Decreased Expression of Slc26a4 (Pendrin) and Slc26a7 in the Kidneys of Carbonic Anhydrase II-Deficient Mice

Xuming Sun^{1,2}, Manoocher Soleimani^{1,2} and Snezana Petrovic^{1,2}

¹Department of Medicine, University of Cincinnati, ²Research Services, Veterans' Affairs Medical Center, Cincinnati

Key Words

Chloride bicarbonate exchangers • Kidney collecting duct • Metabolic acidosis

Abstract

Background/Aims: Intercalated cells (ICs) of the kidney collecting duct are rich in carbonic anhydrase II (CAII), which facilitates proton and bicarbonate transport. Bicarbonate secretion is mediated via Pendrin (Slc26a4), which is expressed on the apical membrane of B-ICs and nonA-nonB ICs in the cortical collecting ducts (CCD). Bicarbonate absorption is mediated via anion exchanger 1 (AE1-Slc4a1) in the CCD and via AE1 and possibly Slc26a7 in the OMCD. Both exchangers are expressed on the basolateral membrane of A-ICs. The aim of this study was to examine the expression of pendrin, Slc26a7, and AE1 in the kidneys of CAII-deficient (CAR2-null) mice. **Methods:** For the expression studies, we used real-time RT-PCR, Northern hybridization, immunolabeling, and immunoblotting. **Results:** Pendrin mRNA expression was reduced 63% along with decreased pendrin immunolabeling in the cortex of CAR2-null mice present predominantly in nonA-nonB ICs.

Slc26a7 mRNA expression was decreased by 73% and Slc26a7 immunolabeling, present in A-ICs, severely reduced in the outer medulla of CAR2-null mice. AE1 mRNA expression was decreased to a similar degree (62%) along with reduced AE1 immunolabeling. The expression of aquaporin 2 (AQP2) water channel, exclusively present in principal cells of the collecting duct, was comparable in the wild type and CAR2-null mice. **Conclusion:** CAII deficiency results in a significant decrease in the gene and protein expression of bicarbonate transport proteins from Slc26 gene family – Slc26a4 (pendrin) and Slc26a7. These results emphasize the critical role of CAII for the maintenance of the intercalated cell phenotype.

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Introduction

Heterogeneous cell population of the kidney collecting duct consists of principal and intercalated cells. The principal cells regulate salt and water balance, and the intercalated cells regulate acid-base balance. There are

at least two types of intercalated cells. The acid-secreting, A-type intercalated cells (A-ICs) extrude protons through their apical membrane via a vacuolar type H^+ -ATPase and, to an extent, via H^+/K^+ ATPase, and reabsorb bicarbonate through a basolateral Cl^-/HCO_3^- exchanger [1]. The B-type intercalated cells (B-ICs) secrete bicarbonate through an apical Cl^-/HCO_3^- exchanger, while protons leave the cell through a basolateral H^+ -ATPase [1]. The number and activity of intercalated cells is regulated in response to overall acid-base status [1].

Members of Solute-Linked Carrier gene families 4 (SLC4) and 26 (SLC26) encode Cl^-/HCO_3^- exchangers in the intercalated cells. The A-ICs express Cl^-/HCO_3^- exchanger AE1 (SLC4A1) on their basolateral membrane [1]. We demonstrated that SLC26A7, a member of SLC26 family of versatile anion transporters, co-localized with AE1 on the basolateral membrane of A-ICs in the outer medullary collecting duct of the rat kidney [2]. SLC26A7 and AE1 are differentially regulated in response to alterations in osmolarity of the kidney medulla, where SLC26A7 is most abundant [3, 4]. A recent study, which used antibodies generated against different epitopes, detected SLC26A7 subapically in proximal tubules and on the basolateral membrane of thick ascending limbs, indicating that SLC26A7 distribution in the kidney is more widespread [5].

Several members of SLC26 gene family (SLC26A3, SLC26A4, SLC26A6, and SLC26A9) mediate Cl^-/HCO_3^- exchange [6-11]. SLC26A7 also mediates Cl^-/HCO_3^- exchange when expressed in *Xenopus oocytes* [2, 12] and in cultured cells (data not shown). Kim et al. demonstrated that SLC26A7 can function as a pH-sensitive chloride channel [13]. This discrepancy in reports from the *in vitro* studies awaits a resolution from *in vivo* studies, demonstrating physiological relevance of the two transport modes. As reviewed in [14, 15], the transporters encoded by genes from SLC26 gene family can function in multiple anion exchange modes.

Pendrin (SLC26A4) is another member of SLC26 gene family, which mediates chloride/bicarbonate [7], chloride/iodide [16-18], and chloride/formate exchange [19, 20]. There is ample evidence supporting the role of pendrin in bicarbonate secretion and chloride reabsorption in the kidney collecting duct [21-30]. This is consistent with pendrin mediating Cl^-/HCO_3^- exchange in the apical membrane of B-ICs. Pendrin is also expressed in the apical membrane of nonA-nonB ICs, an intercalated cell subtype, which expresses H^+ -ATPase on the apical membrane, but no known Cl^-/HCO_3^- exchanger on the basolateral side [23, 31].

Consistent with their rate-limiting role in acid-base regulation, the ICs are rich in the cytosolic enzyme carbonic anhydrase II (CAII) [1, 32]. CAII catalyzes conversion of CO_2 and H_2O to proton and bicarbonate, thus facilitating acid and bicarbonate secretion in the ICs [32].

In humans, CAII deficiency is characterized by renal tubular acidosis, osteopetrosis, cerebral calcification, and severe growth retardation [33]. Lewis et al. developed a strain of mice devoid of CAII by a chemically-induced point mutation (CAR-2 null mice) that results in the premature termination of translation of CAII [34]. Similar to humans, CAR-2 null mice have renal tubular acidosis, which can be rescued by gene therapy [35]. By using immunofluorescent labeling of the anion exchanger AE1 and of the 56-kDa subunit of the H^+ -ATPase to identify the intercalated cells, Breton et al. demonstrated that the cells positive for AE1 and H^+ -ATPase were nearly absent from the medulla of CAR-2 null mice [36].

The purpose of this study was to examine gene and protein expression of the newly characterized transporters of intercalated cells, pendrin and Slc26a7, in CAR2-null mice. Kidneys of wild type and CAR-2 null mice were harvested and the expression of pendrin, Slc26a7, AE1, and the water channel AQP2, as a marker of principal cells, was analyzed by Northern hybridization, real-time RT-PCR, immunoblotting, and immunocytochemistry. We found that CA II deficiency results in marked downregulation of pendrin, Slc26a7, and AE1, and has no effect on AQP2 abundance.

Materials and Methods

Animals and Tissue Preparation

Carbonic anhydrase II-deficient (CAR2-null) and the wild type mice were obtained from the Jackson Laboratories (Bar Harbor, MA), and raised in the animal facility of the University of Cincinnati. Carbonic anhydrase deficiency and phenotype of these mice were previously confirmed and characterized by several laboratories [34, 36-38]. The animals used for the experiments were 12 to 14 weeks old and maintained on tap water and a standard mouse diet. Mice were euthanized with an overdose of pentobarbital sodium according to institutional guidelines. All the procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. After euthanasia the kidneys were removed and snap frozen in liquid nitrogen, stored at $-80^\circ C$ for RNA and protein extraction. To prepare kidney tissues for immunostaining, after euthanasia, the animals were perfused through the heart with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Kidneys

were removed and post fixed in cold 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C, then soaked in 30% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4°C. The tissues were embedded with M-1 Embedding Matrix (Shandon, Pittsburgh, PA) and frozen. 6 µm thick serial sections were sliced on a cryostat, mounted onto Fisherbrand® Superfrost/Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C.

RNA Isolation and Northern Blot Hybridization

Total cellular RNA was extracted from the mouse kidneys using TriReagent according to the established methods [39], quantitated by a spectrophotometer, and stored at -80°C until use. Total RNA samples (25 µg/lane) were fractionated on a 1.2% agarose formaldehyde gel, transferred to Magna NT nylon membranes, and cross-linked by UV light. Hybridization was performed according to Church and Gilbert [40]. The membranes were washed and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). For pendrin a ³²P-labeled 488-bp PCR fragment based on the rat pendrin cDNA (GenBank Accession Number AF167412), was used for Northern experiments [7]. The PCR fragment was generated using the following primers: 5'-CAT TCT GGG GCT GGACCT C-3' (sense), and 5'-CCT TCG GGA CAT TCA CTT TCA C-3' (antisense). For AE1, a ³²P-labeled 650-bp PCR fragment based on the rat AE1 cDNA was used as specified [4]. For SLC26A7, a ³²P-labeled 550-bp PCR fragment was generated using the following primers: 5'-CTC ACC ACC GAA CCT ATT AC-3' (sense), 5'-AAC TCG GAT AAG CCC AAC AC (antisense) (GenBank Accession Number BB666404) and used as a probe for the Northern hybridization. For AQP2 probe, a 296-bp PCR fragment was generated using the following primers: 5'-AGC GCG CAG AAG TCG GAG CA-3' (sense), and 5'-CAG CCA CAT AGA AGG CAG CT-3' (antisense), (GenBank Accession Number NM_012909) [3].

RNA Isolation and Real-Time RT-PCR

Total cellular RNA was extracted from the mouse kidney stored at -80°C with RNeasy® Mini Kit (QIAGEN, Maryland) according to the manufacturer's protocol. DNase I (QIAGEN, Hilden, Germany) was used to digest genomic DNA from the tissues during total RNA extraction and purification. RNA gel was run to assess purity and quality of the extracted RNA. Quantitative analysis of total RNA was done with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

One µg of total RNA was reverse-transcribed to the first-strand cDNA with oligo (dT)₁₂₋₁₈ primer using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufactures' protocol. Real-time RT-PCR was run in triplicates for the quantitative analysis of mRNA expression levels and carried out using a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The primers for AE1 and the housekeeping gene, β-actin, were designed according to the sequence of mouse AE1 (GenBank Accession Number: AY296129) and mouse β-actin (GenBank Accession Number: NM_007393). AE1 primer sequences were: 5'-GCT CTT CCC ACA GAG CAAAC -3' (sense), and 5'-CTG

CCT CCA CCC ATT GTA GT -3' (antisense). β-actin primer sequences were: 5'-TTG CTG ACA GGA TGC AGA AG -3' (sense), and 5'-CAG TGA GGC CAG GAT GGA GC -3' (antisense). The size of PCR products for AE1 was 142 bp and for β-actin 122 bp. We routinely used β-actin as a control for sampling errors.

Quantitative real-time RT-PCR reaction consisted of 1 µl of first-strand cDNA, 0.1 µM primers, 1x PCR buffer, 5.0 mM Mg²⁺, 200 µM dNTP mix, 1 unit of Platinum Tag DNA polymerase (Invitrogen, Carlsbad, CA) and 0.25x SYBR® Green I Nucleic Acid Gel Stain (Molecular Probes, Invitrogen, Eugene, OR) in a total volume of 25 µl. After an initial denaturation at 95°C for 10 min, 45 cycles of PCR were performed with denaturation at 95°C for 10 sec, annealing at 64°C for 15 sec, and extension at 72°C for 35 sec. The saturation curves and threshold line for β-actin and Slc26a7 are shown in Fig. 7B.

The absolute mRNA levels in each sample were calculated based on a standard curve. The standard curve was set up by using serial dilutions of known amounts of specific templates against corresponding cycle threshold values. Then, we calculated the ratio of AE1 over β-actin in each sample to normalize AE1 expression. The specificity of PCR products for both AE1 and β-actin was verified by examining the melting curve, by electrophoresis, and sequencing of the real-time RT-PCR products.

Preparation of Membrane Protein from Mouse Kidneys

For the membrane fraction isolation, tissue samples were homogenized in the ice-cold isolation solution containing 10 mM HEPES, pH 7.4, 350 mM sucrose, 5 mM EDTA, 10 µl/ml Protease Inhibitor Cocktail (Sigma, Louis, MO), and Phosphatase Inhibitor Cocktail II (Sigma, Louis, MO) in glass-homogenizer (Kontes®, Vineland, NJ). The homogenates were centrifuged at 2,000 x g for 10 min at 4°C to remove nuclei and cell debris. The supernatants were centrifuged at 100,000 x g for 1 hr at 4°C. The pellets that contained plasma membrane and intracellular vesicles were suspended in the same buffer. The protein concentration was measured by using BCA™ Protein Assay Kit (PIERCE, Rockford, IL) and stored at -80°C.

Electrophoresis and Immunoblotting

15 µg of the membrane protein from each sample was resolved with 8-16% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) followed by transfer to nitrocellulose membranes. The membranes were blocked overnight with 3% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6, 137 mM Sodium Chloride, 0.1% Tween-20) at 4°C. The blocked membranes containing the membrane protein were incubated with the anti-AQP2 antibody diluted 1:500 in 3% nonfat dry milk in TBS-T for 2 hrs at room temperature. The membranes were washed with TBS-T, incubated for 1 hr with Peroxidase-Labeled affinity purified secondary antibody to rabbit IgG (KPL, Gaithersburg, MD) at room temperature, and subsequently washed again. The secondary antibody was diluted 1:5000 in 3% nonfat dry milk in TBS-T. The protein bands on the membranes were developed with One Component TMB Membrane Peroxidase Substrate (KPL, Gaithersburg, MD). The images were taken by

computerized scanning densitometry, on Foto/Eclipse™ (FOTO DYNE Incorporated, Hartland, WI). After that, the membranes were stripped, blocked, and incubated with anti- β -actin antibody at 1:2500 dilution.

Primary Antibodies

A) *AE1*. The affinity-purified, rabbit polyclonal antibody that recognizes anion exchanger 1 (AE1) antibody was purchased from Chemicon International (Temecula, CA; Catalog #: AB3500P).

B) *AQP2*. The affinity-purified, rabbit polyclonal antibody that recognizes aquaporin 2 was purchased from Alomone Labs (Jerusalem, Israel; Catalog #: AQP-002).

C) β -actin. The mouse monoclonal antibody that recognizes β -actin was purchased from Abcam Inc (Cambridge, MA; Catalog #: ab8226).

D) *SLC26A7*. The affinity-purified, rabbit polyclonal antibody raised to a peptide corresponding to the amino acid residues SHIHSNKNLSKLSHSEV of the human SLC26A7 was a gift of Dr. Juha Kere, Karolinska University Hospital, Sweden. The antibody has been previously characterized [41].

E) *Pendrin*. The affinity-purified, rabbit polyclonal antibody raised to two peptides corresponding to the amino acid residues KSREGQDSLLETVARIRDC and RERRLPERRTRLDLARSC of the mouse pendrin was previously characterized [7].

F) *H⁺-ATPase*. The rabbit polyclonal immune serum raised against the ATP6V0A4 subunit of H⁺-ATPase was a gift from Carsten Wagner, University of Zurich, Switzerland. The antibody has been previously characterized [42].

Immunofluorescent Labeling

For immunofluorescent labeling, cryosections were thawed at room temperature and washed twice with 0.01 M PBS (pH 7.4). The sections were blocked with Image-iT™ FX Signal Enhancer (Molecular Probes, Eugene, OR) for 30 min at room temperature. The primary antibodies were diluted as follows: anti-pendrin antibody 1:40, anti-AE1 1:100, and anti-Slc26a7 1:100. The antibodies were diluted in 0.01 M PBS (pH 7.4) with 0.2% triton X-100 and 10% normal goat serum. The sections were incubated with antibodies overnight at 4°C. The labeling was visualized with Alexa Fluor 488 (green) or Alexa Fluor 594 (red) goat anti-rabbit secondary antibodies (Invitrogen, Molecular Probes, Eugene, OR) used at 1:200 dilution. The sections were incubated with the secondary antibodies for 2 hrs at room temperature, washed, and incubated with the nuclear dye Hoechst 33342 (Invitrogen, Molecular Probes, Eugene, OR) at 1:10,000 dilution for 20 min. The sections were washed, air-dried, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

For double labeling anti-SLC26A7 and anti-pendrin primary antibodies, and anti-H⁺-ATPase serum were used at 1:100, 1:20 and 1:40 dilution. Slc26a7 and pendrin labeling was visualized with secondary Alexa Fluor 594 (red) goat anti-rabbit antibody (Invitrogen, Molecular Probes, Eugene OR) at 1:400 dilution. Anti-ATP6V0A4 serum was pre-labeled with Zenon® Alexa Fluor® 488 (green) rabbit IgG-labeling kit (Invitrogen, Molecular Probes, Eugene OR) as previously described [2].

Fig. 1. Expression of pendrin mRNA in kidneys of wild type and CAR2-null mice. A: A representative Northern blot hybridization experiment indicates the down regulation of pendrin mRNA in the whole kidneys of CAR2-null mice compared to the wild types. B: Results of the densitometric analysis of the Northern hybridization experiments demonstrate that the expression of pendrin mRNA is decreased by 63% in CAR2-null mice compared to wild types ($p < 0.04$; $n = 3$ for each bar; * indicates statistical significance).

The sections were examined and images acquired on the epifluorescent microscope Nikon E600 (Nikon Bioscience, Melville, NY) equipped with SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Digital images were acquired using Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI).

Statistical analyses

Values are arithmetic means $\pm$ SE. Comparisons were done using unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Expression of pendrin (*Slc26a4*) is decreased in CAR2-null mice

We examined the mRNA expression of pendrin in the kidneys of CAR-2 null mice and their wild type littermates by Northern hybridization. Figure 1A is a

Fig. 2. Pendrin labeling in the kidney cortex of wild type and CAR2-null mice. Cryostat sections of the kidney cortex were labeled with the primary antibody that recognizes pendrin and the red fluorescent secondary antibody. In the wild type mice, pendrin labeling is present in a subpopulation of cells in cortical collecting ducts suggestive of intercalated cell staining (panel A; 200x magnification), and characteristically concentrated at the apical pole (panel C; 600x magnification). Fewer pendrin-positive cells are present in kidney sections from CAR2-null mice as evident from panels B (200x magnification) and D (600x magnification). In the panels C and D, differential interference contrast images were merged with red (pendrin) and blue (nuclear labeling) fluorescent images.

Fig. 3. Pendrin and H^+ -ATPase double labeling in kidneys of wild type and CAR2-null mice. Kidney cryosections were labeled with the anti-pendrin primary and a red fluorescent secondary antibody. The sections were also labeled with immune serum that recognizes ATP6V0A4 subunit of H^+ -ATPase. The immune serum was prelabeled with Zenon reagent conjugated to a green fluorescent dye. Panel A illustrates pendrin-positive cells in WTs (arrows). The same cells express H^+ -ATPase on their basolateral side, which identifies them as B-ICs (arrows; panel B). The merged image (panel C) illustrates the double labeled B-ICs. In CAR2-null mice, pendrin labeling is less intense (arrows; panel D) and almost invariably present in nonA-nonB ICs (arrows; panel F), which also express H^+ -ATPase on the apical side (arrows; panel E). In the panels C and F, differential interference contrast images were merged with fluorescent ones. Stars in panels B, C, E, and F identify A-ICs.

representative Northern blot hybridization experiment that illustrates a marked reduction in the expression of pendrin

mRNA in kidneys of CAR2-null mice compared to their wild type littermates. Densitometric analysis of the

Fig. 4. Expression of Slc26a7 mRNA in the kidneys of wild type and CAR-2 null mice. A: A representative Northern blot hybridization experiment indicates a decrease in Slc26a7 mRNA levels in the kidneys of CAR2-null mice compared to the wild types. B: Corresponding densitometric analysis of the Northern hybridization experiments demonstrates that the expression of Slc26a7 mRNA is decreased by 73% in CAR2-null mice compared to the wild types ($p < 0.01$; $n = 3$ for each bar; ** indicates statistical significance).

Northern hybridization experiments demonstrates that pendrin mRNA in the kidneys of CAR2-null mice decreased by 63% compared to the wild types. The ratio of pendrin mRNA/28S ribosomal RNA was 0.4 ± 0.07 ($n = 3$) in wild types vs. 0.15 ± 0.02 ($n = 3$) in CAR2-null mice ($p < 0.05$; summarized in Fig. 1B). The samples were taken from separate animals and the experiments run in duplicates.

Next, we examined pendrin labeling in the kidneys of CAR2-null and wild type animals by indirect immunofluorescence. In the wild type mice, pendrin labeling was present in a subpopulation of cells in cortical collecting ducts (Fig. 2A). The pendrin-positive cells showed labeling concentrated at the apical pole (Fig. 2C), the pattern resembling the staining of B and nonA- nonB intercalated cells, as previously reported [23, 26, 31]. The labeled cells were numerous in the kidney cortex characteristically concentrated in the areas of the cortical part of medullary rays where the collecting ducts occur in bundles. In the kidney sections of CAR2-null mice (Fig. 2B and 2D), the number of pendrin positive cells was lower than in wild type mice.

To examine if these remaining pendrin-positive cells also express vacuolar H^+ -ATPase, we performed double labeling studies presented in Figure 3. In agreement with published studies [31] pendrin-positive cells can be identified as B-ICs, expressing apical pendrin and basolateral or diffuse H^+ -ATPase labeling (Fig. 3A, 3B, and 3C) or as nonA-nonB ICs expressing both pendrin and H^+ -ATPase on the apical side. Previous reports described that nonA-nonB ICs positive for pendrin and H^+ -ATPase never express AE1 on the basolateral side [31]. In the CAR2 null mice however, pendrin-positive cells were fewer and almost exclusively present in nonA-

nonB ICs (Fig. 3D, E and F). Lack of pendrin positive B-ICs may also reflect adaptation to metabolic acidosis. It is well established that bicarbonate secretion, the primary mode of B-ICs function, significantly decreases in metabolic acidosis [1]. Our findings suggest that the remaining ICs have proper polarity albeit lower expression of the key acid-base transport proteins.

Taken together, Northern hybridization experiments and immunolabeling indicate that the deficiency of CA II significantly decreases the expression of pendrin. These data are in agreement with the report from Breton et al., demonstrating depletion of both A and B-ICs in CA II-deficient mice [36]. A decrease in the expression of pendrin is also expected because of metabolic acidosis [22, 24, 28], which is present in CAR2-null mice [34, 38].

Expression of Slc26a7 is decreased in CAR2-null mice

By using Northern blot hybridization, we examined mRNA expression of Slc26a7 in the kidneys of CAR-2 null mice and their wild type littermates. Figure 4A is a representative Northern blot hybridization experiment illustrating a severe reduction in the expression of Slc26a7 mRNA in the kidneys of CAR2-null mice compared to the wild types. Densitometric analysis of the Northern

Fig. 5. Slc26a7 labeling in the kidney cortex of wild type and CAR2-null mice. Kidney cryosections were labeled with the primary antibody that recognizes Slc26a7 and the red fluorescent secondary antibody. Slc26a7 labeling was concentrated at the basolateral pole of a subpopulation of cells in the collecting ducts. The number of basolaterally labeled, Slc26a7-positive cells in the kidney sections taken from the CAR2-null mice (panel B, magnification 100x and panel D, magnification 600x) was markedly decreased compared to the number of the basolaterally labeled, Slc26a7-positive cells in the kidney sections from wild type mice (panel A, magnification 100x and panel C, 600x magnification).

Fig. 6. Slc26a7 and H⁺-ATPase double labeling in kidneys of wild type and CAR2-null mice. Kidney cryosections were labeled with the anti-Slc26a7 primary and a red fluorescent secondary antibody. The sections were also labeled with immune serum that recognizes ATP6V0A4 subunit of H⁺-ATPase. The immune serum was pre-labeled with Zenon reagent conjugated to a green fluorescent dye. Panel A illustrates Slc26a7-positive cells in WTs (arrows). The same cells express H⁺-ATPase on their apical side, which identifies them as A-ICs (arrows; panel B). The merged image (panel C) illustrates the double labeled A-ICs. In CAR2-null mice, very few Slc26a7-labeled cells can be identified, the labeling is dim, and often a substantial part of the labeling is intracellular (arrows, panel D). Slc26a7-labeled cells also express H⁺-ATPase on the apical side (arrows; panel E and F). In the panel C, differential interference contrast image was merged with the fluorescent ones.

hybridization experiments demonstrated that Slc26a7 mRNA in the kidneys of CAR2-null mice decreased by

73% compared to the wild types. The ratio of Slc26a7 mRNA/28S ribosomal RNA was 0.014 ± 0.003 (n=3) in

Fig. 7. Expression of AE1 mRNA in wild type and CAR2-null mice. We used quantitative real-time RT-PCR to assess AE1 mRNA levels. Based on the standard curve, we calculated the number of template molecules for AE1 mRNA and mRNA of the housekeeping gene, β -actin, and expressed the results as AE1/ β -actin ratio. A. The expression of AE1 mRNA is decreased by 62% in the kidneys of CAR2-null mice ($p < 0.03$; $n = 3$ for each bar; * indicates statistical significance). B. The original saturation curves.

Fig. 8. AE1 labeling in the kidney cortex of wild type and CAR2-null mice. Kidney cryosections were labeled with the primary antibody that recognizes AE1 and the green fluorescent secondary antibody. The green fluorescent images were merged with the differential interference contrast (DIC) images. AE1 labeling was concentrated at the basolateral pole of a subpopulation of cells in the collecting duct identifying these as A-ICs. Numerous A-ICs are present in the outer medulla of the wild type mice (panel A); whereas few cells can be observed in the outer medulla of CAR2-null mice (panel B).

CAR2-null mice vs. 0.05 ± 0.007 ($n = 3$) in wild types ($p < 0.01$; Fig. 4B). The samples were taken from separate animals, and the experiments were run in duplicates.

We used indirect immunofluorescence to assess Slc26a7 labeling in kidneys of CAR2-null and wild type animals. Figure 5A shows Slc26a7 labeling in the kidney sections of the wild type mice. Anti-Slc26a7 antibody stained the basolateral domain of the labeled cells and was most prominent in the outer medulla (Fig. 5, panel A and C), as we previously reported in the rat kidney [2, 4].

The number of Slc26a7-positive cells in the kidney sections from CAR2-null mice (Fig. 5, panel B and D) was markedly reduced compared to kidney sections of the wild type mice (Fig. 5, panel A and C). The decreased expression of Slc26a7 in CAR2-null mice is consistent with the decreased number of A-ICs.

To examine if few remaining Slc26a7-positive cells also express vacuolar H^+ -ATPase, we performed double labeling studies presented in Figure 6. In agreement with published studies [2, 4], Slc26a7-positive cells can be

Fig. 9. Expression of AQP2 mRNA in wild type and CAR2-null mice. A: A representative Northern blot hybridization experiment indicates comparable expression of AQP2 mRNA in the kidneys of CAR2-null and the wild type mice. B: Corresponding densitometric analysis of Northern hybridization experiments demonstrates no difference in the ratios of AQP2/ 28S ribosomal RNA expression (0.54 ± 0.08 in wild types vs. 0.58 ± 0.07 in CAR2-null mice; $p > 0.5$; $n = 3$ for each bar).

Fig. 10. Western blot and the corresponding densitometric analysis of AQP2 protein levels in the kidneys of wild type and CAR2-null mice. A: In preparations of membrane protein extracted from the kidneys of the wild type and CAR2-null mice, the anti-AQP2 antibody recognizes ~ 29 and 36-kDa bands that correspond to non-glycosylated and glycosylated forms of AQP2. A representative Western blot experiment indicates comparable AQP2 protein abundance in the wild type and CAR2-null mice. B: The same membranes were stripped and re-probed with anti- β actin antibody followed by densitometric analysis. The ratios of AQP2/ β -actin expression were similar in the wild type and CAR2-null mice (0.42 ± 0.03 in wild types and 0.44 ± 0.03 in CAR2-null mice; $p > 0.5$; $n = 3$ for each bar).

identified as A-ICs, expressing basolateral Slc26a7 and apical H^+ -ATPase labeling (Fig. 6A, 6B, and 6C). In the CAR2 null mice, few Slc26a7-positive cells could be identified. The labeling was dim and often substantial part of Slc26a7-labeling was retained intracellularly (Fig. 6D, 6E, and 6F). These findings are consistent with the severe depletion of ICs in the kidney medulla and demonstrate that Slc26a7-positive cells also express H^+ -ATPase on the apical membrane, identifying them as A-ICs.

The expression of Slc4a1 (AE1) is decreased in CAR2-null mice

Slc26a7 co-localizes with AE1 in the basolateral membrane of the A-type intercalated cells in the rat kidney medulla [2]. For comparison with Slc26a7, we examined AE1 gene expression in the kidneys of CAR-2 null mice and their wild type littermates. The Northern blot hybridization experiments indicated a decrease in the expression of AE1 mRNA in the kidneys of CAR2-null

mice compared with their wild type littermates. Densitometric analysis of the Northern hybridization experiments showed marked decrease, however, the decrease did not reach statistical significance. The ratio of AE1 mRNA/28S ribosomal RNA was 0.06 ± 0.01 ($n=3$) in wild types vs. 0.034 ± 0.004 ($n=3$) in CAR2-null mice ($p>0.05$). Because variability of the samples could have precluded the detection of a true difference, we repeated these analyses with a more sensitive method, the quantitative real-time RT-PCR. Based on a standard curve, we calculated the number of template molecules for AE1 mRNA and mRNA of the housekeeping gene, β -actin, and expressed the results as AE1/ β -actin ratio. As illustrated in Fig. 7A, in the wild types, AE1/ β -actin ratio was $4.8 \times 10^{-3} \pm 0.8 \times 10^{-3}$ ($n=3$) vs. $1.8 \times 10^{-3} \pm 0.1 \times 10^{-3}$ ($n=3$) in CAR2-null mice ($p<0.05$). The expression of AE1 mRNA in CAR2-null mice was significantly decreased (62%) compared to the wild types. The samples were taken from separate animals and the real-time measurements were run in triplicates. The Figure 7B shows β -actin and Slc26a7 saturation curves.

We correlated the gene expression with AE1 immunofluorescent labeling in the kidneys of CAR2-null and wild type animals. In the wild type mice, anti-AE1 antibody stained the basolateral domain of all AE1-positive ICs, identifying them as A-ICs. The labeled cells were evident in the kidney cortex and numerous in the kidney medulla (Fig. 8, panel A), in which B ICs are typically absent [1]. In keeping with the gene expression results and a previous report [36], it is apparent that the number of cells positive for AE1 labeling is significantly depleted (Fig. 8, panel B) in the kidney medulla of CAR2-null mice. Consistent with our previous report demonstrating that Slc26a7 and AE1 co-localize in the rat kidney [2, 4], Slc26a7 (Fig. 5, panels A and B) and AE1 (Fig. 8 panels A and B) labeling patterns were similar in both CAR2-null and wild type mice. The number of AE1-labeled cells in the kidney cortex of CAR2-null mice was also decreased compared with the wild types (data not shown), although the decrease was not as marked as in the medulla. We conclude that the significant decrease in AE1 gene expression correlates with severely decreased AE1 labeling in CAR2 null mice, consistent with depletion of A-ICs and severe down regulation of AE1 in the remaining intercalated cells.

Effect of CA II deficiency on the expression of water channel AQP2

To determine if changes in the gene expression of key transport proteins in collecting ducts of CAR2-null

mice were limited to the intercalated cells or included principal cells as well, we examined gene and protein expression of AQP2, a water channel present exclusively in the principal cells. We examined AQP2 mRNA levels with Northern hybridization and protein levels with immunoblotting in the kidney tissue of CAR2-null and wild type mice. Figure 9A is a representative Northern blot hybridization experiment, which indicates no difference in the expression of AQP2 mRNA in the kidneys of CAR2-null mice compared to the wild types. The accompanying densitometric analysis of the Northern hybridization experiments demonstrates that AQP2 mRNA expression is comparable in kidneys of CAR2-null and wild type mice. As illustrated in Fig. 9B, the ratio of AQP2 mRNA/28S ribosomal RNA was 0.54 ± 0.08 ($n=3$) in wild types vs. 0.58 ± 0.07 ($n=3$) in CAR2-null mice ($p>0.5$). The samples were taken from separate animals and the experiments were run in duplicates. Next, we examined the expression of AQP2 protein in these animals by immunoblotting. A representative Western blot in Figure 10A and the accompanying densitometric analysis (Fig. 10B) show that AQP2 protein levels were similar in CAR2-null and wild type mice. The ratios of AQP2/ β -actin expression were nearly identical: 0.42 ± 0.03 ($n=3$) in the wild types and 0.44 ± 0.03 ($n=3$) in CAR2-null mice ($p>0.5$). We concluded that CAII deficiency did not affect AQP2 gene and protein expression.

Discussion

We examined the expression of chloride bicarbonate exchangers from SLC26 and SLC4 gene families in kidney collecting ducts of the carbonic anhydrase II-deficient (CAR2-null) mice and their wild type littermates. Our results indicated that the deficiency of CAII decreases the mRNA level of Slc26a4 (pendrin) by 63% (Fig. 1), Slc26a7 by 73% (Fig. 4), and Slc4a1 (AE1) by 62% (Fig. 7). Along with the reduction in mRNA expression, the immunolabeling of pendrin, Slc26a7, and AE1 was markedly reduced in the collecting ducts of CAR2-null mice (Figs. 2, 5, and 8). We also find that few remaining intercalated cells that express pendrin or Slc26a7 also express H⁺-ATPase (Figs. 3 and 6). This suggests that, although decreased in number, the remaining cells retain the key characteristics of intercalated cells. We also observed that the remaining pendrin positive cells in CAR2-null mice are of nonA-nonB type (Fig. 3 D, 3E, and 3F). We did not find any difference in the abundance of AQP2 water channel present exclusively in the principal

cells of the collecting duct (Fig. 10). Since pendrin, Slc26a7, and AE1 are expressed in the intercalated cells, these findings are consistent with the selective loss of the intercalated cells or downregulation of their key transport proteins, not with the replacement of intercalated cells with principal cells as has previously been suggested [36].

CAII facilitates transepithelial bicarbonate transport and urine acidification in the collecting duct [1, 32]. CAII is also critical for intracellular pH regulation in the intercalated cells, which have to maintain intracellular pH homeostasis in face of significant transepithelial bicarbonate fluxes. It is possible that the intercalated cells become necrotic or undergo apoptosis, in the absence of CAII because of inability to maintain intracellular pH homeostasis. The depletion of intercalated cells is most prominent in the medullary collecting duct, which sustains bicarbonate fluxes several fold higher than the cortical collecting duct [1]. We did not detect a difference in the number of apoptotic cells in CAR2-null mice compared to wild types (data not shown). It is possible that the increased levels of apoptosis occur during the development. An increased rate of apoptosis is sometimes difficult to detect because of a typically short clearance time of the apoptotic cells [43].

Ridderstrale et al. [44], who used the morphological criteria and histochemical carbonic anhydrase activity to detect intercalated cells, observed the intercalated cell type in CAR-2 null mice. These intercalated cells exhibited intense membrane histochemical activity, which the authors attributed to the membrane-bound isoform of carbonic anhydrase CAIV. While some intercalated cells may undergo apoptosis or necrosis, it appears that in the surviving intercalated cells, CAII deficiency leads to severe down regulation of the key transport proteins. Severe down regulation of H⁺-ATPase in intercalated cells was previously reported in humans with renal tubular acidosis [45].

Changes in gene expression caused by a change in pH are specific to certain tissues and pathophysiologic states. For example, the effect of pH on ammonium production in the kidney proximal tubule is well documented. Low intracellular pH regulates gene expression of enzymes involved in ammonium production. This includes pH-dependent stabilization of glutaminase mRNA and regulation of phosphoenolpyruvate carboxykinase (PEPCK) transcription [46]. pH decrease activates p38 stress-activated protein kinase, which phosphorylates a transcription factor ATF-2. ATF-2 then binds to cAMP-response element 1 within the PEPCK promoter to increase PEPCK transcription [46]. These

effects converge to mediate adaptation to acidosis through increase in ammonium ion production. Less is known about the signaling mechanisms that mediate the effects of pH on gene expression in the kidney collecting duct. We know that metabolic acidosis down regulates the expression of pendrin [24] and up regulates the expression of AE1 [21]. Exposure to low pH of cultured inner medullary cells, causes up regulation of CAII mRNA [47]. However, a signaling cascade that mediates the effects of low pH on gene transcription in these cells is not known.

Our double labeling studies with pendrin or Slc26a7, and H⁺-ATPase demonstrated that the remaining intercalated cells retained their usual phenotype. The cells that expressed pendrin or Slc26a7 also expressed H⁺-ATPase on their apical side in CAR2-null mice. Therefore, in CAR2-null mice, pendrin was present almost exclusively in nonA-nonB ICs. In the wild types, pendrin was detected in both B and nonA-nonB ICs.

Microperfusion studies in the isolated kidney collecting ducts demonstrated that acidosis entails a decrease in function and number of B-ICs and an increase in function and number of A-ICs [24, 48, 49]. During metabolic acidosis, the expression of pendrin is decreased, consistent with reduced B-IC function [22, 24, 28], while the expression of AE1 is increased, indicating an increased activity of A-ICs [1, 21, 50-52]. In addition, trafficking of these transporters to the membrane plays an important role in the adaptive response to metabolic acidosis [22, 28, 50, 51]. The decrease in the expression of pendrin in CAR2-null mice is consistent with a decrease in the absolute number of B-ICs in CAR2-null mice, but may also reflect the adaptive response to metabolic acidosis. A preliminary report on Slc26a7 knockout mice indicates that Slc26a7 is involved in bicarbonate transport in the medullary collecting duct [53]. A dramatic decrease in the expression of Slc26a7 and AE1 is unexpected in view of metabolic acidosis in CAR2-null mice, although consistent with the depletion of intercalated cells [36]. Low expression of AE1 and Slc26a7 likely contributes to the development of renal tubular acidosis in CAR2-null mice.

We have demonstrated that Slc26a7 is expressed in the intercalated cells where it co-localizes with AE1 [2, 4], while Dudas et al. [5] detected Slc26a7 subapically in proximal tubules and on the basolateral side of thick ascending limbs. The results presented in this study are consistent with Slc26a7 being expressed in the collecting duct intercalated cells, and may explain the severe decrease of Slc26a7 expression.

We previously reported that the expression of AE1

and Slc26a7 was differentially regulated in pathologic states such as water deprivation and in Brattleboro rats [3, 4]. In CAR2-null mice, the expression of both exchangers was decreased. We observed the differential regulation of AE1 and Slc26a7 in states of altered medullary osmolarity, whereas changes in medullary osmolarity and water homeostasis in CAR2-null mice have not been reported. By demonstrating that the expression of AQP2 is similar in the CAR2-null and wild type mice, our results argue against changes in water homeostasis. Breton et al [36] found that the depletion of intercalated cells was concomitant with the increase in the number of principal cells in CAR2-null mice. We did not detect a change in either AQP2 RNA message or protein abundance. This discrepancy may originate from differences in the methods used. We relied on densitometric analysis of immunoblots and Breton et al [36] on the analysis of cell numbers. Since the previous study [36] did not determine the absolute number of cells in the collecting duct, the increase in the number of principal cells might be relative, resulting from the selective deletion of intercalated cells.

We conclude that in CAR2-null mice gene and protein expression of the chloride bicarbonate exchangers

in the intercalated cells - pendrin, Slc26a7, and AE1 - is decreased significantly and to a similar extent. Lack of the effect of CAII-deficiency on AQP2 gene and protein expression argues against the possibility that the deleted intercalated cells are replaced with the principal cells. Our data are consistent with the selective deletion of intercalated cells in the kidneys of CAR2-null mice, although some of the decrease likely reflects a severe down regulation of chloride bicarbonate exchangers in the surviving intercalated cells. These results emphasize the importance of CAII for the maintenance of the intercalated cell phenotype.

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