7–8). (H) Correlation between *Chrebp* mRNA levels and fecal neutral sterol excretion in L-*G6pc*^{–/–} and L-*G6pc*^{+/+} mice (n = 8). Data represent Tukey box plots. ***P < 0.001; **P < 0.01; *P < 0.05 indicates significance compared to WT littermates or controls treated with scrambled shRNA. ##P < 0.01 indicates significance compared to WT littermates. See also Supporting Fig. S4. Abbreviation: BW, body weight.

(Fig. 4B). We confirmed that hepatic *Cyp8b1* expression was positively correlated to biliary bile acid hydrophobicity in *L-G6pc*^{-/-} mice⁽³⁷⁾ (Supporting Fig. S4A) and hypothesized that altered hydrophobicity in response to G6P-ChREBP-CYP8B1 signaling impacts intestinal sterol absorption.^(34,36) Hepatic *Cyp8b1* expression indeed negatively correlated with fecal neutral sterol excretion⁽³⁶⁾ (Supporting Fig. S4B). Fecal neutral sterol excretion was reduced in *L-G6pc*^{-/-} mice (Fig. 4C and Supporting Fig. S4C) and, as expected, increased upon hepatic ChREBP knockdown (Fig. 4D and Supporting Fig. S4C). The plasma campesterol/cholesterol ratio, a marker of intestinal cholesterol absorption,⁽³⁸⁾ showed similar patterns (Fig. 4E). Bile acid hydrophobicity and fecal neutral sterol excretion were found to be negatively correlated (Fig. 4F) and hepatic *Chrebp* mRNA expression showed a positive correlation to hydrophobicity index (Fig. 4G), whereas it was negatively correlated with fecal neutral sterol excretion (Fig. 4H). Fecal energy and fatty acid excretion remained unchanged in response to hepatic G6P accumulation or ChREBP knockdown (Supporting Fig. S4D,E).

Discussion

In the current study, we characterized an important regulatory role of glucose, independent of insulin,

in the control of hepatic bile acid synthesis. Using the monogenetic disease GSD I as a model to establish the contribution of intrahepatic glucose, we show that G6P controls hepatic bile acid synthesis through ChREBP-dependent induction of *Cyp8b1* in mice. Our findings furthermore indicate that the G6P-ChREBP axis regulates hepatic CYP8B1 expression through H4 acetylation dynamics. As a consequence, G6P-ChREBP signaling increases the relative abundance of CA-derived bile acids and induces physiologically relevant shifts in bile composition. We confirmed that the human *CYP8B1* gene is regulated by a similar mechanism. Importantly, our work also demonstrates the physiological relevance of this regulatory mechanism: The G6P-ChREBP-dependent change in bile acid hydrophobicity in mice associates with reduced fecal neutral sterol loss and lower plasma campesterol/cholesterol ratios, compatible with enhanced intestinal cholesterol absorption (Fig. 5).

Besides G6P-dependent regulation of CYP8B1, we found that hepatic levels of CYP7A1 protein, the supposedly rate-controlling enzyme in bile acid synthesis,⁽⁶⁾ as well as the plasma concentrations of its product C4 correlated with circulating glucose levels. Several studies have reported altered hepatic *Cyp7a1* mRNA expression in response to changes in hepatic glucose availability.^(9,39) We and others have shown that type 1 and type 2 diabetic rodents

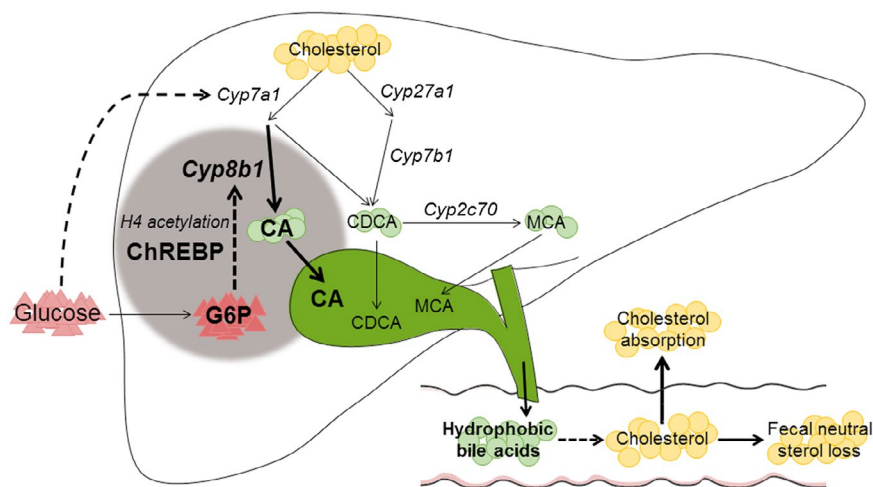


FIG. 5. Working model of the mechanism by which intrahepatic glucose controls bile acid synthesis and intestinal cholesterol handling in mice. Intrahepatic glucose (G6P) controls bile acid synthesis through a ChREBP-dependent induction of *Cyp8b1* by H4 acetylation, whereas hepatic *Cyp7a1* expression is regulated by blood glucose levels. Hepatic G6P-ChREBP-CYP8B1 hence induces corresponding shifts in bile composition, which subsequently promotes intestinal cholesterol absorption.

exhibit increased hepatic expression of *Cyp7a1*⁽³⁹⁾ and an enlarged bile acid pool.^(40,41) On the other hand, prolonged fasting decreases hepatic *Cyp7a1* mRNA expression in mice, with a concomitant reduction of total bile acid pool size,⁽³⁹⁾ consistent with our finding that hypoglycemia is associated with lower hepatic CYP7A1 protein levels. These data indicate that blood glucose level regulates CYP7A1 protein levels independently of hepatic G6P accumulation, possibly through its effect on the insulin-to-glucagon ratio,^(8,9) but independently of hepatic FOXO1,3,4 or ChREBP expression (Supporting Fig. S2A-D).

We thus show that hepatic CYP7A1 expression is partly controlled by circulating glucose levels, whereas intrahepatic glucose (G6P) appears to be the major regulator of CYP8B1 expression. Previous studies have reported an induction of *Cyp8b1* by glucose *in vitro*⁽⁹⁾ and an insulin-mediated suppression of the gene *in vivo*.^(2,4) We now show, in insulin-sensitive mice, that glucose-mediated induction of *Cyp8b1* requires hepatic ChREBP. Importantly, we observed that ChREBP-dependent regulation of *Cyp8b1* expression in response to intracellular glucose signaling was rapid (i.e., within 6 hours in S4048-exposed mouse liver) and also occurred in cultured human hepatocytes (IHH cells). Thus, the observed reduction of *CYP8B1* mRNA levels upon ChREBP knockdown in IHH cells indicates a cell-autonomous relationship between ChREBP and CYP8B1 expression that is independent of circulating factors or potential changes in hepatic inflammation or injury.

Although we did not identify a direct transcriptional regulation of the CYP8B1 promoter by ChREBP, the G6P-ChREBP-dependent changes in hepatic *Cyp8b1* expression were paralleled by altered H4 acetylation patterns in the CYP8B1 promoter and more downstream in the gene. Increased H4 acetylation levels in response to hepatic G6P-ChREBP signaling likely promoted chromatin relaxation in these regions, resulting in an induction of *Cyp8b1* transcription. ChREBP is a key determinant of glycolysis and a direct transcriptional regulator of *ACLY*,^(28,42) the essential enzyme for glucose-induced histone acetylation.^(9,33) In the current study, we observed consistent changes in *Acly* expression, H4 acetylation patterns, and CYP8B1 expression in response to G6P-ChREBP signaling. In contrast, expression of other potential mediators of the G6P-ChREBP-dependent *Cyp8b1* regulation (i.e., FXR, SHP, LRH-1, HNF4 α ,

and MAFG) did not consistently follow the pattern of *Cyp8b1* expression in response to G6P-ChREBP signaling. We therefore propose that the G6P-ChREBP axis controls the CYP8B1-mediated pathway in bile acid synthesis through H4 acetylation dynamics.

Hydrophobic bile acids effectively promote the absorption of dietary lipids and sterols,⁽³⁴⁻³⁶⁾ whereas a more hydrophilic bile acid pool is associated with enhanced intestinal cholesterol excretion.⁽²¹⁾ Our data strongly suggest that ChREBP activity contributes to cholesterol homeostasis in mice through its effect on CYP8B1 and hence on bile acid composition. The ChREBP-mediated increase in CA and decrease in β -MCA synthesis resulted in more hydrophobic bile that was paralleled by reduced fecal neutral sterol excretion. Because dietary cholesterol intake (data not shown), biliary cholesterol excretion (Table 1), and jejunal and ileal mRNA expression of Niemann-Pick C1-like 1, ATP binding cassette subfamily G member 5, ATP binding cassette subfamily G member 8, and acetyl-CoA acetyltransferase 2 (data not shown) were similar in *L-G6pc*^{-/-} mice and WT littermates, the reduction in neutral sterol excretion is most likely related to enhanced fractional cholesterol absorption as a consequence of the more hydrophobic bile acid pool in *L-G6pc*^{-/-} mice. We also show that normalization of bile composition upon hepatic ChREBP knockdown reverses reduced fecal neutral sterol excretion, consistent with the phenotype of *Cyp8b1*^{-/-} mice and with the effect of *Cyp8b1* inhibition in mice.^(34,36,43) However, in contrast to what was reported for *Cyp8b1*^{-/-} mice fed a high-fat diet,^(34,36) fecal fatty acid and energy loss remained unaltered in the current study. In accord with our findings, *Cyp8b1* heterozygous knockout mice displaying an intermediate phenotype with regard to bile acid pool composition also did not present changes in fecal calorie loss.⁽³⁴⁾ The absence of a change in fecal excretion of nonsterol dietary fat could furthermore be attributed to the relatively low fat content of the chow diet used, the high efficiency of intestinal fatty acid absorption under normal conditions,⁽⁴⁴⁾ and the fact that intestinal sterol absorption shows a larger dependency on bile acid hydrophobicity as compared to dietary fatty acids.⁽³⁵⁾ Therefore, we conclude that activation of the hepatic G6P-ChREBP-CYP8B1 axis selectively reduces fecal cholesterol excretion in chow-fed mice.

A major difference in bile acid metabolism between mice and humans is the presence of MCAs in murine bile, attributed to rodent-specific C6 hydroxylation.⁽⁴⁵⁾

Given that MCAs are very hydrophilic,⁽²²⁾ the human bile acid pool is more hydrophobic as compared to mice. G6P-ChREBP-mediated induction of *Cyp8b1*, promoting CA synthesis at the expense of dihydroxylated CDCA, would result in a more hydrophilic, rather than a more hydrophobic, bile acid pool in humans, with a potentially opposite effect on intestinal cholesterol absorption. There are no reports focusing on disturbed bile acid metabolism in GDS Ia patients, yet it is well known that bile acid metabolism is perturbed in type 2 diabetes.^(2,4,5) Although deviations in blood glucose are opposite in GSD Ia and diabetes, intrahepatic glucose metabolism is enhanced in both diseases and the hepatic phenotypes are very similar, rendering GSD Ia a “model” for diabetic liver disease.^(10–15) Type 2 diabetic mice exhibit elevated hepatic *Cyp8b1* expression and a corresponding increase in 12-hydroxylated bile acids,^(4,41) which has been attributed to IR and consequent FOXO activation.⁽⁴⁾ Given that hepatic ChREBP is also activated in type 2 diabetic mice and humans,^(46–48) increased G6P-ChREBP signaling potentially contributes to perturbed bile acid metabolism in type 2 diabetes. Therefore, our current data underscore the need to establish the impact of intrahepatic G6P-ChREBP signaling on bile acid pool composition in mice with a humanized bile acid pool⁽⁴⁵⁾ and GSD I patients, as well as its contribution to perturbed bile acid metabolism in type 2 diabetes.

In conclusion, we present a mechanism by which intracellular glucose controls hepatic bile acid synthesis and intestinal cholesterol handling. The G6P-ChREBP-CYP8B1 signaling cascade that we have identified likely contributes to altered bile acid metabolism and its (patho)physiological consequences in conditions coinciding with excessive intrahepatic glucose signaling such as GSD I and type 2 diabetes.

Acknowledgment: We thank A. Jurdinski, R. Havinga, T. Boer, M. Koehorst, R. Boverhof, Y. van der Veen, K. Tholen, C. van der Leij, S.X. Lee, and Z. Unal for excellent technical assistance. We are thankful for receiving plasmids from M. Herman (pcDNA3.1/ChREBP α , pcDNA3.1/ChREBP β , and pcDNA3.1/Mlx), J.W. Jonker (pcDNA3.1/Hnf4 α), H. Towle (minimal promoter PGL3/ChREBP luciferase reporter), and J. Chiang (human and mouse PGL3/Cyp8b1 promoter luciferase reporters). We thank A. Herling and D. Schmoll (Sanofi) for providing S4048 and L. Chan for sharing the ChREBP ChIP-seq data set.

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Author names in bold designate shared co-first authorship.

Supporting Information

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