Adaptation by the collecting duct to an exogenous acid load is blunted by deletion of the proton-sensing receptor GPR4

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Sun X, Stephens L, DuBose TD Jr., Petrovic S. Adaptation by the collecting duct to an exogenous acid load is blunted by deletion of the proton-sensing receptor GPR4. Am J Physiol Renal Physiol 309: F120–F136, 2015. —We previously reported that the deletion of the pH sensor GPR4 causes a non-gap metabolic acidosis and defective net acid excretion (NAE) in the GPR4 knockout mouse (GPR4+/−) (Sun X, Yang LV, Tieggs BC, Arend LJ, McGraw DW, Penn RB, and Petrovic S. J Am Soc Nephrol 21: 1745–1755, 2010). Since the major regulatory site of NAE in the kidney is the collecting duct (CD), we examined acid-base transport proteins in intercalated cells (ICs) of the CD and found comparable mRNA expression of the kidney anion exchanger 1 (kAE1), pendrin, and the a4 subunit of Na+/H+ ATPase in GPR4+/− vs. +/+ . However, NH4Cl loading elicited adaptive doubling of AE1 mRNA in GPR4+/+, but a 50% less pronounced response in GPR4+/−. In GPR4+/+, Na+/H+ ATPase evoked a cellular response characterized by an increase in AE1-labeled and a decrease in pendrin-labeled ICs similar to what was reported in rabbits and rats. This response did not occur in GPR4−/−. Microperfusion experiments demonstrated that the activity of the basolateral Cl−/HCO3− exchanger, kAE1, in CDs isolated from GPR4−/− failed to increase with NH4Cl loading, in contrast to the increase observed in GPR4+/+. Therefore, the deficiency of GPR4 blunted, but did not eliminate the adaptive response to an acid load, suggesting a compensatory response from other pH/CO2/bicarbonate sensors. Indeed, the expression of the calcium-sensing receptor (CaSR) was nearly doubled in GPR4−/− kidneys, in the absence of apparent disturbances of Ca2+ homeostasis. In summary, the expression and activity of the key transport proteins in GPR4+/− mice are consistent with spontaneous metabolic acidosis, but the adaptive response to a superimposed exogenous acid load is blunted and might be partially compensated for by CaSR.

acid sensor; CasR; collecting duct; GPR4 knockouts; metabolic acidosis

THE REMARKABLE CAPACITY OF acid-secreting kidney epithelial cells to respond to an acid challenge has been a focus of intense research since its first description (39) and is an essential element of the role of the kidney to defend against metabolic acidosis (30). An intriguing aspect of this response is how avidly and accurately kidney epithelial cells adjust proton and bicarbonate secretion to match wide variation in the daily dietary net acid load (14, 39, 56). This observation eventually led to the hypothesis that pH sensors may be involved in the afferent response by the kidney to a reduction in pH that sets acid excretion to the appropriate level (14, 39). Recently a number of clinical observations have suggested that perpetual excretion of high exogenous acid loads, as a result of ingestion of a typical Western diet, enables progression of kidney disease (7, 40–42, 84). This association underscores a potentially important translational perspective in the elucidation of fundamental mechanisms by which the kidney responds to the relatively high acid load of a contemporary Western diet (85).

Kinas [Pyk2 (34, 58) and Erb1/2 (107)], ion channels [like TASK2 (101)], soluble adenylyl cyclase (12, 64, 93), H+ ATPase (62), the endothelin B receptor (45, 57, 66, 67, 104, 105), and several other G protein-coupled receptors (28, 63, 73, 88) have all been implicated in pH sensing in the kidney. Different kinetic properties and differences in subcellular distribution of these sensors may allow for pH sensing in different compartments [e.g., intracellular (pHi) or interstitial or luminal pH], but also complicate attempts to delineate a role of an individual sensor as well as to elucidate the integrated response of various sensors. For example, the finding that the calcium-sensing receptor (CaSR) is exquisitely pH sensitive is intriguing in view of the tight relationship between calcium and acid-base homeostasis (28, 73). Moreover, hereditary defects in acidification have been designated for associated impairments of calcium homeostasis such as rickets, urolithiasis, and nephrocalcinosis (30). Ligand binding to CaSR is so profoundly modulated by pH that CaSR can function as a pH sensor at constant extracellular calcium levels (28, 73). However, variation in the subcellular distribution of CaSR in different nephron segments and its concomitant role in the regulation of calcium transport render the understanding of its possible role in regulation of acid-base homeostasis challenging (76).

We focused on the proton-sensing receptor GPR4 as a candidate pH sensor in the kidney, because of its high expression in organs involved in acid-base regulation, namely, the lung, brain, and kidney, relative to other tissues (49). Proton-sensing receptors (GPR4, OGR1, TDAG8) constitute a small, unique family of G protein-coupled receptors that accept protons as ligands and are activated by protons in a physiological pH range to induce production of cAMP (GPR4 and TDAG8) or inositol 1,4,5-trisphosphate (IP3; OGR1) in a pH-dependent manner (51, 59). Recently this unique family of GPCRs has been linked to pH sensor function in a number of tissues and organs, including bone (36, 37), developing blood vessels and endothelial cells (106), airway smooth muscle cells (79), kidney (23, 88), and to pathological conditions such as cancer (17, 22).
mice exhibit only mild metabolic acidosis. In addition, GPR4 deletion also impairs the expression of CaSR in the kidneys of metabolic acidosis. In the current study, we demonstrated that deletion of GPR4 alters the well-appreciated IC bicarbonate transporters in ICs. In the current study, we dem-

In the mouse collecting duct, three types of ICs have been described: acid-secreting A-ICs, bicarbonate-secreting B-ICs, and non-A-non-B-ICs (60, 61, 78, 91). A-ICs express proton pumps on the apical membrane (8, 97, 98) and a CI-/HCO₃⁻ exchanger (AE1), encoded by Slc4a1 on the basolateral side (81, 96). In contrast, B-ICs express pendrin, encoded by Slc26a4, on the apical membrane (70, 100), and an H⁺-ATPase in the cytoplasm and on the basolateral membrane (8, 9, 97). Non-A-non-B-ICs express both H⁺-ATPase and pendrin on the apical side and may participate in acid-base regulation and chloride reabsorption (60, 78, 89, 91, 95).

Since ICs mediate net acid excretion by the collecting duct, the aim of the current study was to determine the effect of deletion of GPR4 on the expression and activity of proton and bicarbonate transporters in ICs. In the current study, we demon-

METHODS

Animals. GPR4⁺/− mice were a gift from the laboratory of Dr. Owen Witte (Univ. of California Howard Hughes Medical Institute, Los Angeles, CA) and Dr. Li Yang, ECU (106). We established a colony at the Animal Facility of the Veterans Affairs (VA) Medical Center in Cincinnati, OH, and Wake Forest School of Medicine, Winston-Salem, NC, and used published protocols (106) for genotyping. All animal procedures employed in the study were approved by the Institutional Animal Care and Use Committee of the Cincinnati VA Medical Center and Wake Forest School of Medicine. Mice were fed a standard laboratory mouse diet and had free access to tap water. Acid loading was performed by adding 280 mM NH₄Cl with 5% sucrose to the drinking water for 4 days (88). The control group drank tap water. Blood-gas, electrolyte, and urine analyses were also performed to verify appropriate acid loading. Blood samples were obtained in the same manner as in our previous studies (88) from a small nick of the tail artery in nonanesthetized mice; the samples were immediately read on the IRMA TruPoint Blood Analysis System (International Technidyne, Edison, NJ), which measures blood pH, PCO₂, PO₂, and calculates blood bicarbonate using the Henderson-Hasselbach equation. Mice were euthanized with pentobarbital sodium, and kidneys were harvested for expression and functional studies.

RNA isolation and quantitative real-time RT-PCR. Total cellular RNA was extracted from the mouse kidneys with an RNeasy Mini Kit (Qiagen, Germantown, MD) 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. Quantitative analysis of total RNA was done with a NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, DE). One microgram of total RNA was reverse-transcribed to the first-strand cDNA with oligo (dT)₁₂₋₁₈ primer using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Real-time RT-PCR was run in triplicate and carried out on the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). The primers for β-actin, AE1, pendrin, the a₄ subunit of H⁺-ATPase (ATP6V0A4), aquaporin-2 (AQP2), and type 3 Na/H exchanger (NHE3) were designed according to the mRNA sequence of mouse β-actin (GenBank accession no. NM_007393), AE1 (GenBank accession no. AY296129), pendrin (GenBank accession no. NM_011867), a₄ subunit of H⁺-ATPase (GenBank accession no. NM_080467), AQP2 (GenBank accession no. NM_009699), and NHE3 (GenBank accession no. NM_001081060). The primer sequences were: β-actin: 5'-TTG CTG ACA AGG TGA AG-3' (sense), and 5'-CAG TGA GCC CAG GAT GAC GC-3' (antisense); AE1: 5'-GCT CCC ACA GAG CAA AC-3' (sense), and 5'-CTG CCT CCA CCC ATT GTA GT-3' (antisense); pendrin: 5'-TCA TTG CTT TGG GGA TAA AG-3' (sense), 5'-GGC AAC CAT CAC AAT AG-3' (antisense); a₄ subunit of H⁺-ATPase: 5'-GAA CAC GGA CGT GGA ATA CT-3' (sense), and 5'-TTG AAC CCA GGG TCC AAA TC-3' (antisense); AQP2: 5'-TTG CCA TGT CTC CCT TT-3' (sense), and 5'-TTG TGG AGA GGA TCA TGA GT-3' (antisense); and NHE3: 5'-TATT GTC CGG CTT CCT GT-3' (sense), 5'-GCT AGA TGT TCC CTT GC-3' (antisense). The PCR product for β-actin is a size of 122 bp, for AE1 142 bp, for pendrin 149 bp, for a₄ subunit of H⁺-ATPase 129 bp, for AQP2 135 bp, and for NHE3 191 bp. We routinely used β-actin as a housekeeping gene and a control for sampling errors. Quantitative real-time RT-PCR consisted of 1 µl of first-strand cDNA, 0.1 µM primers, 1× PCR buffer, 5.0 mM Mg²⁺, 200 µM dNTP mix, 1 unit of Platinum Tag DNA polymerase (Invitrogen), and 0.25× SYBR Green I Nucleic Acid Gel Stain (Molecular Probes, Invitrogen, Eugene, OR) in a total volume of 25 µl. After initial denaturation at 95°C for 10 min, 40 cycles of PCR were performed with denaturation at 95°C for 10 s, annealing at 64°C for 15 s, and extension at 72°C for 35 s. The absolute mRNA levels in each sample were calculated based on a standard curve. The standard curve was developed by using serial dilutions of known amounts of specific templates against corresponding cycle threshold values. To normalize gene expression, we then calculated the ratio of a specific gene’s mRNA levels over β-actin in each sample. We verified the specificity of PCR products for β-actin, AE1, pendrin, AQP2, a₄ subunit of H⁺-ATPase, and NHE3 by examining the melting curve, electrophoresis, and sequencing of the real-time RT-PCR products.

Nephron segment RNA isolation and quantitative RT-PCR. These experiments were performed according to Schreiner et al. (80). In short, cortical collecting ducts (CCDs) and thick ascending limbs were hand dissected from the kidneys of GPR4⁺/+ and −/− mice before and after acid loading. The length of each nephron segment was measured and collected in a 0.5-mL tube with <5 µl of dissection solution and 50 µl of RNAlater RNA stabilization reagent (Qiagen) and stored at −80°C until the time of RNA isolation. Total RNA was
isolated from each nephron segment with the RNasey Mini Kit (Qiagen) according to the manufacturer’s protocol without using homogenization to limit loss of sample. DNase I (Qiagen) was used to digest genomic DNA from the nephron segments during RNA isolation and purification. The purified RNA sample was eluted in 20 μl of water, and 10 μl of total RNA from each nephron segment was reverse-transcribed to the first-strand cDNA with oligo (dT)2-18 primer using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time RT-PCR was performed in triplicate for each sample and carried out on the 7500 Real Time PCR System (Applied Biosystems). One microliter of first-strand cDNA from each sample was used to perform each real-time PCR (see above). We used the same primers for mouse β-actin as described above as a control for sampling errors. The primers for mouse CaSR were based on the mRNA sequence of mouse CaSR (GenBank accession no. NM_013803), and the primer sequences were as follows: 5′-GGC ATG GTT TGG CTA CTG TT-3′ (sense), 5′-TCG GAA TCC ACG GAA GTT AT-3′ (antisense). The transcript of PCR products for CaSR consisted of 205 bp. The specificity of PCR products for CaSR was verified by examining the melting curve, electrophoresis, and sequencing of the real-time RT-PCR products. To normalize gene expression, we calculated the ratio of CaSR mRNA levels over β-actin in each sample, and then divided the ratio by the tubule length for each sample.

**Immunohistochemistry.** Mice were euthanized with a pentobarbital sodium overdose. The kidneys were immediately perfused with 0.01 M PBS (pH 7.4), then with periodate-lysine-2% paraformaldehyde (PLP) fixative for 10 min. The kidneys were removed and postfixed in the same fixative at 4°C for 48 h, then embedded in paraffin. Four-micrometer-thick sections were cut and used for indirect immunofluorescence.

**Antibodies.** AE1: affinity-purified rabbit polyclonal antibody (AB3000P, Chemicon, Temecula, CA). Pendrin: affinity-purified rabbit polyclonal antibody (sc-50346, Santa Cruz Biotechnology, Santa Cruz, CA). H1-ATPase B1 subunit: affinity-purified goat polyclonal antibody (sc-21206, Santa Cruz Biotechnology).

H1-ATPase a4 subunit: affinity-purified rabbit polyclonal antibody was a gift from Dr. Fiona Karet (Cambridge, UK).

**RhoGAP family.** By immunoprotein, amniotic fluid transporter, (Rhbg): affinity-purified rabbit polyclonal antibody was a gift from Dr. David Weiner (Univ. of Florida, Gainesville, FL). We used Rhbg labeling as an additional criterion for identification of ICs subtypes. As reported previously by Verlander and Weiner (48, 99), the non-A-non-B-ICs, unlike B-ICs, express the ammonium transporter Rhbg on the basolateral side; therefore, Rhbg labeling greatly facilitates the distinction between B- and non-A-non-B-IC subtypes.

AQP2: affinity-purified rabbit polyclonal antibody was purchased from Alomone Labs (Jerusalem, Israel).

NHE3: rabbit polyclonal antibody previously characterized (69).

**Immunofluorescent labeling.** All sections were deparaffinized in xylene and then rehydrated in decreasing concentrations of ethanol and 0.01 M PBS. For AE1 or NHE3 single labeling, the sections were first blocked with blocking buffer (0.2% Triton X-100 and 5% normal goat serum in PBS) for 30 min, and then incubated overnight with AE1 or NHE3 antibodies diluted 1:100 in blocking buffer at 4°C. The sections were subsequently incubated with Alexa Fluor 488 or 594-labeled goat anti-rabbit IgG (Invitrogen, Eugene, OR) at 1:400 dilution for 1 h at room temperature. For double-labeling of AE1 and the B1 subunit of H1-ATPase, 1% SDS in PBS was used for antigen retrieval per published protocols (13). The sections were incubated with AE1 antibodies (1:100 dilution) at 4°C overnight and subsequently washed with high-salt PBS containing 2.7% NaCl, followed by PBS, and incubated with Alexa Fluor 594-labeled goat anti-rabbit IgG (Invitrogen) at 1:400 dilution for 1 h at room temperature. After extensive washing, the sections were incubated with the B1 subunit of V-ATPase antibody prelabeled with a Zenon Alexa Fluor 488 goat IgG-labeling kit (Invitrogen) at 1:40 dilution for 1.5 h at room temperature. The sections were then washed and postfixed with 4% paraformaldehyde for 15 min to stabilize Zenon labeling, and subsequently incubated with the nuclear dye Hoechst 33342 (Invitrogen) at 1:10,000 dilution for 20 min, washed, and mounted in ProLong Gold antifade reagent (Invitrogen). For double-labeling of the a4 subunit of H1-ATPase and AQP2, we used the same protocol as above, except that the a4 subunit of H1-ATPase antibody was used at 1:2,000 dilution and the AQP2 antibody was prelabeled with Zenon Alexa Fluor 488 rabbit IgG-labeling kit (Invitrogen) at 1:2,000 dilution. For pendrin and Rhbg double labeling, the sections were blocked and incubated with the pendrin antibody at 1:20 dilution at 4°C overnight, followed by an amplification step to enhance the relatively weak blue dye fluorescence: biotinylated goat anti-rabbit secondary antibody (at 1:100) was incubated for 30 min, followed by avidin D conjugated with the blue dye 7-amino-4-methylcoumarin-3-acetic acid (ACMA; Vector Laboratories, Burlingame, CA) at 1:200 dilution. Sections were subsequently incubated with the Rhbg antibody prelabeled with Zenon Alexa Fluor 594 rabbit IgG-labeling kit (Invitrogen) at 1:50 dilution, washed, postfixed, and mounted in ProLong Gold antifade reagent.

Labeled kidney sections from GPR4+/+ and GPR4−−/− at baseline (n = 3 each) and after acid loading (n = 3 each) were used to determine distribution of IC and principal cell types: 10 images/kidney section were acquired at ×400 magnification, each encompassing an area of 100 × 100 μm and including the cortical labyrinth, medullary rays, and corresponding tubule segments [connecting tubules (CNTs), CCDs]. We spaced 10 squares of 100 × 100 μm evenly across the section to cover most of the cortex or outer or inner medulla, since different zones were counted separately. Only nucleated cells were counted, and 800–1,500 cells/section were analyzed. Each section was examined by three persons in a blinded manner; the results were compared, checked for consistency, and then averaged. The results are expressed as numbers of cells/100 × 100 μm. In addition, we counted ICs and principal cells in the connecting segments and initial collecting ducts, cortical and outer and inner medullary collecting ducts (OMCDs and IMCDs), expressing cell numbers per specific nephron segment. The purpose of the additional analysis was to determine whether deletion of GPR4 or NH4Cl loading affected the ratio of ICs and principal cells in those mice. As ratios of the ICs to principal cells are different in different nephron segments, we expressed the results in numbers of cells per nephron segment. Nephron segments were distinguished based on the position in the cortical labyrinth or medullary rays and the differential expression of the water channel AQP2: initial collecting tubules and connecting segments are situated in the cortical labyrinth, whereas the cortical collecting ducts are situated in the cortical part of medullary rays, and OMCDs and IMCDs in the respective areas of the medulla. Differential expression of AQP2 helps to distinguish distal convoluted tubules, which do not label for AQP2, from CNTs and initial collecting tubules, which label weakly for AQP2, and CCDs, which typically exhibit strong AQP2 labeling (54). This difference is apparent as long as the image acquisition parameters (camera gain, exposure time, black level, and binning) are kept constant. Only nucleated cells were counted, which helped eliminate the ambiguity of cell boundaries, polarity of labeling, overlapping cells, etc. On average, we analyzed 1,500–2,000 cells/kidney section.

**In vitro microperfusion.** Animals were euthanized by a pentobarbital sodium overdose, and kidneys were quickly removed and placed in ice-cold dissection medium (solution 1, Table. 1) (83) Thin coronal slices (~1 mm) were obtained and transferred to the cooled dissection chamber. CCDs were dissected from medullary rays typically 0.3–0.5 mm in length. CCDs were distinguished from proximal straight tubules by the smaller diameter (about 2/3 of the diameter of the proximal straight tubules) and somewhat turbid appearance; distinct from the typical “ground-glass” appearance of proximal straight tubules or slightly shiny appearance of thick ascending limbs. Dis-
Table 1. Chemical composition of solutions used for microperfusion

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>Solution 2</th>
<th>Solution 3</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>140</td>
<td>115</td>
</tr>
<tr>
<td>Na-glucuronate</td>
<td>0</td>
<td>115</td>
</tr>
<tr>
<td>NaHCO₃</td>
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<td>25</td>
</tr>
<tr>
<td>K₂HPO₄</td>
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<td>2.5</td>
</tr>
<tr>
<td>MgSO₄</td>
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<td>1.2</td>
</tr>
<tr>
<td>d-glucose</td>
<td>5.5</td>
<td>5.5</td>
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<tr>
<td>Na-citrate</td>
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<td>1</td>
</tr>
<tr>
<td>Na-lactate</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>l-alanine</td>
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<td>6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ca-acetate</td>
<td>0</td>
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Values are expressed in mM. Solutions containing bicarbonate were bubbled with 95% O₂-5% CO₂, so that pH was 7.4 at 37°C, osmolality was 290 ± 3 mosmol/kgH₂O. Solution without bicarbonate was gassed with 100% O₂. Solutions containing gluconate salts had 4 mM Ca-acetate to account for complexing of Ca²⁺.

Table 2. Immunoblotting. Kidney tissue was homogenized in ice-cold lysis buffer containing 20 mM Tris, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS, 1% of protease inhibitor cocktail solution (Sigma), and 1% of phosphatase inhibitor cocktail II solution (Sigma). Lysates were cleared by centrifugation at 10,000 × g for 10 min at 4°C. The concentration of total protein from the supernatant was determined by using a BCA Protein Assay Kit (Pierce, Rockford, IL). One hundred micrograms of total protein from each sample was run on 8–16% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) and was transferred to nitrocellulose membranes. The membranes were first blocked with blocking buffer containing 3% nonfat dry milk in TBS-T (20 mM Tris, pH 7.6, 137 mM sodium chloride, 0.1% Tween 20) at 4°C overnight, then incubated in mouse monoclonal β-actin antibody (ab8226, Abcam, Cambridge, MA) at 1:2,500 dilution or 1:200 diluted polyclonal rabbit CaSR antibody (ACR-004, Alomone Labs) in blocking buffer separately for 2 h at room temperature. After washing, the membranes were incubated for 1 h at room temperature with peroxidase-labeled affinity-purified mouse or rabbit secondary antibodies (KPL, Gaithersburg, MD) diluted 1:5,000 in blocking buffer. Protein bands on the membranes were visualized with TMB Membrane Peroxidase Substrate (KPL). Images were taken by computerized scanning densitometry, Foto/Eclipse TM (FOTO DYNE, Hartland, WI), and ImageQuant (GE Healthcare, Piscataway, NJ) software was used for the densitometric analysis.

Statistical analysis. Data are presented as means ± SE. Comparisons were done using Microsoft Excel and GraphPad Prism software packages with a two-tailed Student’s t-test. *P < 0.05 was considered statistically significant.

RESULTS

Acid-base status. GPR4⁺/+ mice adapted quickly to a high net acid load with minimal decrease in serum bicarbonate, but a substantial fall in urine pH, which was more pronounced in GPR4⁻⁻ than in GPR4⁺/+ (Fig. 1). After 4 days of NH₄Cl loading, blood bicarbonate in GPR4⁺/+ was 23 ± 1.1 mM/l, P<0.02. Blood bicarbonate in GPR4⁻⁻ after acid loading was 21.5 ± 0.4 mM/l (P = 0.08 vs. +/+), P<0.02. Blood bicarbonate in GPR4⁻⁻ after acid loading was 21.5 ± 0.4 mM/l (P = 0.08 vs. +/+), and blood pH was 7.34 ± 0.02 (P = 0.03 vs. +/+). Therefore, GPR4⁻⁻ mice exhibited slightly lower blood pH after acid loading and a less pronounced drop in urine pH compared with GPR4⁺⁺, consistent with an acidification defect in the kidney.

Defective response of kAE1 mRNA to acid challenge in GPR4⁻⁻ mice. Using quantitative real-time RT-PCR, we assessed the expression of representative acid-base transporters in ICs: AE1, the a4 subunit of H⁺-ATPase, and pendrin in kidneys harvested from GPR4⁺/+ or −/− mice (Table 2).

The cell population of the collecting duct is heterogeneous, and ICs are interspersed between principal cells (78, 81), so we included the water channel AQP2 in this analysis as a transport protein originating solely from the principal cells (65). Moreover, the expression of AQP2 in the rat kidney is known to increase in response to acid loading, consistent with the increase in urine osmolality that accompanies the adaptive increase in acid excretion by rodent species (5),

BCECF-AM is introduced from the luminal side of the tubule (10, 22), the bath solution was switched to a chloride-free solution 2 (Table 1, solution 2), and the bath solution was switched to a chloride-free solution 3 (Table 1, solution 3) to distinguish between the IC types (70, 83). Removal of bath Cl⁻ causes reversible alkalization of A-ICs due to blocking and/or reversing the basolateral Cl⁻/HCO₃⁻ exchanger. Removal of luminal Cl⁻ blocks/reverses the apical Cl⁻/HCO₃⁻ exchanger in B-ICs only causing a reversible cell alkalization (reversible increase in pH); pHₕ of A-ICs does not change because A-ICs do not possess apical Cl⁻/HCO₃⁻ exchange or Cl⁻ conductance (70, 81, 83, 102).

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After acid loading, pendrin mRNA expression decreased ~50%, \( P = 0.06 \) in GPR4+/+ and 29%, \( P = 0.2 \) in GPR4−/− (Table 2; Fig. 2B). \( \Delta \text{H}^+\text{ATPase} \) mRNA abundance after acid loading was comparable in GPR4+/+ and GPR4−/− (Table 2).

The response of AQP2 mRNA to acid loading was highly significant in GPR4+/+ (AQP2/β-actin mRNA ratio increased 77% from 2.6 × 10^{-2} ± 4 × 10^{-3} to 4.6 × 10^{-2} ± 3 × 10^{-3}, \( P = 0.005 \)), but was less pronounced in GPR4−/− (the ratio of AQP2/β-actin mRNA increased 62% from 2.1 × 10^{-2} ± 4 × 10^{-3} to 3.4 × 10^{-2} ± 6 × 10^{-3}, \( P = 0.08 \)).

Deletion of GPR4 alters cellular distribution of the collecting duct. A number of studies reported that NH₄Cl loading alters the cellular distribution in the collecting duct in rabbits, rats, and mice, such that relative numbers of A-ICs increase and numbers of B-ICs decrease, as part of an adaptive cellular response to acidosis (33, 72, 82, 103). We, therefore, used indirect immunofluorescence to label \( \Delta \text{H}^+\text{-ATPase} \), pendrin, AE1, and AQP2 in the kidney sections of GPR4+/+ and GPR4−/− mice before and after NH₄Cl loading to identify different cell types. All pendrin-labeled sections were double-labeled with Rbgb to aid in identification of non-A-non-B-ICs (see METHODS for further details). We also double-labeled \( \Delta \text{H}^+\text{-ATPase} \) and AE1 and \( \Delta \text{H}^+\text{-ATPase} \) and AQP2, to assess overall numbers of ICs and relative ratio of ICs and principal cells.

Figure 4 shows AE1-labeled kidney sections harvested from GPR4+/+ (A) and illustrates more prominent AE1 labeling in GPR4+/+ after acid loading (Fig. 4A, i vs. ii). In Fig. 4B, however, AE1-labeled kidney sections harvested from GPR4−/− show comparable labeling before and after acid loading (Fig. 4B, i vs. ii). Figures 4, C and D, illustrates changes in pendrin labeling observed after acid loading, which are similar in GPR4+/+ and −/− mice, essentially identical to what has been observed in rabbits, with fewer numbers of cells per tubule/section and shorter “cell caps” caused by endocytosis of this bicarbonate-secreting transporter from the cell surface (71, 83).

Double-labeling of kidney sections harvested from GPR4+/+ and −/− with antibodies against \( \Delta \text{H}^+\text{-ATPase} \) (all ICs) and AQP2 (principal cells) as presented in Fig. 4E illustrates that the ratio of...
ICs and principal cells is similar in GPR4+/+ and −/− at baseline (Fig. 4E, i vs. iii), and no different after NH4Cl loading (Fig. 4E, ii vs. iv, summarized in Fig. 4F).

Analysis of AE1 and pendrin cell numbers is summarized in Table 3; after 4 days of NH4Cl loading, GPR4+/+ mice increased the number of AE1-labeled, acid-secreting A-ICs (Table 3, rows 1 and 2, Fig. 4, A and G). In contrast, the numbers of non-A-ICs, including B-ICs and non-A-non-B-ICs, decreased (Table 3, rows 1 and 2, Fig. 4, C and G). This response (Fig. 4G) was remarkably similar to the response reported recently in the rabbit and rat kidney (33, 72, 103). Nevertheless, we did not observe this response by A-ICs in acid-loaded GPR4−/− (Table 3, rows 4 and 5, Fig. 4, B and G).

At baseline, however, in GPR4−/− the number of A-ICs was higher, while numbers of non-A-non-B- and B-ICs were lower compared with GPR4+/+ (Table 3, rows 7 and 8), compatible with mild chronic acidosis present in GPR4−/−.

We also examined the ratio of ICs to principal cells in GPR4+/+ and −/−, as it typically differs among specific segments of the collecting duct (54). We, therefore, ascertained the numbers of principal and ICs, normalized to the number of total cells per section. The numbers were obtained from specific nephron segments from kidney sections double-labeled for AQP2 and H+;ATPase (see METHODS for details on identification of specific nephron segments) (54). There was no difference in the ratios of ICs to principal cells between GPR4+/+ and −/− either at baseline or after acid loading (Fig. 4, E and F).

Analysis of activity of Cl−/HCO3− exchangers in ICs indicates that AE1 in GPR4−/− mice responds less robustly to acid challenge compared with GPR4+/+ mice. When exposed to NH4Cl loading, acid-base transport proteins in ICs are controlled by phosphorylation and trafficking in addition to changes in expression levels (1–3, 10, 22, 47, 71, 78). Changes in expression and/or phosphorylation and trafficking are re-

Fig. 2. Response of kidney anion exchanger 1 (kAE1) mRNA to acid loading in GPR4−/− mice is defective. Quantitative, real-time RT-PCR was used to assess the response of AE1 (A) and pendrin (B) mRNA expression in the kidney tissue harvested from GPR4+/+ (n = 5) or −/− mice (n = 5) before and after 4 days of NH4Cl loading. We assessed AE1 and pendrin expression as representative Cl−/HCO3− exchangers in A- and B- (and non-A-non-B) intercalated cells (ICs), respectively. Acid loading increased kAE1 mRNA and decreased pendrin mRNA. Percent increase in kAE1 mRNA was significantly lower in GPR4−/− compared with GPR4+/+ (A). A trend for smaller change in pendrin mRNA in GPR4−/−, however, was not significantly different between GPR4+/+ and −/− (B).

Fig. 3. kAE1 mRNA response to NH4Cl loading in individual GPR4−/− and wild-type animals. The ratio of kAE1 mRNA vs. β-actin mRNA from individual animals is plotted at baseline and after 4 days of NH4Cl loading in GPR4+/+ (A) and GPR4−/− (B) illustrating the difference in mRNA response to acid loading between GPR4+/+ and −/−. The numbers on the x-axis indicate the number of an individual animal, and the graph shows the associated ratio values for each animal.

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flected by changes in the activity of the transporters. Therefore, the activity of the transporters was measured in individual ICs as the rate of Cl⁻/HCO₃⁻ exchange. The rate of Cl⁻/HCO₃⁻ exchange was determined as a function of the slope of change in pHᵢ upon removal of Cl⁻ from the perfusate or bath in perfused CCDs isolated from GPR4⁺/⁺ or −/− mice before or after NH₄Cl loading.

The rate of basolateral Cl⁻/HCO₃⁻ exchange increased significantly in GPR4⁺/⁺ mice from 0.28 ± 0.06 pH units/min (n = 11 cells; N = 4 animals) at baseline to 0.45 ± 0.05 pH units/min after NH₄Cl loading.

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A

B

C

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Expression of NHE3 in GPR4+/+ vs. −/−. We examined the expression of NHE3 in kidneys of GPR4+/+ and GPR4−/− because GPR4−/− exhibit relatively mild acidosis and the response of the sodium hydrogen exchanger in the proximal tubule and thick ascending limb (NHE3) to chronic metabolic acidosis would be expected to remain intact in GPR4−/−. In our previous studies, we noted that GPR4 mRNA expression in the proximal tubule was extremely low, and we did not find evidence of bicarbonate wasting either at baseline (88) or after bicarbonate loading (data not shown); therefore, direct control of NHE3 by GPR4 is unlikely. This, however, did not preclude the expected adaptive response of NHE3 to metabolic acidosis in GPR4−/−. The expression of NHE3 mRNA was comparable in GPR4+/+ vs. −/− at baseline: the NHE3/β-actin mRNA ratio was 4.0 × 10−4 ± 2.1 × 10−4 in GPR4+/+ vs. 4.4 × 10−4 ± 2.0 × 10−4 in GPR4−/−, \( P = 0.9, n = 4 \) of each. Similarly, after NH4Cl loading, the NHE3/β-actin mRNA ratio was 4.4 × 10−4 ± 1.4 × 10−4 in GPR4+/+ vs. 4.7 × 10−4 ± 1.7 × 10−4 in GPR4−/−, \( P = 0.9, n = 4 \) of each. Previous reports indicated that the NHE3 response to acid loading did not involve changes in mRNA, but rather increased protein abundance and trafficking to the brush-border membrane of the proximal tubules and thick ascending limbs, which increased gradually with prolonged acid challenge and became significant after 7 days of acid loading (4). We therefore also examined NHE3 immunofluorescent labeling. As expected, NHE3 labeling appeared more prominent and more concentrated at the brush border in the proximal tubules of GPR4−/− than GPR4+/+ at baseline (Fig. 7). Immunofluorescent data also indicated increased labeling after acid loading in both GPR4+/+ and GPR4−/−, suggesting a preserved response of NHE3 to acidosis in GPR4. It appears that NHE3 labeling is more prominent in the luminal membranes of proximal tubule cells and in thick ascending limbs in GPR4−/− at baseline, and after acid loading (Fig. 7), suggesting that an adaptive response of NHE3 to acidosis might have moderated the acidification defect present in GPR4−/−.

Deletion of GPR4 upregulates expression of the CaSR in the kidney. If a receptor functions as an acid sensor in the kidney, we would expect it to mediate the adaptive response of the kidney to an acid challenge. This response is blunted in GPR4−/− mice, but not eliminated. We therefore considered the possibility that redundancy of other sensors might offer a plausible explanation. We had previously examined the expression of other proton receptors (OGR1 and TDAG8) in the kidney and found no change in their expression in GPR4+/+ vs. −/−, suggesting that OGR1 and TDAG8 do not compensate for the loss of GPR4 in the kidney (88).

The CaSR is another pH-sensitive G protein-coupled receptor, which has been implicated in acid-base regulation and is widely expressed in the kidney, including ICS (28, 73, 75). We therefore examined CaSR expression in kidneys of GPR4+/+ vs. −/− to explore a possibility that CaSR may compensate for the loss of GPR4. Quantitative real-time RT-PCR analysis demonstrated that deletion of GPR4 doubled the expression of CaSR mRNA in the kidney tissue. The number of CaSR mRNA copies was normalized to the expression of β-actin. The ratio of CaSR mRNA to β-actin was 1 × 10−3 ± 0.3 × 10−3 in GPR4+/+ compared with 2 × 10−3 ± 0.3 × 10−3 in GPR4−/− mice, a 100% increase in GPR4−/−, \( P = 0.04 \) (Fig. 8A). Analysis of protein expression by immunoblotting indicated that CaSR expression was similarly increased in GPR4−/− compared with GPR4+/+ mice (Fig. 8, B and C).

Fig. 4. Cellular response to acid loading in GPR4+/+ and −/− mice. To identify different cell types in the collecting ducts, we used indirect immunofluorescence to label H−-ATPase (ICs), pendrin (B-ICs and non-A-non-B-ICs), AE1 (A-ICs), and aquaporin-2 (AQ2P; principal cells; PCs) on 4-μm paraffin kidney sections. Kidneys were harvested from GPR4+/+ and GPR4−/− mice before (\( n = 3 \) of each) and after NH4Cl loading (\( n = 3 \) of each). All pendrin-labeled sections were double-labeled with Rhbg to aid in identification of non-A-non-B-ICs (99). AQ2P-labeled sections were also double-labeled with H−-ATPase, so that ICS could be distinguished from the principal cells. Ten images per kidney section were acquired at ×400 magnification; each image encompassed an area of 100 μm², including the cortical labyrinth, medullary rays, and corresponding tubule segments (connecting tubules (CNTs), cortical collecting ducts (CCDs), outer medullary collecting ducts (OMCDs)) so that different distribution of neprhon segments in each of these structures was accounted for. Only nucleated cells were counted, and 800-1,500 cells/slide were analyzed. The results are expressed as numbers of cells/100 μm² (scale bar = 10 μm). A: representative images of AE1 labeling of the acid-secreting A-ICs on the kidney sections of GPR4+/+ mice before (i) and after NH4Cl loading (ii). B: representative images of AE1-labeled acid-secreting A-ICs on kidney sections from GPR4−/− mice harvested before (i) and after NH4Cl loading (ii). C: representative images of pendrin (blue)- and Rhbg (red)-labeled bicarbonate-secreting B-ICs and non-A- non-B-ICs on kidney sections harvested from GPR4+/+ mice before (i) and after NH4Cl loading (ii). D: representative images of pendrin (blue)- and Rhbg (red)-labeled bicarbonate-secreting B-ICs and non-A- non-B-ICs on kidney sections harvested from GPR4−/− mice before (i) and after NH4Cl loading (ii and iv). Arrows point to several B-ICs. E: representative images of H−-ATPase (red)-labeled ICs and AQ2P (green)-labeled principal cells on kidney sections harvested from GPR4+/+ (i and ii) and GPR4−/− (iii and iv) mice before and after acid loading. F: ratio of principal cells to total cells in different collecting duct segments before and after NH4Cl loading is comparable between GPR4+/+ and GPR4−/−. [Similarly, ratio of ICs to total cells in different segments of the collecting duct before and after NH4Cl loading is comparable in GPR4+/+ vs. GPR4−/− (data not shown)]. G: summary of the differences in the number of A-ICs and B-ICs and non-A-non-B-ICs in GPR4+/+ (top) and GPR4−/− (bottom) before and after NH4Cl loading.
It is therefore plausible that increased expression of CaSR in the kidneys of GPR4−/− may partially compensate for the loss of GPR4. This finding is compatible with the view that CaSR may play a role in acid-base regulation (28, 73, 77). Moreover, we also found that 4 days of acid loading significantly increased expression of CaSR mRNA (Fig. 9A) and protein expression (Fig. 9, B and C) in wild-type mice, further supporting the notion that CaSR might play a role in response of the kidney to acid challenge.

Since CaSR is widely expressed in the kidney (76), we also wanted to determine whether the robust upregulation of CaSR in response to the increase in oral acid load involves the collecting duct. We therefore determined CaSR mRNA expression in CCDs. The data indicated a very similar response as the
24 h of acid loading, \( P = 0.9 \). CaSR expression at baseline, however, increased significantly in GPR4\(^{-/-}\) vs. \(+/-\): \(4.89 \times 10^{-3} \pm 1.2 \times 10^{-3} (n = 3)\) vs. \(1.98 \times 10^{-3} \pm 0.4 \times 10^{-3} (n = 4)\), respectively, \( P = 0.04 \) (Fig. 10).

**DISCUSSION**

In the current study, we sought to account more completely and mechanistically for the observation by our laboratory of a mild spontaneous non-gap metabolic acidosis in GPR4\(^{-/-}\)-mice (88). The approach in the current study was to document alterations in acid-base transporter expression and activity in ICs of the kidney collecting duct that might explain these findings. The results reveal a heterogeneous response in the distribution of cell type in the collecting ducts of GPR4\(^{-/-}\) vs. GPR4\(+/-\). We observed 1) an increase in the number of A-ICs as well as a reduction in the numbers of B-ICs and non-A-non-B cells in GPR4\(^{-/-}\) vs. GPR4\(+/-\) at baseline; 2) the distribution of cell type in the collecting ducts of GPR4\(^{-/-}\) did not change in response to acid loading as was observed in GPR4\(+/-\); 3) for AE1 mRNA expression and its Cl\(^{-}/\)HCO\(_3\)\(^{-}\) activity, the response to an acid challenge in GPR4\(^{-/-}\) was only 50 and 19%, of the corresponding response in GPR4\(+/-\); 4) NHE3 labeling increased as expected with acidosis in the proximal tubules and thick ascending limbs of GPR4\(^{-/-}\); 5) deletion of GPR4 nearly doubled mRNA and protein expression of CaSR in the kidney; and 6) acid loading per se increased the expression of CaSR in the kidney tissue and in the kidney collecting duct of wild-type mice.

Changes in the distribution of cell types in the collecting ducts from GPR4\(^{-/-}\) vs. \(+/-\) mice, as evidenced by significantly higher numbers of A-ICs and lower numbers of B-ICs and non-A-non-B-ICs, are comparable to the changes observed in acid-loaded GPR4\(+/-\). Furthermore, these findings are remarkably similar to recent reports regarding the heterogeneity of the distribution of cell types in the collecting duct of acid-loaded rabbits and rats (33, 72, 103). Acid loading did not, however, further alter the distribution of cell type in the collecting ducts in GPR4\(^{-/-}\) mice.

A growing number of reports have documented changes in the distribution of the subtypes of ICs in mice, rats, and rabbits during acid loading (72, 82, 103), lithium intoxication (21, 92), acetazolamide administration (6), with kidney-specific hensin deletion (38), inactivation of notch signaling (46), or foxo1 transcription factor deficiency (11). Some of these changes are developmental, whereas others occur under certain conditions in adult animals. Whether the observed changes in the distribution of different cell types in the collecting ducts of GPR4\(^{-/-}\) originate from hypertrophy or involution of specific cell types (60) (that made them easier or more difficult to detect), interconversion of specific phenotypes (82, 83), or selective proliferation/apoptosis of cell subpopulations (33), was not addressed in this study. While each of these options has been supported by experimental evidence in other studies, technical challenges make this issue difficult to resolve unequivocally.

Consistent with our previous observation that GPR4\(^{-/-}\) mice do not increase urinary net acid excretion to levels observed in wild-type animals in response to an acid load (88), in the current study we found that both mRNA and activity of the basolateral Cl\(^{-}/\)HCO\(_3\)\(^{-}\) exchanger AE1 increased signifi-

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**Fig. 4—Continued**

one observed in the whole kidney tissue: CaSR mRNA (expressed as the CaSR/β actin ratio/1 mm of tubule length) was \(1.98 \times 10^{-3} \pm 0.4 \times 10^{-3}\) at baseline in tubules dissected from GPR4\(+/-\) \((n = 4)\) vs. \(4.04 \times 10^{-3} \pm 0.4 \times 10^{-3} (n = 3)\) after 24 h of acid loading, \( P = 0.04 \). CaSR expression at baseline, however, increased significantly in GPR4\(+/-\) vs. \(+/-\): \(4.89 \times 10^{-3} \pm 1.2 \times 10^{-3} (n = 3)\) vs. \(1.98 \times 10^{-3} \pm 0.4 \times 10^{-3} (n = 4)\), respectively, \( P = 0.04 \) (Fig. 10).
We did not observe such differences in the response (B-ICs and non-A-non-B-ICs), AE1 (A-ICs), and AQP2 (principal cells; PCs) on 4-μm paraffin kidney sections of GPR4/H9262/H11001/H11002 and GPR4/H11005. AQP2-labeled sections were also double-labeled with H cells. Ten images/kidney section were acquired at perfused kidney collecting ducts isolated from GPR4 mice. The Cl− exchange rate was measured as the rate of intracellular pH change upon the removal of the bath or luminal (perfusate) chloride, which elicited reversible intracellular alkalinization in A-ICs (after removal of bath chloride) or B-ICs (after removal of perfusate chloride) in the acid-loaded GPR4 mice, before and after NH4Cl loading. The Cl−/HCO3− exchange in A-ICs and B-ICs, respectively, before and after NH4Cl loading in GPR4+/+ and GPR4−/− mice before (n = 3 of each) and after NH4Cl loading (n = 3 of each) to identify different cell types. All pendrin-labeled sections were double-labeled with Rb + to aid in identification of non-A-non-B-ICs. AQP2-labeled sections were also double-labeled with H+−ATPase so that ICs could be distinguished from the principal cells. Ten images/kidney section were acquired at ×400 magnification; each image encompassed an area of 100 μm², including the cortical labyrinth, medullary rays, and corresponding tubule segments (connecting tubules, cortical collecting ducts, outer medullary collecting ducts) so that a different distribution of nephron segments in each of these structures was accounted for. Only nucleated cells were counted, and 800–1,500 cells/section were analyzed.

### Table 3. Changes in cellular profile of the kidney collecting duct elicited by acidosis with or without deletion of GPR4

<table>
<thead>
<tr>
<th></th>
<th>ICs</th>
<th>A-ICs</th>
<th>Non-A-ICs</th>
<th>B-ICs</th>
<th>Non-A-non-B-ICs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>26.5 ± 0.6</td>
<td>15.3 ± 0.2</td>
<td>11.2 ± 0.5</td>
<td>6.4 ± 0.25</td>
<td>4.8 ± 0.26</td>
</tr>
<tr>
<td><strong>Acid-loaded</strong></td>
<td>30.3 ± 0.9</td>
<td>23.7 ± 1.3</td>
<td>6.6 ± 0.6</td>
<td>3.9 ± 0.4</td>
<td>2.6 ± 0.21</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.02</td>
<td>0.003</td>
<td>0.004</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>27.1 ± 1</td>
<td>20.2 ± 1.1</td>
<td>6.8 ± 0.1</td>
<td>5.1 ± 0.35</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td><strong>Acid-loaded</strong></td>
<td>25.3 ± 1</td>
<td>20 ± 1.3</td>
<td>5.4 ± 0.6</td>
<td>3.6 ± 0.4</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td><em>P</em> value</td>
<td>0.3</td>
<td>0.9</td>
<td>0.1</td>
<td>0.04</td>
<td>0.8</td>
</tr>
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</tr>
<tr>
<td><strong>Baseline</strong></td>
<td>27.1 ± 1</td>
<td>20.2 ± 1.1</td>
<td>6.8 ± 0.08</td>
<td>5.1 ± 0.35</td>
<td>1.7 ± 0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as numbers of cells/100 μm². Indirect immunofluorescence was used to label H+−ATPase (intercalated cells; ICs), pendrin (B-ICs and non-A-non-B-ICs), AE1 (A-ICs), and AQP2 (principal cells; PCs) on 4-μm paraffin kidney sections of GPR4+/+ and GPR4−/− mice before and after NH4Cl loading (n = 3 of each) to identify different cell types. All pendrin-labeled sections were double-labeled with Rb + to aid in identification of non-A-non-B-ICs (99). AQP2-labeled sections were also double-labeled with H+−ATPase so that ICs could be distinguished from the principal cells. Ten images/kidney section were acquired at ×400 magnification; each image encompassed an area of 100 μm², including the cortical labyrinth, medullary rays, and corresponding tubule segments (connecting tubules, cortical collecting ducts, outer medullary collecting ducts) so that a different distribution of nephron segments in each of these structures was accounted for. Only nucleated cells were counted, and 800–1,500 cells/section were analyzed.

It seems unlikely to anticipate identification of a single or unique acid sensor in the kidney because current evidence suggests a complex network of kinases [e.g., Pyk2 adhesion kinase (34, 39, 58), ErbB1/2 tyrosine kinase (87), and AMPK (2)], enzymes [e.g., ammoniagenic enzymes (24, 25, 43) and soluble adenylyl cyclase (14, 64)], G protein-coupled receptors like GPR4 (23, 88) or OGR1 (63) or CaSR (28, 73), the insulin receptor-related receptor (InsR-RR) (68), ion channels [e.g., vATPase (2, 62)], which may all be involved in the response to a reduction in pH. Furthermore, multiple sensors may offer advantages based on different ranges of pH sensitivity, or cellular and subcellular localization. Importantly, it has not yet been established how these distinct subordinate signaling networks interact to detect fluctuations in proton and bicarbonate concentration and CO2 tension to elicit a sequence of adaptive changes in the expression, membrane trafficking, and phosphorylation of the major epithelial transport proteins involved in proton and bicarbonate secretion and reabsorption. It is presumed that such a sequence underlies the adaptive response of the kidney to daily changes in net dietary acid load or, in pathological situations, respiratory or metabolic acidosis, or alkalosis.

Our interest in elucidating a role for an acid sensor in the kidney has been based on the relatively high expression of the proton-sensing receptor GPR4 in the kidney and the responses of other members of the proton-sensing family of GPCR to a decrease in pH in various tissues [e.g., OGR1 in bone (36, 37, 55) and lung], TDAG8 in lung carcinoma (50), and GPR4 in blood vessels (106). We found that the deletion of GPR4 impairs net acid excretion in the kidney, and therefore a spontaneous non-gap metabolic acidosis develops (88). GPR4−/− mice do not respond to an increase in exogenous acid load as efficiently as GPR4+/+, as demonstrated by...
Fig. 6. Representative tracings illustrating the rates of basolateral and apical Cl⁻/HCO₃⁻ exchange in A-ICs and B-ICs before and after NH₄Cl loading in GPR4+/+ and GPR4−/− mice. The Cl⁻/HCO₃⁻ exchange rate was measured as the rate of intracellular pH change upon the removal of Cl⁻ in the perfusate, which elicited reversible intracellular alkalinization in A-ICs (after removal of bath Cl⁻) or B-ICs (after removal of luminal Cl⁻) in the perfused kidney collecting ducts isolated from GPR4+/+ or GPR4−/− mice, before and after NH₄Cl loading. Additionally, removal of Cl⁻ from the bath elicits reversible acidification in B-ICs because of the presence of basolateral Cl⁻ channels: Cl⁻ removal increases the cell-to-bath concentration gradient for basolateral Cl⁻ exit from B-ICs, which lowers intracellular Cl⁻ concentration, and lower intracellular Cl⁻ in turn increases the driving force for the luminal Cl⁻/HCO₃⁻ exchange, facilitates luminal HCO₃⁻ exit, and reversibly lowers intracellular pH. As is apparent from the tracings, luminal Cl⁻ removal does not produce reversible intracellular acidification in A-ICs, presumably because of the lack of physiologically active apical Cl⁻ channels. Tracings reflect the summary provided in Fig. 5, showing the blunted response to acid challenge in GPR4−/− vs. +/+: the rate of apical Cl⁻/HCO₃⁻ exchange in A-IC sin GPR4+/+ was 0.26 pH units/min at baseline (A) and increased to 0.45 pH units/min after NH₄Cl loading (B), whereas in GPR4−/− the rate of apical Cl⁻/HCO₃⁻ exchange in A-ICs was 0.3 pH units/min at baseline (C) and only slightly increased to 0.35 pH units/min after NH₄Cl loading (D). Although we observed higher variability in the response of B-ICs in GPR4−/−, the response of B-ICs in GPR4+/+ and GPR4−/− was similar: the rate of apical Cl⁻/HCO₃⁻ exchange in B-ICs in GPR4+/+ was 0.16 pH units/min at baseline (E) and decreased to 0.11 pH units/min after NH₄Cl loading (F), whereas in GPR4−/− the rate of apical Cl⁻/HCO₃⁻ exchange in B-ICs was 0.2 pH units/min at baseline (G) and decreased to 0.1 pH units/min after NH₄Cl loading (H).
significantly lower urinary NAE after chronic NH₄Cl administration (88). In addition, in the present study we demonstrate that the expected increase in AE1 abundance with acidosis was also impaired in GPR4⁻/⁻ mice. In addition, the previously observed reduction in the adaptive increase in ammonium excretion with acidosis (88) should be addressed in future studies examining ammonia-generating enzymes and transport proteins. Data from this study suggest that ammonium transport in the proximal tubule is likely preserved. Namely, NHE3, which is involved in transport of ammonium in the proximal tubule (30), appears even more abundant in GPR4⁻/⁻ compared with GPR4⁺/+. Rhbg is one of the ammonium transporters in the collecting duct and was employed here to help in discrimination between non-A-non-B and B-ICs; its labeling did not seem to differ between GPR4⁻/⁻ and GPR4⁺/+. The adequate NHE3 response to acid loading in GPR4⁻/⁻ also suggests that the adaptive response of NHE3 may partially ameliorate acidosis in GPR4⁻/⁻. Preserved expression of NHE3 is consistent with our previous observation that GPR4⁻/⁻ do not exhibit bicarbonate wasting (88) at baseline or after bicarbonate challenge (data not shown).

That the adaptive response to an acid challenge was impaired, but not abolished, in GPR4⁻/⁻ is in keeping with the present results and further implies that other sensors might compensate for the loss of GPR4. The other two proton-sensing receptors, OGR1 or TDAG8, are expressed at very low levels in the kidney, several orders of magnitude lower than GPR4; OGR1 and TDAG8 expression did not change after deletion of GPR4 (88). Therefore, in the present study, we examined another pH-sensitive GPCR implicated in acid-base regulation, the calcium-sensing receptor (CaSR), and found that its expression was doubled in GPR4⁻/⁻ mice. The pH sensitivity of CaSR has been established using different techniques by Brown and colleagues (28) and Waldeger and colleagues (73). These studies indicate that pH modulates the affinity of CaSR for its agonists, likely by an allosteric effect. Moreover, several activating mutations of CaSR found in patients with hereditary hypocalcemia and hypercalciuria modulate the activity of CaSR by altering its pH sensitivity, such that the mutant receptors are activated more robustly than wild-type receptors at physiological pH, augmenting CaSR-mediated inhibition of transepithelial calcium transport in thick ascending limbs, resulting in concomitant hypercalciuria (73). This remarkable pH sensitivity of CaSR led to the hypothesis that at stable extracellular Ca²⁺ levels, CaSR may function as an acid sensor in the kidney (28, 73, 75). Interestingly, in this study we found that acid loading upregulates CaSR levels in the GPR4⁺/+ mice. Studies by Renkema et al. (74) and Capasso et al. (16) have already demonstrated that CaSR...
participates in the regulation of H\(^+\)-ATPase in the collecting duct and NHE3 in the proximal tubule. However, these studies investigated conditions of altered Ca\(^{2+}\) levels in the urine, not alterations of acid-base status primarily. Widespread expression of CaSR in different nephron segments (77) with rate-limiting functions and high mortality and short life span of the CaSR\(^{-/-}\) mice (19) have so far made it difficult to ascertain to which extent CaSR may be involved in the day-to-day regulation of acid-base homeostasis, if at all. Potential involvement of CaSR in acid-base homeostasis is particularly intriguing in view of the tight link between pH and calcium homeostasis, apparent in hereditary renal acidification defects (29, 32).

The range of pH sensitivity for GPR4, with peak activity at \(\sim\)pH 7.0 in kidney epithelial cells, renders this sensor uniquely poised to sense fluctuations in interstitial pH (88). Our observation that the deletion of GPR4 appears to influence the response of AE1, but not of pendrin or the a4 subunit of H\(^+\)-ATPase, to an acid challenge suggests that GPR4 may be involved in sensing of interstitial pH and may affect basolateral and not apical acid-base transporters. In fact, a differential effect of a sensor on different transporters in the same cells has been reported; e.g., Pyk2 stimulates the activity of H\(^+\)-ATPase, and of H\(^+\)-K\(^+\)-ATPase in the outer medullary collecting duct cells (mOMCD1) exposed to acidosis, but the former Pyk2 response signals through ERK1/2 and the latter through the MAPK, p38 (34, 35).

Fig. 8. Expression of calcium-sensing receptor (CaSR) mRNA and protein is increased in the kidneys of GPR4\(^{-/-}\) mice. CaSR mRNA was examined with quantitative real-time RT-PCR. The number of CaSR mRNA copies was normalized to the expression of β-actin (A). Analysis of protein expression was performed by immunoblotting (Fig. B). CaSR and β-actin were detected as a band of \(\sim\)160 and \(\sim\)42 kDa, respectively. C: summary of results of densitometric analysis.

Fig. 9. Acid loading upregulates CaSR mRNA and protein expression in the kidney. CaSR mRNA was examined with quantitative real-time RT-PCR in the kidneys of wild-type mice exposed to 4 days of NH\(_4\)Cl loading. The number of CaSR mRNA copies was normalized to the expression of β-actin (A). Analysis of protein expression was performed by immunoblotting (B) and densitometric analysis (C).

Fig. 10. CaSR mRNA expression in kidney collecting ducts isolated from GPR4\(^{+/+}\) and GPR4\(^{-/-}\) before and after NH\(_4\)Cl loading. CaSR mRNA was measured by RT-PCR in isolated collecting ducts. CaSR expression was normalized to tubule length.
In conclusion, our previous metabolic studies and the current analysis of the expression and activity of collecting duct acid-base transport proteins indicate that GPR4−/− is required for maintenance of normal acid-base homeostasis. Our studies involving gene expression and activity of acid-base transport proteins in the collecting duct, and the cellular response to acidosis, all suggest that the response to an acid load in GPR4−/− mouse appears consistent with this notion. In future studies, it will be important to identify the signaling networks involved among these sensors and how these networks link acid-base regulation with other homeostatic systems (e.g., acid-base regulation and calcium homeostasis, acid-base regulation and inflammation and endothelial cell homeostasis), a difficult task that may necessitate a combination of proteomics, phosphoproteomics, and metabolomics. Most importantly, a better understanding of such complex interactions may provide insight into recent clinical observations suggesting that the adaptation by the kidney to protect against metabolic acidosis, namely, the adaptive increase in net acid excretion in response to the high net endogenous acid production induced by a typical Western diet, may become maladaptive and harmful in patients with early stage chronic kidney disease and may, thereby, contribute independently to its progression.

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AUTHOR CONTRIBUTIONS


REFERENCES


