Bilateral ureteral obstruction induces early downregulation and redistribution of AQP2 and phosphorylated AQP2

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Production of concentrated urine depends partly on the high concentration of AQP2 and phosphorylated AQP2.


PRODUCTION OF CONCENTRATED urine depends partly on the high water permeability of the collecting duct in response to the antidiuretic hormone vasopressin, primarily through increased expression of the water channel aquaporin-2 (AQP2) on the apical side of inner medullary collecting duct (IMCD) principal cells (33). During normal physiological conditions, the amount of AQP2 is regulated by vasopressin through trafficking of AQP2-containing intracellular vesicles to the apical membrane (33). This trafficking is regulated by the phosphorylation of AQP2 on serine 256 by protein kinase A (11, 21), and it has been shown that vasopressin increases this phosphorylation, thereby increasing water reabsorption (11, 21, 33, 34). AQP2 contains four serine residues in the C terminal, namely, ser256, ser261, ser264, and ser269. Recently, it has been shown that phosphorylation of all four sites is regulated by vasopressin (8, 15–17). Vasopressin treatment increases the phosphorylation of ser256, -264, and -269 (8, 15, 16) and decreases the phosphorylation of ser261 (16). It has also been demonstrated that phosphorylation of ser256 is a prerequisite for the phosphorylation of ser264 and -269 (15), thereby indicating a coherent function of the four sites in AQP2 trafficking.

It has been speculated whether phosphorylation of ser261 may be involved in regulation of the subcellular localization of AQP2 due to the differential subcellular pools of phosphorylated ser256 and ser261 in the presence of vasopressin (16). However, it was recently shown that mutations of ser261 in LLC-PK1 cells hindered the phosphorylation and dephosphorylation did not affect the AQP2 trafficking pattern (27). In agreement with this, recent studies demonstrated that in Madin-Darby canine kidney (MDCK) cells the same mutated forms of ser261 are still able to accumulate on the plasma membrane in response to forskolin stimulation, although to a lesser extent than wild-type (WT) in the case of the mutant AQP2S261A (32). This indicates that ser261 phosphorylation is unlikely to play an important role in the vasopressin- or cAMP-induced membrane accumulation of AQP2 in this model. The specific role of phosphorylation of ser261 remains unclear.

Obstructive kidney diseases are characterized by an impairment of the urine flow from the kidneys (22). It has previously been shown in rat models of ureteral obstruction that the polyuria and impaired urinary concentrating ability observed after release of the obstruction are associated with significant changes in the expression of renal aquaporins (9, 10). Moreover, it has been shown that AQP2 phosphorylated at ser256 is reduced in response to 24-h unilateral ureteral obstruction (UUO) (24) and 24-h bilateral ureteral obstruction (BUO) followed by release (35). These models clearly demonstrate an involvement of AQP2 in the observed impairment of urine concentrating capacity in obstructed rats, but yet the exact mechanisms behind the downregulation of AQP2 are unknown. To examine the causative effects of downregulation of AQP2 in BUO, the time cause of the onset is important. Most studies to date regarding AQP2 downregulation in BUO models have been performed in animals obstructed for 24 h or longer. One recent study from our group showed reduced AQP2 protein abundance after 12-h BUO, but did not address AQP2 phosphorylation (36). One could speculate that because vasopressin levels are high in BUO (38), the AQP2 phosphorylated at ser261 would be decreased as it is inversely regulated by vasopressin. AQP2 phosphorylated at ser256, which is the only investigated phosphorylation in BUO models, would be...
expected to be increased due to the high vasopressin levels, but this is not the case. In the present study, the early dynamics of AQP2 regulation were examined in rats subjected to BUO in a time-dependent pattern and the expression of AQP2 and selected phosphorylated isoforms of AQP2 were investigated.

MATERIALS AND METHODS

Experimental animals. All procedures conformed to the Danish national guidelines for the care and handling of animals and the published guidelines from the National Institutes of Health. The animal protocols were approved by the board of the Institute of Clinical Medicine at the University of Aarhus according to the licenses for use of experimental animals issued by the Danish Ministry of Justice.

Male Munich-Wistar rats (Moellegaard Breeding Center, Eiby, Denmark), initially weighing 250 g, were used in the experiments. The rats had free access to water and to a standard rodent diet (Altromin, Lage, Germany). During the experiment, the rats were kept in cages with a 12:12-h artificial light cycle, a temperature of 21°C, and a humidity of 55 ± 2%.

The rats were anesthetized with isoflurane (Abbott Scandinavia), and during the surgery the rats were placed on a heating pad to maintain rectal temperature at 37–38°C. Through a midline abdominal incision, both ureters were exposed and a 3-0 silk ligature was used to occlude the ureters (BUO). Age- and time-matched sham-operated controls were prepared and were observed in parallel with each BUO group.

Rats were allocated to the protocols indicated below.

Protocol 1. BUO was induced for 2, 6, 12, or 24 h (n = 6 for each time point), or rats were sham operated (n = 6 for each time point) as controls. The two kidneys were removed, and the inner medullas were isolated. The right kidney inner medulla was used for semiquantitative immunoblotting, and the left kidney inner medulla was used for quantification of mRNA using Q-PCR.

At the end of each protocol, 2–3 ml of blood was collected into a heparinized tube for determination of plasma electrolytes and osmolality. The plasma concentrations of sodium, potassium, creatinine, and urea were determined (Vitros 950, Johnson & Johnson). The osmolality of plasma was determined by freezing-point depression (Advanced Osmometer, model 3900, Advanced Instruments, Norwood, MA, and Osmomat 030-D, Gonotec, Berlin, Germany).

Protocol 2. BUO was induced for 2, 6, 12, or 24 h (n = 6 for each time point), or rats were sham-operated (n = 6 for each time point) as controls. The rats were used for immunohistochemistry and prepared as described below.

Q-PCR. For quantitative PCR, 100 ng cDNA served as a template for PCR amplification using Brilliant SYBR Green QPCR Master Mix according to the manufacturer’s instruction (Stratagene). Serial dilution (1 ng–1 fg/μl) of cDNA was used as a template for generation of a standard curve. Nested primers were used to amplify standards and kidney cDNA samples. TATA box binding protein (TBP): sense GAC TCC TGT CTC CCC TAC CC, antisense CTC ACG GAG GAG GGA AC, GenBank accession no. NM_001004198; AQP: sense CTT CCT TCG AGC TGC CTT CC, antisense CAT TGT TGT GAG GAG CAT TGA C, GenBank accession no. NM_012909; and vasopressin 2 receptor (V2R): V2R NH2 terminal, sense ATGCTCTGGTGTCTACCGTGTTCCG, antisense GCGTCCAGCGCCGCGCCCGCCAT.

Standards and unknown samples were amplified in duplicate in 96-well plates, and PCR was performed for 40 cycles consisting of denaturation for 30 s at 95°C followed by annealing and polymerization at 60°C for 1 min. Emitted fluorescence was detected during the annealing/extension step in each cycle. Specificity was ensured by postrun melting curve analysis.

Membrane fractionation for immunoblotting. The tissue (IM) was homogenized in dissecnting buffer [0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing the following protease inhibitors: 8.5 μM leupeptin (serine and cysteine protease inhibitor, Sigma-Aldrich) and 0.4 mM pefabloc (serine protease inhibitor, Roche)]. The tissue was homogenized for 30 s by an Ultra-Turrax T8 homogenizer (IKA Labortechnik) and then centrifuged at 1,500 g at 4°C for 15 min. Gel samples were prepared from the supernatant in Laemmli sample buffer containing 2% SDS. The total protein concentration of the homogenate was measured using a Pierce BCA protein assay kit (Roche).

Electrophoresis and immunoblotting. Samples of the membrane fraction were run on a 12% polyacrylamide gel (Bio-Rad Mini) or on a 4–15% Criterion precast gel (Bio-Rad). For each gel, an identical fraction were run on a 12% polyacrylamide gel (Bio-Rad Mini) or on a 4–15% Criterion precast gel (Bio-Rad). For each gel, an identical gel was run in parallel and subjected to Coomassie staining. The Coomassie-stained gel was used to confirm identical loading or to allow for correction for minor variations in loading.

Proteins were transferred to a polyvinylidene difluoride membrane (Hybond enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) or Immobilon-P PVDF (Millipore)) for the V2R antibody. After transfer, the blots were blocked with 5% nonfat dry milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, adjusted to pH 7.4 with 10 M NaOH). After washing with PBS-T, the blots were incubated with primary antibodies overnight at 4°C on a tilting table. The antigen-antibody complex was visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000, Dako) using the ECL system (Amersham Pharmacia Biotech).

Table 1. Biochemical values from plasma samples from Sham and BUO rats at different time periods

<table>
<thead>
<tr>
<th></th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
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<tr>
<td>Creatinine, μmol/l</td>
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<tr>
<td>Sham</td>
<td>10.2 ± 3.4</td>
<td>17.6 ± 3.2</td>
<td>16.3 ± 2.9</td>
<td>14.8 ± 2.6</td>
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<tr>
<td>BUO</td>
<td>37.8 ± 6.2*</td>
<td>131.2 ± 6.2*</td>
<td>231.6 ± 11.8*</td>
<td>329.3 ± 5.0*</td>
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<td>Osmolality, mosmol/kgH2O</td>
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<td></td>
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<tr>
<td>Sham</td>
<td>302.5 ± 2.1</td>
<td>309.0 ± 1.1</td>
<td>302.7 ± 1.1</td>
<td>303.8 ± 1.0</td>
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<tr>
<td>BUO</td>
<td>300.3 ± 1.5</td>
<td>315.6 ± 2.5</td>
<td>309.0 ± 6.3</td>
<td>345.3 ± 1.7*</td>
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<tr>
<td>Urea, mmol/l</td>
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<tr>
<td>Sham</td>
<td>7.2 ± 0.3</td>
<td>7.3 ± 0.7</td>
<td>5.4 ± 0.3</td>
<td>5.8 ± 0.3</td>
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<tr>
<td>BUO</td>
<td>9.0 ± 0.3*</td>
<td>16.0 ± 0.5*</td>
<td>26.4 ± 1.3*</td>
<td>44.3 ± 1.7*</td>
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<tr>
<td>Sodium, mmol/l</td>
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<tr>
<td>Sham</td>
<td>136.0 ± 2.3</td>
<td>138.8 ± 0.4</td>
<td>136.7 ± 0.6</td>
<td>140.2 ± 0.8</td>
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<tr>
<td>BUO</td>
<td>135.6 ± 0.9</td>
<td>135.5 ± 1.1*</td>
<td>125.6 ± 4.3*</td>
<td>135.3 ± 0.8*</td>
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<tr>
<td>Potassium, mmol/l</td>
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<td></td>
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<tr>
<td>Sham</td>
<td>4.4 ± 0.2</td>
<td>4.5 ± 0.3</td>
<td>3.9 ± 0.1</td>
<td>4.2 ± 0.1</td>
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<tr>
<td>BUO</td>
<td>5.2 ± 0.2*</td>
<td>5.8 ± 0.3*</td>
<td>6.8 ± 0.5*</td>
<td>6.8 ± 0.3*</td>
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Values are means ± SE; n = 6/group. Sham, sham-operated control rats, BUO, bilateral ureteral obstruction. *P < 0.05 for BUO compared with Sham.
Densitometric analysis was performed on the specific protein bands, and the abundance in the BUO animals was calculated as a fraction of the mean of the sham-operated rats. For AQP2, both the glycosylated and nonglycosylated bands were used for densitometry. Primary antibodies. For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized monoclonal and polyclonal antibodies as summarized below.

AQP2 (LL127AP): an affinity-purified rabbit polyclonal antibody to AQP2 has previously been described (4).

AQP2pS256 (AN224-pp-AP): an affinity-purified rabbit polyclonal antibody to AQP2pS256 has previously been described (3).

AQP2pS261: an affinity-purified rabbit polyclonal antibody to AQP2pS261 has previously been described (7).

Commercial antibodies used were a mouse monoclonal antibody against early endosome-associated protein (EEA1; BD Transduction Laboratories, Franklin Lakes, NJ) and a goat polyclonal antibody against cathepsin D (Research Diagnostics).

Immunohistochemistry. The kidneys from BUO rats and sham-operated control rats were fixed by retrograde perfusion via the abdominal aorta with 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Moreover, the kidneys were immersion fixed for 1 h and washed for 3 × 10 min with 0.1 M cacodylate buffer. The kidney blocks were dehydrated and embedded in paraffin. The paraffin-embedded tissues were cut into 2-μm sections on a rotary microtome (Leica Microsystems, Herlev, Denmark).

For immunoperoxidase labeling, the sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 5% H2O2 in absolute methanol for 10 min at room temperature. To expose antigens, kidney sections were boiled in a target retrieval solution (1 mmol/l Tris, pH 9.0 with 0.5 mM EGTA) for 10 min. After cooling, nonspecific binding was prevented by incubating the sections in 50 mM NH4Cl in PBS for 30 min, followed by blocking in PBS containing 1% BSA, 0.2% gelatin, and 0.05% saponin. Sections were incubated with primary antibodies diluted in PBS with 0.1% BSA and 0.3% Triton X-100 overnight at 4°C. After being washed for 3 × 10 min with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin, the sections were incubated with HRP-conjugated secondary antibody (P448, goat anti-rabbit immunoglobulin, Dako, Glostrup, Denmark) for 1 h at room temperature. After rinsing with PBS wash buffer, the sites of antibody-antigen reactions were visualized with 0.05% 3, 3′-diaminobenzidine tetrachloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2. The light microscopy was carried out with Leica DMRE (Leica Microsystems).

Confocal laser-scanning microscopy. For confocal laser-scanning microscopy studies, the following secondary fluorescent antibodies were used at a 1:1,000 dilution: goat anti-rabbit IgG, Alexa Fluor 555; goat anti-mouse IgG, Alexa Fluor 488; and goat anti-sheep IgG, Alexa Fluor 546 (Molecular Probes, Invitrogen). Laser confocal microscopy was carried out with a Leica TCS-SP2 laser confocal microscope.

Statistics. Values are presented as means ± SE. Statistical comparisons between experimental groups were made by a standard unpaired t-test or one-way ANOVA with Bonferroni correction. Values <0.05 were considered significant.

RESULTS

BUO is associated with an acute impairment of plasma water and salt balance. In rats subjected to BUO for varying durations between 2 and 24 h, osmolality and electrolyte concentrations in plasma were determined (Table 1). Two-hour BUO resulted in a significant increase in the levels of plasma creatinine (37.8 ± 6.22 vs. 10.2 ± 3.44 μmol/l), urea (9.0 ± 0.32 vs. 7.2 ± 0.27 mmol/l), and potassium (5.2 ± 0.18 vs. 4.4 ± 0.19 mmol/l) compared with sham-operated controls. Rats subjected to 6, 12, and 24 h of obstruction also had decreased plasma sodium levels (135.5 ± 1.12 vs. 138.8 ± 0.37, 125.6 ± 4.27 vs. 136.7 ± 0.61, and 135.3 ± 0.76 vs. 140.2 ± 0.79 mmol/l, respectively) compared with sham-operated controls. Furthermore, plasma osmolality was higher after 24 h BUO compared with sham-operated control rats (345.3 ± 1.67 vs. 303.8 ± 0.95 mosmol/kgH2O).

Decreased expression of total AQP2 mRNA and protein abundance after BUO. To determine the time frame of the known AQP2 regulation in a BUO model (10), AQP2 mRNA expression and total AQP2 protein abundance were investigated at different durations of obstruction. Q-PCR showed that as early as after 2 h of obstruction and at all the following time points investigated (6, 12, and 24 h), the expression of AQP2 mRNA normalized for TBP was significantly lower in BUO rats compared with the sham-operated control rats (52, 11, 4, and 2%, respectively) (Fig. 1A). The mRNA level for TBP was not significantly different between groups. The protein abundance of AQP2 after 12-h BUO was significantly decreased (1.67 vs. 303.8 ± 103.7 mosmol/kgH2O).

Fig. 1. Aquaporin-2 (AQP2) mRNA expression and protein abundance in the inner medulla (IM) from rats subjected to sham and bilateral ureteral obstruction (BUO) for different time periods. A: representative quantitative PCR (Q-PCR) for AQP2 and TATA box-binding protein (TBP). For Q-PCR, 100 ng of cDNA was used. Quantification showed reduced AQP2 expression after 2-h BUO. B: semiquantitative immunoblot reacted with anti-AQP2 antibody revealed 29- and 35- to 50-kDa AQP2 bands. Densitometric analyses of the data demonstrated a significantly decreased AQP2 protein abundance after 12-h BUO compared with sham-operated control rats (345.3 ± 1.67 vs. 303.8 ± 0.95 mosmol/kgH2O).

Values are means ± SE. *P < 0.05 BUO vs. sham.
remained constant in sham rats at all investigated time points (data not shown). Semiquantitative immunoblotting was performed on inner medulla homogenates. AQP2 protein abundance did not change in kidneys from 2- and 6-h BUO rats, but after 12 and 24 h BUO significant downregulation was observed (68 and 21%, respectively) compared with sham-operated rats (Fig. 1B).

Consistent with this, immunohistochemical analysis showed a much weaker labeling of anti-AQP2 antibody in kidney IMCD principal cells in rats subjected to 12- and 24-h BUO compared with sham-operated control rats (Fig. 2). There was no difference in labeling intensity between sham rats at the four time points.

AQP2 phosphorylated at ser256 is acutely regulated in the BUO model. The contribution of different durations of obstruction on AQP2 phosphorylated at ser256, a site important for AQP2 exocytosis (11, 21, 43), was investigated. AQP2pS256 protein was significantly downregulated after 6-, 12-, and 24 h-BUO (69, 55, and 4% of sham, respectively) (Fig. 3). In rats subjected to 2-h BUO, the level of AQP2pS256 protein was 81% of sham levels (P = 0.089). Consistent with this, immunohistochemistry showed a reduced intracellular labeling of AQP2pS256 at the apical membrane of the IMCD principal cells in obstructed animals compared with sham-operated rats (Fig. 4, A–J). Already at 2-h BUO there was a markedly weaker labeling at the apical membrane and a more pronounced intracellular distribution of the protein (Fig. 4G) compared with sham (Fig. 4H). The labeling became weaker in proportion with the longer duration of obstruction, and after 24-h obstruction it was difficult to observe any labeling of the IMCD cells (Fig. 4, E and J).

Regulation of abundance of AQP2 phosphorylated at ser261 mimics total AQP2 regulation. The abundance and localization of AQP2 phosphorylated at ser261 were examined and showed a significant downregulation of AQP2pS261 protein after 12- and 24-h BUO (57 and 0.64%) compared with sham-operated rats (Fig. 5). Hence the changed protein abundance for AQP2pS261 mimics the pattern of the total AQP2 regulation in response to BUO.

Consistent with the results from immunoblotting, the anti-AQP2pS261 antibody labeled strongly at the apical membrane of IMCD principal cells in sham-operated rats (Fig. 6, A and E). Analysis of rats subjected to 2-h BUO (Fig. 6, B and F) demonstrated a more dispersed and less apical labeling of IMCD principal cells compared with sham, and after 6-h BUO (Fig. 6, C and G) an overall decreased staining was observed. In IMCD principal cells from rats subjected to 12 h of obstruction, there was a weaker labeling in general (Fig. 6D), and the labeling was more pronounced located in small clusters (Fig. 6H).

Total AQP2 and AQP2pS261 are redistributed to early endosomes and lysosomes. To explore the intracellular localizations of AQP2, AQP2pS256, and AQP2pS261 in more detail, confocal laser-scanning microscopy using antibodies against AQP2 or against the two phosphorylated isoforms and antibodies either for known endosomal protein (EEA1) or known lysosomal protein (cathepsin D) was performed. In sham-operated rats, total AQP2 was located mostly apically but also intracellularly (Fig. 7). After 2-h BUO, AQP2 redistributed to a more intracellular location, and a much weaker apical labeling was observed as well as some colocalization...
with EEA1. After 6- and more pronounced after 12-h BUO, a clustered labeling pattern but generally a weaker labeling was observed and a clearer colocalization with first EEA1 and then cathepsin D was demonstrated. Similarly, AQP2pS261 showed both apical and intracellular labeling in sham rats although not as strong as an apical labeling as seen with total AQP2 but obviously redistributed after BUO into clusters. A weak localization with early endosomes after 6 h and lysosomes after 12 h was observed (Fig. 8). AQP2pS256 showed a very distinct apical labeling in sham rats. After obstruction, a weaker overall staining was observed but this AQP2 isoform did not display the same colocalization as seen with total AQP2 and AQP2pS261 (see Supplemental Fig. S1; all supplementary material for this article is available online at the journal web site).

**V2R is not acutely downregulated in response to BUO.** Previously, it was demonstrated that 24-h BUO or UUO followed by 2 days release result in a decreased abundance and expression of V2R, the basolateral vasopressin receptor, in the inner medulla (18). In this study, the expression of V2R was reduced after 6 h ($P = 0.06$) and significantly after 12 and 24 h (Fig. 9A). The abundance of V2R protein was not statistically altered after 2- and 6-h BUO (Fig. 9B) but 12- or 24-h BUO resulted in a significant reduction in V2R protein abundance compared with sham rats (49 and 19%, respectively).

**DISCUSSION**

The main findings in the present study are that BUO results in an early significant downregulation of AQP2 mRNA after 2 h, followed by significantly reduced AQP2 protein abundance after 12- and 24-h BUO. Also, AQP2 and AQP2 phosphorylated at ser261 redistribute as a consequence of BUO to more intracellular and more clustered localizations and colocalize with the early endosomal marker EEA1 and the lysosomal marker cathepsin D, suggesting that early downregulation of AQP2 in response to BUO could in part be caused by degradation of AQP2 through a lysosomal degradation pathway.

AQP2 can be regulated long term via the regulation of AQP2 mRNA expression as previously demonstrated in response to UUO (9). Consistent with a half-life of 6-12 h for the existing AQP2 protein (14), the reduced protein abundance demonstrated in this study may at least in part be due to the reduced mRNA expression, which we also demonstrate. Administration of vasopressin is known to cause an increase in the expression of AQP2 mRNA (13) and protein (33) in kidney collecting ducts, and recently it was demonstrated in cultured immortalized mouse collecting duct principal cells (mpkCCDcl4) that this increase occurs in a concentration- and time-dependent manner (12). The plasma level of vasopressin is elevated in BUO models (38), but nevertheless this study demonstrated a clear reduction of AQP2 mRNA expression and protein abundance, suggesting the presence of vasopressin-independent regulatory mechanisms in BUO.

In the present study, immunohistochemistry showed a predominant apical labeling of AQP2 in kidney IMCD principal cells of sham-operated rats. This labeling was abolished in the rats subjected to 12- or 24-h BUO. The water permeability is increased in isolated, perfused tubules treated with vasopressin due to increased AQP2 abundance in the apical plasma membrane of the tubules (33) Therefore, it could be speculated that the reduced apical AQP2 labeling seen in this BUO model may at least partly explain the impaired urinary concentrating capacity observed in both BUO rats and patients with congenital
or acquired obstruction of the urinary tract and that this is an early consequence of obstruction.

Early BUO-induced reduction of phosphorylation of AQP2 at ser256 may play a role in impairment of water permeability and urinary concentrating ability. Phosphorylation of AQP2 at four serine residues in the C-terminal tail has been shown to be mediated by vasopressin (8, 15–17, 34), and the roles of these in AQP2 localization and redistribution are under intense investigations. Phosphorylation of ser256 is well characterized and is known to play an important role in the redistribution of AQP2 from intracellular vesicles to the apical membrane (11). This study showed that already after 2-h BUO (and at all later time points investigated) reduced AQP2pS256 protein abundance and targeting of AQP2pS256 to the apical membrane were seen. This finding is consistent with the previous observations in a mouse strain holding a mutation at ser256 demonstrating that the animals suffer from congenital progressive hydronephrosis and massive polyuria, underlining the importance of intact phosphorylation at ser256 in the urinary concentrating mechanism (29). The early AQP2pS256 downregulation observed in this study may therefore be involved in the early impairment of plasma electrolyte balance also demonstrated in this study and the urinary concentrating defect observed in other studies with BUO or UUO (9, 25) where alterations in AQP2pS256 have been demonstrated (24, 35).

Stimulation with vasopressin induces PKA-dependent phosphorylation at ser256 on AQP2 (21, 34), leading to increased water permeability of the apical membrane of principal cells (11). The importance of this specific phosphorylation site for the regulation of AQP2 trafficking is highly debated, and contradictory studies regarding the relevance have been made. Most studies have been done in cell lines where the ser256 residue is substituted with an alanine or an aspartic acid, thereby mimicking nonphosphorylated and constitutively phosphorylated AQP2, respectively. Substitution of serine by alanine results in an inability to translocate the AQP2-S256A mutant to the apical plasma membrane in response to cAMP-elevating agents, whereas the AQP2-S256D mutant only resides in the apical plasma membrane (21, 43), supporting the view that phosphorylation of ser256 is necessary and sufficient to induce trafficking of AQP2 to the membrane. On the contrary, it was suggested that AQP2 membrane accumulation is independent of phosphorylation at ser256 by demonstrating that AQP2pS256D accumulates at the cell surface after inhibition of endocytosis (26). Interestingly, recent studies demonstrate that forskolin, an adenylyl cyclase activator, is able to increase the amount of AQP2pS256D at the apical membrane, but still less than in the WT cell line. Moreover, the level of membrane-bound AQP2pS256D is higher than in the WT under control situations but shows no difference after forskolin stimulation, suggesting that ser256 is necessary for AQP2 exocytosis (32). If ser256 is required for AQP2 maturation or exocytosis, the early decrease in ser256 observed in this model of ureteral obstruction may lead to the later decrease in total AQP2 protein. Combined with the early down-regulation of AQP2 mRNA expression, this will lead to a decreased membrane accumulation of AQP2 and subsequently to an impairment of urinary concentrating ability consistent with observations in other studies (25).

Reduced phosphorylation of AQP2 on ser261 is not an early event in BUO but a consequence of reduced total AQP2 abundance. Ser261 is another vasopressin-regulated residue in the C terminus of AQP2 (17). Stimulation with the vasopressin analog dDAVP for 15 min decreases the phosphorylation of this residue in all collecting duct segments (16), but the exact role of the phosphorylation is yet to be clarified. The phosphorylation of ser261 is not presupposed by the phosphorylation of ser256 as is the case with ser264 and ser269 (15). Therefore, it is important to explore the influence of BUO on the phosphorylation of this particular site, since this may also help elucidate the role of this residue under normal physiological conditions. The present study demonstrated that acute ureteral obstruction regulates phosphorylation of ser261 very early but differently than ser256. In our study, the abundance of AQP2pS261 protein is significantly decreased after 12 and 24 h in parallel with the reduction pattern of total AQP2, suggesting that ureteral obstruction does not specifically alter phosphorylation at this site and the observed reduction is merely a consequence of the reduced AQP2 abundance in...
general. On the contrary we see a clear and early alteration in AQP2pS261 staining of IMCD principal cells, with more dispersed and less apical localization. A recent study has shown that AQP2-S261A mutants in an LLC-dd cell line do not show altered trafficking of AQP2, impaired membrane accumulation, or internalization of AQP2 (27), suggesting that the phosphorylation of ser261 is not crucial in any of these events, at least in this cell line. Also, both oocytes and proteolipids transfected with a mutant of AQP2 unable to be phosphorylated at ser261 do not experience altered water permeability (6, 31), questioning the importance of this particular site in vasopressin-induced trafficking and increased water transport. Moreover, it has recently been confirmed that phosphorylation of ser261 is not required for trafficking or recycling of AQP2 and the biological significance of phosphorylation at this specific residues remains unclear (32). Our in vivo study is in agreement with the earlier studies made on ser261 apparently demonstrating no specific role of the residue in renal water permeability. We demonstrate no evidence for a BUO-induced regulation of protein abundance, and the decrease seen in this study after 12- and 24-h BUO is likely a result of decreased AQP2 in total. However, we do see an early effect of BUO on the localization of AQP2pS261, and therefore the biological significance of ser261 phosphorylation needs to be further examined. Alternatively, the rise in plasma vasopressin level known to be present in BUO models (38) may in part contribute to the BUO-induced downregulation of AQP2pS261, similar to previous demonstration that vasopressin reduced the expression of AQP2pS261 in IMCD suspensions and in the inner medulla of kidneys from Brattleboro rats (16).

AQP2 and AQP2 phosphorylated at ser261 redistribute after BUO, suggesting a lysosomal degradation pathway. Interestingly, this study demonstrated that AQP2 and AQP2pS261 as a consequence of BUO redistribute from the apical membrane to intracellular vesicular compartments and colocalize partly with EEA1 and cathepsin D, an early endosomal and lysosomal marker, respectively. This was most pronounced after 6- and 12-h obstruction. AQP2 is known to be endocytosed after

Fig. 7. Confocal scanning microscopy of AQP2 in rat sham and BUO kidneys. Double labeling of AQP2 (green) and early endosome-associated protein (EEA1; red) or cathepsin D (red) in sham, 2-h BUO, 6-h BUO, and 12-h BUO rats, demonstrating the expression of AQP2 in the apical membrane of collecting duct principal cells and the redistribution into intracellular vesicles colocalizing with EEA1-containing early endosomes after 2, 6, and 12 h and with cathepsin D-containing lysosomes after 12 h as a consequence of BUO.
Buo reduces AQP2 expression and abundance

Vasopressin retrieval into clathrin-coated vesicles (1, 26) and to early endosomes (40), where it can be degraded, exocytosed into urine (37), or recycled (20). This fits very well with our model and suggests a degradation pathway for AQP2 in BUO. We demonstrate a clear reduction in overall apical labeling of AQP2 after 2-h BUO, but only a weak colocalization with early endosomes. Combined with the findings that total AQP2 protein abundance is not significantly reduced after 2-h BUO, this finding suggests that AQP2 upon retrieval from the membrane is located in clathrin-coated vesicles or in endocytic vesicles yet to recruit EEA1 and become early endosomes. This is known to be the initial step in the sorting system leading to degradative lysosomes (30). At longer obstruction times, the retrieved AQP2 protein is moved further along the endocytic degradation pathway and becomes compartmentalized with early endosomes containing EEA1 and finally with cathepsin D-containing lysosomes. AQP2 phosphorylated at ser261 demonstrates a similar redistribution pattern as total AQP2, suggesting that AQP2pS261 is subjected to the same degradation pathway as total AQP2 as a consequence of BUO. In a recent paper by Hoffert et al. (16), an analysis of the subcellular distribution of AQP2pS261 demonstrated that the majority of cytoplasmic AQP2pS261 under normal conditions is not located in the endoplasmic reticulum, Golgi, or lysosomes (16), suggesting that the increased colocalization between lysosomes and both total AQP2 and AQP2pS261 observed in this study is a specific BUO-induced degradation of AQP2 through a lysosomal degradation pathway. Kamsteeg et al. (19) have recently demonstrated that AQP2 contains a site for ubiquitination in the C terminal and that removal of vasopressin causes increased ubiquitination of AQP2, thereby enhancing endocytosis and degradation. Also, it has very recently been demonstrated that the membrane localization of AQP2 is determined by a balance between phosphorylation and ubiquitination (41). A similar mechanism is seen in MDCK cells, where Chen et al. (2) observed ubiquitination of the vasopressin-regulated urea transporter UT-A1 in IMCD cells and degradation through a proteasomal pathway. Increased ubiquitination and subsequently proteasomal degradation could play a role in the BUO-induced downregulation of AQP2 because AQP2 only partly colocalizes with lysosomes, but the available proteasomal markers are too poor to test this in the BUO model, where the amount of AQP2 is very low. AQP2 phosphorylated at ser256 has a very rapid and early downregulation as a result of BUO, but colocalization studies do not demonstrate localization in intracellular degradation compartments as seen with AQP2 and AQP2pS261. The obtained differences in distribution between AQP2pS256 and AQP2pS261 are in agreement with earlier findings by the Knepper group (16) demonstrating different intracellular pools for the two phosphorylated forms.
BUO reduces AQP2 expression and abundance

![Graph A](image1.png)

**Fig. 9.** Semiquantitative immunoblot reacted with anti-V2 receptor (V2R) antibody revealing a 47-kDa band. Densitometric analyses of the data demonstrated a significantly decreased abundance of V2R after 12- and 24-h BUO compared with sham rats; n = 6/group. Values are means ± SE. *P < 0.05 BUO vs. sham.

of AQP2, suggesting different roles for the two phosphorylated residues and a potential involvement of ser261 in the regulation of the subcellular localization of AQP2. The presence of such different regulatory mechanisms was also suggested by Valenti and coworkers (42) by demonstrating the existence of different intracellular pools of AQP2-bearing vesicles due to the finding that okadaic acid increases water translocation independently of AQP2 phosphorylation.

**Early downregulation of V2R is not the mechanism behind early reduced AQP2.** It is of great interest to identify the initiator of the BUO-induced early downregulation of AQP2 mRNA and ser265 phosphorylation. A possible contributor could be the basolateral V2R, well known to be the first step in the vasopressin-induced membrane accumulation of AQP2. This study showed that BUO after 12-24 h leads to reduced V2R mRNA and protein abundance, whereas there is no early change in the V2R and hence not an acute regulation of the receptor. Increased circulating vasopressin leads to increased cAMP (5, 23). Paralleled with the aforementioned regulation of AQP2 phosphorylation, cAMP will also regulate the AQP2 abundance at the transcriptional level via a CREB-mediated mechanism (28). It could be speculated that the observed downregulation of V2R will result in a reduced AQP2 transcription and hence be responsible for the lowered AQP2. However, because of a half-life of ~6-12 h for AQP2, any transcriptional regulatory mechanisms at the early time points may be minimal. However, the lately reduced V2R abundance could play a role in the later transcriptional regulation and reduced abundance of AQP2.

It may still be possible, however, that BUO leads to a reduction in the binding capacity or the activity of the receptor, as seen in some forms of hereditary nephrogenic diabetes insipidus, where a mutation in the V2R gene causes reduced activity (39).

In conclusion, the results of this study add to our understanding of the time course of AQP2 regulation by demonstrating an early significant downregulation of AQP2 mRNA already after 2 h followed by significantly reduced AQP2 protein abundance after 12- and 24-h BUO. Furthermore, there is an early regulation of AQP2pS256 after 2-h BUO. AQP2 and AQP2pS261 redistribute as a consequence of BUO to more intracellular and more clustered localizations and colocalize with the early endosomal marker EEA1 and the lysosomal marker cathepsin D, suggesting that the early downregulation of AQP2 in response to BUO could be caused by degradation of AQP2 through a lysosomal degradation pathway.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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