Cyclooxygenase 2 inhibition exacerbates AQP2 and pAQP2 downregulation independently of V2 receptor abundance in the postobstructed kidney

Anja M. Jensen,1,2 Eun Hui Bae,3 Rikke Norregaard,1,2 Guixian Wang,1,2 Søren Nielsen,1,3 Horst Schweer,5 Soo Wan Kim,4 and Jørgen Frokiaer1,2

1The Water and Salt Research Center, 2Institute of Clinical Medicine, and 3Institute of Anatomy, University of Aarhus, Aarhus, Denmark; 4Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Korea; and 5Department of Pediatrics, Philips University of Marburg, Marburg, Germany

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Jensen AM, Bae EH, Norregaard R, Wang G, Nielsen S, Schweer H, Kim SW, Frokiaer J. Cyclooxygenase 2 inhibition exacerbates AQP2 and pAQP2 downregulation independently of V2 receptor abundance in the postobstructed kidney. Am J Physiol Renal Physiol 298: F941–F950, 2010. First published January 27, 2010; doi:10.1152/ajprenal.00605.2009.—Previously we demonstrated that ANG II receptor (AT1R) blockade attenuates V2 receptor (V2R), AQP2, and pS256-AQP2 downregulation in the postobstructed kidney and partially reverses obstruction-induced inhibition of cAMP generation and cyclooxygenase 2 (COX-2) induction. Therefore, we speculated whether the effects of AT1R blockade on V2R and the vasopressin-regulated pathway are attributable to attenuated COX-2 induction. To examine this, rats were subjected to 24-h bilateral ureteral obstruction (BUO) followed by 48-h release and treated with the COX-2 inhibitor parecoxib or saline. Control rats were sham-operated. Parecoxib treatment significantly reduced urine output 24 h after release of BUO whereas urine osmolality and solute-free water reabsorption was comparable between saline- and parecoxib-treated BUO rats. Immunoblotting revealed a significant decrease in AQP2 and pS256-AQP2 abundance to 20 and 23% of sham levels in parecoxib-treated BUO rats compared with 40 and 55% of sham levels in saline-treated BUO rats. Immunohistochemistry confirmed the exacerbated AQP2 and pS256-AQP2 downregulation in parecoxib-treated BUO rats. Finally, parecoxib treatment had no effect on V2R downregulation and the inhibited, vasopressin-stimulated cAMP generation in inner medullary membrane fractions from the postobstructed kidney. In conclusion, COX-2 inhibition exacerbates AQP2 and pS256-AQP2 downregulation 48 h after release of 24-h BUO independently of V2R abundance and vasopressin-stimulated cAMP generation. The results indicate that COX-2 inhibition does not mimic AT1R blockade-mediated effects and that AT1R-mediated AQP2 regulation in the postobstructed kidney collecting duct is independent of COX-2 induction.

prostaglandin E2; AQP2; vasopressin; ureteral obstruction

THE POSTOBSTRUCTED KIDNEY exhibits a markedly reduced urinary concentrating capacity due to decreased abundance of major renal transport proteins (15, 22, 27, 28). Importantly, the vasopressin type 2 receptor (V2R) and vasopressin-regulated transport proteins are downregulated, causing vasopressin-resistant polyuria (20, 51, 55). A number of hormone/signaling systems contribute to postobstructive kidney disease, including the prostaglandin system (13, 26, 32, 36) and the renin-angioten
din-system (RAS) (21, 40, 56).

The most abundant prostaglandin in the kidney, prostaglan
din E2 (PGE2), is involved in the regulation of kidney water and salt handling. In the medullary thick ascending limb (TAL), PGE2 acutely blunts vasopressin-stimulated NaCl transport and cAMP generation (9, 46, 49) and the long-term effect of PGE2 is downregulation of the vasopressin-regulated Na-K-2Cl cotransporter (NKCC2) (12). Similar PGE2-mediated effects are observed in the isolated rabbit cortical collecting duct (CD) (3, 19, 45, 49), but parallel studies in rat collecting ducts have not yet been able to confirm an inhibitory effect of PGE2 on cAMP generation (3, 4, 31, 49). However, PGE2 does inhibit vasopressin-stimulated water permeability in rat inner medullary collecting duct (IMCD) independently of cAMP (33, 48) by a mechanism not yet fully understood. The mechanism may involve Rho activation by EP3 stimulation or increased Ca2+ levels (33, 48). Moreover, cAMP levels remain unchanged in rat IMCD upon PGE2 stimulation in the absence of vasopressin (31, 33). In vivo studies have shown aquaporin-2 (AQP2) downregulation and a reduced cAMP generation in response to vasopressin stimulation of inner medullary membrane fractions in kidneys from indomethacin-treated rats (24), which may be explained by a concurrent decrease in V2R abundance (30). Despite the reduced total AQP2 abundance in the indomethacin-treated rats, they exhibit a reduced urine output (18, 30) consistent with the observed increased AQP2 targeting to the apical plasma membrane (24).

Interestingly, ureteral obstruction causes cyclooxygenase (COX)-2 induction in the base of the inner medulla (IM) (7, 35) and enhances PGE2 production in medullary and cortical tubules (52). The COX-2 induction persists in the postobstructed kidney 3 days after release of 24-h bilateral ureteral obstruction (BUO) (36). A number of studies suggest that COX-2 plays a role in dysregulation of renal transport proteins in both the obstructed and postobstructed kidney, although most evident in the obstructed kidney (5, 36, 37). COX-2 inhibition during 24-h BUO attenuates downregulation of AQP2, NKCC2, and the type 3 Na+/H+ exchanger (NHE3) (5, 37), whereas the effect of COX-2 inhibition during 24-h BUO followed by 3-day release is limited to AQP2 (36).

In addition to COX-2 induction, ureteral obstruction activates the intrarenal RAS and increases intrarenal ANG II generation (14, 39, 56). Previous studies showed that at least part of the COX-2 induction is attributable to ANG II since ANG II receptor type 1A (AT1) blockade significantly reduced COX-2 abundance in the postobstructed kidney (21). Furthermore, AT1 receptor blockade attenuates downregulation of V2R and the vasopressin-regulated transport proteins pS256-AQP2, AQP2, and NKCC2 48 h after release of 24-h BUO (20, 21).
Since both COX-2 inhibition and AT1 receptor blockade reduce postobstructive polyuria and mainly influence vasopressin-regulated transport proteins, we hypothesize that the effect of AT1 receptor blockade on V2R and the downstream pathways regulating AQP2 may at least partly be the result of attenuated COX-2 induction. Therefore, we wanted to test the hypothesis that COX-2 inhibition mimics the effects of AT1 receptor blockade. Thus the present study aimed at exploring the effect of COX-2 inhibition on 1) the abundance of V2R, AQP2, and pS256-AQP2 and 2) vasopressin-stimulated cAMP generation in the kidney 48 h after release of 24 h BUO.

MATERIALS AND METHODS

Experimental Animals

The animal protocols were approved by the board at the Institute of Clinical Medicine, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice and the Institutional Guidelines of Experimental Animal Care and Use, Korea. Studies were performed in male Munich-Wistar rats [immunoblotting, immunohistochemistry, gas chromatography-tandem mass spectrometry (GC-MS-MS) analysis, Mollegaard Breeding Center, Eiby, Denmark] and male Sprague-Dawley rats (membrane preparation), initially weighing 250 ± 10 g. The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water. During the entire experimental period, the rats were kept in individual metabolic cages with a 12:12-h artificial light-dark cycle, a temperature of 21 ± 2°C, and a humidity of 55 ± 2%. Rats were allowed to acclimatize to the cages for 2 days before surgery.

Surgical Procedures

The rats were anesthetized with isoflurane (Abbott), and through a midline abdominal incision a 5-mm-long piece of bisected polyethylene tubing (PE-50) was placed around each ureter. The ureter was then occluded by tightening the tubing with a 5-0 silk ligature and 24 h later, the ligature and PE tubing were removed.

Protocol. BUO was induced for 24 h following by 48-h release (n = 16, 16) (Fig. 1). Osmotic minipumps (Alzet, Scanbur, Denmark) with saline (n = 8) or parecoxib (6 mg·kg⁻¹·day⁻¹; Pfizer, Kent, Great Britain) dissolved in physiological saline (n = 8) were surgically implanted subcutaneously when the obstruction was performed. Sham-operated controls were prepared in parallel (n = 7). Kidneys were prepared for immunoblotting and GC-MS-MS analysis. An identical protocol was used for membrane preparation (n = 19) and immunohistochemistry (n = 13). A parecoxib dose of 6 mg·kg⁻¹·day⁻¹ was previously shown to completely block de novo synthesis of prostanoids (38). Furthermore, we found a compensatory enhanced COX-2 induction in kidney IM from parecoxib-treated rats compared with saline-treated rats (data not shown) consistent with COX-2 inhibition.

Electrophoresis and Immunoblotting

Protein was isolated and quantitated as described previously (21). Samples of membrane fractionation were run on 12% polyacrylamide gels (Criterion Tris-HCl, Bio-Rad). To ascertain identical loading and to avoid for correction, an identical gel was run in parallel and subjected to Coomassie staining. The proteins were transferred to either a PVDF membrane (Immobilon-P PVDF, Millipore); V2R antibody or to a nitrocellulose membrane (Hybond ECL RPN3032D, Amersham Pharmacia Biotech); AQP2, pAQP2, and NKCC2 antibody. After transfer, the blots were then blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated overnight at 4°C with the following primary antibodies: V2R antibody (no. 7251 AP) previously characterized (11); NKCC2 (no. 1495 AP), a new antibody raised against the primary antibodies: V2R antibody (no. 7251 AP) previously characterized (11); NKCC2 (no. 1495 AP), a new antibody raised against the
Table 1. Changes in renal function 48 h after release of 24-h BUO

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 7)</th>
<th>BUO (n = 8)</th>
<th>BUO+P (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_furerences, μmol·min⁻¹·kg⁻¹</td>
<td>5.1 ± 0.3</td>
<td>2.7 ± 0.2*</td>
<td>2.1 ± 0.3*</td>
</tr>
<tr>
<td>T³-H₂O, μmol·min⁻¹·kg⁻¹</td>
<td>173 ± 7</td>
<td>123 ± 12*</td>
<td>103 ± 10*</td>
</tr>
<tr>
<td>U_som, mosmol/kg·H₂O</td>
<td>2,199 ± 243</td>
<td>886 ± 36*</td>
<td>739 ± 54*</td>
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<tr>
<td>U_urea, mM</td>
<td>1,287 ± 154</td>
<td>541 ± 16*</td>
<td>403 ± 35*</td>
</tr>
<tr>
<td>U_urea·K¹·U_urea·K¹·μmol·min⁻¹·kg⁻¹</td>
<td>2.9 ± 0.2</td>
<td>1.1 ± 0.2*</td>
<td>2.0 ± 0.3*†</td>
</tr>
<tr>
<td>FL_K, μmol·min⁻¹·kg⁻¹</td>
<td>691.6 ± 35</td>
<td>373.3 ± 26*</td>
<td>263.3 ± 49*</td>
</tr>
<tr>
<td>Paretaxels, μmol·min⁻¹·kg⁻¹</td>
<td>688.6 ± 34</td>
<td>372.2 ± 26*</td>
<td>261.3 ± 49*</td>
</tr>
<tr>
<td>F_EK, %</td>
<td>0.4 ± 0.02</td>
<td>0.3 ± 0.1</td>
<td>1.1 ± 0.3†</td>
</tr>
<tr>
<td>U_K⁺·U_urea, μmol·min⁻¹·kg⁻¹</td>
<td>9.8 ± 0.1</td>
<td>6.0 ± 0.2*</td>
<td>7.4 ± 0.2*</td>
</tr>
<tr>
<td>FL_K⁺·μmol·min⁻¹·kg⁻¹</td>
<td>20.9 ± 2</td>
<td>12.1 ± 1*</td>
<td>8.1 ± 1*</td>
</tr>
<tr>
<td>Paretaxels, μmol·min⁻¹·kg⁻¹</td>
<td>11 ± 2</td>
<td>6.1 ± 1*</td>
<td>0.8 ± 1*</td>
</tr>
<tr>
<td>F_EK⁺, %</td>
<td>49 ± 4</td>
<td>48 ± 6</td>
<td>107 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Sham, sham-operated control rats; BUO, bilateral ureteral obstruction and saline treatment; BUO+P, bilateral ureteral obstruction and parecoxib treatment; n, no. of rats; C_furerences, creatinine clearance; T³-H₂O, solute-free water reabsorption; U_som, urine osmolality; U_urea, urine urea concentration; U_urea·K¹·U_urea·K¹, sodium/potassium excretion; FL_K⁺, filtered load of sodium/potassium; Paretaxels, net reabsorption of sodium/potassium; F_EK⁺, fractional excretion of sodium/potassium. †P < 0.05, statistically significant compared with sham-operated rats. ‡P < 0.05, statistically significant compared with saline-treated BUO rats.

RPSLQEC as previously described (10); AQP2 antibody (H7661) previously characterized (34); and pS256-AQP2 (KO307), a new antibody raised against the same sequence of immunizing peptide [GRRRQ(pS)VELHISPC] as the previous characterized antibody (8). The specificity was evaluated by 1) detection of the immunizing peptide on immunoblots using immune serum and as a negative control preimmune serum from the same rabbit; and 2) detection of pS256-AQP2 in a protein sample prepared from rat kidney homogenates on immunoblots using affinity-purified anti-pS256-AQP2 antibody showing 29- and 35- to 50-kDa bands identical to those seen in rat tissue with the previously characterized antibody. The antigen-antibody complex was visualized with horseradish peroxidase-conjugated secondary antibodies (P448, DAKO, Glostrup, Denmark) using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

The band densities were quantified by using a bizhub C550 scanner and ImageJ. The specific bands were normalized to the mean of control bands.

Immunohistochemistry

Kidneys were fixed by retrograde perfusion via the abdominal aorta with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. After removal, the midregion of the kidney was sectioned into 2- to 3-mm transverse sections and immersion-fixed for an additional 1 h, followed by 3 × 10-min washes with 0.1 M cacodylate buffer, pH 7.4. The tissue was dehydrated and embedded in paraffin, and 2-μm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark). For immunolabeling, the sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked by 0.5% H₂O₂ in absolute methanol for 10 min. To expose antigens, kidney sections were boiled in a target retrieval solution (1 mM Tris solution, pH 9.0 with 0.5 mM EGTA) for 10 min. After cooling, nonspecific binding was blocked with 50 mM NH₄Cl in PBS for 30 min followed by 3 × 10-min washing with PBS blocking buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated with primary antibodies diluted in PBS with 0.1% BSA and 0.3% Triton X-100 overnight at 4°C. The sections were washed 3 × 10 min with PBS buffer containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with horseradish peroxidase-conjugated secondary antibodies (P448, goat anti-rabbit immunoglobulin, DAKO) for 1 h at room temperature. After 3 × 10 rinses with PBS wash buffer, the antibody-antigen reactions were visualized with 0.05% 3,3′-diaminobenzidine tetrachloride (DAB, Kemen Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H₂O₂.

Light microscopy was carried out with a Leica DMRE (Leica Microsystems).

Membrane Preparation and Adenylate Cyclase Activity

The membrane preparation and adenylate cyclase measurements have previously been described. (23, 25) Briefly, kidney tissue was homogenized in ice-cold buffer (50 mM Tris-HCl, pH 8.0, with 1 mM ethylenediaminetetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, 250 mM sucrose), and centrifuged at 1,000 and 100,000 g. Adenylate cyclase activity in response to vasopressin and forskolin stimulation was measured in the membrane fractions (the pellet) by the slightly modified method of Bar (2). The reaction was initiated by adding membrane fractions to a working solution (50 mM Tris-HCl, pH 7.6, with 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl₂, 1 mM 3-isobutyl-1-methylxanthine, 0.02 mM GTP), and after 15 min blocked by addition of a cold 50 mM sodium acetate solution (pH 5.0). Finally, the mixture was centrifuged at 1,000 g for 10 min at 4°C, and cAMP was measured in the supernatant by equilibrated radioimmunoassay as previously described. (23) Protein concentrations were determined using a biocinchonic acid assay kit (Bio-Rad, Hercules, CA). Results are expressed as nanomoles cAMP per milligram protein per minute.

Measurement of Urinary Prostanoid Excretion and Kidney Tissue Prostanoid Levels

In an isotope-dilution assay, PGE₂, PGE-M, 6-keto-PGF₁α, 2,3-dinor-6-keto-PGF₁α, thromboxane (Tx) B₂, and 2,3-dinor-TxB₂ were measured as nanomoles cAMP per millgram protein per minute.
determined in urine by GC/MS/MS. After addition of deuterated internal standards, the prostanoids were derivatized to methoximes and extracted with ethyl acetate-hexane. The samples were further derivatized to trimethylsilyl ethers, and the products were quantified using GC/MS/MS. Three zones were scraped from the TLC. The prostanoid derivates were converted to the trimethylsilyl ethers and the products were quantified using GC/MS/MS.

Kidney tissue was homogenized in 4 volumes water (wt/vol). Further sample cleanup and derivatization was as described for urine.

**GC-MS-MS analysis.** A GC/MS/MS (Finnigan MAT TSQ700) equipped with a gas chromatograph (Varian 3400) and an autosampler (model CTC A200S) was used. Gas chromatography of prostanoid derivates was carried out on a DB-1 (20m, 0.25-mm ID, 0.25-μm film thickness) capillary column (Analyt, Mühlheim, Germany) in the splitless mode. GC/MS/MS parameters were as described by Schweer et al. (43).

**Presentation of Data and Statistical Analyses**

Quantitative data are presented as means ± SE. Statistical comparisons were accomplished by one-way ANOVA and a post hoc Tukey honestly significant difference test. Data were analyzed by a Mann-Whitney rank-sum test when variables were not normally distributed. *P* values <0.05 were considered statistically significant.

**RESULTS**

**COX-2 Inhibition Transiently Reduces Postobstructive Polyuria**

Consistent with previous studies, COX-2 inhibition significantly reduced postobstructive polyuria 24 h after release of 24-h BUO whereas the polyuria was comparable in saline-treated BUO rats and rats treated with a COX-2 inhibitor 48 h after release of 24-h BUO rats (Fig. 2). In parallel, the parecoxib-treated BUO rats exhibited a significantly lower water intake than the saline-treated BUO rats 24 h after release of the obstruction (Fig. 2). COX-2 inhibition had no influence on reduced creatinine clearance, urine osmolality, and solute-free water reabsorption in the postobstructed kidney (Table 1). The filtered load and the net reabsorption of sodium and potassium remained comparable in saline- and parecoxib-treated BUO rats (Table 1). However, the fractional excretion of both sodium and potassium increased significantly in parecoxib-treated BUO rats compared with saline-treated BUO rats (Table 1).

**COX-2 Regulates AQP2 and pS256-AQP2 Abundance 48 h after Release of 24-h BUO**

COX-2 inhibition clearly attenuates AQP2 and pS256-AQP2 downregulation in kidney IM during 24-h BUO whereas it only slightly attenuates AQP2 downregulation and has no effect on pS256-AQP2 abundance in the postobstructed kidney IM 3 days after release of BUO. COX-2 inhibition caused additional downregulation of AQP2 in kidney IM 48 h after release of 24-h BUO to 20.2 ± 4% of sham levels compared with 40.1 ± 3% of sham levels in saline-treated BUO rats (*P* < 0.01, Fig. 3). Similarly, pS256-AQP2 abundance decreased significantly to 22.5 ± 5% of sham levels in parecoxib-treated BUO rats compared with 55.4 ± 5% of sham levels in saline-treated BUO rats (*P* < 0.01, Fig. 4). These findings were confirmed by immunohistochemistry showing

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**Fig. 4.** pS256-AQP2 protein abundance using IM from saline-treated BUO rats (*n* = 8), BUO + parecoxib-treated BUO rats (*n* = 8), and sham-operated control rats (*n* = 7). A: immunoblots were reacted with an affinity-purified anti-pS256-AQP2 antibody revealing a 29- and 35- to 50-kDa bands representing nonglycosylated and glycosylated forms of pS256-AQP2. B: densitometric analysis of all samples from BUO, BUO + parecoxib, and sham-operated control rats (corrected according to loading) revealed a significant decrease in pS256-AQP2 protein abundance in kidney IM from saline-treated BUO rats compared with sham rats. Parecoxib treatment significantly exacerbated pS256-AQP2 downregulation. Values are means ± SE. *Statistically significant compared with sham-operated rats, *P* < 0.05. #Statistically significant compared with saline-treated BUO rats, *P* < 0.05.

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**Fig. 5.** Immunoperoxidase labeling of AQP2 in the base of kidney IM from sham-operated control rats (*n* = 4), saline-treated BUO rats (*n* = 5), and BUO + parecoxib-treated BUO rats (*n* = 4). AQP2 labeling is seen in the apical plasma membrane and intracellularly in the collecting duct principal cells in sham-operated control rats. In saline-treated BUO rats, the AQP2 labeling is weak and AQP2 is primarily seen in the apical plasma membrane of the collecting duct principal cell. The labeling of AQP2 is further weakened in BUO + parecoxib-treated BUO rats compared with saline-treated BUO rats.
reduced inner medullary AQP2 and pS256-AQP2 labeling in saline-treated BUO rats compared with sham-operated rats (Figs. 5 and 6). The AQP2 and pS256-AQP2 labeling was further reduced in sections from parecoxib-treated BUO rats (Figs. 5 and 6). AQP2 and pS256-AQP2 showed enhanced apical targeting to a similar degree in saline- and parecoxib-treated BUO rats.

**NKCC2 Abundance is Comparable in Parecoxib- and Saline-Treated BUO Rats**

NKCC2 downregulation in kidneys from rats subjected to 24-h BUO is partly prevented by COX-2 inhibition whereas NKCC2 remains equally downregulated in parecoxib-treated BUO rats compared with saline-treated BUO rats 3 days after release of 24-h BUO. Consistently, we found a comparable reduction in NKCC2 abundance to 47.2 ± 3% of sham levels and 42.2 ± 4% of sham levels in saline- and parecoxib-treated rats, respectively (P < 0.01, Fig. 7).

**COX-2 Inhibition Has No Influence on V2R Abundance in Postobstructed Kidney IM**

The V2R is significantly downregulated in the postobstructed kidney (20) and since PGE2 interacts with AVP-stimulated water reabsorption and V2R abundance in the IMCD (30, 33, 48), COX-2 inhibition may potentially also influence V2R abundance. However, we found comparable, profoundly reduced V2R levels to below 10% of sham levels in postobstructed kidney IM from both saline- and parecoxib-treated BUO rats (P < 0.01, Fig. 7).

**Vasopressin-Stimulated cAMP Generation Remains Impaired in Parecoxib-Treated Rats**

The amount of cAMP generated in response to vasopressin stimulation of IM membrane fractions from saline-treated BUO rats is clearly reduced (22), which we confirmed in the present studies at increasing vasopressin concentrations. Moreover, vasopressin-stimulated cAMP generation remained impaired in parecoxib-treated rats (P < 0.05, Fig. 9).

**COX-2 Inhibition Has No Influence on Prostanoid Levels in Kidney IM Tissue**

Measurements of prostanoid levels in kidney IM revealed significantly reduced (PGE2α and PGD2) or unchanged (PGE2, TxB2, and 6-keto-PGF1α) prostanoid levels (Table 2) in the postobstructed kidney IM, although COX-2 abundance was significantly enhanced (data not shown). COX-2 inhibition tended to inhibit PGE2 levels, whereas no effect was observed with regard to the other prostanoids (Table 2).

**Urinary Prostanoid Excretion**

The excretion of primary prostanoids and their metabolites were measured in the urine 24 h before BUO (day −1) and 24 (day 1) and 48 h (day 2) after release of BUO (Table 3). It is generally accepted that the primary prostanoids reflect renal concentrations whereas the metabolites reflect systemic prostanoid activity (44). In saline-treated BUO rats, the excretion of the primary prostanoids PGE2, 6-keto-PGF1α, and TxB2 was significantly enhanced at day 2, and a similar tendency was found at day 1. COX-2 inhibition tended to reduce the excretion of PGE2, 6-keto-PGF1α, and TxB2 at day 2 whereas the effect at day 1 was more subtle. The major metabolite of PGE2, PGE-M, reflecting systemic PGE2, was clearly enhanced at days 1 and 2 in saline-treated BUO rats although only statis-

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**Fig. 6.** Immunoperoxidase labeling of pS256-AQP2 in the base of kidney IM from sham-operated control rats (n = 4), saline-treated BUO rats (n = 5) and BUO + P rats (n = 4). sP256-AQP2 labeling is seen in the apical plasma membrane and intracellularly in the collecting duct principal cells in sham-operated control rats. In saline-treated BUO rats, the p-S256-AQP2 labeling is weak and pS256-AQP2 is primarily seen in the apical plasma membrane of the collecting duct principal cell. The labeling of pS256-AQP2 is further weakened in BUO + P rats compared with saline-treated BUO rats.

**Fig. 7.** Na-K-2Cl cotransporter (NKCC2) protein abundance using the inner stripe of the outer medulla (ISOM) from saline-treated BUO rats (n = 8), BUO + P rats (n = 8), and sham-operated control rats (n = 7). A: Immunobots were reacted with a specific anti-NKCC2 antibody revealing a single band, ~63 kDa. B: densitometric analysis of all samples from BUO, BUO + P, and sham-operated control rats (corrected according to loading) revealed a significant decrease in NKCC2 protein abundance in kidney ISOM from saline-treated BUO rats compared with sham rats. NKCC2 abundance was comparable in BUO + P and saline-treated BUO rats. Values are means ± SE. *Statistically significant compared with sham-operated rats, P < 0.05.
tically significant at day 1 due to high variance. The enhanced PGE-M excretion was attenuated by parecoxib treatment, indicating sufficient COX-2 inhibition. Similarly, the 6-keto-PGF1α metabolite 2,3-dinor-6-keto-PGF1α tended to increase in saline-treated BUO rats at day 1, and the levels tended to be reduced in the parecoxib-treated BUO rats.

**DISCUSSION**

It is generally accepted that PGE2 works as a negative feedback system antagonizing the antidiuretic action of vasopressin in the kidney (17). Therefore, COX-2 induction and COX-2-derived PGE2 may be speculated to account for the vasopressin-resistant postobstructive polyuria observed after release of BUO. In the present study, treatment with the specific COX-2 inhibitor parecoxib transiently reduced postobstructive polyuria, consistent with previous studies (5, 36). However, we found sustained V2R downregulation and impaired vasopressin-stimulated cAMP generation in the postobstructed kidney IM from BUO rats treated with the selective COX-2 inhibitor parecoxib. Parecoxib treatment caused additional downregulation of vasopressin-regulated AQP2 and pS256-AQP2 in the postobstructed kidney IM 48 h after release of 24-h BUO, and PGE2 excretion was clearly increased although not significantly. COX-2 inhibition tended to reduce the excretion. This discrepancy between kidney and urine prostanoid levels may indicate de novo prostanoid synthesis in the urothelium (1, 37, 54). Furthermore, we measured only pro-BUO rats. The reasons for these results are unclear. The analysis does not discriminate between COX-1- and COX-2-derived prostanoids, and studies by Qi et al. (41, 42) have demonstrated that prostaglandins derived from COX-1 and COX-2 have different effects in the kidney medulla; COX-2- but not COX-1 inhibition reduces the medullary blood flow and attenuates ANG II-stimulated prostanoid synthesis and natriuresis. Thus it is possible that the relative abundance of prostaglandins derived from COX-1 and COX-2 are different in the kidney IM from the three groups of animals, which may influence kidney function. Urinary excretion of the primary prostanoids is believed to reflect renal concentrations (44). In contrast to the findings in kidney IM tissue, urinary excretion of TxB2 and 6-keto-PGF1α were significantly enhanced 48 h after release of 24-h BUO, and PGE2 excretion was clearly increased although not significantly. COX-2 inhibition tended to reduce the excretion. The analysis does not discriminate between COX-1- and COX-2-derived prostanoids, and studies by Qi et al. (41, 42) have demonstrated that prostaglandins derived from COX-1 and COX-2 have different effects in the kidney medulla; COX-2- but not COX-1 inhibition reduces the medullary blood flow and attenuates ANG II-stimulated prostanoid synthesis and natriuresis. Thus it is possible that the relative abundance of prostaglandins derived from COX-1 and COX-2 are different in the kidney IM from the three groups of animals, which may influence kidney function. Urinary excretion of the primary prostanoids is believed to reflect renal concentrations (44). In contrast to the findings in kidney IM tissue, urinary excretion of TxB2 and 6-keto-PGF1α were significantly enhanced 48 h after release of 24-h BUO, and PGE2 excretion was clearly increased although not significantly. COX-2 inhibition tended to reduce the excretion. This discrepancy between kidney and urine prostanoid levels may indicate de novo prostanoid synthesis in the urothelium (1, 37, 54). Furthermore, we measured only pro...
stanoid concentrations in kidney IM, and since urinary prostanoid concentrations derive from the total kidney and previous studies found a significantly enhanced prostanoid production by isolated glomeruli from BUO rats (53), the enhanced urinary PGE₂ excretion may mainly derive from kidney cortex.

Previous studies revealed that COX-2 inhibition attenuates AQP2 and NKCC2 downregulation during 24-h BUO and transiently reduces postobstructive polyuria (5, 35, 36). Furthermore, downregulation of AQP2 was slightly attenuated by parecoxib treatment 3 days after release of 24-h BUO, whereas p5256-AQP2 and NKCC2 levels remained comparable in kidneys from parecoxib- and saline-treated BUO rats at this time point (36), suggesting that COX-2-derived prostanoids differentially influence renal transport proteins during obstruction and after release of obstruction. Our results confirmed the transiently reduced polyuria and the unchanged urine osmolality 24 h after release of 24-h BUO in parecoxib-treated rats. Contrary to the previous studies, the present findings showed an exacerbated AQP2 and p5256-AQP2 protein downregulation in postobstructed kidney IM in response to parecoxib treatment 48 h after release of 24-h BUO. However, the hemodynamic and hormonal conditions during BUO and in the postobstructed kidney are quite different, which may very likely explain the discrepancy between the findings in the obstructed kidney and the results of the present study. In particular, the dehydration and high vasopressin levels in postobstructed kidney IM (27, 51, 55) may contribute to the opposite actions of parecoxib treatment during and after release of BUO since PGE₂ differentially regulates V2R abundance dependent on vasopressin levels. Unspecific inhibition of COX-1 and COX-2 by indomethacin treatment reduces V2R abundance in control rats whereas V2R abundance is increased in indomethacin-treated dehydrated rats (30). However, COX-2 inhibition had no influence on V2R abundance in the postobstructed kidney IM, emphasizing that regulation of V2R abundance by COX-2-derived PGE₂ plays no major role in AQP2 dysregulation in the postobstructed kidney. In vitro studies have revealed that V2R mRNA levels are enhanced by hyperosmolality (30), and as previously demonstrated, the profound decrease in IM osmolality in the postobstructed kidney persists in rats treated with a COX-2 inhibitor (36). Therefore, it may be speculated that the persistent hypoosmolality in postobstructed kidney IM is responsible for the distinct and comparable V2R downregulation in both saline- and parecoxib-treated BUO rats. The persistent V2R downregulation in the parecoxib-treated BUO rats is reflected in the functional studies revealing a sustained impairment of vasopressin-stimulated cAMP generation in IM membrane fractions from parecoxib-treated BUO rats. Furthermore, the sustained NKCC2 downregulation in the postobstructed kidney during COX-2 inhibition indicates that the COX-2-mediated AQP2 regulation in the postobstructed kidney is mediated by a non-vasopressin-dependent mechanism.

In contrast to the present findings, it was previously demonstrated that parecoxib treatment slightly attenuated AQP2 downregulation in postobstructed kidney IM 72 h after release of 24-h BUO (36). This discrepancy may be due to the different time points of examination. The renal medullary blood flow as well as formation/degradation of various growth factors, vasoactive peptides, and signaling proteins may very likely differ at 48 and 72 h after the release of obstruction. Moreover, protein synthesis/degradation/trafficking of various membrane proteins may also vary between the different time points of examination. Finally, in the present study parecoxib treatment significantly reduced water intake compared with saline-treated rats at day 1, which may aggravate dehydration.

Table 2. Profile of prostanoid production in kidney IM

<table>
<thead>
<tr>
<th></th>
<th>Kidney IM Weight, mg</th>
<th>PGF₂α</th>
<th>6-keto-PGF₁α</th>
<th>PGE₂</th>
<th>TXB₂</th>
<th>PGD₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 7)</td>
<td>29 ± 1.9</td>
<td>3.2 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td>5.7 ± 0.6</td>
<td>1.9 ± 0.3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>BUO (n = 8)</td>
<td>48 ± 2.5</td>
<td>1.7 ± 0.2 *</td>
<td>2.7 ± 0.3</td>
<td>4.2 ± 0.6</td>
<td>2.9 ± 0.4</td>
<td>1.4 ± 0.2 *</td>
</tr>
<tr>
<td>BUO + P (n = 8)</td>
<td>52 ± 3.2</td>
<td>2.1 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.3 *</td>
<td>2.5 ± 0.2</td>
<td>1.5 ± 0.1 *</td>
</tr>
</tbody>
</table>

Values are means ± SE. Prostanoid production is expressed as ng/mg tissue. n, No. of rats; IM, inner medulla; TXB₂, thromboxane B₂. *P < 0.05 respective prostanoid in BUO vs. sham rats. 

Table 3. Profile of prostanoid excretion in urine

<table>
<thead>
<tr>
<th></th>
<th>PGE₂</th>
<th>PGE-M</th>
<th>TXB₂</th>
<th>2,3-dimor-TXB₂</th>
<th>6-keto-PGF₁α</th>
<th>2,3-dimor-6-keto-PGF₁α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham (n = 7)</td>
<td>256 ± 50</td>
<td>12,050 ± 3,080</td>
<td>21 ± 6</td>
<td>79 ± 12</td>
<td>70 ± 10</td>
<td>114 ± 15</td>
</tr>
<tr>
<td>BUO (n = 8)</td>
<td>250 ± 59</td>
<td>12,976 ± 1,859</td>
<td>19 ± 4</td>
<td>103 ± 38</td>
<td>39 ± 8</td>
<td>104 ± 21</td>
</tr>
<tr>
<td>BUO + P (n = 8)</td>
<td>251 ± 59</td>
<td>15,707 ± 1,607</td>
<td>22 ± 2</td>
<td>130 ± 29</td>
<td>38 ± 5 *</td>
<td>120 ± 14</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham (n = 7)</td>
<td>284 ± 60</td>
<td>13,662 ± 3,113</td>
<td>18 ± 5</td>
<td>74 ± 13</td>
<td>86 ± 4</td>
<td>156 ± 24</td>
</tr>
<tr>
<td>BUO (n = 8)</td>
<td>583 ± 124</td>
<td>76,376 ± 17,877 *</td>
<td>60 ± 12</td>
<td>38 ± 13</td>
<td>220 ± 56</td>
<td>198 ± 30</td>
</tr>
<tr>
<td>BUO + P (n = 8)</td>
<td>576 ± 65 *</td>
<td>19,432 ± 5,462 *</td>
<td>76 ± 17 *</td>
<td>40 ± 10</td>
<td>128 ± 31</td>
<td>83 ± 13 *</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as ng·mg⁻¹·day⁻¹. n, No. of rats. *P < 0.05 respective prostanoid in BUO vs. sham rats. 

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and the subsequent hypotension, which, in turn, may augment kidney injury.

AQP2 regulation is complex and in addition to the “classic” vasopressin-stimulated cAMP generation and PKA activation, AQP2 regulation involves several other mechanisms, including Rho activation and intracellular Ca\(^{2+}\) levels (6, 47, 50). Interestingly, the PGE\(_2\) receptors (EP1, EP3, and EP4) are localized in the CD and specifically influence intracellular Ca\(^{2+}\) levels, Rho activity, and cAMP generation (for a review, see Ref. 16). Several in vitro studies have demonstrated that EP3 stimulation inhibits vasopressin-mediated cAMP generation and CD water permeability, which have been argued to be the molecular mechanism explaining the antidiuretic action of prostaglandins (3, 19, 45, 49). These studies were mainly conducted in rabbit cortical CD, and parallel studies using rat CD have not been able to confirm an inhibitory effect of PGE\(_2\) on vasopressin-stimulated cAMP generation in rat IM (3, 4, 31, 49). Consistently, we found a sustained impairment in cAMP generation in vasopressin-stimulated IM membrane fraction from rats treated with a COX-2 inhibitor. However, PGE\(_2\) does inhibit the IMCD water permeability independently of cAMP by a mechanism not yet fully understood, but it may involve Rho activation via EP3 stimulation (48) or changes in intracellular Ca\(^{2+}\) levels (33). Furthermore, the EP4 receptor directly stimulates cAMP generation in the CD (16), and a recent study revealed that treating V2 receptor knockout mice with an EP4-receptor agonist reduces the urine output and increases AQP2 abundance (29). Thus it could be speculated that multiple vasopressin-independent, EP receptor-mediated mechanisms regulate AQP2 abundance and/or phosphorylation and thereby influence IMCD water permeability in the postobstructed kidney IM. The exacerbated AQP2 and pS256-AQP2 downregulation in parecoxib-treated animals in the present study may indirectly be caused by altered EP receptor-mediated signaling in postobstructed kidney IM.

It is proposed that PGE\(_2\) stimulates endocytic retrieval of AQP2 from the apical plasma membrane (57), which may explain the paradoxical downregulation of AQP2 and the concomitantly reduced urine output in indomethacin-treated rats as enhanced AQP2 targeting to the apical plasma membrane is observed in response to indomethacin treatment (24). A similar mechanism may explain the significantly reduced urine output in the parecoxib-treated rats 24 h after release of BUO. Immunohistochemistry in the present study showed comparable, increased AQP2 labeling in the apical plasma membrane in both parecoxib- and saline-treated rats 48 h after release of BUO, consistent with the comparable urine output at this time point. Moreover, the total urine output depends on the glomerular filtration rate, which may be influenced by COX-2 inhibition as prostanoids are vasoactive. Actually, the glomerular filtration rate tended to be reduced 48 h after release of BUO in the parecoxib-treated rats, potentially preventing polyuria. In addition to AQP2-mediated water reabsorption, the final urine volume depends on the renal handling of different solutes, including urea and sodium. Solute-free water reabsorption may be a more accurate indicator of AQP2-mediated water reabsorption and indeed, solute-free water reabsorption clearly tended to be decreased in the parecoxib-treated BUO rats compared with saline-treated control rats.

As mentioned above, the urine output and urine concentration capacity are highly dependent on renal urea and sodium handling. In the present study, we chose to focus on water handling. However, it should be noted that the fractional excretion of sodium was increased fourfold in the parecoxib-treated BUO rats compared with the saline-treated BUO rats. A possible explanation could be exacerbated downregulation of sodium transport proteins other than NKCC2.

In conclusion, we have shown that COX-2 inhibition exacerbates AQP2 and pS256-AQP2 downregulation independently of V2R abundance and vasopressin-stimulated cAMP generation in postobstructed kidney IM 48 h after release of 24-h BUO. Furthermore, COX-2 inhibition seems not to influence the increased AQP2 targeting to the apical plasma membrane of CD principal cells. As COX-2 inhibition does not mimic the previously demonstrated AT1 receptor blockade-mediated effects on transport protein abundance and cAMP generation, the present study indicates that ANG II and COX-2 regulate AQP2 by alternative/separate pathways in the postobstructed kidney IM, at least at the time point examined.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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