AT₁ Receptors in the Collecting Duct Directly Modulate the Concentration of Urine

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ABSTRACT
Mice lacking AT₁ angiotensin receptors have an impaired capacity to concentrate the urine, but the underlying mechanism is unknown. To determine whether direct actions of AT₁ receptors in epithelial cells of the collecting duct regulate water reabsorption, we used Cre-Loxp technology to specifically eliminate AT₁A receptors from the collecting duct in mice (CD-KOs). Although levels of AT₁A receptor mRNA in the inner medulla of CD-KO mice were significantly reduced, their kidneys appeared structurally normal. Under basal conditions, plasma and urine osmolalities and urine volumes were similar between CD-KO mice and controls. The increase in urine osmolality in response to water deprivation or vasopressin administration, however, was consistently attenuated in CD-KO mice. Similarly, levels of aquaporin-2 protein in inner and outer medulla after water deprivation were significantly lower in CD-KO mice compared with controls, despite its normal localization to the apical membrane. In summary, these results demonstrate that AT₁A receptors in epithelial cells of the collecting duct directly modulate aquaporin-2 levels and contribute to the concentration of urine.


The renin-angiotensin system (RAS) has myriad physiologic actions including the regulation of water homeostasis through modulation of thirst, vasopressin release, and urinary concentrating mechanisms.1–5 Pharmacologic and gene targeting studies suggest that these actions are mediated primarily by type 1 (AT₁) receptors.5,6 Mice have two AT₁ receptor isoforms, AT₁A and AT₁B, which are highly homologous. The AT₁A receptor is predominantly expressed in most tissues including the kidney and is the murine homologue to the single human AT₁ receptor. Mice completely lacking AT₁A receptors develop polyuria and an impaired urinary concentrating capacity, with an attenuated increase in urine osmolality after water deprivation or vasopressin administration.5,6 On the other hand, gene targeting studies have demonstrated distinct roles for the AT₁B receptor in the regulation of thirst.7,8 Nonetheless, the precise mechanisms and cellular targets of AT₁A receptors responsible for regulating urine concentration have not been precisely documented.

Evidence from in vivo and in vitro studies suggests that the collecting duct is an important target for the modulation of water handling by the RAS. For example, in cell culture experiments, it

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has been suggested that AT1A receptors regulate aquaporin-2 (AQP2) expression and trafficking to the apical membrane in cortical or inner medullary collecting duct cells.9–11 Moreover, Li et al.6,11 found increased AQP2 protein expression levels after angiotensin II stimulation in renal collecting duct cells and decreased AQP2 membrane fractions in inner medullary kidney homogenates from mice, completely lacking AT1A receptors. However, AT1A receptors are expressed throughout the kidney, including the proximal tubule, the thick ascending limb, distal tubule, medullary interstitial cells, and renal vasculature, where they may affect solute and water reabsorption, thereby modifying renal concentrating mechanisms.12–14 For example, it has been shown that AT1 receptors directly influence the abundance and activity of amiloride-sensitive epithelial sodium channels (ENaCs), thereby potentially influencing sodium and water reabsorption by the collecting duct.15–18 Furthermore, complete AT1A receptor-deficient mice may have anatomical changes in the inner medulla that could independently affect urinary concentrating ability.5,19

To address the contributions of AT1A receptors in the collecting duct to water homeostasis, we generated transgenic mice lacking AT1A receptors only in the collecting duct (CD-KO) with Cre/loxP technology. Using this new model, we demonstrate that the AT1A receptor exerts direct actions in collecting duct epithelia to enhance urinary concentrating capacity.

RESULTS

Generation of Mice Lacking AT1A Receptors in Collecting Duct

We generated mice with a conditional Agtr1a allele on an inbred 129/SvEv genetic background by inserting loxp sites flanking exon 3 of the Agtr1a gene (Agtr1a^flox) as described.20,21 To delete the Agtr1a^flox allele in collecting duct, we took advantage of an existing transgenic line expressing Cre recombinase in the collecting duct under the control of a Hoxb7 promoter (Hoxb7-Cre)^22 that had been back-crossed multiple generations onto the 129/SvEv background. We verified the pattern of Cre expression by crossing the Hoxb7-Cre mouse with a double-fluorescence reporter mouse (mT/mG). At baseline, tissues in the mT/mG animals express a red fluorescence protein, whereas in the presence of Cre recombinase, green fluorescent protein (GFP) expression is triggered. As shown in Figure 1B, there was substantial GFP expression in tubules in the renal medulla in Hoxb7-Cre+ mT/mG mice. To determine whether Cre expression is restricted to the collecting duct, we performed double immunofluorescent histochemistry com-
paring patterns of expression of GFP with AQP2, which is expressed exclusively in the collecting duct, and these patterns had almost complete overlap (Figure 1C).

We next carried out a series of crosses between 129-Hoxb7-Cre and Agtr1a^flox/flox animals to generate 129-SvEv-Hoxb7-Cre^-Agtr1a^flox/flox mice with deletion of AT1A receptors specifically from the collecting duct (CD-KOs). To determine whether expression of AT1A receptors was altered in the CD-KOs, we measured AT1A mRNA expression by reverse transcription-quantitative PCR in different regions of the kidney. In preparations of renal cortex or outer medulla, there were no differences in AT1A receptor mRNA expression levels between CD-KOs and controls. By contrast, in isolated preparations of inner medulla (Figure 1A), levels of AT1A mRNA were reduced by 43 ± 6.5% in CD-KO mice compared with controls (P < 0.01), confirming successful deletion of the receptor.

**AT1A Receptors in the Collecting Duct Are Essential for Achieving Maximal Urinary Concentration**

To determine whether urinary concentrating capacity was altered in CD-KOs, we measured urinary volumes and osmolalities under baseline conditions during ad libitum water intake and after 18 hours of water deprivation (Figure 2). Basal 24-hour urine volumes tended to be higher (3.06 ± 0.23 versus 2.71 ± 0.16 ml/d; n = 13) (Figure 2A), and urine osmolalities tended to be lower (1810 ± 142 versus 1951 ± 169 mOsmol/kg) in CD-KOs compared with controls, but these differences did not achieve statistical significance. As shown in Figure 2A, urine flow rates decreased significantly in both groups after water deprivation (P < 0.001) but were significantly higher in the CD-KOs than controls (1.07 ± 0.14 versus 0.61 ± 0.12 ml/18 h; P < 0.05). Similarly, although urine osmolalities increased significantly in both groups with thirsting, they remained significantly lower in CD-KOs (2979 ± 69 versus 3517 ± 116 mOsmol/kg; P < 0.01) (Figure 2B).

To elucidate whether the impaired ability to concentrate urine in CD-KO mice during water deprivation reflected an attenuated renal response to vasopressin, we measured urinary osmolalities at baseline (2300 ± 117.4 versus 2289 ± 122.9 mOsmol/kg; n = 12) and 4 hours after the injection of 1 μg/kg of the V2 receptor agonist, dDAVP. As shown in Figure 2C, urinary osmolalities increased significantly and to a similar extent in both groups with thirsting, they remained significantly lower in CD-KOs (2757 ± 58 versus 3103 ± 130 mOsmol/kg; n = 12; #P < 0.05). The data are presented as the means ± SEM.

**AT1A Receptors in the Collecting Duct Regulate AQP2 Expression**

The actions of vasopressin to influence water permeability of the collecting duct are determined by levels of AQP2 water channels and their recruitment to the apical plasma membrane.5,8,9,10 To examine...
whether the AT1A receptors in collecting duct affect AQP2 levels, we compared total AQP2 protein in the renal outer and inner medulla in CD-KO and control mice at baseline (control: n = 4; CD-KO: n = 4) and after 18 hours of water deprivation (control: n = 4; CD-KO: n = 5). In water-deprived control mice, outer medullary AQP2 expression levels were significantly increased compared with baseline controls and water-deprived CD-KO mice. (A) Outer medullary AQP2 abundance tended to be increased in water-deprived CD-KO mice compared with baseline. (B) Inner medullary AQP2 expression levels were similar between CD-KO and control mice at baseline. After 18 hours of water deprivation, inner medulla AQP2 expression levels increased significantly in both groups. AQP2 expression levels were significantly lower in the inner medulla of CD-KO mice compared with control mice. For the densitometric analysis, AQP2 expression levels were normalized to the corresponding β-actin expression and further evaluated by calculating the fold induction relative to the AQP2/β-actin ratio observed in the sample from mouse #1 (baseline control). The sample from mouse #1 is present as a reference on all Western blots to allow comparison of all samples. †P < 0.01 versus baseline control; #P < 0.05 versus baseline CD-KO; *P < 0.05 versus water deprivation control. The data are presented as the means ± SEM.

Figure 3. Increases of AQP2 with thirsting are significantly attenuated in CD-KO mice compared with controls. Representative immunoblots and densitometric analysis of aquaporin-2 (AQP2) in renal outer and inner medulla in CD-KO and control mice at baseline (control: n = 4; CD-KO: n = 4) and after 18 hours of water deprivation (control: n = 4; CD-KO: n = 5). In water-deprived control mice, outer medullary AQP2 expression levels were significantly increased compared with baseline controls and water-deprived CD-KO mice. (A) Outer medullary AQP2 abundance tended to be increased in water-deprived CD-KO mice compared with baseline. (B) Inner medullary AQP2 expression levels were similar between CD-KO and control mice at baseline. After 18 hours of water deprivation, inner medulla AQP2 expression levels increased significantly in both groups. AQP2 expression levels were significantly lower in the inner medulla of CD-KO mice compared with control mice. For the densitometric analysis, AQP2 expression levels were normalized to the corresponding β-actin expression and further evaluated by calculating the fold induction relative to the AQP2/β-actin ratio observed in the sample from mouse #1 (baseline control). The sample from mouse #1 is present as a reference on all Western blots to allow comparison of all samples. †P < 0.01 versus baseline control; #P < 0.05 versus baseline CD-KO; *P < 0.05 versus water deprivation control. The data are presented as the means ± SEM.

whether the AT1A receptors in collecting duct affect AQP2 levels, we compared total AQP2 protein in the renal outer and inner medulla in CD-KOs and controls at baseline and after water deprivation (Figure 3, A and B; see also Supplemental Figure 1). AQP2 protein levels were similar in CD-KO and control mice at baseline and increased after water deprivation. However, the extent of the increase of AQP2 levels with water deprivation was significantly attenuated in CD-KO mice in both the outer (2.00 ± 0.15 versus 3.06 ± 0.40; P < 0.05) (Figure 3A) and inner medulla (1.20 ± 0.13 versus 2.10 ± 0.37; P < 0.05) (Figure 3B). Thus, AT1A receptors in the collecting duct act to enhance AQP2 expression levels during water deprivation.

Cellular Localization of AQP2 in CD-KO and Control Mice

Previous studies have suggested that AT1A receptors may also affect AQP2 trafficking.10,11,23 To determine whether cellular localization of AQP2 to the apical membrane is altered in the CD-KOs, immunofluorescent labeling of AQP2 was analyzed by confocal microscopy. As shown in Figure 4, under baseline conditions with free access to water, AQP2 immunostaining in the inner medulla showed a weak and diffuse staining pattern that was similar in control (Figure 4, A and E) and CD-KO (Figure 4, B and F) mice. In contrast, with water deprivation, there was intense and defined localization of AQP2 labeling to the apical membrane of principal cells in the inner medulla with a complete shift of AQP2 staining to the apical membrane in both groups (Figure 4, C, D, G, and H). These findings suggest that the major effect of AT1A receptors in collecting duct on vasopressin-dependent water flux is not caused by impaired localization of AQP2 to the apical membrane but rather is mediated through regulation of AQP2 protein levels (Figure 3, A and B).

Elimination of AT1A Receptors from Collecting Duct Does Not Affect Abundance of ENaC Proteins

Along with alterations in water permeability, fluid delivery to the medullary collecting duct can also affect the capacity of the urinary concentrating mechanism.24,25 Changes in sodium transport in the cortical collecting duct will affect downstream fluid delivery to the medullary portions of the
Because it has been suggested that AT1A receptors can modulate expression and activity of the ENaC in the CD, we measured the abundance of all three subunits of ENaC (H9251, H9252, and H9253) in the renal cortex as well as outer and inner medulla under baseline conditions and after 18 hours of water deprivation.16,17 As shown in Supplemental Figure 2, there were no differences in abundance of any of the ENaC subunits between CD-KOs and controls in any region of the kidney at baseline or during water deprivation. In line with these findings, there was likewise no difference in urinary sodium excretion between the groups (Table 1).

Elimination of AT1A Receptors from Principal Cells Impairs Urinary Concentration

The Hoxb7-Cre transgene is expressed by all cells of ureteric bud lineage including both principal and intercalated cells of the collecting duct, as well as ureteral epithelia.22,26 To confirm that the physiologic effect of the AT1A receptors on the regulation of water homeostasis is due to direct effects in principal cells, we used a second transgenic mouse line where Cre expression is driven by the promoter of the Aqp2 gene.27 We confirmed specific expression of Cre in principal cells of the collecting duct by crossing with the mTmG line as above (data not shown). We then generated AQP2-Cre Agtr1a flox/flox mice to eliminate expression of AT1A receptors in principal cells. Expression of AT 1A-receptor mRNA was significantly reduced in the inner medulla of AQP2-Cre Agtr1a flox/flox mice compared with controls (1 ± 0.08 versus 0.49 ± 0.09 P < 0.05; n = 4 to 8) (Figure 5A). After 18 hours of water deprivation, urine osmolalities increased significantly in both groups but remained significantly lower in AQP2-Cre Agtr1a flox/flox mice compared with controls (Table 1).
with controls (3081 ± 90 versus 3535 ± 148 mOsmol/kg; 
P < 0.05, n = 9) (Figure 5B).

DISCUSSION

Gene targeting experiments have clearly highlighted the role of the RAS in regulating urinary concentrating mechanisms. For example, mice homozygous for targeted disruptions of the angiotensinogen or angiotensin-converting enzyme genes were found to have polyuria with marked defects in urinary concentrating capacity.22,28,29 A similar phenotype was found in mice lacking both AT1 receptor isoforms, the AT1A and AT1B receptors, indicating that the RAS primarily uses AT1 receptor pathways to mediate this process.30 In addition, the angiotensin-converting enzyme-deficient, angiotensinogen-deficient, and combined AT1A and AT1B receptor-deficient mouse lines also share the common phenotype of marked atrophy of the inner medulla of the kidney, which may also contribute to their concentrating defect. On the other hand, mice lacking only the AT1A receptor isoform also have polyuria and impaired urinary concentrating capacity, but with normal kidney structure.5,6 Furthermore, pharmacologic blockade of AT1 receptors with losartan attenuates maximal urine osmolality in normal mice, suggesting that physiologic actions of AT1 receptors modulate water handling.5,31

Our previous studies using mice lacking only the AT1A receptor isoform have suggested that their urinary concentrating defect is due to renal resistance to vasopressin because we found normal plasma vasopressin levels at baseline that increase with thirsting and blunted response to exogenous dDAVP. However, AT1A receptor-deficient mice also have marked hypotension, impaired renal sodium handling, and reduced expression of key sodium transporters in the kidney, all of which may have effects on water handling that are independent of alterations in water permeability of the collecting duct. Furthermore, Li et al.6 have suggested that reduced vasopressin levels might contribute to the impaired urinary concentration in the AT1A receptor KO mice.

To further examine the mechanism underlying regulation of urinary concentrating mechanisms by AT1A receptors, we generated mice lacking AT1A receptors only in the collecting duct using Cre/loxP technology. This approach allows us to define actions of AT1 receptors in collecting duct in isolation in the intact animal. Unlike mice completely lacking AT1A receptors, which have marked polydipsia and polyuria,5,6 baseline water intake and urine volumes were similar in CD-KOs and controls, and their kidneys and collecting systems appear grossly normal. Moreover, CD-KO mice have normal BP at baseline and during water deprivation. With thirsting, urine osmolalities increase in CD-KO mice but never reach the levels seen in control mice. A similar phenotype is observed when AT1A receptors influence the urinary concentrating mechanism through direct effects on collecting duct epithel-
lia, independent of any of the systemic perturbations seen in mice completely lacking AT1A receptors.

Activation of the V2 receptor promotes water reabsorption in collecting duct epithelia by increasing the levels of AQP2 water channels and promoting their trafficking to the apical membrane. Previous studies have indicated that AT1 receptors can modulate AQP2 levels. For example, in cultured cells from the medullary collecting duct, activation of AT1 receptors stimulated expression and trafficking of AQP2 proteins. Conversely, pharmacologic blockade of the AT1 receptor reduces AQP2 expression in experimental heart failure. In addition, Li et al. have recently shown that angiotensin II stimulates expression and trafficking of AQP2 proteins to the apical surface of immortalized principal cells. Our finding of an impaired response to vasopressin in CD-KOs supports a key interaction between AT1A receptors in the collecting duct and V2 receptor activation of AQP2. Expression of V2 receptor was unaffected in the CD-KOs. On the other hand, abundance of AQP2 proteins was significantly diminished in both the outer and inner medulla of water-deprived CD-KO mice compared with controls. On the other hand, we found normal and virtually complete localization of AQP2 proteins to the apical surfaces of principal cells in water-deprived CD-KO mice. Thus, AT1A receptors in the collecting duct do not seem to affect trafficking of AQP2 in vivo. Our data suggest that AT1A receptors in collecting duct epithelia promote water permeability primarily by enhancing expression of AQP2 proteins.

Altered water permeability of the collecting duct mediated by AQP2 proteins is a major determinant of urinary concentrating capacity, but there are other factors affecting concentration of urine that could be relevant to the CD-KOs. These include delivery of fluid into the medullary collecting duct from more proximal nephron segments. Previous studies have shown that AT1 receptors modulate expression and activity of the epithelial sodium channel (ENaC). Such effects on ENaC could enhance sodium reabsorption in the cortical collecting duct, reducing fluid flow to the medulla and thereby affecting urinary concentration. However, we found that the deletion of AT1A receptors from collecting duct did not influence either ENaC expression or urinary sodium excretion. Therefore, this study provides convincing evidence that the impaired urinary concentration ability in CD-KOs is not related to differences in salt delivery. Frokiaer et al. have shown that increased intrapelvic pressure associated with urinary tract obstruction suppresses AQP2 expression. Because the Hoxb7-Cre is expressed in cells of ureteric bud lineage including renal pelvis ureter, and it has also been suggested that AT1 receptor activation affect contractility of the ureters, it is theoretically possible that such a mechanism might contribute to the phenotype of the CD-KOs. Nonetheless, a similar defect in maximal urinary concentration was also seen in the AQP2-Cre+Agtr1afox/fox mice where AT1A receptors are eliminated only from the collecting duct.

In conclusion, we show that AT1A receptors in collecting duct epithelia make a critical contribution to achieving maximal urinary concentration. The effects of AT1A receptors are most dramatically manifested in the presence of vasopressin receptor activation, where they promote increased AQP2 mRNA expression and elevated levels of AQP2 protein. However, AT1A receptors in the collecting duct do not appear to play a major role in controlling AQP2 trafficking.

CONCISE METHODS

Experimental Mice.

A mouse line with a conditional Agtr1afox allele was generated using homologous recombination in embryonic stem cells as described previously. To delete the AT1A receptors from the collecting duct, we crossed an inbred 129SvEv transgenic mouse lines expressing Cre recombinase specifically in the collecting duct under the control of a Hoxb7 promoter (Hoxb7-Cre) or an AQP2 promoter (AQP2-Cre) with Agtr1afox/fox mice to generate Hoxb7-Cre+Agtr1afox/fox (CD-KO) or AQP2-Cre+Agtr1afox/fox (CD-KO AQP2-Cre) mice.

Membrane-targeted tdTomato (mT)/membrane-targeted EGFP (mG) mice with loxP sites flanking the membrane-targeted tdTomato cassette followed by an N-terminal membrane-tagged version of EGFP were purchased from the Jackson Laboratory and crossed with the two Cre recombinase transgenic lines. mTmG mice normally express red fluorescence protein in all tissues. When Cre is present, the mT cassette is deleted, triggering expression of the membrane-targeted EGFP.

All of the experimental mice, maintained on a 129/SvEv background, were bred in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility at the Duke University and the Durham Veterans Affairs Medical Centers under National Institutes of Health Guidelines for Care and Use of Laboratory Animals and housed with free access to standard rodent chow and water unless specified.

Quantification of AT1A receptor mRNA levels. The renal cortex, outer medulla, and inner medulla were microscopically dissected, and total RNA was isolated by using the RNeasy mini kit (Qiagen). RNA expression levels were then determined for AT1A receptor (forward 5'-GCTTGGTGTTGATCGTACC-3' and reverse 5'-GGGCGAGATTAGAAGAACG-3').

Chronic Measurement of Intra-arterial Pressure by Radiotelemetry.

BP levels were measured in conscious, unrestrained mice after the implantation of a pressure-sensing catheter (TA11PA-C10; Transome Medical) via the left common carotid artery as described previously. During the measurement period, the mice were housed in a monitoring room where quiet is maintained, and BP levels were recorded over a 10-second interval every 5 minutes; allowing 5 days for re-establishment of diurnal BP variation, baseline BP measurements were recorded for 5 days. At day 6, the mice were thihrmed for 18 hours.
Water Deprivation.
To examine the effect of AT1a receptor deletion from the CD on the ability to concentrate urine, CD-KO and control mice were housed in metabolic cages as described previously in detail. During an initial 24-hour period, water intake and urine volume were measured while animals had free access to water and food. Water bottles were when removed, and urine outflow was measured during 18 hours of water deprivation. Urine samples were obtained by bladder massage just before and 18 hours after water deprivation. Urinary osmolality was measured immediately using a vapor pressure osmometer (Wescor Instruments).

Desmopressin (dDAVP) Administration.
To examine the effects of dDAVP (Sigma-Aldrich, St. Louis, MO) on urinary osmolality in CD-KO and control mice, dDAVP (1.0 μg/kg of body weight), a selective vasopressin-2 receptor agonist with strong antidiuretic potency but modest vascular actions, was administered as described previously. Four hours after dDAVP administration, urine samples were collected and measured as described above.

Immunofluorescence Staining.
Kidneys were fixed in 4% paraformaldehyde and incubated in 30% sucrose overnight. 5-μm longitudinal cryostat sections of kidney from CD-KO and control mice were hydrated through graded ethanol solutions (100 to 50%), equilibrated to water, subjected to microwave antigen retrieval, and then quenched with 3% peroxide for 30 minutes. Sections were blocked with 5% goat serum in PBS, 0.1% Triton X-100 for 60 minutes and incubated with either a polyclonal rabbit anti-GFP antibody (1:1000; Invitrogen) and a polyclonal goat anti-AQP2 antibody (1:500 (C-17); Santa Cruz Biotechnology, Santa Cruz, CA) or a polyclonal rabbit anti-AQP2 antibody (1:2000) (Novus Biologicals [NB110-74682], Littleton, CO) diluted in 1% goat serum in PBS overnight at 4°C. Adjacent, sections were washed extensively in PBS and subsequently incubated with Alexa 568 and Alexa 568 as secondary antibodies (1:1000; Invitrogen). Confocal fluorescence images were taken using a confocal Zeiss LSM 510 microscope and analyzed with AxiosVision LE software (Carl Zeiss MicroImaging Solutions GmbH).

Western Blot Analysis for AQP2.
Renal outer medulla and inner medulla were dissected and placed into ice-cold isolation buffer (10 mM Tris, 250 mM sucrose, and 5 mM EDTA, pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich) and immediately homogenized. The lysates were centrifuged at 4000 × g for 10 minutes at 4°C. Protein concentrations from the supernatant were determined by a Bradford assay (Bio-assay Systems). Twenty micrograms of total protein were loaded onto 12% SDS-PAGE gels and then transferred to nitrocellulose membranes according to the manufacturer’s instructions (X-Cell Blot Module; Invitrogen). The membranes were blocked in blocking buffer (5% dry milk, and 0.1% Tween 20 in PBS) for 1 hour at room temperature and then incubated with primary polyclonal rabbit anti-AQP2 antibody (1:2000) (Novus Biologicals [NB110-74682], Littleton, CO), and mouse anti-β-actin (1:1000) (Sigma-Aldrich) overnight. Bound primary antibody was detected with either donkey anti-rabbit or sheep anti-mouse horseradish peroxidase-conjugated secondary antibodies (donkey anti-rabbit or sheep anti-mouse [1:10,000]; Amersham Life Sciences). The intensities of the bands were analyzed using densitometry and quantified with a digital imaging system with a CCD camera (FluorChem FC3 System, AlphaEase Software 6.0; Alpha Innotech, San Leandro, CA). Immunoblots from each tissue sample were performed in triplicate.

Western Blot Analysis for ENaC Expression.
The kidney cortex, outer medulla, and inner medulla were separately harvested and immediately homogenized in ice-cold K-HEPES buffer (200 mM mannitol, 80 mM K-HEPES, 41 mM KOH, 2 mM K-EDTA, 2 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride, pH 7.5) as described by Wagner et al. The samples were first centrifuged at 1000 × g for 10 minutes at 4°C, and the supernatant was subsequently ultracentrifuged at 100,000 × g for 1 hour at 4°C. The pellet containing the plasma membrane fraction was again resuspended in ice-cold K-HEPES buffer. Fifty μg of the crude membrane proteins were solubilized in Laemmli sample buffer resolved by 8% SDS-PAGE and visualized by Western blotting. The antibodies against α-ENaC, β-ENaC, and γ-ENaC (generous gifts from Susan Wall, Johannes Loffing, and Lawrence Palmer) were diluted 1:2000 (α-ENaC) or 1:20,000 (β-ENaC and γ-ENaC) and incubated overnight at 4°C. The anti-β-actin antibody was obtained from Sigma-Aldrich and diluted 1:5000. After washing, the blots were incubated with the secondary antibodies (donkey anti-rabbit or sheep anti-mouse 1:10,000; Amersham Life Sciences) for 1 hour at room temperature. The intensities of the bands were quantified by densitometric analysis as described above (AlphaEase Software 6.0; Alpha Innotech). Immunoblots from each tissue were performed in triplicate.

Statistics
The values for each parameter within a group are expressed as the means ± SEM. For comparisons between CD-KO and control mice, statistical significance was assessed using ANOVA after either paired or unpaired two-tailed t test. P values less than 0.05 were considered significant.

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None.
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See related editorial, “Role of Collecting Duct AT1a Receptors in Concentrating Urine,” on pages 2144–2145.

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