AT1a receptor signaling is required for basal and water deprivation-induced urine concentration in AT1a receptor-deficient mice

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1Laboratory of Receptor and Signal Transduction, Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi; 2Center of Excellence for Cardiovascular-Renal Research, University of Mississippi Medical Center, Jackson, Mississippi; and 3Division of Nephrology, Department of Internal Medicine, University of Mississippi Medical Center, Jackson, Mississippi

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Li XC, Shao Y, Zhuo JL. AT1a receptor signaling is required for basal and water deprivation-induced urine concentration in AT1a receptor-deficient mice. Am J Physiol Renal Physiol 303: F746–F756, 2012. First published June 27, 2012; doi:10.1152/ajprenal.00644.2011.—It is well recognized that ANG II interacts with arginine vasopressin (AVP) to regulate water reabsorption and urine concentration in the kidney. The present study used ANG II type 1a (AT1a) receptor-deficient (Agtr1a−/−) mice to test the hypothesis that AT1a receptor signaling is required for basal and water deprivation-induced urine concentration in the renal medulla. Eight groups of wild-type (WT) and Agtr1a−/− mice were treated with or without 24-h water deprivation and 1-desamino-8-arginine vasopressin (DDAVP; 100 ng/h ip) for 2 wk or with losartan (10 mg/kg ip) during water deprivation. Under basal conditions, Agtr1a−/− mice had lower systolic blood pressure (P < 0.01), greater than threefold higher 24-h urine excretion (WT mice: 1.3 ± 0.1 ml vs. Agtr1a−/− mice: 5.9 ± 0.7 ml, P < 0.01), and markedly decreased urine osmolality (WT mice: 1,834 ± 86 mosM/kg vs. Agtr1a−/− mice: 843 ± 170 mosM/kg, P < 0.01), without significant changes in 24-h urinary Na+ excretion. These responses in Agtr1a−/− mice were associated with lower basal plasma AVP (WT mice: 105 ± 8 pg/ml vs. Agtr1a−/− mice: 67 ± 6 pg/ml, P < 0.01) and decreases in total lysate and membrane aquaporin-2 (AQP2; 48.6 ± 7% of WT mice, P < 0.001) and adenylyl cyclase isoform III (55.6 ± 8% of WT mice, P < 0.01) proteins. Although 24-h water deprivation increased plasma AVP to the same levels in both strains, 24-h urine excretion was still higher, whereas urine osmolality remained lower, in Agtr1a−/− mice (P < 0.01). Water deprivation increased total lysate AQP2 proteins in the inner medulla but had no effect on adenylyl cyclase III, phosphorylated MAPK ERK1/2, and membrane AQP2 proteins in Agtr1a−/− mice. Furthermore, infusion of DDAVP for 2 wk was unable to correct the urine-concentrating defects in Agtr1a−/− mice. These results demonstrate that AT1a receptor-mediated ANG II signaling is required to maintain tonic AVP release and regulate V2 receptor-mediated responses to water deprivation in the inner medulla.

angiotensin II; angiotensin II type 1a receptor; adenylyl cyclases; aquaporin 2; arginine vasopressin; inner medullary collecting ducts; urine concentration

WATER REABSORPTION by the inner medulla plays an important role in maintaining basal body electrolytes and fluid balance, urine concentration, and blood pressure homeostasis. Physiologically, water transport in the inner medulla is primarily regulated by the neuropeptide hormone arginine vasopressin ([Arg8]-AVP), which is released from the posterior pituitary in response to changes in extracellular fluid volume and plasma and urine osmolality (1, 23, 40). In the inner medulla of the kidney, AVP binds to Gs protein-coupled type 2 (V2) receptors expressed on basolateral membranes of principal cells, which activates adenyl cyclases III and/or V/VI to increase intracellular cAMP concentrations (2, 8). The primary action of cAMP is to activate PKA and MAPK ERK1/2, which, in turn, induces the phosphorylation of the water channel protein aquaporin-2 (AQP2) and its subsequent translocation from intracellular vesicles to cell membranes (5, 8). AQP2 is one of key water channel proteins responsible for water transport and urine concentration in the inner medulla of the kidney (23, 36).

In addition to AVP, the vasoactive peptide ANG II is also involved in regulating water transport and urine concentration in the kidney by interacting with AVP (24, 25, 37, 50). ANG II type 1a (AT1a) and 1b (AT1b) receptors are expressed in the posterior pituitary and other brain regions, where ANG II activates these receptors to modulate AVP release (6, 49). In the inner medulla of the kidney, ANG II appears to interact with V2 receptors to control water reabsorption and concentrate urine via the activation of AT1 receptor signaling (13, 25). In animal studies, administration of physiological concentrations of ANG II decreased urine excretion and increased urine osmolality, and, conversely, chronic administration of angiotensin-converting enzyme inhibitors or AT1 receptor blockers led to increased excretion of diluted urine (12, 24, 29). In vitro, ANG II stimulates the translocation of AQP2 from intracellular vesicles to cell membranes in cultured inner medullary collecting duct cells (25) or potentiates AVP-induced CAM production and AQP2 expression in CHO cells coexpressing dual AT1a and V2 receptors (21). Furthermore, we and others (30, 37) have recently shown that deletion of AT1a receptors is associated with the development of polyuria and a urine-concentrating defect in AT1a receptor-deficient (Agtr1a−/−) mice. However, the cellular mechanisms responsible for this defect remain poorly understood. In the present study, we used Agtr1a−/− mice as a model to test the hypothesis that AT1a receptor signaling is required for basal and water deprivation-induced water reabsorption and urine concentration in the renal medulla.

METHODS

Animals. Eight groups of adult male wild-type C57BL/6J (Agtr1a+/+) mice and eight groups of Agtr1a−/− mice were used in the present study (body weight: ~25 g, n = 7–10 mice/group). Agtr1a+/+ mice were purchased from Jackson Laboratories and, upon arrival, were maintained on a normal rodent chow and had free access to tap water. Agtr1a−/− mice were bred in our laboratory from breeding pairs of heterozygous AT1a mice (Agtr1a+/−, stock no. 002682), which were originally deposited in Jackson Laboratories by Dr. Oliver Smithies of
the University of North Carolina (B6.129P2-Agrtl<sup>tm1oiw/J</sup>) (18, 39). All animal protocols were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University Medical Center. Genotyping of Agrtl<sup>−/−</sup> mice was performed by PCR duplicate on tail DNA samples using standard protocols from Jackson Laboratories, as previously described (18, 29, 30). All wild-type and Agrtl<sup>−/−</sup> mice were trained for 1 wk for blood pressure monitoring via a computerized tail-cuff method (Visitec, Cary, NC) and were housed in metabolic cages for urine collection before the experiment began (29, 30).

**Twenty-four-hour water deprivation.** One cohort of wild-type and Agrtl<sup>−/−</sup> mice was continuously given free access to tap water after their basal systolic pressure, 24-h water intake, 24-h urinary Na<sup>+</sup> and K<sup>+</sup> excretion, and plasma and urine osmolality were measured, as previously described (35, 37). All parameters were measured again in these animals and used as time controls. The second cohort of wild-type and Agrtl<sup>−/−</sup> mice was completely denied access to tap water for 24 h after their basal parameters had been measured as described above, serving as water-deprived groups. The third cohort of wild-type and Agrtl<sup>−/−</sup> mice was first studied under basal conditions and then 24 h after water deprivation for paired comparisons. Urine samples were collected for 24 h to determine 24-h urine excretion, urinary Na<sup>+</sup> and K<sup>+</sup> excretion, and urine osmolality before and after 24-h water deprivation (29–31, 37).

**Effects of 24-h water deprivation on plasma and urine osmolality.** Urine osmolality was measured in all urine samples (spot or 24-h collections) under basal conditions as well as after 24-h water deprivation using a vapor pressure osmometer (30, 37). At the end of the experiments, mice were decapitated without anesthesia, and trunk blood samples were collected into a tube without heparin and other peptidase inhibitors for measurements of plasma osmolality (30, 34, 37).

**Effects of 24-h water deprivation on plasma [Arg<sup>8</sup>]-AVP concentrations.** To determine whether Agrtl<sup>−/−</sup> mice have partial central AVP deficiency, plasma [Arg<sup>8</sup>]-AVP levels and their responses to 24-h water deprivation were measured in wild-type and Agrtl<sup>−/−</sup> mice. Briefly, mice were decapitated at the end of the experiments without anesthesia to avoid anesthesia-induced AVP release (34). To further determine the role of AT<sub>1b</sub> receptors, separate groups of wild-type (n = 8) and Agrtl<sup>−/−</sup> (n = 7) mice were treated with losartan (10 mg/kg ip) during water deprivation. Trunk blood samples were collected into a tube containing EDTA and a peptidase inhibitor cocktail and centrifuged for 15 min at 3,000 rpm (37). Plasma was collected, and peptides were extracted as we have previously described (29, 31). Plasma [Arg<sup>8</sup>]-AVP was measured using a sensitive ELISA kit (Peninsula Laboratories, San Carlos, CA). The assay is 100% specific for [Arg<sup>8</sup>]-AVP and does not cross-react with other neuropeptides, such as [Arg<sup>8</sup>]-vasotocin, [Lys<sup>5</sup>]-vasopressin, oxytocin, ACTH, or met-enkephalin. The assay has an intra-assay variation of <5% and an interassay variation of <14% (30).

**Effects of 24-h water deprivation on 24-h urine excretion and urinary Na<sup>+</sup> and K<sup>+</sup> excretion.** Basal 24-h water intake and urine samples were collected from wild-type and Agrtl<sup>−/−</sup> mice before 24-h water deprivation. Urine samples were collected for an additional 24 h in control mice and complete water-deprived wild-type and Agrtl<sup>−/−</sup> mice. Urine volumes were determined gravimetrically, and plasma and urine concentrations of Na<sup>+</sup> and K<sup>+</sup> were measured by flame photometry as previously described (29–31).

**Effects of 24-h water deprivation on 125I-labeled V<sub>2</sub> receptor binding in the inner medulla.** To determine whether AVP V<sub>2</sub> receptor levels are altered under basal conditions and respond to 24-h water deprivation in the inner medulla of wild-type and Agrtl<sup>−/−</sup> mice, V<sub>2</sub> receptor binding in the cortex and medulla of all mice was measured by quantitative in vitro autoradiography using an 125I-labeled V<sub>2</sub> ([125I]V<sub>2</sub>) receptor analog (kindly provided by Dr. Robert Speth, University of Mississippi), as previously described (30, 45, 50). [125I]V<sub>2</sub> receptor binding was separately quantitated in the cortex and medulla of wild-type and Agrtl<sup>−/−</sup> mice under basal conditions and during 24-h water deprivation (30).

**Effects of 24-h water deprivation on adenyl cyclase III proteins in the inner medulla.** Adenyl cyclase III is a major isoform of adenyl cyclase in the inner medulla and mediates [Arg<sup>8</sup>]-AVP-induced cAMP accumulation in the loop of Henle and collecting ducts (1, 14). To determine whether there is a V<sub>2</sub> receptor signaling defect that may contribute to the development of polyuria and low urine osmolality in Agrtl<sup>−/−</sup> mice, we measured basal adenyl cyclase III protein expression and its response to 24-h water deprivation in wild-type and Agrtl<sup>−/−</sup> mice (30). Inner medullary proteins (100 μg) from each mouse were incubated for 3 h at room temperature with an affinity-purified rabbit polyclonal antibody raised targeting the COOH-terminus of adenyl cyclase III of mouse origin (1:200, Santa Cruz Biotechnology). Western blot signals were detected with a goat anti-rabbit secondary antibody and enhanced chemiluminescence (Amersham), as described above for AQP2 (30).

**Effects of 24-h water deprivation on the activation of MAPK ERK1/2 in the inner medulla.** MAPK ERK1 and ERK2 comprise important downstream signaling proteins for AT<sub>1</sub> receptor-mediated cell growth and protein synthesis (26–28). ERK1 and ERK2 are also major downstream signaling proteins for PKA in renal cells (26–28). AVP stimulates V<sub>2</sub> receptors to increase cAMP production in the inner medulla, which activates PKA (23) and induces the insertion of AQP2 proteins in apical membranes (19). We measured total and phosphorylated ERK1/2 proteins in the inner medulla to determine whether a decrease in adenyl cyclase III expression after the deletion of AT<sub>1</sub> receptor signaling in Agrtl<sup>−/−</sup> mice decreases phosphorylated ERK1/2, as previously described (26, 27, 30).

**Effects of 24-h water deprivation on total lysate and membrane AQP2 proteins in the inner medulla.** To determine whether the urine-concentrating defect in Agrtl<sup>−/−</sup> mice is due to a decrease in total lysate AQP2 expression or membrane insertion, all wild-type and Agrtl<sup>−/−</sup> mice were decapitated, and their kidneys were quickly removed at the end of the experiments. The inner medulla was carefully dissected out from each kidney and divided in two portions, with one portion for preparing total protein lysates to measure total AQP2 proteins in the inner medulla and the other portion for preparing membrane fractions to measure membrane AQP2 proteins, as previously described (35, 37). Protein samples (100 μg) from each mouse were size separated by SDS-PAGE using 8–16% Tris-glycine gels, transferred to Millipore Immobilon-P membranes, and blotted overnight at 4°C with 5% nonfat dry milk. Membranes were then incubated for 3 h at room temperature with an affinity-purified goat polyclonal antibody raised to target the COOH-terminus of AQP2 of human origin (1:200, Santa Cruz Biotechnology). After being washed, membranes were further incubated with a donkey anti-goat secondary antibody at 1:10,000 for 1 h at room temperature (sc-2020, Santa Cruz Biotechnology). Western blot signals were detected using enhanced chemiluminescence (Amersham) and analyzed using a microcomputer imaging device with a digital camera (MCID, Imaging Research). To ensure equal protein loading, the same membranes were treated with a stripping buffer (Pierce) for 20 min, blocked with 5% nonfat dry milk, and reprobed with a mouse anti-β-actin monoclonal antibody at 1:2,000 (Sigma-Aldrich) (28, 30, 32).

**Effects of 1-desamino-8-D-AVP infusion on urine osmolality, lysate and membrane AQP2, MAPK ERK1/2, and adenylate cyclase III proteins in the inner medulla.** To determine whether a central mechanism, i.e., partial [Arg<sup>8</sup>]-AVP deficiency, may be involved in the urine-concentrating defects of Agrtl<sup>−/−</sup> mice, two groups each of wild-type and Agrtl<sup>−/−</sup> mice (n = 8) were infused with vehicle or the [Arg<sup>8</sup>]-AVP analog 1-desamino-8-D-AVP (DDAVP) via an osmotic minipump for 2 wk (100 ng/h) (30, 37). DDAVP is commonly used to treat central diabetes insipidus due to [Arg<sup>8</sup>]-AVP deficiency (40). DDAVP binds to V<sub>2</sub> receptors in the collecting ducts of the kidney to stimulate water reabsorption, leading to increased urine osmolality without altering blood pressure (23). Basal and weekly
blood pressure and 24-h urine excretion were measured as previously described. At the end of the experiments, urine osmolality was measured, and inner medullary lysate and membrane AQP2, MAPK ERK1/2, and adenylyl cyclase III proteins were determined as previously described.

Statistical analysis. Data are presented as means ± SE of 7–10 animals/group. Two-way ANOVAs were used to compare the differences between wild-type and Agtr1a mice under basal conditions and after 24-h water deprivation. A paired t-test was used to compare the differences between basal and after 24-h water deprivation in the same mouse, whereas an unpaired t-test was used to compare the differences in the same parameter between wild-type and Agtr1a mice. Significance was set at P values of <0.05.

RESULTS

Effects of 24-h water deprivation on urine excretion and urinary Na⁺ and K⁺ excretions. Table 1 shows basal water intake, systolic blood pressure, and renal excretory phenotypes and their responses to 24-h water deprivation in wild-type and Agtr1a mice. As expected, basal systolic blood pressure was ~28 mmHg lower in Agtr1a mice than in wild-type control mice (P < 0.01). Although basal body weight was not statistically different between two different strains of mice, Agtr1a mice drank three times more water over the 24-h period under basal conditions (P < 0.01). After 24-h water deprivation, Agtr1a mice lost 19% of their body weight compared with ~10% for wild-type mice (P < 0.05). Kidney weight was lighter in Agtr1a mice before and after 24-h water deprivation, and after water deprivation, the kidney-to-body weight ratio was increased by ~30% in Agtr1a mice compared with ~6% in wild-type mice (P < 0.01). Under basal conditions, Agtr1a mice had three times higher urine excretion over the 24-h period than wild-type controls (P < 0.01; Fig. 1, top). After 24-h water deprivation, urine excretion was decreased by ~39% in wild-type control mice compared with ~58% in Agtr1a mice (P < 0.01). In contrast, basal 24-h urinary Na⁺ excretion was not different between wild-type and Agtr1a mice, but it was decreased by ~37% in the latter mice compared with 12% in wild-type mice after 24-h water deprivation (P < 0.01; Fig. 1, middle). Basal 24-h urinary K⁺ excretion appeared to be slightly lower, and it was decreased more in Agtr1a mice after 24-h water deprivation (Table 1 and Fig. 1, bottom).

Effects of 24-h water deprivation on plasma [Arg⁸]-AVP levels. Figure 4 shows that basal plasma [Arg⁸]-AVP levels were ~40% lower in Agtr1a mice than in wild-type mice (wild-type mice: 92.1 ± 3.7 pg/ml vs. Agtr1a mice: 59.8 ± 11.5 pg/ml, P < 0.01). In response to 24-h water deprivation, plasma [Arg⁸]-AVP levels increased by 25% in wild-type mice, whereas they were increased by ~63% in Agtr1a mice (wild-type mice: 115.2 ± 2.5 pg/ml vs. Agtr1a mice: 98.0 ± 5.4 pg/ml, P < 0.01 vs. their controls; Fig. 4). Losartan partially attenuated the AVP response to 24-h water deprivation in wild-type (101.2 ± 3.3 pg/ml, P < 0.05) and Agtr1a mice (86.1 ± 5.6 pg/ml, P < 0.01). Losartan treatment attenuated urine osmolality response to basal levels in wild-type mice but not in Agtr1a mice (Fig. 3, middle). Clearly, urine-concentrating ability was markedly impaired in Agtr1a mice under both basal conditions and after it was challenged by 24-h water deprivation or losartan treatment.

Morphological structures of the inner medulla of wild-type and Agtr1a mice. The anatomic and histological structures of the inner medulla of wild-type and Agtr1a mice are shown in Fig. 5. With the exception that the kidneys of age- and body weight-matched Agtr1a mice were smaller than those of wild-type mice (Table 1 and Fig. 5), comparisons of

Table 1. Basal systolic blood pressure, water intake, body weight, and kidney weight as well as their responses to 24-h water deprivation in wild-type and Agtr1a mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>24-h Water Deprivation</th>
<th>Agtr1a⁻/⁻ Mice</th>
<th>24-h Water Deprivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.1 ± 0.25</td>
<td>24.3 ± 0.24*</td>
<td>26.2 ± 0.83</td>
<td>21.2 ± 0.78**††</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.32 ± 0.01</td>
<td>0.32 ± 0.01</td>
<td>0.24 ± 0.01**</td>
<td>0.25 ± 0.01**</td>
</tr>
<tr>
<td>Kidney-to-body weight ratio, ×100</td>
<td>1.24 ± 0.02</td>
<td>1.32 ± 0.02*</td>
<td>0.93 ± 0.01**</td>
<td>1.21 ± 0.04**</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>3.60 ± 0.50</td>
<td>9.03 ± 0.90††</td>
<td>10.83 ± 0.90††</td>
<td>10.83 ± 0.90††</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>122 ± 6</td>
<td>118 ± 5</td>
<td>94 ± 5††</td>
<td>88 ± 6††</td>
</tr>
<tr>
<td>Urine Na⁺, mmol/l</td>
<td>149.7 ± 3.6</td>
<td>187.3 ± 2.1*</td>
<td>58.4 ± 4.3††</td>
<td>75.4 ± 2.7††</td>
</tr>
<tr>
<td>Urine K⁺, mmol/l</td>
<td>329.9 ± 8.1</td>
<td>345.2 ± 5.2</td>
<td>100.0 ± 6.7††</td>
<td>147.0 ± 8.1††</td>
</tr>
</tbody>
</table>

Values are means ± SE. Agtr1a⁻/⁻ mice, ANG II type 1a receptor-deficient mice. *P < 0.05 and **P < 0.01 vs. the respective baselines of wild-type or Agtr1a⁻/⁻ control mice; ††P < 0.01 vs. the corresponding control or 24-h water-deprived wild-type mice.
hematoxylin and eosin-stained fresh sections of wild-type and Agtr1a*/H11002/H11002 kidneys showed no apparent atrophy, fibrosis, or malformation in the inner medulla of Agtr1a*/H11002/H11002 mice (Fig. 5, B and D). Furthermore, the widths of the entire cortical (wild-type mice: 1.67 ± 0.08 mm vs. Agtr1a*/ */ mice: 1.60 ± 0.05 mm, NS) and renal medulla (wild-type mice: 3.74 ± 0.09 mm vs. Agtr1a*/ */ mice: 3.66 ± 0.07 mm, NS) were not statistically different between wild-type and Agtr1a*/ */ mice (Fig. 5, E and F).

Effects of 24-h water deprivation on [125I]V2 receptor binding and intrarenal distribution in the cortex and medulla. The effects of [Arg8]-AVP on water transport in the inner medulla were mediated primarily by the activation of V2 receptors (23, 36, 45, 50). Figure 6 shows the intrarenal distribution of [125I]V2 receptor binding in the cortex and medulla in wild-type and Agtr1a*/ */ mice under basal conditions and after 24-h water deprivation, as revealed by quantitative in vitro autoradiography. In wild-type mice, [125I]V2 receptor binding predominated in the inner stripe of the outer medulla and inner medulla under basal conditions (cortex: 56 ± 6 disintegrations·min⁻¹·mm⁻² vs. medulla: 123 ± 8 disintegrations·min⁻¹·mm⁻¹, P < 0.01).

In response to 24-h water deprivation, levels of [125I]V2 receptor binding were significantly increased in the cortex (101 ± 10 disintegrations·min⁻¹·mm⁻¹, P < 0.01 vs. basal) and medulla (165 ± 16 disintegrations·min⁻¹·mm⁻¹, P < 0.01 vs. basal), respectively. In Agtr1a*/ */ mice, [125I]V2 receptor binding was not statistically different between the cortex (236 ± 12 disintegrations·min⁻¹·mm⁻¹) and medulla (258 ± 10 disintegrations·min⁻¹·mm⁻¹) under basal conditions. However, binding was significantly increased in the cortex and medulla compared with wild-type mice (P < 0.01). In response to 24-h water deprivation, [125I]V2 receptor binding was significantly increased in the medulla (366 ± 12 disintegrations·min⁻¹·mm⁻¹, P < 0.01 vs. basal) but not in the cortex (225 ± 10 disintegrations·min⁻¹·mm⁻¹, NS vs. basal). Binding affinity was not altered in both wild-type and Agtr1a*/ */ mice (not shown).

Effects of 24-h water deprivation on adenylyl cyclase III proteins in the inner medulla. cAMP plays an important role in mediating AQP2 expression as well as the translocation of AQP2 proteins from the intracellular vesicles to cell membranes in collecting duct cells (1, 8, 23, 30, 36). Adenylyl cyclase III is a major adenylyl cyclase in the inner medulla and mediates intracellular cAMP accumulation in response to V2 receptor activation by [Arg8]-AVP (1, 8, 13–15). Figure 7 shows Western blots of adenylyl cyclase III proteins in the inner medulla of wild-type and Agtr1a*/ */ mouse kidneys at
the basal level and in response to 24-h water deprivation. Under the same condition of free access to tap water, adenylyl cyclase III proteins were decreased by 67% in Agtr1a/−/− mice compared with wild-type control mice (wild-type mice: 100% vs. Agtr1a/−/− mice: 33.3 ± 4.2%, P < 0.001). Twenty-four-hour water deprivation increased the expression of adenylyl cyclase III proteins by 76% in wild-type mice (175.8 ± 30%, P < 0.01), but it had no significant effect on this cyclase in Agtr1a/−/− mice (44.0 ± 4.2%, NS; Fig. 7).

Effects of 24-h water deprivation on MAPK ERK1/2 activation in the inner medulla. MAPK ERK 1 and ERK2 are the downstream signaling proteins for the activation of AT1a receptors as well as PKA in response to AVP stimulation (43, 45, 46). We determined whether the deletion of AT1a receptors would decrease adenylyl cyclase III expression in the inner medulla, which in turn alters MAPK ERK1/2 activation. Figure 8 shows Western blots of total and phosphorylated MAPK ERK1/2 proteins in the inner medulla of wild-type and Agtr1a/−/− mouse kidneys under basal conditions and after 24-h water deprivation. At the basal level, phosphorylated ERK1/2 proteins were decreased by 64% in the inner medulla of Agtr1a/−/− mice compared with wild-type control mice (wild-type mice: 100 ± 0% vs. Agtr1a/−/− mice: 35.6 ± 6.3%, P < 0.001). After 24-h water deprivation, phosphorylated ERK1/2 proteins were increased by 39% in the inner medulla of wild-type mice but not in Agtr1a/−/− mice (wild-type mice: 138.9 ± 13.4% vs. Agtr1a/−/− mice: 39.9 ± 4.8%, P < 0.01; Fig. 8).
from 6 animals/group. Western blots of AQP2 revealed two bands with the unglycosylated form of 29 kDa and the glycosylated form of 45 kDa in the inner medulla (Fig. 9). Under the same condition that all mice had free access to tap water, AQP2 protein expression was ~43% lower in the inner medulla of Agtr1a−/− mice than in wild-type mice (wild-type mice: 100 ± 0% vs. Agtr1a−/− mice: 57.1 ± 3.5%, P < 0.01). As expected, total AQP2 protein expression was increased by 123% in wild-type mice in response to 24-h water deprivation, but it was also increased by 99% in Agtr1a−/− mice (wild-type mice: 222.7 ± 15.6% vs. Agtr1a−/− mice: 199.3 ± 20%, P < 0.01 vs. their controls). However, AQP2 proteins in freshly isolated membrane fractions were increased to a similar extent to total lysate AQP2 in wild-type mice after 24-h water deprivation, whereas this response was markedly attenuated in Agtr1a−/− mice (Fig. 9, bottom).

**Effects of DDAVP infusion on urine osmolality, lysate and membrane AQP2, MAPK ERK1/2, and adenylyl cyclase III proteins in the inner medulla.** Table 2 shows basal blood pressure, plasma and urine osmolality levels, and their responses to 2-wk DDAVP infusion (100 ng/h ip) in wild-type and Agtr1a−/− mice. Unlike native [Arg8]-AVP, which has vascular effects, DDAVP did not alter systolic blood pressure in wild-type mice but slightly elevated systolic blood pressure in Agtr1a−/− mice. As expected, DDAVP significantly increased urine osmolality in wild-type mice by an average of 984 mosmol/kgH2O (P < 0.01). In contrast, DDAVP increased urine osmolality by ~280 mosmol/kgH2O in Agtr1a−/− mice (P < 0.05). The maximal level of increased urine osmolality during DDAVP infusion remained way below the basal level of wild-type mice (Table 2). Figure 10 shows signaling protein responses in the inner medulla to 2-wk DDAVP infusion. Under basal conditions, adenylyl cyclase III, phosphorylated ERK1/2, and total lysate and membrane AQP2 proteins in the inner medulla of Agtr1a−/− mice were only ~30% of their wild-type counterparts. In wild-type mice, DDAVP infusion increased adenylyl cyclase III by 1.6-fold (P < 0.01), phosphorylated ERK1/2 by 3-fold (P < 0.01), total lysate AQP2 by 2-fold (P < 0.01), and membrane AQP2 proteins by 3-fold (P < 0.01), respectively (Fig. 10). In contrast, although DDAVP infusion also significantly increased these responses to levels above their control levels (P < 0.05), the maximal responses reached only ~30% of those observed in wild-type mice (Fig. 10).

**DISCUSSION**

The results of the present study extend our previous findings and those of others by showing that mice with global deletion of AT1a receptors (Agtr1a−/− mice) developed polydipsia, polyuria, and urine-concentrating defects (29, 30, 37) and that neither acute AVP replacement (acute or long term) nor complete 24-h water deprivation fully corrected these defects. These phenotypes in Agtr1a−/− mice are consistent with the results of Western blot analysis with actin reprobed in the same membrane serving as an index of equal loading. Fig. 9, bottom, shows semiquantitative results.
in the inner medulla (Fig. 9). We further showed that wild-type mice responded robustly to 24-h water deprivation by markedly reducing urine excretion and increasing urine osmolality and the expression of adenylyl cyclase III, activated MAPK ERK1/2, and AQP2 proteins in the inner medulla. Similarly, long-term infusion of DDAVP for 2 wk led to similarly robust responses in wild-type mice. In contrast, although 24-h water deprivation and DDAVP also significantly increased inner medullary lysate AQP2 proteins and urine osmolality in Agtr1a−/− mice, both approaches failed to completely correct urine-concentrating defects in Agtr1a−/− mice. These findings strongly suggest that both partial AVP deficiency (central) and impaired inner medullary responsiveness to AVP or water deprivation (nephrogenic) may contribute to urine-concentrating defects in Agtr1a−/− mice. Thus, AT₁a receptor signaling may be necessary for maintaining physiological AVP release and the urine-concentrating capacity in the renal medulla of the kidney.

Whether a central mechanism that mediates AVP release is involved in the development of urine-concentrating defects in Agtr1a−/− mice remains debatable. The present study was not specifically designed to address this issue, and, instead, the level of plasma AVP was used as an indirect function of AVP release. It is well recognized that AVP is primarily synthesized in the hypothalamic neurohypophyseal tract of the brain and...
released from the posterior pituitary in response to changes in extracellular volume and plasma osmolality (6, 10, 16, 40). Thus, comparisons of plasma AVP levels under basal conditions as well as after 24-h water deprivation may allow us to evaluate the role of AT1a receptors in regulating circulating AVP levels in wild-type and Agtr1a<sup>−/−</sup> mice. We further evaluated the role of AT1b receptors by using losartan treatment to block AT1b receptors in Agtr1a<sup>−/−</sup> mice. We found that under the condition that all mice had free access to water, basal plasma AVP levels were ~30% lower in 70% of Agtr1a<sup>−/−</sup> than in wild-type mice, with another 30% of Agtr1a<sup>−/−</sup> mice having AVP levels not different from wild-type mice (n = ~20 each; Fig. 4). This finding may partly explain why Agtr1a<sup>−/−</sup> mice had higher basal urine excretion with markedly reduced urine osmolality (Figs. 2 and 3) and lower expression of adenylyl cyclase III, activation of MAPK ERK1/2, and expression of AQP2 proteins in the inner medulla than wild-type mice (Figs. 7–9). However, the basal AVP levels of our Agtr1a<sup>−/−</sup> mice were different from those reported in angiotensinogen knockout mice (20) or in Agtr1a<sup>−/−</sup> mice (30, 34, 37). In those studies, basal plasma AVP levels were either three to four times higher in angiotensinogen knockout mice (12 pg/ml) than in wild-type mice (3 pg/ml) (20) or similar between Agtr1a<sup>−/−</sup> and wild-type mice, ranging

Table 2. Basal systolic blood pressure, water intake, body weight and kidney weight, and plasma and urine osmolality as well as their responses to DDAVP infusion for 2 wk in wild-type and Agtr1a<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-Type Mice</th>
<th>Agtr1a&lt;sup&gt;−/−&lt;/sup&gt; Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DDAVP</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>22.5 ± 0.4</td>
<td>23.2 ± 1.2</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.34 ± 0.02</td>
<td>—</td>
</tr>
<tr>
<td>Kidney-to-body weight ratio, ×100</td>
<td>1.56 ± 0.01</td>
<td>—</td>
</tr>
<tr>
<td>Water intake, ml/24 h</td>
<td>4.7 ± 0.4</td>
<td>3.1 ± 0.4**</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>124 ± 6</td>
<td>122 ± 9</td>
</tr>
<tr>
<td>Urine excretion, ml/24 h</td>
<td>1.63 ± 0.2</td>
<td>0.34 ± 0.13**</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kgH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>316 ± 2</td>
<td>330 ± 4††</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>2,366 ± 113</td>
<td>3,350 ± 223**</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 wild-type mice and 9 Agtr1a<sup>−/−</sup> mice. 1-Desamino-8-D-arginine vasopressin (DDAVP) was infused at 100 ng/h ip via an osmotic minipump. *P < 0.05 and **P < 0.01 vs. the baselines of control wild-type or Agtr1a<sup>−/−</sup> mice; ††P < 0.01 vs. the corresponding control or DDAVP-treated wild-type mice.
from ~3 to ~300 pg/ml (7, 34, 37). These widely different basal plasma AVP levels in Agtr1a−/− mice are difficult to reconcile but may result from the use of different sample collection and assays, such as anesthetized versus conscious animals or radioimmunoassay versus ELISA.

It is well documented that ANG II, via activation of AT1 receptors in the posterior pituitary and other brain structures, physiologically regulates AVP release from the brain (12, 17, 40). It was expected that the plasma AVP response to 24-h water deprivation would be markedly impaired in Agtr1a−/− mice. Instead, we found that plasma AVP was significantly increased in both wild-type and Agtr1a−/− mice after 24-h water deprivation. This finding may be best explained by the fact that there are two different subtypes of AT1 receptors, AT1a and AT1b, which are coexpressed in the hypothalamic neurohypophyseal tract and posterior pituitary of the brain, with a predominance of AT1b receptors (6, 12, 16, 34, 35, 49). In wild-type animals, the role of AT1a receptors dominates, whereas that of AT1b receptors may be redundant. AT1b receptors may be upregulated in the absence of AT1a receptors and likely mediate the effect of ANG II on AVP synthesis and release from the brain after 5-day water deprivation (41, 35). However, it remains difficult to test the role of AT1b receptors in AVP release in the present study, because AT1a and AT1b receptors share almost 95% genetic homology (6) and there is no antibody or antagonist that specifically targets the AT1b receptor. Alternatively, we used losartan to block AT1b receptors in water-deprived Agtr1a−/− mice. Losartan attenuated AVP responses to water deprivation in both wild-type and Agtr1a−/− mice but had no further effect on urine osmolality in the latter mice (Fig. 3). These results suggest that in the absence of AT1a receptors, AT1b receptors may regulate AVP responses to water deprivation in Agtr1a−/− mice. Mutant mice with knockin or overexpression of the AT1b receptor selectively in the hypothalamus and posterior pituitary of Agtr1a−/− mice may be necessary to further determine the role of AT1b receptors in AVP release.

The results of the present study strongly suggest that nephrogenic mechanisms, namely, functional V2 receptor signaling defects in the inner medulla, play a more dominant role in the development of polyuria and urine-concentrating defects in Agtr1a−/− mice. Previous studies in mice with genetic deletion of the renin gene Ren1c (41), angiotensinogen gene (20), angiotensin-converting enzyme gene (9), or AT1a and AT1b genes (38, 47) have all suggested that renal structural defects are responsible for the urine-concentrating defects in those animals. Structural abnormalities, especially medullary and papillary malformation or atrophy, were clearly manifested in those mice, which were accompanied by significantly impaired urine-concentrating responses to AVP administration or water deprivation (9, 20, 38, 47). Deletion of AT1b receptors alone, however, had no effect on renal structures in AT1b receptor-deficient mice (4), and Agtr1a−/− mice as well as collecting duct-specific Agtr1a−/− mice appear to have normal microscopic cortical and inner medullary structures (18, 37, 43). In the present study, we did not find remarkable structural abnormalities in the papilla of Agtr1a−/− mice or significant differences in the widths of the cortex and medulla between wild-type and Agtr1a−/− mice (Fig. 5). However, it may still be necessary to use high-resolution electron microscopy to systemically examine cortical and medullary structures at the cellular level.

Several nephrogenic mechanisms should be considered to explain the urine-concentrating defects in Agtr1a−/− mice. First, the role of pressure diuresis and natriuresis may be ruled out, because Agtr1a−/− mice have hypotension, which is unlikely to lead to pressure diuresis. Second, the increased water intake in these mice was also unlikely to be a major factor, because the urine-concentrating defects were not corrected in Agtr1a−/− mice by restricting their 24-h water intake to the level of wild-type mice (30). This is supported by the fact that ANG II stimulates thirst via AT1 receptors, and deletion of this receptor is expected to reduce rather than increase water intake in these mice (6, 33). Third, increased filtered load and decreased proximal tubular reabsorption of Na+ and fluid may lead to increases in the end-proximal tubular delivery of water into distal nephron segments, which may lead to diuresis and natriuresis. However, Cervenka et al. (3) found that the glomerular filtration rate and proximal tubular reabsorption rate were not different between wild-type and Agtr1a−/− mice. Similarly, we (30) found no differences in basal FITC-inulin clearance rates between wild-type and Agtr1a−/− mice. Finally, impairment of solute transports in the loop of Henle in the absence of AT1a receptors may diminish osmotic gradients in the renal medullary interstitium, which, in turn, inhibits water absorption in the inner medulla (10, 17, 23,
signaling due to AT1a receptor deletion may be the predominant factor for the urine-concentrating defects in Agtr1a−/− mice. First, basal AQP2 (the key water channel protein in the collecting ducts) proteins (10, 23, 48) were decreased by ~50% in the inner medulla of Agtr1a−/− mice (Fig. 9). Although 24-h water deprivation increased total lysate AQP2 proteins to similar levels and membrane AQP2 proteins in the inner medulla were accompanied by decreases in the basal expression of adenylyl cyclase III and MAPK ERK1/2 signaling proteins in Agtr1a−/− mice (Figs. 7 and 8). These reduced signaling proteins failed to respond to 24-h water deprivation or 2-wk DDAVP infusion in these mice (Figs. 7, 8, and 10). The lack of adenylyl cyclase III and ERK1/2 responses to water deprivation or DDAVP in Agtr1a−/− mice is significant, because adenylyl cyclase III is expressed primarily in inner medulla collecting tubules and appears to mediate V2 receptor-induced cAMP production in collecting duct cells (5, 15, 21, 22). cAMP is the second messenger that mediates the translocation of AQP2 from intracellular vesicles to apical membranes (10, 11, 22). cAMP also activates PKA and MAPK ERK1/2 (19, 22, 27, 28). Activation of ERK1/2 may induce AQP2 phosphorylation, facilitating water transport in the collecting ducts. Taken together, our study suggests that the defects in V2 receptor signaling due to AT1a receptor deletion may be the predominant factor for the urine-concentrating defects in Agtr1a−/− mice.

In summary, the present study demonstrates that global deletion of AT1a receptors and therefore complete removal of AT1a receptor signaling in Agtr1a−/− mice led to impairments of both ANG II/AT1 receptor-regulated basal circulating AVP levels and interactions between ANG II and AVP receptor signaling in the inner medulla. The defects in V2 receptor signaling in Agtr1a−/− mice under basal conditions and in response to water deprivation or DDAVP infusion play a more dominant role than a moderate fall in basal AVP levels. We conclude that the urine-concentrating defects in Agtr1a−/− mice are unlikely to be solely due to AQP2 synthesis, but most likely due to the decreases in AQP2 translocation to apical membranes as a result of decreased adenylyl cyclase III and activation of MAPK ERK1/2 in the inner medulla.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: X.C.L. and J.L.Z. conception and design of research; X.C.L., Y.S., and J.L.Z. performed experiments; X.C.L., Y.S., and J.L.Z. analyzed data; X.C.L. and J.L.Z. interpreted results of experiments; X.C.L., Y.S.; and J.L.Z. prepared figures; X.C.L. and J.L.Z. drafted manuscript; X.C.L. and J.L.Z. edited and revised manuscript; X.C.L., Y.S., and J.L.Z. approved final version of manuscript.

REFERENCES


