Indomethacin Enhances Shuttling of Aquaporin-2 Despite Decreased Abundance in Rat Kidney

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Abstract. The effect of nonsteroidal antiinflammatory drugs on the regulation of aquaporin-2 (AQP2) water channels in the kidney was determined. Male Sprague-Dawley rats were injected with indomethacin (5 mg/kg twice a day intraperitoneally) for 2 d. The control group was injected with vehicle. The expression of AQP2 proteins was determined in the kidney by immunoblotting and immunohistochemistry. The expression of Gsα and type VI adenyl cyclase was determined by immunoblotting. The activity of adenyl cyclase complexes was determined by stimulated accumulation of cAMP. Immunoblotting revealed that indomethacin markedly decreased the expression of AQP2. Accordingly, however, the ratio of AQP2 expression in the membrane fraction versus that in the cytoplasmic fraction was increased. The urinary excretion of AQP2 proteins also increased. Immunohistochemistry demonstrated almost exclusive apical labeling of AQP2 with scanty cytoplasmic localization along the collecting duct. The expression of Gsα and adenyl cyclase VI proteins was decreased. The generation of cAMP provoked by arginine vasopressin, sodium fluoride, or forskolin was blunted. These results suggest that indomethacin increases the shuttling of AQP2 while it decreases its abundance in the collecting duct.

It has long been known that inhibition of prostaglandin synthesis with nonsteroidal antiinflammatory drugs (NSAID) may be complicated by various functional alterations. Among others, indomethacin enhances the antidiuretic activity of arginine vasopressin (AVP) (1). It markedly reduces urine flow with no measurable changes of systemic BP, GFR, or effective renal plasma flow (2). However, mechanisms underlying the increased urinary concentrating ability have not been fully understood.

Over the last decade, it has been well known that epithelial water transport occurs through aquaporin (AQP) channels. Among its multiple isoforms, aquaporin-2 (AQP2) is mainly expressed in the collecting duct, which plays an important role in determining the final urinary output of water and solutes. It is regulated by AVP/cAMP pathway in the short and long term to increase osmotic water reabsorption in the principal cell (3–5). Moreover, alterations of its regulation have been implicated in various pathophysiological states mainly associated with altered urinary concentrating ability, such as acute ischemic renal failure (6), urinary tract obstruction (7), gentamicin-induced nephropathy (8), chronic renal failure induced by surgical renal mass reduction (9), and cisplatin-induced nephropathy (10).

It has been also demonstrated that acute oral intake of ibuprofen increases the urinary excretion of AQP2 in healthy humans (11). In this context, an altered regulation of AQP2 may be causally related with the altered urinary concentration ability associated with the use of NSAID. The study presented here sought to examine the effects of NSAID on the regulation of AQP2 channels in the kidney. Rats were treated with indomethacin, and their expression of AQP2 was determined in the kidney by Western blot analysis and immunohistochemistry. To specify the point of impairment in AVP/cAMP pathway, the activity and expression of adenyl cyclase complexes were also determined.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 200 to 250 g were used. Rats were given indomethacin (2.5 to 5.0 mg/kg dissolved in 10 mM Na2CO3, every 12 h intraperitoneally) for 2 d. The control group was injected with the solvent, i.e., Na2CO3. During the experiment, the rats were kept in a metabolic cage. The amount of food in the control group was adjusted according to that consumed in the experimental group. Another experimental group was injected with diclofenac (100 mg/kg dissolved in saline, single dose, intraperitoneally), and kept for 2 d. Its control group was injected with saline. The whole experimental procedure conformed to the Institutional Guidelines of Experimental Animal Care and Use.

Renal Functional Data and Plasma AVP Level

On the experimental day, the rats were decapitated in a conscious state to collect the trunk blood. Serum levels of creatinine and osmolality were determined. The plasma AVP concentration was measured by means of a commercial RIA kit (Incstar, Stillwater, MN).
Urinary AQP2 excretion

To determine the urinary excretion of AQP2, the urine was collected in 15 ml of ice-cold 1 M Tris-Cl (pH 6.8) containing 1 mg/ml leupeptin, 1 mM sodium azide, and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF). The debris was removed by centrifugation at 1000 \( \times g \) for 5 min. The supernatant was centrifuged again at 100,000 \( \times g \) for 1 h at 4°C, and the resultant pellet was suspended with 30 \( \mu l \) of isolation buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 \( \mu M \) leupeptin, 1 mM PMSF, pH 7.2). The sample was stabilized by heating at 95°C for 5 min in sample buffer (4% SDS, 20% glycerol, 0.125 M Tris [pH 6.8], 0.2% bromophenol blue, 10% 2-mercaptoethanol) and then subjected to AQP2 determination.

Differential Centrifugation

Differential centrifugation of the kidney was carried out as described by previous investigators (12). Inner medullary homogenates were initially centrifuged at 4000 \( \times g \) for 10 min at 4°C to remove incompletely homogenized fragments and nuclei. The pellets were resuspended in ice-cold isolation solution with protease inhibitors and centrifuged again at 4000 \( \times g \) for 10 min. The supernatants were collected and centrifuged at 17,000 \( \times g \) for 20 min. The pellets were retained (high-density fraction, HD), and the supernatants were then pelleted by centrifugation at 200,000 \( \times g \) for 1 h (low-density fraction, LD). The expression of AQP2 was then determined in HD and LD fractions. The shuttling was noted by the ratio of AQP expression in HD fraction over that in LD fraction. An increase of HD/LD represents an enhanced shuttling.

Western Blot Analysis

Amounts of AQP2 in the kidney and the urine were determined by Western blot analysis as described previously (7). Antibodies used were rabbit polyclonal AQP2 (Alomone Lab; Jerusalem, Israel), \( G_{\alpha} \) (Calbiochem-Novabiochem; San Diego, CA), and type V1 adenylyl cyclase antibodies (Santa Cruz Biochemicals; Santa Cruz, CA). Heat-shock protein (HSP) 25 and HSP70 antibodies were purchased from StressGen (Victoria, BC, Canada). The bound antibody was detected by enhanced chemiluminescence (Amersham; Little Chalfont, Buckinghamshire, UK) on hyperfilm. Relative protein levels were determined by analyzing the signals of autoradiograms with a transmittance-scanning videodensitometer.

Immunohistochemistry

Kidneys were fixed in rats under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally) by in vivo perfusion through the abdominal aorta with 2% paraformaldehyde-lysine-periodate in PBS, pH 7.4. They were removed and additionally fixed by immersion in the same fixative overnight at 4°C. Fifty-\( \mu m \) sections were made and processed via a horseradish peroxidase preembedding technique. Tissues were dehydrated and embedded in wax (polyethylene glycol 400 disterate; Polysciences, Warrington, PA). Four 4- to 6-\( \mu m \)-thick wax sections were prepared for postembedding immunohistochemistry.

Before Embedding

Vibratome sections were washed three times with 50 mM NH₄Cl in PBS for 15 min. The sections were incubated for 3 h with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution A), and incubated with primary antibodies. They were then incubated overnight at 4°C in a solution of AQP2 antibodies (1:500) in PBS containing 1% bovine serum albumin (solution B). After several washes with solution A, the tissue sections were incubated for 2 h in peroxidase-conjugated donkey anti-rabbit IgG, Fab fragment (Jackson ImmunoResearch Lab, West Grove, PA; 1:100 diluted in solution B). The sections were then rinsed, first in solution A and subsequently in 0.05 M Tris(hydroxyethyl)aminomethane (Tris) buffer (pH 7.6). For the detection of horseradish peroxidase, the sections were incubated in 1% 3,3′-diaminobenzidine (brown color) in 0.05 M Tris buffer for 5 min, after which H₂O₂ was added to a final concentration of 0.01%, and the incubation was continued for 10 min. After washing with 0.05 M Tris buffer three times, the sections were dehydrated in a graded series of ethanol and embedded in Epon 812 resin, and photographed.

After Embedding

Wax sections were processed for immunohistochemistry with the avidin-biotin-horseradish peroxidase technique (Elite Vectastain ABC kit; Vector Laboratories; Burlington, CA). The sections were dewaxed with xylene and ethanol. After rinsing in tap water, they were treated with methanolic H₂O₂ for 30 min to inhibit the intrinsic peroxidase. They were then permeabilized by incubation in 0.5% Triton X-100 in PBS for 15 min, and subsequently blocked with normal goat serum (1:10 diluted in PBS) for 15 min, and incubated with rabbit polyclonal AQP2 antibodies (1:500) overnight at 4°C. After rinsing in PBS, the sections were incubated with biotinylated goat anti-rabbit antibodies for 1 h at room temperature. After further washing, the sections were incubated with 0.3% avidin-biotin-peroxidase complex for 1 h at room temperature. The site of the antigen-antibody reaction was visualized by 3,3′-diaminobenzidine tetrahydrochloride reaction. After dehydration with increasing concentrations of ethanol, the sections were covered with glass slips and photographed.

Membrane Preparation and Adenylyl Cyclase Activity

The membrane preparation was obtained as described previously (6). Adenylyl cyclase activity was assayed by the method of Bar with a slight modification (13). AVP was used to activate V2 receptors, sodium fluoride was to stimulate adenylyl cyclase in a receptor-independent but G protein–dependent manner, and forskolin was used to directly stimulate the catalytic unit of the complex. cAMP was measured in the supernatant by equilibrated RIA. Iodinated 2′-O-monomosuccinyl-adenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester was prepared as described by previous investigators (14). All samples in one experiment were analyzed in a single assay. Nonspecific binding was <2.0%. The 50% intercept was at 16.5 ± 0.8 fmol/tube (n = 10). The intra- and interassay coefficients of variation were 5.0 ± 1.2% (n = 10) and 9.6 ± 1.9% (n = 10), respectively. Results were expressed as moles of cAMP generated per milligram protein per minute.

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

Statistical Analyses

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

Results

Water Balance and Other Functional Parameters

Table 1 shows the functional data. There were no significant differences in the food and water intake between the groups. In the experimental group treated with indomethacin (5 mg/kg), the urinary flow rate significantly decreased in association with a positive water balance. However, there were no significant
changes in the plasma clearance of creatinine. Neither the plasma level of AVP nor the serum osmolality was significantly affected.

Expression of AQP2 Proteins

The expression of AQP2 proteins was determined in different segments of the kidney. The anti-AQP2 antibody recognized 29-kD and 35- to 50-kD bands, corresponding to nonglycosylated and glycosylated AQP2, respectively. After the treatment with indomethacin (5 mg/kg), the expression was significantly decreased in the cortex, outer medulla, and inner medulla (Figure 1). Despite the changes in AQP proteins, there were no significant differences in the expression of HSP25 and HSP70 between the control and experimental groups (Figure 2). The expression of AQP2 in the membrane fraction was preserved while that in the cytoplasmic fraction markedly decreased, so that HD/LD increased significantly (Figure 3). The urinary excretion of AQP2 was slightly increased (Figure 4). A lower dose of indomethacin (2.5 mg/kg) and diclofenac (100 mg/kg, single dose) similarly decreased the expression of AQP2 in the kidney (Figure 5).

Immunohistochemistry

Figures 6 to 9 show light micrographs of kidney sections from control and indomethacin-treated rats. The AQP2 immunoreactivity was present on the apical membrane and throughout the cytoplasm along the entire collecting duct as well as in the connecting tubule (CNT). The treatment with indomethacin consistently decreased the AQP2 immunoreactivity, being most prominent in the inner medullary collecting duct (Figure 6). Moreover, almost exclusive apical labeling with scanty cytoplasmic localization was noted (Figures 7 and 8). In CNT, the immunoreactivity was predominantly labeled on the basolateral membrane (Figure 9).

Expression of $G_{sa}$ and Adenylyl Cyclase VI and Activities of Adenylyl Cyclase

Figure 10 shows immunoblots of $G_{sa}$ and adenylyl cyclase VI in the inner medulla. The anti-$G_{sa}$ antibody recognized a doublet at 50 and 45 kD. The $G_{sa}$ expression was significantly decreased after the indomethacin treatment. The expression of type VI adenylyl cyclase, recognized as a broad band at ap-
proximately 160 kD, was significantly decreased. The generation of cAMP evoked by AVP, forskolin, or sodium fluoride also decreased significantly (Figure 11).

**Discussion**

The treatment with NSAID has been known to markedly reduce the urinary flow with no measurable changes of systemic BP, GFR, or effective renal plasma flow (2). It was also demonstrated in the study presented here that indomethacin did not significantly alter the plasma creatinine clearance. However, there was a decreased urinary excretion of sodium. These changes may in part be related with an altered regulation of natriuretic hormones in the kidney (15), accounting for the extracellular volume expansion associated with the use of NSAID (16,17). However, there have been no documents related with regulation of water channels, although an altered sodium handling may result in an altered water handling.

In the study presented here, there was a decrease in the abundance of AQP2 after the treatment with indomethacin, as shown by immunoblotting and immunohistochemistry. Furthermore, indomethacin diminished the generation of cAMP in response to forskolin and that to sodium fluoride in the kidney, along with decreased expression of Gs\textsubscript{a} and adenylyl cyclase VI. Therefore, not only Gs\textsubscript{a} proteins but also the catalytic unit of adenylyl cyclase may have been functionally impaired by indomethacin, to which the dysregulation of AQP2 channels may in part be attributable.

Despite the decrease of its total abundance, however, the shuttling of AQP2 was rather increased. Immunohistochemistry also demonstrated that the residuum of AQP2 immunoreactivity along with scanty cytoplasmic localization was chiefly seen in the apical region of the principal cell in the collecting duct. The urinary excretion of AQP2 was also slightly increased, being consistent with an increased expression in the apical plasma membrane.

An increased shuttling of AQP2 associated with a decrease of its total abundance is seemingly contradictory. However, the urinary excretion of AQP2 does not necessarily correlate with total AQP2 levels in the kidney (18), but closely follows conditions known to change AQP2 in the apical membrane (19,20). Therefore, the increase of its urinary excretion may represent an enhanced shuttling. Furthermore, the enhanced AQP2 shuttling was apparently translated into a diminished urine volume. The positive water balance may be a reflection of decreased urinary excretion of sodium and concomitantly enhanced shuttling of water channels.

It has been found that PGE\textsubscript{2} does not affect AQP2 phosphorylation, but increases the retrieval of AQP2 from the membrane fraction to the intracellular fraction, thereby counteracting the action of AVP (21). Conversely, Pedersen et al. (11) observed that inhibition of prostaglandin synthesis by ibuprofen in humans results in increases of urinary AQP2 excretion. A diminished production of PGE\textsubscript{2} by NSAID would thus lead to a reduction of AQP2 endocytosis, maintaining the apical distribution of AQP2.

It has been known that both short-term and long-term regulation of AQP2 is exerted by AVP/cAMP pathway (3,5). However, the dissociation of short-term and long-term regulation of AQP2 suggests that the regulatory mechanisms
may differ between them. Indomethacin may differentially affect cAMP-dependent and -independent intracellular signaling mechanisms. It is also likely that the short-term regulation of water permeability may be more complex than generally recognized and may involve regulation of both exocytosis and endocytosis of AQP2, possibly via multiple signaling pathways (22,23). In addition, the plasma level of AVP was not significantly altered by the treatment with indomethacin in our study. Therefore, the altered regulation of AQP2 cannot be attributed to changes in AVP levels, but to a direct effect of indomethacin on the adenylyl cyclase

Figure 5. Abundances of aquaporin-2 (AQP2) in the high-density (HD) and low-density (LD) fractions of inner medullary extracts. Legend as in Figure 1.

Figure 6. Light micrographs illustrating immunostaining of aquaporin-2 (AQP2) of 50-µm-thick sections of kidneys of control (A) and indomethacin-treated (5 mg/kg) experimental (B) rats. Immunolabeling is present along the entire collecting duct from cortex to inner medulla as well as in connecting tubule in both groups. Original magnification, ×16.

Figure 7. Aquaporin-2 (AQP2) localization in the cortical collecting duct. (A) AQP2 labeling is exclusively shown in the principal cell, both in the apical region and throughout the cytoplasm in the control. (B) Almost exclusive apical labeling with scanty cytoplasmic localization is noted in the indomethacin-treated tissue. Double small arrows, apical labeling of AQP2; arrowheads, AQP2-negative intercalated cells. Original magnification, ×700.

Figure 8. Aquaporin-2 (AQP2) localization in the outer (A and B) and inner (C and D) medullary collecting duct. (A and C) AQP2 immunolabeling is seen in both apical membrane and supranuclear region in the principal cell of outer and inner medullary collecting duct in the control. (B and D) AQP2 labeling is seen mainly on the apical plasma membrane (double small arrows) with scanty cytoplasmic localization in the indomethacin-treated tissue. Arrowheads, AQP2-negative intercalated cells. Original magnification, ×700.
complex. Mechanisms underlying the dissociation should be further studied.

It may be of interest to note that indomethacin caused differential changes in AQP2 targeting in different portions of the tubule: an exclusive apical labeling with scanty cytoplasmic localization was noted in the collecting duct while an

Figure 9. Aquaporin-2 (AQP2) localization in the connecting tubule. (A) AQP2 labeling is seen predominantly on the apical membrane, and weakly on the basolateral membrane in the control. (B) More intense labeling in the basolateral region in the indomethacin-treated tissue. Double small arrows, basolateral AQP2 labeling; arrowheads, AQP2-negative intercalated cells. Original magnification, ×700.

Figure 10. Immunoblots of stimulatory G proteins and type VI adenylyl cyclase in the inner medulla. Legends as in Figure 1.

Figure 11. cAMP production in response to arginine vasopressin (AVP), sodium fluoride, and forskolin in the inner medulla. Open circles, control; solid circles, indomethacin treated (5 mg/kg). Each point represents mean ± SEM of six experiments.
enhanced labeling was shown in the basolateral domain in CNT. It is not clear whether basolaterally located AQP2 acts as a novel exit of water in CNT, although AQP3 and AQP4 have been considered as main exit in the basolateral domain of the collecting duct (24,25). The regulation of AQP2 may differ in different portions of the tubule.

Finally, our study also demonstrated that indomethacin in a lower dose or diclofenac, another NSAID, similarly decreased the expression of AQP2 channels. In addition, there were no significant differences in the expression of HSP25 and HSP70 between the control and experimental groups. The HSP may be induced by exposure to heat or other stresses and play a protective role in minimizing the damaging effect of such stresses. A clear protective effect of over-expression of HSP70 has been demonstrated against subsequent challenge with hypoxia (26,27). The diminished expression of AQP channels cannot be ascribed to a non-specific effect of indomethacin per se.

In summary, indomethacin markedly reduced the expression of AQP2 water channels in the collecting duct along the activity of AVP/cAMP pathway. Accordingly, however, the shuttling of AQP2 was rather enhanced. The altered regulation of AQP2 may in part be causally related with the altered urinary concentrating ability and body water balance associated with the use of NSAID.

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References


