Diminished Renal Expression of Aquaporin Water Channels in Rats with Experimental Bilateral Ureteral Obstruction

SOO WAN KIM, SAM HYEON CHO, BONG SUK OH, CHUNG HO YEUM, KI CHUL CHOI, KYU YOUN AHN, and JONGUN LEE

Chonnam National University Research Institute of Medical Sciences, Gwangju, Korea.

Abstract. Whether postobstructive diuresis could be related to altered regulation of aquaporin (AQP) water channels in the kidney was investigated. Male Sprague-Dawley rats underwent bilateral obstruction of the proximal ureters for 48 h. The renal expression of AQP1 to AQP4 proteins was then determined by Western blot and immunohistochemical analyses. For elucidation of the primary impairment in the upstream pathway leading to the expression of cAMP-mediated AQP channels, the expression of Gsα and that of adenylyl cyclase were also determined. For some rats, the obstruction was released for collection of urine samples. After the ureteral obstruction, the urinary flow rate was increased and free water reabsorption was decreased. In the obstructed kidneys, the expression of AQP1 to AQP3 was decreased in the cortex, outer medulla, and inner medulla, whereas that of AQP4 was decreased in the inner medulla. Immunoreactivities for AQP1 to AQP4 were also decreased in the obstructed kidneys. The protein expression of Gsα was decreased in the cortex, outer medulla, and inner medulla, whereas that of adenylyl cyclase VI was decreased in the outer and inner medullae. cAMP generation stimulated by arginine vasopressin was decreased in the cortex, outer medulla, and inner medulla. cAMP generation in response to forskolin was decreased in the outer and inner medullae, whereas that in response to sodium fluoride was decreased in the cortex, outer medulla, and inner medulla. These results suggest that a reduced abundance of AQP water channels in the kidney accounts in part for postobstructive diuresis. The primary impairment of AQP channels that are regulated via the arginine vasopressin/cAMP pathway may lie at the level of G proteins and adenylyl cyclase itself.

Obstruction of the urinary tract is a common cause of the loss of renal function. Although the obstruction is potentially reversible with treatment, marked and sometimes prolonged diuresis associated with an impaired ability to concentrate the urine may follow relief of the obstruction (1). Among patients with chronic partial urinary tract obstructions or with recently relieved partial or complete urinary tract obstructions, decreases in renal concentrating ability can usually be demonstrated (2).

The recent discovery of aquaporin (AQP) channels has increased our understanding of water transport across the permeable epithelial cell membrane. In the kidney, at least six isoforms of AQP proteins have been detected (AQP1, -2, -3, -4, -6, and -7). Among these isoforms, AQP1 is primarily responsible for the constitutive water permeability of proximal tubules and thin descending limbs of Henle (3). Studies using AQP1-knockout mice revealed a dual role of AQP1 in proximal tubular reabsorption and medullary hypertonicity construction (4,5). However, AQP2 to AQP4 are mainly expressed in the collecting duct. AQP2 is specifically located in the principal cells of the collecting duct (6) and is regulated in the short term and long term via the arginine vasopressin (AVP)/cAMP pathway (7,8). In addition, the constitutive localization of AQP3 and AQP4 in the basolateral membrane of principal cells confers to the epithelium high water permeability, in concert with AQP2 insertion into the apical membrane (9,10).

Frøkiaer et al. (11) recently observed a decrease in the expression of AQP2 channels in the kidney after a bilateral ureteral obstruction. However, detailed mechanisms underlying the altered regulation of AQP2 have not been established. In addition, whether there is altered regulation of AQP channels other than AQP2 has not been determined. This study was designed to investigate the regulation of AQP channels in postobstructive kidneys. Rats underwent bilateral ureteral ligation, and the expression of AQP1 to AQP4 in the kidneys was determined by Western blot and immunohistochemical analyses. To further elucidate the primary impairment of AQP channels that are regulated via the AVP/cAMP pathway, the catalytic activities of different parts of adenylyl cyclase complexes were separately examined.

Materials and Methods

Animals and Renal Function

Male Sprague-Dawley rats weighing 200 to 250 g were used. The experimental procedure conformed to the institutional guidelines for experimental animal care and use. The abdominal cavity was opened, and 2-0 silk ligatures were proximally placed on both ureters, using anesthesia with ketamine (50 mg/kg, intraperitoneally). After closure of the abdomen, the animals were maintained for 48 h while being given food and water ad libitum. Control rats were treated in the same way, except that no ligation was made.
On the experimental day, the rats were considered to have a successful ureteral obstruction when the ureteral diameter was >2 mm and evidence of hydronephrosis was present. The kidneys were collected, without release of the ligature, using ketamine anesthesia (50 mg/kg, intraperitoneally). For some rats, the ureteral ligature was released for collection of a urine sample. The urinary bladder was exposed through a low midline abdominal incision and was cannulated with PE-50 tubing. The urine was collected for 1 h (postobstructive hours 3 to 4). At the end of the urine collection, arterial blood was collected for measurements of creatinine clearance and free water reabsorption. The free water reabsorption (TćeH2O) was calculated using the following equation: TćeH2O/V[Uosm/Posm − 1], where V is the urine volume, Uosm is the urine osmolality, and Posm is the plasma osmolality.

Protein Preparation and Western Blot Analyses

The collected kidneys were rapidly frozen and kept at −70°C until assayed. The cortex, outer medulla, and inner medulla from each frozen kidney were dissected and homogenized at 3000 rpm in a solution containing 250 mM sucrose, 1 mM ethylenediaminetetraacetate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl buffer (pH 7.6). Large tissue debris and nuclear fragments were removed by two low-speed centrifugations (1000 g for 10 min and 10,000 × g for 10 min). Protein samples were loaded and electrophoretically size-separated with a discontinuous system consisting of a 12.5% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed with Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST) and were blocked for 1 h with 5% nonfat milk in TBST. The membranes were then incubated for 1 h at room temperature with affinity-purified, anti-rabbit polyclonal antibodies against AQP1 (diluted 1:750), AQP2 (1:750), AQP3 (1:200; Alomone Laboratories, Jerusalem, Israel), AQP4 (1:500; Alpha Diagnostics, San Antonio, TX), heteromeric G protein subunit Gs (1:1000; Calbiochem-Novabiochem, San Diego, CA), or type VI adenylyl cyclase (1:200; Santa Cruz Biochemicals, Santa Cruz, CA) or a 1:750 dilution of a monoclonal mouse IgG (1:1000) in TBST with 2% nonfat milk. The bound antibody was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) with Hyperfilm. The relative protein levels

![Figure 1](https://example.com/figure1.png)
were determined by analyzing the signals with a transmitter scanning video-densitometer.

**Differential Centrifugation**

Comparisons of the magnitudes of AQP2 expression in the membrane-enriched and cytoplasmic fractions allowed assessment of AQP2 trafficking. Differential centrifugation was performed as described previously (12). Total-kidney homogenates were centrifuged at low speed (1000 × g for 10 min) for removal of cellular debris and nuclear fragments. The supernatant was further centrifuged at 17,000 × g for 20 min, to yield a membrane-enriched pellet. Trafficking was determined as the ratio of AQP expression in the high-density fraction (17,000 × g) to that in the low-density fraction (100,000 × g). A decrease in the high-density/low-density ratio reflects inhibited trafficking.

**Immunohistochemical Analyses**

The expression of AQP1, AQP2, AQP3, and AQP4 was also assessed by immunohistochemical analyses using an immunoperoxidase procedure (Vector Laboratories, Burlingame, CA). The rats were anesthetized with sodium thiopental (50 mg/kg, intraperitoneally), and the kidneys were fixed by in vivo perfusion, via the abdominal aorta, with 4% paraformaldehyde for 10 min. The kidneys were then excised and cut into 2-mm coronal slices, which were immersed overnight at 4°C in the same fixatives. The slices were washed in phosphate-buffered saline (PBS), dehydrated in a graded series of ethanol washes, and embedded in paraffin. Tissue sections (6 μm) were cut and mounted on gelatin-coated glass slides.

The tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol washes, rinsed twice with PBS, and then treated for 30 min with 3% H2O2 in 60% methanol, to quench endogenous peroxidase activity. After being washed twice (5 min each) with PBS, the sections were blocked for 1 h in PBS containing 5% normal goat serum. The sections were incubated for 12 to 14 h with AQP1-, AQP2-, AQP3-, or AQP4-specific antibodies diluted (1:100 to 1:3600) in PBS with 0.3% bovine serum albumin. As a negative control, sections were incubated in PBS containing 5% normal goat serum only. The sections were then rinsed three times in PBS and incubated sequentially, for 30 min each time, with biotinylated secondary antibody and avidin-biotin-peroxidase complex reagents (Vector Laboratories, Burlingame, CA), followed by a 6-min incubation with the peroxidase substrate diaminobenzidine. The sections were examined and photographed with a light microscope.

**Membrane Preparation and Adenylyl Cyclase Activity**

The membrane preparation was obtained as described previously (13). The cortex, outer medulla, and inner medulla were separated,
homogenized in ice-cold buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM ethylenediaminetetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose), and centrifuged successively at 1000 g and 100,000 g. The resulting pellet was used as the membrane preparation. Protein concentrations were measured by using a bicinchoninic acid assay kit (BioRad, Hercules, CA).

Adenylyl cyclase activity was assayed by using the method described by Bar (14), with a slight modification. Activity was stimulated with AVP, sodium fluoride, or forskolin. AVP was used to activate V2 receptors, sodium fluoride to stimulate adenylyl cyclase in a receptor-independent but G protein-dependent manner (15,16), and forskolin to directly stimulate the catalytic unit of the adenylyl cyclase complex (17). The reaction was initiated by addition of the membrane fraction (the protein contents of which were 20, 10, and 10 μg for the renal cortex, outer medulla, and inner medulla, respectively) in 100 μl of working solution (50 mM Tris-HCl, pH 7.6, containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine, and 0.02 mM GTP). After 15 min, the reaction was stopped by addition of a cold solution consisting of 50 mM sodium acetate (pH 5.0), and the mixture was centrifuged at 1000 × g for 10 min at 4°C.

cAMP levels in the supernatant were measured by equilibrated RIA. Iodinated 2′-O-monomethyl-cAMP tyrosyl methyl ester was

Figure 4. Immunoblots of AQP3 in the cortex (C), outer medulla (OM), and inner medulla (IM). Protein samples loaded in each lane were as follows: cortex, 30 μg; outer medulla, 40 μg; inner medulla, 40 μg. BUO, bilateral ureteral obstruction. □, control; □, experimental. Each column represents the mean ± SEM for six rats. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.

Figure 5. Immunoblots of AQP4 in the inner medulla. BUO, bilateral ureteral obstruction. □, control; □, experimental. A total of 20 μg of protein was loaded in each lane. Each column represents the mean ± SEM for six rats. *P < 0.05 versus control.

Figure 6. Immunoblots of endothelial nitric oxide synthase (eNOS) protein in the cortex (C), outer medulla (OM), and inner medulla (IM). Protein samples loaded in each lane were as follows: cortex, 100 μg; outer medulla, 80 μg; inner medulla, 80 μg. BUO, bilateral ureteral obstruction. □, control; □, experimental. Each column represents the mean ± SEM for six rats.
Figure 7. Immunohistochemical localization of AQP1, AQP2, and AQP3 in the outer stripe of the outer medulla and of AQP4 in the inner medulla. AQP1 immunoreactivity was most prominent in the apical membrane of proximal tubules (A) and was decreased in the obstructed kidneys (B). AQP2 labeling was observed exclusively in the principal cells of the collecting ducts, both in the apical region and throughout the cytoplasm (C), and was decreased in the obstructed kidneys (D). AQP3 was localized to the principal cells of the collecting ducts (E), and its labeling was decreased in the obstructed kidneys (F). AQP4 was localized to the inner medullary collecting ducts (G), and its labeling was decreased in the obstructed kidneys (H). •, S3 segment of the proximal tubule; ◊, outer medullary collecting duct; ★, inner medullary collecting duct. Magnifications, ×250.
prepared as described previously (18). Standards or samples were taken up in a final volume of 100 μl of 50 mM sodium acetate buffer (pH 4.8). One hundred microliters of dilute anti-cAMP antiserum (Calbiochem-Novabiochem) and 125I-2’-O-monomosuccinyl-cAMP tyrosyl methyl ester (10,000 cpm/100 μl) were then added and incubated for 15 h at 4°C. The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in a gamma counter (Packard Instruments, Meriden, CT). All samples in each experiment were analyzed in a single assay. Nonspecific binding was 2.0%. The 50% intercept was at 16.50.8 fmol/tube (n = 10).

The intra- and interassay coefficients of variation were 5.0 1.2 (n = 10) and 9.6 1.9% (n = 10), respectively. Results were expressed as moles of cAMP generated per milligram of protein per minute.

Drugs were purchased from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise.

**Statistical Analyses**

Results are expressed as mean ± SEM. The statistical significance of differences between groups was determined by using the unpaired t test.

**Results**

**Renal Functional Parameters**

Table 1 presents the renal functional data for the control and experimental groups of rats. A renal failure was evidenced by increases in blood urea nitrogen and serum creatinine levels in the experimental group. The plasma clearance of creatinine was also significantly decreased. The urinary flow rate was increased approximately sevenfold, with increased excretion of sodium in the postobstructive kidneys. The urine/plasma osmolality ratio and tubular free water reabsorption were decreased.

**Expression of AQP Water Channels**

The anti-AQP1 antibody recognized 29-kD and 35- to 50-kD bands, corresponding to nonglycosylated and glycosylated AQP1, respectively. AQP1 expression was decreased in the cortex, outer medulla, and inner medulla (Figure 1). The anti-AQP2 antibody recognized 29-kD and 35- to 50-kD bands, corresponding to nonglycosylated and glycosylated AQP2, respectively. AQP2 expression was decreased in the cortex, outer medulla, and inner medulla, with the decrease being more pronounced in the medulla than in the cortex (Figure 2). The decreases in AQP2 protein expression in the membrane and cytoplasmic fractions were parallel (Figure 3).

The anti-AQP3 antibody recognized 27-kD and 33- to 40-kD bands, corresponding to nonglycosylated and glycosylated AQP3, respectively. AQP3 expression was also decreased in the cortex, outer medulla, and inner medulla, with the decrease again being more pronounced in the medulla than in the cortex (Figure 4). AQP4 expression was detected only in the inner medulla and was also significantly decreased (Figure 5).

Figure 6 presents immunoblots of eNOS protein in the cortex, outer medulla, and inner medulla. In contrast to AQP
channels, there were no significant changes in eNOS expression in either the cortex, outer medulla, or inner medulla in the obstructed kidneys.

**Immunohistochemical Analysis of AQP Channels**

In light-microscopic analyses, renal tubules in the obstructed kidneys demonstrated atrophy and thinning, which were most prominent in the inner medullary collecting ducts. The proximal tubules also demonstrated thinning and loss of brush borders. The labeling of AQP1 to AQP4 was consistently decreased in the obstructed kidneys. AQP1 immunoreactivity was prominently expressed in the apical membrane of proximal tubules and was markedly decreased in the obstructed kidneys (Figure 7, A and B). Abundant AQP2 labeling was observed in the principal cells of collecting ducts, both in the apical region of the cell and throughout the cytoplasm (Figure 7C). Labeling was decreased in the obstructed kidneys, in which residual AQP2 was expressed both in the cytoplasmic region and in the apical membrane (Figure 7D). The expression of AQP3 was localized to the principal cells in the collecting ducts and was also decreased in the obstructed kidneys (Figure 7, E and F). The expression of AQP4 was localized mainly to the inner medullary collecting ducts and was decreased in the obstructed kidneys (Figure 7, G and H). Marked heterogeneity was observed in the collecting ducts of the obstructed kidneys, especially for AQP4. As demonstrated in Figure 7H, some collecting ducts revealed extensive AQP4 labeling, whereas others exhibited weak or no labeling.

**Expression of G_sα and Adenylyl Cyclase VI Proteins**

Figure 8 presents immunoblots of G_sα proteins in the cortex, outer medulla, and inner medulla. Anti-G_sα antibody recognized a doublet at 50 and 45 kD. Expression was significantly decreased in the cortex, outer medulla, and inner medulla of the obstructed kidneys. Figure 9 presents immunoblots of type VI adenylyl cyclase, the major isoform expressed in collecting ducts (19). Its antibody recognized a broad band at approximately 160 kD; expression was decreased in the outer medulla and inner medulla but not in the cortex.

**Adenylyl Cyclase Activity**

Figure 10 demonstrates cAMP generation in response to increasing doses of AVP in the cortex, outer medulla, and inner medulla. The AVP-evoked generation of cAMP was attenuated in the cortex, outer medulla, and inner medulla of the obstructed kidneys, and attenuation was more pronounced in the medulla than in the cortex. The cAMP generation stimulated by sodium fluoride was significantly decreased in the cortex, outer medulla, and inner medulla (Figure 11). The cAMP generation in response to forskolin was not significantly affected in the cortex but was decreased in the outer medulla and inner medulla of the obstructed kidneys (Figure 12).

**Discussion**

After the relief of ureteral obstruction, urinary flow rates were increased sevenfold, with decreases in tubular free water reabsorption and urinary osmolality. These findings are in accord with observations of nephrogenic diabetes insipidus, with polyuria and hypotonic urine, among patients with ureteral obstructions (20,21). This study further examined whether the postobstructive failure of urinary concentration could be related to altered regulation of AQP water channels in the kidney. After ureteral obstruction, the expression of AQP1 to AQP4 proteins was indeed decreased in the cortex, outer medulla, and inner medulla of obstructed kidneys.

Among AQP channels in the kidney, AQP2 is specifically regulated via the AVP/cAMP pathway (7,8). This study demonstrated that cAMP generation in response to AVP was blunted in the obstructed kidneys, with the decrease being more pronounced in the medulla than in the cortex. Furthermore, cAMP generation in response to forskolin, which directly activates the catalytic unit of adenylyl cyclase (17), was decreased in the medulla of the obstructed kidneys. The cAMP generation in response to sodium fluoride, which activates
adenylyl cyclase in a receptor-independent but G protein-dependent manner (15,16), was also significantly blunted. Accordingly, the expression of Gs\textsubscript{\text{H9251}} and that of adenylyl cyclase VI were significantly decreased. Not only Gs\textsubscript{\text{H9251}} protein but also the catalytic unit of adenylyl cyclase itself may be impaired in the obstructed kidneys, and the dysregulation of AQP2 channels may be attributable to these impairments. The minimal or absent changes in adenylyl cyclase activity in the cortex may be in line with the previous hypothesis that the cortex is normally not heavily loaded with adenylyl cyclase activity (22). It is also likely that the impairment may become more extensive in the obstructed kidneys when the degree of local changes, such as tubular pressure changes, becomes greater.

However, the reduction of AQP2 channels was parallel in the membrane-enriched and cytoplasmic fractions, suggesting preserved trafficking. Similar findings were noted in rats with several acquired forms of nephrogenic diabetes insipidus syndromes, such as cisplatin-induced nephropathy (23), lithium-induced nephropathy (24), and chronic renal failure induced by surgical renal mass reduction (25).

AQP3 is mainly localized to the basolateral membrane of collecting duct principal cells in the cortex and outer medulla (26). Our study demonstrated that AQP3 expression was decreased in the cortex, outer medulla, and inner medulla of the obstructed kidneys, more prominently in the medulla. Recent studies demonstrated long-term regulation of AQP3 (with a marked increase in its expression in the collecting ducts), but not AQP1 or AQP4, in response to water restriction or AVP infusion (8,26,27). This finding suggests that the AVP/cAMP pathway also has a role in the long-term regulation of AQP3. The decrease in AQP3 abundance may also be attributable to an impairment in the activity of the AVP/cAMP pathway.

AQP1 is present in large amounts in both apical and basolateral membranes of proximal tubules and descending thin limbs (3). AQP1 gene-knockout mice demonstrated 80 to 90% reductions in osmotic water permeability in the proximal tu-
bules and descending thin limbs and became severely dehydrated (4,5). We demonstrated in this study that AQP1 expression was significantly reduced in the obstructed kidneys, as revealed by both immunohistochemical and Western blot analyses. The decrease in AQP1 expression was more prominent in the cortex and outer medulla than in the inner medulla. Although the altered expression of AQP1 could be causally related to postobstructive diuresis, it may also indicate differential regulation of AQP1, compared with AQP2 and AQP3. Mechanisms regulating AQP1 remain to be further elucidated.

Although it is primarily expressed in the brain, AQP4 is also expressed in the basolateral membrane of inner medullary collecting ducts (28). It presumably provides the exit during AVP-dependent water reabsorption. Recently, transgenic knockout mice lacking AQP4 demonstrated a mild urinary concentration defect and a fourfold reduction of water permeability in the inner medulla (29,30). The decreased expression of AQP4 in the inner medulla may also contribute to postobstructive diuresis.

The uniform decrease in AQP water channels, as well as other proteins such as Gs, and adenyl cyclase, might indicate the possibility that ureteral obstruction causes generalized cellular dysfunction and protein downregulation, rather than a specific effect. However, the expression of eNOS protein (a positive control) was not altered by the ureteral obstruction, which is consistent with previous observations in unilaterally obstructed kidneys (31).

In summary, the expression of AQP water channels was decreased in the ureter-obstructed kidneys, which may at least in part account for the urinary concentration defect associated with postobstructive diuresis. The primary impairment of AQP channels that are regulated via the AVP/cAMP pathway may lie at the level of G proteins and adenyl cyclase itself.

Acknowledgments

This work was supported by Korea Research Foundation Grant KRF-2001-041-F00142.

References


Access to UpToDate on-line is available for additional clinical information at http://www.jasn.org/