Enteral bile acid treatment improves parenteral nutrition-related liver disease and intestinal mucosal atrophy in neonatal pigs

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OVER 30,000 patients in the United States are permanently dependent on parenteral nutrition (PN) for survival, and many more require PN for varying duration during hospital stay or home care. In pediatric patients, PN-associated liver disease (PNALD) is a major but still poorly understood complication of PN. Infants that receive PN for at least 2 mo may experience liver injury, and PNALD occurs in up to 50% of infants. The clinical spectrum includes steatosis, disruption in glucose and lipid metabolism, cholestasis (PN-associated cholestasis), cholelithiasis, hepatic fibrosis, biliary cirrhosis, development of portal hypertension, and liver failure. The histological features of PNALD are intracellular and intracanalicular cholestasis, steatosis and periportal inflammation, and eventually fibrosis. The risk factors associated with PNALD in infants are lack of enteral feeding, developmental immaturity in the hepatic transport and metabolism of bile acids, and sepsis or infection. Despite these known risk factors, the etiology of PNALD, especially in infants and children, is not established, and presently there are no proven effective therapeutic treatments.

Among a number of proposed mechanisms to account for the pathology of PNALD is the lack of enteral feeding, leading to gut atrophy and disruption of enterohepatic circulation of bile acids. We have shown that PNALD induces both intestinal mucosal atrophy and PNALD in neonatal piglets. Intestinal mucosal atrophy induced by chronic TPN is strongly linked to the loss of enteral nutrient-mediated glucagon-like peptide-2 (GLP-2) secretion, and GLP-2 replacement markedly augments mucosal growth. During normal enterohepatic circulation, bile acid absorption in the ileum is associated with activation of the nuclear bile acid receptor farnesoid X receptor (FXR), leading to induction of the recently described metabolic hormone fibroblast growth factor-19 (FGF19) secretion. Enterohepatic secretion of FGF19 acts in the liver to repress bile acid production and improve lipid metabolism and glycemic control. Bile acid activation of the G protein-coupled receptor TGR-5 in intestinal endocrine cells results in activation of GLP-1 secretion. This also reduces bile acid production in the liver and peripheral metabolic profile but could also, via cosreceptor GLP-2, result in a trophic effect in the gut. We hypothesized that disruption of the enterohepatic bile flow and gut signaling processes occur during TPN, which results in loss of FXR-mediated intestinal secretion of FGF19, GLP-1, and GLP-2 and thereby contributes to PNALD and mucosal atrophy. We therefore evaluated the role of enteral supplementation with both PNALD and gut atrophy, which were associated with increases in both FGF19 and GLP-1 secretion in the gut.

MATERIALS AND METHODS

Animals. The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS publication no. (NIH) 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. Newborn (2-day-old) pigs were obtained from the Texas Department of Criminal Justice (Huntsville, TX), transported to the animal facility at the Children’s Nutrition Research Center, and housed in the animal facility at the Children’s Nutrition Research Center (Houston, TX). Animals were housed in individual cages with free access to food and water, and the room temperature was maintained at 22°C. Pigs were weighed daily and fed according to their body weight.

PNALD is a major but still poorly understood complication of PN. The clinical spectrum includes steatosis, disruption in glucose and lipid metabolism, cholestasis (PN-associated cholestasis), cholelithiasis, hepatic fibrosis, biliary cirrhosis, development of portal hypertension, and liver failure. The histological features of PNALD are intracellular and intracanalicular cholestasis, steatosis and periportal inflammation, and eventually fibrosis. The risk factors associated with PNALD in infants are lack of enteral feeding, developmental immaturity in the hepatic transport and metabolism of bile acids, and sepsis or infection. Despite these known risk factors, the etiology of PNALD, especially in infants and children, is not established, and presently there are no proven effective therapeutic treatments.

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TX) and immediately placed in cages in a heated room (~30°C) until surgery the following day.

**Surgery.** The piglets were anesthetized using isoflurane general anesthesia, and silastic catheters were inserted into the jugular vein and carotid artery as previously described (4, 23). Intragastric and intraduodenal catheters were also implanted. Catheters were secured in pockets of a jacket that was attached to a tether, which allowed free movement and secure administration of TPN to the piglets in their cages. During the initial 24 h postoperatively, all pigs received TPN at 50% of full intake providing (in g·kg⁻¹·day⁻¹) 12.5 glucose, 6.5 L-amino acids, 2.5 lipid, and 412 kJ·kg⁻¹·day⁻¹ at a volume of 120 ml·kg⁻¹·day⁻¹. Thereafter, intakes were increased to 100% within 48 h according to their respective treatment. Pre- and postoperatively on each day, piglets received enrofloxacin (2.5 mg/kg; Bayer, Shawnee Mission, KS). Postoperatively, each piglet received one dose of analgesic (0.1 mg/kg butorphenol tartrate; Fort Dodge Laboratories, Fort Dodge, IA).

**Study design.** Piglets (N = 15) were randomly allocated to three groups, enteral nutrition (EN), TPN, and TPN + CDCA group. Each group received nutrition for 14 days as follows. EN pigs were fed a liquid cow’s milk-based formula (Litter Life; Merrick, Middleton, WI) at a rate of 50 g·kg⁻¹·body wt⁻¹·day⁻¹, suspended in 240 ml water providing in g·kg⁻¹·body wt⁻¹·day⁻¹, 25 lactose, 12.5 protein, 5 fat, and electrolytes, (trace) minerals, and vitamins (815 kJ·kg⁻¹·body wt⁻¹·day⁻¹). TPN piglets were administered TPN providing per 240 ml fluid in g·kg⁻¹·body wt⁻¹·day⁻¹, 25 glucose, 13 amino acids, 5 lipid (Intralipid 20%; Fresenius Kabi, Bad Homburg, Germany), and electrolytes, (trace) minerals, and vitamins (823 kJ·kg⁻¹·body wt⁻¹·day⁻¹). TPN + CDCA piglets were administered TPN as described above and also received CDCA via the duodenal catheter at 30 mg·kg⁻¹·body wt⁻¹·day⁻¹, in doses of 10 mg/kg·body wt dissolved in ethanol at 0.15 ml/kg·body wt every 8 h. TPN pigs above were administered ethanol alone via duodenal catheter. EN was administered every 4 h intragastrically, and TPN was administered continuously. Piglets were weighed daily, and their intake was adjusted accordingly.

**Intravenous glucose tolerance test.** On days 7 and 14 after an 8-h fast, pigs received an intravenous glucose tolerance test (IVGTT, 1.0 g glucose/kg body wt) with arterial blood sampled over 60 min for assay of plasma glucose and insulin as described previously (23).

**Plasma analysis.** Blood samples were obtained on day 14 for plasma analysis for liver parameters, which was done by automated analyzer at Baylor College of Medicine core facility. Plasma FGF19 levels were assayed using ELISA (Quantitative ELISA Kit by R&D Systems, Minneapolis, MN). For GLP-1 and GLP-2 assays, blood samples were collected into chilled tubes containing EDTA, kept on ice, and centrifuged within 30 min at 3.000 revolution/min for 10 min. Plasma was collected and analyzed for GLP-1 by radioimmunoassay using antiserum 89 390 reacting with the amidated COOH terminus of GLP-1, and therefore mainly reacting with fully processed GLP-1. In the circulation, GLP-1 is metabolized by the enzyme dipeptidyl peptidase IV to the NH₂-terminally truncated metabolite GLP-1 9–36 amide; this metabolite crossreacts 100% in the GLP-1 assay. The assay measures the sum of degraded and undegraded GLP-1 as described previously (17). Plasma GLP-2 concentrations were quantified as described previously using an antibody raised against human GLP-2 that recognizes both the human and porcine full-length GLP-2 (1–33) peptide (4).

**Tissue collection.** At the end of the study, all pigs were euthanized with an intravenous injection of pentobarbital sodium (50 mg/kg) and phenytoin sodium (5 mg/kg; Beutanasia-D; Schering-Plough Animal Health, Kenilworth, NJ). The abdomen was opened, and the entire small intestine distal to the ligament of Treitz to the ileocecal junction was immediately flushed with ice-cold saline. After being flushed with ice-cold saline, the small intestine was divided into two equal portions; the proximal half was designated the jejunum, and the distal half, the ileum. Liver and small intestine were weighed, and tissue was snap-frozen in liquid nitrogen and stored at ~80°C until further analysis.

**Histological analysis and morphometry.** A 2–3-cm segment of fresh tissue from the proximal and distal small intestine was fixed in 4% buffered formalin for 24 h and then stored in 70% ethanol at room temperature for 24 h. Samples were embedded in paraffin, sectioned (5 mm), and stained with eosin and hematoxylin. The mean villus height and crypt depth were quantified by an Axiohot microscope (Zeiss, Jena, Germany) and NIH Image 1.60 (National Institutes of Health, Kenilworth, NJ). The abdomen was opened, and the entire small intestine distal to the ligament of Treitz to the ileocecal junction was immediately flushed with ice-cold saline. After being flushed with ice-cold saline, the small intestine was divided into two equal portions; the proximal half was designated the jejunum, and the distal half, the ileum. Liver and small intestine were weighed, and tissue was snap-frozen in liquid nitrogen and stored at ~80°C until further analysis.
Health, Bethesda, MD) in at least 15 vertically well-oriented villus-crypt columns as described previously (4).

RESULTS

We have previously described the development of hepatic steatosis and inflammation in neonatal piglets maintained on TPN (23, 28). To test the potential effects of bile acid supplementation in this model, we compared groups of piglets \( n = 5 \) per group that were fed enterally or parenterally with or without CDCA (EN, TPN, TPN + CDCA). CDCA was administered via a duodenal catheter at 30 mg·kg⁻¹·day⁻¹, in doses of 10 mg/kg body weight every 8 h. EN was administered every 4 h intragastrically, and TPN was administered continuously. Piglets were weighed daily, and their intake was adjusted accordingly.

At baseline, all three groups EN, TPN, TPN + CDCA were comparable for age and weight with mean weight in grams and standard deviation (± SD) of 2,063 ± 302, 2,092 ± 460, and 2,015 ± 501, respectively, for EN, TPN, TPN + CDCA. At the end of 14 days, mean weight gain (g/day) was 51.1 ± 3.7, 45.3 ± 5.7, and 44.7 ± 5.2, respectively, for the three groups. The liver weights (g/kg body wt) were lower \( P < 0.05 \) in EN vs. TPN and TPN + CDCA (27.8 ± 3.8 vs. 38.0 ± 5.3 and 40.0 ± 6.7, respectively). Although isocaloric nutrition was provided to each group, the EN group had the highest weight gain. Diarrhea was observed in two of the five piglets in the CDCA treatment group. No other adverse effects were documented.

Plasma lipid profiles were determined for each of the groups. The EN group had higher \( P < 0.05 \) total cholesterol and HDL.

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**Fig. 3.** Macroscopic and microscopic images of liver and steatosis in pigs fed EN, TPN, or TPN + CDCA. Treatments as in Fig. 1. Steatosis is evident from microvesicular lipid droplets stained by oil-red-O (right panels).

**Fig. 4.** Plasma concentrations of fibroblast growth factor-19 (FGF19) and glucagon-like peptide (GLP)-1 and GLP-2 in neonatal pigs fed EN, TPN, or TPN + CDCA. Treatments as in Fig. 1. Levels of FGF19 (A) and GLP-1 and -2 (B) are shown. Differences between groups are based on one-way ANOVA and Tukey’s test; *\( P < 0.05 \) vs. enteral, **\( P < 0.05 \) CDCA vs. TPN.
levels compared with the TPN group or the TPN + CDCA group (Fig. 1). No differences in LDL levels were evident in either group. Plasma triglycerides were higher ($P < 0.05$) in the TPN and TPN + CDCA compared with the EN group (Fig. 1). No significant differences were documented in alanine aminotransferase or albumin values in any group.

As expected, the TPN group showed steatosis and liver dysfunction after 2 wk, as indicated by elevated liver triglycerides and elevated plasma levels of direct and total bilirubin, as well as bile acids (Fig. 2). Cholestasis and steatosis in the TPN group were also evident macroscopically in yellow liver appearance and microscopically in microvesicular lipid droplets by oil-red O staining, whereas EN and TPN + CDCA groups appeared normal (Fig. 3). In accord with our initial hypothesis that TPN would decrease, and bile acid signaling would increase expression of FGF19 and GLP-1 and GLP-2, levels of all three peptides were significantly decreased in plasma from the TPN group and increased in the TPN + CDCA group (Fig. 4).

The restoration of gut peptide secretion was associated with marked differences in small intestinal weight and morphology. Small bowel from piglets in the TPN + CDCA group was remarkably preserved compared with their thin and friable counterparts from the TPN group (Fig. 5). Total small bowel weight in the TPN + CDCA group was almost double that of the TPN group ($P < 0.05$). These differences were more prominent in the proximal small bowel. Histological assessment of distal small intestine showed evidence of severe villus blunting in the TPN group. Morphometric analysis of the distal small bowel revealed a villus/crypt ratio (V/C) that was significantly reduced in the TPN group compared with EN group. However, the CDCA treatment markedly ($P < 0.05$) improved this effect (Fig. 5).

Given our previous evidence that TPN induces insulin resistance and that CDCA-augmented FGF19 and GLP-1 secretion might improve this condition, we performed IVGTT in all piglets at 7 and 14 days (Fig. 6). As expected, the results show that TPN resulted in insulin resistance compared with EN pigs at both time points marked by increased mean insulin AUC values. However, CDCA treatment did not improve this outcome at either time point.

**DISCUSSION**

Enteral nutrition protects against the development of PNALD (14, 22, 30). This study was based on the hypothesis...
that TPN associated pathologies, particularly PNALD and gut atrophy, are at least in part a consequence of the loss of the normal enterohepatic circulation of bile acids that occurs with enteral nutrition. This hypothesis is based on the ability of bile acids to induce gut expression and secretion of key signaling peptides, including FGF19 and GLP-1 and GLP-2. The FGF19 pathway is based on activation of the bile acid nuclear receptor FXR, which directly induces FGF19 gene expression and secretion in enterocytes (12, 13). The GLP-1 and GLP-2 pathway is based on activation of the bile acid membrane, G protein-coupled receptor TGR-5, which induces GLP-1/2 secretion by enteroendocrine cells (25).

FGF19 has recently emerged as a key regulator of hepatic bile acid production (6). Moreover, FGF19 treatment of obese mice prevented or reversed diabetes, improved glycemic control, and reduced triglyceride levels (8, 10, 18, 29). Thus we predicted that restoration of FGF19 expression in response to bile acid treatment would improve pathologies associated with PNALD. In addition, evidence of gut mucosal atrophy was observed upon initiation of TPN, with effects evident as early as 24 h in neonatal pigs (16). Because GLP-2 is a well established, gut-trophic factor, we predicted that the reported induction of GLP-1 and -2 in response to bile acid treatment would prevent such atrophy and perhaps the insulin resistance we previously showed in this TPN piglet model (4, 23).

To test these hypotheses, we treated neonatal pigs in a well-established model of TPN administration (16) with or without enteral infusion of the bile acid CDCA. Our results confirmed that TPN markedly decreased levels of FGF19, GLP-1, and GLP-2 in the circulation, whereas CDCA treatment significantly induced all three peptides (Fig. 4). In accord with our predictions, liver pathologies were markedly improved, as indicated by decreased steatosis and decreased plasma levels of bile acids and bilirubin. The finding that TPN results in reduced FGF19 secretion is novel and may provide a mechanism to explain the cholestasis and steatosis observed with PNALD. Because FGF19 production in the small intestine causes suppression of CYP7A1 in hepatocytes (12), diminished FGF19 levels in TPN could increase CYP7A1 expression in the liver, resulting in persistent activation of bile acid synthesis and accumulation in hepatocytes.

A clear prediction of our results is that systemic treatment with FGF19 should result in similar beneficial effects, particularly with respect to the liver. Because we have found that the injected protein is quite unstable, we have used increasing
doses but so far have not observed such responses to our treatments with recombinant FGF19. Although this negative result may be due to technical limitations, it raises the possibility that additional factors are required to account for the beneficial effects of CDCA.

Despite this negative result, results from mice suggest that bile acid induction of FGF19 could also contribute to the prevention of steatosis. Overexpression or infusion of FGF19 in mice reduced adiposity, serum triglycerides, and hepatic acetyl coenzyme A carboxylase and increased metabolic rate and glycemic control (8, 27). Conversely, FGF receptor 4-deficient mice exhibit increased white adipose tissue mass, glucose intolerance, insulin resistance, and hyperlipidemia, further confirming an important role of FGF19 in glucose and lipid metabolic regulation (10). The effect of FGF19 on hepatic lipid metabolism is poorly understood but likely increases fatty acid oxidation and suppresses triglyceride synthesis, both outcomes that could reduce hepatic steatosis.

We also expected that enteral bile acid induction of GLP-1 secretion might improve the dysregulation of glucose homeostasis and insulin resistance that we recently reported in piglets given chronic TPN. GLP-1 is a potent incretin hormone that is known to augment pancreatic insulin secretion and insulin sensitivity, and expression of its precursor glucagon is well known to be stimulated by nutrients (2). In addition, it was possible that increased circulating FGF19 could improve glucose homeostasis by suppression of hepatic gluconeogenesis via a recently described mechanism that functions in parallel with insulin (18). Surprisingly, despite the fact that both GLP-1 and FGF19 secretion were substantially restored by CDCA treatment, this did not translate into improved insulin sensitivity based on IVGTT results.

The other striking observation in this study was the dramatic stimulation of intestinal growth and reversal of mucosal atrophy in piglets treated with CDCA. This was evident by macroscopic appearance, as well as a significant improvement in histology documented by the strong increase in V/C ratio. The idea that bile and bile acids in particular can stimulate intestinal mucosal growth has been reported previously. Studies in rodents show that feeding bile salts induced mucosal proliferation, and biliary diversion after small bowel resection leads to impaired intestinal adaptation and growth (1, 20, 26); we have also shown that bile acid administration induces precocious gut maturation (11). Interestingly, differences in intestinal adaptation associated with pancreaticobiliary diversion were ascribed to changes in circulating levels of enteroglucagon, the previously described hormone whose activity is now known to be due to GLP-2 (1). Thus our results suggest a novel mechanism for bile acid-induced mucosal growth that we suggest involves activation of enteroendocrine L cell secretion of the intestinal trophic peptide GLP-2. In addition, however, we have not reproduced the trophic effect with GLP-2 treatment alone, and bile acids have been shown to induce proliferation of cultured intestinal epithelial cells (7, 26). Thus we cannot rule out more direct effects or additional factors in the bile acid induced responses.

We conclude that the FXR-FGF19 axis is disrupted by TPN in a neonatal piglet model, as is the TGR-5-GLP axis. We predict that FXR activation by exogenous CDCA induces FGF19, which exerts beneficial metabolic effects. Similarly, we predict that TGR-5 activation may mimic nutrient responses to induce expression of both GLP-1 and -2, with the former complementing the metabolic effects of FGF19 and the latter exerting well-known local intestinal trophic effects. The net result of these responses is a significant improvement in liver function, as revealed by decreased serum bilirubin and bile acids as well as liver triglycerides, and also markedly stimulated intestinal mucosal growth in a piglet TPN model. These initial studies provide a firm basis for further studies to explore the molecular mechanisms for the beneficial responses observed. They also raise the provocative prospect that CDCA or other bile acids, which are generally thought of as toxic agents but have recently emerged as key signaling molecules, may have therapeutic utility in the clinical context of various diseases such as PNALD and short-bowel syndrome in patients receiving PN.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors. AKJ, BS, DGB, and DDM were involved in study concept and design; AKJ, BS, DGB, and JJH in acquisition of data; all authors were involved in analysis and interpretation of data; AKJ, BS, DGB, and DDM were involved in drafting of the manuscript; AKJ, DGB, and DDM obtained funding.

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