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Expression of renal aquaporins 1, 2, and 3 in a rat model of cisplatin-induced polyuria

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Expression of renal aquaporins 1, 2, and 3 in a rat model of cisplatin-induced polyuria.

Background. Cisplatin (CP)-induced polyuria in rats is attributed to decreased medullary hypertonicity and/or an end-organ resistance to vasopressin. However, the roles of renal aquaporins (AQPs) have not yet been explored.

Methods. Male Sprague-Dawley rats (230 to 245 g) received either a single injection of CP (5 mg/kg, $N = 4$) or saline ($N = 4$) intraperitoneally five days before sacrifice. Urine, blood, and kidney samples were analyzed.

Results. Platinum accumulated in the cortex and outer medulla of CP-treated rats (39.05 ± 7.50 and 36.48 ± 12.44 μg/g vs. 2.52 ± 0.43 and 1.87 ± 0.84 μg/g dry tissue in controls, respectively). Histologically, tubular damage and decreased AQP1 immunolabeling were detected in the S3 segment of proximal tubules. CP treatment caused 4.4- and 4.8-fold increases, respectively, in blood urea nitrogen and urine volume, and a 4.4-fold decrease in urine osmolality. Immunoblots showed that AQP2 and AQP3 were significantly reduced to 33 ± 10% ($P < 0.001$) and 69 ± 11% ($P < 0.05$), respectively, in the inner medulla of CP-treated rats. Immunocytochemical analysis showed a decrease in AQP2 labeling in the inner medulla of CP-treated rats. Northern hybridization revealed a 33 ± 11% ($P < 0.002$) decrease in AQP2 mRNA expression in the inner medulla of CP-treated rats. AQP1 protein expression levels were modestly (67 ± 7%, $P = 0.057$) and significantly (53 ± 13%, $P < 0.007$) decreased in outer and inner medullae, respectively, of CP-treated rats.

Conclusions. CP-induced polyuria in rats is associated with a significant decrease in the expression of collecting duct (AQP2 and AQP3) and proximal nephron and microvascular (AQP1) water channels in the inner medulla.

Aquaporins (AQPs) are members of a family of membrane channels that allow rapid movement of water across permeable epithelia, such as renal tubular epithelium. To date, at least 11 different types of AQPs have been cloned and characterized. Of these, the expression and/or physiological regulation of AQP1, AQP2, AQP3, AQP4, and AQP6 are well documented in the kidney [1, 2]. AQP1 is constitutively and very abundantly expressed on both the apical and basolateral domains of proximal tubular cells and the cells of the descending limb of Henle's loop. In this part of the proximal nephron, AQP1 aids the rapid reabsorption of large quantities (70 to 80%) of filtered water isosmotically coupled to the transport of sodium. AQP1 is also expressed in the renal microvasculature, especially the medullary descending vasa recta, which takes part in the counter current multiplication mechanism and thus plays an important role in the urinary concentrating mechanism [3]. AQP2, AQP3, and AQP4 are expressed in the collecting duct principal cells and aid in the osmotic transport of water in this part, which accounts for the reabsorption of 10 to 20% of filtered water. However, the absorption of this small fraction of free water, which is under the control of arginine vasopressin (AVP), determines the osmolality of the final voided urine. AQP3 is expressed on the basolateral aspect of the principal cells throughout the collecting duct. AQP4 is also expressed on the basolateral aspects of the same cells, but is restricted to the distal third of the medullary collecting ducts. On the other hand, AQP2 is expressed only on the apical domain and also on the subapical vesicles of collecting duct principal cells, and is regulated by AVP via the V2 subtype of vasopressin receptor that is coupled to the cAMP second-messenger system. Vasopressin has both short- and long-term effects on the expression of AQP2 [4]. The short-term effect (within a few minutes) consists of the translocation of AQP2 water channel-containing subapical vesicles to the apical plasma membrane. The long-term effect (several hours to few days) promotes the synthesis of AQP2 mRNA, resulting in an increase in the absolute amount of AQP2 protein in the cell. Unlike other members of the AQP family, AQP6 is

Key words: collecting duct, nephrotoxicity, vasopressin, proximal tubules, medullary hypertonicity.

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distributed in intracellular vesicles in multiple types of renal epithelia, where it apparently participates in distinct physiological functions such as glomerular filtration, tubular endocytosis, and acid-base metabolism [2]. Recent studies on animal models of several clinically relevant acquired polyuric conditions, such as lithium- or hypokalemia-induced bilateral ureteral obstruction, hypercalcemia, and ischemic reperfusion injury, revealed that these pathophysiological conditions are associated with a significant reduction in the expression levels of collecting duct water channels [5, 6].

Cisplatin (CP; *cis*-dichlorodiammine platinum II) is a potent antineoplastic agent in which the clinical use is limited because of its potential renal toxicity leading to acute renal failure [7–9]. CP also induces dose-related nephrotoxicity in laboratory animals. The nephrotoxicity manifests as a reduction in glomerular filtration rate [elevation in blood urea nitrogen (BUN) and serum creatinine levels], polyuria, a marked defect in urine concentrating ability, proteinuria, hyperuricemia, and frequently, magnesium wasting [10]. Pathologically, toxicity in both humans and laboratory animals is characterized by damage to the S3 segment (pars recta) of the proximal tubule [11–15]. In addition, while the human kidneys show evidence of necrosis of the distal tubules and collecting ducts [16, 17], there is controversy with respect to the effect of CP on the distal tubules of rodent kidneys, with reports ranging from no damage [18, 19] to moderate or to severe damage [13].

The most prominent feature of CP nephrotoxicity is polyuria, which occurs in two distinctive phases. Early polyuria occurs when the glomerular filtration rate (GFR) is still normal (1 to 2 days following a single dose) and can be reversed by the administration of vasopressin, suggesting impaired release of this hormone from the pituitary gland [10, 20]. The late-phase polyuria, which occurs when the GFR is low (5 to 6 days following a single dose), is of renal origin, and is resistant to vasopressin administration. This end-organ resistance to vasopressin is associated with a decrease in papillary hypertonicity [19, 21] and reduced cAMP generation, secondary, in part or whole, to a defect in the G-protein–mediated signaling in the collecting ducts [22]. The regulation of collecting duct water permeability by vasopressin is mediated by its effect on the translocation and/or expression of AQP2 water channel, which involves an intact cAMP second-messenger system. Hence, we hypothesized that the late polyuria induced by CP is associated with the decreased expression of collecting duct water channels. In the current study, we examined that hypothesis by determining the expression levels of collecting duct water channels (AQP2 and AQP3) and the proximal nephron water channel (AQP1) in a rat model of CP-induced polyuria.

METHODS

Animals and treatment

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN, USA) weighing 230 to 245 g were used in this study. The rats were housed two per cage and were maintained on a 12-hour light/dark cycle and had free access to drinking water and standard rat chow. The animals were acclimated to the housing conditions for a few days prior to the experimental procedures. Baseline urine samples were collected over a period of 24 hours prior to treatment (day 0) by placing the animals in individual metabolic cages. On day 1, the rats were divided into two groups ($N = 4$ per group) and were injected intraperitoneally with either 5 mg/kg body wt CP (Alfa Products, Ward Hill, MA, USA) in a volume of 0.5 to 0.6 mL normal saline (CP-treated group) or with an equal volume of normal saline alone (control group). On day 4, the animals were placed in metabolic cages to collect a second 24-hour urine sample. Rats were euthanized on day 5 by sodium pentobarbital overdose, and whole blood was collected by cardiac puncture while the animals were in deep anesthesia. Both kidneys were quickly removed, placed in ice-cold sterile phosphate-buffered saline (PBS) in Petri dishes, and decapsulated. One kidney was transversely bisected, and one half was immersed in neutral-buffered 10% formalin for fixation for immunohistochemical studies. Cortex and outer and inner medullae were dissected from the remaining one-and-one-half kidneys and flash frozen in liquid nitrogen before storing at −75°C. The sharp dissection of different regions of rat kidneys was performed on ice and under a dissection microscope. Each kidney was cut across into four to five pieces and kept in ice-cold PBS. From each piece, the cortical tissue was excised from the rest of the medulla using the differential coloration of corticomedullary junction as a landmark. Then the reddish outer medulla, including the outer and inner stripes, was separated from the white papillary portion or inner medulla of the kidney.

Analysis of blood and urine samples

Blood urea nitrogen concentration was determined colorimetrically using a commercially available kit (Sigma Chemical Co., St. Louis, MO, USA). Twenty-four–hour urine volumes were measured. Aliquots of urine samples were centrifuged at $10{,}000 \times g$ for five minutes to remove suspended material, if any, and the supernatants were used to measure the osmolality by freezing point depression using a Microosmometer Model 3300 (Advanced Instruments Inc., Norwood, MA, USA).

Assay of tissue platinum levels

Platinum levels in the renal cortex and outer medulla were assayed as described previously [23]. Briefly, tissue

samples (about 150 mg wet wt) were oven dried to constant weight at 70°C and reweighed (dry wt). Dried tissue samples were then digested in nitric acid, and platinum levels in the digest were determined by flameless atomic absorption spectroscopy using a Perkin-Elmer Model 380 Spectrophotometer fitted with a model HGA 400 programmer. A calibration curve was constructed by using appropriate nitric acid dilutions of a commercially available platinum atomic absorption spectroscopy standard solution (Sigma Chemical Co.). Results are expressed as micrograms of platinum per gram dry tissue.

Polyclonal antibodies and cDNA probes for the detection of AQPs

Peptide-derived, affinity-purified polyclonal antibodies to AQPs 1, 2, and 3 were used in this study. The antibodies to AQP1 (L266) and AQP3 (L178) were kindly supplied by Dr. Mark Knepper (NHLBI, NIH, Bethesda, MD, USA) and have been characterized previously [24, 25]. The antibody to AQP2 (GN-762) was commercially raised (Genosys Biotechnologies, Inc., The Woodlands, TX, USA) by using standard methods against a C-terminal peptide sequence designed and published by Dr. Knepper [26]. GN-762 was purified and characterized by us as follows. The antiserum was affinity purified using a column of agarose beads on which the immunizing peptide was immobilized via a covalent linkage (SulfoLink Immobilization Kit # 2; Pierce, Rockford, IL, USA). An IgG fraction of the GN-762 preimmune serum was prepared on a protein A column (Pierce) for use in control experiments. Control immunoblots were prepared by using crude membrane fractions of rat kidney inner medulla as a source of AQP2 protein and were probed with either the affinity-purified antibody alone or affinity-purified antibody preadsorbed with an excess of immunizing peptide or with the purified preimmune IgG fraction at similar concentrations. The blots probed with the affinity-purified AQP2 antibody gave two bands that correspond to the expected molecular mass of the native and glycosylated AQP2 protein. Both of these bands were ablated by the preadsorption of the antibody by the immunizing peptide. No bands could be seen when the preimmune IgG fraction was used to probe the blots (data not shown here). In our immunohistochemical studies, we also used another AQP1 antibody (4835-EXT1; Zymed Laboratories, Inc., South San Francisco, CA, USA) that we commercially raised using the same peptide sequence that Dr. Knepper used for raising L266. This AQP1 antibody was purified and characterized previously [27]. The cDNA probe for AQP2 was obtained from a polymerase chain reaction (PCR) amplification of rat cDNA using rat AQP2 sequence-specific primers. The 370 bp amplification product was subcloned and sequence confirmed. The product extends from nt 281 to 651 of the mRNA (GenBank accession no. D13906). The cDNA probe for β-actin was generated by PCR amplification of rat kidney cDNA using the primers as previously described [27].

Extraction of total RNA and Northern hybridization

These were carried out essentially as described previously [28]. Briefly, total RNA was extracted from the inner medullary tissue using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) as per the manufacturer's protocol. Twenty micrograms of total RNA from each sample were chemically and heat denatured and size fractionated on an 1% formalin agarose gel in 1 × MOPS buffer for 4.5 hours at 96 V. Equality of sample loading was determined by matching the density of the 28S rRNA band visualized by ethidium bromide staining. RNA was transferred to Hybond N+ nylon membrane overnight by capillary transfer method using 10 × standard saline citrate (SSC) buffer and then ultraviolet cross-linked. The total RNA blot was prehybridized in 15 mL ExpressHyb (Clontech Laboratories, Palo Alto, CA, USA) for 30 minutes at 68°C and then hybridized with 1.5×10^6 cpm/mL of ^{32}P random-labeled (High Prime DNA Label; Boehringer-Mannheim, Indianapolis, IN, USA) rat cDNA probe for AQP2 for two hours at 68°C. Membranes were washed two times at room temperature for 15 minutes each in 2 × SSC, 0.05% sodium dodecyl sulfate (SDS), and two times at 50°C for 15 minutes each in 0.1 × SSC, 0.1% SDS and exposed to x-ray film (MS Film; Eastman Kodak Co., New Haven, CT, USA) overnight at −70°C with a Kodak-intensifying screen. The membranes were striped by boiling in 0.5% SDS for 30 minutes and then probed with a ^{32}P-labeled cDNA probe for β-actin as a control for loading.

Preparation of membrane vesicle fractions and immunoblotting

Fractions enriched in both plasma membranes and intracellular vesicles were prepared as described previously [28, 29] with a few modifications. Briefly, tissue samples were homogenized in isolation solution containing protease inhibitors using polytron tissue homogenizer. The homogenates were initially spun at 1000 × *g* for 10 minutes at 4°C to pellet nuclei and cell debris. Then the supernatants were centrifuged at 150,000 × *g* for 90 minutes at 4°C in a Beckman ultracentrifuge to pellet both plasma membrane and intracellular vesicles. The pellets were suspended in isolation solution with protease inhibitors, and the total protein concentration was measured. The membrane vesicle fractions thus prepared were solubilized at 60°C for 20 minutes in Laemmli sample buffer. The solubilized membrane proteins were electrophoresed on 12% polyacrylamide minigels (Novex, San Diego, CA, USA) under denaturing conditions. Equality of protein loading was checked by running loading gels and staining the separated proteins by Coomassie

blue. The size-fractionated proteins were electrophoretically transferred to nitrocellulose membranes (Novex) using Bio-Rad transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with milk proteins and then probed with affinity-purified polyclonal antibodies to AQP1 (L266) or AQP2 (GN-762) or AQP3 (L178) at an IgG concentration of 0.03 to 0.14 μg/mL. After washing to remove the nonspecifically bound primary antibodies, the membranes were probed with donkey antirabbit IgG conjugated to horseradish peroxidase (Pierce number 31458) at an IgG concentration of 0.16 μg/mL. Membranes were washed again, and the sites of antigen-antibody reaction were visualized by chemiluminescence (SuperSignal Substrate; Pierce). The reaction was captured on light-sensitive imaging film (Kodak no. 165-1579 Scientific Imaging Film). Each immunoblot was run at least twice to confirm the consistency of results obtained.

Quantitation of mRNA and protein levels

Images obtained from both Northern and Western blotting were scanned on a flatbed scanner (Plustek, OptiPro 483P), and the density of the bands was quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Densitometry results are reported as volume integrated values expressed as percentage of the mean values in controls (100%). For Northern hybridization, the band densities of AQP2 were normalized by the corresponding band densities of β-actin and were expressed as percentage of the mean values in controls (100%).

Histology and immunohistochemistry

Kidney samples were fixed in neutral-buffered 10% formalin for two days at room temperature, transferred to methanol, and then embedded in paraffin. Sections (5 μm thickness) were cut on a microtome and processed for immunocytochemical labeling as described previously [27]. Paraffin sections were stained with hematoxylin and eosin for conventional histologic examination. For immunocytochemical labeling, tissue sections were deparaffinized in xylene and rehydrated through a series of graded ethanol. Endogenous peroxidase activity was removed by incubating with hydrogen peroxidase. Nonspecific binding was blocked by incubation of sections with normal goat serum. Sections were then incubated with either AQP1 antibody (4835 EXT1; 0.4 μg/mL) or AQP2 antibody (GN 762; 0.07 μg/mL) overnight at 4°C. Sections were rinsed five times in 0.1 mol/L PBS containing 0.2% Triton X-100 and then incubated for one hour at room temperature with biotinylated goat antirabbit antibody (Vector Labs, Burlingame, CA, USA), followed by incubation with avidin-biotin complex (Vector Labs) for one hour at room temperature. Sections were then washed with PBS, rinsed briefly in 0.1 mol/L acetate buffer, pH 6.0, and then incubated with diaminebenzidine for four minutes and counterstained with 0.1% nuclear fast red in 5% aluminum sulfate. Labeling controls were performed under the same conditions but by omitting either the primary or the secondary antibody, or using preimmune IgG (0.5 μg/mL) in place of primary antibody as described previously in this article. The sections were examined by at least two investigators, of which one was blind to the CP treatment status. However, the differences between the control and CP-treated groups are so striking that a cursory examination under light microscope readily distinguishes these groups, as differences in both staining pattern and cellular and tubular architecture are obvious. Digital microphotographs were taken with a Nikon Coolpix 950 digital camera mounted on a Nikon X-FL microscope.

Statistical analysis

All quantitative data are expressed as mean ± SEM. Differences among the means of multiple parameters were analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer Multiple Comparisons test. Differences between two parameters were analyzed by either unpaired *t*-test or by nonparametric methods (Mann–Whitney test). *P* values of less than 0.05 were considered significant. Instat2 software (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis.

RESULTS

Body weight

Table 1 presents the body weights in control and CP-treated groups of rats. Five days after initiation of the experiment, the control group showed a significant (11%) increase in body weight ($P < 0.001$), whereas the CP-treated groups has a significant loss (5.2%) of initial body weight ($P < 0.05$). Since the initial body weights in both groups on day 0 were similar, it is reasonable to assume that the CP-treated group showed a net loss of 16% in their body weight on day 5 as compared with the control group, indicating a generalized debilitating condition.

Urine analysis

Table 1 shows urine volumes and osmolality at the initiation (day 0) and completion (day 5) of the study. The urine volumes over a period of 24 hours were not significantly different in control group on day 0 versus day 5. However, those collected from the CP-treated group showed a 4.8-fold increase on day 5 as compared with day 0, indicating severe polyuria. The urine osmolality in the control group showed variability from day 0 to day 5, but the mean values on both days are within the normal physiological range. In contrast, the urine osmolality in CP-treated rats showed a 4.4-fold decrease

Table 1. Body weight, urine volume, urine osmolality, blood urea nitrogen (BUN) and tissue platinum levels in control and cisplatin (CP)-treated rats

	Controls		CP-treated	
Parameter	Day 0	Day 5	Day 0	Day 5
Body weight[a]	100.0 ± 1.7	111.0[b] ± 1.2	100.8 ± 1.5	94.8[c] ± 1.7
Urine volume *mL/24 h*	14.8 ± 0.9	11.9 ± 0.7	10.3 ± 1.8	49.8[b] ± 5.5
Urine osmolality *mOsm/kg H_2O*	1313 ± 44	1686 ± 43	1783 ± 123	408[d] ± 39
BUN *mg/dL*	—	17.21 ± 0.39	—	75.22[e] ± 11.73
Platinum in cortex *μg/g dry tissue*	—	2.52 ± 0.43	—	39.05[e] ± 7.5
Platinum in outer med. *μg/g dry tissue*	—	1.88 ± 0.84	—	36.48 ± 12.44

All values are mean ± SEM, $N = 4$ per group.
[a] As percentage of values in controls on day 0, where 100% is equivalent to 235 g
[b] $P < 0.001$ vs. corresponding day 0 values
[c] $P < 0.05$ vs. corresponding day 0 values and $P < 0.001$ vs. day 5 values in controls
[d] $P < 0.001$ vs. day 0 values in CP-treated group or day 5 values in controls
[e] $P < 0.003$ vs. control values

on day 5 as compared with the values in the same group on day 0, thus indicating a severe impairment of urinary concentrating ability.

Blood urea nitrogen and tissue platinum levels

Table 1 shows the BUN and tissue platinum levels at the end of the study (day 5). The BUN values in CP-treated rats are 4.4-fold higher as compared with the values in control group, indicating severe renal functional impairment in CP-treated animals. The mean background levels of platinum in the cortex and outer medulla of control animals by our assay method were 2.52 ± 0.43 and 1.88 ± 36.49 μg/g dry tissue. Thus, the mean platinum levels in the renal cortex and outer medulla of the CP-treated rats were 15- and 19-fold higher than the respective control values, indicating that the renal cortex and outer medulla of these rats accumulated large amounts of the injected drug. Furthermore, there is a significant correlation between levels of platinum in the cortex and outer medulla ($r = 0.93$, $P < 0.04$). For want of sufficient sample size, we could not assay the platinum levels in the inner medulla of these animals.

Immunoblotting studies

A semiquantitative immunoblotting approach was used to examine the effect of CP treatment on the expression levels of AQP proteins in different regions of the kidney. Figures 1–3 show the expression of both native (26 to 29 kD) and glycosylated (33 to 55 kD) protein bands of AQP1 (Fig. 1), AQP2 (Fig. 2), and AQP3 (Fig. 3). Figure 4 represents the percentage changes in protein expression levels quantitated either individually (native or glycosylated) or combined together (total) in CP-treated group as compared with the control group. As seen in Figures 1 and 4, the abundance or levels of AQP1 protein expression was not altered in the cortex ($P = 0.68$), while its total amount was decreased to a modest value with borderline significance (67 ± 7%, $P = 0.057$) in the outer medulla. However, the expression levels of both the native and glycosylated AQP1 protein bands were significantly decreased in the inner medulla. Figures 2 and 4 document the changes in the expression levels of vasopressin-regulated apical AQP2 protein of collecting ducts in the cortex and outer and inner medullae. In the cortex, the expression of the native band showed a modest decrease with borderline significance (42 ± 25%, $P = 0.051$), while the glycosylated band was not altered ($P = 0.24$). In the outer medulla, both the native and glycosylated bands showed modest nonsignificant decreases. However, the levels or abundance of native and glycosylated AQP2 protein expressed in the inner medulla were significantly decreased (42 ± 7%, $P < 0.004$, and 29 ± 12%, $P < 0.001$). Figures 3 and 4 document the changes in the expression levels of AQP3. In the cortex and outer medulla of CP-treated rats, the AQP3 water channel showed a modest, but statistically nonsignificant increase in both native and glycosylated protein bands. However, CP treatment significantly decreased the expression of native (69 ± 11%, $P < 0.05$) and glycosylated (69 ± 10%, $P < 0.03$) AQP3 protein in the inner medulla.

Northern hybridization

To determine whether the changes in the expression levels of AQP2, the vasopressin-regulated apical water channel protein, were accompanied by corresponding changes in the expression levels of AQP2 mRNA levels, we performed Northern analysis on total RNA from the inner medulla using a gene-specific cDNA probe for AQP2. The same membrane was probed with a gene-specific probe for β-actin as an internal control. Figure 5 shows the expression levels of AQP2 mRNA normalized to β-actin mRNA levels. In the CP-treated rats, AQP2 mRNA levels were significantly decreased to 33 ± 11% of the control values ($P < 0.002$).

Histologic examination

Conventional histologic examination using light microscopy on paraffin sections of kidney stained with he-

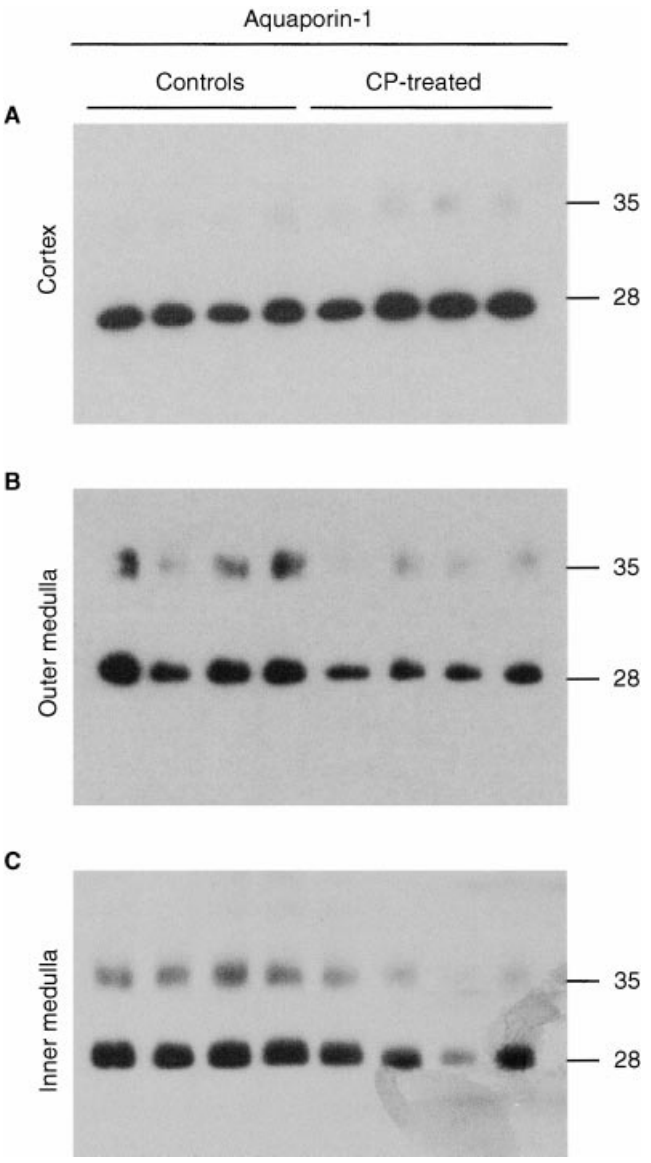

Fig. 1. Representative immunoblots of aquaporin 1 (AQP1) water channel in cortex and outer and inner medullae of saline-treated (controls) and cisplatin (CP)-treated rats. Quantities of 1.5, 1.5, and 5 μg of total protein were loaded in each lane of the gel for cortex, outer, and inner medullae, respectively. Blots were probed with AQP1 antibody (L226) at an IgG concentration of 0.04 μg/mL. Blots were exposed for longer duration to visualize the glycosylated protein bands.

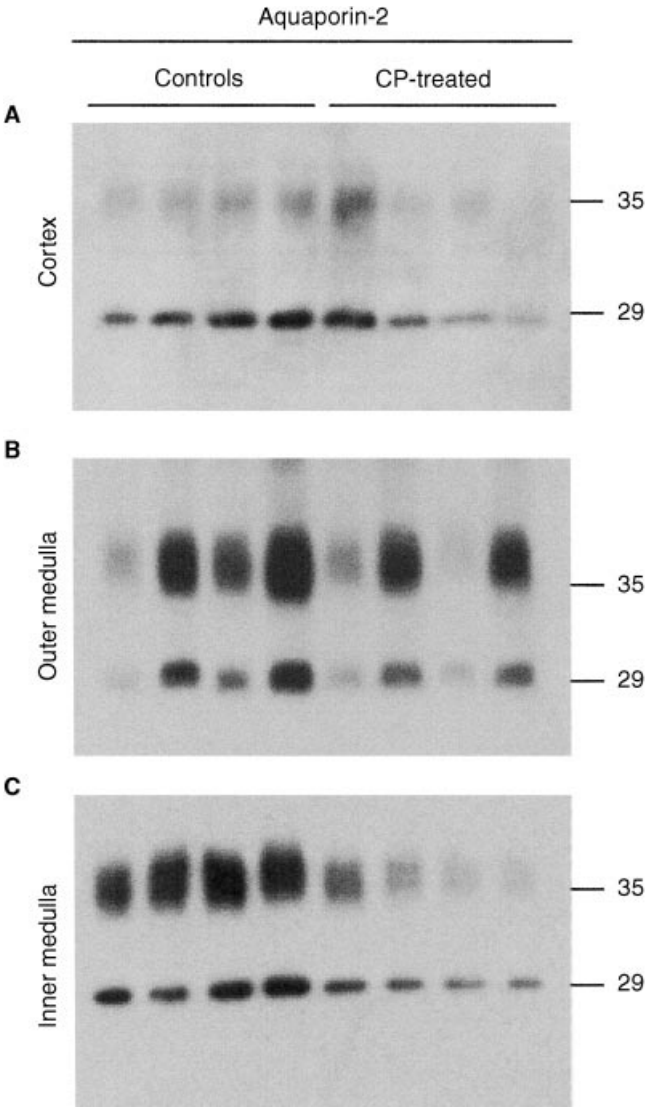

Fig. 2. Representative immunoblots of AQP2 water channel in cortex and outer and inner medullae of saline-treated (controls) and CP-treated rats. Quantities of 10, 5, and 1.5 μg of total protein were loaded in each lane of the gel for cortex, outer, and inner medullae, respectively. Blots were probed with AQP2 antibody (GN-762) at an IgG concentration of 0.14 μg/mL. Blots were exposed to longer duration to visualize the bands from all the lanes.

matoxylin-eosin showed striking and consistent differences between the CP-treated group and the control group in the deep cortex and corticomedullary junction. Specifically, in the CP-treated group, the S3 segments of proximal tubules showed evidence of tubular necrosis, loss of microvilli, and vacuolization, with surrounding interstitial infiltration of blood cells (data not illustrated). The superficial cortex, however, appeared to be free from such alterations (data not illustrated). Additionally, no histologic alterations could be detected in either the outer or inner medulla at the magnifications we could achieve with the light microscope.

Immunocytochemical examination

To examine the cellular and subcellular pattern of AQP expression, we performed immunocytochemistry on paraffin sections using specific polyclonal antibodies to AQP1 and AQP2. The labeling pattern of AQP1 in the superficial cortex of CP-treated rats was not different from the control group (data not illustrated). However, in the deep cortex and corticomedullary junction, consistent alterations in the labeling pattern of AQP1, espe-

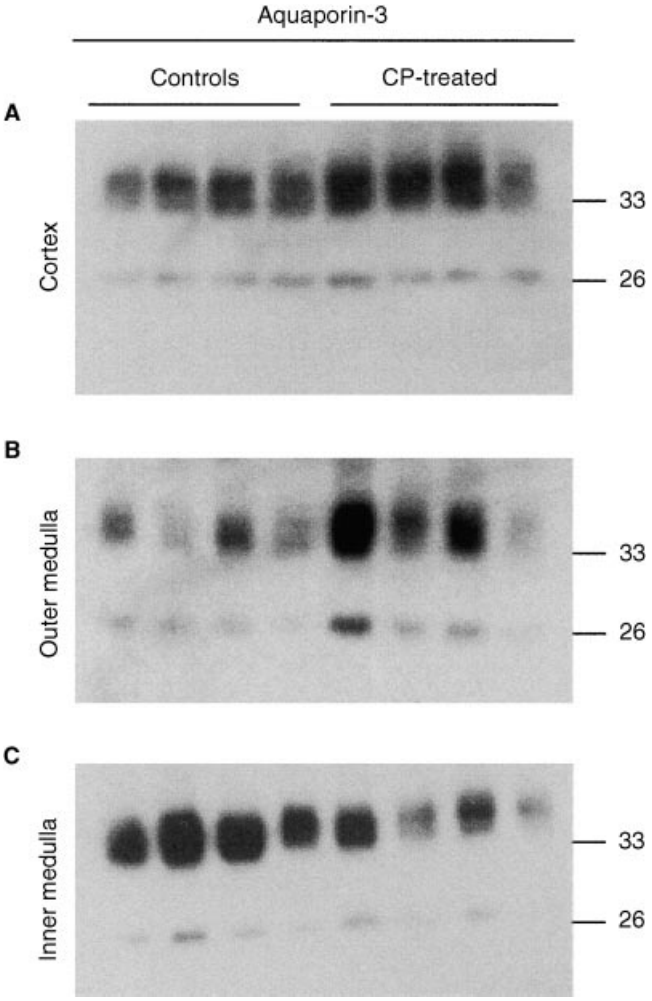

Fig. 3. Representative immunoblots of AQP3 water channel in cortex and outer and inner medullae of saline treated (controls) and CP-treated rats. Quantities of 10, 5, and 3 μg of total protein were loaded in each lane of the gel for cortex and outer and inner medullae, respectively. Blots were probed with AQP3 antibody (L178) at an IgG concentration of 0.03 μg/mL. Blots were exposed to longer duration to visualize the band from all the lanes.

cially in the proximal straight (S3) segment, as compared with the controls, were observed. In the control rats, the proximal nephron segments in the deep cortex and corticomedullary junction were uniformly labeled for AQP1 protein and were structurally intact (Fig. 6A). In CP-treated rats, this uniformity in labeling was conspicuously absent and was associated with a loss of structural integrity of the proximal nephrons (Fig. 6B). Examination of the distal part of the proximal tubule at higher magnification showed further consistent differences between the controls and CP-treated rats, especially in the S3 segment. Figure 6C shows that in control rats, the architecture of the distal part of the proximal tubule and S3 segment was well preserved and that these structures labeled for AQP1 protein both on the apical and basolateral domains. Figure 6D shows that administration of CP caused loss of microvilli and tubular architecture and cell necrosis in S3 segment and distal part of proximal tubules. No consistent alterations in the labeling pattern of AQP1 were observed in outer medulla. No alterations in the immunohistochemical labeling pattern and intensity of AQP2 staining were observed in the cortex or outer medulla of CP-treated rats as compared with the controls. However, consistent alterations were observed in the immunohistochemical labeling pattern of AQP2 in the inner medulla of CP-treated rats. As illustrated in Figure 6E, intense AQP2 labeling is evident in the apical domains (apical plasma membrane and subapical vesicles) in nearly all of the profiles of the inner medullary collecting ducts (IMCDs) of control rats. CP treatment resulted in a loss of uniformity in both the pattern and intensity of AQP2 immunolabeling. As shown in Figure 6F, a subset of IMCD profiles showed low levels of AQP2 labeling restricted to the apical membrane. However, other IMCD profiles showed relatively more AQP2 immunoreaction, although the staining was less intense than that seen in the controls and was limited to the apical membrane (Fig. 6G). However, in a few IMCD profiles, more intense labeling of AQP2 protein could be seen (Fig. 6G). Thus, CP treatment resulted in a distributional heterogeneity of AQP2 immunoreactivity with most of the IMCD expressing very low to moderate levels of AQP2, which is limited to the apical membrane.

DISCUSSION

The purpose of this study was to test the hypothesis that late-phase polyuria associated with the administration of the nephrotoxic anticancer drug CP is due, at least in part, to decreased expression of renal AQPs, specifically AQP1, AQP2, and AQP3. Our experimental model in which rats were administered a nonlethal nephrotoxic dose of CP produced the expected toxic syndrome, including late polyuria. Using different technical approaches, our results support our hypothesis by demonstrating a significant decrease in the expression of collecting duct water channels (AQP2 and AQP3) as well as the proximal nephron water channel (AQP1) in inner medulla. To the best of our knowledge, this is the first report documenting significant alterations of renal AQPs in CP-induced polyuria.

In our model, the CP-treated rats lost a considerable degree of body weight and were afflicted with a severe fall in GFR, as assessed by BUN. These changes in renal function are also associated with a severe degree of polyuria and loss of urinary concentrating ability. In this context, one can argue that the disturbances in food and/or water intake associated with CP administration per se can affect the urinary concentrating ability. However, substantial data exist in the literature demonstra-

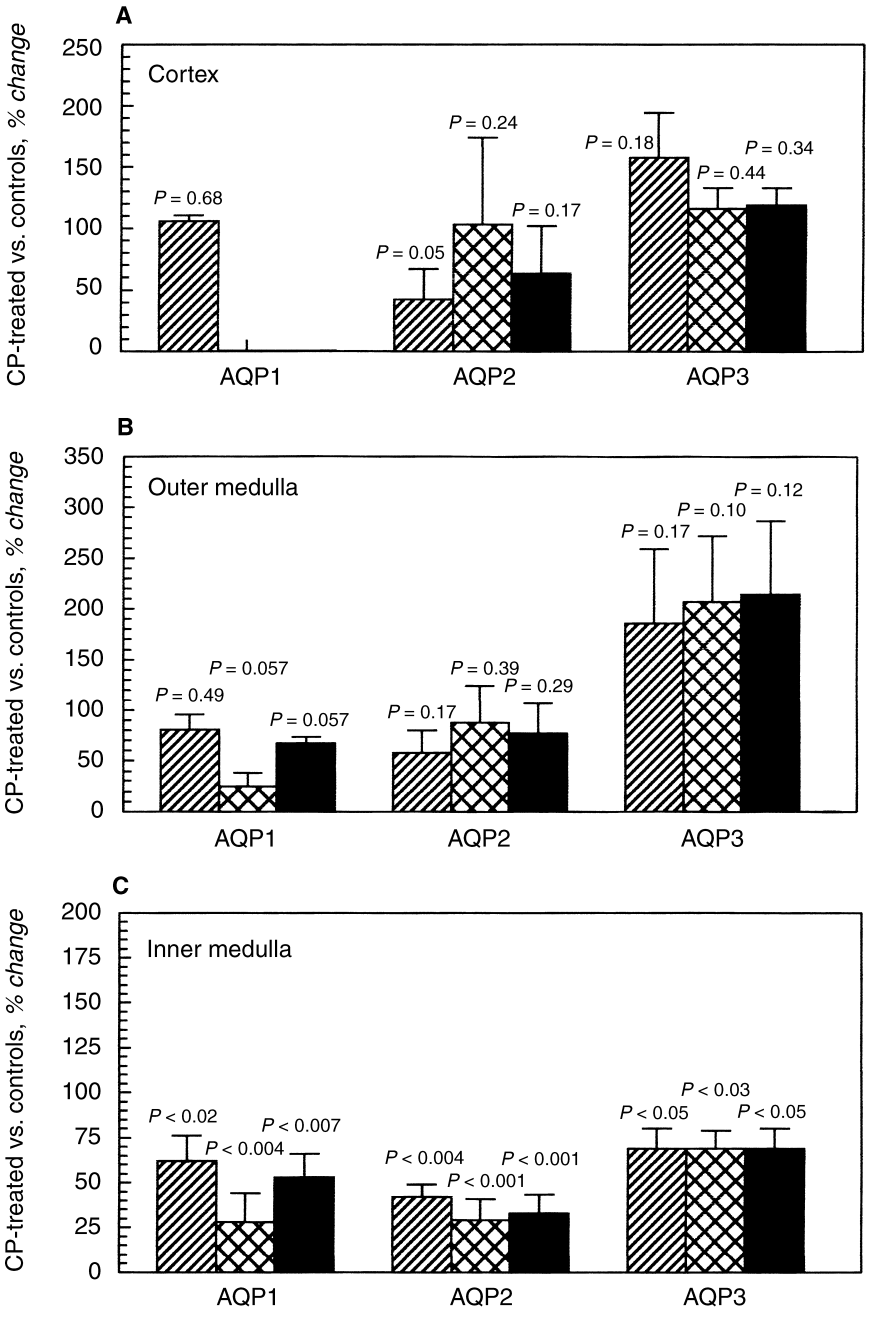

Fig. 4. Quantitative changes in AQP1, AQP2, and AQP3 water channel protein expression in cortex (*A*), outer medulla (*B*), and inner medulla (*C*) in CP-treated rats as determined by densitometry of Western blots. Values are expressed as percentage changes (mean ± SE) of CP-treated rats relative to the mean for the saline-treated controls ($N = 4$ for all bars). Symbols are: (▨) native; (▩) glycosylated; (■) total.

ting that the renal concentrating defect seen in rats exposed to CP is independent of anorexic and polydipsic effects of this drug and is directly related to the effect of CP on the kidney. Using pair-fed and/or pair-watered control rats, at least three different groups of investigators have demonstrated that the urinary concentrating defect observed after CP treatment can be dissociated from the associated changes in food and water intake [19, 21, 30]. Thus, we can confidently exclude the possibility that disturbances in food and/or water intake associated with CP administration may contribute to our observed results.

In our model, the changes in renal function are also associated with high levels of platinum in the kidney tissue. The levels of an accumulation of platinum in the cortex and outer medulla were very similar, and we observed a strong correlation between the platinum levels in the cortex and outer medulla. Our immunoblotting experiments using peptide-derived antibodies specific to AQP1, AQP2, and AQP3 revealed significant to modest alterations in the expression levels of these proteins. Thus, the vasopressin-regulated apical AQP2 water channel protein of the collecting ducts was markedly (33% of control values) decreased in inner medulla, ac-

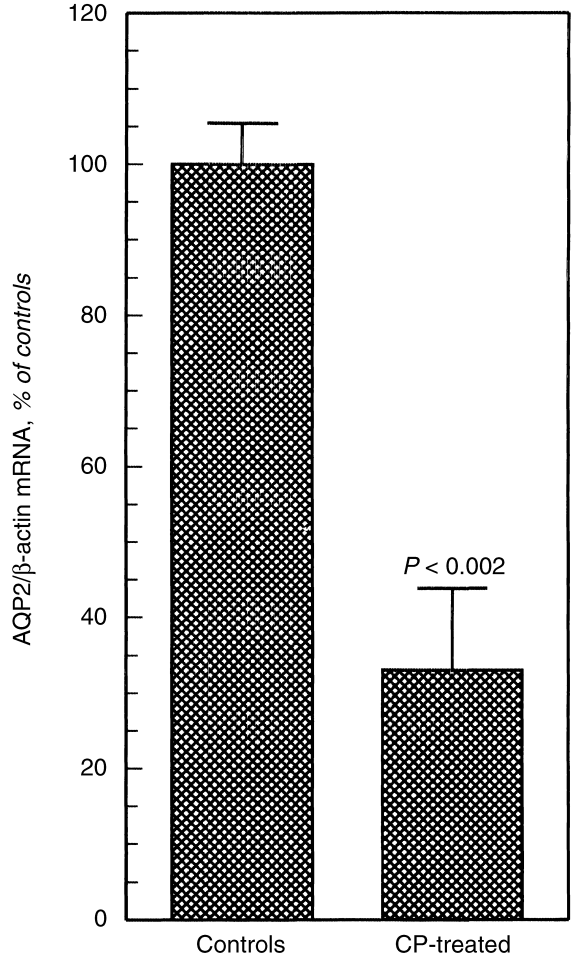

Fig. 5. Quantitative representation of the changes in the expression of AQP2 messenger RNA levels in the inner medulla of CP-treated rats relative to the saline-treated controls determined by densitometry of Northern blots. The densitometric values for AQP2 mRNA were normalized by the corresponding values for β-actin mRNA and expressed as percentage change (mean ± SEM, $N = 4$ for each bar).

companied by its modest decrease in outer medulla (77% of control values) and cortex (63% of control values). AQP2 protein expression levels in the inner medulla of CP-treated rats seen here are in the range of expression of that reported in the medullary collecting ducts of Brattleboro rats [31]. The latter genetically lack vasopressin (central diabetes insipidus) and thus suffer from a severe urinary concentration defect. It is interesting to note that the urine osmolality in Brattleboro rats is typically around 300 mOsm/kg water, a value that is not much different from the mean osmolality observed in our CP-treated rats (408 mOsm/kg H_2O). Furthermore, AQP2 expression levels in CP-treated rats are also similar to those reported in rat models of certain forms of acquired nephrogenic diabetes insipidus, such as hypercalcemia, and acute and chronic renal failure [5]. The decrease in AQP2 protein expression in the inner medulla in CP-treated rats was also associated with a marked and matching decrease in AQP2 mRNA expression (33% of control values), indicating that alterations in AQP2 protein expression are related to alterations in gene expression. This evidence for the altered gene regulation is also strengthened by the fact that in our study, we did not observe any structural damage in the medulla of CP-treated rats at the light miscroscopy level. However, we cannot exclude the possibility of ultrastructural alterations in the medullary structures following CP treatment.

As stated earlier, the amount of water absorbed by the collecting ducts is the determining factor for the osmolality of the final voided urine. Since AQP3 protein, under most conditions, is constitutively expressed on the basolateral aspect of the collecting duct principal cells, the expression of the vasopressin-regulated apical AQP2 protein in these cells is the rate-limiting factor in the transepithelial water flow (assuming that the transepithelial osmotic gradients are effectively maintained). In this context, it is tempting to predict that the marked decrease in the expression of apical AQP2 water channel coupled with a significant decrease in the basolateral AQP3 in the inner medulla per se can account to a large extent for the loss of urinary concentrating ability seen following CP treatment.

Our study also suggests that there is a tendency for an increase in AQP3 protein expression in the outer medulla following CP treatment. Various experimental models of acquired nephrogenic diabetes insipidus demonstrated that the changes in the expression of collecting duct water channels (AQP2 and AQP3) usually run in parallel [5]. However, recently it has been shown at least in two animal models, namely, the escape from vasopressin-induced antidiuresis [32] and diabetes mellitus (abstract; Nejsum et al, *J Am Soc Nephrol* 10:21A(A0109), 1999) that dissociation in the expression of AQP2 and AQP3 can occur. In this context, our CP-induced polyuria is the third such experimental model to demonstrate that such a dissociation is possible under certain pathophysiologic conditions. Finally, our study also reveals a significant decrease in the expression of AQP1 protein in the inner medulla. In this region of the kidney, AQP1 is expressed in the descending vasa recta and descending thin limbs of the Henle's loop, but not the collecting duct segments that make up the bulk of the cell population. Our immunocytochemical examination did not reveal any consistent changes in the expression of AQP1 protein in the inner medulla between the CP-treated and control groups. We consider that this is due to the inherent limitation of the immunocytochemical approach rather than a true dissociation between the immunocytochemical and Western blot data. The moderate decrease observed in the AQP1 protein content in the outer medulla as seen by Western blotting correlates well with

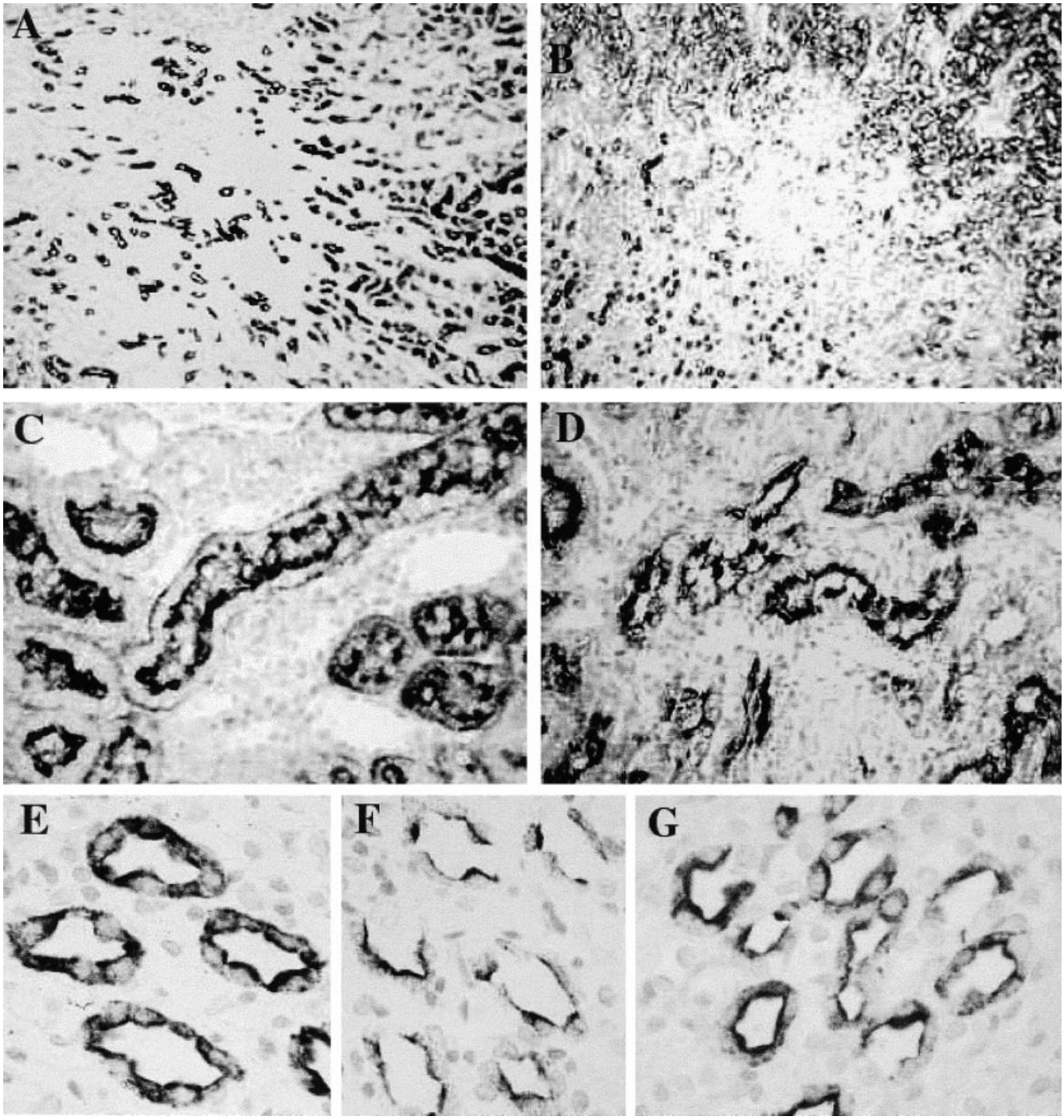

Fig. 6. Representative immunohistochemical labeling for AQP1 (*A–D*) and AQP2 (*E–G*) on paraffin sections (5 μm thickness) of kidneys from control and CP-treated rats. Sections were incubated with either AQP1 antibody (4835 EXT1; 0.4 μg/mL) or with AQP2 antibody (GN-762; 0.07 μg/mL). (A and B) Low-magnification (×40) view of deep cortex and corticomedullary junction in a saline-treated control and a CP-treated rat, respectively. Dark stained structures represent proximal tubular segments labeled for AQP1 protein. (C and D) High magnification (×400) view of AQP1 labeled (dark areas) proximal tubular and S3 segments in the corticomedullary junction of a saline-treated control and a CP-treated rat, respectively. (E–G) Higher magnification (×600) pictures showing AQP2 labeling (dark areas) in the IMCD of control (E) or CP-treated (F and G) rat kidneys.

the immunocytochemical observations of tubular damage and reduced labeling of S3 segments for AQP1 protein.

In conclusion, our study demonstrates that CP-induced polyuria and end-organ resistance are associated with a significant decrease in the expression of collecting duct water channels AQP2 and AQP3, and the proximal nephron and microvascular water channel AQP1 in the inner medulla. While the decreased expression of these water channels alone can account for the loss of urinary concentrating ability, the factors responsible for their decreased expression are yet to be deciphered.

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