Alterations in Lipid Metabolism Mediate Inflammation, Fibrosis, and Proliferation in a Mouse Model of Chronic Cholestatic Liver Injury

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BACKGROUND & AIMS: The liver controls central processes of lipid and bile acid homeostasis. We aimed to investigate whether alterations in lipid metabolism contribute to the pathogenesis of chronic cholestatic liver disease in mice. **METHODS:** We used microarray and metabolic profiling analyses to identify alterations in systemic and hepatic lipid metabolism in mice with disruption of the gene *ATP-binding cassette sub-family B member 4* (*Abcb4*$^{-/-}$ mice), a model of inflammation-induced cholestatic liver injury, fibrosis, and cancer. **RESULTS:** Alterations in *Abcb4*$^{-/-}$ mice, compared with wild-type mice, included deregulation of genes that control lipid synthesis, storage, and oxidation; decreased serum levels of cholesterol and phospholipids; and reduced hepatic long-chain fatty acyl-CoAs (LCA-CoA). Feeding *Abcb4*$^{-/-}$ mice the side chain–modified bile acid 24-*nor*ursodeoxycholic acid (*nor*UDCA) reversed their liver injury and fibrosis, increased serum levels of lipids, lowered phospholipase and triglyceride hydrolase activities, and restored hepatic LCA-CoA and triglyceride levels. Additional genetic and nutritional studies indicated that lipid metabolism contributed to chronic cholestatic liver injury; crossing peroxisome proliferator-activated receptor (PPAR)-α–deficient mice with *Abcb4*$^{-/-}$ mice (to create double knockouts) or placing *Abcb4*$^{-/-}$ mice on a high-fat diet protected against liver injury, with features similar to those involved in the response to *nor*UDCA. Placing pregnant *Abcb4*$^{-/-}$ mice on high-fat diets prevented liver injury in their offspring. However, fenofibrate, an activator of PPARα, aggravated liver injury in *Abcb4*$^{-/-}$ mice. **CONCLUSIONS: Alterations in lipid metabolism contribute to the pathogenesis and progression of cholestatic liver disease in mice.**

Keywords: Cholestasis; Canalicular Phospholipid Flippase; Hepatocellular Cancer; PSC.

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Chronic liver injury is a highly dynamic process leading to liver fibrosis and can progress to cirrhosis with development of portal hypertension, liver failure, and cancer. Thus, chronic liver injury is worldwide one of the major causes of morbidity and mortality.[1] Cholestasis comprises a wide spectrum of chronic liver diseases, including primary sclerosing cholangitis, and is characterized by intrahepatic and systemic accumulation of bile acids (BAs), accompanied by inflammation and fibrosis of the biliary tree.[2,3] Mice lacking the canalicular phospholipid (PL) export pump ABCB4 (*Abcb4*$^{-/-}$) represent a well-suited and highly reproducible in vivo model system to study the complex cellular interplay of chronic cholestatic liver diseases, including primary sclerosing cholangitis.[4] Moreover, mutations in the human *ABCB4* gene are associated with cholestasis and biliary fibrosis.[5]

Under physiologic conditions, the liver controls numerous aspects of lipid metabolism that include synthesis, storage, secretion, and (via BAs) intestinal lipid absorption. The emerging role of BAs and their receptors as master regulators of lipid and energy homeostasis and as essential gatekeepers of liver regeneration and inflammation has evoked a renaissance in BA research.[6] Moreover, the synthesis of side chain–modified BAs such as 24-*nor*ursodeoxycholic acid (*nor*UDCA), which undergoes cholehepatic shunting with pronounced hepatic and bil-

Abbreviations used in this paper: AP, alkaline phosphatase; BA, bile acid; C/EBPα, CCAAT/enhancer binding protein α; CH, cholesterol; DAG, diacylglycerol; DKO, double knockout; FA, fatty acid; FFA, free fatty acid; HDL, high-density lipoprotein; HFD, high-fat diet; LCA-CoA, long-chain acyl-CoA ester; *nor*UDCA, 24-*nor*ursodeoxycholic acid; PL, phospholipid; PPAR, peroxisome proliferator-activated receptor; TG, triglyceride; WT, wild-type.

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iary enrichment, has led to improved therapeutic efficacy above conventional BAs, thus ameliorating liver injury in *Abcb4*$^{-/-}$ mice.[4] Similar to human cholestatic liver diseases, *Abcb4*$^{-/-}$ mice display multiple derangements of lipid metabolism, including alterations in cholesterol (CH) and PL metabolism.[7,8]

We therefore hypothesized that disturbances in lipid homeostasis are causally linked to the pathogenesis and progression of cholangiopathies and biliary fibrosis, while targeting these metabolic alterations may combat liver injury in *Abcb4*$^{-/-}$ mice. The findings of the current study show that the beneficial effects of *nor*UDCA can in part be attributed to its effects on lipid homeostasis in *Abcb4*$^{-/-}$ mice and that genetic and dietary interventions combat/reverse liver injury in *Abcb4*$^{-/-}$ animals. Collectively, these different but mechanistically linked experimental approaches substantiate a critical role of lipid metabolism in the pathobiology of cholangiopathies and biliary fibrosis.

Materials and Methods

Animals and Diets

Abcb4$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). *Pparα* knockout mice were crossbred with *Abcb4*$^{-/-}$ mice to generate *Pparα* × *Abcb4*$^{-/-}$ double knockout (*DKO*) mice. 24-*nor*UDCA (0.5% wt/wt) and fenofibrate (0.05% wt/wt) were mixed into standard chow. High-fat diet (HFD) was obtained from Sniff (Soest, Germany) and is described in Supplementary Materials and Methods. All experiments were performed with 2-month-old animals fed the respective diets for 4 weeks.

Serum Biochemistry, Bile Duct Plastination, and Histologic Analysis and Staining

Serum biochemical analysis, liver histology, bile duct plastination, and Sirius red staining were performed as described previously[4] and are described in detail in Supplementary Materials and Methods.

Sample Preparation and RNA Isolation

Tissues were immediately snap frozen in prechilled 2-methylbutane and RNA was isolated as described in detail in Supplementary Materials and Methods.

Transcriptional Profiling Using Microarray Technology

Mouse Genome Survey Arrays V2.0 (Applied Biosystems, Foster City, CA) were used to determine the transcriptional profiles of wild-type (WT), *Abcb4*$^{-/-}$, and *nor*UDCA-treated *Abcb4*$^{-/-}$ samples as described in detail in Supplementary Materials and Methods. Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO Series accession number GSE26547.

Microarray Data Analysis and Gene Ontology Evaluation

Expression values were imported into GeneSpring 6.2.1 (Silicon Genetics, Redwood, CA) and processed according to Applied Biosystems standard procedure ("per chip" and "per gene" normalization) as described in detail in Supplementary Materials and Methods.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

First-strand complementary DNA was synthesized as described previously.[4] Relative RNA levels were quantified using SYBR Green Master Mix (Applied Biosystems) as described in Supplementary Materials and Methods.

Western Blot Analysis and Immunohistochemistry

Western blots and immunohistochemistry were performed as described previously[4] and are described in Supplementary Materials and Methods.

Extraction and Analysis of Lipids on Lipoprotein Particles, Total Hepatic Lipids, and CH Precursors

Distribution of lipids on lipoprotein particles was determined with fast pressure liquid chromatography. Hepatic lipids were extracted from tissue homogenates and quantified as described in Supplementary Materials and Methods.

Phospholipase Activity Assay

Phospholipase activity from postheparin serum was performed as described previously and is summarized in detail in Supplementary Materials and Methods.

Quantification of Acyl-CoAs

Acyl-CoAs were determined by online solid phase extraction coupled to liquid chromatography with mass spectrometry as described in Supplementary Materials and Methods.

Tissue Triglyceride Hydrolase Activity Assay

Preparation of liver tissue to obtain a lipid-free cytosolic fraction and triglyceride (TG) hydrolase activity was performed using triolein as substrate as described previously.[9]

Molecular Fatty Acid Composition in Hepatic Lipid Species

Preparation of fatty acid (FA) methyl esters of individual hepatic tissue lipids (TG, diacylglycerol [DAG], CH, PL) and free fatty acids (FFAs) was performed as essentially described previously.[10]

Bile Flow, BA Output, and Biliary FA Profile

Bile flow and biliary BA output were determined as described previously.[4] Biliary FA amount and composition were analyzed from bile (30 μL) after Bligh-Dyer extraction using gas chromatography–mass spectrometry in EI mode equipped with a DB5-MS column.

Statistics

Values are expressed as mean ± SD. Statistical analysis was performed using unpaired Student *t* test or analysis of variance comparisons for 3 or more groups using a Bonferroni posttest. Data were analyzed using SigmaStat (Jandel Scientific, San Rafael, CA); a *P* value of $<.05$ was considered significant.

Results

Molecular Effects of norUDCA *on Lipid Metabolism in* Abcb4$^{-/-}$ *Mice*

To identify biologic processes relevant for disease development and progression in the *Abcb4*$^{-/-}$ mouse