Altered expression of COX-1, COX-2, and mPGES in rats with nephrogenic and central diabetes insipidus

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F1053

PROSTAGLANDINS, WHICH FUNCTION as autocrine and paracrine lipid mediators, play important roles in a variety of regulatory homeostatic mechanisms (36). In the kidney, prostaglandins have an important role in affecting renal hemodynamics, renin release, tubular sodium, and water reabsorption (12). The main prostaglandin in the kidney is prostaglandin E2 (PGE2), which is synthesized from arachidonic acid in a two-step enzymatic reaction. Arachidonic acid is first converted to an unstable intermediate PGH2 by one of the two cyclooxygenase isoenzymes, COX-1 or COX-2. This rate-limiting step is then followed by formation of PGE2 by the prostaglandin E synthase (PGES) (8, 21). The synthesis of PGE2 is thus dependent on the presence of COX-1 or COX-2 with PGES. However, the sites of expression in kidney and regulation of these enzymes in pathophysiological states such as altered water balance are still debated.

COX-1 and COX-2 exhibit similar catalytic activity and have a comparable molecular mass of 71 and 73 kDa, respectively; however, their amino acid sequence and localization in the kidney are different (2, 12, 22). COX-1 is believed to be constitutively expressed, providing the majority of prostaglandin production in the kidney (5, 36). In contrast, COX-2 is considered only to be induced during physiological stress such as inflammation and dehydration (39, 48). Particularly, COX-2 is thought to play an important role in the survival of inner medullary interstitial cells during increased interstitial osmolality, whereas expression of inner medullary COX-1 remains unchanged in this condition (48). Moreover, COX-1 and COX-2 in the inner medulla are downregulated in response to low interstitial osmolality induced by furosemide administration or water loading (3). Nevertheless, the role of COX-2 as an inducible stress enzyme has been questioned by the data showing that COX-2 appears to be expressed constitutively in the rat and mouse macula densa region of cortical thick ascending limb and inner medullary interstitial cells (2). Moreover, until recently PGES was thought to be functionally coupled to COX-2 (31), but recent studies show that renal PGES expression is colocalized mainly with COX-1 and not COX-2 in the cortical and outer and inner medullary collecting duct (2, 8, 46).

Although the present understanding of the regulation and sites of expression of the enzymes important for PGE2 synthesis is not completely characterized, the molecular target for the effects of PGE2 on water metabolism is well understood. PGE2 plays an important role in modulating the effect of vasopressin on osmotic water permeability in the renal collecting duct, where it attenuates antidiuretic action. This effect has been attributed to both inhibition of cAMP synthesis and elevation...
of cytosolic Ca$^{2+}$ in rabbit cortical collecting ducts (14, 15, 40) and rat terminal inner medullary collecting ducts, causing decreased trafficking of aquaporin-2 (AQP2) to the apical plasma membrane (32, 49). In addition, it has recently been proposed that the signaling pathway underlying the diuretic effect of PGE2 includes the cAMP- and Ca$^{2+}$-independent activation of the Rho-kinase and formation of F actin (43). Interestingly, PGE2 has also been shown to stimulate epithelial solute and water transport in the absence of antidiuretic hormone (ADH) (38).

The importance of PGE2 in the regulation of water metabolism is further signified by the association between altered urinary PGE2 excretion and conditions of perturbed urinary concentrating ability (18, 30, 41). In nephrogenic diabetes insipidus (NDI) caused either by mutation in the AQP2 genes, hypercalcemia, or lithium treatment, there is a peripheral resistance to ADH (24, 29). Patients suffer from ADH in parallel with normalized urinary concentrating ability (18, 30, 41, 47). These observations have led to the use of COX inhibitors to decrease urine volume in NDI patients (18, 26, 37, 45). Moreover, patients with central diabetes insipidus (CDI), which is caused by the lack of ADH (38), can be increased in response to several days of treatment from congenitally ADH-deficient Brattleboro (BB) rats is low with DDAVP (20). Interestingly, PGE2 has also been shown to stimulate epithelial solute and water transport in the absence of antidiuretic hormone (ADH) (38).

<table>
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<td>P蒌, mmol/l</td>
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</table>

Values are means ± SE. *The change in body weight was measured during 2 days of dehydration. a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z.  

METHODS

Experimental Protocols

Both protocols 1 and 2 (described below) were designed in accordance with the Danish regulations for care and use of experimental animals (Danish Ministry of Justice).

Protocol 1 (low-dose LiCl treatment with a fixed intake of sodium). Male Wistar rats (n = 16; body weight 250–270 g) were obtained from Taconic M&B (Ry, Denmark). All rats were fed a food mix consisting of 21 g of ground rat chow (Altromin no. 1320, sodium content 1.7 mmol/15 g rat chow) mixed in 21 ml of tap water. The lithium-treated rats (n = 8) received 0.8 mmol LiCl (L 4408, Sigma) added to the food mixture. In addition to the water in the food mixture, the rats had free access to a water bottle until 2 days before death. A 12:12-h artificial light-dark cycle was maintained during the entire experiment. Food was offered to the animals at 9:00 A.M. For the first 19 days, the animals were housed individually in normal rat cages. The intake of food and water intake was monitored daily throughout the experiment. The control rats (n = 8) were offered only the amount that the lithium-treated rats ate throughout the experiment to obtain equal daily intake of nutrients and sodium (Table 1). By following this protocol, a steady increase in body weight was achieved in both groups. For the last 7 days, all animals were housed in metabolic cages to allow daily measurement of urine output and sample collections. For the last 2 days, one-half of the animals in each experimental group (n = 4) were randomly selected to undergo water restriction. Their daily water offer was limited to one-third of their average 5-day water intake before the start of water restriction. The water given was

Table 1. Functional data in protocol 1
mixed with the previously described dry food mix. The remaining one-half of the experimental animals continued to have free access to water.

One more set of lithium-treated (n = 5) and control rats (n = 5) was prepared using the same protocol. Right kidneys were subjected to immunoblotting (kidney cortex samples), and left kidneys were perfusion fixed for immunocytochemistry.

Protocol 2 (deaminoo-Cys\textsubscript{1},d-Arg\textsubscript{8}-vasopressin treatment in BB rats lacking endogenous vasopressin). Female homozygous BB rats (n = 10; body weight 195–220; age 12–14 wk) were obtained from Harlan Sprague Dawley (Indianapolis, IN). They were fed 20 g of ground rat chow (Altromin no. 1320, sodium content 1.7 mmol/15 g rat chow) throughout the experiment and had free access to water. A 12:12-h artificial light-dark cycle was maintained during the entire experiment. Food was offered to the animals at 9 AM. Rats were placed into metabolic cages 2 days before insertion of osmotic minipumps (Alzet model 2002, Cupertino, CA). Using halothane inhalation anesthesia, osmotic minipumps containing deaminoo-Cys\textsubscript{1},d-Arg\textsubscript{8}-vasopressin (DDAVP; V-9879, Sigma) were inserted subcutaneously into BB rats (n = 5) and DDAVP was delivered at a rate of 250 ng/day for 6 days. Control BB rats (n = 5) received osmotic minipumps containing only vehicle. Water and food intake and urine output were monitored, and 24-h urine samples were collected.

On the day of harvesting, rats from both protocols underwent the same procedure. They were anesthetized with halothane, and large laparotomy was made. Venous blood was collected for determination of plasma Na\textsuperscript{+}, K\textsuperscript{+}, creatinine, urea, Li\textsuperscript{+}, and osmolality. The right kidney was quickly removed and immediately dissected into regions (inner medulla, inner stripe of outer medulla, and cortex) and further processed as described below (see Immunoblotting). The left kidney was perfusion fixed as described below (see Immunohistochemistry).

**Immunoblotting**

The dissected kidney regions (inner medulla, inner stripe of outer medulla, and cortex) were separately homogenized in ice-cooled isolation solution (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, adjusted to pH 7.2) using an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). The homogenate was centrifuged at 4,000 g for 10 min at 10,000 g for 15 min to remove large cellular debris and nuclei. Protein concentration was measured in the supernatant (Pierce BCA protein assay reagent kit, Pierce, Rockford, IL), and samples were adjusted with isolation solution to achieve the same final protein concentration. The samples were solubilized in Laemmlı sample buffer at 65°C for 15 min, and stored at −20°C. To confirm equal loading of protein, the initial gel was stained with Comassie blue stain reagent (Pierce) as previously described (44).

In addition to this standard preparation of kidney protein, an alternate preparation was carried out to investigate the role of differential centrifugation on immunoblotting band appearance of COX-2. An homogenate of kidney inner medulla from a normal control rat was centrifuged for 10 min at 10,000 g followed by a 60-min centrifugation at 100,000 g of the supernatant to sediment microsomes as described by Harris et al. (13).

Samples were run on 9, 10, 12, or 15% gradient polyacrylamide minigels (Bio-Rad Mini Protein II). With regard to immunoblotting of COX-2, 9% gels were used rather than 12% gels to allow separation of the specific, peptide-ablatable COX-2 band from background bands of which one often comigrated with COX-2 in ordinary 12% gels.

Proteins were transferred electrophoretically to nitrocellulose membranes (Hybond ECL RPN 3032D, Amersham Pharmacia Biotech, Little Chalfont, UK), which were subsequently blocked for 1 h in 5% milk in PBS-T (80 mM Na\textsubscript{2}HPO\textsubscript{4}, 20 mM NaH\textsubscript{2}PO\textsubscript{4}, 100 mM NaCl, 0.1 Tween 20, adjusted to pH 7.4). Membranes were incubated with primary antibodies overnight at 4°C (see Primary Antibodies).

Antigen-antibody interactions were visualized with horseradish peroxidase-conjugated secondary antibodies (P448 or P449 diluted 1:3,000, DAKO, Glostrup, Denmark) using the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech) and exposure to photographic film (Hyperfilm ECL, Amersham Pharmacia Biotech). The band densities were quantitated by scanning the films and normalizing the densitometry values to facilitate comparisons. Results are listed as the relative band densities between the groups and not absolute, hence the term “semiquantitative immunoblotting.”

**Immunohistochemistry**

A perfusion needle was inserted into the abdominal aorta, and the inferior vena cava was cut to establish an outlet after blood sampling. Blood was flushed from the kidneys with cold 0.01 M PBS (pH 7.4) for 15 s, before a switch to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for additionally 1 h, followed by 3 × 10-min washes with 0.1 M cacodylate buffer, pH 7.4. The tissue was dehydrated in graded ethanol and left overnight in xylene. After tissue embedding in paraffin, 2-μm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark).

For immunolabeling, the sections were dewaxed with xylene and rehydrated with graded ethanol. Sections had endogenous peroxidase activity blocked with 0.5% H\textsubscript{2}O\textsubscript{2} in absolute methanol for 10 min. In a microwave oven, the sections were boiled in a target retrieval solution (1 mmol/l Tris, pH 9.0, with 0.5 mM EGTA) for 10 min. After the sections were cooled, nonspecific binding was blocked with 50 mM NH\textsubscript{4}Cl in PBS for 30 min followed by 3 × 10 min in PBS blocking-buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated with primary antibody (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 wash buffer containing 0.1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO P448) for 1 h at room temperature. After 3 × 10-min rinses with PBS wash buffer, the sites of antibody-antigen reaction were visualized with a brown chromogen produced within 10 min by incubation with 0.05% 3,3’-diaminobenzidine tetrachloride (Kem-En Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H\textsubscript{2}O\textsubscript{2}. Mayer’s hematoxylin was used for counterstaining and after dehydration coverslips were mounted with hydrophobic medium (Eukitt, O. Kindler, Freiburg, Germany). Light microscopic examination was carried out with Leica DMRE (Leica Microsystems).

All COX-2-positive cells in the macula densa region of cortical thick ascending limb and all glomeruli were counted in a 90-mm\textsuperscript{2} large region of kidney cortex in immunohistochemical sections from all experimental animals. The ratio of counted COX-2-positive cells and glomeruli was obtained from each experimental animal.

**Primary Antibodies**

For both semiquantitative immunoblotting and immunohistochemistry, the following previously characterized antibodies were used (2): anti-COX-1 (murine) rabbit polyclonal antibody (batch 160109, Cayman Chemical, Ann Arbor, MI); anti-COX-2 (murine) affinity-purified rabbit polyclonal antibody (batch 160126, Cayman Chemical; this antibody was used in all the figures presented); anti-COX-2 (rat) goat polyclonal antibody (batch SC-1747, Santa Cruz Biotechnology, Heidelberg, Germany); and anti-membrane-associated prostaglandin E\textsubscript{2} synthase (mPGES; human) rabbit polyclonal antibody-affinity purified (batch 160140, Cayman Chemical).
COX-1, COX-2, and mPGES Protein Abundances were increased in the inner medulla of kidneys from lithium-treated rats decreased to 35 ± 4% of the controls (P < 0.05, Fig. 1C, Table 2). Immunohistochemistry showed scarce but distinct COX-2 labeling in a characteristic peritubular pattern in inner medullary interstitial cells in kidneys from control rats (Fig. 4, A and B), whereas no labeling was detected in the inner medulla of kidneys from lithium-treated rats (Fig. 4, E and F). mPGES was also significantly downregulated to 55 ± 4% of the controls in lithium-treated rats (P < 0.05, Fig. 1E, Table 2). In control rats, mPGES immunolabeling was associated with the IMCD cells and the interstitial cells (Fig. 5A). In addition, faint labeling was seen in the tubular cells of thin limbs. Chronic lithium treatment was associated with reduced labeling of mPGES in IMCD cells, interstitial cells, and thin limbs compared with controls (Fig. 5C).

**RESULTS**

**Chronic Lithium Treatment in Rats was Associated with Significantly Decreased Urine Concentration, Polyuria, and Increased Urinary PGE2 Excretion**

Treatment of rats with a low dose of LiCl for 4 wk resulted in a large increase in urine output (6-fold) and a marked decrease in urine osmolality and urine-to-plasma osmolality ratio (Table 1). This is consistent with impaired urinary concentration ability, as previously demonstrated (25). The 24-h urinary PGE2 excretion was significantly increased in lithium-treated rats compared with the controls (752 ± 99 vs. 404 ± 21 ng/day, P < 0.05, n = 8/group).

**COX-1, COX-2, and mPGES Protein Abundances were Decreased in Kidney Inner Medulla of Lithium-Treated Rats**

Lithium treatment markedly decreased the expression of COX-1 in the kidney inner medulla to 17 ± 6% of the controls (P < 0.05, Fig. 1A, Table 2). Consistent with this, immunohistochemistry revealed a comparable decrease in the COX-1 labeling of inner medullary interstitial cells, whereas the decreased labeling of inner medullary collecting duct cells was less prominent (Fig. 2). Control experiments revealed that the anti-COX-2 antibody specifically labeled a band of the expected molecular size, consistent with previous reports (Fig. 3A), and this was completely ablated when the antibody previously preincubated with an excess of the immunizing peptide was used (Fig. 3B), confirming the specificity also seen by complete ablation of the signal obtained by immunocytochemistry. It should be mentioned that the lower nonablatable band could be eliminated by an alternative membrane fractionation protocol isolating the microsome fractions by differential centrifugation (see METHODS and Fig. 3C), indicating that the antibody also cross-reacts with an unidentified protein only present in certain membrane fractions. Immunoblotting revealed that COX-2 expression in the inner medulla of kidneys from lithium-treated rats decreased to 35 ± 4% of the controls (P < 0.05, Fig. 1C, Table 2). Immunohistochemistry showed scarce but distinct COX-2 labeling in a characteristic peritubular pattern in inner medullary interstitial cells in kidneys from control rats (Fig. 4, A and B), whereas no labeling was detected in the inner medulla of kidneys from lithium-treated rats (Fig. 4, E and F). mPGES was also significantly downregulated to 55 ± 4% of the controls in lithium-treated rats (P < 0.05, Fig. 1E, Table 2). In control rats, mPGES immunolabeling was associated with the IMCD cells and the interstitial cells (Fig. 5A). In addition, faint labeling was seen in the tubular cells of thin limbs. Chronic lithium treatment was associated with reduced labeling of mPGES in IMCD cells, interstitial cells, and thin limbs compared with controls (Fig. 5C).

**COX-1 Abundance in Kidney Cortex was Decreased, while COX-2 Labeling was Increased in Macula Densa Cells in Kidneys from Lithium-Treated Rats**

Immunoblotting revealed that COX-1 expression was significantly downregulated in the outer medulla of kidneys from lithium-treated rats to 64 ± 6% of the controls (P < 0.05). In contrast, there was no significant change in the expression of mPGES in the outer medulla (82 ± 17% of controls, not significant (NS), Table 2), and COX-2 could not be detected in this region. Immunolabeling of control rats showed that both COX-1 and mPGES immunolabeling was present in the outer medullary collecting duct cells, and COX-1 immunolabeling was weaker in lithium-treated rats (not shown), consistent with the immunoblotting data.

**COX-1 Abundance in Kidney Cortex was Decreased, while COX-2 Labeling was Increased in Macula Densa Cells in Kidneys from Lithium-Treated Rats**

Immunoblotting revealed that COX-1 expression in the kidney cortex was marginally reduced in lithium-treated rats (76 ± 1% of the control levels, P < 0.05, Table 2). This was supported by immunocytochemistry (not shown) showing reduced labeling in connecting tubule, cortical collecting duct, as well as in mesangial cells of the glomerular tuft. In contrast, there was no change in the protein abundance of mPGES. The labeling pattern of mPGES was not changed by lithium treatment and was localized to the connecting tubule and cortical collecting duct, similar to COX-1 (not shown), but mPGES was also detected in the cortical thick ascending limb together with COX-2, as previously described (2). Immunoblotting of COX-2 showed a tendency for COX-2 to increase in abundance in the cortex of kidneys from lithium-treated rats (Table 2), although this did not reach statistical significance in this set of animals. To confirm whether lithium treatment increased COX-2 protein abundance in the cortex, an additional immunoblot was made with samples from another set of identically treated rats. In this set of animals, COX-2 was significantly increased in the kidney cortex of the lithium-treated rats (291 ± 61% of control levels 100 ± 33%, P < 0.05, Fig. 6).

*Immunocytochemistry also revealed an absence of immunolabeling of COX-2 in COX-2 knockout mice obtained from Taconic M&B in contrast to significant labeling in normal mice (medullary interstitial cells and macula densa cells). Data not shown.*
Moreover, immunohistochemistry of COX-2 showed an increased number of COX-2-positive cells in the macula densa region of the cortical thick ascending limb in lithium-treated rats (Fig. 7, E and F) compared with the controls (Fig. 7, A and B). Semiquantitation on sections confirmed the increase: 2.4 ± 0.4 COX-2-positive cells/glomerulus in lithium-treated rats vs. 1.0 ± 0.2 in control rats (Table 2).

Water Restriction in Lithium-Treated Rats was Associated with a Marked Increase in Urine Osmolality But not in Urinary PGE2 Excretion Compared with Control Rats

Based on the changes in rats with lithium-induced NDI, we further investigated the effect of water restriction in lithium-treated rats on the expression of COX-1 and COX-2 and on the functional parameters including urinary excretion of PGE2.

Previous reports have shown that COX-2 but not COX-1 expression in kidney inner medulla increases with high urine osmolality in response to dehydration of normal rats, and similar results have been shown in dehydrated BB rats lacking endogenous vasopressin (48). The effect of dehydration on mPGES expression, however, has not been investigated in either normal or lithium-treated rats.

After 48-h water restriction, lithium-treated rats had a markedly decreased 24-h urine output to 16% of nondehydrated lithium-treated rats and an associated sevenfold increase in urine osmolality (Table 1). The final urine osmolality in dehydrated lithium-treated rats remained significantly lower than in dehydrated control rats (Table 1). The 24-h PGE2 urinary excretion in dehydrated control rats increased significantly compared with nondehydrated con-
trols (1,352 ± 83 vs. 470 ± 82 ng/day, \( P < 0.05 \), \( n = 4 \)/group), whereas urinary excretion of PGE2 in dehydrated lithium-treated rats did not change significantly compared with nondehydrated lithium-treated rats (747 ± 190 vs. 519 ± 35 ng/day; NS, \( n = 4 \)/group). Thus lithium-treated rats responded to dehydration with decreased urine output and increased urine osmolality, but this was not associated with an increased urinary excretion of the PGE2 as seen in dehydrated control rats, suggesting that PGE2 synthesis potentially was unchanged.

![Fig. 2. Immunohistochemistry for COX-1 in kidney inner medulla of control (A), dehydrated control (B), lithium-treated (C), and dehydrated lithium-treated (D) rats. Inner medullary collecting duct (IMCD) and interstitial cells are stained.](http://ajprenal.physiology.org/)

Table 2. Summary of densitometric analysis of immunoblots from protocol 1

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<th>Li (n = 4)</th>
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Values are means ± SE. \( n = \) No. of rats; COX-1 and COX-2, cyclooxygenase-1 and -2, respectively; IM, inner medulla; ISOM, inner stripe of outer medulla; mPGES, membrane-associated PGE2 synthase; CTX, Cortex. ^a\( P < 0.05 \) when Con is compared with Con dehydrated. ^b\( P < 0.05 \) when Li is compared with Li dehydrated. ^c\( P < 0.05 \) when Con is compared with Li. ^d\( P < 0.05 \) when Con dehydrated is compared with Li dehydrated. ^e\( P < 0.05 \) when Con is compared with Li dehydrated.
Water Restriction Increased COX-2 Expression in Inner Medulla of Control Rats and Lithium-Treated Rats but did not Affect COX-1 and mPGES Expression

The increased urine osmolality in dehydrated control rats was associated with markedly increased COX-2 expression in the inner medulla (209 ± 21% of the control levels, P < 0.05, Fig. 1 and Table 2), consistent with previous evidence (48). The increased COX-2 expression in response to dehydration in control rats was also observed with immunohistochemistry, showing markedly increased COX-2 labeling in inner medullary interstitial cells (Fig. 4, C and D). In dehydrated lithium-treated rats, COX-2 abundance was markedly increased compared with nondehydrated lithium-treated rats (Fig. 1C, Table 2), suggesting that COX-2 expression was enhanced in response to dehydration in lithium-treated rats. Importantly, the undetected or very weak COX-2 immunolabeling in the nondehydrated lithium-treated rats (Fig. 4, E and F) was replaced by strong labeling of inner medullary interstitial cells in response to dehydration in lithium-treated rats (Fig. 4, G and H). In contrast, the expression levels of COX-1 and mPGES in the inner medulla were not significantly affected by dehydration in control rats, and they remained decreased in dehydrated lithium-treated rats compared with nondehydrated controls (Figs. 1, 3, and 5, Table 2).

DDAVP Treatment in Vasopressin-Deficient BB Rats Decreased Urine Output and Increased Urinary Excretion of PGE2

Treatment of BB rats with DDAVP caused a marked decrease in urine volume (215 ± 15 vs. 11 ± 1 ml/day, P < 0.05, Table 3) with a concomitant 20-fold increase in urinary osmolality, consistent with functional urinary concentrating ability. The 24-h urinary excretion of PGE2 was increased ninefold in DDAVP-treated BB rats compared with nontreated control BB rats (994 ± 69 vs. 109 ± 10 ng/day, P < 0.05, Table 3), suggesting increased prostaglandin synthesis.

DDAVP Treatment in Vasopressin-Deficient BB Rats Increased COX-1, COX-2, and mPGES Protein Abundances in the Inner Medulla

The increased urinary PGE2 excretion in DDAVP-treated BB rats was associated with a 2- to 3-fold increase in the expression of COX-1 and mPGES in the kidney inner medulla, whereas COX-2 was increased over 100-fold (Fig. 8, Table 4). Immunohistochemistry showed increased COX-1 labeling in both IMCD cells and inner medullary interstitial cells, with the labeling intensity appearing strongest in the latter (arrows in Fig. 9). Contrary to COX-1, the immunolabeling of COX-2 in DDAVP-treated BB rats was exclusively localized to the inner medullary interstitial cells (Fig. 10, C and D), whereas no labeling was detected in the untreated BB rats (Fig. 10, A and B). The increased mPGES protein abundance was associated

Figure 3. Immunoblots of inner medullary (IM) homogenates from control rats (C), lithium-treated (Lic), dehydrated lithium-treated rats (Lid), and dehydrated control rats (Cd). The blot was reacted with anti-COX-2 antibody and revealed a 73-kDa band (arrow) in addition to other bands suspected to represent background (A). This was confirmed by using anti-COX-2 antibody preabsorbed with an excess of immunizing peptide, which revealed complete ablation of the band (B, arrow). The nonablating lower band on the COX-2 blot was only observed by using protein prepared from supernatant (sup.) of a 4,000-g centrifugation of the homogenate but not protein prepared by a 10-min centrifugation at 10,000 g to sediment microsomes (C, arrow).
Fig. 4. Immunohistochemistry for COX-2 in kidney inner medulla of control (A and B), dehydrated control (C and D), lithium-treated (E and F), and dehydrated lithium-treated (G and H) rats at low (left) and high magnification (right). Only interstitial cells (arrows) of inner medulla are labeled. There is faint labeling at the tip of the papilla in control rats (A and B) and virtually absent labeling in inner medulla of lithium-treated rats (E and F). After 2 days of dehydration, a dramatic increase in COX-2 labeling was observed in both dehydrated control (C and D) and dehydrated lithium-treated rats (G and H). Magnification: ×50 (A, C, E, and G) and ×400 (B, D, F, and H).
with a marked increase in IMCD cells and a less pronounced increase in interstitial cells and thin limb cells. In control rats, the labeling was observed primarily in the IMCD cells (Fig. 11).

**DDAVP Treatment in Vasopressin-Deficient BB Rats Decreased COX-2 Expression in Cortex but Increased mPGES Expression in the Outer Medulla**

COX-1 expression in the outer medulla was unchanged, whereas the expression in the cortex was marginally increased in DDAVP-treated BB rats (Table 4). COX-2 could not be detected in the outer medulla but was decreased 1.6-fold in the cortex in response to DDAVP treatment. The mPGES enzyme was increased 1.9-fold in the outer medulla but was unchanged in the cortex (Table 4). Immunochemistry demonstrated unchanged COX-1 labeling in response to DDAVP in both the outer medulla and cortex (not shown). Moreover, the labeling intensity in these two regions was lower compared with the inner medulla in the same rats. For COX-2, the labeling intensity in COX-2-positive cells located at the macula densa region appeared to be decreased, consistent with the immunoblotting data (Fig. 12). mPGES labeling in the outer medulla was increased in outer medullary collecting duct cells and unchanged in the cortex (not shown), consistent with immunoblotting. The overall mPGES intensity in the outer medulla and cortex was much less compared with the inner medulla in the same rats.

**DISCUSSION**

In the present study we demonstrated that 1) lithium-induced NDI in rats was associated with decreased expression of COX-1, COX-2, and mPGES in kidney inner medulla; 2) COX-1 expression was also moderately reduced in ISOM and...
Fig. 7. Immunohistochemistry for COX-2 in kidney cortex of control (A and B), dehydrated control (C and D), lithium-treated (E and F), and dehydrated lithium-treated (G and H) rats at low (left) and high magnification (right). COX-2 labeling is localized to single cells or small groups of cells in the macula densa region of the cortical thick ascending limb. In lithium-treated rats (E and F), the number of stained cells per section increased compared with control rats (A and B); similarly, the number of COX-2-stained cells was increased in dehydrated lithium-treated rats (H) compared with dehydrated control rats (D). Magnification: ×50 (A, C, E, and G) and ×400 (B, D, F, and H).
kidney cortex of lithium-treated rats, whereas COX-2 protein expression and immunolabeling at the macula densa region in cortex were increased; 3) dehydration of both lithium-treated rats and control rats resulted in a marked increase in COX-2 immunolabeling in inner medullary interstitial cells paralleled by increased urine concentration. In the cortex, COX-2 protein expression was equally increased in the dehydrated control rats, whereas the increase in dehydrated lithium-treated rats was not significant; 4) there was no significant change in COX-1 and mPGES expression after dehydration in any kidney zones in both lithium-treated rats and control rats; 5) chronic DDAVP treatment of BB rats was associated with markedly increased expression of COX-1, COX-2, and mPGES in the kidney inner medulla paralleled by an increased urine concentration, whereas there was no upregulation of COX-1 in other kidney zones; and 6) COX-2 was downregulated in kidney cortex in response to DDAVP treatment in BB rats.

Changes in COX-1, COX-2, and mPGES Expression in Lithium-Induced NDI

We examined the altered expression of renal COX-1, COX-2, and mPGES in a model of NDI induced by chronic lithium treatment in rats where the expression of collecting duct water channel AQP2 and AQP3 was markedly decreased (25, 28). Special attention was addressed to the changes in the inner medulla, as it was previously shown that the inner medulla is a main site of renal PGE2 synthesis (10, 19). In addition, expression of all three investigated enzymes in the kidney is the greatest in this zone (22, 46, 48).

We observed that COX-1 expression in kidney inner medulla was significantly decreased in response to chronic lithium treatment. It has previously been reported that inner medullary expression of COX-1 mRNA as well as protein levels were downregulated by furosemide treatment or water loading in rats where renal interstitial osmolality was reduced (3). The downregulation of mRNA and protein levels was paralleled by a decrease in tissue PGE2 concentrations in the papilla (3). Moreover, in vitro studies demonstrated that the expression of COX-1 mRNA changed in parallel with the changes in osmolality of the culture medium for IMCD cells (3), suggesting that the changes in interstitial osmolality may be a possible mediator of the effects of furosemide on the COX-1 expression. Similarly, lithium treatment reduces inner medullary interstitial osmolality (4) and urinary osmolality compared with controls.

![Image](http://ajprenal.physiology.org/)

**Fig. 8.** Semiquantitative immunoblots of kidney inner medullary homogenates from untreated Brattleboro rats (BB) and BB rats treated with BB+DDAVP for 6 days (DDAVP). A: band density of the ~71-kDa COX-1 band (A) and the ~73-kDa COX-2 band (B, arrow) were markedly increased in DDAVP-treated BB rats as was the density of the mPGES band at 16 kDa (C). *P < 0.05 when BB+DDAVP is compared with BB.

### Table 3. Functional data in protocol 2

<table>
<thead>
<tr>
<th></th>
<th>BB Con (n = 4)</th>
<th>BB+DDAVP (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>224±5</td>
<td>223±8</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>237.5±15.9</td>
<td>21.8±10</td>
</tr>
<tr>
<td>Urine output, ml/day</td>
<td>214.8±15.3</td>
<td>10.8±1.0</td>
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<tr>
<td>Food intake, g/day</td>
<td>17.3±0.8</td>
<td>16.8±1.1</td>
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<td>Na intake, mmol/day</td>
<td>2.0±0.1</td>
<td>1.9±0.1</td>
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<tr>
<td>Uosmol, mosmol/kgH2O</td>
<td>67±6</td>
<td>1434±82*</td>
</tr>
<tr>
<td>U_PGE2, ng/day</td>
<td>109±10</td>
<td>994±69*</td>
</tr>
<tr>
<td>Posmol, mosmol/kgH2O</td>
<td>312.5±1.9</td>
<td>306.8±2.9*</td>
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<tr>
<td>U/Posmol ratio</td>
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</tr>
<tr>
<td>Pw, mmol/l</td>
<td>143.5±1.1</td>
<td>137.8±1.3*</td>
</tr>
<tr>
<td>Pp, mmol/l</td>
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<td>4.5±0.1</td>
</tr>
<tr>
<td>Pcreatinin, μmol/l</td>
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<td>32.0±1.6</td>
</tr>
<tr>
<td>Purea, mmol/l</td>
<td>6.1±0.1</td>
<td>14.4±0.3*</td>
</tr>
<tr>
<td>Ccreatinin, ml/min</td>
<td>0.03±0.001</td>
<td>0.034±0.002</td>
</tr>
<tr>
<td>CH2O, ml/min</td>
<td>0.118±0.011</td>
<td>−0.027±0.002</td>
</tr>
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</table>

Values are means ± SE. n, No. of rats; BB, Brattleboro rats; BB Con, control BB rats; DDAVP, DDAVP-treated BB rats. U_PGE2, daily urinary PGE2 excretion. *P < 0.05 when DDAVP is compared with Con.
This would suggest that chronically decreased interstitial osmolality in NDI could be an important factor in decreasing COX-1 expression in kidney inner medulla. Immunohistochemistry demonstrated that COX-1 immunolabeling in the inner medulla was associated with both IMCD cells and inner medullary interstitial cells and that the decrease in labeling appeared to be greater in interstitial cells. In contrast, in ISOM and kidney cortex, COX-1 labeling was restricted to the collecting duct. Therefore, the observed more pronounced decrease in COX-1 expression in the kidney inner medulla compared with ISOM and kidney cortex may suggest that inner medullary interstitial cells were more affected by the decreased interstitial osmolality than principal cells of the collecting duct.

We have also demonstrated that COX-2 expression in kidney inner medulla was decreased. This was shown both by immunoblotting and by immunohistochemistry. The consistent downregulation observed raises the possibility that decreased interstitial osmolality in the inner medulla may play a role in the downregulation of both COX-1 and COX-2. In contrast, COX-2 expression in the kidney cortex was significantly increased in lithium-induced NDI, and the number of COX-2-labeled cells in the macula densa region of ascending thick ascending limbs was increased moderately by lithium treatment. In the macula densa region, COX-2 expression has been demonstrated to increase in high-renin states, e.g., sodium restriction, angiotensin-converting enzyme inhibition, and renovascular hypertension, and selective COX-2 inhibitors significantly decrease plasma renin levels and renal renin activity (11). Thus it is possible that the control of renin secretion by macula densa could be dependent on COX-2-derived prostanooids. We have previously demonstrated that chronic lithium treatment in rats is associated with an increase in urinary sodium excretion in addition to impaired urinary concentration ability and polyuria (25, 33), and thereby the animals are prone to the development of extracellular fluid volume contraction and high renin secretion. Therefore, the increased number of COX-2-labeled cells in the macula densa region is consistent with the state of extracellular fluid volume contraction in lithium-induced NDI.

The downstream enzyme in PGE2 synthesis, mPGES, was also investigated. In the kidney inner medulla, it colocalized with the expression sites of both COX-1 and COX-2 in IMCD cells and interstitial cells. However, the expression of mPGES was noticeably more pronounced in IMCD cells than in interstitial cells, indicating the more predominant coupling of mPGES with COX-1 in IMCD cells. Chronic lithium treatment was associated with decreased expression of mPGES in the inner medulla. On immunohistochemistry, mPGES labeling was moderately decreased in IMCD cells and absent in interstitial cells. In contrast, there was no significant change in mPGES immunoreactivity in ISOM and kidney cortex. Similar to COX-1 and COX-2, the significant downregulation of mPGES in the inner medulla may imply that decreased interstitial osmolality could also regulate mPGES expression in this region.

**Effect of Dehydration on the Expression of COX-2 in Lithium-Induced NDI**

Dehydration of lithium-treated rats resulted in a distinct increase in inner medullary COX-2 expression paralleled by increased urine concentration, consistent with the previously observed increased COX-2 expression in the renal inner medulla of Sprague-Dawley rats by dehydration (48). Immunohistochemistry revealed that the increased COX-2 labeling in response to dehydration was exclusively associated with the inner medullary interstitial cells, but not with IMCD cells. It has been demonstrated that COX-2 expression in the inner medulla is regulated by the interstitial osmolality both in vivo and in vitro (48). Moreover, COX-2 expression is upregulated by dehydration in both normal rats (48) and rats with lithium-induced NDI (present study), which is consistent with the previous findings of the effect of renal medullary osmolality.
and body fluid volume on COX expression (3, 44). Thus these findings support that COX-2-derived PGs participate in the regulation of urinary water excretion. In accordance, a number of studies have demonstrated that PGE2 directly inhibits solute reabsorption in in vitro microperfused thick ascending limbs (TAL), as well as water and solute reabsorption in the collecting ducts (15, 16, 40). Moreover, acute infusion of PGE2 into the intact animals has been shown to cause water diuresis (1) and to inhibit water absorption in collecting ducts (14, 38). PGE2 therefore appears to antagonize the hydrosmotic effect of vasopressin, and recently Zelenina et al. (49) demonstrated that PGE2 may counteract vasopressin action by activation of AQP2 retrieval from the apical plasma membrane of collecting duct principal cells, and this has subsequently been demonstrated by Nejsum et al. (34) to be independent on AQP2 dephosphorylation. Since dehydration (high interstitial hyperosmolality) and vasopressin treatment (DDAVP-treated BB rats in the present study) increase COX-2 expression in the inner medulla (and thereby presumably results in an increased PG production), this would be consistent with the notion that PGs serve as local feedback regulators of the antidiuretic action of vasopressin. Thus the existence of the two opposing signaling pathways of vasopressin and PGs may play a key role in the fine regulation of net water excretion in collecting ducts.

**Urinary Excretion of PGE2 in Lithium-Induced NDI**

The observed decrease of COX-1, COX-2, and mPGES expression in the kidney inner medulla, which is considered to be the major site of PGE2 synthesis, would suggest that kidney PGE2 synthesis and urinary PGE2 excretion should be decreased on lithium treatment. However, the 24-h urinary excretion of PGE2 in lithium-treated rats was significantly increased compared with control rats. This is consistent with previous findings showing increased daily urinary PGE2 excretion in lithium-induced NDI in both experimental animals.

**Fig. 10. Immunohistochemistry for COX-2 in kidney inner medulla of untreated BB rats (A and B) and BB rats treated with DDAVP for 6 days (C and D) at low (top) and high magnification (bottom). Arrows, interstitial cells. Labeling is absent in control BB rat (A and B). After 6 days of DDAVP treatment, there is a dramatic increase in labeling intensity for COX-2 in inner medullary interstitial cells (C and D). Magnification: ×50 (A and C) and ×400 (B and D).**

**Fig. 11. Immunohistochemistry for mPGES in kidney inner medulla of untreated BB rats (A) and BB rats treated with DDAVP for 6 days (B). mPGES labeling is mainly localized to the IMCD, and a much weaker signal is seen in inner medullary interstitial cells (filled arrows). With DDAVP treatment, the mPGES staining was increased in the IMCD, interstitial cells, and thin limb tubular cells (open arrows). Magnification: ×400.**
and human patients as well as in patients with congenital forms of NDI (17, 18, 41). Thus these observations suggest that measurements of PGE2 in the urine do not accurately reflect inner medullary renal PGE2 synthesis. The high tubular flow rate in lithium-treated rats could partly be responsible for this discrepancy, which, it is very important to emphasize, has similarly been observed after furosemide treatment where urinary excretion of prostanoid is also increased despite down-regulation of COX-1 and COX-2 expression in the inner medulla (9, 35). Interestingly, the changes in cortical COX-2 protein expression in response to lithium and dehydration in control rats paralleled the changes in PGE2 excretion, whereas dehydration of lithium-treated rats did not result in a significant increase in 24-h urinary PGE2 excretion despite elevated COX-2 labeling in the inner medulla. These results also suggest that cortical COX-2 expression and PGE2 production may affect urinary PGE2 excretion. In addition, the expression of other enzymes involved in PGE2 production, such as cytosolic PGES and phospholipase A2, could be increased with lithium treatment. The discrepancy could also be a consequence of activation of the cyclooxygenase-independent prostaglandin synthesis pathway (7). Thus further studies are needed to understand the discrepancy between increased urinary PGE2 excretion and decreased expression of inner medullary COX-1, COX-2, and mPGES in lithium-induced NDI.

Changes in COX-1, COX-2, and mPGES Expression in Vasopressin-Deficient BB Rats

BB rats treated with the V2 receptor-selective agonist DDAVP for 6 days showed a marked increase in COX-1, COX-2, and mPGES expression in the kidney inner medulla, paralleled by decreased urine output and increased urine osmolality, compared with nontreated BB rats. Immunohistochemistry demonstrated that the increased expression was most pronounced in inner medullary interstitial cells, further supporting the role of these cells as important kidney osmolality sensors. In contrast, COX-1 was not significantly changed in ISOM and kidney cortex. COX-2 expression in the cortex was even moderately downregulated, and COX-2 labeling in the macula densa region was decreased by the DDAVP treatment in BB rats. This is consistent with the finding that DDAVP treatment in BB rats is associated with low plasma renin activity compared with nontreated control BB rats (23). Upregulation of COX-1 and COX-2 in the inner medulla was accompanied by a 2.5-fold increase in mPGES expression in the kidney inner medulla. Immunohistochemistry revealed that the increase in mPGES labeling was most noticeable in IMCD cells, indicating the functional coupling between COX-1 and mPGES.

Summary

We have demonstrated that lithium-induced NDI was associated with decreased expression of all three main enzymes of prostaglandin synthesis in the inner medulla. This is likely due to chronically decreased interstitial osmolality. The observation that water restriction of lithium-treated rats resulted in a marked increase in COX-2 immunolabeling in inner medullary interstitial cells, paralleled by an increased urine concentration, further supports this view. Moreover, chronic treatment with a vasopressin V2-receptor agonist in BB rats increased the expression of COX-1, COX-2, and mPGES in the kidney inner medulla. Observed changes in expression are again most likely due to the increase in interstitial osmolality after DDAVP treatment. This further implicates both cyclooxygenases and mPGES as major factors in protecting kidney functional integrity in physiological and pathophysiological conditions of urinary osmolality changes.

Fig. 12. Immunohistochemistry for COX-2 in kidney cortex of untreated BB rats (A and B) and DDAVP-treated BB rats (C and D) at low (top) and high magnification (bottom). COX-2 is localized to single cells or small groups of cells (arrows) in the macula densa region of the cortical thick ascending limb. In DDAVP-treated BB rats (C and D), the labeling intensity of COX-2-positive cells appeared decreased compared with untreated BB rats (A and B). Magnification: ×50 (A and C) and ×400 (B and D).
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