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GLYCEMIC REGULATION AND INSULIN SECRETION ARE ABNORMAL IN CYSTIC FIBROSIS PIGS DESPITE SPARING OF ISLET CELL MASS

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Abstract

Diabetes is a common and significant comorbidity in cystic fibrosis (CF). The pathogenesis of CF-related diabetes (CFRD) is incompletely understood. Because exocrine pancreatic disease is similar between humans and pigs with CF, the CF pig model has the potential to contribute significantly to the understanding of CFRD pathogenesis. We determined the structure of the endocrine pancreas in fetal, newborn and older CF and non-CF pigs and assessed endocrine pancreas function by intravenous glucose tolerance test (IV-GTT). In fetal pigs, pancreatic insulin and glucagon density was similar between CF and non-CF. In newborn and older pigs, the insulin and glucagon density was unchanged between CF and non-CF per total pancreatic area, but increased per remnant lobular tissue in CF reflecting exocrine pancreatic loss. Although fasting

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glucose levels were not different between CF and non-CF newborns, CF newborns demonstrated impaired glucose tolerance and increased glucose area under the curve during IV-GTT. Second phase insulin secretion responsiveness was impaired in CF newborn pigs and significantly lower than that observed in non-CF newborns. Older CF pigs had elevated random blood glucose levels compared to non-CF. In summary, glycemic abnormalities and insulin secretion defects were present in newborn CF pigs and spontaneous hyperglycemia developed over time. Functional changes in CF pig pancreas were not associated with a decline in islet cell mass. Our results suggest that functional islet abnormalities, independent of structural islet loss, contribute to the early pathogenesis of CFRD.

Keywords

cystic fibrosis-related diabetes; porcine; glucagon; pancreas; glucose tolerance test; exocrine pancreas

INTRODUCTION

CF is the most common life threatening recessive genetic disorder in Caucasians. It is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (*CFTR*) [1, 2]. *CFTR* is expressed in epithelial cells of various organs including sweat ducts, airways, pancreatic ducts, intestines, and it functions as an apical membrane anion channel, involved primarily in anion secretion [3–6]. The exocrine pancreas is affected early and universally in CF [6–8]. Despite treatment with pancreatic enzymes that prevent severe malnutrition, exocrine pancreatic involvement impairs growth and accelerates the progression of lung disease [9–12], the major cause of mortality in CF [13]. CF patients develop diabetes mellitus as they age: ~10% of patients have Cystic Fibrosis Related Diabetes Mellitus (CFRD) by 10 years of age, and ~50% of CF patients over 30 years of age have CFRD [14, 15]. CFRD is associated with a rapid decline in pulmonary function, higher morbidity and greater mortality [16, 17]. The diagnosis of CFRD is preceded by a decline in body weight and lung function, ascribed to insulin deficiency [15, 18].

Insulin secretory defects occur in CF, with diminished and/or delayed insulin responses to oral glucose demonstrated in CF patients both with and without diabetes [19–24]. This partial loss of insulin secretion in CF is present at least by the end of the first decade of life. In CF, there is a structural loss of beta-cells and islets, especially in those with CFRD [25–28]. However this loss is partial and insufficient to cause diabetes on its own [25]. The relationship of CF-related structural changes in the exocrine and endocrine pancreas to beta-cell functional impairments is not known. Understanding the pathogenesis of CFRD would allow for better therapies to reduce the incidence of CFRD.

We recently generated *CFTR*^{-/-} [29] and *CFTR*^{dF508/dF508} [30, 31] pigs, which we will refer to as CF pigs. This porcine CF model recapitulates many aspects of human CF disease including lung pathology [29, 32]. At birth, CF pig pancreata have reduced number of acini, decreased cytoplasmic zymogen granules, and ectatic and plugged ducts surrounded by degenerative exocrine tissue. Over time, the exocrine pancreas is replaced by fat and fibrous

tissue [29–33], as it occurs in humans with CF [8, 34–36]. With multi-organ disease and exocrine pancreatic disease that is similar to human disease, the CF pig model offers a unique opportunity to investigate the pathogenesis of CFRD.

We investigated the structure and function of the endocrine pancreas in CF pigs over time as the exocrine pancreas was progressively damaged. We found that CF pigs had abnormal insulin secretion in the newborn period and subsequently developed spontaneous hyperglycemia. Interestingly, the functional impairments in insulin secretion were not associated with structural loss of islets or decreased insulin content in CF pigs at any age. These findings suggest that functional abnormalities in the islets play a role in the early pathogenesis of CFRD despite sparing of the islet cell mass in the face of marked exocrine pancreas disease.

MATERIALS AND METHODS

CF pigs

Animal experiments were reviewed and approved by the University of Iowa Institutional Animal Care and Use Committee. CF (*CFTR*^{-/-}, *CFTR*^{F508/F508}) and non-CF (*CFTR*^{+/+}, *CFTR*^{+/-}, *CFTR*^{+/-} *F508*) piglets were obtained from Exemplar Genetics (Sioux Center, IA, USA) and studied within 24 h after birth. Fetuses (54, 83–90 day gestation) were collected via C-section (pig gestation is ~114 days). Newborn pigs were either euthanized at 6–24 hour following birth or euthanized due to lung disease postnatally at 2–12 months of age (average age 5 ½ months for CF pigs, 3–6 months for control pigs). For IV-GTT, piglets were euthanized immediately after completion of the study.

Histopathology

Following euthanasia, the pancreas was quickly prosected and placed into 10% neutral buffered formalin and after 48–96 hour the tissues were routinely processed and paraffin-embedded. Since islet size and subcellular composition may not be uniformly distributed within the whole pancreas [37, 38], we consistently took samples from the splenic lobe of CF and non-CF pig pancreas.

Immunohistochemistry

Formalin-fixed, paraffin-embedded pancreas blocks were sectioned at 4 µm on positively charged slides. Sections were deparaffinized and rehydrated. Slides were retrieved with proteinase K (5 min) followed by an endogenous peroxidase block (3% hydrogen peroxide, 8 min) and a background block (Background Buster, Innovex Biosciences, Richmond, CA). Insulin (1:4000, MP Biomedicals, Santa Ana, CA #65104) and glucagon (1:100, MP Biomedicals, Santa Ana, CA #11184) primary antibodies were applied for 1 hour at room temperature. Slides were rinsed then incubated with DAKO Rabbit Envision HRP System reagent for 30 min. Slides were then developed with DAKO DAB plus for 5 min followed by DAB Enhancer for 3 min. Slides were rinsed in distilled water, counterstained and cover slipped. A negative control slide was stained using the same procedure, omitting the primary antibody.

Immunofluorescence

Pancreatic tissues were excised from newborn piglets and immediately frozen in OCT with liquid N₂. Tissue segments were kept at -80°C . Tissues were cut into $8\text{ }\mu\text{m}$ sections, fixed in 10% cold Z-fix for 5 min, permeabilized in 0.2% Triton X-100 for 10 min and blocked in 10% goat serum for 30 min at room temperature. Tissue sections were incubated for 1 hour at 37°C with anti-beta-III tubulin (1:250 dilution, Millipore MAB1637, Temecula, CA), anti-chromogranin A (1:400 dilution, Abcam ab43861, Cambridge, MA), anti-von Willebrand (1:1000 dilution, Abcam ab68545, Cambridge, MA), protein gene product 9.5 (1:100 dilution, Abcam ab8189, Cambridge, MA) followed by secondary antibody (Alexa-fluor 488 and Alexa-fluor 568 (1:500 dilution, Life Technologies, Grand Island, NY) for 30 min at room temperature. Sections were mounted with Vectashield containing DAPI (Vector Labs, Burlingame, CA) to visualize nuclei. Images were acquired using identical parameters on a Zeiss 710 confocal microscope.

Morphometric analysis

All tissues were digitized with an Aperio ScanScope CS at 20X. Images were analyzed using ImageJ software (NIH, National Institute of Health, Bethesda, MD). Total area of pancreas was defined by the exterior borders in each tissue section. Lobular area was defined by the remaining parenchymal pancreatic tissue as previously described [35]. Images were converted to an RGB stack and staining thresholded in the blue channel. The percent staining for insulin and glucagon was defined as the percent staining within either the total or lobular areas of the pancreas. The density of discrete insulin-positive islets and islet diameters were calculated from a thresholded image using the particle count function in ImageJ. The particle count included the total number of thresholded particles and the diameter of the particle. The islet number was normalized to total pancreatic area to obtain the islet density.

Blood glucose measurements

Blood glucose was measured with a glucometer (LifeScan, Milpitas, CA) and recorded immediately after blood was drawn from the animal. Blood glucose was measured randomly in non-fasting pigs (older animals) or fasting pigs (baseline lab draws- IV-GTT from newborn animals).

IV glucose tolerance test

The IV glucose tolerance test was done using a protocol similar to Kobayashi *et al* [39]. Pigs were initially sedated with IM Ketamine (10–20 mg/kg) and xylazine (0.2–2.2 mg/kg). Then a peripheral IV line was placed and further sedation was provided with propofol (0.8–1.6 mg/kg). One dose was sufficient to provide sedation and a second dose was rarely needed. Once the animal was appropriately sedated, a 3.5 Fr Argyle™ umbilical vessel catheter (Kendall-Covidien, Mansfield, Massachusetts # 8888160333) was placed into the umbilical vein and advanced ~5–6 cm until venous blood return was obtained. Blood was collected via the umbilical vein at baseline for glucose and insulin measurements, followed by IV dextrose infusion (0.5 g/kg over 2–3 min, followed by 5 ml of normal saline bolus with 1 unit per ml heparin to avoid clotting). Blood was collected at 1, 5, 10, 20, 40, 60 min after

infusion. Before each blood draw, 0.5 ml was wasted, as it would reflect old blood in the tubing. After each blood collection, the catheter was flushed with 5 ml normal saline and ¼ unit per ml heparin to avoid clotting. Animals tolerated the procedure well without any complications. Second phase insulin responsiveness was defined as the proportionality between the integrated increase in insulin at 40–60 min above base levels ($AUC_{\text{insulin},40-60\text{min}}$) and the integrated increase in antecedent glycemia above base levels ($AUC_{\text{glucose},0-20\text{min}}$) [40].

ELISA

Plasma insulin and glucagon: Whole blood was collected in 4 ml K2 EDTA tubes, gently rocked and immediately centrifuged at 4°C for 15 min at 1,600 RPM (Beckman Coulter, Allegra X-22R, Indianapolis, IN). Plasma was collected, immediately snap frozen in liquid N₂ and stored at –80° C. A two-site ELISA was performed using a porcine insulin ELISA kit (Mercodia, Winston Salem, NC # 10-1200-01). This porcine insulin ELISA has virtually no cross-reactivity with porcine pro-insulin or C-peptide. A colorimetric glucagon ELISA was performed using a glucagon ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA # EK-028-02). All ELISA procedures were performed according to manufacturer recommendations. Plates were read on VersaMax ELISA Microplate Reader (Molecular Devices, v5, Sunnyvale, CA).

Pancreatic Insulin Content: Pancreata were harvested and placed in 1.5% HCl 70% EtOH [41]. After overnight incubation at –20°C they were polytron homogenized, volume adjusted to 20 ml per g of pancreas, and reincubated overnight at –20°C. Insulin content was measured in supernatant from 15 min centrifugation at 2000 rpm. Sample was neutralized with an equal volume of 1M Tris pH 7.5 and diluted 200 times further in Insulin ELISA sample diluent. Insulin concentration was measured using a Porcine Insulin ELISA kit (#10-1223-01, Mercodia, Uppsala, Sweden). Protein content was measured by Bradford kit (Bio-rad, #500-0002).

Proinsulin: Proinsulin was measured by Porcine Proinsulin ELISA (#MBS031568, MyBiosource Inc., San Diego, USA) according to the manufacturer's instructions.

HOMA-IR and HOMA%B: HOMA-IR and HOMA%B were determined from fasting newborn pigs samples as $\text{plasma glucose (mmol/L)} * \text{insulin (}\mu\text{M/mL)} / 22.5$ and $(20 - \text{insulin}) / (\text{glucose} - 3.5)$ [42]. Note that the constants in these formulae are derived from humans but are largely unknown for newborn pigs. Thus the derived values should be considered in a relative, not absolute, context.

Serum Electrolytes

Blood was collected via the umbilical vein of non-CF and CF newborn pigs into heparinized tubes. Plasma was separated and calcium, chloride, sodium and potassium were measured by the Clinical Biochemistry Lab at the University of Iowa, using a Roche 7000 ISC. Sodium, potassium and chloride were measured by ion selective electrodes and measurement of calcium was photometric.

Statistical Analysis

Data from each group were summarized with the mean and standard error of the mean (SEM). Statistical analysis was performed by Mann-Whitney test. When appropriate unpaired Student's *t* test was used. When evaluating the differences in variance between glycemia of CF and non-CF newborns, the variance ratio test (F test) was utilized. Unless otherwise specified, statistical significance was determined as $P < 0.05$.

RESULTS

Islets are spared as the exocrine pancreatic disease progresses in CF pigs

As in humans [7, 22, 34–36, 43–46], the exocrine pancreatic damage starts *in utero* in CF pigs [33] and progresses over time [29]. We have previously demonstrated that fetal CF pig pancreata (83–90 day, pig gestation is ~114 days) have patchy loss of zymogen-filled acini, inflammation and tissue destruction and these lesions progress into the newborn period [33]. Eventually, the exocrine pancreas is replaced by fibrous and adipose tissue in older CF pigs [29–33]. To determine whether the islets were similarly affected in CF pigs, we immunostained fetal, newborn, and older CF and non-CF pig pancreata for insulin and glucagon.

Fetal pancreas—We examined 2 fetal time points, 54 day gestation, which is a time point before overt exocrine pancreatic destruction begins, and 83–90 day gestation, a time point when patchy, modest exocrine pancreatic damage is present [33]. Immunostaining of insulin and glucagon were scattered in small clusters within the developing lobules of fetal pigs in both genotypes (Figure 1A, B). The insulin and glucagon cellular immunostaining as a percentage of total pancreatic area was not significantly different between genotypes at 54 or 83–90 days gestation (Figure 1C, D). These findings demonstrate that the islet cell mass was not reduced and the islets were structurally intact in fetal CF and non-CF pigs.

Newborn pancreas—In newborn (<24 hours old) pigs, immunostaining of insulin and glucagon occurred as variable-sized cellular aggregates in the pancreatic parenchyma. CF pigs had immunostaining restricted to the pancreatic lobules (Figure 2A), which were diminutive and separated by loose connective tissue as previously described. The insulin and glucagon cellular densities as a percentage of total pancreatic parenchymal tissue were similar between the genotypes (Figure 2B) whereas cellular densities in the lobular tissue were increased in the pancreas of CF pigs (Figure 2C). The discrepancies between the lobular and total cellular density are not unexpected given the loss and fibrotic/fatty replacement of exocrine tissue in the CF animals.

Older pig pancreas—Insulin and glucagon stains in older (2–12 months of age) non-CF and CF pig pancreas were detected in large distinct islets to smaller cellular aggregates. Within the islets, the majority of the cells were positive for insulin whereas glucagon-positive cells localized along the periphery of the islet (Figure 3A). In the pancreas of older CF pigs, the insulin and glucagon cellular aggregates were concentrated in the remnant lobular tissue. There was no difference in the cellular density of insulin or glucagon when measured within the total pancreatic area, including the area of fatty infiltration (Figure 3B).

However, the cellular densities of insulin and glucagon were higher in the pancreas of CF pigs when measured within the remnant lobular tissue (Figure 3C). This was again consistent with the CF islets populating the remnant lobular tissue.

To evaluate possible structural changes in endocrine pancreas, discrete islet numbers and size were assessed by quantitative morphometry on insulin stained pancreas. This was done for the adult pig pancreas only, because the islets in the neonatal pig pancreas are immature and characterized as small clusters of cells that often merge together and are difficult to distinguish from each other as discrete islets, regardless of the genotype. The density of discrete islets (3.8 ± 1.4 islets per mm^2 in non-CF versus 9.5 ± 9.2 islets per mm^2 in CF, $n=7$, $p=0.15$, Student t test) and mean islet diameter (67 ± 6.7 μm in non-CF versus 60 ± 13 μm in CF, $n=7$, $p=0.27$, Student t test) were not different between the genotypes.

These results suggest that the endocrine pancreas structure remains intact while the exocrine pancreas is destroyed and replaced by fibrous and adipose tissue in CF pigs.

Glycemic control is abnormal in CF pigs

Blood glucose levels are frequently elevated, albeit often only in the sub-diabetic range, in humans with CF by mid-childhood. We thus tested blood glucose in CF and non-CF pigs at various ages (ranging 2–12 months). Due to the limited number of surviving older CF pigs and the ensuing limitations on disruptive/invasive protocols, testing was limited to small random-fed capillary blood samples. The mean blood glucose levels measured per pig were significantly higher in CF pigs compared to non-CF pigs (Fig. 3D). These results suggest that CF pigs experience spontaneous hyperglycemia.

Newborn pigs—Humans with CF have impaired insulin secretion, which manifests as a blunted first phase, found in both those with and without CFRD [19–24]. However, the correlation of this defect to exocrine pancreatic disease is unknown. CF newborn pigs have exocrine pancreatic disease and are currently more amenable to invasive and controlled studies than older CF pigs. We thus sought to determine whether insulin secretion and glucose homeostasis were abnormal in newborn CF pigs as compared to their non-CF littermates. To this end, fasting glycemia and intravenous glucose tolerance tests (IV-GTTs) were performed on newborn non-CF and CF pigs. The body weights of CF and non-CF piglets were not significantly different (Figure 4A). Although the mean fasting glucose did not differ between genotypes (Figure 4B), CF piglets as a group exhibited 6.5-fold greater glycemic variance compared to non-CF piglets ($p=0.02$ by F test) with a portion of the CF piglets being hypoglycemic or hyperglycemic. Following an IV glucose bolus, the CF piglets had significantly higher blood glucose levels at 20–60 minutes (Figure 4C), and increased glucose area under the curve (AUC) (Figure 4D) thus impaired glucose tolerance compared to non-CF piglets.

Despite having higher glucoses, CF piglets did not exhibit increased insulin levels compared to non-CF piglets. In fact, total insulin AUC during the IV-GTT trended lower in CF (1.71 ± 0.42 ng·min/mL versus 2.66 ± 0.56 in non-CF, $p=0.09$) and CF piglets exhibited lower mean insulin values at all post-glucose administration time points during the IV-GTT (Figure 4E). Neither genotype exhibited first phase insulin secretion, in that insulin levels

during the first 20 minutes did not differ statistically from baseline levels. The non-CF piglets did exhibit a second phase insulin response, in that insulin levels at 40 and 60 minutes were statistically higher than baseline levels. By contrast, CF piglets did not exhibit second phase insulin response, in that insulin levels at 40 and 60 minutes did not differ from baseline levels. Accordingly, calculated second phase insulin responsiveness was significantly higher in non-CF than CF piglets (Figure 4F). Gender did not influence the glycemic and insulinemic outcomes in newborn piglets, as there were no differences between genders in these when assessed by 2-way ANOVA on gender and CF status.

HOMA%B, another indicator of beta-cell secretory action, showed a trend towards diminished function in CF compared to non-CF piglets (Figure 5A). The ratio of plasma proinsulin to insulin was not different between CF and non-CF pigs in the fasted state. However, at 60 minutes during ivGTT, the ratio of proinsulin to insulin was elevated in CF compared to non-CF newborn piglets (Figure 5B). Nonetheless, the pancreatic insulin content was not different between CF and non-CF newborn pigs when normalized to pancreatic weight (Figure 6A). The pancreatic insulin mRNA content between the genotypes was also not different (Figure S1). However, when expressed per pancreatic protein amount, insulin content was increased in CF piglets compared to non-CF (Figure 6B). Despite this, there was a trend towards reduced total insulin content per piglet (Figure 6C) owing to the smaller total pancreatic mass in CF newborns.

Because the above data collectively indicate beta-cell dysfunction in newborn CF pigs despite relative sparing of the endocrine pancreas, we sought to determine whether there was vascular or neural element compromise in CF islets. We assessed CF and non-CF newborn pig pancreas with von Willebrand Factor (VWF) (endothelial cell marker) [47] or beta-III tubulin and protein gene product 9.5 (PGP 9.5) (neuronal cell markers) [48, 49] immunostaining. The islets were visualized with chromogranin A (CGA) (neuroendocrine cell marker) [50]. The islet vascular supply was similar between the genotypes (Figure S2A and B). In newborn non-CF pancreas, beta-III tubulin and PGP 9.5 stains identified neural fibers that were moderately abundant in the exocrine pancreas and some fibers traversed adjacent to CGA positive islets (Figure S3A, C, E). By contrast, in the newborn CF pancreas, the number of neural fibers was reduced, with typically only one fiber per several high power views (Figure S3B, F). Just as in non-CF pancreas, some of these fibers coursed near islets (Figure S3D). In addition, beta-III tubulin and PGP 9.5 staining showed a fragmented, single-cell staining pattern not clearly associated with neural fibers in the CF pancreas (Figure 3B, D, F) that was only rarely observed in the non-CF pancreas (Figure 3A, C, E).

HOMA-IR was assessed in newborn piglets as an index of insulin resistance, to determine if this might contribute to the impaired glucose tolerance in CF pigs. There was no difference in HOMA-IR between CF and non-CF pigs (Figure S4A). Likewise, there was no difference between CF and non-CF plasma glucagon in fasting newborn pigs, suggesting that glucagon was not causing the glycemic dysregulation in CF pigs (Figure S4B). Serum calcium, chloride, sodium and potassium of CF newborns were similar between genotypes, indicating that fluid-electrolyte status was not different between CF and non-CF pigs (Table 1).

DISCUSSION

Herein, we describe the endocrine pancreas and glycemic abnormalities in the CF pig model from the fetal period into adulthood. Newborn CF pigs have abnormal insulin secretion with reduced responsiveness to hyperglycemia. CF newborn pigs have widely variant fasting glycemia and impaired glucose tolerance. CF adult pigs develop spontaneous hyperglycemia. Interestingly, the abnormal glycemic regulation and insulin secretion is associated with an increase in the insulin-positive cellular area when compared to lobular pancreas, indicating a relative sparing of the endocrine pancreas despite marked exocrine destruction and replacement.

Impaired insulin secretion is considered to be the primary defect underlying CFRD. An important question is to what extent the impairment in insulin secretion is due to beta cell loss versus functional defects in beta cell. The pancreatic structural correlates of impaired insulin secretion have been difficult to address in humans, due to barriers coupling functional beta cell tests with structural pancreatic studies. Our morphometric, total insulin protein and mRNA studies are consistent with sparing of beta-cells compared to loss of exocrine pancreatic components. This suggests that in newborn pigs with CF, the functional impairments in insulin secretion are major contributors to glycemic abnormalities.

There are several indicators of functional insulin secretory defects in CF pigs, including reduced second phase insulin secretion and a trend towards reduced HOMA%B. CF pigs demonstrate an increased proinsulin:insulin ratio, just as has been shown in humans with CF [22, 51]. Elevated proinsulin to insulin ratio can be a reflection of beta-cell stress [52], suggesting that the beta-cells in CF newborn piglets are experiencing an adverse environment. An alternative explanation could be that the beta-cells in CF animals are experiencing overwork conditions, due to loss of total beta-cell mass, leading to release of immature secretory granules. An ensuing question is whether the impaired beta-cell function originates from the modestly reduced total insulin-positive mass versus from functional deficits independent of beta-cell mass. A mechanistic example of the former would be overwork of the remaining beta-cells, whereas mechanisms of the latter could be a reduced beta-cell function due to a toxic pancreatic milieu or lack of CFTR to support the islet function. Although we cannot rule out a contribution from the modest reduction of total pancreatic insulin content per pig, the observed insulin secretory functional defects in the CF pigs are more severe than the reduced total pancreatic insulin content. This suggests that CF islets are unable to properly release their insulin content in response to glucose.

Another mechanism for reduced insulin secretion in CF pigs could be due to low oxygen tension secondary to reduced blood flow to the islets, forcing them to go dormant, also called: “sleeping islets” [53]. This could be expected in CF pig islets that reside in a hostile environment surrounded by a degenerating exocrine pancreas. However, there was not reduced vascularity in CF pig islets compared to non-CF.

In some animal models of diabetes, islet-associated neural elements may show hypertrophy possibly as an attempt to raise insulin secretion [54]. In chronic pancreatitis and pancreatic cancer, swelling of intra- and peri-insular nerves occurs, possibly in response to the loss of

beta-cells [48]. In CF pigs, the nerve staining pattern was different compared to non-CF, but the nerve cells were found outside and around the periphery of the newborn pig islets and not in the islets as described in humans [48, 55]. These changes are most likely caused by the inflammation and damage in the exocrine pancreas of CF pigs. Their contribution to the insulin secretion defects is unknown.

Our data suggest that whole-body insulin resistance does not contribute to the impaired glycemia in newborn CF piglets. However, we cannot exclude the possibility of tissue specific insulin resistance. Indeed, humans with CF and pancreatic insufficiency demonstrate insulin resistance localized to the liver that is compensated by enhanced peripheral insulin sensitivity [56].

A major advantage of our study is the ability to directly compare the progression of exocrine pancreas lesions, structure of the islets and functional studies in the animals. Because the pancreas cannot be easily obtained from human fetuses, infants or children with CF, such evaluation cannot be done in humans. Thus, the CF pig model represents a unique opportunity to interrogate insulin secretion as affected by CF exocrine pancreatic disease, but in the presence of relatively preserved beta cell mass. Our studies are in concordance with human studies that show islet cell loss as a late phenomenon in individuals with CF. For example, islets are relatively spared in young children, even when the adjacent exocrine pancreas is destroyed [57]. However, with progressive loss of exocrine pancreas, increasing amounts of fibrosis and degeneration within the islets may occur [25, 58].

Diminished and/or delayed insulin responses to glucose are common in CF patients both with and without diabetes [19–23]. Early autopsy studies have shown that islet insulin staining in CF patients with normoglycemia was similar to controls, but significantly lower once diabetes developed [26, 27]. We have not observed a deficiency of insulin or glucagon producing cells in the CF pig pancreas up to one-year of age. It is not known whether the islet mass or insulin/glucagon staining would decline at later time points.

Fasting blood glucose was not different between CF and non-CF newborn pigs, but CF newborns had higher glucose values at later time points (20–60 minutes) during the GTT with significantly higher glucose AUC. This finding is similar to early glucose tolerance abnormalities in human CF patients where elevations of plasma glucose are present at the mid-time points (20-, 60-, and 90-minute) of the oral glucose tolerance test [59].

Normal plasma insulin levels in unfed newborn pigs are largely unknown, as the youngest reported values in the literature are from 2–3 week old pigs following IV-GTT testing [39]. Insulin levels in the fasted newborn pigs were at the lower limits of detection. Because insulin levels were very low during the earliest time points of the IV-GTT, it is not possible to definitively conclude whether first phase insulin secretion was impaired or not in CF newborns. However, minimal first phase insulin secretion observed in both CF and non-CF groups is not unexpected, as newborn pigs are known to exhibit virtually no first phase insulin secretion [60] similar to human infants [61, 62].

The older CF pigs in our study had surgical correction of the meconium ileus to be able to survive beyond the neonatal period, with diminished availability of older animals for

dynamic endocrine studies. With the recent availability of pigs with transgenic intestinal CFTR expression and alleviation of the meconium ileus phenotype [63], CF pigs can now survive beyond the newborn period without the need for intestinal surgery. This will open the door for studies in older pigs and also in newborns at later time points in addition to immediately after birth.

Another animal model, CF ferrets, have minimal exocrine pancreatic disease at birth, but nonetheless manifest glycemic dysregulation and impaired first phase insulin secretion [64, 65]. In contrast, CF pigs have significant exocrine pancreatic damage at birth [29–33, 63, 66, 67], glycemic dysregulation, and impaired insulin secretion. In CF ferrets, exocrine pancreatic disease progresses very rapidly after birth; islet mass is lost and spontaneous hyperglycemia develops [65]. In contrast, CF pigs have slower progression of pancreatic disease with replacement of the pancreas with fat and fibrous tissue which initiates *in utero* [30, 63], while islet structure is not affected (Figs 1, 2, 3). In humans, prior to overt CFRD, patients with abnormal glucose tolerance have decreased first phase insulin secretion indicating beta-cell dysfunction, which can progress to decreased total insulin secretion over time [68–70]. There is a correlation between exocrine pancreatic disease and CFRD because CF patients with exocrine pancreatic insufficiency have significantly impaired plasma insulin, glucagon and pancreatic polypeptide levels compared to those with normal exocrine pancreatic function [19]. The significant impairment in plasma hormone levels was interpreted as possible injury to the islets. Our study in CF pigs suggests that impairments in insulin secretion in newborn CF pigs are not due to islet cell loss, but may correlate with exocrine pancreatic disease. With varying degrees of exocrine pancreatic involvement and glycemic regulation abnormalities, these two animal models offer a unique and complementary opportunity to evaluate the mechanisms of insulin secretory defects in CFRD.

The prevalence of diabetes in cystic fibrosis is higher in women than men, despite higher relative insulin secretion in CF women [71]. Although we found no differences between genders of pigs, the number per gender was small and thus our studies were poorly powered to pick up gender differences.

Based on these findings, we propose that the CF pig will be a useful model to study the pathophysiology of CFRD. Taken together, islet morphometry and functional studies in CF humans, ferrets, and pigs suggest that there are at least two endocrine pancreas determinants of CFRD that are independent of beta-cell loss. One determinant is a loss of insulin secretion capacity limited to only the first phase, which occurs independently of severe exocrine pancreas disease in newborn ferrets [65]. Another determinant is a loss of overall insulin secretion capacity, likely a pathophysiologic defect as it occurs in the presence of sufficient islet mass as was observed in newborn pigs in this study. Further understanding of these functional insulin secretory defects will aid in development of new therapeutic approaches for CFRD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVES

- As the overall care and life expectancy of patients with CF has improved over the years, CFRD has become increasingly prevalent. CFRD is associated with a rapid decline in pulmonary function, higher morbidity and greater mortality.
- The pathogenesis of CFRD is poorly understood, but insulin secretion declines during early phases of this disease. Therapies that can restore insulin secretion will reduce the incidence of CFRD and thus morbidity and mortality. However, there are no such therapies, because the mechanisms underlying impaired insulin secretion in CF are not known.
- Our findings have implications for understanding how functional islet abnormalities, independent of structural islet loss, contribute to the early pathogenesis of CFRD.

Summary Statement

In the Cystic Fibrosis pig model, exocrine pancreatic disease starts *in utero* and progresses over time while the endocrine pancreas remains relatively spared. CF piglets exhibit abnormal glycemic tolerance and reduced insulin secretion and develop spontaneous hyperglycemia with age.

Figure 1. Islet cell mass is intact in fetal CF pigs

Representative images showing insulin and glucagon immunostaining in fetal non-CF and CF pancreas at (A) 54d and (B) 83–90d gestation. The percent insulin and glucagon staining was not different between non-CF and CF at (C) 54d (CF n=3, non-CF n=4; p=0.7 and 0.2 respectively) or (D) 83–90d gestation (CF n=12, non-CF n=7; p=0.4 and 0.9 respectively). Statistical analysis was performed by Mann Whitney test. Bar=100µm.

Figure 2. Islet cells are intact in newborn CF pigs with islets populating the remnant lobular tissue

(A) Representative images showing insulin and glucagon immunostaining in newborn pancreas from CF and non-CF pigs. (B) The insulin and glucagon density in the whole pancreas was not different between genotypes (non-CF=9, CF=11; $p=0.3$ and $p=0.89$ respectively). (C) However, insulin and glucagon density were significantly increased in the remnant lobular tissue (* $p=0.02$ and 0.008 respectively). Statistical analysis was performed by Mann Whitney test. Bar=40 μ m.

Figure 3. Islets remain intact in older CF pig pancreas, predominantly in the remnant lobular tissue

(A) Representative images showing insulin and glucagon immunostaining in the pancreas from older CF and non-CF pigs. (B) The insulin and glucagon density in the whole pancreas was not different between genotypes when normalized to the total pancreatic area (non-CF=8, CF=8; $p=0.5$ and $p=0.5$ respectively). (C) However, insulin and glucagon density were significantly increased in the remnant lobular tissue ($*p=0.001$ and $p=0.01$ respectively). (D) Randomly fed blood glucose levels were significantly higher in older CF pigs ($n=6$) compared to non-CF pigs ($n=10$) ($*p=0.016$). Statistical analysis was performed by Mann Whitney test. Bar=100 μ m.

Figure 4. Abnormal glycemic control and insulin secretion in newborn CF pigs

(A) The birth weights ($p=0.13$, CF vs. non-CF, Student t test). (B) Fasting blood glucose at baseline of IV-GTT ($p=0.15$, CF vs. non-CF, Student t test; variance ratio for CF versus non-CF, 95% confidence range 1.3–29.5, $p=0.02$, F test). (C) Blood glucose levels following IV-GTT (* $p<0.05$, CF vs. non-CF, Student t test). (D) Glucose area under the curve (* $p=0.05$, CF vs. non-CF, Student t test). (E) Insulin levels during IV-GTT (* $p<0.05$, insulin levels in non-CF piglets at 40 and 60 minutes compared to baseline, Student t test); (F) Second phase insulin secretion responsiveness during IV-GTT (* $p=0.015$, CF vs. non-CF, Student t test). $N=8$ non-CF, $N=9$ CF for all groups.

Figure 5. Assessment of beta-cell function in newborn CF pigs

(A) The HOMA%B in CF and non-CF newborn pigs, ($p=0.10$, CF vs. non-CF, Student t test). (B) The ratio of proinsulin to insulin measured just before the start and 60 min during ivGTT in newborn pigs (* $p<0.01$ CF vs. non-CF, two-way ANOVA); Non-CF: open circles; CF: closed squares. N=8 non-CF, N=9 CF for all groups.

Figure 6. Pancreatic insulin content of newborn CF pigs

Pancreatic insulin content was measured in newborn pigs after extraction in acidified-ethanol. Results are expressed as (A) normalized to pancreatic weight ($p=0.9$, CF vs. non-CF, Student t test); (B) normalized to pancreatic protein, ($p=0.08$, CF vs. non-CF, Student t test); and (C) as the total insulin per pig, ($*p<0.05$ CF vs. non-CF, Student t test). $N=16$ non-CF, $N=8$ CF for all groups.

Table 1

Serum electrolytes from CF and non-CF pigs are not significantly different.

Ion	Non-CF	CF	
Calcium	11.30 ± 0.29	11.86 ± 0.56	ns
Chloride	98.88 ± 2.71	101.80 ± 1.60	ns
Sodium	149.10 ± 3.24	152.60 ± 2.26	ns
Potassium	2.74 ± 0.24	3.40 ± 0.37	ns

Results are expressed as mean ± SEM; ns: not statistically significant (Statistical analysis was performed by Student t test).