Cisplatin Decreases the Abundance of Aquaporin Water Channels in Rat Kidney

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Abstract. The present study examined whether the cisplatin-induced urinary concentration defect can be related to an altered regulation of aquaporin (AQP) water channels in the kidney. Cisplatin (8 mg/kg) was injected intraperitoneally into male Sprague-Dawley rats. The control group was without cisplatin treatment. Four d later, the expression of AQP1, AQP2, and AQP3 proteins was determined in the kidney. To specify further the primary point of derangement in the pathway that activates the arginine vasopressin–mediated AQP channels, different components of adenylyl cyclase complex were examined separately. The cisplatin treatment caused a polyuric renal failure in association with decreases of free water reabsorption. The expression of AQP1 and AQP2 was decreased in the cortex, the outer medulla, and the inner medulla, whereas that of AQP3 was decreased in the outer medulla and the inner medulla. The expression of AQP2 proteins in the apical membrane-enriched fraction decreased in parallel with that in the subapical vesicle-enriched fraction, indicating a preserved targeting. Immunohistochemistry of the outer medulla also revealed that cisplatin decreased immunoreactivity for AQP1, AQP2, and AQP3. The arginine vasopressin–evoked generation of cyclic adenosine monophosphate was attenuated by cisplatin, being most prominent in the outer medulla. However, the cyclic adenosine monophosphate generation in response to forskolin was not affected, whereas that to sodium fluoride was diminished significantly. Cisplatin also decreased the expression of Gsα proteins in the outer medulla and the inner medulla. These results suggest that a reduced expression of AQP water channels accounts at least in part for the cisplatin-induced urinary concentration defect.

Although cisplatin is used widely as an antineoplastic agent, its therapeutic usefulness may be limited by the potential toxicity that leads to acute renal failure. Among others, the most prominent feature associated with the cisplatin-induced renal failure is polyuria, of which urinary concentration defect has been attributed to an inability of the collecting duct to respond to arginine vasopressin (AVP) (1–3). The AVP resistance in turn has been related to a reduced cyclic adenosine monophosphate (cAMP) generation secondary to a defect in a G protein–mediated mechanism (4). However, detailed mechanisms that underlie the AVP resistance have not been established.

It has been established recently that the epithelial water transport occurs through distinct channels called aquaporins (AQP) (5,6). Among multiple isoforms of the AQP family, AQP1 is present in the cells of the proximal tubule and the descending limb of Henle’s loop (7). Recent evidence from AQP1 gene knockout mice demonstrated that 80 to 90% of osmotic permeability in the proximal tubule and descending thin limb can be attributed to AQP1 channels (8,9). This points to an important role of AQP1 in concentrating the urine. A decreased expression of AQP1 may then result in a decreased medullary hypertonicity that is produced by countercurrent multiplication.

Conversely, AQP2 is expressed on the subapical vesicles as well as the apical membranes of the collecting duct principal cells (10). It is regulated short term and long term by the AVP/cAMP pathway. A short-term regulation of AQP2 represents trafficking of cytoplasmic vesicles to the apical membrane (11,12), and a long-term regulation is to augment the abundance of its protein in the cell (13). On the contrary, AQP3 is localized mainly to the basolateral membrane of collecting duct principal cells (14). Recent studies demonstrated a long-term regulation of AQP3 with a marked increase in its expression in the collecting duct in response to water restriction or AVP infusion but not of AQP1 or AQP4 (13,14). Certain pathophysiologic conditions associated with an altered urinary concentration have been related causally to an altered regulation of AQP water channels (15–18).

The present study aimed to investigate whether the cisplatin-induced urinary concentration defect may be related to an altered regulation of AQP channels. Rats were treated with cisplatin, and their expression of AQP1, AQP2, and AQP3 proteins in the kidney was determined by Western blot analysis and immunohistochemistry. To specify further the point of primary impairment in the pathway that leads to the stimulation of AVP-mediated AQP channels, the expression of stimulatory G proteins and the activity of adenylyl cyclase were also determined.
Materials and Methods

Animals and Renal Function

Male Sprague-Dawley rats weighing 200 to 250 g were used. They were kept in accordance with the Institutional Guidelines of Experimental Animal Care and Use. They received an injection of cisplatin (4 or 8 mg/kg intraperitoneally; Boryung, Ansan, Korea) and were kept for 4 d. Rats that received an injection of vehicle only served as control.

On the experimental day, the rats were anesthetized with thiopental sodium (50 mg/kg intraperitoneally). The urinary bladder was cannulated with PE 50 tubings through a low abdominal incision to collect urine samples. At the end of urine collection, the arterial blood was taken. Creatinine clearance was determined. Free water reabsorption (T^3H_2O) was calculated by the following formula:

\[ T^3H_2O = V \times (\text{Uosm/Posm} - 1) \]

where \(V\) is urine volume, Uosm is urine osmolality, and Posm is plasma osmolality.

Protein Preparation and Western Blot Analysis

The kidneys were isolated rapidly after the rats were decapitated under a conscious state. They were kept at \(-70°C\) until assayed. The cortex, outer medulla, and inner medulla were dissected and homogenized at 3000 \(x\) \(g\) in a solution containing 250 mM sucrose, 1 mM ethylenediaminetetraacetate, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl buffer at pH 7.6. Large tissue debris and nuclear fragments were removed by two low-speed spins in succession (1000 \(x\) \(g\), 10 min; 10,000 \(x\) \(g\), 10 min). Protein samples were loaded and electrophoretically size-separated with a discontinuous system consisting of 12.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 0.1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for 1 h, and incubated with affinity-purified anti-rabbit polyclonal AQP1 (diluted 1:750), AQP2 (1:750), and AQP3 (1:200) antibodies (Alomone Laboratories, Jerusalem, Israel), or heteromeric G-protein subunit Gso (1:1000) antibody (Calbiochem-Novabiochem, San Diego, CA) in 2% nonfat milk/TBST for 1 h at room temperature. The membranes were then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:1200) in 2% nonfat milk in TBST for 2 h. The bound antibody was detected by enhanced chemiluminescence (Amersham, Little Chalfont, Buckinghamshire, UK) on hyperfilm. The relative protein levels were determined by analyzing the signals of autoradiograms using the transmitter scanning videodensitometer.

Immunohistochemistry

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

The tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, rinsed twice in PBS, and then treated with 3% \(H_2O_2\) in 60% methanol for 30 min to quench endogenous peroxidase activity. After the sections were washed twice (5 min each) in PBS, they were blocked in PBS containing 5% goat serum for 1 h. The sections were incubated for 12 to 14 h with AQP1, AQP2, or AQP3 antibodies diluted 1:1000 in PBS with 0.3% bovine serum albumin. For a negative control, the sections were incubated in PBS containing 5% goat serum only. The sections were then rinsed thrice in PBS and incubated sequentially for 30 min each with biotinylated secondary antibody and ABC reagents, followed by a 6-min incubation with the peroxidase substrate solution diaminobenzidine. The sections were examined and photographed on a light microscope.

Differential Centrifugation

To differentiate the apical membrane-enriched versus subapical vesicle-enriched fractions, we conducted centrifugation as described by previous investigators (19). The homogenates of the cortex, outer medulla, and inner medulla were centrifuged at low-speed spins (1000 \(x\) \(g\) for 10 min) to remove cell debris and nuclear fragments. The supernatant was centrifuged at 17,000 \(x\) \(g\) for 20 min to yield apical membrane-enriched pellets (high-density fraction [HD]). The supernatant was centrifuged again at 100,000 \(x\) \(g\) for 1 h to obtain a vesicle-enriched pellet (low-density fraction [LD]). Comparing the magnitude of its expression in the two fractions assessed the trafficking of AQP2. A decrease in HD/LD represents an inhibited trafficking.

Membrane Preparation and Adenylyl Cyclase Activity

The membrane preparation was obtained as described previously (15). The cortex, outer medulla, and inner medulla were separated. They were homogenized in ice-cold homogenizing buffer (50 mM Tris-HCl [pH 8.0] containing 1 mM ethylenediaminetetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose) and centrifuged at 100,000 \(x\) \(g\) and 100,000 \(x\) \(g\) in succession. The resulting pellet was used as membrane preparation. Protein concentrations were measured by bichinchonic acid assay kit (BioRad, Hercules, CA).

Adenylyl cyclase activity was assayed by the method of Bar (20), with a slight modification. Segments of adenylyl cyclase complex were examined separately using different drugs. AVP was used to activate V2 receptor, sodium fluoride was used to stimulate adenylyl cyclase in a receptor-independent but G-protein-dependent manner (21), and forskolin was used to provoke directly the catalytic unit of adenylyl cyclase complex (22). The reaction was started by adding the membrane fraction, of which protein contents were 20, 10, and 10 \(\mu g\) for the cortex, outer medulla, and inner medulla, respectively, in 100 \(\mu l\) of working solution (50 mM Tris-HCl [pH 7.6], containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase, 6.4 mM MgCl\(_2\), 1 mM 3-isobuty-1-methylxanthenine, 0.02 mM GTP). After 15 min, the reaction was stopped by cold application of solution consisting of 50 mM sodium acetate (pH 5.0) and centrifuged at 1000 \(x\) \(g\) for 10 min at 4°C.

cAMP was measured in the supernatant by equilibrated RIA. iodinated 2-O-monosuccinyl-adenosine 3',5'-cyclic monophosphate tyrosyl methyl ester (125I-ScAMP-TME) was prepared as described by previous investigators (23). Standards or samples were taken up in a final volume of 100 \(\mu l\) of 50 mM sodium acetate buffer (pH 4.8). Dilute cAMP antiserum (Calbiochem-Novabiochem) and 125I-ScAMP-TME (10,000 cpn/100 \(\mu l\)), 100 \(\mu l\) each, were added and incubated for 15 h at 4°C. The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in a \(\gamma\) counter (Packard Instrument, Meriden, CT). 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 (\(n = 10\)). Intra- and interassay coefficients of variation were 5.0 ± 1.2 (\(n = 10\)) and 9.6 ± 1.9% (\(n = 10\)), respectively. Results were expressed as moles of cAMP generated per milligram of protein per
minute. Drugs were purchased from Sigma Chemical Company (St. Louis, MO), unless stated otherwise.

**Statistical Analyses**

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

**Results**

**Renal Function**

Table 1 summarizes the renal functional data. After treatment with cisplatin, serum levels of creatinine increased while its renal clearance decreased. The urinary volume significantly increased in association with decreases in its osmolality and tubular free water reabsorption. The degree of polyuria was more prominent with 8 mg/kg than with 4 mg/kg cisplatin.

**Expression of AQP Water Channels**

The expression of AQP proteins was determined in the cortex, outer medulla, and inner medulla of the kidney. The anti-AQP1 antibody recognized 29-kD and 35- to 50-kD bands, corresponding to nonglycosylated and glycosylated forms of the protein.
AQP1, respectively. The anti-AQP2 antibody recognized 29-kD and 35- to 50-kD bands, corresponding to nonglycosylated and glycosylated AQP2, respectively. The anti-AQP3 antibody recognized 27-kD and 33- to 40-kD bands, corresponding to nonglycosylated and glycosylated AQP3, respectively. After treatment with cisplatin (8 mg/kg), the expression of AQP1 was decreased significantly in the cortex, outer medulla, and inner medulla (Figure 1). The AQP2 expression was also decreased in the cortex, outer medulla, and inner medulla (Figure 2). The expression of AQP3 was decreased in the outer medulla and the inner medulla but not in the cortex (Figure 3). By the use of a lower dose of cisplatin (4 mg/kg), the expression of AQP1 and AQP2 was decreased only in the outer medulla while that of AQP3 was not affected significantly (Figures 1 through 3).

**Cellular Distribution of AQP2**

The expression of AQP2 channels was determined differentially in the apical membrane-enriched and the subapical vesicle-enriched fractions to examine whether their targeting was changed. Cisplatin decreased the expression of AQP2 proteins in the apical membrane-enriched fraction in parallel with that in the subapical vesicle-enriched fraction, so HD/LD remained unchanged (Figure 4).

**Immunohistochemistry of AQP Channels**

Histologic changes induced by cisplatin were most prominent in the outer stripe of the outer medulla. Partial to complete desquamation and necrosis of individual cells or short sections of the tubular epithelium was variably present along the straight portion of the proximal tubule. In contrast, the glomerulus, proximal convoluted tubule, loop of Henle, and collecting duct were well preserved. The immunoreactivity for AQP1 was expressed prominently in the apical membrane of the S3 segment of proximal tubules but was markedly decreased by cisplatin (Figure 5, A and B). The abundance of AQP2 labeling was observed in the principal cells of collecting duct, both in the apical region of the cell and throughout the cytoplasm (Figure 5C). Cisplatin decreased the AQP2 labeling, and the residual AQP2 was expressed in both the cytoplasmic region and the apical membrane (Figure 5D). The expression of AQP3, which was localized to the basolateral membrane of principal cells in the outer medullary collecting duct, was decreased by cisplatin (Figure 5, E and F).

![Figure 3](image1)

**Figure 3.** Immunoblots of AQP3 in the cortex (C), outer medulla (OM), and inner medulla (IM). Each column represents mean ± SEM of six rats. □, control; □, cisplatin 4 mg/kg; □, cisplatin 8 mg/kg; *, P < 0.05; ***, P < 0.01 versus control.

![Figure 4](image2)

**Figure 4.** The abundance of AQP2 proteins in the high-density (HD) and low-density fractions (LD) in the cortex (C), outer medulla (OM), and inner medulla (IM). Each column represents mean ± SEM of eight rats. □, control; □, cisplatin 8 mg/kg.
Figure 5. Immunohistochemical localization of AQP1, AQP2, and AQP3 in the outer medulla in control and cisplatin-treated (8 mg/kg) rats. (A) Immunoreactivity for AQP1 was most prominent in the apical membrane of proximal tubules. (B) The AQP1 labeling was decreased markedly by the treatment with cisplatin. (C) The abundance of AQP2 labeling was shown exclusively in the collecting duct principal cells, both in the apical region and throughout the cytoplasm. (D) The AQP2 labeling was decreased by cisplatin. (E) AQP3 was localized to the basolateral membrane of the collecting duct principal cells. (F) The AQP3 labeling was decreased in the outer medullary collecting duct by cisplatin. ★, S3 segment of the proximal tubule; ●, collecting duct. Magnification: ×350.
Expression of Stimulatory G Proteins

Figure 6 shows immunoblots of $G_s$ expressed in the cortex, outer medulla, and inner medulla. The anti-$G_s$ antibody recognizes a doublet at 50 kD and 45 kD. The expression of $G_s$ proteins was decreased significantly in the outer medulla and inner medulla after cisplatin treatment but was not affected in the cortex.

Adenylyl Cyclase Activity

Figures 7 and 8 show cAMP generation in the cortex, outer medulla, and inner medulla. The generation in response to AVP was blunted by the cisplatin treatment and the effect was most prominent in the outer medulla. Conversely, the cAMP generation evoked by forskolin was not affected by cisplatin treatment, whereas that stimulated by sodium fluoride was attenuated significantly (Figure 8).

Discussion

The treatment with cisplatin caused renal failure associated with polyuria. The serum creatinine level was increased along with a decrease of its renal clearance. The urinary osmolality and tubular free water reabsorption were decreased. Accordingly, cisplatin (8 mg/kg) decreased the expression of AQP1 and AQP2 proteins in the cortex, outer medulla, and inner medulla of the kidney and that of AQP3 in the outer medulla and the inner medulla. Immunohistochemistry also revealed markedly decreased immunoreactivity for AQP1, AQP2, and AQP3. The decreased expression of AQP water channels in the kidney may be causally related to the cisplatin-induced polyuria.

Among the different regions of the kidney, the outer medulla may be most vulnerable to cisplatin. There was a dose-dependency in the degree of polyuria and in the extent of the reductions of AQP channels. By the use of a lower dose (4 mg/kg), the expression of AQP1 and AQP2 was decreased only in the outer medulla, whereas that of AQP3 was not affected. This finding is in line with that in a previous investigation (24), in which cisplatin-induced histopathologic features were limited primarily to the S3 segment of the proximal tubule located in the outer medulla.

AQP2 channels are regulated short term as well as long term (10). Although the expression of AQP2 was reduced, the reductions were in parallel in the apical membrane-enriched and subapical vesicle-enriched fractions, suggesting a pre-
It has been suggested that AVP has a regulatory role on the expression of AQP2 and AQP3 (13,14). Therefore, an abnormality in the AVP/cAMP pathway may result in a diminished generation of cAMP and hence AQP2 and AQP3 proteins.

One may argue that there may also be some derangement upstream affecting the Gs, such as the expression of V2 receptors or the binding of AVP to the receptor. In addition, Gs may not be a rate-limiting factor because its expression is relatively high compared with that of V2 receptor. Further studies will be needed to examine these possibilities in the cisplatin-induced nephropathy.

In summary, our results suggest that a reduced expression of AQP water channels accounts at least in part for the cisplatin-induced urinary concentration defect.

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**References**


**Figure 8.** cAMP production stimulated by forskolin and sodium fluoride in the outer medulla in control and cisplatin-treated rats. ○, control; ●, cisplatin (8 mg/kg)-treated rats. Each point represents mean ± SEM of five experiments. *, P < 0.05; **, P < 0.01 versus control.


