Aliskiren restores renal AQP2 expression during unilateral ureteral obstruction by inhibiting the inflammasome

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1Institute of Hypertension, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China; 2Division of Renal Diseases and Hypertension, Anschutz Medical Campus, University of Colorado, Aurora, Colorado; and 3Department of Medicine, University of Utah, and Veterans Affairs Medical Center, Salt Lake City, Utah

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Wang W, Luo R, Lin Y, Wang F, Zheng P, Levi M, Yang T, Li C. Aliskiren restores renal AQP2 expression during unilateral ureteral obstruction by inhibiting the inflammasome. *Am J Physiol Renal Physiol 308: F910–F922, 2015. First published February 18, 2015; doi:10.1152/ajprenal.00649.2014.—Ureteral obstruction is associated with reduced expression of renal aquaporins (AQPs), urinary concentrating defects, and an enhanced inflammatory response, in which the renin-angiotensin system (RAS) may play an important role. We evaluated whether RAS blockade by a direct renin inhibitor, aliskiren, would prevent the decreased renal protein expression of AQPs in a unilateral ureteral obstruction (UUO) model and what potential mechanisms may be involved. UUO was performed for 3 days (3UUO) and 7 days (7UUO) in C57BL/6 mice with or without aliskiren injection. In 3UUO and 7UUO mice, aliskiren abolished the reduction of AQP2 protein expression but not AQP1, AQP3, and AQP4. mRNA levels of renal AQP2 and vasopressin type 2 receptor were decreased in obstructed kidneys of 7UUO mice, which were prevented by aliskiren treatment. Aliskiren treatment was also associated with a reduced inflammatory response in obstructed kidneys of UUO mice. Aliskiren significantly decreased mRNA levels of several proinflammatory factors, such as transforming growth factor-β and tumor necrosis factor-α, seen in obstructed kidneys of UUO mice. Interestingly, mRNA and protein levels of the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome components apoptosis-associated speck-like protein containing a caspase recruitment domain, caspase-1, and IL-1β were dramatically increased in obstructed kidneys of 7UUO mice, which were significantly suppressed by aliskiren. In primary cultured inner medullary collecting duct cells, IL-1β significantly decreased AQP2 expression. In conclusions, RAS blockade with the direct renin inhibitor aliskiren increased water channel AQP2 expression in obstructed kidneys of UUO mice, at least partially by preventing NLRP3 inflammasome activation in association with ureteral obstruction.

Urinary tract obstruction is a serious disorder that may potentially result in irreversible kidney damage associated with impaired renal function, such as a compromised ability to regulate urinary excretion of water and Na+. Release of a ureteral occlusion is characterized by a reduced urinary concentrating capacity, and patients suffer from natriuresis and polyuria. It has been previously demonstrated that the abundance of renal aquaporins (AQPs), Na+ transporters, and urea transporters was significantly reduced in response to ureteral obstruction, suggesting that these proteins at least partly contribute to the urinary concentrating defect in response to urinary tract obstruction (28–32). However, the mediators and intracellular signaling pathways responsible for this downregulation of membrane transporters remain to be elucidated.

Recently, the presence of an intrarenal renin-angiotensin system (RAS) has been demonstrated, and it plays an important role on cellular homeostasis in the kidney. Local RAS activation and its components lead to structural and functional changes in kidney cells, which are independent of those elicited by the classical renin-angiotensin endocrine system (12). Local intracrine/intracellular ANG II is profoundly involved in cell proliferation, oxidative stress, nitric oxide production, and electrolyte metabolism (53). The RAS may play an important role in the progression of obstructive nephropathy. ANG II is synthesized de novo in the kidney and may have direct actions on tubular transport function (6, 40). An increase of intrarenal ANG II content has been shown in the obstructed kidney (15), which may mediate the decrease of glomerular filtration rate (GFR) and renal blood flow (RBF) in ureteral obstruction. Angiotensin-converting enzyme (ACE) inhibitors or ANG II type 1 receptor (AT1R) blockers (ARBs) partly prevented reductions in GFR and RBF (14) and improved expression and trafficking of renal AQP2 and several key Na+ transporters in ureteral obstruction (19, 20, 45), indicating a potential role of ANG II in the urinary concentrating defect of obstructed kidneys. Several studies have also demonstrated that blockade of the RAS attenuates the expression of an array of cytokines and growth factors with important roles in the impairment of renal functions in rats with unilateral ureteral obstruction (UUO) (8, 18, 52). Currently, drugs targeting the RAS, including direct renin inhibitors (DRIs), ACE inhibitors, ARBs, and aldosterone blockers have been most widely used for slowing or preventing renal damage in chronic kidney diseases (49).

DRIs are a new type of RAS inhibitors that target the initial rate-limiting step of the RAS, by blocking the conversion of angiotensinogen to ANG I, which inhibits plasma renin activity (PRA) and reduces the production of ANG II and aldosterone. Direct renin inhibition provides an additional approach for achieving a full blockade of RAS activity without increasing PRA, and renin inhibitors possess an expanded potential for therapy in addition to ACE inhibitors or ARBs. Aliskiren, the only available orally effective DRI, has been demonstrated to be effective in blood pressure reduction and end-organ protection, such as cardiovascular and renal protection in preclinical and clinical experiments. In addition, aliskiren has been shown to protect kidneys from acute kidney injuries induced by either contrast (23) or ischemia-reperfusion (17, 50) or by chronic kidney diseases (e.g., in diabetic nephropathy) (22, 49) as well.
as by doxorubicin-induced nephrotoxicity (39). Aliskiren alone or in combination with other drugs has also been shown to attenuate renal fibrosis and inflammation induced by ureteral obstruction (8, 42, 52).

Accumulating evidence indicates that inflammation in the absence of pathogens, also called sterile inflammation, is mediated through the inflammasome, a large cytosolic multiple protein complex regulating proinflammatory cytokine IL-1β production (1, 10). The inflammasome contains NOD-like receptors (NLRs) associated with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which recruits caspase-1 and induces its activation. Caspase-1 then processes pro-IL-1β into its mature form IL-1β, causing inflammation and tissue damage (25). Recent data now document a role for the NLR family pyrin domain-containing 3 (NLPR3) inflammasome and IL-1β/IL-18 in kidney diseases. Activation of the NLPR3-ASC-caspase-1-IL-1β axis has been demonstrated to play a substantial role in renal injury and fibrosis induced by UUO (47).

The purpose of the present study was to investigate whether aliskiren prevents downregulation of renal AQPs in obstructed kidneys and whether inflammasome inhibition plays a substantial role in this protection.

MATERIALS AND METHODS

Materials. For semiquantitative immunoblot analysis and immunocytochemistry, previously characterized affinity-purified polyclonal antibodies to AQP2, AQP3, and AQP4 were used (44). Antibodies to AQP1 were obtained from Chemicon, IL-1β from Cell Signaling Technology, caspase-1 from Abcam, and ASC from Santa Cruz Biotechnology. Human IL-1β was obtained from Peprotech, and 8-arginine deaminovasopressin (dDAVP) and enalapril were purchased from Sigma-Aldrich.

Animals and treatments. All animal procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University (Guangzhou, China). In brief, 3-month-old male C57BL/6 mice were purchased from the animal facility center of Sun Yat-sen University and maintained on a 12:12-h light-dark cycle at 24°C and received food and water ad libitum before experimentation.

Before surgery, mice were anesthetized with pentobarbital sodium. During surgery, mice were placed on a heated table to maintain rectal temperature at 37–38°C. UUO was established as previously described (31). In brief, UUO was established through a midline abdominal incision, where the left ureter was exposed. The ureter was then occluded by tightening the tubing with a 5-0 silk suture at the midportion. Thirty minutes before UUO surgery, mice were intraperitoneally injected aliskiren, and, in the following days, aliskiren was given twice a day (20 mg·kg body wt−1·day−1). Aliskiren and valsartan were kindly provided by Novartis (Basel, Switzerland).

After either 3 or 7 days, mice were euthanized. Mice were allocated to the protocols described below. Age- and time-matched sham-operated control mice were prepared and observed in parallel with each UUO group in protocols 1–3. Protocol 1 consisted of UUO for 3 days (3UUO), UUO with aliskiren injection (3UUO + Ali), and sham operation (sham) groups (n = 9 mice/group). Protocol 2 consisted of UUO for 7 days (7UUO), UUO with aliskiren injection (7UUO + Ali), and sham operation (sham) groups (n = 9 mice/group). Protocol 3 consisted 7UUO, UUO with ARB valsartan treatment (8 mg·kg−1·day−1 by gavage; 7UUO + Val), UUO with ACE inhibitor enalapril (10 mg·kg−1·day−1 by gavage; 7UUO + Ema), and sham groups (n = 4 or 6 mice/group). In all protocols, the two kidneys in each animal were removed and separately prepared for protein and mRNA measurements or for immunohistochemistry.

Blood and urine chemistry. Urine was collected, and clearance experiments were performed during 24-h periods throughout the study of UUO. At the end of each protocol, blood samples were collected into heparinized tubes for the determination of serum creatinine and osmolality when mice were euthanized. The osmolality of urine and serum was determined by freezing-point depression (OM 806, Omometer, Loser, Germany). Serum and urine concentrations of creatinine were determined using an EIA kit according to the manufacturer’s instructions (BioAssay System). Solute-free water reabsorption was calculated based on the following formula: solute-free water reabsorption = urine output × (urine osmolality/serum osmolality − 1).

Electrophoresis and immunoblot analysis. On the day of euthanization, mice were anesthetized with pentobarbital sodium, and kidneys were frozen in liquid nitrogen immediately after removal. Tissue was minced finely and homogenized in dissection buffer [0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA (pH 7.2)] containing the following protease inhibitors: 8.5 μM leupeptin and 1 mM PMSF. The homogenate was centrifuged at 4,000 g for 15 min at 4°C. Supernatants were assayed for protein concentration using the BCA method (Pierce, Rockford, IL). Gel samples were made from this supernatant. Samples of membrane fractions were run on 12% polyacrylamide minigels. After proteins had been transferred by electroelution to polyvinylidene difluoride membranes, blots were blocked with 5% milk in PBS with Tween 20 (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated with primary antibodies overnight at 4°C. After being washed with PBS-Tween 20, blots were incubated with horseradish peroxidase-conjugated secondary antibody (Pierce). After a final wash as described above, the corresponding secondary antibodies were visualized using enhanced chemiluminescence (Pierce). Signals were quantified with a chemiluminescence detector and the accompanying densitometry software (UVP, Upland, CA).

Immunohistochemistry. Kidneys from UUO and sham mice were fixed by retrograde perfusion via the left ventricle with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. For immunoperoxidase microscopy, sections (4 μm thick) cut from paraffin-embedded kidney samples were used for immunostaining of AQP2 and ASC (29). Briefly, after dewaxing and rehydration, sections were treated with 0.1% sodium citrate in 12% acrylamide minigels. After proteins had been transferred by electroelution to polyvinylidene difluoride membranes, blots were blocked with 5% milk in PBS with Tween 20 (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated with primary antibodies overnight at 4°C. After a rinse in PBS, slides were exposed for 1 h to secondary antibody. Sections were later washed with PBS followed by an incubation with diamobenzidine for 10 min. Microscopic examination was carried out using a Leica DM2000 light microscope (Leica, Heidelberg, Germany).

RNA extraction and quantitative real-time PCR. Total RNA was extracted from kidneys according to the manufacturer’s instructions for TRIzol reagent (Invitrogen). Total RNA (500 ng) was used for reverse transcription using the PrimeScript RT reagent Kit Perfect Real Time kit (Takara Bio). cDNA was used for quantitative real-time PCR analysis using SYBR Premix Ex Taq (Perfect Real Time kit (Takara Bio)). Target mRNA was determined using the comparative cycle threshold method of relative quantitation. The calibrator sample was selected from PBS-treated tissue, and GAPDH was used as an internal control. The primer sequences used are shown in Table 1.

Primary cell culture experiments. Primary cultures of renal inner medullary collecting duct (IMCD) cells were generated with a modification of a previous study (3). In brief, Wistar rats (8–10 wk) were killed by cervical dislocation, and kidneys were quickly removed under sterile conditions. The renal medulla was dissected, minced, and digested for 60 min in 10 ml DMEM containing 0.2% collagenase type I, 0.2% hyaluronidase, and 0.025% trypsin-EDTA at 37°C with shaking. After incubation, cells were then centrifuged at 1,000 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in the modified medium (DMEM and 100 U/ml penicillin G-streptomycin sulfate). Cell suspensions were treated with either IL-1β or...
### Table 1. Primer sequences for real-time PCR (mouse)

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<th>Target Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>5'-GGAGACTGCTATGAAAGGAG-3'</td>
</tr>
<tr>
<td>V2R</td>
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<td>5'-GGAGACTGCTATGAAAGGAG-3'</td>
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<td>5'-AAAGGGACATCATGGCAAG-3'</td>
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<td>5'-CATGCGGAGTGAGATGTC-3'</td>
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<td>5'-GAGATTGCAGCTTCAAGAGTGGAA-3'</td>
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**AQP**, aquaporin; **V2R**, vasopressin type 2 receptor; **PRR**, (pro)renin receptor; **ACE**, angiotensin-converting enzyme; **AT1R**, ANG II type 1 receptor; **AT2R**, ANG II type 2 receptor; **MasR**, Mas receptor; **NLRP3**, NOD-like receptor, pyrin domain-containing 3; **ASC**, apoptosis-associated speck-like protein containing a caspase recruitment domain; **MCP-1**, monocyte chemotactic protein-1; **TGF-β**, transforming growth factor-β.

Aliskiren prevented reduced expression of AQP2 in the obstructed kidneys of mice with 3UUO. As shown in Fig. 1, no significant difference was observed in urinary output, urinary osmolality, serum osmolality, and creatinine clearance among sham, 3UUO, and 3UUO + Ali groups, since in mice with UUO, the unobstructed kidney could fully compensate. In obstructed kidneys of mice with 3UUO, the protein abundance of renal AQP2 was dramatically decreased compared with sham mice; aliskiren significantly prevented such a reduction (Fig. 2, A and B). Protein expression of AQP1, AQP3, and AQP4 was also significantly reduced in obstructed kidneys; however, aliskiren treatment failed to increase AQP1, AQP3, and AQP4 abundance in mice with 3UUO (Fig. 2, A and B). mRNA levels of AQP1, AQP2, and AQP3 were significantly decreased in obstructed kidneys of 3UUO mice (Fig. 3A). Vasopressin type 2 receptor (V2R) in the collecting duct is essential for the regulation of AQP2 trafficking and synthesis. In 3UUO, V2R mRNA was significantly downregulated. Aliskiren treatment did not pre-

Fig. 1. Urine output, urine and serum osmolality, and clearance of creatinine (C<sub>c</sub>) in mice with 3 days of unilateral urethral obstruction (3UUO) after aliskiren treatment. There were no significant changes of urine output (A), urine osmolality (B), serum osmolality (C), and clearance of creatinine (D) among sham-operated (sham), 3UUO, and 3UUO and aliskiren (3UUO + Ali) groups. BW, body weight.
vent the reduced mRNA expression of AQP2 and V2R in obstructed kidneys (Fig. 3A).

Effects of aliskiren on RAS components in obstructed kidneys of mice with 3UUO. Expression levels of RAS components were examined by quantitative real-time PCR (Fig. 4A). Renal mRNA expression of (pro)renin receptor and renin was significantly increased in 3UUO mice treated with aliskiren compared with untreated 3UUO and sham control mice. ACE and ANG(1–7) Mas receptor (MasR) mRNA expression were reduced in obstructed kidneys independent of aliskiren treatment. Aliskiren treatment was associated with increased mRNA expression of AT1R and ANG II type 2 receptors (AT2Rs) in 3UUO mice.

Aliskiren improved the inflammatory response in obstructed kidneys of mice with 3UUO. 3UUO was associated with increased inflammation in the kidneys, as shown in Fig. 5, A and B. mRNA levels of inflammasome components (NLRP3, caspase-1, and IL-1β), monocyte chemotactic protein (MCP)-1, transforming growth factor (TGF)-β, and TNF-α were significantly increased in obstructed kidneys of 3UUO mice, and these increase were attenuated by aliskiren treatment. mRNA expression of ASC and IL-6 was also increased in obstructed kidneys of mice with 3UUO, which was not prevented by aliskiren (Fig. 5, A and B).

Aliskiren prevented reduced expression of AQP2 in obstructed kidneys of mice with 7UUO. Similar to 3UUO, there were no significant differences in urinary output, urine osmolality, and creatinine clearance among the three groups in 7UUO (Fig. 6, A, B, and D). Serum osmolality was slightly higher in 7UUO mice but did not reach statistical significance (Fig. 6C). Solute-free water reabsorption, on the other hand, was significantly reduced in mice with 7UUO, which was prevented by aliskiren treatment (Fig. 6E). Urine samples collected from the pelvis of obstructed kidneys exhibited higher urinary osmolality in 7UUO + Ali groups than those from 7UUO groups (Fig. 6F).

In obstructed kidneys of mice with 7UUO, the protein abundance of renal AQP2 was dramatically decreased compared with sham mice, which was significantly prevented by aliskiren treatment (Fig. 7, A and B). Protein expression of AQP1, AQP3, and AQP4 was also significantly decreased in obstructed kidneys; however, aliskiren treatment did not increase their abundance in mice with 7UUO (Fig. 7, A and B). Immunohistochemistry confirmed an overall decreased labeling in AQP2 expression in collecting ducts of the cortex (Fig. 8, A–C) and the inner medulla (Fig. 8, D–F) of obstructed kidneys, whereas aliskiren treatment was associated with increased AQP2 labeling intensity in obstructed kidneys (Fig. 8, C and F). mRNA levels of AQP1, AQP2, AQP3, and V2R were significantly decreased in kidneys of 7UUO mice. The downregulation of AQP2 and V2R mRNA expression was clearly prevented by aliskiren (Fig. 3B).

Effects of aliskiren on RAS components in obstructed kidneys of mice with 7UUO. mRNA expression of renin, AT1R, and MasR was dramatically increased in obstructed kidneys of 7UUO mice treated with aliskiren compared with untreated 7UUO mice. Unlike 3UUO, aliskiren treatment was associated with downregulated mRNA levels of (pro)renin receptors, renin receptors, and ACE in obstructed kidneys of 7UUO mice (Fig. 4B). mRNA expression of other RAS components did not change among the three groups.

Aliskiren reduced IL-1β mRNA and protein expression in obstructed kidneys of mice with 7UUO. In obstructed kidneys of 7UUO mice, mRNA levels of NLRP3, ASC, caspase-1, IL-1β, IL-18, MCP-1, TGF-β, TNF-α, and IL-6 were significantly increased compared with sham mice. Interestingly, aliskiren prevented the increased mRNA expression of ASC, caspase-1, IL-1β, TGF-β, and TNF-α, and IL-6, but not other inflammatory factors (Fig. 5, C and D). Consistent with the changes of mRNA expression in obstructed kidneys, IL-1β protein expression was significantly upregulated and aliskiren clearly prevented such an increase (Fig. 9, A and B). Express-

Fig. 2. Aliskiren treatment prevented the reduction of water channel aquaporin (AQP)2 protein abundance, but not AQP1, AQP3, and AQP4, in obstructed kidneys of 3UUO mice. A: semiquantitative immunoblots reacted with anti-AQP2, anti-AQP1, anti-AQP3, and anti-AQP4 antibodies. B: corresponding densitometric analyses of protein expression levels of AQP2, AQP1, AQP3, and AQP4 corrected by GAPDH. *P < 0.05 compared with the sham control groups; #P < 0.05 compared with 3UUO groups.
Enalapril treatment in obstructed kidneys compared with untreated 7UUO mice did not reach statistical significance. Valsartan treatment was associated with reduced mRNA expression of IL-1β and IL-6 in obstructed kidneys of 7UUO compared with untreated 7UUO groups (Fig. 12, A and B).

**IL-1β reduced AQP2 expression in primary cultured IMCD cells.** To examine whether increased intrarenal IL-1β during obstruction downregulates AQP2 expression, primary cultured IMCD cells were treated with IL-1β for 6 h. As shown in Fig. 13, A and B, IL-1β treatment led to a significant decrease of AQP2 protein abundance in cultured IMCD cells, which was independent of dosage of IL-1β. When cultured IMCD cells were treated with the V2R agonist dDAVP (Fig. 13, C and D), the induced AQP2 expression was also abolished by IL-1β treatment at different dosages of 1, 5, 10, and 15 ng/ml.

**DISCUSSION**

In the present study, we clearly demonstrated that the DRI aliskiren improved reduced mRNA and protein levels of AQP2 and AQP3 in obstructed kidneys of 7UUO mice. We also showed that aliskiren prevented upregulation of the NLRP3 inflammasome component ASC in obstructed kidneys and, accordingly, inhibited caspase-1 activation and subsequent IL-1β processing and release, which likely mediates downregulation of AQP2 in UUO mice.

**AQP2, V2R, AQP1, and AQP3 in 3UUO (A) and 7 days of 7UUO (B) mice with or without aliskiren treatment. A: in 3UUO groups, mRNA levels of AQP2, V2R, AQP1, and AQP3 in obstructed kidneys were significantly reduced compared with sham mice. Aliskiren treatments did not prevent the reduction of these mRNA expressions in obstructed kidneys of 3UUO mice. B: aliskiren treatment significantly prevented the reduced mRNA levels of AQP2 and V2R, but not AQP1 and AQP3, in obstructed kidneys of mice with 7UUO compared with sham control mice. 7UUO + Ali, 7UUO group with aliskiren treatment. *P < 0.05 compared with sham control mice; #P < 0.05 compared with 7UUO mice.

**Effects of valsartan and enalapril on RAS components in obstructed kidneys of mice with 7UUO.** As shown in Fig. 11B, valsartan and enalapril showed increased renin mRNA levels compared with the sham control group, although they did not reach statistical significance. Valsartan treatment was associated with increased mRNA expression of AT2R and MasR in obstructed kidneys compared with untreated 7UUO mice, which was similar to aliskiren. Enalapril treatment induced increased mRNA levels of renin receptor in obstructed kidneys of 7UUO mice, but it did not reach statistical significance (Fig. 11B).

**Valsartan improved the inflammatory response in obstructed kidneys of mice with 7UUO.** The increased mRNA levels of inflammasome components (NLRP3, ASC, and IL-1β), TGF-β, TNF-α, and IL-6 in obstructed kidneys of 7UUO mice were significantly attenuated by valsartan treatment (Fig. 12, A and B). Enalapril treatment was associated with reduced mRNA expression of IL-1β and IL-6 in obstructed kidneys of 7UUO compared with untreated 7UUO groups (Fig. 12, A and B).

**Fig. 3. Analysis of mRNA expression by quantitative real-time PCR for AQP2, vasopressin V2 receptor (V2R), AQP1, and AQP3 in 3UUO (A) and 7 days of 7UUO (B) mice with or without aliskiren treatment. A: in 3UUO groups, mRNA levels of AQP2, V2R, AQP1, and AQP3 in obstructed kidneys were significantly reduced compared with sham mice. Aliskiren treatments did not prevent the reduction of these mRNA expressions in obstructed kidneys of 3UUO mice. B: aliskiren treatment significantly prevented the reduced mRNA levels of AQP2 and V2R, but not AQP1 and AQP3, in obstructed kidneys of mice with 7UUO compared with sham control mice. 7UUO + Ali, 7UUO group with aliskiren treatment. *P < 0.05 compared with sham control mice; #P < 0.05 compared with 7UUO mice.

**Fig. 4. Analysis of mRNA expression by quantitative real-time PCR for renin-angiotensin system (RAS) components in obstructed kidneys of 3UUO (A) and 7UUO (B) mice with or without aliskiren treatment. PRR, pro(renin) receptor; reninR, renin receptor; ACE, angiotensin-converting enzyme; AT,R, ANG II type 1 receptor; AT,R, ANG II type 2 receptor; MasR, ANG(1–7) Mas receptor. *P < 0.05 compared with sham control groups; #P < 0.05 compared with 3UUO or 7UUO groups.
It is well documented that ureteral obstruction reduces expression levels of AQPs and several key Na+/H+-transporters along the nephron, providing an explanation for the polyuria and Na+/H+-loss observed after the release of ureteral obstruction (30, 31). Candesartan, an ARB, significantly prevented the decrease in AQP2, Na+/K+-2Cl⁻ cotransporter, and Na+/K+-ATPase protein expression in the postobstructed kidneys (20) and in neonatal UUO kidneys (45). Candesartan also prevented

Fig. 5. Analysis of mRNA expression by quantitative real-time PCR for inflammasone components (A and C) and several other proinflammatory factors (B and D) in obstructed kidneys of 3UUO (A and B) and 7UUO (C and D) mice. NLRP3, Nod-like receptor, pyrin domain-containing 3; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; MCP-1, monocyte chemotactic protein-1; TGF-β, transforming growth factor-β. *P < 0.05 compared with sham control groups; #P < 0.05 compared with 3UUO or 7UUO group.

Fig. 6. Urine output, urine and serum osmolality, clearance of creatinine, and solute-free water reabsorption (TcH₂O) in mice with 7UUO after aliskiren treatment. There were no significant changes of urine output (A), urine osmolality (B), serum osmolality (C), and clearance of creatinine (D) among all three groups. E: aliskiren treatment was associated with significantly increased solute-free water reabsorption compared with that in 7UUO mice. F: urine samples collected from the pelvis of obstructed kidneys in 7UUO mice treated with aliskiren exhibited a higher urine osmolality than those in obstructed kidneys of 7UUO mice without aliskiren treatment. *P < 0.05 compared with sham control groups; #P < 0.05 compared with 7UUO groups.
downregulation of the V2R complex in collecting ducts and reversed the obstruction-induced inhibition of stimulated cAMP generation in the inner medulla of obstructed kidneys (19). These findings highlight an important role of RAS activation in the regulation of AQPs and Na+/H+ transporters in obstructive nephropathy.

The present study demonstrated increased AQP2 protein expression independent from changes of AQP2/V2R mRNA in obstructed kidneys at the third day after aliskiren treatment. The observed recovery of AQP2 protein abundance in the obstructed kidney was at least partially due to RAS blockade-associated amelioration of inflammation, in particular, reduced IL-1β levels, as IL-1β directly inhibited AQP2 protein expression in IMCD cells. RAS blockade with aliskiren significantly prevented the downregulation of AQP2 mRNA and protein abundance in collecting ducts of obstructed kidneys with 7UUO. Aliskiren also partially reversed reduced mRNA levels of V2R in obstructed kidneys. These findings indicate a potential improvement of the vasopressin-V2R-AQP2 network by aliskiren, likely via inhibiting IL-1β production, which not only inhibits AQP2 expression but also reduces vasopressin binding (16) in the inner medulla. Urine samples collected from the pelvis of obstructed kidneys exhibited moderately increased urinary osmolality after aliskiren treatment, likely

![Fig. 7. Aliskiren treatment prevented reduction of water channel AQP2 protein abundance, but not AQP1, AQP3, and AQP4 in the obstructed kidneys of 7UUO mice. (A). Semi-quantitative immunoblots reacted with anti-AQP2, AQP1, AQP3, AQP4, and GAPDH antibodies; (B) Corresponding densitometric analyses of protein expression levels of AQP2, AQP1, AQP3, and AQP4 corrected by GAPDH. Sham: sham-operated groups; 7UUO: unilateral ureteral obstruction for 7 days; 7UUO+Ali: 7UUO group with aliskiren treatment. * P < 0.05 compared with Sham controls; # P < 0.05 compared with 7UUO groups.](http://ajprenal.physiology.org/)

![Fig. 8. Immunohistochemistry of AQP2 in obstructed kidneys of 7UUO mice treated with or without aliskiren. Compared with sham-operated control mice, labeling of AQP2 was much weaker in cortical (B) and inner medullary (IM; E) collecting duct principal cells of 7UUO mice, whereas aliskiren treatment was associated with increased staining of AQP2 in obstructed kidneys. B and C: cortical staining of 7UUO (B) and 7UUO + Ali (C) groups. E and F: IM staining of 7UUO (E) and 7UUO + Ali (F) groups. Magnification: ×1,000.](http://ajprenal.physiology.org/)
indicating that increased AQP2 in response to aliskiren treatment actually functioned well. However, the release of UUO would be a better way to evaluate fast recovery of AQP2 and improvement of urinary concentrating ability in response to aliskiren treatment.

In contrast to AQP2, the reduction of AQP3 and AQP4 protein and mRNA levels in UUO kidneys was not prevented by aliskiren, indicating a V2R-independent signaling pathway in AQP3 and AQP4 regulation in response to obstruction. Basolaterally located AQP3 and AQP4 in collecting duct principal cells are thought to represent exit pathways for water reabsorbed via AQP2. The molecular mechanism of AQP3 and AQP4 regulation in the kidney is not fully understood. Alteration of AQP2 expression is not always paralleled with AQP3 in some conditions. Hyperosmolality induced by NaCl upregulated AQP2 expression in vasopressin-deficient Brattleboro rats but not AQP3 (33). The ARB candesartan prevented the reduced expression of AQP2 in obstructed kidneys of rats with bilateral ureter obstruction and release, but the reduction of AQP3 expression persisted (20). These studies indicate a different signaling mechanism of AQP3 regulation from AQP2 in the kidney. AQP2 is also found in the basolateral plasma membrane in principal cells of collecting ducts (9, 36). Although AQP3/AQP4 double-knockout mice have a greater impairment of urinary concentrating ability than AQP3 single-knockout mice, AQP3/AQP4 double-knockout mice are able to modestly raise their urine osmolality after water deprivation or administration of dDAVP, which might be due to the expression of basolateral AQP2 in the same cells (34).

Extrarenal factors may contribute to increased AQP2 expression in response to aliskiren treatment after UUO, e.g., plasma arginine vasopressin (AVP) levels. Urinary tract obstruction is a relatively common condition in acquired nephrogenic diabetes insipidus, characterized as the inability of the kidney to respond to AVP stimulation. Therefore, elevated AVP levels, if any, are unlikely to play an important role in upregulating renal AQP2 after obstruction. Also, a major determinant of AVP release is plasma osmolality. In the present study, serum osmolality among all three groups was not different, probably due to the compensation of the contralateral kidney. AVP levels therefore may not be changed between UUO and UUO + Ali groups. Finally, ANG II type 1a receptors are expressed in the posterior pituitary and other brain regions, where ANG II activates these receptors to modulate AVP release (11). Reduced systemic ANG II induced by aliskiren may not promote AVP release.

The RAS is activated in ureteral obstruction, as evidenced by the fact that ACE inhibitor, ARB, or DRI suppressed renal fibrosis and inflammation induced by UUO (18, 21, 24, 42, 52). Interestingly, studies have demonstrated that ANG II stimulates V2R expression in inner medullary collecting ducts (51). Vasopressin-modulated AQP2 trafficking and expression can be enhanced by ANG II (27, 32), and vasopressin, together with ANG II, determines urinary concentration via coregulation with collecting duct water channels (26, 48). The present findings indeed showed a paradoxical, opposite effect of ANG II on the water transport properties of obstructed kidneys. A previous study (41) has suggested a biphasic response to ANG II on Na+ and water transport in proximal tubules depending on the concentration of the hormone. Physiological concentrations of ANG II may stimulate fluid transport in the kidney, whereas pathological concentrations, as seen in ureteral obstruction, may inhibit it.

Similar to aliskiren, our data showed that both valsartan (8 mg·kg⁻¹·day⁻¹) and enalapril (10 mg·kg⁻¹·day⁻¹) prevented

Table 2. Renal functional data in sham and 7UUO mice with or without valsartan or enalapril treatment

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>7UUO</th>
<th>7UUO + Valsartan</th>
<th>7UUO + Enalapril</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum osmolality, mosmol/kg H₂O</td>
<td>304 ± 2</td>
<td>309 ± 1</td>
<td>310 ± 4</td>
<td>312 ± 11</td>
</tr>
<tr>
<td>Clearance of creatinine, ml·min⁻¹·kg body wt⁻¹</td>
<td>2.25 ± 0.64</td>
<td>2.08 ± 0.61</td>
<td>1.79 ± 0.54</td>
<td>2.34 ± 0.52</td>
</tr>
<tr>
<td>Urine output, µl·24 h⁻¹·g body wt⁻¹</td>
<td>32.2 ± 3.3</td>
<td>44.1 ± 9.5</td>
<td>37.9 ± 8.8</td>
<td>41.9 ± 11.6</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kg H₂O</td>
<td>2047 ± 101</td>
<td>2013 ± 280</td>
<td>2058 ± 288</td>
<td>1912 ± 345</td>
</tr>
<tr>
<td>Solute-free water reabsorption, ml/24 h</td>
<td>5.0 ± 0.5</td>
<td>4.4 ± 0.3</td>
<td>5.3 ± 0.6</td>
<td>5.5 ± 0.8</td>
</tr>
</tbody>
</table>

There were no significant differences of serum osmolality, clearance of creatinine, urine output, urine osmolality, or solute-free water reabsorption among sham-operated (sham), unilateral ureteral obstruction for 7 days (7UUO), 7UUO + valsartan, and 7UUO + enalapril groups.
the downregulation of renal AQP2 at mRNA and protein levels after UUO. At dosage in the present study, valsartan in particular suppressed mRNA expression on most of inflammasome components and proinflammatory factors in obstructed kidneys after UUO, although not as well as aliskiren in some aspects. These results suggest that blockade of the local RAS with DRI, ARB, or ACE inhibitor is beneficial for the recovery of renal AQP2, which is likely via suppressing the inflammatory re-

![Fig. 10. Valsartan (Val) or enalapril (Ena) significantly prevented the reduction of AQP2 protein abundance in obstructed kidneys of 7UUO mice. A: semiquantitative immunoblots reacted with anti-AQP2, anti-AQP1, anti-AQP3, anti-AQP4, and anti-GAPDH antibodies. B: corresponding densitometric analyses of protein expression levels of AQP2, AQP1, AQP3, and AQP4 corrected by GAPDH. *P < 0.05 compared with sham control groups, #P < 0.05 compared with 7UUO groups.](image)

![Fig. 11. Analysis of mRNA expression by quantitative real-time PCR for AQP2, V2R, AQP1, and AQP3 (A) as well as RAS components (B) in obstructed kidneys of 7UUO mice with or without valsartan or enalapril treatment. *P < 0.05 compared with sham control groups; #P < 0.05 compared with 7UUO groups.](image)
response after UUO. DRI may also provide additional protection over other RAS inhibitors by interfering with the enhanced catalytic activity of (pro)renin after the binding of these molecules to the (pro)renin receptor (43). Further investigations are needed to clarify whether various potential signaling pathways, as seen in a recent study [e.g., cyclooxygenase-PGE2 and ROS (38)] are involved in AQP2 regulation after UUO in response to DRI, ARB, or ACE inhibitor. A very recent study (13), however, showed that in contrast to an ACE inhibitor, aliskiren partitions to and is retained in kidneys, where aliskiren was found to localize not only in afferent arteries but also in the distal nephron and collecting ducts (13). Conceivably, this localization pattern resulted merely from the concentrating function of the renal medulla. It indicates a potential involvement of aliskiren in urinary concentration via directly affecting AQPs or Na\(^{+}\)/H\(^{+}\) transporters in the distal nephron and collecting ducts. Further studies are therefore warranted to clarify the underlying pathophysiological significance of the presence of aliskiren at this site in the kidney.

Complete UUO initiates a rapid sequence of events in the obstructed kidney, leading within 24 h to reduced RBF and GFR. This is followed within several days by interstitial...

**Fig. 12.** Analysis of mRNA expression by quantitative real-time PCR for inflammasone components (A) and several other pro-inflammatory factors (B) in obstructed kidneys of 7UUO mice with or without valsartan or enalapril treatment. *P < 0.05 compared with sham control groups; #P < 0.05 compared with 7UUO groups.

**Fig. 13.** IL-1\(^{\beta}\) significantly decreased AQP2 expression in primary cultured IM collecting duct cells. In primary cultured IM collecting duct cells, a suspension of digested cells was treated with IL-1\(^{\beta}\) (1 or 5 ng/ml) for 6 h. A and B: AQP2 expression was deceased significantly by IL-1\(^{\beta}\) treatment at dosages of 1 and 5 ng/ml. C and D: IL-1\(^{\beta}\) treatment at dosages of 1, 5, 10, and 15 ng/ml also decreased V2R agonist 8-arginine deamino-vasopressin (dDAVP; 10\(^{-10}\) M)-induced AQP2 expression. *P < 0.05 compared with control or dDAVP-treated groups.
infiltration of leukocytes, a significant decrease of the urine concentration, and loss of the capacity to concentrate urine as well as fibrosis and apoptosis. We therefore selected particular time points (3UUO and 7UUO) to observe persistently protective effects of aliskiren in the kidney and a differential activation of the local RAS after UUO.

Given the fact that renin catalyzes the first and rate-limiting step of the RAS, DRIIs (e.g., aliskiren) were proposed to be effective RAS inhibitors. Theoretically, aliskiren could decrease both ANG II formation and PRA, but it may induce an increase in renin secretion as a feedback. Aliskiren exerts various effects on several RAS components with 3UUO and 7UUO, suggesting the activation of the local RAS is dependent on the progression of renal injury after UUO. Renin mRNA expression was increased in obstructed kidneys after UUO, and aliskiren treatment resulted in even greater increase of renin mRNA levels than in UUO, indicating a reactive increase in renin or (pro)renin secretion with aliskiren (52). With 7UUO, both (pro)renin receptor and renin receptor mRNA were significantly downregulated by aliskiren, consistent with a recent study (8), probably due to the feedback-suppressive mechanism of (pro)renin and renin receptor expression by a high concentration of (pro)renin.

The regulatory mechanism of other RAS components and their involvement in renal water handling after UUO are not yet fully understood. One interesting finding in the present study is the increased mRNA levels of AT2R and MasR in response to aliskiren treatment after 7UUO. Emerging evidence has shown a protective RAS comprising of the AT2R and ACE2-ANG(1–7)-Mas axis. Both the AT2R and MasR mediate a broad array of tissue-protective effects, including anti-inflammation, anti-fibrosis, anti-apoptosis, and favorable metabolic effects. Increased expression of AT2R and MasR in the obstructed kidney in response to aliskiren treatment may counterbalance the adverse effect of local RAS activation-associated inflammation after UUO. Interestingly, ANG(1–7), via MasR, interacts with the vasopressin V2 system and controls water transport in IMCD cells (35), suggesting an important physiological link between AVP and the RAS in the kidney. More studies are warranted to clarify a role of protective components of the RAS in regulating renal AQP2s in response to UUO.

Our data provide evidence showing that increased inflammatory cytokines that are associated with RAS activation during UUO may play a key role in the regulation of AQP2 expression of the obstructed kidney. UUO is known to be associated with inflammation, which is characterized as infiltration of inflammatory cells and release of cytokines from macrophages and other kidney cells, among which are MCP-1, TGF-β, TNF-α, IL-6, and IL-1β. Interestingly, aliskiren significantly inhibited mRNA expression of these proinflammatory cytokines. In one other study (42), aliskiren also reduced MCP-1 mRNA levels in UUO in the obstructed kidney. The effect of aliskiren on cytokine levels, as observed from the present study, indicated its persistently protective action as an anti-inflammatory agent, which improves the inflammatory response and ameliorates the compromised tubular function that is associated with UUO. This property of aliskiren can be largely attributed to its renin-blocking action, which, in turn, prevented the detrimental effects of ANG II, which is known to play a key role in the UUO-associated inflammatory response. Studies have documented that endotoxemia induced by lipopolysaccharide led to diuresis, downregulation of AQP2 (37, 46), and an impaired V2R/AQP2 network as well (7, 16), suggesting that proinflammatory cytokines may be involved in the regulation of AQP2 and urinary concentrating defects seen in endotoxemia. IL-1β, a key proinflammatory cytokine, is produced by macrophages, monocytes, endothelial cells, tubular epithelial cells, and mesangial cells in the kidney. Recent studies have revealed that chronic administration with IL-1β induced marked increases in urine flow (5) and reduced AQP2 expression via cyclooxygenase-2 in medullary collecting ducts (4) of mice. In the present study, we demonstrated, for the first time, that IL-1β may directly inhibit AQP2 expression in inner medullary suspensions, although the detailed signaling pathway remains to be investigated. Therefore, prevention of the AQP2 decrease by aliskiren in UUO may be, at least partially, mediated through reduced IL-1β production in the kidney.

IL-1β production during kidney injury is known to be regulated by the inflammasome (2), in which ASC recruits and activates caspase-1. Caspase-1 cleaves pro-IL-1β (and pro-IL-18) into mature forms and induces the release of IL-1β and IL-18. It has been documented that NLRP3 deficiency (47) or ASC deficiency (25) improves renal inflammation and fibrosis after ureteral obstruction; in particular, ASC was shown to be upregulated in collecting duct cells after UUO (25). We speculate that the increased ASC after UUO may activate caspase-1; accordingly, more mature IL-1β is released, which mediates the downregulation of V2R and AQP2 in collecting ducts. Consistent with a very recent study (25), mRNA expression of inflammasome components (NLRP3, ASC, caspase-1, and IL-1β) was time dependently (3UUO and 7UUO) increased in obstructed kidneys compared with sham controls. Importantly, aliskiren prevented increased mRNA and protein expression of ASC, caspase-1, and IL-1β in the obstructed kidney, indicating that local RAS activation may play a role in the initiation and maturation of the inflammasome in the kidney during UUO. UUO was associated with increased mRNA expression of NLRP3. Unlike other components of the inflammasome, aliskiren did not reduce NLRP3 mRNA expression with 7UUO. The reason for this is unclear; it may indicate that aliskiren interacts with the inflammasome but not NLRP3. Obviously, further studies are needed to elucidate the molecular link between the inflammasome and UUO-associated kidney injury.

In conclusion, we have shown that RAS blockade with the DRI aliskiren prevented the severely reduced V2R mRNA levels and AQP2 mRNA and protein expression in obstructive kidneys from UUO mice. We also demonstrated that aliskiren significantly inhibited upregulation of the NLRP3 inflammasome component ASC in obstructed kidneys after UUO and, accordingly, suppressed caspase-1 expression and IL-1β production, by which aliskiren may prevent the reduction of AQP2 expression after UUO. Our results indicate a potential role of renal RAS activation in inflammasome maturation during ureteral obstruction. For the first time, we demonstrated a therapeutic potential of aliskiren in the management of obstruction-associated dysregulation of fluid metabolism.

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

AUTHOR CONTRIBUTIONS
Author contributions: W.W. and C.L. conception and design of research; W.W. and C.L. interpreted results of experiments; W.W., R.L., and C.L. drafted manuscript; W.W., M.L., T.Y., and C.L. edited and revised manuscript; R.W. and C.L. performed experiments; R.L., Y.L., F.W., and C.L. analyzed data; R.L. and C.L. prepared figures.

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