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Critical role of cholic acid for development of hypercholesterolemia and gallstones in diabetic mice

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Abstract

We studied bile acid and cholesterol metabolism in insulin-dependent diabetes utilizing genetically modified mice unable to synthesize cholic acid (Cyp8b1−/−). Diabetes was induced in Cyp8b1−/− and wild type animals (Cyp8b1+/+) by alloxan, and the mice were fed normal or cholesterol-enriched diet for 10 weeks. The serum levels of cholesterol were strongly increased in diabetic Cyp8b1+/+ mice fed cholesterol, while diabetic Cyp8b1−/− mice did not show any aberrations regardless of the diet. Diabetic cholesterol-fed Cyp8b1+/+ mice had much higher biliary cholesterol and cholesterol saturation index than all other groups, their bile contained a large number of cholesterol crystals, and their canalicular cholesterol transporter Abcg5/g8 mRNA levels were much higher. Cyp7a1 mRNA levels were similar in all diabetic mice but higher compared to non-diabetic animals. The results indicate a critical role for cholic acid for the development of hypercholesterolemia and gallstones in our animal model.

Keywords: Cholic acid; Diabetes; Abcg5/g8; Gallstone; Biliary cholesterol; Hypercholesterolemia

Diabetes mellitus is either due to insulin deficiency (insulin-dependent diabetes or type I) or decreased responsiveness of the target cell to insulin (insulin-independent diabetes or type II). The increased morbidity connected with diabetes is secondary to a number of pathological conditions like coronary heart disease, renal insufficiency, cerebrovascular disorders, dyslipidemia, and neuropathy. Abnormalities of the plasma lipids, associated with angiopathy and atherosclerosis, include increased concentrations of cholesterol, mainly confined to the VLDL- and LDL-fraction, decreased levels of HDL-cholesterol, and elevated TG [1–3]. Also hepatobiliary functions seem to be affected and an increased incidence of cholelithiasis is reported among diabetic patients [4]. Many studies of the hepatobiliary system in diabetes have used animal models, in which diabetes is induced by treatment with streptozotocin (STZ) or alloxan (ALX), thereby abolishing insulin secretion. Alterations in production and composition of the bile have been described [5,6] including enhanced secretion of biliary cholesterol and phospholipids [7]. Increased intestinal cholesterol absorption has also been documented in animal experimental models [1,6].

Mice with alloxan-induced diabetes can develop gallstones when given a cholesterol-enriched diet for 8–10 weeks [7]. Healthy animals are resistant to produce gallstones, but do develop gallstones when fed a special lithogenic diet containing cholesterol and cholate [8], although the outcome is also depending on the genetic background. However, in diabetic mice it is possible to induce gallstone formation by cholesterol feeding even without the need for external supply of cholic acid (CA). CA facilitates intestinal absorption of lipids, especially cholesterol, and will

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suppress endogenous bile acid synthesis in mice, probably by interaction with the nuclear receptor FXR [9]. Sterol 12α-hydroxylase (CYP8B1), a P450 cytochrome expressed exclusively in the liver, is required for the CA-synthesis. In the bile of diabetic rats and mice, CA and its secondary product deoxycholic acid (DCA) are reported to increase while the amount of β-muricholic acid (β-MCA) is reduced [6,7]. In accordance, Cyp8b1 mRNA levels are elevated in rats with streptozotocin-induced diabetes, and insulin administration was able to suppress the Cyp8b1 gene expression [10].

In the present study, we have utilized Cyp8b1−/− mice to further characterize the mechanisms responsible for generating gallstones in alloxan-treated mice, and, in addition, we have investigated the role of CA for the bile acid/cholesterol homeostasis under diabetic conditions.

Materials and methods

Chemicals. The following items were purchased from Sigma–Aldrich Corp., St. Louis, Missouri, USA: Cholesterol (>99% purity), alloxan monohydrate.

Animals and procedures. Male age-matched mice (2–6 months of age) deficient for the Cyp8b1 gene (Cyp8b1−/−) and the corresponding wild type animals (Cyp8b1+/+) were bred from a mixed genetic background (C57BL6/129). Both Cyp8b1−/− and +/+ mice were housed at 22–24 °C at a light circle from 8 a.m. to 8 p.m. in groups of 4–8 animals. Diabetes was induced by a single intravenous injection of alloxan monohydrate (65 mg/kg body weight) dissolved in phosphate-buffered saline (PBS). After the injections, the mice were fed for 9–10 weeks either normal chow (R36 powder, Lactamin, Vadstena, Sweden with a declared content of 0.025% (w/w) cholesterol) or a diet containing 0.5% cholesterol (w/w). Control animals (Cyp8b1−/− and +/+ mice) were injected with a corresponding volume of PBS and fed either chow or cholesterol-enriched diet. Water was available ad libitum.

To follow the development of the diabetic condition during the experimental period urinary glucose was monitored once a week using Uristix dipsticks (Bayer Healthcare LLC, Tarrytown, NY, USA). Serum glucose levels were analysed from blood drawn by heart puncture immediately before the sacrifice of the animals. The condition of diabetes mellitus was defined from a plasma glucose level >12 mmol/L. Animals were killed by cervical dislocation following CO_2-anesthesia, whereafter liver and gallbladder were washed in physiological saline and immediately frozen in liquid nitrogen. Prior to sampling of gallbladder bile mice were fasted for 10 h. The experiments were approved by the Ethics Committee for Animal Experimentation in Karolinska University Hospital.

Determination of serum glucose and lipid levels. Serum was obtained by centrifugation of the blood by centrifugation at 2800 rpm for 15 min. Serum levels of glucose, total cholesterol, and triglycerides were determined by standard enzymatic procedures. Lipoproteins were separated by FPLC and cholesterol assayed continuously on-line as described [11].

Analysis of biliary lipids in gallbladder bile. Five microliter aliquots of bile from gallbladder were examined for cholesterol crystals and concrements on glass slides by polarized light microscopy. The composition of bile acids was determined by gas liquid chromatography as reported [12]. Biliary cholesterol and total bile acid concentration were quantitated by enzymatic methods [13,14], and phospholipids by the method described by Rouser et al. [15]. The relative concentrations of biliary lipids were expressed as molar percentages of the total biliary lipids. Cholesterol saturation index (CSI) was calculated according to Carey [16].

Liver mRNA quantitation by real-time PCR. To elucidate the effects of alloxan on the hepatobiliary system, liver mRNA levels for specific genes were determined by quantitative real-time PCR, essentially as described [17]. The following genes were analysed: mouse Cyp7a1, Cyp8b1, Cyp27a1, SHP, Abcg5, Abcg8, Bsep, Mdr2, HMG-CoA reductase, and the LDL receptor. As an internal standard mouse hypoxanthine phosphoribosyl transferase (HPRT) was utilized. All PCR products were DNA-sequenced to confirm that they represented the correct fragments.

Statistical analysis. Data are presented as means ± SEM. Statistical analysis was performed with *STATISTICA* software. The significance of differences was tested by Fractional two-way ANOVA, followed by planned comparisons. A value of $p < 0.05$ was considered to be statistically significant.

Results

Alterations of the plasma glucose levels

Generally, the urine of the alloxan-treated animals started to give positive reactions for glucose 1 week after the injection. A strong hyperglycemia was observed in all 4 groups, with serum glucose values ranging between 20 and 27 mmol/L. Diabetic mice that were subjected to overnight fasting before sacrifice generally displayed slightly lower serum glucose levels but always well over 12 mmol/L (data not shown). Use of lower doses of alloxan (50 mg/kg), as have been suggested previously [18], allowed the mice in many cases to recover from the diabetic condition after 3–4 weeks.

Alterations of the hepatobiliary functions

The total quantity of bile acids in the bile is shown in Table 1, where the highest levels were found in groups with Cyp8b1+/+ animals. When analyzing the composition of bile from the Cyp8b1−/− mice, small insignificant peaks were observed with retention times as CA and its secondary product DCA, probably representing unknown compounds. Instead, muricholic acids (MCAs) made up the major part of the bile acids (Table 2). The levels of UDCA were higher in all Cyp8b1−/− groups compared to their Cyp8b1+/+ equivalents in accordance with previous reports [17]. Alloxan-treated Cyp8b1+/+ mice displayed significantly higher levels of CA than the Cyp8b1+/+ mice fed either normal chow or cholesterol-enriched diet. However, the data also suggested that cholesterol feeding reduced the amount of CA in bile.

The Cyp8b1+/+ mice treated with ALX/Ch displayed the highest molar percentage of cholesterol and CSI (13% and 167%, respectively), while the bile from Cyp8b1+/+ animals treated with PBS/Ch was found to have 8.2% and 112%, respectively. The animals in all other groups showed very modest deviations from the values of molar percentage of cholesterol and CSI in the controls injected with PBS and fed normal chow. Likewise, only modest variations were seen in the phospholipid (PL) levels between all groups. However, total lipid concentrations were found to be lower in all Cyp8b1−/− mice compared to their +/+ equivalents. None of the different groups had gallstones in the gallbladders, but the bile from the Cyp8b1+/+ mice treated with ALX/Ch contained plenty