AT$_1$ Receptors in the Collecting Duct Directly Modulate the Concentration of Urine

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ABSTRACT

Mice lacking AT$_1$ angiotensin receptors have an impaired capacity to concentrate the urine, but the underlying mechanism is unknown. To determine whether direct actions of AT$_1$ receptors in epithelial cells of the collecting duct regulate water reabsorption, we used *Cre-Loxp* technology to specifically eliminate AT$_{1A}$ receptors from the collecting duct in mice (CD-KOs). Although levels of AT$_{1A}$ 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 AT$_{1A}$ 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 (AT$_1$) receptors.[5,6] Mice have two AT$_1$ receptor isoforms, AT$_{1A}$ and AT$_{1B}$, which are highly homologous. The AT$_{1A}$ receptor is predominantly expressed in most tissues including the kidney and is the murine homologue to the single human AT$_1$ receptor. Mice completely lacking AT$_{1A}$ 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

AT$_{1B}$ receptor in the regulation of thirst.[7,8] Nonetheless, the precise mechanisms and cellular targets of AT$_{1A}$ 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
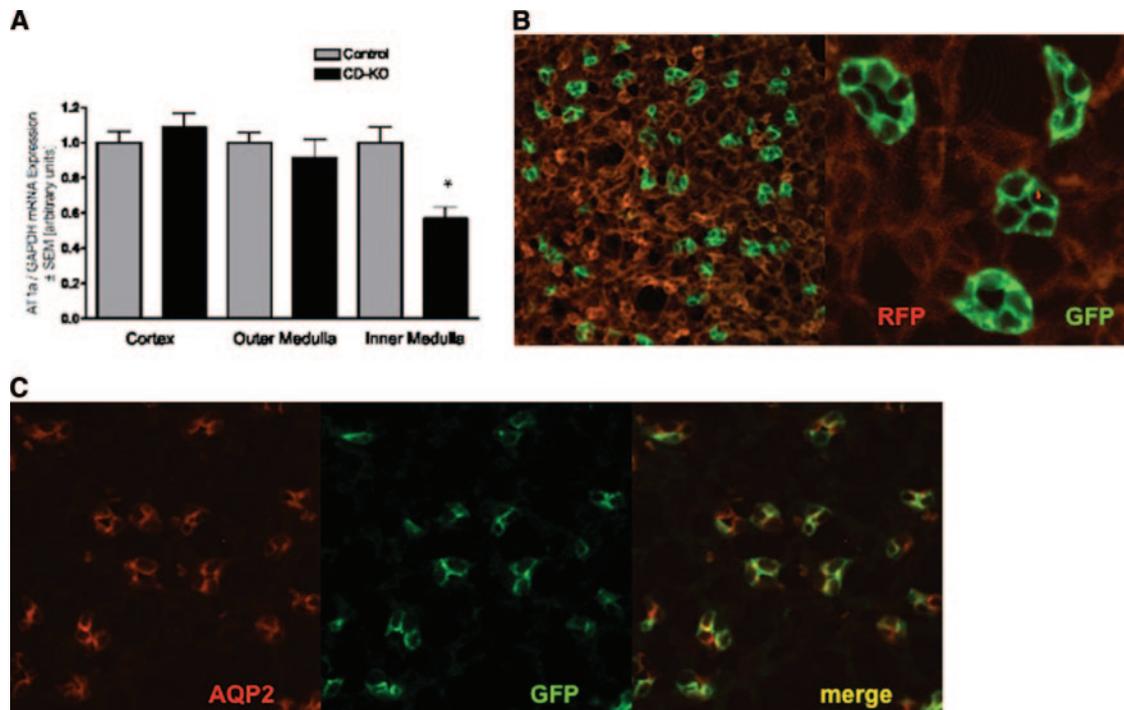
Figure 1. The increased level of AQP2 with thirsting was significantly attenuated in CD-KO mice. (A) AT_{1A} receptor mRNA expression is reduced in the renal inner medulla of CD-KO mice compared with control mice as determined by real time PCR (1.00 ± 0.09 *versus* 0.56 ± 0.07; $n = 12$; $*P < 0.01$). No differences in AT_{1A} receptor expression were detected in the cortex and the outer medulla of CD-KO and control mice. Representative confocal laser-scanning microscopy ($20\times$ and $40\times$) of the inner medulla of *Hoxb7-Cre*$^+$ *mT/mG* mice are shown. (B) Green fluorescence indicates the presence of *Hoxb7 Cre* expression, whereas red fluorescence indicates the absence of *Hoxb7 Cre* expression. (C) Double immunofluorescence with green fluorescent protein (GFP) and aquaporin-2 (AQP2) confirms that *Hoxb7 Cre* is restricted to the collecting duct in the kidney.

has been suggested that AT_{1A} 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 AT_{1A} receptors. However, AT_{1A} 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 AT_1 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 AT_{1A} 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 AT_{1A} receptors in the collecting duct to water homeostasis, we generated transgenic mice lacking AT_{1A} receptors only in the collecting duct (CD-KO) with *Cre/loxp* technology. Using this new model, we demonstrate that the AT_{1A} receptor exerts direct

actions in collecting duct epithelia to enhance urinary concentrating capacity.

RESULTS

Generation of Mice Lacking AT_{1A} 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-
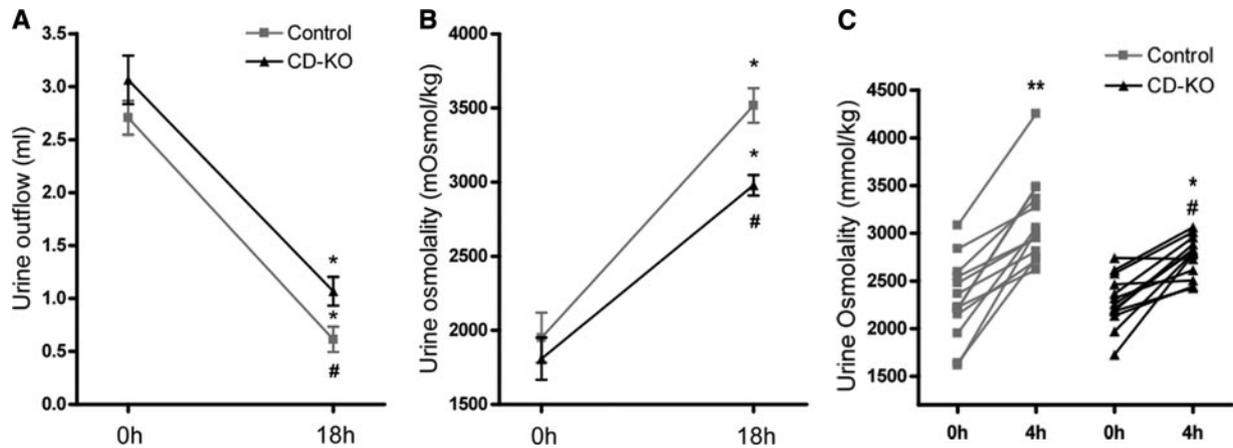
Figure 2. Basal urine volumes were similar in CD-KO and control mice (3.06 ± 0.23 *versus* 2.71 ± 0.16 ml/d; $n = 13$). (A) Urinary flow rate decreased significantly during 18 hours of water deprivation (*$P < 0.001$) but remained significantly higher in CD-KO mice (1.07 ± 0.14 *versus* 0.61 ± 0.12 ml/18 hours; $n = 13$; #$P < 0.05$). No differences in urine osmolalities were observed under basal conditions between CD-KO and control mice (1810 ± 142 *versus* 1951 ± 169 mOsmol/kg; $n = 13$). After 18 hours of water deprivation, urine osmolality increased significantly in CD-KO and control mice (*$P < 0.001$). (B) In accordance with the urine outflow, urine osmolalities remained significantly lower in CD-KO mice compared with control mice (2979 ± 69 *versus* 3517 ± 116 mOsmol/kg; $n = 13$; #$P < 0.01$). Shown are urine osmolality levels 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. (C) Administration of dDAVP significantly increases urinary osmolality (*$P < 0.01$; **$P < 0.001$), but osmolalities remained significantly lower in CD-KO mice compared with control mice (2757 ± 58 *versus* 3103 ± 130 mOsmol/kg; $n = 12$; #$P < 0.05$). The data are presented as the means ± SEM.

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 AT$_{1A}$ receptors specifically from the collecting duct (CD-KOs). To determine whether expression of AT$_{1A}$ receptors was altered in the CD-KOs, we measured AT$_{1A}$ 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 AT$_{1A}$ receptor mRNA expression levels between CD-KOs and controls. By contrast, in isolated preparations of inner medulla (Figure 1A), levels of AT$_{1A}$ mRNA were reduced by 43 ± 6.5% in CD-KO mice compared with controls ($P < 0.01$), confirming successful deletion of the receptor.

AT$_{1A}$ 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) 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 *versus* 2289 ± 122 mOsmol/kg) and 4 hours after injection of 1 µg/kg of the vasopressin-2 (V2) receptor agonist dDAVP. As shown in Figure 2C, urinary osmolalities increased in both groups after dDAVP administration but remained significantly lower in CD-KOs compared with controls (2757 ± 58 *versus* 3103 ± 130 mOsmol/kg; $P < 0.05$), recapitulating the pattern seen during water deprivation (Figure 2B). To determine whether the resistance to vasopressin could be explained by reduced levels of V2 receptor expression in the CD-KOs mice, we measured V2 receptor mRNA levels in inner medulla under baseline conditions and found that they were similar in both groups (1 ± 0.22 *versus* 1.06 ± 0.21 arbitrary units). V2 receptor mRNA levels increase significantly and to a similar extent in both groups with water deprivation (9.09 ± 2.41 and 10.55 ± 3.57 arbitrary units; $P < 0.001$ *versus* baseline), and there is no difference in V2 mRNA levels between thirsted CD-KOs and controls.

AT$_{1A}$ 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.[6,9,10] To examine
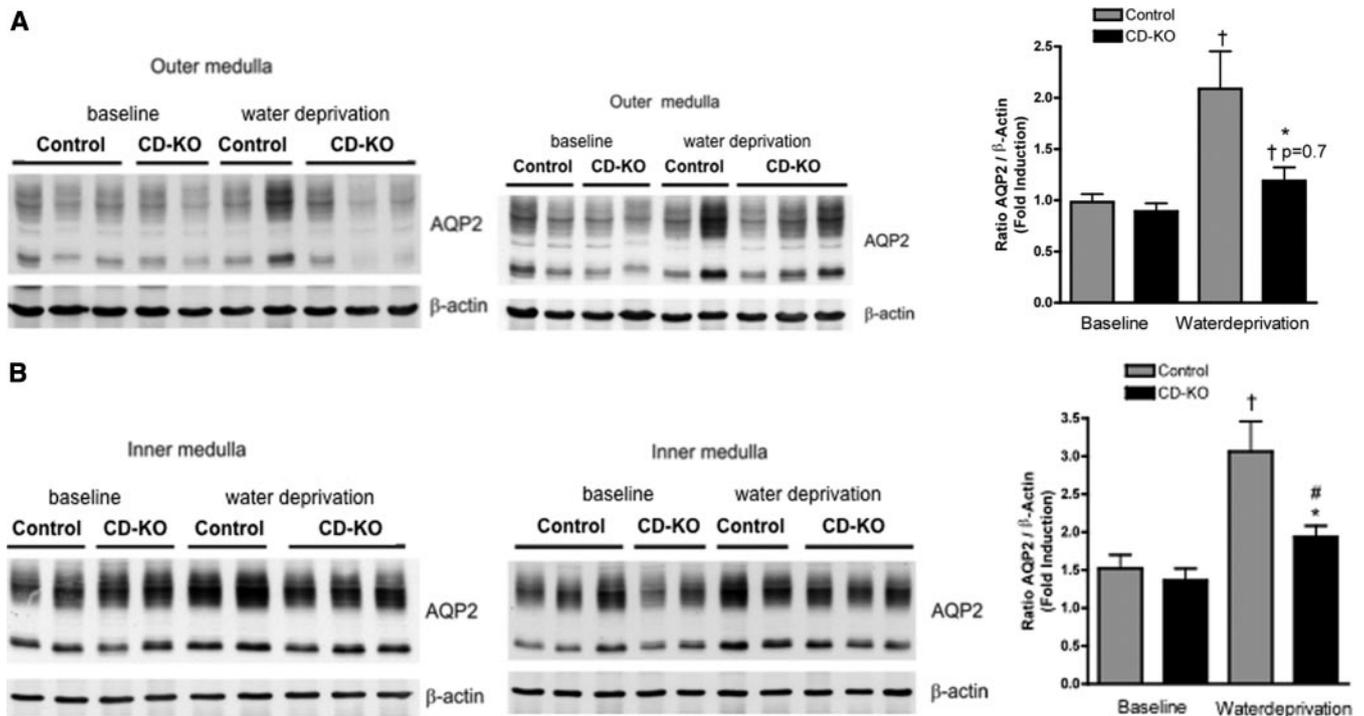
Figure 3. Increases of AQP2 with thirsting are significantly attenuated in CD-KO mice compared with controls. Representative immunoblots and densitometric analysis of aquaporin-2 (AQP2) in renal outer and inner medulla in CD-KO and control mice at baseline (control: $n = 4$; CD-KO: $n = 4$) and after 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. $\dagger P < 0.01$ *versus* baseline control; $\# P < 0.05$ *versus* baseline CD-KO; $*P < 0.05$ *versus* water deprivation control. The data are presented as the means $\pm$ SEM.

whether the AT_{1A} 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, AT_{1A} 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 AT_{1A} 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 AT_{1A} 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 AT_{1A} 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
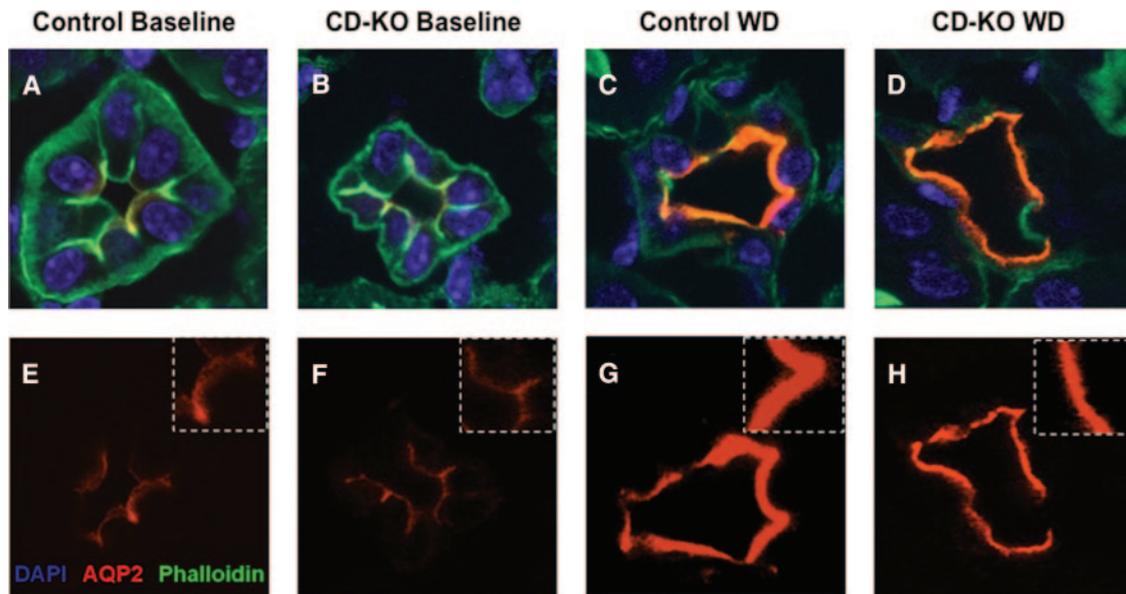
Figure 4. Normal cellular localization of AQP2 in CD-KO mice. Representative confocal laser-scanning microscopy (64×) of the inner medulla from CD-KO and control mice at baseline and after 18 hours of water deprivation. Under baseline conditions, aquaporin-2 (AQP2) (red) is diffusely localized around the apical membrane of the collecting duct in control (A and E) and CD-KO mice (B and F). After 18 hours of water deprivation, intense AQP2 expression strictly defined to the apical membrane of principle cells were detected in both control (C and G) and CD-KO (D and H) mice (C and D). However, similar to AQP2 protein expression levels (Figure 3), the extent of apical AQP2 labeling after 18 hours of water deprivation appeared to be stronger in control mice compared with CD-KO mice. DAPI, 4,6,diamidino-2-phenylindole; WD, water deprivation.

Table 1. Blood pressure and excretory function in CD-KO and control mice

Physiological Parameters	Control Mice (n = 7 to 14)		CD-KOs (n = 7 to 14)	
	Baseline	Water Deprivation	Baseline	Water Deprivation
Mean arterial pressure (mmHg)	120 ± 6 (n = 6)	130 ± 6 (n = 6)[a]	121 ± 5 (n = 6)	134 ± 6 (n = 6)[a]
Water intake (ml/day)	4.2 ± 0.8 (n = 13)	N/A	4.2 ± 1.0 (n = 13)	N/A
Osmolar excretion rate (mOsmol/kg H_2O * ml/h)	133.1 ± 8.9 (n = 13)	128.1 ± 11.34 (n = 13)	138.7 ± 7.3 (n = 13)	163.5 ± 11.6 (n = 13)[b]
Urinary sodium excretion (mM/24 h)	0.25 ± 0.03 (n = 13)	0.14 ± 0.02 (n = 13)[c]	0.29 ± 0.02 (n = 13)	0.16 ± 0.03 (n = 13)[c]

Shown are the baseline characteristics in CD-KO and control mice under baseline conditions and after 18 hours of water deprivation. The data are presented as the means ± SEM. N/A, not applicable.
[a]$P < 0.05$ *versus* baseline control and baseline CDKO.
[b]$P < 0.05$ *versus* water deprivation control.
[c]$P < 0.01$ *versus* baseline control and baseline CD-KO.

collecting duct. Because it has been suggested that AT_{1A} receptors can modulate expression and activity of the ENaC in the CD, we measured the abundance of all three subunits of ENaC (α, β, and γ) 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 AT_{1A} 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 AT_{1A} 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 AT_{1A} 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
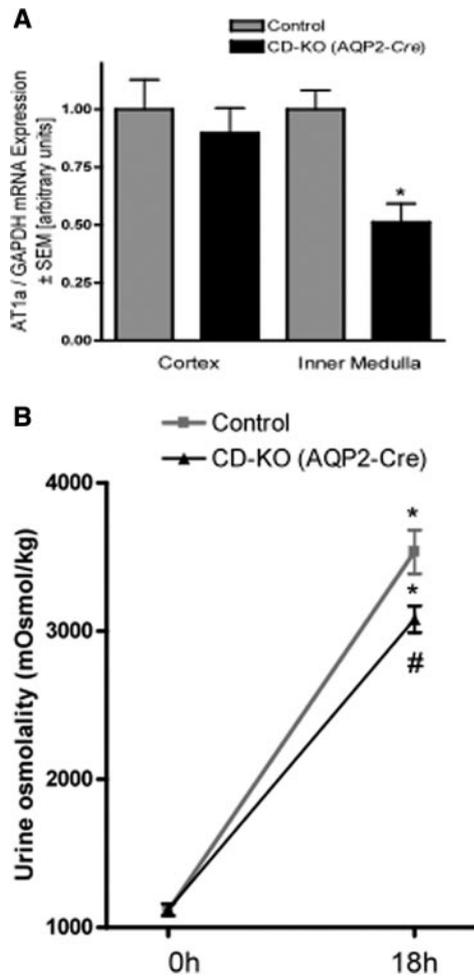
Figure 5. AT$_{1A}$ receptor mRNA expression is reduced in the renal inner medulla of CD-KO (*AQP2-Cre*) mice compared with control mice as determined by real time PCR (1 ± 0.08 *versus* 0.49 ± 0.09; *P < 0.05; n = 4 to 8). (A) No differences in AT$_{1A}$ receptor expression were detected in the cortex of CD-KO (*AQP2-Cre*) and control mice (cortex 1.00 ± 0.1285 *versus* 0.89 ± 0.11; P = 0.56; n = 4 to 8). Basal urine osmolalities in CD-KO (*AQP2-Cre*) and control mice were similar (1120 ± 41 *versus* 1114 ± 18 mOsmol/kg; n = 8). After 18 hours of water deprivation, urine osmolality increased significantly in CD-KO (*AQP2-Cre*) and control mice (*P < 0.001). (B) Urine osmolalities remained significantly lower in CD-KO (*AQP2-Cre*) mice compared with control mice (3081 ± 90 *versus* 3535 ± 147 mOsmol/kg; #P < 0.05; n = 9). The data are presented as the means ± SEM.

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.[3,28,29] A similar phenotype was found in mice lacking both AT$_1$ receptor isoforms, the AT$_{1A}$ and AT$_{1B}$ receptors, indicating that the RAS primarily uses AT$_1$ receptor pathways toy mediate this process.[30] In addition, the angiotensin-converting enzyme-deficient, angiotensinogen-deficient, and combined AT$_{1A}$ and AT$_{1B}$ 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 AT$_{1A}$ receptor isoform also have polyuria and impaired urinary concentrating capacity, but with normal kidney structure.[5,6] Furthermore, pharmacologic blockade of AT$_1$ receptors with losartan attenuates maximal urine osmolality in normal mice, suggesting that physiologic actions of AT$_1$ receptors modulate water handling.[5,31]

Our previous studies using mice lacking only the AT$_{1A}$ 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, AT$_{1A}$ 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 AT$_{1A}$ receptor KO mice.

To further examine the mechanism underlying regulation of urinary concentrating mechanisms by AT$_{1A}$ receptors, we generated mice lacking AT$_{1A}$ receptors only in the collecting duct using *Cre/loxp* technology. This approach allows us to define actions of AT$_1$ receptors in collecting duct in isolation in the intact animal. Unlike mice completely lacking AT$_{1A}$ 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 AT$_{1A}$ receptors are deleted only from principal cells with *AQP2-Cre*. Likewise, the increase in urine osmolality after administration of dDAVP is significantly blunted in CD-KOs compared with controls, indicating renal resistance to vasopressin. These features of the concentrating defect in the CD-KOs are similar to that seen in global AT$_{1A}$ receptor-deficient mice but are significantly less severe including the absence of a concentrating defect at baseline.[5,6] On the basis of the nature and specificity of the genetic alteration, our findings suggest that AT$_{1A}$ receptors influence the urinary concentrating mechanism through direct effects on collecting duct epithe-

lia, independent of any of the systemic perturbations seen in mice completely lacking AT_{1A} 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 AT_1 receptors can modulate AQP2 levels. For example, in cultured cells from the medullary collecting duct, activation of AT_1 receptors stimulated expression and trafficking of AQP2 proteins.[10,11] Conversely, pharmacologic blockade of the AT_1 receptor reduces AQP2 expression in experimental heart failure.[9,23] In addition, Li et al.[11] 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 AT_{1A} 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, AT_{1A} receptors in the collecting duct do not seem to affect trafficking of AQP2 in vivo. Our data suggest that AT_{1A} receptors in collecting duct epithelium 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.[32] Previous studies have shown that AT_1 receptors modulate expression and activity of the epithelial sodium channel (ENaC).[15–18] 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 AT_{1A} 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.[33,34] 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 AT_1 receptor activation affect contractility of the ureters, it is theoretically possible that such a mechanism might contribute to the phenotype of the CD-KOs.[33–35] Nonetheless, a similar defect in maximal urinary concentration was also seen in the $AQP2\text{-}Cre^+ Agtr1a^{flox/flox}$ mice where AT_{1A} receptors are eliminated only from the collecting duct.

In conclusion, we show that AT_{1A} receptors in collecting duct epithelia make a critical contribution to achieving maximal urinary concentration. The effects of AT_{1A} 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, AT_{1A} 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 $Agtr1a^{flox}$ allele was generated using homologous recombination in embryonic stem cells as described previously.[20,21] To delete the AT_{1A} 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 $Agtr1a^{flox/flox}$ mice to generate $Hoxb7\text{-}Cre^+ Agtr1a^{flox/flox}$ (CD-KO) or $AQP2\text{-}Cre^+ Agtr1a^{flox/flox}$ (CD-KO AQP2-Cre) mice.[22,27] Membrane-targeted tdTomato (mT)/membrane-targeted EGFP (mG) mice with loxP sites flanking the membrane-targeted dtTomato 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.[37]

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 AT_{1A} 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 AT_{1A} receptor (forward 5′-GCTTGGTGGTGATCGTCACC-3′ and reverse 5′-GGGCGA-GATTTAGAAGAACG-3′ and GAPDH (forward 5′-TCACCAC-CATGGAGAAGGC-3′ and reverse 5′-GCTAAGCAGTTGGTGGT-GCA-3′) using SYBR Green (Bio-Rad) in real time PCR.

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; Transoma Medical) via the left common carotid artery as described previously.[36] 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 thirsted for 18 hours.

Water Deprivation.
To examine the effect of AT_{1A} 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.[5,38] 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, und 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 administrated as described previously.[5] 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 1× PBS and subsequently incubated with Alexa 568 and Alexa 568 as secondary antibodies (1:1000) (Invitrogen) for 1 h at room temperature. After being washed in PBS, the sections were mounted using Fluoromount-G (Southern Biotech, Birmingham, AL) containing 2 μg/ml 4,6,diamidino-2-phenylindole (Sigma). Confocal fluorescence images were taken using a confocal Zeiss LSM 510 microscope and analyzed with AxioVision 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 (Bioassay 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.*[39] 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.

DISCLOSURES
None.

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