Carbonic anhydrase 2 deficiency leads to increased pyelonephritis susceptibility

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1Division of Nephrology, Le Bonheur Children’s Hospital, Memphis, Tennessee; 2Center for Clinical and Translational Medicine, The Research Institute at Nationwide Children’s Hospital, Columbus, Ohio; 3Division of Nephrology, Nationwide Children’s Hospital, Columbus, Ohio; 4Department of Pediatrics, The Ohio State University, Columbus, Ohio; 5Department of Pediatrics, The University of Rochester, Rochester, New York

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Hains DS, Chen X, Saxena V, Barr-Beare E, Flemming W, Easterling R, Becknell B, Schwartz GJ, Schwaderer AL. Carbonic anhydrase 2 deficiency leads to increased pyelonephritis susceptibility. Am J Physiol Renal Physiol 307: F869–F880, 2014. First published August 20, 2014; doi:10.1152/ajprenal.00344.2014.—Carbonic anhydrase 2 regulates acid-base homeostasis, and recent findings have indicated a correlation between cellular control of acid-base status and the innate defense of the kidney. Mice deficient in carbonic anhydrase 2 (Car2−/− mice) have metabolic acidosis, impaired urine acidification, and are deficient in normal intercalated cells. The objective of the present study was to evaluate the biological consequences of carbonic anhydrase 2 deficiency in a murine model of pyelonephritis. Infection susceptibility and transcription of bacterial response components in Car2−/− mice were compared with wild-type littermate controls. Car2−/− mice had increased kidney bacterial burdens along with decreased renal bacterial clearance after inoculation compared with wild-type mice. Standardization of the urine pH and serum HCO3− levels did not substantially alter kidney infection susceptibility between wild-type and Car2−/− mice; thus, factors other than acid-base status are responsible. Car2−/− mice had significantly increased neutrophil-gelatinase-associated lipocalin mRNA and protein and expression at baseline and a marked decreased ability to upregulate key bacterial response genes during pyelonephritis. Our findings provide in vivo evidence that supports a role for carbonic anhydrase 2 and intercalated cells in promoting renal bacterial clearance. Decreased carbonic anhydrase expression results in increased antimicrobial peptide production by cells other than renal intercalated cells, which is not sufficient to prevent infection after a bacterial challenge.

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ICs (4, 12). Lower CA-II levels of expression are present in collecting duct principal cells (PCs), the loop of Henle, and proximal tubules (3, 5). Mice that lack CA-II have metabolic acidosis, a high urine pH, and are severely depleted in kidney ICs compared with wild-type mice (4).

During an ascending pyelonephritis, pathogens initially encounter the collecting tubule. The collecting tubule contains ICs and PCs. PCs mediate Na+ and water transport, and ICs regulate acid-base transport (20, 38). Escalating evidence shows that IC function as innate immunity effectors in addition to their traditional role in the maintenance of acid-base homeostasis. We (42) have previously characterized ribonuclease 7, a potent antimicrobial peptide with a broad range of activity against uropathogens in the human kidney. Renal ribonuclease 7 expression is limited to ICs, constitutively expressed at baseline, upregulated in response to infection, and secreted into the urine (42, 43). ICs have been demonstrated to express other innate immune proteins, including neutrophil gelatinase-associated lipocalin, also known as lipocalin 2 (gene: Lcn2; protein: Ngal), IL–18 (gene: Il18), and human α-defensin 5 (15, 28, 41). Additionally, uropathogenic Escherichia coli (UPEC) preferentially adhere to the luminal surface of medullary ICs (10). ICs are ideally positioned to defend the kidney from ascending urinary tract infections (UTIs) and activate systemic inflammatory responses (42, 43).

Based on their phenotype of metabolic acidosis, impaired urine acidification, and IC depletion, we hypothesized that CA-II has a critical role in the innate defense of the kidney. The objective of the present study was to evaluate the functional consequences of CA-II depletion on the innate immune response during experimental murine pyelonephritis.

METHODS

Mice. The Institutional Animal Care and Use Committee of the Nationwide Children’s Hospital approved murine research protocol AR12–00035. All animal experiments adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Car2−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME). The initial coding sequence of the Car2 gene was replaced with a LacZ marker under the direction of the Car2 promoter (25). Heterozygous (Car2+−/) mice were rederived on a C57BL/6 genetic background at the Transgenic Core at the Research Institute of Nationwide Children’s Hospital. Homozygous Car2−/− mice were obtained by crossing Car2+−/ mice. Car2+−/ littermates were used as controls. For mouse growth experiments, Car2+−/ control mice were also used. The mouse genotype was determined by PCR using tail DNA using the following primer sequences: Car2 wild-type forward and reverse primers: forward, 5′-GTTCTTCTCCTGTGCTGCTG-3′; reverse, 5′-CGCTTGGTTACCCAGTATC-3′; reverse deletion primers: forward, 5′-GTTCTTCTCCTGTGCTGCTG-3′; reverse, 5′-CGCTTGGTACCCAGTATC-3′.

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primer, 5'-ATGAAGGCTGAACTTTAATC-3'; Car2 mutant forward primer, 5'-TCCACATACTTACCTGTC-3', and common reverse primer, 5'-AGGCCGTAAGTTATCATC-3'.

**Infection.** UPEC strain CFT073 was used to infect mice. CFT073 was grown statically in Luria broth (LB) medium overnight at 37°C. Bacteria were pelleted and resuspended in PBS. Female mice aged 7–10 wk were infected by inoculating 1 x 10⁸ CFT073 in 50 μL PBS transurethrally. A second inoculum was processed 3 h later, as previously described (45). Twenty-four hours after the first infection, kidneys and bladders were harvested. Kidneys were homogenized in a bullet blender (Next Advance, Averill Park, NY). The bacterial burden was determined by plating serial dilutions of kidney or bladder homogenates on the LB plate. If UTI confirmation was needed for a PCR experiment, kidneys were bisected longitudinally. Infection was confirmed as half of the kidney, and the other longitudinal half of each kidney was processed for immunostaining or mRNA extraction. Kidney or bladder clearance of bacteria was defined as <50 colony-forming units (CFU)/tissue (44).

**Base supplementation.** NaHCO₃ (100 mM/l) was added to the drinking water of mice assigned base supplementation from the time of weaning (~4 wk) until euthanasia (7–10 wk) based on prior reports (32, 37).

**LacZ staining.** After euthanasia, mice were perfused with PBS and periodate-lysine-paraformaldehyde (PLP) fixative (2% paraformaldehyde, 75 mM lysine, and 75 mM NaIO₄). Kidneys were bisected, fixed in cold PLP fixative for 2 h, and then soaked in 30% sucrose overnight. Kidneys and bladders were embedded in optimal cutting temperature embedding media after treatment. Frozen sections were dried at room temperature for 10 min and then fixed in PLP fixative for 10 min. Kidney slides were incubated with the LacZ substrate X-galactosidase at 4°C overnight. Slides were counterstained with nuclear fast red.

**Immunostaining.** After deparaffinization, rehydration, antigen retrieval, and blocking, specimens were incubated with primary antibody overnight at 4°C and incubated for 30 min at room temperature with horseradish peroxidase for immunohistochemistry or fluorophore-conjugated secondary antibody for immunofluorescence. Slides for immunohistochemistry were developed with 3,3'-diaminobenzidine and counterstained with hematoxylin. Slides for immunofluorescence were mounted in Vectashield Mounting Media with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Imaging was performed with a Keyence BZ-9000 “all in one” microscope and camera (Keyence, Osaka, Japan). Cells were quantified with the automated “dynamic cell count” and “cell count” features of BZ-II Analyzer software (Keyence). Images were adjusted with the BZ-II Analyzer and/or a GNU image manipulation program (GNU Free Software Foundation, Boston, Massachusetts). The antibodies and fluorochromes used are shown in Table 1.

**Blood and urine analysis.** Blood was collected through the submandibular vein and injected into iSTAT EC8+ for HCO₃⁻, K⁺, and pH measurements (Abbot Point of Care, Princeton, NJ). Urine was collected by bladder massage. Urine was transferred into 1.5-mL tubes containing corn oil. Urine pH was measured with a micro-pH electrode (Mettler Toledo, Columbus, OH). The Comparative Pathology and Mouse Phenotyping Shared Resource at The Ohio State University (Columbus, OH) performed complete blood counts and differentials.

**ELISA.** Murine urine Ngal levels were quantified by ELISA (catalog no. AF1857, R&D Systems) and run in duplicate. Urine creatinine levels were measured by a colorimetric assay to account for differences in urine concentration (catalog no. 500701, Cayman Chemical).

**Quantitative real-time PCR.** For cDNA generation, RNA was purified from kidneys using the RNeasy kit (Qiagen, Valencia, CA) and quantified with spectrometry. RNA quality/purity was also determined by the ratio of 260 to 280 nm. A value of 2.0 ± 0.1 was considered to be pure. cDNA was generated using the RT First Strand kit (Qiagen) per the manufacturer’s protocol.

For gene profiling, cDNA was amplified using the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The RT² profiler antibacterial response (catalog no. PAM148z, Qiagen) was performed according to the manufacturer’s instructions. Analysis and data quality control were performed using RT² profiler PCR Array Data Analysis software (version 3.5, Qiagen). Only results that passed quality checks in PCR array reproducibility, RT efficiency, and genomic DNA contamination were included. Gene expression was normalized using a panel of five housekeeping genes [β-actin (Actb), β₂-microglobulin (B2m), Gapdh, glucuronidase-β (Gusb), and heat shock protein 90 kDa (cytosolic) class B member 1 (Hsp90ab1)].

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**Table 1. Antibodies used in the present study**

<table>
<thead>
<tr>
<th>Antibody (Species)</th>
<th>Company</th>
<th>Catalog Number</th>
<th>Dilution</th>
</tr>
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<tr>
<td>Aquaporin 2 (rabbit, anti-human, ATTO 550 fluorescent label)</td>
<td>Alomone Labs (Jerusalem, Israel)</td>
<td>AQP-002-AO</td>
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<td>CA-II (rabbit, anti-human)</td>
<td>Epitomics (Burlingame, CA)</td>
<td>S2774</td>
<td>1:1,000</td>
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<tr>
<td>Lcn2/Ngal (goat, anti-mouse)</td>
<td>R&amp;D Systems (Minneapolis, MN)</td>
<td>AF1857</td>
<td>1:50</td>
</tr>
<tr>
<td>Ly6G (rabbit, anti-mouse)</td>
<td>Biologend (San Diego, CA)</td>
<td>127607</td>
<td>1:50</td>
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<tr>
<td>V-ATPase (goat, anti-human)</td>
<td>Santa Cruz Biotechnology (Santa Cruz, CA)</td>
<td>2109</td>
<td>1:50</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
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<td></td>
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<tr>
<td>Alexa fluor 488 (donkey, anti-goat)</td>
<td>Jackson ImmunoResearch (West Grove, PA)</td>
<td>705-546-147</td>
<td>1:250</td>
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<tr>
<td>Cy3 (donkey, anti-rat)</td>
<td>Jackson ImmunoResearch</td>
<td>712-296-150</td>
<td>1:1,000</td>
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<tr>
<td>Horseradish peroxidase (goat, anti-rabbit)</td>
<td>Jackson ImmunoResearch</td>
<td>111-055-003</td>
<td>1:500</td>
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</table>

CA-II, carbonic anhydrase 2 (gene: Car2); Lcn2/Ngal, lipocalin 2/neutrophil gelatinase-associated lipocalin.
Statistical analysis. Statistical analysis was performed, and graphs were made with GraphPad Prism software (GraphPad Software, La Jolla, CA). For the purpose of graphs, negative kidney culture results were assigned a value of 0.1 so that all data points were represented on log-scale scatter plots; the zero value was used for statistical analysis. Groups were analyzed for differences with a two-tailed Student’s t-test. Welch’s correction was used for bacterial burden analysis, as comparable SDs were not assumed. Proportions and percentages were compared using the VassarStats 2 × 2 contingency table, which assigned the χ²-value, or, if the expected cell frequency was less than five, the Fisher
exact probability test was applied (26). Two-way ANOVA was used to 
measure the effects of two or more variables in weight time course data. 
If significant differences were identified with ANOVA, post hoc analysis 
with the Tukey test was used to determine which groups within the 
sample were different. For the mouse antibacterial response arrays, fold 
changes of expression between groups and treatments were calculated 
using SA Biosciences RT² profiler PCR Array Data Analysis software 
(11). Statistical significance was assigned for P values of <0.05, and 
results are expressed as means ± SD.

RESULTS

The phenotype of Car2−/− mice. To confirm CA-II deletion 
and phenotype as previously reported by Breton et al. (4), 
absent CA-II expression was demonstrated with LacZ staining 
and CA-II antibody immunohistochemistry (Fig. 1). At 8 wk 
of age, Car2−/− mice were significantly smaller than Car2+/+ 
mice (14.49 ± 1.66 vs. 20.95 ± 3.68 g, respectively, P < 
0.0001). Enumeration of V-ATPase-positive cells demon-
strated threefold IC depletion in Car2−/− mice compared with 
Car2+/+ mice in the cortex and medulla (Fig. 2). Laboratory 
evaluation (Table 2) demonstrated similar complete blood 
counts and leukocyte differentials but higher urine pH along 
with lower serum pH, HCO₃⁻, and K⁺ in Car2−/− versus 
Car2+/+ mice. Car2−/− mice had similar weights (20.49 ± 
2.97 g, n = 35), serum pH (7.28 ± 0.05, n = 17), and urine pH 
(5.86 ± 0.25, n = 5) compared with Car2+/+ mice (P = 0.651, 
0.447, and 0.57, respectively).

Bacterial burden and renal bacteria clearance post-UPEC 
inoculation. Infections were variable based on kidney laterality 
with mean CFU differences between left and right kidneys of 
3.892 (range: 0–109,580) in Car2+/+ kidneys and 60,261 
(range: 0 to 2.4 × 10⁶) in Car2−/− kidneys (n = 16 mice/ 
genotype). Thus, individual rather than pooled kidneys were 
evaluated, as previously done by Tittel et al. (45). Figure 3 
shows 24- and 48-h kidney and bladder bacterial burdens along 
with kidney bacterial clearance. Twenty-four hours after inoc-
ulation, Car2−/− mice had a mean bacterial burden that was 
5-fold higher in the bladder and 15-fold higher in the kidneys 
compared with Car2+/+ mice. Furthermore, Car2−/− mice 
were three times less likely to clear bacteria from the kidney at 
24 h compared with control mice, and they were eight times 
less likely to clear at 48 h. Bacteria were largely retained in the 
bladder of both genotypes. Histological confirmation of infec-
tion in UPEC-inoculated mice was confirmed by the presence 
of neutrophils (Fig. 4).

Fig. 2. Quantification of intercalated cells (ICs). ICs were identified by immunofluorescence for V-ATPase immune reactivity (green) and colabeled with DAPI 
(blue) to identify nuclei and aquaporin 2 (red) to identify collecting duct principal cells. Isolated V-ATPase-positive cells (arrows) were identified in the collecting 
ducts of the cortex (A) and medulla (D) of Car2+/+ mice. ICs (arrows) in the cortex and medulla were present in tubules positive for aquaporin 2, consistent 
with collecting ducts, and also in isolated tubules negative for aquaporin 2 in the cortex, consistent with connecting segments. In contrast, Car2−/− mice had 
infrequent cells that stained for V-ATPase in the cortex (B) and medulla (E). C: ICs comprised 1.27 ± 0.40% and 0.32 ± 0.13% of cortical cells in Car2+/+ 
and Car2−/− kidneys, respectively (P = 0.004). F: medullary ICs decreased from 2.21 ± 0.58% of total cells in Car2+/+ kidneys to 0.83 ± 0.23% in Car2−/− 
kidneys (P = 0.004). The automated cell count of ICs was performed on four ×20 images from 2 mice. The presented images are at ×40 magnification, with 
20-μm scale bars.
Table 2. Laboratory values

<table>
<thead>
<tr>
<th></th>
<th>Car2+/+ Mice</th>
<th>Car2−/− Mice</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td><strong>Urinary tests</strong></td>
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<tr>
<td>pH</td>
<td>5.73 ± 0.11</td>
<td>6.38 ± 0.34</td>
<td>0.013*</td>
</tr>
<tr>
<td>Serum chemistries</td>
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<tr>
<td>pH</td>
<td>7.30 ± 0.07</td>
<td>7.12 ± 0.03</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td>19.93 ± 2.61</td>
<td>17.24 ± 1.43</td>
<td>0.007*</td>
</tr>
<tr>
<td>K⁺, mmol/l</td>
<td>6.32 ± 1.01</td>
<td>4.90 ± 1.47</td>
<td>0.017*</td>
</tr>
<tr>
<td><strong>Complete blood counts and differentials</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>White blood cells, K/μl</td>
<td>9.13 ± 0.84</td>
<td>8.69 ± 1.06</td>
<td>0.754</td>
</tr>
<tr>
<td>Neutrophils, K/μl</td>
<td>1.08 ± 0.10</td>
<td>0.96 ± 0.07</td>
<td>0.356</td>
</tr>
<tr>
<td>Lymphocytes, K/μl</td>
<td>7.43 ± 0.74</td>
<td>7.21 ± 0.94</td>
<td>0.861</td>
</tr>
<tr>
<td>Monocytes, K/μl</td>
<td>0.61 ± 0.08</td>
<td>0.49 ± 0.07</td>
<td>0.292</td>
</tr>
<tr>
<td>Eosinophils, K/μl</td>
<td>0.01 ± 0.00</td>
<td>0.02 ± 0.01</td>
<td>0.347</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>44.93 ± 1.12</td>
<td>44.17 ± 1.90</td>
<td>0.579</td>
</tr>
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</table>

Values are means ± SD. For urinary tests, n = 3 Car2+/+ mice and 7 Car2−/− mice except for serum chemistries, where n = 11 mice/genotype; for complete blood counts and differentials, n = 3 mice/genotype. *Statistically significant difference.

Normalization of serum HCO₃⁻. Because Car2−/− had a lower serum HCO₃⁻ and pH than Car2+/+ mice (Table 2), mouse growth, serum laboratory, and 24-h bacterial burden experiments were repeated with NaHCO₃ supplemented to Car2−/− mice in their drinking water and compared with a control group of Car2+/+ mice with no base supplementation. The highest tolerable NaHCO₃-supplemented water concentration was 100 mmol/l. Serum HCO₃⁻ normalized from 17 mmol/l in Car2−/− mice to 20 mmol/l in base-supplemented Car2−/− mice (Table 1 and Fig. 5A). Because mean weights did not differ between Car2+/+ and Car2−/− mice, these genotypes were pooled for time course growth data. The growth of Car2−/− mice with and without base supplementation was compared with that of pooled Car2+/− and Car2+/+ mice from 3 to 12 wk. Car2−/− mice demonstrated improved growth with base supplementation, whereas growth was not affected in control mice (Fig. 5C).

To analyze the effect of correction of the acid-base disturbances, we performed experimental UTI on base-supplemented Car2−/− mice and nontreated control mice. The mean 24-h CFU was 2.4 times higher in base-supplemented Car2−/− bladders. Compared with Car2+/+ mice, the mean CFU was 16.5 times higher in base-supplemented Car2−/− kidneys (Fig. 3). Despite missing statistical significance, presumably because of variability in the controls, it was consistent with nonbase-supplemented Car2−/− kidneys (Fig. 3B). Base-supplemented Car2−/− kidneys were six times less likely to be clear of bacteria than Car2+/+ kidneys (Fig. 5E).

Standardization of urine pH. Because urine pH may affect bacterial growth more than host serum acid-base status, we standardized urine pH between Car2−/− and control mice. To standardize urine pH secondary to the relatively high baseline urine pH in Car2−/− mice (Table 1), control Car2+/+ mice had NaHCO₃ added to their drinking water, and the 24-h bacterial burden experiment was repeated (Fig. 6). Car2−/− mice had a mean bacterial burden that was 10.4 times higher in the bladder.
changes in mRNA bacterial response gene expression after UPEC inoculation demonstrated a diminished response in Car2−/− compared with Car2+/+ mice (Fig. 7). Genes that displayed at least a twofold change and that were statistically significantly different (P < 0.05) between infection status and genotype are shown in Table 3. Uninfected Car2−/− mice had increased Lcn2 and cathelicidin expression. The antibacterial response was diminished in infected Car2−/− mice; specifically, Car2−/− mice had a relative inability to upregulate Cd14, Tnf, and Mediterranean fever (Mefv) expression along with relatively decreased interferon regulatory factor 5 (Irf5), NLR family CARD domain-containing protein 4 (Nlrc4), and C-reactive protein (Crp) expression during kidney infection. Although Lcn2 expression was increased in baseline, Car2+/+ mice but not Car2−/− mice could significantly upregulate Lcn2 expression after infection.

Ngal immunoreactivity and ELISA. Ngal (protein product of Lcn2) immunostaining in Car2+/+ and Car2−/− mice with and without infection is shown in Fig. 7. Isolated Car2+/+ and Car2−/− proximal tubular cells were positive for Ngal at baseline and during infection. Scattered immunoreactivity for Ngal was present in focal areas of Car2−/− but not Car2+/+ medullas at baseline. Medullary Ngal staining increased in both genotypes with infection but remained focal. Staining was often present in cells with apical aquaporin 2 staining, consistent with PCs (Fig. 8, I and J). In the absence of infection, urine Ngal/creatinine levels were higher in Car2−/− mice than in Car2+/+ mice at 20.81 ± 5.032 pg/mg (n = 6) versus 10.28 ± 1.393 pg/mg (n = 10), respectively (P = 0.026).

**DISCUSSION**

We have demonstrated a significant increase in kidney bacterial burden and diminished antibacterial response in Car2−/− mice compared with Car2+/+ mice. We further confirmed the previous findings that Car2−/− mice have at least a threefold decrease in the percentage of ICs. CA-II helps generates the needed H+ for H+ V-ATPase; thus, even ICs that are present should concep-tually have impaired function (9). Of note, avascular macrophage antibacterial activity is impaired with V-ATPase inhibition, providing an example of overlap between acid-base and immune regulation (2).

A primary component of human renal CA-II deficiency and/or IC dysfunction is distal renal tubular acidosis (dRTA). dRTA results when urinary acidification in the collecting tubule is impaired, resulting in systemic metabolic acidosis and total body K+ depletion (33). Furthermore, urine pH remains inappropriately high due to the inability to secrete protons distally. Car2−/− mice have metabolic acidosis and hypokalemia along with relatively alkaline urine, consistent with dRTA. The association of CA-II deficiency and UTI risk is supported by reports of increased UTIs in humans with dRTA. First, Halperin et al. (17) reported that “most” of 10 unrelated dRTA patients had a history of UTI. Next, children and adults with familial hypomagnesaemia-hypercalciuria, a rare condition in which dRTA is a component, had UTIs in 3 of 4 subjects (75%) and 10 of 18 subjects (55%) in case series (34, 35). Finally, in a case series of patients with medullary sponge, a condition associated with dRTA, 14 of 21 female patients (67%) and 4 of 26 of male patients (15%) presented with bacteriuria (7, 19). The increased UTI susceptibility in

![Image](https://via.placeholder.com/150)

**Fig. 4. Neutrophil aggregation.** A and B: representative pictures of neutrophil aggregation in Car2+/+ mice (A) and Car2−/− mice (B) 24 h after transurethral inoculation with UPEC (magnification: ×40; scale bars = 20 μm). Neutrophils are labeled with Ly6g antibody (red), and nuclei are counterstained with DAPI (blue). Neutrophil aggregation (arrows) was seen in both genotypes, mostly around the periphery of the renal papilla, but neutrophils appeared more numerous and diffuse in Car2−/− kidneys. Images were obtained from 2 kidneys/genotype with 2 sections analyzed per kidney.

1 Supplemental Material for this article is available at the American Journal of Physiology-Renal Physiology website.

10.1152/ajprenal.00344.2014 - www.ajprenal.org
Car2−/− mice we have identified appears consistent with our initial hypothesis that proper IC number and function is a crucial component of the innate defense of the urinary tract. Potential mechanisms for the increased UTI risk in Car2−/− mice include an increased infection risk from metabolic acidosi, changes in bacterial growth secondary to urine pH, increased infection susceptibility due to CA-II deficiency in other organ systems, IC depletion, or abnormal renal expression of CA-II across the kidneys and urinary tract. In the present study, we attempted to address all possible mechanisms to establish the significance of CA-II and ICs in the innate defense of the kidney.

Whether altered acid-base homeostasis contributes to infection risk is controversial. Immune function is supported by increased human neutrophil activation and macrophage phagocytosis during acidosis (29, 46). However, decreased neutrophil chemotaxis and increased infection-related hemodialysis mortality with acidosis support increased infection susceptibility (39, 47). In the present study, we demonstrated impaired renal bacterial clearance and an approximately >15-fold increased bacterial burden in Car2−/− compared with Car2+/+ kidneys with and without base supplementation. Additionally base-supplemented Car2−/− bladders had increased bacterial burdens, despite negligible CA-II bladder expression. Thus, factors other than base supplementation or local expression of CA-II are likely responsible for the infection risk. The increase in serum HCO$_3^-$ was biologically relevant as base supplementation of 24 mice, 8.3%) than lower in base-supplemented Car2−/− mice (12,965, but just missed significance with a P value of 0.056. F: the proportion of kidneys clear of bacteria was lower in base-supplemented Car2−/− mice (2 of 24 mice, 8.3%) than Car2+/+ mice (8 of 16 mice, 50%, P = 0.007).
Car2 organ systems is a potential mechanism for UTI risk in Car2−/− mice. Although systemic metabolic acidosis, impaired urine acidification, and extrarenal abnormalities associated with CA-II deficiency might contribute to infection risk, our findings suggest that they are not the primary etiology of UTI risk in Car2−/− mice. Therefore, we propose that loss of innate immune effectors produced by ICs contributes to UTI risk in Car2−/− mice. We (42, 43) have previously demonstrated that human ICs secrete RNase 7 into the urine and that inactivation with IC deficiency. However, no known mouse ortholog of RNase 7 has been identified (36). Car2−/− mice have increased mRNA expression of antibacterial peptides (Lcn2) at baseline. Lcn2/Ngal has previously been demonstrated to be expressed postrenal injury by renal ICs, the loop of Henle, and, in some reports, the proximal tubule (18, 28). Regardless of the etiology of the baseline increased Lcn2 expression in Car2−/− mice, it is not sufficient to prevent increased infection susceptibility and is associated with a decreased ability to upregulate Lcn2 expression in response to infection. A marked inability to upregulate the bacterial response after UPEC inoculation was identified. In addition to their antimicrobial roles, cathelicidin and Ngal have previously been effective (16). Furthermore, strains of uropathogens may have variable pH sensitivity (22). Our group has demonstrated that certain urinary antimicrobial peptides can have a wide variety of activity at different urinary pHs. Specifically, activity is reduced at extremes of pH and optimum around physiologic 7.4 (48). The host must balance altering growth conditions for bacteria while not detrimentally affecting its innate defenses. Car2−/− and base-supplemented Car2+/+ mice had similar mean pH readings, yet Car2−/− mice had significantly higher kidney and bladder bacterial burdens. Either factors other than urine pH are responsible for the UTI susceptibility in Car2−/− mice or the microenvironment around the collecting duct is altered by “local” pH adjustments not reflected by the overall urine pH.

Infection susceptibility due to CA-II deficiency in other organ systems is a potential mechanism for UTI risk in Car2−/− mice. The phenotype of Car2−/− mice has previously been reported to include impaired bone formation along with gastric cysts and pit cell hyperplasia (24, 27). The likelihood is low that these extrarenal manifestations contribute to UTI risk. Neutrophils contain CA-I and CA-II at a 3:1 concentration; however, the similar serum neutrophil number in Car2+/* and Car2−/− mice does not suggest an inherent neutrophil deficiency in these cells (6).
demonstrated to have anti-inflammatory properties. Cathelicidin blocks dendritic cell Toll-like receptor 4 activation (13). During pneumococcal pneumonia, Ngal attenuates the inflammatory response and bacterial clearance (49). Historically, CRP production was presumed restricted to the liver, but renal cortical epithelial cells have been identified as a second site for production (21). CRP mediates phagocytosis; therefore, the decreased Crp expression in Car2−/− mice is consistent with increased bacterial burden postinfection. Bacterial lipopolysaccharides induce inflammation by Cd14, Tnf, and Mefv (14, 23, 31). Inflammasomes, including those assembled by Nlcr4, are cytoplasmic complexes that recognize bacterial infections and

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**Table 3. RT2 bacterial response array results**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Functional Gene Grouping</th>
<th>Expression in Car2−/− mice relative to Car2+/+ mice</th>
<th>Fold Change</th>
<th>P Value</th>
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<td>Lipocalin 2</td>
<td>Lcn2</td>
<td>Antimicrobial peptide</td>
<td>+4.05</td>
<td>0.03</td>
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<tr>
<td>Cathelicidin antimicrobial peptide</td>
<td>Camp</td>
<td>Antimicrobial peptide</td>
<td>+2.03</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein, pentraxin-related</td>
<td>Crp</td>
<td>Bacterial pattern recognition receptors and the inflammatory response</td>
<td>−3.9</td>
<td>0.045</td>
<td></td>
</tr>
</tbody>
</table>

Expression in infected Car2−/− mice relative to infected Car2+/+ mice

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Functional Gene Grouping</th>
<th>Expression in infected Car2−/− mice relative to infected Car2+/+ mice</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipocalin 2</td>
<td>Lcn2</td>
<td>Antimicrobial peptide</td>
<td>+4.5</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>CD14 antigen</td>
<td>Cd14</td>
<td>Inflammatory response</td>
<td>+4.5</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>Tnf</td>
<td>Inflammatory response</td>
<td>+4.4</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Mediterranean fever</td>
<td>Mefv</td>
<td>Inflammatory response</td>
<td>+20.9</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Expression in infected Car2−/− mice relative to infected Car2+/+ mice

<table>
<thead>
<tr>
<th>Gene Name</th>
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<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon regulatory factor 5</td>
<td>Ifn5</td>
<td>Cytokines and chemokines</td>
<td>−3.5</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>NLR family, CARD domain containing 4</td>
<td>Nlr4</td>
<td>Inflammasomes and the inflammatory Response</td>
<td>−3.5</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein, pentraxin-related</td>
<td>Crp</td>
<td>Bacterial pattern recognition receptors and the inflammatory response</td>
<td>−3.5</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
activate cytokine production (50). The ability of Car2+/+ but not Car2−/− mice to significantly upregulate Cd14, Tnf, Nlcr4, and Mev after UPEC inoculation further implicates a decreased inflammatory response with CA-II deficiency.

Renal ICs have several similarities to innate immune cells from other organs. Examples of epithelial cells with important innate immunity roles include type II pneumocytes of the lung and Paneth cells of the small intestine. ICs share common structural features with these aforementioned epithelial cells, including optimal strategic locations to prevent infection dissemination, apical microvilli, prominent endoplasmic reticulum and/or mitochondria, and the production of innate immune proteins (1, 8).

While our study offers an exciting new correlation between CA-II deficiency and pyelonephritis risk, we do acknowledge some limitations. First, although serum HCO3− in Car2−/− mice normalized with base supplementation, the serum pH only partially corrected. Our antibacterial response PCR array evaluated whole kidney mRNA for differences between Car2−/− and wild-type mice. Because ICs, the primary site for renal CA-II expression, only account for a small percentage of total kidney cells, significant differences may be diluted out by the overall contribution of other cells. Inflammatory infiltrates during renal function have focal differences in occurrence and intensity; thus, we often had relatively large SDs, which resulted in a marked change in fold expression between genotypes and infection status but did not reach statistical significance. It is possible that key regulatory genes with statistically nonsignificant and/or lower fold changes have considerable biological relevance. The antibacterial response arrays only evaluate for differences between 84 key genes. While these genes are important in general in response to infection, IC-specific pathways and genes may not have been captured on the arrays. Because CA-II deficiency is global, extrarenal contributions to the observed phenotype cannot be completely ruled out. An important future research direction will be the generation of conditional knockout animals in which CA-II deficiency is limited to ICs. Such model systems can be used to conclusively demonstrate whether ICs are kidney epithelial innate immune cells, similar to Paneth cells of the small intestine. Additionally, whether altered CA-II expression impairs circulating neutrophil, macrophage, or dendritic cell function warrants extensive evaluation.

In conclusion, after transurethral UPEC inoculation, Car2−/− mice have increased kidney and bladder bacterial burdens, decreased kidney bacterial clearance, and a marked decreased

**Fig. 8.** Neutrophil gelatinase-associated lipocalin (Ngal) immunoreactivity. Kidney Ngal (green) immunolabeling staining at baseline and during infection labeled for collecting ducts with aquaporin 2 (red). In the Car2+/+ cortex at baseline (A) and during infection (B), occasional proximal tubules demonstrated low immunoreactivity (arrows), but collecting ducts were negative (arrowheads). At baseline (C), no Car2+/+ medullary staining for Ngal was found in collecting ducts (arrowhead), but during infection (D), some collecting ducts stained for Ngal (arrows), whereas others did not (arrowheads). In Car2−/− mice at baseline (E) and during infection (F), cortical Ngal immunoreactivity was present in occasional proximal tubules (arrows) but not collecting ducts (arrowheads). At baseline (G), some occasional Car2−/− medullary collecting ducts immunolabeled with Ngal, with the intensity of the fluorescence increasing during infection (H). Areas of marked Ngal staining were scattered, usually in the area of neutrophil aggregations (*). Aquaporin-2-positive cells were also positive for Ngal (arrows) in Car2+/+ (I) and Car2−/− (J) medullas, indicating that principal cells can express Ngal in response to infection. Magnification: ×40 in A–D and ×100 in I and J. Three sections from 2 mice per genotype and infection status were reviewed. All scale bars = 20 μm.
inflammatory response. The findings of this study expand on the past descriptions of acid-base regulation and renal innate immunity to determine the functional immune consequences of CA-II deficiency. Car2<sup>/−/−</sup> mice had decreased IC endowment, which indicates that Car2 is important to not just acid-base homeostasis but also to proper IC development and/or function. In light of the previous data, we propose that CA-II deficiency, potentially due to IC depletion, increases pyelonephritis risk.

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DISCLOSURES

G. J. Schwartz has consulted for Novartis. Otherwise, the authors have no conflicting financial interests.

AUTHOR CONTRIBUTIONS


REFERENCES


