Original Paper

Downregulation of the $\text{Cl}^-/\text{HCO}_3^-$ Exchanger Pendrin in Kidneys of Mice with Cystic Fibrosis: Role in the Pathogenesis of Metabolic Alkalosis

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Key Words

Metabolic Alkalosis • Cystic Fibrosis • Pendrin • CFTR

Abstract

Background/Aims: Patients with cystic fibrosis (CF) are prone to the development of metabolic alkalosis; however, the pathogenesis of this life threatening derangement remains unknown. We hypothesized that altered acid base transport machinery in the kidney collecting duct underlies the mechanism of impaired bicarbonate elimination in the CF kidney. **Methods:** Balance studies in metabolic cages were performed in WT and CFTR knockout (CF) mice with the intestinal rescue in response to bicarbonate loading or salt restriction, and the expression levels and cellular distribution of acid base and electrolyte transporters in the proximal tubule, collecting duct and small intestine were examined by western blots, northern blots and/or immunofluorescence labeling. **Results:** Baseline parameters, including acid-base and systemic vascular volume status were comparable in WT and CF mice, as determined by blood gas, kidney renin expression and urine chloride excretion. Compared with WT animals, CF mice demonstrated a significantly higher serum HCO_3^- concentration (22.63 in WT vs. 26.83 mEq/L in CF mice; $n=4$, $p=0.013$) and serum pH (7.33 in WT vs. 7.42 in CF mice; $n=4$, $p=0.00792$) and exhibited impaired kidney HCO_3^- excretion (urine pH 8.10 in WT vs. 7.35 in CF mice; $n=7$, $p=0.00990$) following a 3-day oral bicarbonate load. When subjected to salt restriction, CF mice developed a significantly higher serum HCO_3^- concentration vs. WT animals (29.26 mEq/L in CF mice vs. 26.72 in WT; $n=5$, $p=0.0291$). Immunofluorescence labeling demonstrated a profound reduction in the apical expression of the $\text{Cl}^-/\text{HCO}_3^-$ exchanger pendrin in cortical collecting duct cells and western and northern blots indicated diminished plasma membrane abundance and mRNA expression of pendrin in CF kidneys. **Conclusions:** We propose that patients with cystic fibrosis are prone to the development of metabolic alkalosis secondary to the inactivation of the bicarbonate secreting transporter pendrin, specifically during volume depletion, which is a common occurrence in CF patients.

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Introduction

Cystic fibrosis (CF) is the most common life-threatening genetic disease in the United States. It is caused by mutations in CFTR, a cAMP/PKA and ATP-regulated apical chloride channel, which is widely expressed in epithelial tissues [1, 2]. Due to its wide-spread distribution in epithelial cells, patients with a CFTR mutation develop tissue damage in multiple organs, including lung injury and/or infection, pancreatic inflammation (pancreatitis) and/or intestinal obstruction [3–5]. However, no apparent kidney phenotype has been identified in CF patients, despite the abundant expression of CFTR in the kidney tubules, including the collecting duct intercalated cells [6].

Patients with CF are prone to the development of metabolic alkalosis [7–9], which creates a physiologic diminution of respiratory effort that can be detrimental in CF patients, who suffer from chronic lung injury. Metabolic alkalosis could also be the initial presentation or complication of cystic fibrosis in infants and children [10]. The pathogenesis of metabolic alkalosis in CF remains unknown. A common feature of patients with cystic fibrosis is the presence of dehydration/volume depletion. However, it should be noted that the severity of metabolic alkalosis in CF patients is disproportionately greater than the magnitude of volume depletion [7–10], indicating that volume depletion, while a contributing factor, is not the sole determinant of the pathogenesis of metabolic alkalosis in CF patients.

The nephron segment which plays an essential role in regulating bicarbonate elimination is the cortical collecting duct (CCD), which is comprised of bicarbonate secreting B-intercalated cells, bicarbonate absorbing A-intercalated cells, and the salt absorbing principal cells [11]. Bicarbonate secretion in the kidney occurs predominantly via pendrin (SLC26A4), a $\text{Cl}^-/\text{HCO}_3^-$ exchanger located on the apical membrane of B-intercalated cells in the CCD and the connecting tubules (CT) [12–16]. Due to its ability to reabsorb chloride and secrete bicarbonate, pendrin has been proposed to be a major contributor to acid base and vascular volume homeostasis [12–16]. This exchanger belongs to the family of SLC26 anion/bicarbonate transporters, which is comprised of 11 members (SLC26A1–11) with distinct distributions throughout the body [17–19]. The apical chloride channel CFTR interacts with three major bicarbonate transporting members of the SLC26 family, SLC26A3, SLC26A6 and SLC26A9, consistent with a vital role for CFTR in HCO_3^- secretion in the duodenum and the pancreatic duct by mobilizing these apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers/anion channels [20, 21].

Bicarbonate reabsorption in the proximal tubule is predominantly mediated via the apical Na^+/H^+ exchanger NHE-3, and the basolateral $\text{Na}^+:\text{HCO}_3^-$ co-transporter NBC-e1 (NBC-1), acting in series [22–27]. Both NHE-3 and NBC-e1 play important roles in bicarbonate reabsorption and could, therefore, contribute to the maintenance of metabolic alkalosis once they become overactive. Both NHE-3 and NBC-e1 are also present in the thick ascending limb of Henle; however, no report has demonstrated their presence in the CCD.

The purpose of these studies was to ascertain the impact of impaired CFTR function on the expression and activity of acid base transporters in the kidney collecting duct as they pertain to the development of metabolic alkalosis.

Materials and Methods

Animal models

Breeding pairs of wild type and CFTR KO mice (C57/BL6) harboring a wild type CFTR in their intestine [28] were obtained from Jackson Laboratory and their colonies established at the University of Cincinnati. The wild type CFTR transgene allows for the rescue of the gastrointestinal electrolyte transport and nutrition [28]. Wild type and mutant animals were housed and cared for in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati. All animal handlers were IACUC-trained. Animals had access to food and water *ad libitum*, were housed in humidity-, temperature-, and light/dark-controlled rooms, and were inspected daily. Animals were terminated with the use of excess anesthetics (pentobarbital sodium) according to institutional guidelines and approved protocols.

Bicarbonate loading

Animals (4–5 WT and CFTR KO mice) were placed in metabolic cages and after acclimation were subjected to bicarbonate loading (140 mM NaHCO₃ added to their drinking water) for 3 days. Urine output, water intake, food intake and body weight were measured daily. Experiments were terminated after 3 days, animals were sacrificed, blood samples were collected for acid base and electrolyte concentration measurements, and kidneys were removed for western and northern hybridizations and for immunofluorescence labeling.

Salt loading

Animals (4 WT and CFTR KO mice with the intestinal rescue) were subjected to 3 days of bicarbonate loading (140 mM NaHCO₃ added to their drinking water) followed by salt loading (140 mM NaCl added to their drinking water) for an additional 3 days. Animals were terminated and their blood was collected for acid base and electrolyte concentration measurement.

Salt restriction

5 WT and CF mice were placed on a salt restricted diet (0.1% salt, Envigo, Indianapolis, IN) for 4 days. Thereafter, the animals were terminated and their blood was analyzed for acid base parameters.

Antibodies

Polyclonal antibodies against pendrin, NBC-e1, H⁺-ATPase and Slc26a6 (PAT-1) for immunofluorescence labeling were developed in our laboratory [29–31]. NHE-3 antibody was developed in Dr. Alicia McDonough's lab. The pendrin antibody for Western blots was a generous gift from Dr. Peter Aronson [32]. Renin antibodies were purchased from MyBiosource (San Diego, CA) and ENaC antibodies were obtained from Stressmarq (Victoria, BC). Actin and AQP-2 antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA; antibodies against ^{Ser256}p-AQP-2 were purchased from Assay Biotech, Sunnyvale, CA; and antibodies for ^{Ser261}p-AQP-2 were from Taipei, Taiwan.

Balance (metabolic cage) studies

Animals (4–5 WT and CFTR KO mice per study) were placed in metabolic cages and after acclimation were subjected to bicarbonate loading (140 mM NaHCO₃ added to their drinking water) or salt restriction (0.1% salt) and compared to control. Urine output, water intake, food intake and body weight were measured daily.

Immunofluorescent microscopy

Animals were terminated with an overdose of pentobarbital sodium, and kidneys were removed, cut in tissue blocks, perfused with saline at room temperature, and then, fixed in paraformaldehyde solution overnight at 4 °C. The tissue was transferred to 70% ethanol. Fixed tissues were embedded in paraffin, and 5-micron sections were cut and stored until used. The expression of pendrin, NBC-e1, NHE-3 and H⁺-ATPase in the kidney was assessed in WT and CFTR KO mice receiving normal or high bicarbonate in their drinking water. The expression of NHE-3 and Slc26a6 (PAT-1) in the intestine was examined according to established protocol. Immunofluorescent single- and double- labeling studies were performed according to established methods [30, 31].

Western blot analysis

Plasma membrane proteins were extracted from mouse kidneys as previously described, size-fractionated by SDS/PAGE (50 µg/lane) and transferred to nitrocellulose membrane [29–31]. Western blot analyses were performed using anti-pendrin, ENaC gamma subunit, renin, AQP-2, ^{S256}-P-AQP-2, ^{S261}-P-AQP-2 or H⁺-ATPase antibodies. Appropriate horseradish peroxidase-conjugated IgGs (Thermo Scientific, Rockford, IL) were used as secondary antibodies. The bands were visualized by chemiluminescence method (Invitrogen, Carlsbad, CA) and captured on light-sensitive imaging film (Denville Scientific Inc, Metuchen, NJ).

RNA isolation and Northern hybridization

Total cellular RNA was extracted from kidneys, quantitated spectrophotometrically; and stored at -80°C. Hybridization was performed according to established protocols [29–31]. Gene specific DNA fragments were generated by RT-PCR and used as specific probes for Northern blot hybridization. For pendrin, a fragment encoding nucleotides 1883–2217 from a mouse kidney cDNA was used. For IRR (insulin

receptor-related receptor, gene bank accession number NM_011832), a PCR fragment encompassing nucleotides 2185-2863; for glutamine dehydrogenase (gene bank accession number NM_008133), a PCR fragment encoding the nucleotides 347 to 843; and for renin (gene bank accession number NM_031192), a PCR fragment encoding nucleotides 290 to 599 were used. For human CFTR (gene bank accession number NM_000492), a PCR fragment encoding nucleotides 3651 to 4618 and for mouse CFTR (gene bank accession number NM_021050), a PCR fragment spanning nucleotides 3317 to 3752 were used. The DNA fragments were labeled with ^{32}P and used as probes for northern hybridization.

Urine and serum electrolytes analysis

Mice were housed in metabolic cages and had free access to rodent chow and water. Food intake, water intake, and urine volume were measured daily. Urine was collected under mineral oil. Urine chloride and sodium concentrations were measured using an EasyLyte Plus Urine Analyzer (Medica Corp, Bedford, MA). Blood was collected from the animals by direct cardiac puncture and processed to obtain serum. Serum concentration of Na^+ , K^+ , Ca^{2+} , and HCO_3^- were measured using i-STAT[®]-1 analyzer with i-STAT EG7+ cartridges (Abbott Laboratories, Abbott Park, IL).

Statistical analysis

The results for serum bicarbonate and pH and urine sodium and chloride excretion and urine pH are presented as means $\pm$ SE. Statistical significance between the results in wild-type and CF mice was determined by Student's unpaired *t*-test or Anova and $p < 0.05$ was considered significant.

Results

Animal models

Breeding pairs of wild type mice and CFTR KO mice harboring a wild type human CFTR transgene in their intestine [28] were obtained from Jackson Laboratory and their colonies established at the University of Cincinnati. The wild type CFTR transgene allows for the rescue of the intestinal electrolyte transport and nutrition in CFTR KO mice [28].

HCO_3^- loading

In the first series of experiments we examined urine pH, serum bicarbonate and arterial pH at baseline and in response to a 3 day oral bicarbonate load (140 mM/lit sodium bicarbonate added to the drinking water) in WT and CF mice. As shown, urine pH (Fig. 1A) in CF mice was mildly lower at baseline (6.18 ± 0.03 in WT vs. 5.98 ± 0.02 in CF mice, $n=7$, $p=0.001$), but was significantly reduced vs. WT mice after a 3-day bicarbonate load (8.10 ± 0.16 in WT vs. 7.35 ± 0.10 in CF mice; $n=7$, $p=0.00990$). Serum HCO_3^- concentration (Fig. 1B) was comparable in CF and WT mice at baseline (25.10 ± 0.38 in WT vs. 26.53 ± 1.11 mEq/l in CF mice, $p=0.287$). However, compared to WT animals, CF mice demonstrated a significant increase in their serum HCO_3^- concentration (26.83 ± 0.90 mEq/l in CF mice vs. 22.63 ± 0.80 in WT mice; $n=4$, $p=0.013$) after the bicarbonate load (Fig. 1B). The increase in serum bicarbonate was associated with an elevation in arterial blood pH in CF mice (7.33 ± 0.01 in WT vs. 7.42 ± 0.02 in CF mice; $n=4$, $p=0.00792$) (Fig. 1C). Serum sodium (Na^+) and potassium (K^+) concentrations were 137 ± 1 and 5.8 ± 0.2 mEq/l in WT and 138 ± 1 and 5.8 ± 0.2 mEq/l in CF mice ($n=5$ /group, $p=0.585$ and $p=0.882$, respectively). Fluid intake was 0.201 and 0.213 ml/gm body weight at baseline and increased to 0.271 and 0.284 ml/gm body weight after bicarbonate loading in CF and WT mice, respectively ($p>0.05$, $n=4$).

The above results strongly suggest that CF mice display an impaired ability to excrete excess bicarbonate, as judged by a reduction in urine pH and the generation of metabolic alkalosis, as shown by an elevated serum bicarbonate and arterial pH in response to a bicarbonate load (Fig. 1). Baseline chloride excretion was not different between CF and WT mice (0.34 ± 0.03 in WT vs. 0.31 ± 0.02 in CF mice; $n=5$, $p=0.417$) (Fig. 2A). Urine chloride excretion was also comparable in WT and CF mice following bicarbonate loading (urine chloride was 0.34 ± 0.05 mM/day in WT and 0.31 ± 0.04 in CF mice; $n=5$, $p=0.603$) (Fig.

2A). Urine sodium excretion was lower in CF mice, both at baseline and in response to bicarbonate loading (Fig. 2B).

In separate studies, we tested the effect of high salt or regular water intake on acid base parameters following a 3-day bicarbonate load. Mice were subjected to 3 days of sodium bicarbonate added to the drinking water and then switched to either a salt - containing water (140 mM NaCl/lit added to the drinking water) or regular water for 72 hrs. Our results demonstrated that switching to a NaCl-containing water abrogated the differences in urine pH in bicarbonate loaded animals (6.51 ± 0.25 in CF vs. 6.38 ± 0.17 in control mice; $n=8$, $p=0.878$) and serum bicarbonate concentration (HCO_3^- concentration was 23.23 ± 0.65 in CF mice vs. 22.93 ± 0.90 mEq/l in WT mice on saline, $n=5$, $p=0.625$). These results indicate that switching to NaCl following the NaHCO_3 loading results in enhanced bicarbonate excretion, which was verified by a decline in urine pH in both genotypes vs. their bicarbonate loaded states.

Fig. 1. Effect of bicarbonate loading on acid base parameters in WT and CF mice. A). Effect of bicarbonate loading on urine pH in WT and CF mice. Urine pH measured in WT and CF mice at baseline and following a 3-day sodium bicarbonate loading. Urine pH was lower in CF KO mice under baseline conditions (6.18 ± 0.03 in WT vs. 5.98 ± 0.02 in CF mice, $n=7$, $p=0.001$), and showed a significant difference vs. WT mice following bicarbonate loading (8.10 ± 0.16 in WT vs. 7.35 ± 0.10 in CF mice; $n=7$, $p=0.00990$); B). Effect of bicarbonate loading on blood HCO_3^- concentration in WT and CF mice. Three days of bicarbonate loading caused a significant increase in serum bicarbonate concentration in CF mice vs. WT mice (26.83 ± 0.90 mEq/l in CF mice vs. 22.63 ± 0.80 in WT mice; $n=4$, $p=0.013$). Baseline serum bicarbonate concentration was comparable in both genotypes (25.10 ± 0.38 in WT vs. 26.53 ± 1.11 mEq/l in CF mice, $n=3$, $p=0.287$). C). Effect of bicarbonate loading on blood pH in WT and CF mice. Three days of bicarbonate loading increased the blood pH in CF mice vs. WT mice (7.33 ± 0.01 in WT vs. 7.42 ± 0.02 in CF mice; $n=4$, $p=0.00792$).

Fig. 2. Urine chloride and sodium excretion rates in WT and CF mice. Urine chloride excretion was comparable in WT and CF mice under baseline conditions (0.34 ± 0.03 in WT vs. 0.31 ± 0.02 in CF mice; $n=6$, $p=0.417$) and following a 3-day bicarbonate load (urine chloride was 0.34 ± 0.05 mM/day in WT and 0.31 ± 0.04 in CF mice; $n=5$, $p=0.603$) (A, left and right panels). Sodium excretion was lower in CF mice, both under baseline conditions and following a 3-day bicarbonate load (urine sodium 0.6378 ± 0.415 vs. 0.4708 ± 0.0247 ; $n=5$, $p=0.00859$) (B, left and right panels).

Fig. 3. Expression of the Na^+/H^+ Exchanger (NHE-3) and the $\text{Cl}^-/\text{HCO}_3^-$ exchangers Slc26a6 (PAT-1) and Slc26a3 (DRA or CLD) in intestines of WT and CF mice. A, B and C). Northern hybridization of NHE-3, PAT-1 and DRA in the jejunum of WT and CF mice. D). Immunofluorescence labeling of NHE-3 in intestine of WT and CF mice. E). Immunofluorescence labeling of PAT-1 in intestine of WT and CF mice.

The correction of metabolic alkalosis was also observed when CF mice were switched to a regular drinking water, as verified by a significant improvement in serum HCO_3^- concentration (HCO_3^- concentration was 24.42 ± 0.91 mEq/l in CF mice and 25.32 ± 1.02 in WT mice; $n=5$, $p=0.530$) after 3 days of regular water intake. The urine pH also decreased after 3 days of water intake in bicarbonate-loaded CF mice (urine pH was 7.52 ± 0.21 after 3 days of bicarbonate loading and decreased to 5.64 ± 0.03 after 3 days of normal water intake in CF mice, $n=5$, $p=0.008$), consistent with the correction of alkalosis. In WT mice, urine pH was elevated during bicarbonate loading (8.00 ± 0.12 , $n=5$) and returned to baseline levels following 3 days of regular water intake (6.14 ± 0.08 , $n=5$, $p=0.000363$). These results demonstrate the correction of metabolic alkalosis in CF mice following the cessation of bicarbonate intake and suggest that this correction is independent of the nature of replacement liquid (salt or regular water). The correction of metabolic alkalosis following switching to a bicarbonate free drinking water strongly supports the conclusion that the systemic vascular volume in CF mice was normal. Fluid intake increased to 0.34 and 0.31 ml/gm body weight after switching to NaCl in CF and WT mice, respectively ($p>0.05$, $n=4$).

Food intake, when adjusted for body weight, was comparable in both groups under baseline conditions (food intake: 0.182 ± 0.005 gm food/gm body weight in WT vs. 0.168 ± 0.011 in CF mice; $n=5$, $p=0.250$), indicating that the low urine pH in CF mice is not due to reduced oral intake. CF mice exhibited lower water intake (6.62 ± 0.26 ml/day in WT vs. 5.05 ± 0.69 in CF mice; $n=5$, $p=0.0475$) but elevated urine osmolality (Uosm 2253 ± 150 in WT, $n=5$ and 3220 ± 180 in CF mice; $n=5$, $p=0.00867$) under baseline conditions (please see western blots on AQP-2, $\text{S}^{256}\text{-P-AQP-2}$ and $\text{S}^{261}\text{-P-AQP-2}$ later discussed in Result section and (for all online suppl. material, see www.karger.com/doi/10.1159/000487691) shown in Supplemental data). When adjusted for their body weight, water intake was comparable in both genotypes.

In addition to the pulmonary disease, cystic fibrosis presents with several gastrointestinal manifestations, including mal-absorption and mal-digestion of nutrients, reduced motility, and diminished activity of sodium and chloride transporters in the small intestine [1–5]. To

determine whether the impaired renal HCO_3^- excretion could be due to decreased activity of acid base or electrolyte transporters in the intestine, the expression of major sodium and chloride transporters in the intestine of WT or CF mice was examined. Toward this goal, northern blot analysis and/or immunofluorescence labeling were performed to examine the expression of the Na^+/H^+ exchanger NHE-3 and the $\text{Cl}^-/\text{HCO}_3^-$ exchangers Slc26a6 (PAT1) and Slc26a3 (DRA or CLD) in the small intestine. The results indicate that WT and CF mice display comparable expression levels for NHE-3 (Fig. 3A and D) and PAT-1 in their intestines (Fig. 3B and E). Similarly, the expression of Slc26a3 (DRA or CLD) in the small intestine was comparable in both genotypes (Fig. 3C).

Expression of pendrin

Diminished bicarbonate excretion in response to bicarbonate loading (Figs. 1 and 2) is likely consequent to impaired bicarbonate secretion in the collecting duct. We therefore examined the expression of pendrin likely consequent to impaired bicarbonate secreting transporter in CF mice. First, we tested the specificity of our pendrin antibody by comparing the expression of pendrin in WT and pendrin KO mice, which were developed in our laboratory [29]. (See online suppl. material) Fig. S1 is an immunofluorescence experiment and shows the labeling of pendrin in kidneys of WT mice (see online suppl. material, Fig. S1A, left panel) is completely ablated in pendrin KO mice (see online suppl. material, Fig. S1B, right panel), indicating the specificity of the antibody. In normal mouse kidney, pendrin localizes on the apical membrane of a subset of cells in the CCD (see online suppl. material, Fig. S1A, arrows in left panel). These are B-intercalated cells based on the expression of H^+ -ATPase on their basolateral membrane, which distinguishes these

Fig. 4. Expression of Pendrin in kidneys of WT and CF mice. A). Expression of pendrin in WT vs. CF mice (low magnification). In CF mice (right images in upper and lower panels) pendrin expression shows significant reduction in the apical membrane vs. WT mice (left images in upper and lower panels). B). Pendrin expression in WT and CF mice (high magnification). Pendrin labeling is detected in cytoplasmic compartments in CF mice (right panel). No basolateral or cytoplasmic labeling with pendrin antibody was detected in WT mice (left panel). C). Pendrin expression in kidneys of WT and CF mice stained with DAPI. D). Double immunofluorescence labeling of pendrin and H^+ -ATPase in kidneys of WT mice (upper panels) vs. CF mice (lower panels).

Fig. 5. Expression of Pendrin and other collecting duct transporters in kidneys of CF mice. A. Pendrin Northern hybridization. B. Pendrin Western blot. C. H⁺-ATPase Western blot. D. ENaC γ subunit Western blot. Northern hybridization and Western blot analysis demonstrate significant downregulation of pendrin in kidneys of CF mice (normalized pendrin RNA WT 1.0765 ± 0.0798 vs CF 0.7104 ± 0.0053 ; $n=3$, $p=0.0102$) (normalized pendrin protein WT 0.9201 ± 0.0351 vs CF 0.4589 ± 0.0503 ; $n=4$, $p=0.000287$) (5A and 5B). Western blot analysis of H⁺-ATPase shows comparable abundance in WT and CF mice (normalized H⁺-ATPase protein WT 1.4574 ± 0.0468 vs. CF 1.5051 ± 0.2216 ; $n=4$, $p=0.840$) (Fig. 5C). ENaC γ subunit abundance was comparable in WT and CF mice by western blot analysis (normalized ENaC γ protein WT 0.7434 ± 0.0604 vs CF 0.7233 ± 0.1681 ; $n=4$, $p=0.914$) (Fig. 5D).

cells from A-intercalated cells that express H⁺-ATPase and AE-1 on their apical and basolateral membranes, respectively [13–15]. When compared to WT mice, immunofluorescence labeling studies demonstrated profound reduction in the apical expression and increased cytoplasmic localization of pendrin in B-intercalated cells of CCD in CF mice (Fig. 4A, right panels) vs. WT mice (Fig. 4A, left panels). The arrows in Fig. 4A, right panels, show cytoplasmic labeling of pendrin in CF kidney. Fig. 4B is a higher magnification image and indicates decreased apical expression and enhanced intracellular localization of pendrin in CF mice during bicarbonate loading. Fig. 4C is an immunofluorescence image of pendrin in the kidney which is stained with the diamidino-2-phenylindole (DAPI) and confirms enhanced intracellular localization of pendrin in CF mice. Fig. 4D depicts double immunofluorescence label-

ing of pendrin and H⁺-ATPase in kidneys of WT mice (top panels) vs. CF mice (bottom panel).

To better estimate the expression of pendrin in kidneys of CF mice, northern and western blot analyses were performed on RNA and plasma membrane proteins isolated from kidneys of WT and CF mice. As shown in Fig. 5A, the mRNA expression of pendrin decreased considerably in kidneys of CF mice (normalized WT 1.0765 ± 0.0798 vs CF 0.7104 ± 0.0053 ; $n=3$, $p=0.0102$). Western blot analysis (Fig. 5B) indicated that pendrin abundance was significantly diminished in kidneys of CF mice when the intensity of pendrin band was adjusted for B-actin (normalized WT 0.9201 ± 0.0351 vs CF 0.4589 ± 0.0503 ; $n=4$, $p=0.000287$). To determine whether the reduction in pendrin expression reflects a generalized downregulation of acid base transporters in CF kidney, western blot analysis of H⁺-ATPase and ENaC was performed on kidney plasma membrane proteins. As shown in Fig. 5C, H⁺-ATPase abundance was comparable in CF and WT kidneys (normalized WT 1.4574 ± 0.0468 vs. CF 1.5051 ± 0.2216 ; $n=4$, $p=0.840$). Fig. 5D indicates that the abundance of ENaC γ subunit was comparable in WT and CF mice (normalized ENaC γ protein WT 0.7434 ± 0.0604 vs CF 0.7233 ± 0.1681 ; $n=4$, $p=0.914$). Taken together, these results indicate that pendrin expression is specifically diminished, whereas H⁺-ATPase and ENaC remain unchanged in kidneys of CF mice.

Salt restriction

The next series of experiments examined the effect of salt restriction on acid base homeostasis in WT and CF mice. Toward this goal, WT and CF mice were placed on a salt restricted diet (0.1% salt) for 4 days. Thereafter, animals were anesthetized and their blood was obtained and analyzed for acid base parameters. The results (Fig. 6A) indicate that CF mice exhibited a significant elevation in their serum bicarbonate when subjected to salt restriction (29.26 ± 0.53 in CF mice vs. 26.72 ± 0.80 mEq/l in WT; $n=5$, $p=0.0291$), and as compared to baseline conditions (Fig. 1B). The arterial blood pH, while elevated in CF vs. WT mice on salt restricted diet (7.34 ± 0.015 in CF mice vs. 7.37 ± 0.006 in WT mice; $n=5$, Fig. 6B) did achieve statistical significance ($p=0.0469$).

Induction of NHE-3 and NBC-e1 in cortical collecting duct cells

In the last series of experiments, we examined the expression of NBC-e1 and NHE-3, the main bicarbonate absorbing transporters in the kidney proximal tubule [22–27]. Immunofluorescence double-labeling of NBC-e1 with H⁺-ATPase demonstrated a weaker labeling of NBC-e1 on the basolateral membrane of kidney proximal tubules in CF mice (Fig. 7A, lower left panel) as compared to the WT mice (Fig. 7A, upper left panel). Unexpectedly, NBC-e1 showed a significant induction in the cortical collecting duct (CCD) of CF mice (Fig.

Fig. 6. Effect of salt restriction on acid base parameters in WT and CF mice. A). Effect of salt restriction on serum HCO₃⁻ concentration in WT and CF mice. Four days of salt restriction caused a significant increase in serum bicarbonate concentration in CF mice vs. WT mice (29.26 ± 0.53 mEq/L in CF mice vs. 26.72 ± 0.80 in WT; $n=5$, $p=0.0291$). B). Effect of salt restriction on blood pH in WT and CF mice. The blood pH tends to be slightly higher in CF mice following 4 days of salt restriction (7.34 ± 0.015 in CF mice vs. 7.37 ± 0.006 in WT mice; $n=5$, $p=0.0469$).

Fig. 7. Expression of NHE-3 and NBC-e1 in kidneys of WT and CF mice. Double immunofluorescence labeling with NBC-e1 and H⁺-ATPase antibodies (7A) or NHE-3 and H⁺-ATPase antibodies (7B) in WT and CF mice. Fig. 7A (upper left panels) shows the expression of NBC-e1 on the basolateral membrane of proximal tubule cells in WT mice when merged images with H⁺-ATPase were acquired (Fig. 7A, upper middle panel). Fig. 7A (lower panels) are from CF mice and show a strong basolateral and cytoplasmic staining of NBC-e1 in the CCD of CF mice (7A, left lower panel). Merged images with H⁺-ATPase (Fig. 7A, middle panels) confirms the presence of H⁺-ATPase and NBC-e1 in the CCD but in adjacent cells, consistent with the NBC-e1 localization in principal cells. The immunofluorescence double labeling with NHE-3 and H⁺-ATPase antibodies

demonstrated the induction of NHE-3 in the CCD in CF mice (Fig. 7B, left lower panel) vs. WT mice, which do not show any NHE-3 labeling in their CCD (Fig. 7B, left upper panel). The cells expressing NHE-3 in the CCD of CF mice are principal cells, based on co-localization studies with H⁺-ATPase (Fig. 7B, middle lower panel)..

7A, lower left panel). There was no expression of NBC-e1 in the CCD of WT mice (Fig. 7A, upper left panel). The cells expressing NBC-e1 in the CCD of CF mice are the principal cells, based on merged images with H⁺-ATPase (Fig. 7A, middle panels).

An expression pattern similar to NBC-e1 was observed for NHE-3 when immunofluorescence double-labeling images of NHE-3 with H⁺-ATPase were acquired (Fig. 7B). In WT mice (Fig. 7B, left upper panel), NHE-3 was mainly detected in the proximal tubule. However, in CF mice (Fig. 7B, left lower panel), NHE-3 was also observed in cells adjacent to H⁺-ATPase-expressing cells in the CCD (merged image in the middle), suggesting the induction of NHE-3 in the principal cells of the collecting duct.

To investigate the possibility of systemic vascular volume depletion, the mRNA expression and protein abundance of renin were examined kidneys of WT and CF mice. The CF mice did not exhibit any enhanced expression of renin in their kidneys under baseline conditions, as judged by northern hybridization and western blotting, indicating the absence of systemic vascular volume depletion (see online suppl. material, Fig. S2).

To determine the basis of elevated urine osmolality in CF mice, the expression of total and phosphorylated AQP-2 variants (S256 and S261 AQP-2) were examined by western blotting. The results showed that while the total AQP-2 abundance is mildly increased, the abundance of the AVP-regulated, membrane bound S256-AQP-2 was decreased in CF mice (see online suppl. material, Fig. S3). The reduction in S256-AQP-2 was associated with a reciprocal increase in the cytosolic S261-AQP-2 (see online suppl. material, Fig. S3). These results indicate that the increased urine osmolality in CF mice is independent of AQP-2 activity and reflects an AVP-independent, enhanced water reabsorption consequent to CFTR deletion.

The CF mice show the complete absence of mouse CFTR in their kidneys (see online suppl. material, Fig. S4, top panel). The expression of human CFTR transgene was comparable in both WT and CF mice, indicating that the very low signal which was present in both genotypes may reflect a background signal (see online suppl. material, Fig. S4, bottom panel). Expression of Insulin Receptor Related Receptor (IRR) was comparable in kidneys of WT and CF mice (see online suppl. material, Fig. S5, top panel) as well as in kidneys of WT and pendrin KO mice (see online suppl. material, Fig. S5, bottom panel).

Discussion

The results of current studies demonstrate the impaired kidney ability to excrete excess bicarbonate by CF mice. As shown, 3-days of bicarbonate loading caused a significant increase in blood bicarbonate concentration and pH (metabolic alkalosis) in CF mice, subsequent to the impaired kidney excretion of HCO_3^- (Results). CF mice subjected to salt restriction showed a similar pattern. CF mice exhibited a significant reduction in the expression of pendrin in B-intercalated cells vs. wild type mice. Wild type mice displayed enhanced bicarbonate excretion in response to bicarbonate loading, and did not develop metabolic alkalosis either during bicarbonate loading or salt restriction (Results).

The most salient features of our studies are the down-regulation of pendrin and the development of metabolic alkalosis in CF mice in response to bicarbonate loading. The impaired ability to excrete bicarbonate following the bicarbonate load in CF mice (lower urine pH) correlates with the downregulation of pendrin and resembles the phenotype that is observed in pendrin deficient mice [13]. Published reports demonstrate that pendrin expression is decreased in acidosis and increased in alkalosis, consistent with its critical role in regulating bicarbonate excretion and acid base homeostasis [16, 33–35].

Pendrin plays an important role in vascular volume homeostasis, specifically during volume depletion [13, 17, 33, 34]. Under conditions of salt restriction or dehydration, pendrin KO mice or humans with Pendred Syndrome exhibit impaired ability to absorb chloride and secrete bicarbonate into the collecting duct, therefore resulting in hypotension and the elevation of serum bicarbonate (metabolic alkalosis) [33, 36]. Wild type mice do not develop hypotension or metabolic alkalosis in response to salt restriction [33].

Metabolic alkalosis could be the initial presentation or complication of cystic fibrosis in infants and children [10]. Recent reports indicate that metabolic alkalosis could also be a frequent complication of cystic fibrosis in adults living in hot climate or following a rigorous exercise [37], strongly suggesting that volume depletion may be the precipitating factor in the generation of metabolic alkalosis. Our results demonstrating impaired pendrin trafficking and activity provide a molecular basis for the role of volume depletion (dehydration) in the pathogenesis of metabolic alkalosis in cystic fibrosis. Accordingly, patients with cystic fibrosis will develop significant impairment in chloride absorption and bicarbonate excretion during volume depletion, secondary to the downregulation of pendrin, which becomes a vital player in acid base and volume regulation during dehydration, which is encountered very frequently in CF patients.

CFTR is abundantly expressed in the kidney proximal tubule [38, 39]; however, no evidence has been uncovered to demonstrate altered bicarbonate absorbing capacity of proximal tubule cells in CF mice. The induction of the proximal tubule apical Na^+/H^+ exchanger, NHE-3, and the basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC-e1) [22–27] in the principal cells of CCD is intriguing, and points to the possibility that principal cells are acquiring a bicarbonate absorbing phenotype, which could facilitate the generation of metabolic alkalosis in volume depletion.

A number of SLC26 anion transporters, besides being involved in the transport of anions such as HCO_3^- and I^- , mediate the transfer of Cl^- across the plasma membrane [40]. CFTR (a Cl^- channel) may be involved in providing the chloride ion needed for the activity of these transporters [40]. The interactions between CFTR and SLC26 transporters in different

epithelia might explain how PKA-induced stimulation of CFTR can give rise to tissue-specific secretory products, as well as the diverse transport phenotypes observed when CFTR is absent or impaired in CF. Additional findings indicate that CFTR and SLC26 bicarbonate transporters activate each other in the formation of a bicarbonate-secreting complex via binding at their PDZ-binding ligands [20, 21].

The signals responsible for the downregulation of pendrin in CF mice remain speculative. Recent studies in parotid ducts and cultured airway serous cells demonstrated that non-mutated CFTR and the apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger, pendrin (SLC26A4), mutually activate each other [41, 42]. It is plausible that a synergistic interaction, parallel to that seen in airway serous cells [42], could synchronize the actions of CFTR and pendrin in B-intercalated cells of kidney CCD. Given the fact that B-intercalated cells have the highest expression of CFTR in the kidney collecting ducts [6], and given the regulation of CFTR and pendrin by cAMP [1, 2, 43, 44], a functional coupling between CFTR and pendrin in CCD is highly plausible.

Our current studies do not demonstrate any direct physical interaction between pendrin and CFTR. Pendrin, as well as other SLC26 isoforms contain a carboxy-terminal cytoplasmic fragment organized around a central Sulfate Transporter and Anti-Sigma factor antagonist (STAS) domain [19, 40]. Published reports indicate that STAS domain plays an important role in surface trafficking and functional expression for SLC26 isoforms [19]. As with the CFTR, several SLC26A family members possess PDZ-domain-interacting motifs at their carboxyl termini [19, 20, 40]. This motif allows SLC26A isoforms to associate with CFTR via scaffolding proteins such as NHERF 1 and 2 and CAP70 in macromolecular complexes that are likely to include cytoskeleton-interacting proteins (e.g., ezrin) [45–48] and links to regulatory proteins such as PKA [47]. Recent studies suggest that mutual stimulatory interactions between SLC26A3, SLC26A6, and CFTR involve regulated association between subdomains of these proteins, the R region of CFTR, and the carboxy-terminal STAS domain, allowing their stimulatory reciprocity to be enhanced by PKA phosphorylation of the R region of CFTR [28]. Whether CFTR and pendrin directly interact with each other remain to be determined.

The expression of IRR (insulin receptor regulated receptor), which is abundantly expressed in B-intercalated cells and functions as an alkali receptor [49], remained

Fig. 8. A schematic diagram depicting the impaired trafficking of pendrin and aberrant induction of NBC-e1 and NHE-3 in the kidney cortical collecting duct cells of CF mice. The $\text{Cl}^-/\text{HCO}_3^-$ exchanger pendrin shows reduced expression on the apical membrane and increased localization in the cytoplasmic compartment of B-intercalated cells in CF mice (right panel) vs. WT mice (depicted in left panel). In addition, the Na^+/H^+ exchanger, NHE-3, and the $\text{Na}^+/\text{HCO}_3^-$ cotransporter, NBC-e1, show aberrant induction on the apical and the basolateral membrane of principal cells in the CCD.

unchanged in kidneys of CF mice (or pendrin null mice) indicating that the impaired ability to excrete excess bicarbonate in CF mice was not due to the downregulation of IRR (see online suppl. material, Fig. S3). Our results showed that the expression of human CFTR transgene in kidneys of CF mice was negligible, indicating that the rescue of intestinal defects with human CFTR had no impact on CFTR expression in the kidney (see online suppl. material, Fig. S2).

Conclusion

CF mice exhibit impaired ability to excrete excess bicarbonate and develop metabolic alkalosis subsequent to the downregulation of pendrin in the collecting duct. In addition, the sodium hydrogen exchanger, NHE-3, and the sodium bicarbonate cotransporter, NBC-e1, show aberrant induction in principal cells of the cortical collecting duct. The schematic diagram in Fig. 8 depicts the impaired trafficking of pendrin and the induction of NHE-3 and NBC-e1 in the kidney cortical collecting duct cells in cystic fibrosis. We propose that patients with cystic fibrosis are prone to the development of metabolic alkalosis secondary to the dysregulation of pendrin in B-intercalated cells, which would interfere with bicarbonate secretion in the collecting duct, specifically during volume depletion, which is a common occurrence in CF patients. Whether the induction of NHE-3 and NBC-e1 in principal cells contributes to bicarbonate absorption in the collecting duct in CF remains to be determined.

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Disclosure Statement

The authors declare no Disclosure Statement.

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