Pancreatic and biliary secretion are both altered in cystic fibrosis pigs

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Pancreatic and biliary secretion are both altered in cystic fibrosis pigs. Am J Physiol Gastrointest Liver Physiol 303: G961–G968, 2012. First published August 30, 2012; doi:10.1152/ajpgi.00030.2012.—The pancreas, liver, and gallbladder are commonly involved in cystic fibrosis (CF), and changes in biliary function can be observed from the time of birth. In CF pigs, biliary and pancreatic secretory function is abnormal in response to secretin stimulation. The goal of this study was to examine whether biliary and pancreatic secretory function were affected in CF. The model of CF pigs has the advantage of allowing the examination of both biliary and pancreatic function in a single study. We found that biliary and pancreatic secretory function were affected in CF. Biliary and pancreatic secretions are characteristic findings in patients with CF.
composition was also affected in CF pigs (normal baseline fluid secretion, relatively lower pH and higher protein concentration, and lack of response to secretin). Our results suggest that abnormal pancreatic and biliary secretion in CF may have important implications in disease pathogenesis.

METHODS

Animals. All studies were approved by the University of Iowa Animal Care and Use Committee. Four wild-type (WT, CFTR+/+, 2 males and 2 females), 3 CFTR+/− (1 male and 2 females), and 2 CFTRΔF508/ΔF508 (1 male and 1 female) piglets were obtained from Exemplar Genetics (Sioux Center, IA) for pancreatic fluid and bile collection within 24 h after birth. At the end of the study, animals were euthanized with pentobarbital sodium-phenytoin sodium (Euthanol, Virbac, Fort Worth, TX), and the pancreas was collected. For gallbladder pH studies, separate animals were used: 8 WT (CFTR+/+, 5 males and 3 females) and 12 CF (CFTR+/−, 6 males and 6 females) piglets were studied at the time of euthanasia.

Tissues and pathology examination. After euthanasia, pancreata were collected and immersed in fixative for 48–96 h, routinely processed, embedded, sectioned (4 μm), and stained with hematoxylin and eosin.

Immunofluorescence. Pancreatic tissues were excised from newborn piglets, immediately placed in ice-cold 30% sucrose, and quick-frozen in optimal cutting temperature compound with liquid N2. Tissue segments were kept at −80°C. Tissues were cut into 7-μm sections, fixed in 100% methanol at −20°C for 10 min, permeabilized in 0.2% Triton X-100 (Thermo Scientific) in PBS, and blocked in SuperBlock (Thermo Scientific) with 10% normal goat serum (Jackson ImmunoResearch). Tissue sections were incubated for 2 h at 37°C in anti-CFTR-596 (21, 56) (Chemicon) and polyclonal antibody to β-catenin (Zymed), all diluted at 1:100, and then in secondary antibodies (goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568; Molecular Probes/Invitrogen), diluted 1:1,000. Sections were mounted with VECTASHIELD HardSet containing 4',6-diamidino-2-phenylindole (Vector Laboratories) to visualize nuclei.

Collection of pancreatic and biliary fluid with the blind-loop technique. Pancreaticobiliary anatomy in pigs is slightly different from that in humans. In humans, the pancreatic duct converges with the CBD shortly before it opens at the duodenum. In pigs, the CBD is located distal to the pylorus, and the pancreatic duct opens to the duodenum distal to the CBD (Fig. 1) (42). We collected the pancreatic fluid using a blind-loop technique similar to the method described by Freedman et al. (36) and Meyerholz et al. (60). After sedation with ketamine (10–20 mg/kg im) and acepromazine (0.1–2.2 mg/kg im), piglets were anesthetized with inhaled halothane, endotracheally intubated, and ventilated. Heart rate, pulse oximetry, and level of anesthesia were monitored continuously. After the animals were fully anesthetized, a laparotomy was done, and three sutures were placed to create the intestinal blind loops. A biliary blind loop was created by placing the first suture at the distal end of the pylorus and the second suture 1 cm caudally to the first suture. A third suture was placed 1 cm distal to the pancreatic duct, and the intestinal loop was created between the second and third suture as the pancreatic blind loop. The blind loops were entered with 22-gauge Angiocaths that were secured in place with tissue adhesive. After a basal collection for 30 min, secretin (0.2 μg/kg iv; ChirRhoClin, Burtonsville, MD; generously donated by Dr. Edward D. Purich) was administered, and bile and pancreatic fluid were collected for 30 min. Animals were euthanized upon completion of the procedure, as described above. Volume was measured with micropipettes, pH was measured with pH strips, and protein concentrations were measured in 2 μl of fluid using a spectrophotometer (NanoDrop 1000, Thermo Scientific). Because CF pancreatic secretions and bile were not homogenous and contained pieces of mucoid material, we could not measure protein concentration in each sample; therefore, our data may underestimate the effect of CF on protein concentration. The pH of bile and pancreatic secretions was measured using colorpHast pH test strips (catalog no. 9590, EMD Chemicals, Gibbstown, NJ). The pH of gallbladder bile was measured using a needle-type fiber-optic pH meter (catalog no. 502123, World Precision Instruments, Sarasota, FL).

Pancreatic enzyme expressions. Pancreas tissue samples were homogenized in phosphate-buffered saline (20 mM Na2HPO4 and 140 mM NaCl, pH 7.4) containing 0.1% BSA and protease inhibitors. Homogenates were centrifuged at 13,000 g at 4°C for 15 min, and the supernatant was collected. Protein concentrations were estimated with a bichinchoninic acid (BCA) assay.

Amylase activity. Pancreatic amylase activity (IU/μg total protein) was determined using blue starch as a substrate (Phadebas Amylase Kit, Mule Life Sciences, Cambridge, MA) (29).

Lipase activity. Lipase activity in the pancreas homogenates was measured by the Clinical Biochemistry Laboratory at the University of Iowa using a commercially available chromogenic substrate (Roche).

Trypsin activity. Pancreatic trypsin activity was determined using N-α-benzoyl-l-Arg-p-nitroanilide as the substrate in the presence of enterokinase (Sigma-Aldrich, St. Louis, MO) (53). Absorbance at 405 nm was measured every minute for 10 min at room temperature. Enzyme activity was expressed as units and defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per minute.

Immunoblot. Snap-frozen tissues from the pancreas were homogenized in lysis buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris·Cl, and 100 μM PMSF), and protein was quantified with a Micro BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS-PAGE using 12% acrylamide resolving gels. After electrophoretic transfer to a nitrocellulose membrane, blots were blocked in TTBS buffer (10 mM Tris, pH 7.4, 130 mM NaCl, 0.8 mM disodium EDTA, and 1% Tween 20) with 5% nonfat dry milk for ≥1 h and subsequently incubated for 1 h with pancreatic elastase antibody (1:2,000 dilution; Assay Designs, Ann Arbor, MI) or chymotrypsinogen antibody (diluted 1:2,000 in TTBS buffer). The protein of interest was detected using goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:10,000 dilution).
dilution; Upstate Biotechnology, Lake Placid, NY). Blots were washed several times with TTBS buffer. Antibody-labeled bands were visualized by incubation of the blots for 1 min with ECL chemiluminescent substrate (Amersham, Arlington Heights, IL) and exposure of Kodak XAR film for 1–5 min.

Statistics. Values are means ± SE. Differences between groups were analyzed using two-way ANOVA with Bonferroni’s posttest analysis; \( P < 0.05 \) was considered significant. Results from 3 CFTR\(^{-/-}\) and 2 CFTR\(^{AF508/AF508}\) pigs were similar, and they were grouped together as CF pigs.

RESULTS

Loss of CFTR function reduces pancreatic enzyme levels in pigs. Our previous studies suggested that the CF pigs have pancreatic disease at birth, with reduced number of acini, decreased cytoplasmic zymogen granules, and ectatic and plugged ducts surrounded by degenerative exocrine tissue (2, 61, 66, 72, 81). To further test for pancreatic disease in CF pigs, we assayed enzyme levels in the pancreas. Activities of pancreatic amylase, lipase, and trypsin were significantly lower in newborn CF pigs (2, 61, 66, 72, 81). To further test for pancreatic disease in CF pigs was significantly different from that in WT pigs, we created intestinal blind loops to collect pancreatic and biliary fluids. Pancreatic fluid volume was 35 ± 9.5 and 22 ± 18 \( \mu l/h \) in WT and CF pigs, respectively, at baseline (\( P = 0.6 \)). After stimulation with secretin, pancreatic fluid volume increased to 700 ± 91 \( \mu l/h \) in WT pigs (\( P < 0.01 \), before vs. after secretin), but it did not significantly increase in CF pigs (44 ± 36 \( \mu l/h \), \( P = 0.3 \), before vs. after secretin; Fig. 4A).

Pancreatic fluid \( \text{pH} \) was 8.4 ± 0.1 and 5.7 ± 0.1 in WT and CF pigs, respectively, at baseline (\( P < 0.001 \)). After secretin stimulation, \( \text{pH} \) of WT pancreatic fluid increased to 9.5 ± 0 (\( P < 0.01 \), before vs. after secretin) but was unchanged in CF pigs (5.4 ± 0.1, \( P = 0.5 \), before vs. after secretin; Fig. 4B). We did not observe a higher secretion of pancreatic fluid volume or less dramatic \( \text{pH} \) changes in CFTR\(^{AF508/AF508}\) pigs.

A sufficient quantity of pancreatic fluid was obtained from three WT and two CF pigs to measure the protein concentration. Protein concentration in pancreatic fluid was 7.9 and 4.2 mg/ml before and after secretin, respectively, in WT pigs and 44.2 and 22.7 mg/ml, respectively, in CF pigs (Fig. 4C). These results indicate defective pancreatic secretion in CF and are consistent with the reduced volume of secretions.

Biliary secretion in newborn CF pigs. To assess biliary secretions, we took advantage of the separate openings of biliary and pancreatic systems in the pig to collect the bile separate from the pancreatic fluid. The baseline bile volume and \( \text{pH} \) were not significantly different between WT and CF pigs. At baseline, bile volume was 135 ± 65 and 131 ± 73 \( \mu l/h \)}
in WT and CF pigs, respectively \((P = 0.82)\). After secretin, bile volume doubled in WT pigs to \(267 \pm 33 \mu l/h\) \((P = 0.05,\) before vs. after secretin), but it was unchanged in CF pigs \((118 \pm 62 \mu l/h, P = 0.49,\) before vs. after secretin; Fig. 5A).

Although the differences were not as dramatic as in pancreatic fluid, bile pH was also lower in CF than WT pigs at baseline, but the difference did not reach statistical significance \((8.7 \pm 0.1\) and \(8.2 \pm 0.05\) in WT and CF, respectively, \(P = 0.18)\). After secretin, bile pH was unchanged in both groups: \(8.7 \pm 0.1\) and \(8.2 \pm 0.05\) in WT and CF pigs, respectively (Fig. 5B). We did not observe a higher secretion of biliary fluid volume or less dramatic pH changes in \(CFTR^{F508/F508}\) pigs.

A sufficient quantity of bile was obtained from three WT and two CF pigs to measure protein concentration; CF samples were collected only after secretin stimulation. Bile protein concentration was, on average, 8.6 and 16.4 mg/ml in WT and CF pigs, respectively, after secretin (Fig. 5C).

These data indicate that the CF biliary system had impaired fluid secretion into the intestine with secretin stimulation. In addition, there was a tendency for a lower pH and increased protein concentration in the bile. These data show similarity to the data obtained with pancreatic secretions.

Contents of gallbladder are more acidic in CF pigs. CFTR is expressed on the apical membrane of gallbladder epithelium and plays a significant role in fluid, Cl\(^{-}\), and HCO\(_3\)^{-} secretion (22, 25, 67). The gallbladder is affected in CF pigs, with an
almost universal presence of microgallbladder (61, 66, 72). To determine whether the pH of gallbladder luminal contents was altered in CF pigs, we measured bile pH from the gallbladder of WT and CF pigs. Sampling was done at baseline only. WT pig gallbladder contents flowed easily, whereas they were very thick and tenacious in the CF pigs. The pH of the bile in the gallbladder was lower in newborn CF than WT pigs (6.42 ± 0.04 vs. 7.01 ± 0.08, *P < 0.0001; Fig. 6). The pH was also lower in gallbladder bile than in bile collected from the intestinal blind loops, as expected on the basis of earlier studies (76).

**DISCUSSION**

In this study, we took advantage of the anatomically separate biliary and pancreatic drainage systems to assess differences between CF and non-CF secretions from the two organs. We found that secretions from both systems were altered in CF. It is interesting that, under basal conditions, the protein concentration of pancreatic secretions was nearly fivefold greater in CF than WT pigs. This can be partially, but not completely, explained by a secretory rate that was reduced by ~40% in CF. We do not know the reason for the rest of the difference in protein concentration. Given the destruction of the CF pancreatic acini and the resulting decreased pancreatic enzyme content of the pancreatic tissue, it is not likely due to increased enzyme secretion. However, it might have been due to intermittent fluid secretion by WT, but not CF pigs, that was missed in our assay and would have magnified a difference in secretory volume. Another possibility is the genotype-dependent difference in liquid absorption. Identifying the proteins in CF pancreatic fluid and further studying the pancreatic duct function might provide an explanation for, and an insight into, the pathogenesis of obstructive ductal plugs.

As suggested earlier in humans (41, 45, 50, 51) and mice (36) with CF, the volume of CF pig pancreatic secretions was reduced, and the fluid had decreased pH and increased protein concentration. The studies in humans used secretin (which induces volume- and HCO3−-rich secretions) and CCK (which induces pancreatic enzyme-rich secretions). We used only secretin in our studies, because pancreatic secretion does not increase in response to CCK in anesthetized pigs (20) and CCK induces pancreatic protein and trypsin, not volume secretion, only when directly delivered to the gastroduodenal circulation (gastric and right gastroepiploic arteries) in pigs (30). These arteries are very small and difficult to cannulate in the newborn pigs. Because loss of acinar mass does not lead to acidic and dehydrated pancreatic secretions [i.e., Shwachman-Diamond syndrome (47, 50)], these secretory defects in CF pigs are most likely due to the loss of CFTR function in the pancreatic ducts.

Bile volume and pH have not been individually analyzed in CF previously. Although there are differences between the mechanisms of fluid and electrolyte transport in biliary and pancreatic ducts, they do have some similarities, including stimulation by secretin and involvement of CFTR and Cl−/HCO3− exchange (11, 15, 33, 43, 54, 71, 79). In addition, constituents of bile, such as bile acids and ATP, also stimulate cholangiocyte secretion (4, 33, 58, 73). It has been proposed that a CFTR- and CAMP-independent Cl− secretory pathway in the biliary tract may account for the relatively low incidence of liver disease in patients with CF (32, 33). Studying the fluid and anion secretion in CF pig cholangiocytes and pancreatic duct epithelial cells will be an important approach to further understand the physiological differences between the liver and the pancreas.

The pH of gallbladder bile was significantly lower in CF pigs, similar to an observation in CF mice (38). Because CFTR...
is expressed on the apical membrane of gallbladder epithelium and plays a significant role in HCO₃⁻ secretion (22), a lower pH is expected in CF than WT pig gallbladder luminal contents. However, we do not know whether these pH changes are due to reduced HCO₃⁻ secretion from a defective CFTR vs. an increased H⁺ secretion from CF gallbladder epithelium. Nevertheless, our results are consistent with the evidence that CFTR is involved in HCO₃⁻ secretion in the pancreas (48) and other organs (14, 59, 78). We also found a trend for reduced bile pH from the CBD of CF pigs, although the difference was not statistically significant. This difference may be due to the lower number of pigs that underwent the blind-loop studies relative to the number of samples from gallbladders.

It is possible that the acidic pH during storage in the gallbladder may change physical characteristics of the bile, alter the epithelium to a mucus-producing phenotype, or produce other changes. Identification of pH differences between CF and non-CF gallbladder will allow studies of pathogenesis. Importantly, these findings may also be applicable to other gallbladder diseases.

Our study had advantages and limitations. A major advantage was the ability to separately obtain pancreatic and biliary secretions. Previous studies of human samples collected from the jejunum included a mixture of bile and pancreatic fluid (26, 34, 46, 51). Moreover, studies in CF mice have not separated pancreatic and biliary secretions (36). Another advantage is that CF pigs develop pancreatic and liver disease remarkably similar to that of humans with CF (2, 61, 66, 72, 81). We also were able to study newborn pigs, which minimizes the possibility that nutritional differences following birth might have altered hepatic or pancreatic function or disease state. Limitations of our study include the possibility that differences between pancreatic fluid and bile from CF pigs might be secondary to disease in these organs, because the newborn porcine CF liver shows only mild involvement (61, 66, 72). As in studies in humans, we also cannot exclude the possibility that the intestine might have altered the samples collected from the blind loops. However, the marked differences in fluid collected from adjacent pancreatic and biliary segments suggest that modifications by the intestine do not likely account for all the differences. Another consideration is that secretions were collected during laparotomy, which required general anesthesia. Anesthesia with pentobarbital sodium and chloralose-urethane reduces exocrine pancreatic responses to secretin and CCK in dogs (7), but it is not known whether the propofol or isoflurane we used has similar effects. Nevertheless, because anesthesia was the same for all the pigs, these anesthetics are not likely the cause of differences between CF and non-CF.

The difference in disease severity between the porcine CF pancreas and liver is striking. Results from our separate collections of their secretions may allow some speculation as to the pathogenic factor. Despite the large discrepancy in the degree of organ histopathology in CF pancreas and liver, secretion failed to increase secretion from either CF organ, and the protein concentration was increased in CF fluid from both organs. These results suggest that impaired fluid secretion may not be the key factor accounting for the different severity of liver and pancreatic disease. The greatest difference between the pancreas and the liver was in the effect of CF on the pH of their secretions. With secretin stimulation, the pH of pancreatic secretions was 9.5 in non-CF and 5.4 in CF, an H⁺ concentration >10,000 times higher in CF. In contrast, bile pH was 8.7 in WT pigs and 8.2 in CF pigs, an approximately threefold difference. Specialization of the pancreatic duct for HCO₃⁻ secretion allows it to generate those very high pH values, and CFTR is critical for that HCO₃⁻ secretion. Thus we speculate that the marked difference in pH caused by loss of CFTR is responsible for the greater destruction in the CF pancreas than liver. The finding that an acute extracellular acid load sensitizes acinar cells to injury, causes pancreatitis, or worsens disease in animals (8, 62) would be consistent with our speculation. A reduced luminal pH and/or HCO₃⁻ concentration as the cause of pancreatic disease would also be congruent with data suggesting that such abnormalities initiate CF lung and intestinal disease (44, 68).

Beyond the conclusion that the CF pig mimics many aspects of the human disease, it may also be helpful in studying the mechanisms of chronic pancreatitis. Despite advances in understanding the pathogenesis of chronic pancreatitis, there are no well-established treatments to prevent the progression or treat complications of the disease (3). With features typical of chronic pancreatitis (chronic inflammation, acinar atrophy, and fibrosis) (1), the porcine CF model may be instrumental to further study this disease and possibly develop treatment options that could be applicable to humans.

In summary, we have shown that exocrine pancreatic secretion and bile were affected in CF vs. WT pigs. It is possible that the abnormal pancreatic and biliary secretion in CF may have important implications in disease pathogenesis. Additional studies in fetal pigs are required to elucidate the pathogenesis of the pancreatic and biliary disease in CF. The CF pig model may also be helpful in unraveling the role of ductal cell defects in acinar cell physiology and studying the mechanisms of chronic pancreatitis.

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DISCLOSURES
M. Welsh holds equity in Exemplar Genetics, which is licensing materials and technology related to this work.

AUTHOR CONTRIBUTIONS

REFERENCES

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00030.2012 • www.ajpgi.org
56. Mall M, Kreda SM, Mengos A, Jensen TJ, Hirtz S, Seydewitz HH, Maggee DF, Naruse S.

Kopelman H, Corey M, Gaskin K, Durie P, Weizman Z, Forstner G.


Mall M, Kreda SM, Mengos A, Jensen TJ, Hirtz S, Seydewitz HH, Yankaskas J, Kunzelmann K, Riordan JR, Boucher RC.


