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Deletion of the Cl^-/HCO_3^- exchanger pendrin downregulates calcium-absorbing proteins in the kidney and causes calcium wasting

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Abstract

Background. The epithelial calcium channel (ECaC) (TRPV5) and the Cl^-/HCO_3^- exchanger pendrin (SLC26A4) are expressed on the apical membrane of tubular cells in the distal nephron and play essential roles in calcium re-absorption and bicarbonate secretion, respectively, in the kidney.

Methods. A combination of functional and molecular biology techniques were employed to examine the role of pendrin deletion in calcium excretion.

Results. Here, we demonstrate that deletion of pendrin causes acidic urine [urine pH 4.9 in knockout (KO) versus 5.9 in wild-type (WT) mice, $P < 0.03$)] and downregulates the calcium-absorbing molecules ECaC and Na/Ca exchanger in the kidney, as shown by northern hybridization, immunoblot analysis and/or immunofluorescent labeling. These changes were associated with a ~100% increase in 24-h urine calcium excretion in pendrin null mice. Subjecting the pendrin WT and KO mice to oral bicarbonate loading for 12 days increased the urine pH to ~8 in both genotypes, normalized the expression of ECaC and Na/Ca exchanger and reduced the urine calcium excretion in pendrin-null mice to levels comparable to WT mice.

Conclusions. We suggest that pendrin dysfunction should be suspected and investigated in humans with an otherwise unexplained acidic urine and hypercalciuria.

Keywords: acid-base transporters; acidic urine; calcium excretion; distal nephron; urine alkalinization

Introduction

SLC26 proteins are members of a conserved family of anion transporters, which display distinct and/or limited tissue expression [1–8]. They transport an assortment of anions, including chloride, bicarbonate, sulfate and oxalate, with variable specificity [9, 10] and show a specific cellular or subcellular localization pattern [1–8]. Several SLC26A members can function predominantly as chloride/bicarbonate exchangers, including SLC26A3 (DRA) and SLC26A4 (pendrin) [9–16]. Genes coding for these transporters are mapped to chromosome 7 and are arranged in a head to head orientation [11–13]. Other members such as SLC26A6 (PAT1), SLC26A7 and SLC26A9 can function in multiple anion exchange modes, including chloride/bicarbonate exchange [14–20]. SLC26A7 and SLC26A9 can also function as chloride channels [20–23].

Pendrin or SLC26A4 is abundantly expressed in the thyroid, inner ear and the kidney [4, 12, 24–26]. Pendrin expression in the kidney is limited to the apical membrane of non-A-intercalated cells in the cortical collecting duct (CCD), connecting tubules and the distal convoluted tubules [12, 24–26] and plays an important role in bicarbonate secretion in the distal nephron [24, 27]. Animals lacking pendrin produce very acidic urine as a result of decreased apical Cl^-/HO_3^- exchanger activity in their CCDs [27, 28].

The bulk of filtered calcium is reabsorbed in the proximal tubule and the thick ascending limb of Henle's loop *via* a passive paracellular pathway [29, 30]. Calcium delivered to the distal nephron is reabsorbed through an active transcellular pathway that includes epithelial calcium channel (ECaC), calbindin and basolateral Na/Ca exchanger acting in series [31–39]. ECaC, also known as TRPV5, is expressed on the apical membrane of epithelial cells in the distal convoluted and connecting tubules of the kidney [31–33]. Downregulation or ablation of ECaC has been associated with profound calcium wasting by the kidney, indicating that this molecule is essential for calcium re-absorption in the distal nephron [34, 35]. ECaC is known to be inhibited by testosterone and extracellular acidic pH [35–37]. In addition to the apical ECaC, the cytoplasmic calcium-binding protein calbindin carries calcium ions from the apical to the basolateral side of the cells, while the basolateral Na/Ca exchanger mediates the exit of calcium from the cells to the peritubular space. This pathway plays an important role in vectorial re-absorption of calcium in the distal nephron [38, 39].

Given the important role of pendrin in urinary pH regulation, we sought to examine the impact of pendrin ablation on the rate of urinary calcium excretion and the expression of the calcium-absorbing transport proteins in the distal

nephron. Our studies demonstrate that the expression of calcium-absorbing pathway molecules (apical ECaC and basolateral Na/Ca exchanger) is downregulated in pendrin knockout (KO) mice. These changes were associated with a significant renal calcium wasting. We further demonstrate that urine alkalinization in pendrin KO mice increased the expression of calcium-absorbing molecules and reduced calcium excretion to levels observed in wild-type (WT) mice. The significance of the results will be discussed.

Materials and methods

Animal models

Mice were cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati. All animal handlers are IACUC trained. Pendrin KO ($Pds^{-/-}$) and WT ($Pds^{+/+}$) mice were used for these studies. Animals were allowed free access to water and food. The use of anesthetics (pentobarbital sodium) and the method of euthanasia (pentobarbital sodium overdose) were approved according to the institutional guidelines.

Urine alkalinization was performed by subjecting $Pds^{+/+}$ and $Pds^{-/-}$ mice to oral sodium bicarbonate (280 mM) added to their drinking water for 12 days. In separate studies, animals were placed in metabolic cages, subjected to 100 mM oral bicarbonate and received daily acetazolamide (ACTZ), a carbonic anhydrase inhibitor, at 20 mg/kg/day subcutaneously for 4 days, to ensure the generation of alkaline urine pH and prevent the induction of metabolic acidosis by ACTZ, which could downregulate calcium absorbing molecules in the distal nephron.

Genotyping of $Pds^{+/+}$ and $Pds^{-/-}$ mice

The genotype of the pups was determined by polymerase chain reaction (PCR) amplification and electrophoretic analysis of DNA extracted from their tail clippings as previously described [27]. The PCR reaction on isolated tail DNA to identify WT mice was performed using the following primers: 5′-AGGTAAGATGCTGCTGGATAGG-3′ (forward) and 5′-GCAGGCAAGCATTCTACCAC-3′ (reverse), which amplify a 1.9-kb band. The PCR reaction to identify KO mice was performed using the following primers: 5′-GGAACTTCGCTAGACTAGTACGCGTG-3′ (forward) and 5′-GGCAGGCAAGCATTCTACCACTAAG-3′ (reverse), which amplify a 1.8-kb band. The PCR conditions were as follows: Segment 1, 2 min at 94°C (denature) 1 cycle; Segment 2, 35 cycles of 30 s at 94°C (denature), 30 s at 65°C (annealing), 2 min at 68°C (extension) and Segment 3, link to 68°C for 5 min (1 cycle).

RNA isolation and northern blot hybridization

Total cellular RNA was extracted from mouse kidney cortex and medulla according to established methods, quantitated spectrophotometrically and stored at −80°C. Total RNA samples (30 μg per lane) were fractionated on a 1.2% agarose–formaldehyde gel, transferred to Magna NT nylon membranes, cross-linked by ultraviolet light and baked. ^{32}P-labeled rat (or mouse) probes were used for northern blot analyses. Complementary DNA (cDNA) fragments spanning nucleotides1148–1586 of ECaC (accession number AF209196), nucleotides 120–629 of calbindin (accession number NM031984) and nucleotides 1949–2812 of Na/Ca exchanger (accession number NM019268) were used as gene-specific probes. A mouse cDNA fragment spanning nucleotides 1883–2217 of pendrin was used for northern hybridization. Hybridization was performed according to established methods. The membranes were washed, blotted dry and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). The signal strength of hybridization bands was quantitated by densitometry using ImageQuaNT software (Molecular Dynamics).

Immunoblot analysis

Membrane proteins isolated from the mouse kidney cortex were size fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (30 μg per lane) and transferred to nitrocellulose membrane. The membrane was blocked with 5% milk proteins and then incubated for 6 h with desired concentrations of specific antibodies. The secondary antibody was a donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL) for polyclonal antibodies and a goat anti-mouse IgG conjugated to horseradish peroxidase for monoclonal antibodies. The recognized bands (proteins) were visualized using the chemiluminescence method (RapidStep ECL Reagent, San Diego, CA) and captured on light-sensitive imaging film (MidSci, St Louis, MO). The antibodies utilized for western blot analyses were pendrin [27], Na/Ca exchanger (Abcam, Cambridge, MA), ECaC (Novus Biologicals, Littleton, CO) and calbindin (Abcam). The dilutions for ECaC, calbindin and basolateral Na/Ca exchanger antibodies were 1/600, 1/800 and 1/1000, respectively.

Immunofluorescence labeling studies

Mice were euthanized with an overdose of pentobarbital sodium and perfused through the left ventricle with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Kidneys were removed, cut in tissue blocks, fixed in formaldehyde solution overnight at 4°C and then transferred to 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C. The tissues were frozen in liquid nitrogen, and 6-μm sections were cut using a cryostat. Frozen sections were stored at −80°C until used. Single immunofluorescence labeling was performed as described [16–18] using either Alexa Fluor 488 (green) or Alexa Fluor 594 (red) antibody (Invitrogen, Carlsbad, CA) as secondary antibodies. For single-labeling studies with pendrin antibody, we utilized Alexa Fluor 594-conjugated (red color) goat anti-mouse IgG labeling kit.

For immunofluorescence double labeling, polyclonal antibodies for pendrin (at 1:25 dilution) or calbindin (at 1:50 dilution) were used in conjunction with the monoclonal Na/Ca exchanger antibodies (1:100 dilution). Pendrin or calbindin antibodies were detected by Alexa Fluor 488-conjugated (green color) anti-rabbit IgG labeling kit and Na/Ca exchanger antibodies were detected using Alexa Fluor 594-conjugated (red color) goat anti-mouse IgG labeling kit (Invitorgen Molecular probes, Eugene, OR) according to the manufacturer's instructions.

For immunofluorescent microscopy studies, paraffin-embedded slides were subjected to antigen retrieval protocol. Frozen kidney sections were allowed to thaw at room temperature and were subsequently rehydrated in phosphate-buffered saline (PBS) for 15 min and permeabilized in PBS containing 0.3% Triton X-100 [Phosphate Buffered saline with Triton (PBT)] for 20 min at room temperature. Non-specific binding was blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min at room temperature. Primary antibodies in a diluent of 0.3% Triton X-100 and 10% bovine serum albumin in 0.1 M PBS were applied to the sections overnight at 4°C in a humidified chamber. Sections underwent three PBS washes of 10 min each on the orbital shaker. Sections were then briefly allowed to dry and coverslipped with the anti-fade fluorescent mounting medium (Vectashield, Burlingame, CA). Sections were examined and images were acquired on a Zeiss Axio-plan fluorescent microscope.

Balanced studies in experimental animals

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 calcium concentration was measured *via* a Calcium Assay Kit (BioChain Institute, Hayward, CA). Serum Ca^{2+} concentration was measured with an i-STAT®-1 analyzer using i-STAT EG7+ cartridges (Abbott Laboratories, Abbott Park, IL).

Materials

[^{32}P]dCTP was purchased from Perkin Elmer (Shelton, CT). Nitrocellulose filters and other chemicals were purchased from Sigma (St Louis, MO). Probes were labeled with [^{32}P]dCTP *via* QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA).

Statistic analysis

The results for northern hybridization, western blotting, calcium excretion or urine pH studies are expressed as means ± SE. Statistical significance between various experimental groups was determined by Student's unpaired *t*-test or analysis of variance, and $P < 0.05$ was considered significant.

Results

Animal models

The generation of $Pds^{-/-}$ mice used in these studies was recently reported from our laboratory [27]. Figure 1a is a representative genotype analysis by PCR on tail DNA and depicts the identification of WT, heterozygote and $Pds^{-/-}$

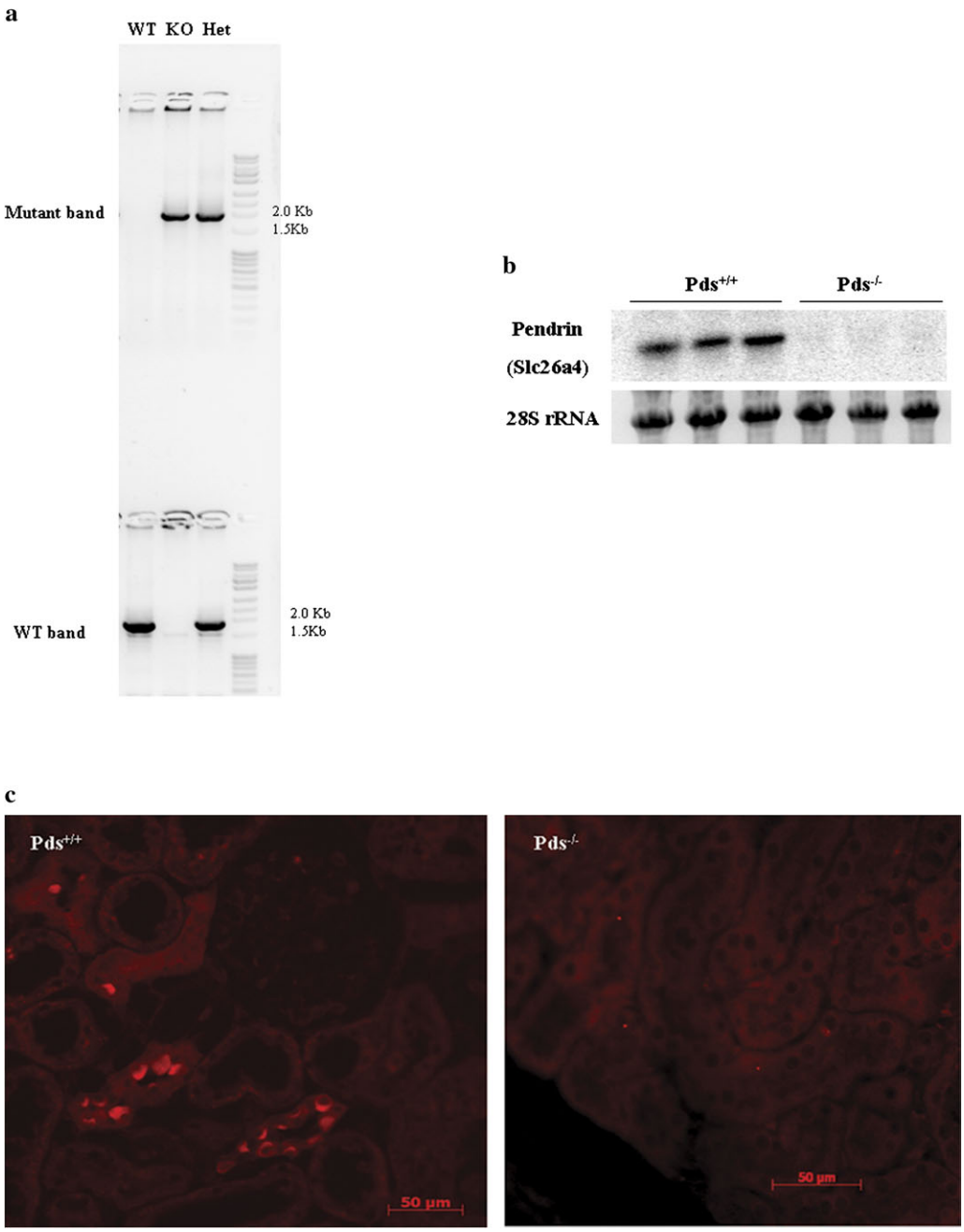


Fig. 1. Genotyping and expression of pendrin in $Pds^{+/+}$ and $Pds^{-/-}$ mice. (**a**) Genotyping of $Pds^{+}/^{+}$ and $Pds^{-}/^{-}$ mice. A representative ethidium bromide staining of agarose gel demonstrates the identification of wild-type ($Pds^{+/+}$), heterozygote ($Pds^{+/-}$) and pendrin-deficient ($Pds^{-/-}$) mice. (**b**) Northern blot analysis. Pendrin mRNA was abundantly expressed in the kidney cortices of $Pds^{+/+}$ mice but was absent in $Pds^{-/-}$ mice. The faint larger band in $Pds^{-}/^{-}$ mice is the neocassette-containing untranslatable mutant transcript (see immunolabeling studies below). (**c**). Immunofluorescent labeling of pendrin in kidneys of $Pds^{+/+}$ and $Pds^{-/-}$ mice. Immunofluorescence labeling with pendrin-specific antibodies (red color) detected the expression of pendrin on the apical membrane of cells in the CCD in WT mice but did not detect any expression in pendrin KO mice.

mice. The sequences of primers used in genotyping are included in Materials and methods.

Northern blot analysis and immunofluorescent detection of pendrin in the kidneys of $Pds^{+/+}$ and $Pds^{-/-}$ mice

Northern blot analysis results indicate the complete absence of pendrin messenger RNA (mRNA) in kidney cortices of $Pds^{-/-}$ mice (Figure 1b). Immunofluorescent labeling with pendrin antibodies demonstrated its specific localization to the apical membrane of cells in CCD in $Pds^{+/+}$ mice. Pendrin staining was not detected in CCD cells of $Pds^{-/-}$ mice (Figure 1c).

Localization of pendrin and calcium-absorbing molecules in the distal nephron

Published reports indicate that $Pds^{-/-}$ mice have significantly lower urine pH compared to their $Pds^{+/+}$ littermates

[27, 28]. In the next series of experiments, we examined possible co-localization of pendrin and calcium-absorbing molecules in the distal nephron. Toward this end, double immunofluorescent labeling studies were performed using pendrin and Na/Ca exchanger antibodies. As demonstrated in Figure 2a and b (merged images in middle panels), the Na/Ca exchanger and pendrin show distinct localization patterns. In distal convoluted tubules and connecting tubules (Figure 2a and b, see yellow and white arrows), the two transporters are expressed by distinct cell types within the same segment (Figure 2a and b). These results suggest that distal convoluted and connecting tubules express both pendrin and calcium absorbing molecules, with pendrin appearing on the apical membrane of B (should this be β)-intercalated cells and Na/Ca exchanger localizing to the basolateral membrane of non-intercalated cells. In addition, pendrin labeling is also detected on the apical membrane of B-intercalated cells of CCD, which do not express any Na/Ca exchanger (Figure 2b).

Given the localization of pendrin and calcium absorbing molecules to the distal convoluted and connecting tubules (above) and given the role of luminal pH in regulating ECaC expression and/or activity [36, 37], we entertained the possibility that the acidic urine pH in pendrin KO ($Pds^{-/-}$) mice might downregulate ECaC activity in the distal nephron, therefore decreasing calcium re-absorption. To test this possibility, animals were placed in metabolic cages and their water and food intake as well as body weight and urine output was recorded on a daily basis (Figure 3a). After acclimatization, daily urine output was collected and analyzed. Urine pH was determined with a microelectrode and urine calcium was measured with a calcium assay kit (Materials and methods). As shown in Figure 3b (top panel), urine calcium excretion was significantly increased in pendrin KO mice ($P < 0.05$ versus WT mice). The increase in urine calcium excretion was paralleled by a reduction in urine pH in pendrin KO mice (Figure 3b, bottom panel).

Effect of pendrin ablation on the expression of ECaC, Na/Ca exchanger and calbindin

Given the increased urine calcium excretion in pendrin KO mice (Figure 3), we examined the expression of ECaC, the apical calcium-absorbing channel in the distal nephron [29–32]. The ECaC mRNA expression and protein abundance decreased significantly in the kidney cortex of $Pds^{-/-}$ mice (Figure 4a and b). Northern blot analysis of RNA isolated from the kidneys of four separate animals showed that the expression of ECaC transcript decreased by 58% in the cortices of $Pds^{-/-}$ mice (right panel in Figure 4a) ($P < 0.05$ versus $Pds^{+/+}$). The protein levels of ECaC were also diminished by ~45% in $Pds^{-/-}$ mice (right panel in Figure 4b) ($P < 0.05$ versus $Pds^{+/+}$). Our attempts at obtaining immunofluorescent labeling of ECaC in the kidneys of experimental animals were not successful.

The apical ECaC works in tandem with the cytoplasmic calbindin and the basolateral Na/Ca exchanger to reabsorb calcium in the distal nephron. In the next series of experiments, we examined the mRNA expression and protein abundance of Na/Ca exchanger (Figure 5A) and calbindin (Figure 5B) in the kidneys of $Pds^{+/+}$ and $Pds^{-/-}$ mice. Our results demonstrated that the mRNA expression of the basolateral Na/Ca exchanger was significantly reduced in the kidneys of $Pds^{-/-}$ mice (Figure 5A, a) ($P < 0.05$ versus $Pds^{+/+}$). Immunofluorescent labeling on kidney sections and western blot analysis on membrane proteins showed significant reduction in the expression of the Na/Ca exchanger in $Pds^{-/-}$ mice (Figure 5A, b and c).

Northern blot analyses further indicated that the expression of calbindin mRNA was reduced in the kidneys of $Pds^{-/-}$ mice versus $Pds^{+/+}$ mice (Figure 5B, a; $P < 0.05$). Immunofluorescent labeling studies on kidney sections (Figure 5B, b) and western blot analysis on membrane proteins showed significant reduction in the expression of calbindin in $Pds^{-/-}$ mice (Figure 5B, c).

Effect of urine alkalinization on calcium excretion and the expression of calcium-reabsorbing molecules $Pds^{-/-}$ mice

The purpose of the next series of experiments was to examine the role of urine pH on the expression of calcium-absorbing molecules in the kidney and its impact on urine calcium excretion. Toward this end, $Pds^{+/+}$ and $Pds^{-/-}$ mice were placed in metabolic cages and after acclimatization on normal food and water for 3 days were switched to oral sodium bicarbonate (280 mM) added to their drinking water. Animals were maintained on oral bicarbonate for 12 days. The water intake, urine output, food intake and body weight were measured daily. Figure 6a shows body weight, food intake, water intake and urine output at baseline and in response to oral bicarbonate loading. As shown, animals on bicarbonate solution maintained their food intake but increased their water intake and urine output. Figure 6b depicts calcium excretion rate and urine pH in WT and $Pds^{-/-}$ mice at baseline and in response to bicarbonate loading. As indicated, urine pH was significantly lower in $Pds^{-/-}$ mice at baseline state compared to $Pds^{+/+}$ mice (Figure 6b, bottom panel). Urine pH increased in both $Pds^{+/+}$ and $Pds^{-/-}$ mice immediately following switching to oral bicarbonate therapy and reached comparable values in both genotypes on Days 11 and 12 (Figure 6b, bottom panel).

The 24-h calcium excretion rate in $Pds^{-/-}$ mice was increased at basal state but decreased significantly in response to oral bicarbonate loading. In $Pds^{+/+}$ mice, urine calcium excretion was lower at basal state as compared to $Pds^{-/-}$ mice, confirming the studies in Figure 3. The calcium excretion in $Pds^{-/-}$ mice on oral bicarbonate loading decreased to values that were comparable to $Pds^{+/+}$ mice on the same treatment (Figure 6b, top panel).

Oral bicarbonate therapy results in urine alkalization as well as metabolic alkalosis in mice [38]. ACTZ, which is a carbonic anhydrase inhibitor, has several distinct effects on urinary parameters, acid-base status and calcium excretion [38]. It results in bicarbonate wasting, which is manifested by urine alkalinization but also causes metabolic acidosis. In the next series of experiments, $Pds^{-/-}$ mice were placed on 100 mM oral sodium bicarbonate added to their drinking water and received daily subcutaneous doses of ACTZ, a carbonic anhydrase inhibitor, at 20 mg/kg/day for 4 days. Serum bicarbonate concentration was 29.2 ± 0.6 mEq/L, which is not significantly different than 27.6 ± 0.53 mEq/L in $Pds^{-/-}$ mice under

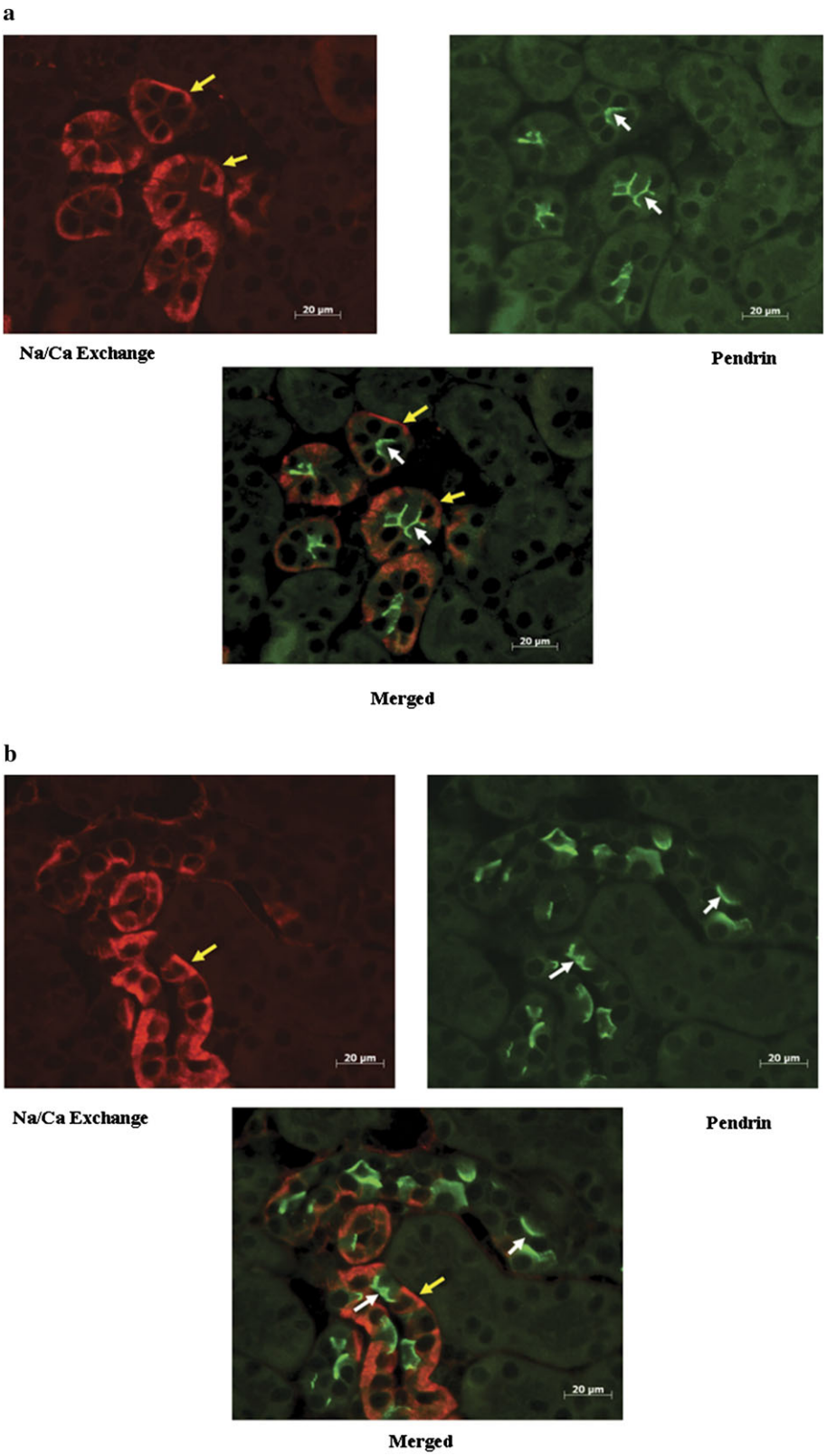


Fig. 2. Localization of pendrin and Na/Ca exchanger in the kidney. (**a**) Co-localization of pendrin and Na/Ca exchanger in the connecting tubule. Immunofluorescent labeling studies showed the expression of pendrin (green) and Na/Ca exchanger (red) in the same tubules (connecting tubules) but in distinct cells (white arrows for pendrin and yellow arrows for Na/Ca exchanger). (**b**). Localization of pendrin and Na/Ca exchanger in the distal nephron. Immunofluorescent labeling studies showed the co-localization of pendrin (green color) and Na/Ca exchanger (red color) in the same distal tubules and connecting tubules but in distinct cells (white arrows for pendrin and yellow arrows for Na/Ca exchanger). Pendrin is also expressed in the collecting duct (arrows), whereas Na/Ca exchanger is not.

a

	Body weight (g)	Food intake (g/day)	Water intake (mL/day)	Urine volume (mL/day)	
Pds$^{+/+}$	22.5 ± 1.9	4.9 ± 0.2	5.1 ± 0.3	2.10 ± 0.25	
Pds$^{-/-}$	21.7 ± 2.1	4.4 ± 0.3	4.9 ± 0.3	2.45 ± 0.25	

b

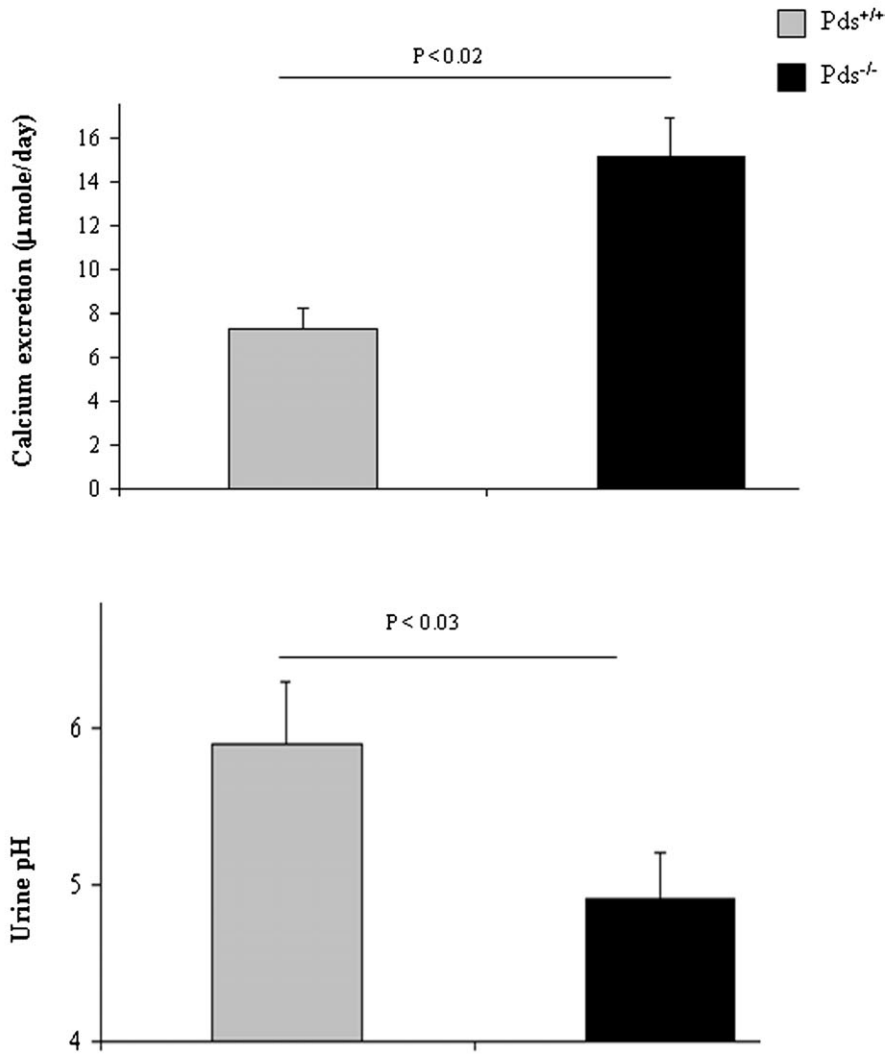


Fig. 3. Effect of pendrin ablation on urine pH and calcium excretion. (**a**). Balanced studies in Pds$^{+/+}$ and Pds$^{-/-}$ mice. Body weight, food intake and urine output were measured at baseline state in animals in metabolic cages. (**b**). Urine pH and calcium excretion in Pds$^{+/+}$ and Pds$^{-/-}$ mice. Urine pH was determined by microelectrode in Pds$^{+/+}$ and Pds$^{-/-}$ mice. As shown, urine pH (lower panel) was significantly decreased and calcium excretion (top panel) was significantly increased in Pds$^{-/-}$ mice at baseline.

basal conditions [27]. Urine pH was 8.2 ± 0.3 in Pds$^{-/-}$ mice on oral bicarbonate and ACTZ (Figure 6c, left panel), which is comparable to oral bicarbonate loading alone in the same genotype (Figure 6b) but is significantly higher than basal conditions in Pds$^{-/-}$ mice (Figures 3 and 6b and c). Calcium excretion was mildly reduced in Pds$^{-/-}$ mice treated with

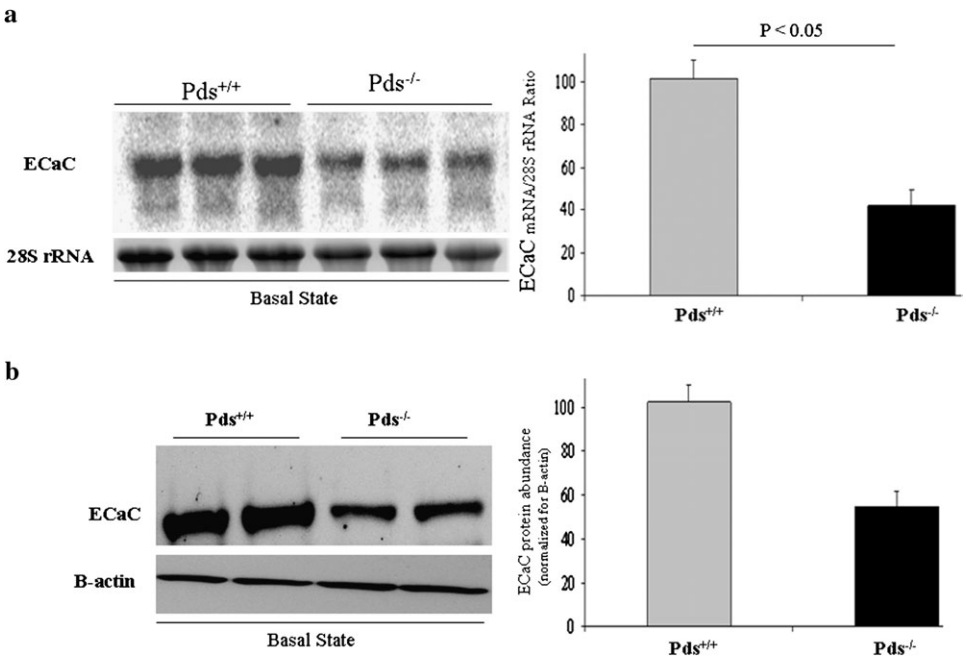


Fig. 4. Effect of pendrin ablation on the expression of ECaC. (**a**). Northern blot analysis. ECaC mRNA expression decreased significantly in the kidney cortices of pendrin KO mice. Our results demonstrate that the mRNA expression of ECaC decreased by ~58% in the cortex of $Pds^{-/-}$ compared to $Pds^{+/+}$ mice ($n = 4$ per genotype). (**b**). Immunoblot analysis. The protein abundance of ECaC decreased by 45% in the kidney cortices of $Pds^{-/-}$ mice.

bicarbonate and ACTZ versus basal conditions (Figure 6c, right panel) but remained significantly higher relative to oral bicarbonate loading alone (Figure 6b).

Finally, we examined the effect of urine alkalinization on the expression of calcium-absorbing molecules in the kidney. Toward that end, WT ($Pds^{+/+}$) and pendrin KO ($Pds^{-/-}$) mice on oral bicarbonate solution for 12 days were euthanized and their kidneys were examined for the expression of ECaC, calbindin and Na/Ca exchanger. As shown in Figure 7A, a, mRNA levels of kidney ECaC in $Pds^{-/-}$ mice receiving oral bicarbonate were comparable to $Pds^{+/+}$ mice subjected to the same treatment. Furthermore, western blot analyses indicated that the ECaC levels in $Pds^{-/-}$ mice on oral bicarbonate loading became comparable to those observed in $Pds^{+/+}$ mice (Figure 7A, b).

The expression levels of basolateral Na/Ca exchanger and calbindin were examined in animals subjected to bicarbonate loading. Our results indicate that the expression of Na/Ca exchanger mRNA and protein in the kidneys of $Pds^{-/-}$ mice receiving oral bicarbonate increased to levels comparable to those of the $Pds^{+/+}$ animals (Figure 7B, a and b). The mRNA expression and protein levels of calbindin in $Pds^{-/-}$ mice on oral bicarbonate were also elevated to levels comparable to those of the $Pds^{+/+}$ mice (Figure 7C, a and b).

Discussion

The vectorial re-absorption of calcium in the distal nephron is mediated *via* ECaC, calbindin and Na/Ca exchanger working in sequence. The apical calcium re-absorption is the rate-limiting step in this process and is mediated *via* ECaC [31–35]. Downregulation or ablation of ECaC has been associated with profound renal calcium wasting, indicating the important role that this channel plays in calcium re-absorption in the kidney [34, 35].

Pendrin or SLC26A4, which functions in Cl^-/HO_3^- exchange mode in the kidney, is located on the apical membrane of non-A (B and non-A, non-B)-intercalated cells in the CCD, connecting tubule and the distal convoluted tubule [24–26] and is the major transporter responsible for bicarbonate secretion in the distal nephron [24, 27]. Pendrin shows adaptive downregulation during metabolic acidosis and potassium depletion and upregulation in response to bicarbonate loading and aldosterone treatment [40–44].

The present studies establish a novel interaction between the pendrin- and the calcium-absorbing pathway in the distal nephron. Our studies (Figure 2) demonstrate that the ablation of pendrin downregulates the expression of ECaC and the protein that regulates the rate-limiting step in calcium re-absorption pathway in the distal nephron. As a result, $Pds^{-/-}$ mice develop calcium wasting (Figure 3). Indeed, the 24-h calcium excretion rate increased by ~100% in $Pds^{-/-}$ mice (Figure 3). In addition to ECaC, pendrin deletion downregulates the cytoplasmic calbindin and the basolateral Na/Ca exchanger in the distal nephron (Figure 5). Our results demonstrate that the decrease in the mRNA levels of ECaC, calbindin and Na/Ca exchanger correlates with a reduction in their protein abundance and increased urinary calcium wasting in $Pds^{-/-}$ mice.

ECaC expression and/or activity are shown to be regulated by extracellular pH in *in vitro* systems [36, 37]. Indeed, it has been shown that ECaC expression is reduced in an acidic extracellular environment; whereas, its cell surface expression increases in an alkaline milieu. These changes were accompanied by alterations of ECaC trafficking to or from the plasma membrane, respectively [36, 37].

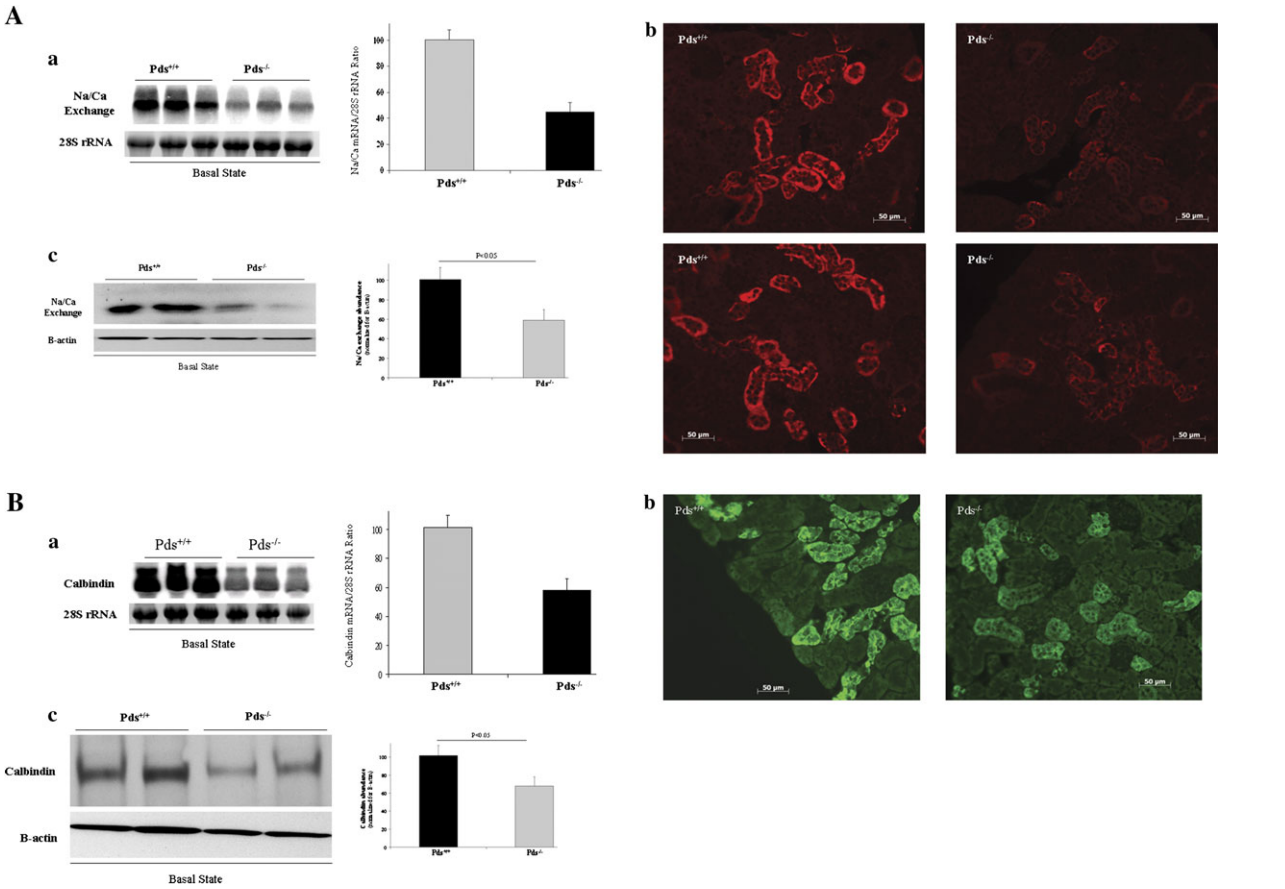


Fig. 5. Effect of pendrin ablation on the expression of Na/Ca exchanger and calbindin. (**A**) Na/Ca exchanger. (a) Northern blot analysis. The mRNA levels of the basolateral Na/Ca exchanger decreased by ~54% in the kidneys of $Pds^{-/-}$ compared to $Pds^{+/+}$ mice. (b) Immunofluorescent labeling. Single immunofluorescent labeling studies demonstrated significant reduction in the Na/Ca exchanger staining in $Pds^{-/-}$ (right panels) compared to $Pds^{+/+}$ mice (left panels). (c) Immunoblot analysis. The protein abundance of the Na/Ca exchanger decreased by 41% in the kidneys of $Pds^{-/-}$ mice. (**B**) Calbindin. (a) Northern hybridization. The mRNA levels of calbindin decreased by ~42% in the kidneys of $Pds^{-/-}$ compared to $Pds^{+/+}$ mice. (b) Immunofluorescent labeling. Single immunofluorescent labeling studies suggested reduction in calbindin labeling in $Pds^{-/-}$ (right panels) compared to $Pds^{+/+}$ mice (left panels). (c) Immunoblot analysis. The protein abundance of calbindin decreased by 32% in the kidneys of $Pds^{-/-}$ mice.

The changes in cell surface expression of ECaC in acidic pH environments were associated with congruent changes in its activity [36, 37]. ECaC expression can also be altered under systemic metabolic acidosis, which is associated with the excretion of acidic urine [34]. Under such condition, renal expression of ECaC decreases and there is a concomitant increase in calcium excretion by the kidney [34]. Our studies demonstrated the downregulation of calcium absorbing proteins both by northern and western blotting in pendrin KO mice, suggesting that the acidic milieu in the distal nephron lumen has additional effects on the synthesis of these proteins.

$Pds^{-/-}$ mice do not display any evidence of systemic metabolic acidosis [24, 27, 44]. Published reports indicate that pendrin-null mice either demonstrate a normal acid-base status or have a mild systemic metabolic alkalosis subsequent to decreased bicarbonate excretion in the distal nephron [24, 27, 44]. As such, we suggest that the down-regulation of ECaC in pendrin-null mice is secondary to the acidic luminal pH in the kidney distal nephron and is not due to any systemic acid-base disturbances. The causal relationship between the acidic urine and ECaC downregulation was further supported by urine alkalinization studies, which showed enhanced expression of ECaC in $Pds^{-/-}$ mice subjected to oral bicarbonate loading (Results).

Oral bicarbonate loading causes urine alkalinization (Results) and results in metabolic alkalosis [38]. We attempted to examine the role of urine alkalinization independent of metabolic alkalosis by subjecting the animals to bicarbonate loading and ACTZ treatment. As shown, ACTZ-treated $Pds^{-/-}$ mice displayed significantly elevated urine pH but only a mild increase in their serum bicarbonate (Results), consistent with urine alkalinization and the absence of metabolic alkalosis. Urine calcium excretion was mildly reduced in $Pds^{-/-}$ mice on oral bicarbonate and ACTZ (Figure 6c). These results are not in conflict with our conclusion that urine pH plays an important role in calcium excretion. ACTZ is a non-specific inhibitor of carbonic anhydrases and has been shown to increase calcium excretion [38], presumably secondary to the inhibition of sodium re-absorption in the proximal tubule, which provides the driving force for calcium re-absorption in that segment. In addition, ACTZ can lower systemic pH, which may contribute to a rise in urinary calcium excretion. We suggest that the calcium-reabsorbing

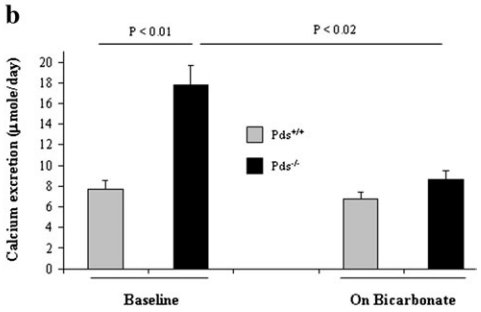
a

Basal

	Body weight (g)	Food intake (g/day)	Water intake (mL/day)	Urine volume (mL/24 h)
$Pds^{+/+}$	22.1 ± 2.1	4.0 ± 0.20	5.0 ± 0.3	2.00 ± 0.20
$Pds^{-/-}$	23.3 ± 1.7	4.2 ± 0.25	5.3 ± 0.3	2.30 ± 0.25

Oral bicarbonate

	Body weight (g)	Food intake (g/day)	Water intake (mL/day)	Urine volume (mL/24 h)
$Pds^{+/+}$	22.9 ± 2.0	4.4 ± 0.25	5.6 ± 0.3	2.40 ± 0.30
$Pds^{-/-}$	24.0 ± 1.8	4.1 ± 0.3	5.9 ± 0.4	2.75 ± 0.35



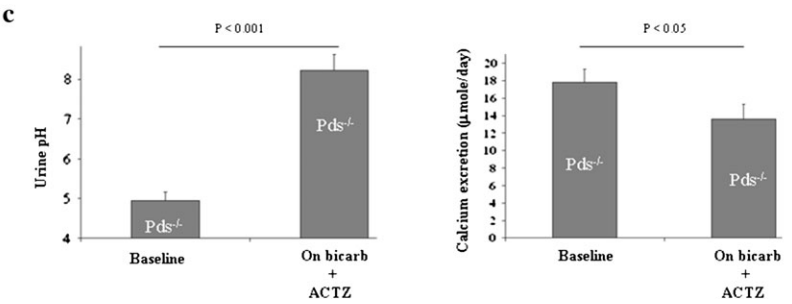


Fig. 6. Effect of oral bicarbonate therapy on urine pH and calcium excretion. (**a**) Balanced studies in $Pds^{+/+}$ and $Pds^{-/-}$ mice. Body weight, food intake, water intake and urine output were measured at baseline (top panel) and in response to oral bicarbonate loading (bottom panel). (**b**) Urine pH and calcium excretion in $Pds^{+/+}$ and $Pds^{-/-}$ mice. As shown, urine pH was low in $Pds^{-/-}$ mice at baseline (bottom panel) and increased in both $Pds^{+/+}$ and $Pds^{-/-}$ mice on oral bicarbonate therapy. Urinary calcium excretion (top panel) was higher in $Pds^{-/-}$ mice at baseline but decreased in response to oral bicarbonate therapy. (**c**). Urine pH and calcium excretion in $Pds^{-/-}$ mice on ACTZ and oral bicarbonate. As shown, urine pH increased in $Pds^{-/-}$ mice on oral bicarbonate therapy and ACTZ versus baseline conditions (left panel). Urinary calcium excretion decreased mildly in $Pds^{-/-}$ mice treated with ACTZ and oral bicarbonate versus baseline conditions (right panel).

effect of urine alkalinization in the distal tubule is likely offset by the calcium wasting effect of ACTZ in the proximal tubule in our $Pds^{-/-}$ mice on oral bicarbonate and ACTZ.

The present studies are the first reports on the impact of a gene deletion causing low urinary pH with subsequent hypercalciuria. Our studies utilizing double immunofluorescence demonstrate that distal nephron bicarbonate secreting cells (manifested by the expression of pendrin on their apical domain) are distinct from calcium-absorbing cells (as shown by Na/Ca exchanger localization on their basolateral membrane) but both are present in the same tubule (Figure 7). These results indicate that impaired bicarbonate secretion will affect the luminal pH in tubules expressing ECaC, suggesting that urine pH is working in a 'paracrine' fashion to regulate ECaC expression and or activity. A recent study indicated that pendrin ablation leads to calcium oxalate stone formation in the inner ear [45], lending support to the possibility that ECaC regulation by extracellular pH (or bicarbonate) is observed in other organs.

It is plausible that the impact of urine alkalinization on reducing the magnitude of hypercalciuria in pendrin KO mice is secondary to several factors, one of which is the upregulation of ECaC/calbindin/Na/Ca exchanger pathway. Recent studies showed that bicarbonate loading increased calcium absorption in the distal nephron in the absence of ECaC, albeit at a lower rate [34].

Hypercalciuria, or increased calcium excretion, plays an important role in the pathogenesis of various kidney stones, such as calcium oxalate, which is the most common kidney stone in humans, and calcium phosphate stones [46–50]. Patients with increased oxalate excretion might be in danger of developing kidney stones if the concentration of calcium in their urine exceeds normal limits [46–50]. In a recently published study, Worcester and Coe [48] found that patients with calcium oxalate stones have acidification defects in their kidney distal tubules.

The important role of urinary pH in the prevention of kidney stones has been well documented in patients with cystine stones who showed significant reduction in the formation of cystine stones in response to oral alkalinization (reviewed in [51]). In addition to its role in renal calcium wasting, acidic urine can contribute to the formation of uric

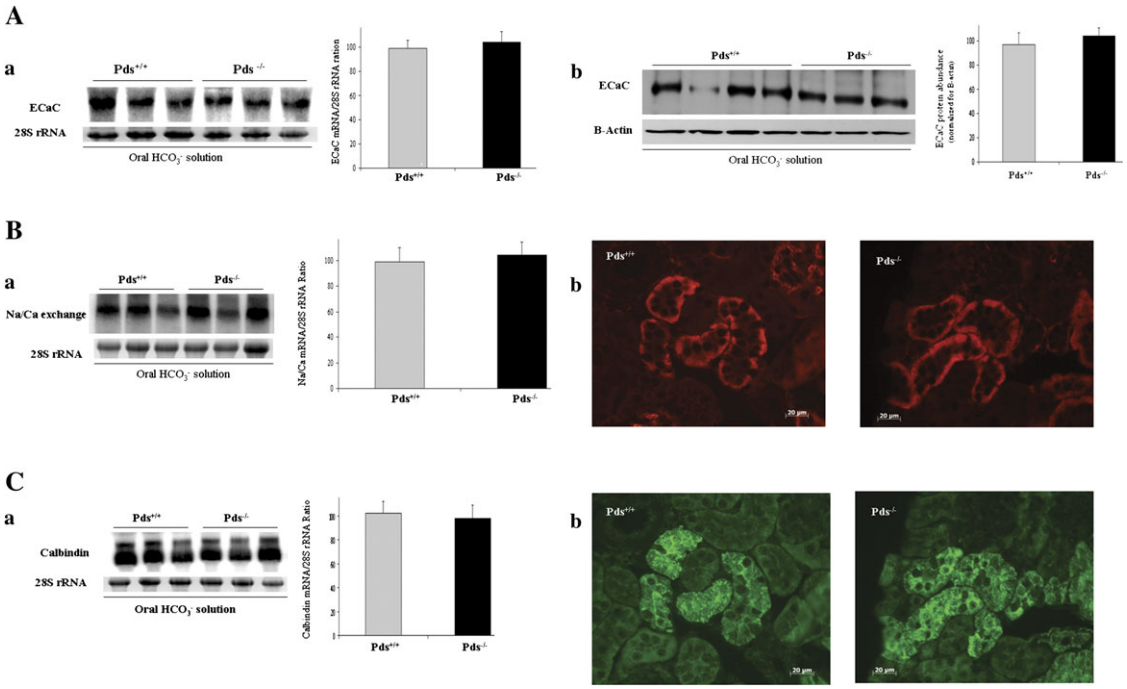


Fig. 7. Effect of oral bicarbonate therapy on the expression of ECaC, Na/Ca exchanger and calbindin in $Pds^{+/+}$ and $Pds^{-/-}$ mice. (**A**) ECaC. (a) Northern blot analysis. The mRNA levels of ECaC were not significantly different in the kidneys of $Pds^{-/-}$ compared to $Pds^{+/+}$ mice receiving oral bicarbonate solution. (b) Immunoblot analysis. Western blot analyses indicated that the ECaC levels were comparable in kidneys of $Pds^{-/-}$ and $Pds^{+/+}$ mice receiving oral bicarbonate solution. (**B**) Na/Ca exchanger. (a) Northern hybridization. The mRNA expression levels of Na/Ca exchanger were not significantly different in the kidneys of $Pds^{-/-}$ compared to $Pds^{+/+}$ mice receiving oral bicarbonate solution. (b) Immunfluorescence labeling. Immunofluorescent labeling studies showed comparable Na/Ca exchanger expression in kidneys of $Pds^{-/-}$ and $Pds^{+/+}$ mice receiving oral bicarbonate solution. (**C**) Calbindin. (a) Northern blot analysis. The mRNA levels of calbindin were comparable in kidneys of $Pds^{-/-}$ and $Pds^{+/+}$ mice receiving oral bicarbonate solution. (b) Immunfluorescence labeling. Immunofluorescent labeling studies showed comparable calbindin staining in kidneys tubules of $Pds^{-/-}$ and $Pds^{+/+}$ mice receiving oral bicarbonate solution.

acid stones in the kidneys, ureters or bladder if uric acid excretion exceeds the normal limit [52, 53]. In convincing studies by Pak *et al.* [52, 53], patients with idiopathic uric acid nephrolithiasis, without secondary causes (such as dehydration or diarrhea), were found to have urinary pH values that were lower than control subjects, suggestive of a primary defect in urinary acidification. While the pathogenesis of acidic urine has not been studied in detail in patients with primary uric acid stone, it is plausible that alterations in acid-base transporters such as pendrin in the distal nephron might contribute to this abnormality.

Based on its location and function, it is speculated that pendrin mediates the post-prandial alkaline tide, a temporary increase in urine pH immediately following a meal. The 'alkaline tide' is important to normal kidney function for the elimination of 'uric acid' generated from meals [54] and is lost in patients who make uric acid stones [54]. It is intriguing to speculate that pendrin-mediated bicarbonate secretion in the distal nephron plays a pivotal role in the excretion of uric acid by preventing its precipitation in the distal tubules.

In summary, deletion of pendrin downregulates the calcium-absorbing pathway molecules (ECaC, calbindin and Na/Ca exchanger) in the distal nephron and causes calcium wasting. The downregulation of ECaC and related molecules is likely secondary to the acidic urine resulting from the inhibition of bicarbonate secretion in the distal nephron in $Pds^{-/-}$ mice. Urine alkalinization reversed the impact of pendrin deficiency on ECaC and calcium excretion by enhancing the expression of ECaC and re-absorption of calcium in the distal nephron.

We propose that acidic urine, as observed in pendrin KO mice, can play an important role in the pathogenesis of calcium, uric acid and possibly cystine stones. By causing acidic urine, pendrin deficiency can cause hypercalciuria, which, in the context of high urine oxalate or phosphate, will provide the right milieu for calcium oxalate or calcium phosphate stone formation. In parallel, by causing acidic urine, pendrin ablation can potentially facilitate the precipitation of uric acid (or cystine) crystals in the tubules if the generation and or excretion of uric acid (or cystine) are enhanced. Future studies should investigate whether single nucleotide polymorphisms in the pendrin gene are associated with the currently unexplained acidic urine in humans with calcium or uric acid stones.

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References

1. Bissig M, Hagenbuch B, Stieger B *et al.* Functional expression cloning of the canalicular sulfate transport system of rat hepatocytes. *J Biol Chem* 1994; 269: 3017–3021

2. Hastbacka J, de la Chapelle A, Mahtani MM *et al.* The diastrophic dysplasia gene encodes a novel sulfate transporter: positional cloning by fine-structure linkage disequilibrium mapping. *Cell* 1994; 78: 1073–1087
3. Hoglund P, Haila S, Socha J *et al.* Mutations of the down-regulated in adenoma (DRA) gene cause congenital chloride diarrhea. *Nat Genet* 1996; 14: 316–319
4. Everett LA, Glaser B, Beck JC *et al.* Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). *Nat Genet* 1997; 17: 411–422
5. Zheng J, Shen W, He DZ *et al.* Prestin is the motor protein of cochlear outer hair cells. *Nature* 2000; 405: 149–155
6. Lohi H, Kujala M, Kerkela E *et al.* Mapping of five new putative anion transporter genes in human and characterization of SLC26A6, a candidate gene for pancreatic anion exchanger. *Genomics* 2000; 70: 102–112
7. Lohi H, Kujala M, Makela S *et al.* Functional characterization of three novel tissue-specific anion exchangers SLC26A7, -A8, and -A9. *J Biol Chem* 2002; 277: 14246–14254
8. Vincourt JB, Jullien D, Amalric F *et al.* Molecular and functional characterization of SLC26A11, a sodium-independent sulfate transporter from high endothelial venules. *FASEB J* 2003; 17: 890–892
9. Romero MF, Chang MH, Plata C *et al.* Physiology of electrogenic SLC26 paralogues. *Novartis Found Symp* 2006; 273: 126–138
10. Soleimani M, Xu J. SLC26 chloride/base exchangers in the kidney in health and disease. *Semin Nephrol* 2006; 26: 375–385
11. Melvin JE, Park K, Richardson L *et al.* Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is upregulated in colon of mice lacking the NHE-3 Na(+)/H(+) exchanger. *J Biol Chem* 1999; 274: 22855–22861
12. Soleimani M, Greeley T, Petrovic S *et al.* Pendrin: an apical Cl-/OH-/HCO3- exchanger in the kidney cortex. *Am J Physiol Renal Physiol* 2001; 280: F356–F364
13. Schweinfest CW, Spyropoulos DD, Henderson KW *et al.* Slc26a3 (dra)-deficient mice display chloride-losing diarrhea, enhanced colonic proliferation, and distinct up-regulation of ion transporters in the colon. *J Biol Chem* 2006; 281: 37962–37971
14. Knauf F, Yang CL, Thomson RB *et al.* Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells. *Proc Natl Acad Sci U S A.* 2001; 98: 9425–9430
15. Wang Z, Petrovic S, Mann E *et al.* Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G573–G579
16. Xie Q, Welch R, Mercado A *et al.* Molecular characterization of the murine Slc26a6 anion exchanger: functional comparison with Slc26a1. *Am J Physiol Renal Physiol* 2002; 283: F826–F838
17. Petrovic S, Ju X, Barone S *et al.* Identification of a basolateral Cl-/HCO3- exchanger specific to gastric parietal cells. *Am J Physiol Gastrointest Liver Physiol* 2003; 284: G1093–G1103
18. Wang Z, Wang T, Petrovic S *et al.* Renal and intestine transport defects in Slc26a6-null mice. *Am J Physiol Cell Physiol* 2005; 288: C957–C965
19. Clark JS, Vandorpe DH, Chernova MN *et al.* Species differences in Cl- affinity and in electrogenicity of SLC26A6-mediated oxalate/Cl-exchange correlate with the distinct human and mouse susceptibilities to nephrolithiasis. *J Physiol* 2008; 586: 1291–1306
20. Xu J, Song P, Nakamura S *et al.* Deletion of the chloride transporter slc26a7 causes distal renal tubular acidosis and impairs gastric acid secretion. *J Biol Chem* 2009; 284: 29470–29479
21. Kim KH, Shcheynikov N, Wang Y *et al.* SLC26A7 is a Cl^- channel regulated by intracellular pH. *J Biol Chem* 2005; 280: 6463–6470
22. Xu J, Song P, Miller ML *et al.* Deletion of the chloride transporter Slc26a9 causes loss of tubulovesicles in parietal cells and impairs acid secretion in the stomach. *Proc Natl Acad Sci U S A.* 2008; 105: 17955–17960
23. Chang MH, Plata C, Zandi-Nejad K *et al.* Slc26a9—anion exchanger, channel and Na+ transporter. *J Membr Biol* 2009; 228: 125–140
24. Royaux IE, Wall SM, Karniski LP *et al.* Pendrin, encoded by the Pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion. *Proc Natl Acad Sci U S A.* 2001; 98: 4221–4226
25. Kim YH, Kwon TH, Frische S *et al.* Immunocytochemical localization of pendrin in intercalated cell subtypes in rat and mouse kidney. *Am J Physiol renal Physiol* 2002; 283: F744–F754
26. Wall SM, Hassell KA, Royaux IE *et al.* Localization of pendrin in mouse kidney. *Am J Physiol Renal Physiol* 2003; 284: F229–F241
27. Amlal H, Petrovic S, Xu J *et al.* Deletion of the anion exchanger Slc26a4 (pendrin) decreases apical Cl-/HCO3- exchanger activity and impairs bicarbonate secretion in kidney collecting duct. *Am J Physiol Cell Physiol* 2010; 299: C33–C41
28. Verlander JW, Kim YH, Shin W *et al.* Dietary Cl(-) restriction upregulates pendrin expression within the apical plasma membrane of type B intercalated cells. *Am J Physiol Renal Physiol* 2006; 291: F833–F839
29. Hoenderop JG, Nilius B, Bindels RJ. Calcium absorption across epithelia. *Physiol Rev* 2005; 85: 373–422
30. Blanchard A, Jeunemaitre X, Coudol P *et al.* Paracellin-1 is critical for magnesium and calcium reabsorption in the human thick ascending limb of Henle. *Kidney Int* 2001; 59: 2206–2215
31. Hoenderop JG, Hartog A, Stuiver M *et al.* Localization of the epithelial Ca(2+) channel in rabbit kidney and intestine. *J Am Soc Nephrol* 2000; 11: 1171–1178
32. Hoenderop JG, Nilius B, Bindels RJ. Molecular mechanism of active Ca^{2+} reabsorption in the distal nephron. *Annu Rev Physiol* 2002; 64: 529–549
33. Mensenkamp AR, Hoenderop JG, Bindels RJ. TRPV5, the gateway to Ca2+ homeostasis. *Handb Exp Pharmacol* 2007; 179: 207–220. Review
34. Gkika D, Hsu YJ, van der Kemp AW *et al.* Critical role of the epithelial Ca2+ channel TRPV5 in active Ca2+ reabsorption as revealed by TRPV5/calbindin-D28K knockout mice. *J Am Soc Nephrol* 2006; 17: 3020–3027
35. Hsu YJ, Dimke H, Schoeber JP *et al.* Testosterone increases urinary calcium excretion and inhibits expression of renal calcium transport proteins. *Kidney Int* 2010; 77: 601–608
36. Nakaya K, Harbidge DG, Wangemann P *et al.* Lack of pendrin HCO3- transport elevates vestibular endolymphatic [Ca2+] by inhibition of acid-sensitive TRPV5 and TRPV6 channels. *Am J Physiol Renal Physiol* 2007; 292: F1314–F1321
37. Bonny O, Rubin A, Huang CL *et al.* Mechanism of urinary calcium regulation by urinary magnesium and pH. *J Am Soc Nephrol* 2008; 19: 1530–1537
38. Nijenhuis T, Renkema KY, Hoenderop JG *et al.* Acid-base status determines the renal expression of Ca2+ and Mg2+ transport proteins. *J Am Soc Nephrol* 2006; 17: 617–626
39. Loffing J, Loffing-Cueni D, Valderrabano V *et al.* Distribution of transcellular calcium and sodium transport pathways along mouse distal nephron. *Am J Physiol Renal Physiol* 2001; 281: F1021–F1027
40. Petrovic S, Wang Z, Ma L *et al.* Regulation of the apical Cl-/HCO3-exchanger pendrin in rat cortical collecting duct in metabolic acidosis. *Am J Physiol Renal Physiol* 2003; 284: F103–F112
41. Wagner CA, Finberg KE, Stehberger PA *et al.* Regulation of the expression of the Cl-/anion exchanger pendrin in mouse kidney by acid-base status. *Kidney Int* 2002; 62: 2109–2117
42. Frische S, Kwon TH, Frøkiaer J *et al.* Regulated expression of pendrin in rat kidney in response to chronic NH4Cl or NaHCO3 loading. *Am J Physiol Renal Physiol* 2003; 284: F584–F593
43. Quentin F, Chambrey R, Trinh-Trang-Tan MM *et al.* The Cl-/HCO3-exchanger pendrin in the rat kidney is regulated in response to chronic alterations in chloride balance. *Am J Physiol Renal Physiol* 2004; 287: F1179–F1188
44. Verlander JW, Hassell KA, Royaux IE *et al.* Deoxycorticosterone upregulates PDS (Slc26a4) in mouse kidney: role of pendrin in mineralocorticoid-induced hypertension. *Hypertension* 2003; 42: 356–362
45. Dror AA, Politi Y, Shahin H *et al.* Calcium oxalate stone formation in the inner ear as a result of an Slc26a4 mutation. *J Biol Chem* 2010; 285: 21724–21735
46. Jiang Z, Asplin JR, Evan AP *et al.* Calcium oxalate urolithiasis in mice lacking anion transporter Slc26a6. *Nat Genet* 2006; 38: 474–478
47. Worcester EM, Coe FL. Clinical practice. Calcium kidney stones. *N Engl J Med* 2010; 363: 954–963
48. Worcester EM, Coe FL. Evidence for altered renal tubule function in idiopathic calcium stone formers. *Urol Res* 2010; 38: 263–269
49. Markadieu N, Bindels RJ, Hoenderop JG. The renal connecting tubule: resolved and unresolved issues in Ca(2+) transport. *Int J Biochem Cell Biol* 2011; 43: 1–4

50. Sakhaee K, Maalouf NM, Kumar R *et al.* Nephrolithiasis-associated bone disease: pathogenesis and treatment options. *Kidney Int* 2011; 79: 393–403
51. Ahmed K, Dasgupta P, Khan MS. Cystine calculi: challenging group of stones. *Postgrad Med J* 2006; 82: 799–801. Review
52. Pak CY, Sakhaee K, Peterson RD *et al.* Biochemical profile of idiopathic uric acid nephrolithiasis. *Kidney Int* 2001; 60: 757–761
53. Sakhaee K, Adams-Huet B, Moe OW *et al.* Pathophysiologic basis for normouricosuric uric acid nephrolithiasis. *Kidney Int* 2002; 62: 971–979
54. Rodman JS. Prophylaxis of uric acid stones with alternate day doses of alkaline potassium salts. *J Urol* 1991; 145: 97–99

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