Effective Treatment of Unconjugated Hyperbilirubinemia With Oral Bile Salts in Gunn Rats

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Background & Aims: **We tested the hypothesis that oral administration of bile salts, which are known to increase the biliary excretion of unconjugated bilirubin (UCB), decreases unconjugated hyperbilirubinemia in the Gunn rat model.** ***Methods:*** **Adult Gunn rats were fed a standard diet or the same diet supplemented with 0.5 weight % ursodeoxycholic acid (UDCA) or cholic acid (CA) for 1 or 6 weeks. UCB and urobilinoids, a family of intestinal UCB breakdown products, were determined in plasma, feces, or both. After 6 weeks of treatment, tracer ^{3}H-UCB was administered intravenously to determine steady-state UCB kinetics over the next 60 hours.** ***Results:*** **One-week treatment with UDCA or CA decreased plasma UCB concentrations by 21% and 30%, respectively (each $P < .01$). During the first 4 days of treatment, both UDCA and CA increased the combined fecal excretion of UCB and urobilinoids (+52% and +32%, respectively; each $P < .01$). Prolongation of treatment to 6 weeks caused a persistent decrease in plasma UCB concentrations to ~40% below baseline (each bile salt $P < .001$). ^{3}H-UCB kinetic studies showed that UDCA and CA administration decreased UCB pool size (−33% and −32%, respectively; each $P < .05$) and increased UCB fractional turnover (+33% and +25%, respectively; each $P < .05$).** ***Conclusions:*** **Dietary bile salt administration induces a large, persistent decrease in plasma UCB concentrations in Gunn rats. Both UDCA and CA enhance UCB turnover by increasing its fecal disposal. These results support the application of oral bile salt treatment in patients with unconjugated hyperbilirubinemia.**

Unconjugated hyperbilirubinemia occurs in conditions such as neonatal hemolytic jaundice and Crigler-Najjar disease. Crigler-Najjar disease is characterized by a genetically absent (type I) or decreased (type II) capacity to conjugate bilirubin in the liver,[1] which is essential for efficient biliary excretion of the pigment. Impaired conjugation results in unconjugated hyperbilirubinemia, due to retention of unconjugated bilirubin (UCB) in the body. Severe unconjugated hyperbilirubinemia can lead to deposition of UCB in the central nervous system, causing bilirubin-induced neurologic dysfunction (BIND), kernicterus, and death.[2] Unconjugated hyperbilirubinemia is conventionally treated by phototherapy, which induces photoisomerization of the hydrophobic UCB to polar isomers that can readily be excreted into the bile.[3] Although generally effective, phototherapy does not always decrease plasma UCB to nontoxic levels. Most importantly, long-term phototherapy, such as needed for patients with Crigler-Najjar disease type I, becomes less effective with age and has a profound impact on social life.[4,5] These considerations favor the development of effective alternative treatments for unconjugated hyperbilirubinemia.

During severe unconjugated hyperbilirubinemia, most UCB does not enter the intestinal lumen via biliary excretion, but rather via direct diffusion across the intestinal mucosa.[6,7] The efficiency of this pathway is decreased, however, by the ability of the intestine to reabsorb UCB from its lumen.[8,9] Several experimental therapies have aimed to prevent reabsorption by oral administration of agents that trap UCB in the intestinal lumen. However, trapping agents tested so far, including agar,[10] cholestyramine,[11] charcoal,[12] amorphous calcium phosphate,[13] zinc salts,[14] and orlistat,[15] have been clinically unsatisfactory because of side effects and inconsistent results.

Since bile salts can stimulate biliary excretion of organic anions,[16] including bilirubin in rats,[17] we reasoned that bile salt administration could be relevant for treatment of unconjugated hyperbilirubinemia. Ursodeoxycholic acid (UDCA) treatment in healthy volunteers decreased the expiration of $^{14}CO_2$ from triolein, suggesting

Abbreviations used in this paper: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BW, body weight; CA, cholic acid; dpm, disintegrations per minute; HPLC, high-performance liquid chromatography; UCB, unconjugated bilirubin; UDCA, ursodeoxycholic acid.

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that UDCA also mildly decreases the absorption of fat.[18] Mild fat malabsorption induced by orlistat decreased plasma bilirubin levels in a subset of Crigler-Najjar patients and in homozygous Gunn rats, their well-established animal model.[15,19] Finally, bile salts associate with UCB in vitro, and bile salt administration could therefore also lower plasma UCB concentrations via enhancement of fecal excretion of UCB–bile salt complexes.[20,21]

In the present study we show that dietary administration of UDCA indeed reduces unconjugated hyperbilirubinemia in homozygous Gunn rats. We studied several dosages and administration periods to evaluate the clinical applicability of this potential treatment. We compared the UDCA effects in Gunn rats with those obtained after administration of cholic acid (CA) to evaluate whether effects were bile salt specific. Finally, we studied steady-state kinetics of intravenously administered ^{3}H-UCB, to gain insight into the underlying mechanisms of both UDCA and CA administration.

Materials and Methods

Animals

Homozygous adult male Gunn Rats (RHA/jj, 240–360 g), obtained from our breeding colony (University Medical Center Groningen, The Netherlands), were housed in an environmentally controlled facility and fed *ad libitum*. The Ethics Committee for Animal Experiments of the University of Groningen approved all experimental protocols.

Materials

Chemicals. UDCA was a generous gift from Dr Falk Pharma GmbH (Freiburg, Germany). CA and heptadecanoic acid (C17:0) were obtained from Sigma-Aldrich (St Louis, MO). Xanthobilirubin-methyl ester was a generous gift from Dr J. Fevery (Leuven, Belgium). Urobilin was obtained from Frontier Scientific (Logan, UT). ^{3}H-labeled UCB (specific activity 6.02 μCi/μmol) was prepared by biosynthetic labeling of 2,3-^{3}H-labeled 5-aminolevulinic acid (specific activity 13 mCi/mmol; Amersham Biosciences, Piscataway, NJ).[22–24] ^{3}H-labeled UCB solution was prepared immediately before injection into Gunn rats as described before.[23]

Diets. The semisynthetic, purified control diet (code 4063.02) was produced by Hope Farms BV (Woerden, The Netherlands) and contained 13 energy% fat and 5.2 weight (wt%) long-chain fatty acids. Diets containing bile salts were identical except for supplementation with UDCA or CA (0.05%–1.5% by chow weight).

Methods

Preliminary dose-response experiment. After a 6-week run-in period on the control diet, Gunn rats were randomly assigned to receive the control diet supplemented with either UDCA or CA (n = 6 per group). All animals were housed and fed by dietary group for a period of 10 weeks, during which the dosage of UDCA and CA was increased every 2 weeks. Used dosages were 0.05 wt%, 0.1 wt%, 0.5 wt%, 1 wt%, and 1.5 wt% (by chow weight). Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before and 2, 4, 6, 8, and 10 weeks after dietary randomization for determination of plasma UCB concentrations and, in the last plasma sample, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations.

Short-term experiment. After a 6-week period on the control diet, individually housed Gunn rats were randomly assigned to receive the control diet or the same diet supplemented with UDCA or CA (each 0.5 wt%; n = 6 per group). Food intake and animal weights were determined daily. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia at day 0, 1, 3, 5, and 8 for determination of plasma UCB concentrations. Feces were collected every 24 hours for 4 days before and for 4 days after dietary randomization to determine fecal excretion of UCB, urobilinoids, and bile acids. Eight days after dietary randomization, the common bile duct was cannulated under pentobarbital anesthesia, and bile was collected for 30 minutes under light-protected conditions. Bile flow was determined gravimetrically, assuming a density of 1 g/mL. A 1-mL blood sample was then obtained by puncture of the inferior vena cava to determine AST and ALT.

Long-term experiment. After 6 weeks on the control diet, individually housed Gunn rats were randomly assigned to receive either the control diet or the same diet supplemented with UDCA or CA (each 0.5 wt%; n = 6 per group). Food intake and animal weights were determined weekly. Heparinized samples of tail vein blood were obtained under isoflurane anesthesia before and at 2, 4, and 6 weeks after dietary randomization to determine plasma UCB concentrations. At 5 weeks, the rats were gavaged with 1 mL (20 mg/mL) carmine red, and stools were examined for red staining to assess intestinal transit time. At 6 weeks, the ^{3}H-labeled UCB solution (~0.29 μCi/100 g body weight [BW]) was administered via the penile vein.[23] Subsequently, heparinized samples of tail vein blood were collected every 12 hours for 60 hours for determination of plasma UCB concentrations, and feces were collected to determine fecal excretion of urobilinoids, ^{3}H-label, bile salts, calcium, phosphate, and fat. At ~60 hours after the ^{3}H-UCB-injection, bile was collected for 30 minutes, followed by vena cava inferior puncture as described above. The intestine was then removed and divided into 5 segments (3 equal parts of small intestine, the cecum, and the remaining colon) that were flushed with phosphate-buffered saline (pH 7.4) for analysis of UCB and urobilinoids.

Plasma analysis. Blood samples were protected from light and processed immediately. Bilirubin, AST, and ALT levels were determined by routine clinico-chem-