Reduced Renal Medullary Water Channel Expression in Puromycin Aminonucleoside–Induced Nephrotic Syndrome

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Abstract. The aquaporins are molecular water channels that mediate transcellular water transport across water-permeable epithelia. To investigate the cause of the concentrating defect in the nephrotic syndrome, immunoblotting using membrane fractions from inner medulla was utilized to assess the level of expression of four aquaporin water channels in vehicle-treated versus puromycin aminonucleoside (PAN)–treated rats. Scanning electron microscopy demonstrating loss of glomerular foot processes and measurements of urinary protein excretion confirmed the efficacy of the PAN treatment. In rats receiving PAN, there was an increase in plasma vasopressin, without a change in plasma sodium concentration. Inner medullary tissue hypertonicity was sustained in PAN-treated rats while the urinary osmolality was low, pointing to defective osmotic equilibration across the collecting ducts in PAN-nephrosis. Among collecting duct aquaporins, there was an 87% decrease in aquaporin-2 expression and a 70% decrease in aquaporin-3 expression in the inner medulla, whereas aquaporin-4 expression was unaltered. Transmission electron microscopy of the inner medullary collecting ducts of PAN-treated rats showed normal-appearing cells. Thus, PAN-nephrosis is associated with an extensive downregulation of collecting duct water channel expression despite increased circulating vasopressin, providing an explanation for the concentrating defect associated with the nephrotic syndrome. (J Am Soc Nephrol 8: 15–24, 1997)

Deranged renal water handling is a cardinal feature of the nephrotic syndrome. Defects in both urinary diluting ability (1–4) and concentrating capacity (1,5) have been documented in nephrotic patients. Decreased diluting ability is thought to be a consequence of nonosmotic increases in plasma vasopressin levels (6), which presumably result in enhanced free water reabsorption in the renal cortex. However, the cause of the concentrating defect is unknown. One possibility is that expression of collecting duct water channels in the renal medulla may be decreased in nephrotic syndrome, preventing a normal rise in collecting duct water permeability in response to vasopressin and preventing osmotic equilibration with the hypertonic medullary interstitium. Such a decrease in water channel expression has already been implicated in the urinary concentrating defects associated with lithium therapy (7,8), prolonged hypokalemia (8), and bilateral ureteral obstruction (9).

A family of molecular water channels referred to as “aquaporins” plays a central role in renal epithelial water transport (10,11). Four aquaporins, presently termed aquaporin-1, -2, -3, and -4, are known to be expressed in the kidney (11,12). Aquaporin-1 (also termed CHIP28 or aquaporin-CHIP) is expressed in the proximal tubule, descending limb of Henle’s loop, and descending vasa recta, and is thought to be responsible for the constitutively high water permeability of these structures (13,14). Vasopressin-regulated water transport across the apical plasma membrane of collecting duct cells is believed to be mediated by aquaporin-2 (also termed aquaporin-CD) (15,16). Water transport across the basolateral membrane of collecting duct cells is thought to be mediated by aquaporin-3 (17–19). Our studies have demonstrated that the expression of both aquaporin-2 (12,16,20) and aquaporin-3 (12,19) is increased after sustained increases in the circulating level of vasopressin. Aquaporin-4 (the “mercurial insensitive water channel” [21,22]) is also expressed in the basolateral membrane of some collecting duct cells (18,23). However, unlike aquaporin-3, its expression is limited chiefly to the inner medullary portion of the collecting duct and its expression is not regulated by vasopressin (23). Thus, it may play a role (with aquaporin-3) in basolateral water transport in the inner medullary portion of the collecting duct system.

To determine whether the concentrating defect in nephrotic syndrome is associated with downregulation of collecting duct water channels, we have utilized a well-characterized animal model, viz. puromycin aminonucleoside (PAN) nephrosis. We utilized mono-specific polyclonal antibodies to all four renal aquaporins (aquaporin-1, -2, -3, and -4) to test the hypothesis that the concentrating defect in PAN nephrosis is associated with a decrease in expression of one or more of the aquaporins found in the inner medullary collecting duct.
Methods

Anti-Aquaporin Antibodies

Polyclonal antibodies prepared against aquaporin-1 (12), aquaporin-2 (16,20), aquaporin-3 (19), and aquaporin-4 (23) have been described previously. These were raised against carrier-conjugated synthetic peptides corresponding to the carboxy terminal tails of each of the aquaporins, which have no sequence similarity. The specificity of each has been demonstrated by showing unique peptide-abloatable bands on immunoblots and a unique distribution of labeling by immunohistochemistry and immuno-electron microscopy. All four antibodies were affinity purified against the immunizing peptides for use in these studies.

Animal Model

Pathogen-free male Sprague-Dawley rats (National Cancer Institute, Frederick, MD, Breeding Facility; body weight, 200 to 300 g) were used in this study. These rats were maintained initially in filter-top microisolator cages with autoclaved feed and bedding, and were allowed free access to drinking water at all times. After equilibration in metabolism cages, the animals were injected intravenously with either puromycin aminonucleoside (Sigma, St. Louis, MO) dissolved in sterile normal saline, or with the vehicle alone. Two different doses of puromycin were used: 50 mg/kg body wt (“low dose”) and 180 mg/kg body wt (“high dose”). The rats were subsequently maintained in the metabolism cages to allow urine collections for monitoring of protein excretion by using protein-sensitive “dipsticks” (Albustix; Miles Inc., Elkhart, IN). In addition, the rats were weighed daily.

Some of the rats given the high dose of PAN were utilized for measurements of maximal urinary concentrating capacity. For this, the rats were deprived of water for 18 h and then were given an injection of aqueous arginine vasopressin (1 nmol/100 g body wt im). Spontaneously voided urine was collected 90 min after the vasopressin injection for determination of osmolality by using a vapor pressure osmometer (Wescor Model 5100C; Logan, UT). In additional experiments, the same protocol was followed except that after the final urine collection, the rats were immediately decapitated and the inner medulla was dissected from the left kidney for estimation of tissue osmolality. To this end, the inner medullas were weighed in pre-tared Eppendorf tubes to which 500 μL of deionized water was added. The tissue was homogenized using a tissue homogenizer (Omni International, Inc., Gainesville, VA) and the osmolality of the homogenate was determined by vapor-pressure osmometry. The original tissue osmolality was estimated based on the nominal dilution factor and the assumption that 80% of the wet weight is water.

Immunoblotting

Kidney dissection and tissue preparation. The rats were killed by decapitation and trunk blood was collected into chilled heparinized beakers for determination of plasma vasopressin (Corning-Hazelton Laboratories, Vienna, VA) and sodium levels (atomic emission spectrophotometry; Thermo Jarrell Ash Corp., Franklin, MA) After both kidneys were rapidly removed, they were washed briefly in isotonic saline and were dissected to obtain the complete inner medulla including papillary and base portions (“white medulla”). The tissue was initially homogenized for 10 s using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) in ice-cold isolation solution containing 250 mM sucrose/10 mM triethanolamine (Calbiochem, La Jolla, CA) with 1 μg/mL leupeptin (Bachem California, Torrance, CA) and 0.1 mg/mL phenylmethyl sulfonyl fluoride (United States Biochemical Corporation, Toledo, OH). Membrane fractions were prepared by centrifuging the homogenate at appropriate speeds as described in the remainder of this paragraph. Initially, homogenates were spun at low speeds (1000 × g) for 10 min to remove nuclei and incompletely homogenized membrane fragments. To increase yields, this pellet was rehomogenized as described above and was spun again at 1000 × g for 10 min. The supernatants from the two 1000 × g centrifugations were pooled to give the “post-nuclear supernatant.” The post-nuclear supernatant was subjected to one of two subsequent fractionation protocols: (1) To obtain a “total membrane fraction,” the supernatant was centrifuged at 200,000 × g. This was used for immunoblots probed for aquaporin-1, -2, and -3. (2) To obtain a plasma membrane–enriched fraction (24), the pooled supernatant was centrifuged at 17,000 × g. This fraction was used for immunoblots probed for aquaporin-4, which has been localized to the plasma membranes of collecting duct cells (23). The 1000 × g and 17,000 × g centrifugations were accomplished using a Sorvall RC2-B refrigerated centrifuge with an SS-34 rotor (DuPont Medical Products, Newtown, CT). The 200,000 × g spin was carried out with a Beckman L8-M ultracentrifuge fitted with a type 80Ti rotor. The resulting pellets were resuspended in isolation solution and total protein concentration in membrane fractions was measured using the Pierce BCA Protein Assay reagent kit.

Electrophoresis and immunoblotting of membrane proteins. The membrane fractions were solubilized at 60°C for 15 min in Laemml sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 12% polyacrylamide minigels, loading equal amounts of total protein per lane. For each set of samples, an initial gel was stained with Coomassie blue to confirm that equal loading had been achieved as described previously (12). Representative bands were quantified by laser densitometry (LKB Ultrascan XL), assuring that loading did not differ for any sample by more than 10% from the mean. For immunoblotting, the proteins were transferred from unstained gels electrophoretically to nitrocellulose membranes. After being blocked with 5 g/dL nonfat dry milk, the blots were probed with the affinity-purified polyclonal antibody to the appropriate water channel at immunoglobulin (lg) G concentrations in the range of 0.15 to 0.46 μg/mL in an antibody dilution buffer solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dL sodium azide, 50 mg/dL Tween-20, and 1 g/dL percent BSA (pH 7.5). The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce #31458) at a concentration of 0.16 μg/mL. Sites of antibody-antigen reaction were visualized using luminol-based enhanced chemiluminescence (LumiGLO; Kirkegaard and Perry Laboratories, Gaithersburg, MD) before exposure to x-ray film (Kodak #165-1579 Scientific Imaging Film; Eastman Kodak, Rochester, NY). Relative quantification of the resulting band densities was carried out by densitometry using a laser densitometer (LKB Ultrascan XL).

Transmission and Scanning Electron Microscopy

Fixation of kidneys. Intact living kidneys from PAN-treated and vehicle-treated rats were fixed either by vascular perfusion of fixative or by immersion, as indicated in the Results section. For perfusion fixation, the rats were anesthetized with pentobarbitol and a laparotomy was performed. The abdominal aorta was cannulated below the renal arteries and, after the vena cava was severed, physiological saline (37°C) was perfused through the kidneys by using a peristaltic pump. After the kidneys were cleared of blood (20 s), and without interruption of perfusion pressure, a phosphate-buffered 2% glutaraldehyde solution (pH 7.2, 410 mosmol) was perfused through the kidneys for approximately 10 min. The fixed kidneys were removed and stored in phosphate-buffered glutaraldehyde for at least 3 to 6 h before further preparation for electron microscopy. For immersion fixation, kidneys were removed from anesthetized rats and inner
medullas, dissected as described above, and immersed in the same phosphate-buffered 2% glutaraldehyde solution.

**Tissue preparation for electron microscopy.** Scanning and transmission electron microscopy were carried out as previously described (25). For scanning electron microscopy (SEM), the glutaraldehyde-fixed samples were dehydrated through graded acetones and dried by the critical point method. The dried samples were mounted on SEM stubs, coated with gold/palladium in a sputter coater, and viewed with a Hitachi 570 scanning electron microscope (Hitachi Scientific, Mountain View, CA) operating at 20 kV.

For transmission electron microscopy (TEM), the glutaraldehyde-fixed samples were rinsed in buffer, postfixed with 2% osmium tetroxide, and stained in Spurr medium. Ultrathin sections (50 to 80 nm) were post-stained with uranyl acetate and lead citrate and viewed in a JEOL1200EX electron microscope (JEOL U.S.A., Peabody, MA) operating at 60 or 80 kV.

**Statistics**

Statistical comparisons were accomplished by unpaired t tests (when variances were the same) or by Mann-Whitney rank-sum tests (when variances were significantly different between groups).

**Animal Use Assurance**

All animal experimentation described in this article was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NHLBI/IR protocol number 5-KE-1).

**Results**

**Documentation of Efficacy of PAN Model**

The efficacy of the PAN injections in creating the nephrotic state was documented in two ways: scanning electron microscopy of the glomeruli and semiquantitative assessment of urinary protein levels. Scanning electron micrographs of glomeruli from vehicle-treated and high-dose PAN-treated rats are shown in Figure 1. As demonstrated previously, PAN administration results in a marked loss of foot processes from the glomerular visceral epithelial cells (Figure 1B). Qualitative assessment of protein excretion was made using “dip sticks.” For the low-dose PAN animals, the urinary protein level was greater than that of the control animals within 5 days and reached a 4+ level within 6 to 7 days. In the high-dose PAN animals, the urinary protein level was greater than that of control animals within 2 to 3 days and reached a 4+ level within 3 to 4 days.

**Concentrating Capacity Measurements**

Maximal concentrating capacity was measured in three high-dose PAN-treated rats and three vehicle-treated control rats to assess whether the urinary concentrating mechanism is impaired in the PAN model as we carry it out. These measurements were performed 7 days after PAN or vehicle injection. The maximal urinary osmolality in the three vehicle-injected rats was 2735 ± 152 (SE) mosmol/kg H2O. In contrast, the maximal urinary osmolality in the PAN-treated rats was significantly suppressed to 509 ± 38 mosmol/kg H2O (P < 0.0001, unpaired t test).

**Water Channel Expression in Inner Medulla**

We assessed the effect of single intravenous injections of PAN at two dose levels, viz. 50 mg/kg body wt (“low dose”) and 180 mg/kg body wt (“high dose”), on the expression levels of individual aquaporins in the inner medulla. With the low-dose protocol, neither plasma sodium concentration (vehicle-infused, 145 ± 3 (SE) mEq/L; PAN-infused, 146 ± 2 mEq/L; P > 0.05) nor the plasma vasopressin concentration (vehicle-infused, 0.74 ± 0.10 pg/mL; PAN-infused, 3.2 ± 2.2 pg/mL; P > 0.05) was significantly altered (N = 6 for both control and PAN-treated rats). With the high-dose protocol (N = 6 for both control and PAN-treated rats), plasma sodium was also unchanged (vehicle-infused, 142 ± 6 mEq/L; PAN-infused, 140 ± 5 mEq/L; P > 0.05) although the plasma vasopressin concentration was significantly increased in response to high-dose PAN infusion (vehicle-infused, 1.0 ± 0.2 pg/mL; PAN-infused, 4.0 ± 1.2 pg/mL; P < 0.02 by Mann-Whitney non-parametric method).

As summarized above, at least three aquaporins are normally expressed in the renal inner medullary collecting duct. Figures 2 through 5 summarize the effects of PAN-treatment on levels of these three water channels in the inner medulla.

Aquaporin-2 is the vasopressin-regulated water channel expressed in the apical plasma membrane and intracellular vesicles of collecting duct principal cells (16). Figure 2 shows immunoblots comparing aquaporin-2 protein level in inner medullary total membrane fractions of PAN-treated rats with total membrane fractions from vehicle-treated control rats. As observed previously (16), the aquaporin-2 antibody labels bands at 29 kd and 35 to 40 kd, corresponding to the nonglycosylated and glycosylated forms of the water channel protein, respectively. With low-dose PAN (Figure 2A), there was a significant 61% reduction relative to vehicle-injected control rats (densitometry of lower band: control rats, 184 ± 27 U; low-dose PAN-treated rats, 72 ± 15 U; P < 0.005). With high-dose PAN (Figure 2B), aquaporin-2 protein expression was reduced to even lower levels, an 87% reduction versus control rats (values from densitometry of lower band: control, 366 ± 40 U; high-dose PAN, 48 ± 17 U; P < 0.0001). We conclude that PAN-nephrosis is associated with a marked dose-dependent decrease in aquaporin-2 water channel expression in the renal inner medulla.

We carried out differential centrifugation of inner medullary homogenates from the same rats studied in Figure 2 to determine whether the distribution of aquaporin-2 between a plasma membrane-enriched fraction (“low-speed” fraction) and an intracellular vesicle-enriched fraction (“high-speed” fraction) is altered in response to low-dose PAN treatment (Figure 3) or high-dose PAN treatment (Figure 4). As shown in both Figures, there was a disproportionately greater reduction in the level of aquaporin-2 in the vesicle-enriched “high-speed” fraction than in the plasma-membrane-enriched “low-speed” fraction. Thus, at both doses, a greater fraction of the total aquaporin-2 in the collecting duct cells is found in the plasma membrane fraction, suggesting that PAN treatment is associated with translocation of aquaporin-2 into the plasma membrane, presumably in response to the rise in circulating vasopressin concentration.
Figure 1. Scanning electron microscopic view of glomerular epithelial podocytes from a vehicle-treated (A) and a PAN (180 mg/kg body wt)-treated rat (B). Note the extensive loss of podocyte foot processes (f), which occurs in response to PAN-induced nephrotic syndrome. (Original magnification, ×3500.)

Figure 5 summarizes effect of PAN treatment on the renal inner medullary expression of aquaporin-3, a basolateral water channel present along the entire length of the collecting duct system (17–19). As observed previously (19), the aquaporin-3 antibody labels a weak 26-kd band and a stronger 34 to 38-kd band. The upper panel of Figure 5 shows an immunoblot
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Figure 2. Immunoblots comparing aquaporin-2 levels in total membrane fractions from inner medullas of vehicle-treated and PAN-treated rats. Each lane represents a sample from a different rat (2 μg/protein per lane). Upper panel, low-dose PAN (vehicle, N = 6; PAN, N = 6). Lower panel, high-dose PAN (vehicle, N = 6; PAN, N = 6). Total membrane fraction is the pellet from a single 200,000 × g centrifugation of post-nuclear supernatant. See text for results of densitometry comparison of band densities.

Figure 3. Immunoblots showing effect of low-dose PAN on aquaporin-2 levels in vesicle-enriched membrane fraction (upper panel) and plasma membrane-enriched membrane fraction (lower panel) from rat inner medullas. Individual lanes were loaded with membrane protein samples from different rats (2 μg protein per lane). Animals were the same as those used for upper panel of Figure 2 (low-dose PAN).

Figure 4. Immunoblots showing effect of high-dose PAN on aquaporin-2 levels in vesicle-enriched membrane fraction (upper panel) and plasma membrane-enriched membrane fraction (lower panel) from rat inner medullas. Individual lanes were loaded with membrane protein samples from different rats (2 μg protein per lane). Animals were the same as those used for lower panel of Figure 2 (high-dose PAN).

Figure 5. Immunoblots comparing aquaporin-3 levels in total membrane fractions from inner medullas of vehicle-treated and PAN-treated rats. Each lane represents a sample from a different rat (5 μg/protein per lane). Upper panel, low-dose PAN (vehicle, N = 6; PAN, N = 6). Lower panel, high-dose PAN (vehicle, N = 6; PAN, N = 6). Total membrane fraction is the pellet from a single 200,000 × g centrifugation of post-nuclear supernatant. See text for results of densitometry comparison of band densities.

comparing the aquaporin-3 protein levels in inner medullas of low-dose PAN-treated rats with those of vehicle-treated control rats. There was a 74% decrease in aquaporin-3 level versus control rats (densitometry of 34 to 38-kd band: control rats, 147 ± 33 U; low-dose PAN-treated rats, 39 ± 12; P < 0.02). With high-dose PAN (Figure 5, lower panel), aquaporin-3 protein expression was reduced to even lower levels, a 70% reduction versus control rats (values from densitometry of 34 to 38-kd band: control rats, 48.8 ± 6.6 U; high-dose PAN-treated rats, 14.5 ± 3.0 U; P < 0.001). We conclude that, as seen for aquaporin-2, PAN-nephrosis is associated with a marked decrease in aquaporin-3 water channel expression in the renal inner medulla.
Assessment of Countercurrent Multiplier Mechanism

The defect in renal concentrating ability demonstrated in the PAN-treated rats could be a result of a failure of the countercurrent multiplication process to raise inner medullary osmolarity or of a failure of the collecting duct fluid to osmotically equilibrate with the hypertonic medullary interstitium. To examine the former possibility, inner medullary tissue osmolality was estimated after 18-h water deprivation and an intramuscular injection of arginine vasopressin as described in the Methods section. In these experiments, the PAN-treated rats displayed a marked diminution of maximal urinary osmolality (vehicle-injected, 2102 ± 341 mosmol/kg H2O; PAN-injected, 836 ± 134 mosmol/kg H2O) similar to the results shown above. However, mean inner medullary tissue osmolality was not as profoundly decreased in the PAN-treated rats (vehicle-injected, 2346 ± 104 mosmol/kg H2O; PAN-injected, 1448 ± 54 mosmol/kg H2O). This result is consistent with the conclusion that the failure of the PAN-treated rats to generate highly concentrated urine was not solely a result of a possible defect in the countercurrent multiplication system, but rather is largely associated with a failure of complete osmotic equilibration across the inner medullary collecting duct.

Collecting Duct Ultrastructure

The upper two panels of Figure 8 show transmission electron micrographs of inner medullary collecting ducts obtained by perfusion fixation of kidneys from a vehicle-treated rat (Panel A) and a rat treated with PAN at the high-dose level (Panel B). Although the collecting duct cells from the PAN-treated rat are normal in appearance, a marked widening of the intercellular space is apparent (Panel B). Because such widening is often seen in association with increased interstitial pressure (K.R.

Figure 6 summarizes results for aquaporin-4, a second basolateral water channel (18,23) that is expressed predominantly in the inner medullary collecting duct (23). The upper panel of Figure 6 shows an immunoblot comparing the aquaporin-4 protein levels in the inner medullas of low-dose PAN-treated rats with those of vehicle-treated control rats. As observed previously (23), the aquaporin-4 antibody labels a predominant 52-kd band. Based on densitometry of the 52-kd band, there is no evident effect of PAN treatment at either the low- or high-dose on aquaporin-4 expression (densitometric analysis for low-dose experiment: control rats, 0.87 ± 0.11 U; low-dose PAN-treated rats, 0.75 ± 0.12 U; P > 0.40; densitometric analysis for high-dose experiment: control rats, 1.16 ± 0.16 U; high-dose PAN-treated rats, 0.86 ± 0.06; P > 0.10).

In the renal inner medulla, aquaporin-1 is expressed in the apical and basolateral membranes of the descending limb of the loop of Henle (13,14). It is not present in the collecting duct (14). We assessed its level in the inner medulla to determine whether the downregulation of water channel expression is limited to collecting duct water channels. As shown in Figure 7, with low-dose PAN, there was no significant change in aquaporin-1 protein level relative to vehicle-injected control rats (densitometry of lower band: control rats, 131 ± 23 U; low-dose PAN-treated rats, 94 ± 30 U; P > 0.34). However, with high-dose PAN, there was a 50% decrease in aquaporin-1 protein expression level (densitometry of lower band: control rats, 262 ± 28 U; high-dose PAN-treated rats, 131 ± 12 U; P < 0.002). Thus, diminution of water channel expression is not limited to collecting duct water channels, at least with the high dose of PAN.

Assessment of Countercurrent Multiplier Mechanism

The defect in renal concentrating ability demonstrated in the PAN-treated rats could be a result of a failure of the countercurrent multiplication process to raise inner medullary osmolarity or a failure of the collecting duct fluid to osmotically equilibrate with the hypertonic medullary interstitium. To examine the former possibility, inner medullary tissue osmolality was estimated after 18-h water deprivation and an intramuscular injection of arginine vasopressin as described in the Methods section. In these experiments, the PAN-treated rats displayed a marked diminution of maximal urinary osmolality (vehicle-injected, 2102 ± 341 mosmol/kg H2O; PAN-injected, 836 ± 134 mosmol/kg H2O) similar to the results shown above. However, mean inner medullary tissue osmolality was not as profoundly decreased in the PAN-treated rats (vehicle-injected, 2346 ± 104 mosmol/kg H2O; PAN-injected, 1448 ± 54 mosmol/kg H2O). This result is consistent with the conclusion that the failure of the PAN-treated rats to generate highly concentrated urine was not solely a result of a possible defect in the countercurrent multiplication system, but rather is largely associated with a failure of complete osmotic equilibration across the inner medullary collecting duct.
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Spring, personal communication), we considered the possibility that it could be an artifact of the perfusion fixation protocol. Consequently, we fixed additional kidneys from rats treated with high-dose PAN using the same fixative, but by immersion fixation, a procedure that would avoid the increase in interstitial pressure seen with perfusion fixation. As shown in Figure 8C, there was no intercellular space dilatation with immersion fixation, demonstrating that the dilation shown in Figure 8B was an artifact of the procedure used to fix the kidneys. The collecting duct cells shown in Figure 8C (high-dose PAN) are entirely normal in appearance (compare with Figure 8A). Presumably, intercellular space dilatation does not occur in perfusion-fixed control kidneys because their collecting ducts are sufficiently permeable to water to allow rapid water flow into the lumen, thus obviating any substantial increase in interstitial hydrostatic pressure.

Discussion

The studies presented here demonstrate that the concentrating defect present in rats with PAN-induced nephrotic syndrome is associated with a marked downregulation of the collecting duct water channels aquaporin-2 and aquaporin-3. Aquaporin-2 is the vasopressin-regulated water channel of the apical membrane, whereas aquaporin-3 is a constitutive water channel found in the basolateral membrane of collecting duct principal cells.

Measurements of tissue osmolality in thirsted rats showed that, although the inner medullary tissue osmolality was somewhat decreased in PAN-treated rats, these rats retained the ability to generate a hypertonic medullary interstitium. Tissue and urinary osmolality were very nearly the same in vehicle-treated control rats, indicating near osmotic equilibration of collecting duct urine with the surrounding interstitium. In contrast, there was a substantial gap between urinary and tissue osmolality in the PAN-treated rats consistent with a defect in osmotic equilibration across the collecting duct, a finding presumably resulting from the observed marked suppression of aquaporin-2 and -3 water channel expression in the inner medullary collecting duct.

The results suggest that PAN treatment causes a derangement of the long-term regulation of water channel expression in the collecting duct. In recent years, considerable knowledge has accrued with regard to long-term regulation of water channel expression in the kidney. Both in vivo water restriction (26) and vasopressin infusions (20) have been found to cause stable increases in the osmotic water permeability of the inner medullary collecting ducts of rats. These effects are associated with a large increase in the expression level of aquaporin-2 protein

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Figure 8. Transmission electron micrographs of rat inner medulla, showing inner medullary collecting duct morphology. (A) Thin section of inner medulla of a vehicle-treated control rat. The kidney was fixed using a vascular perfusion technique as described in the Methods section. The inner medullary collecting duct cells appear normal with relatively straight intercellular borders (large arrows), distinct apical junctional complexes (small arrows), and a paucity of cytoplasmic organelles. N, nucleus. (B) Thin section of inner medulla from a rat treated with high-dose PAN (180 mg/kg body wt). The kidney was fixed by vascular perfusion as in A. Cells appear normal. However, there is marked distention of the intercellular spaces (asterisks). Arrows indicate apical junctional complexes. (C) Thin section of inner medulla from a rat treated with high-dose PAN (180 mg/kg body wt). In contrast to B, the kidney was fixed by immersion in fixative. No intercellular space dilatation is seen. Cells appear normal. Arrows indicate apical junctional complexes. N, nucleus. (All panels, original magnification X5000.)
in the collecting duct (16,20) as well as an increase in aquaporin-2 mRNA levels (27,28). The increase in aquaporin-2 expression allows a greater number of water channels to be shuttled into the apical plasma membrane of principal cells from the reserve pool present in intracellular vesicles (16,20), accounting for the demonstrated increase in collecting duct water permeability.

The recent finding that the 5' flanking region of the aquaporin-2 gene contains a putative cAMP regulatory element (CRE) (29), combined with the physiological data cited above, supports the idea that the long-term regulation of aquaporin-2 expression is mediated at least in part by changes in intracellular cyclic AMP levels. Because circulating levels of vasopressin were higher with PAN-treatment than in vehicle-infused control rats, upregulation—not downregulation—of aquaporin-2 expression could have been expected. The finding of downregulation suggests that either the elevated plasma level of vasopressin was ineffective in increasing intracellular cyclic AMP or that some other unrelated factor may have overridden the cyclic AMP-mediated control process. A similar degree of downregulation of the aquaporin-2 water channel has recently been noted in other pathophysiological models associated with concentrating defects, viz. lithium intoxication (7,8), hypokalemia (8), and bilateral ureteral obstruction (9). Conceivably, a common mechanism could be involved in the suppression of aquaporin-2 water channel expression in all of these pathophysiological states, although further studies will be needed to address this possibility.

Compared with aquaporin-2, little is currently known with regard to the long-term regulation of aquaporin-3 expression. Our recent studies (12,19), however, have demonstrated that aquaporin-3 expression at a protein level is regulated in response to in vivo water restriction and to vasopressin infusion in a manner similar to aquaporin-2. That is, aquaporin-3 is upregulated in association with increased plasma vasopressin levels, leading to the conclusion that changes in aquaporin-3 expression may be important to the overall regulation of collecting duct water permeability. Thus, we speculate that the reduction of aquaporin-3 expression seen with PAN nephrosis is, in part, responsible for the associated concentrating defect.

We have found that in contrast to the other collecting duct water channels, aquaporin-4 does not undergo long-term regulation of expression in response to changes in circulating vasopressin levels (12,23). Interestingly, aquaporin-4 expression was also unaffected by induction of nephrotic syndrome by PAN injection. In contrast, aquaporin-1, which is found in the descending limb of Henle’s loop in the inner medulla rather than in the collecting duct, appears to exhibit a moderate decrease in expression, at least with high-dose PAN. Thus, dysregulation of water channel expression is apparently not restricted to the collecting duct in PAN nephrosis.

How PAN nephrosis leads to decreased water channel expression cannot be ascertained from the present studies. Recent reports (30,31) have demonstrated that PAN-induced nephrotic syndrome is associated with an inflammatory tubulointerstitial nephritis in addition to the well-described glomerulopathy. Similar observations have been made in another model of the nephrotic syndrome, adriamycin-induced nephrosis (32). This tubulointerstitial nephritis is thought to be dependent in some manner on the severe proteinuria associated with the glomerulopathy (33–35) and has been observed to be less severe in Sprague-Dawley strain of rats used in the present study than in the Munich-Wistar strain (30). It is unclear from these studies whether this tubulointerstitial inflammatory process involves the renal medulla or collecting duct cells. Hence, it is unclear to what extent the observed decrease in water channel expression seen in our studies is related to the tubulointerstitial nephritis of PAN-induced nephrosis. Transmission electron microscopy of the inner medullary collecting ducts revealed normal cellular morphology, indicating a lack of large-scale disruption of cellular function. The only abnormality seen was marked dilation of the intercellular spaces of collecting ducts of the PAN-treated rats, an effect that we demonstrated is an artifact resulting from increased interstitial pressure during perfusion of the renal vasculature for tissue fixation. Such dilation did not occur in control kidneys, presumably because their collecting ducts were permeable to water, thus allowing fluid to escape into the tubule lumens rather than to accumulate in the intercellular spaces. Thus, the intercellular space distention seen in the PAN-treated rats provides additional indirect evidence for a low water permeability in the collecting ducts of PAN-treated rats.

The short-term effect of vasopressin to increase water permeability in collecting duct cells occurs as a result of exocytosis of aquaporin-2-laden vesicles, causing translocation of aquaporin-2 into the plasma membrane (36). This translocation process can be assessed by immunoblotting using subcellular membrane fractions from the inner medulla as described by Marples et al. (37). With this technique, fractions enriched in plasma membranes and intracellular vesicles can be obtained by sequential low-speed (17,000 × g) and high-speed (200,000 × g) centrifugations, respectively. In the study presented here, we used this technique to assess whether PAN nephrosis is associated with any deficiency in the distribution of aquaporin-2 between intracellular vesicles and the plasma membrane. Indeed, we found that with PAN nephrosis, there was a marked increase in the proportion of aquaporin-2 protein associated with the low-speed, plasma membrane–enriched fraction. This finding is compatible with the view that the short-term regulation by vasopressin is intact and compensates in part for the decrement in the total amount of aquaporin-2 by an increase in the fraction of aquaporin-2 present in the apical membrane.

In summary, PAN-induced nephrotic syndrome in rats has been demonstrated to be associated with a marked reduction in aquaporin-2 and -3 expression in the renal collecting duct. The reduction in water channel expression provides an explanation for the failure of the urine to equilibrate osmotically with the hypertonic medullary interstitium in these animals, and therefore explains the concentrating defect seen with PAN-induced nephrotic syndrome.

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References


34. Bertani T, Zoa C, Abbate M, Rossini M, Remuzzi G: Age-
related nephropathy and proteinuria in rats with intact kidneys exposed to diets with different protein content. *Lab Invest* 60: 196–204, 1989
