Decreased Abundance of Collecting Duct Aquaporins in Post-Ischemic Renal Failure in Rats

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Abstract. Increased urine flow is often a feature of mild to moderate acute renal failure. This study examines the possible role of dysregulation of collecting duct aquaporins as a factor in this increase. In rats, the left renal pedicle was clamped for 45 min followed by contralateral nephrectomy. Control rats were identical except that the renal pedicle was not clamped. Rats were sacrificed and the kidneys were homogenized at various time points after release of the clamp for semiquantitative immunoblotting of collecting duct aquaporins, as well as the thick ascending limb Na-K-2Cl cotransporter and the proximal tubule water channel, aquaporin-1. Urinary flow rate was significantly increased 18 h after the ischemic insult and remained increased through 72 h. Whole kidney aquaporin-2 protein abundance was 45% of controls at 18 h, 55% of controls at 36 h, and returned to normal 72 h after ischemia. Whole kidney aquaporin-3 protein abundance was 37% of controls at 18 h, 13% of controls at 36 h, and 45% of controls at 72 h. The decline in aquaporin-2 and -3 was confirmed by immunocytochemistry. Abundance of the thick ascending limb Na-K-2Cl cotransporter protein was not significantly decreased. Aquaporin-1 protein abundance was not significantly decreased at 18 h after the ischemic insult, but was significantly reduced after 36 h. Thus, the post-ischemic state is associated with decreased levels of the collecting duct aquaporins, coinciding with an increase in water excretion. It is concluded that decreased aquaporin protein abundance in collecting duct cells is a contributing factor in the increased urine flow seen in moderate post-ischemic acute renal failure.

Polyuria and a failure to concentrate urine maximally are frequent consequences of mild to moderate ischemic acute renal failure (1,2). The defect is not responsive to pharmacologic doses of vasopressin and is not believed to be due to downregulation of the vasopressin V2 receptor (3). In this article, we address the hypothesis that the increased urine flow seen in moderate post-ischemic renal failure is associated with reduced cellular levels of collecting duct aquaporins.

Aquaporins are integral membrane proteins that mediate water transport across plasma membranes in a variety of tissues (4,5). In the collecting duct, three aquaporins are expressed, i.e., aquaporin-2, aquaporin-3, and aquaporin-4. Aquaporin-2 is the chief target for regulation of water transport by vasopressin (6). It mediates water transport across the apical plasma membrane of collecting duct principal cells and connecting tubule cells. Vasopressin regulates aquaporin-2 in two ways: (1) short-term regulation by trafficking of aquaporin-2-containing vesicles to the apical plasma membrane; and (2) long-term regulation by increases in the absolute abundance of aquaporin-2 in the collecting duct. Aquaporin-3 and aquaporin-4 are both basolateral aquaporins. Aquaporin-3 is most abundant in the cortical and outer medullary collecting ducts, and aquaporin-4 is predominantly found in the inner medullary collecting ducts (7). Aquaporin-3 protein abundance appears to be regulated by vasopressin, but there is no evidence for long-term regulation of aquaporin-4 expression in the kidney (8). A fourth renal aquaporin, aquaporin-1, is not expressed in the collecting duct but rather is the predominant aquaporin in the proximal tubule and thin descending limb of Henle’s loop (9).

Abnormal regulation of the collecting duct aquaporins has been implicated in the water balance abnormalities associated with a variety of pathophysiologic states, including congestive heart failure (10,11), nephrotic syndrome (12–14), protein malnutrition (15), nephrogenic diabetes insipidus due to lithium administration (16), hypokalemia (17), ureteral obstruction (18), and hypercalcemia (19,20). Transport abnormalities associated with ischemic acute renal failure are generally attributed to cellular damage in the proximal tubule (2). In general, the collecting ducts are thought to be relatively resistant to ischemic insult.

Although the proximal tubule is the chief site of renal damage following renal ischemia, recent evidence suggests that the thick ascending limbs and collecting ducts may also react extensively to the ischemic insult. The expression of...
several renal-specific genes in the thick ascending limb, such as preproEGF (21) and Tamm-Horsfall protein (22), are significantly downregulated during an ischemic insult. Furthermore, the immediate early genes c-fos and c-jun have been shown to be activated in the collecting duct after renal ischemia (23). These observations suggest that the distal nephron and collecting duct are important sites of the molecular response to ischemia-reperfusion injury and that such responses may have pathophysiologic significance.

In this article, we use a rat model of post-ischemic acute renal injury to address the view that the collecting duct can also be a site of post-ischemic injury, accounting in part for the increased urine flow associated with nonoliguric acute renal failure. We use semiquantitative immunoblotting, differential centrifugation, and immunocytochemistry to investigate the possibility that nonoliguric acute renal failure is associated with a disruption of either short-term or long-term regulation of aquaporin-2, or altered abundance of aquaporin-3 or -4 protein.

Materials and Methods

Antibodies

Polyclonal antibodies prepared against aquaporin-1 (8), aquaporin-2 (24), aquaporin-3 (25), aquaporin-4 (7), and the thick ascending limb Na-K-2Cl cotransporter (26) have been described previously. They were raised against keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to hydrophilic portions of the Na-K-2Cl cotransporter and aquaporin sequences in rat. The specificity of each antibody has been demonstrated by showing unique peptide-ablatable bands on immunoblots and a unique distribution of labeling by immunohistochemistry and immunoelectron microscopy. These antibodies are affinity-purified against their respective immunizing peptides for use in this study.

Animal Models

Pathogen-free male Sprague Dawley rats (200 to 300 g body wt; National Cancer Institute Breeding Facility, Frederick, MD) were used in this study. These rats were maintained initially in filter-top microisolator cages with autoclaved food and bedding, and were allowed free access to drinking water at all times. In preparation for surgery, rats were anesthetized with ketamine (60 mg/kg) administered intramuscularly followed by an intraperitoneal injection of sodium pentobarbital (21 mg/kg). A laparotomy was performed and the viscera were reflected to expose the left kidney. The renal pedicle was then clamped using a 1.5-inch bulldog clamp (Roboz Surgical, Rockville, MD), and the abdominal cavity was temporarily closed with wound clips. After exactly 45 min of ischemia, the abdominal cavity was reopened and the bulldog clamp was removed from the renal pedicle to permit reflow of blood to the kidney. A right contralateral nephrectomy was performed, the abdominal cavity was sutured closed, and rats were placed in cages with free access to food and water. Control animals underwent the same procedure except that the left kidney was not clamped. The rats were killed by decapitation after clamping (18 to 72 h). In additional experiments, a procedure identical to that described above was performed but the right nephrectomy was omitted.

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 no. 5-KE-1).

Blood and Urine Analyses

Urine and serum osmolalities were measured using a vapor pressure osmometer (model 5100C; Wescor, Logan, UT). Creatinine levels in urine and serum samples were quantified colorimetrically (alkaline picrate method, kit no. 555-A; Sigma Diagnostics, St. Louis, MO). The clearance of creatinine (Ccr) was determined by the formula Ccr = V × (U/P)cr, where V is urine volume of flow of urine (ml/min) and (U/P)c is the ratio of the concentration of creatinine in urine to that in serum.

Kidney Tissue Preparation for Immunoblotting

After the kidneys were removed, they were stored at −80°C. All kidneys from a given experimental protocol were processed simultaneously. After thawing on ice, the kidneys were washed briefly in ice-cold isotonic saline. Each kidney was homogenized for 15 s using a tissue homogenizer (Omnium 1000 fitted with a micro-sawtooth generator; Omni International, Warrenton, VA) 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 phenylmethylsulfonyl fluoride (U.S. Biochemical Corp., Toledo, OH). The total protein concentration in this homogenate was measured using the Pierce BCA protein assay reagent kit (Pierce, Rockford, IL). Half of the whole kidney homogenate from each sample was saved for differential centrifugation as follows. The whole kidney homogenates were centrifuged at 1000 × g for 10 min to remove nuclei and incompletely homogenized membrane fragments. To increase yield, 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 “postnuclear supernatant.” The postnuclear supernatant was subjected to two subsequent centrifugations. To obtain a high-density fraction, enriched in plasma membranes (25,27), the pooled supernatant was centrifuged at 17,000 × g for 20 min. Then, the supernatant from the 17,000 × g centrifugation was spun at 200,000 × g for 60 min to obtain a low-density fraction enriched in intracellular vesicles (25,27). The 1000 × g and 17,000 × g centrifugations were carried out using a Sorvall RC2-B refrigerated centrifuge with a 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 these membrane fractions was measured using the Pierce BCA Protein Assay reagent kit before addition of Laemmli sample buffer for immunoblotting.

Electrophoresis and Immunoblotting of Membrane Proteins

The protein samples were solubilized at 60°C for 15 min in Laemmli sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 8 or 12% polyacrylamide. For each set of samples from experimental and control rats, an initial gel was stained with Coomassie blue dye to confirm that equal loading had been achieved as described previously (8). Representative bands were quantified by laser densitometry (Personal Densitometer SI; Molecular Dynamics, San Jose, CA), assuming 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 for 30 min, the blots were probed with the respective antibodies for 24 h at 4°C. After washing, the nitrocellulose membranes were exposed to secondary antibody (donkey anti-rabbit IgG conjugated with horseradish peroxidase; Pierce no. 31458, diluted to
controls and multiplying by 100%.

Density values were normalized by dividing by the mean value for the two bands. The band intensities reported represent the sums of the two bands. The band density values were normalized by dividing by the mean value for the two bands and multiplying by 100%.

**Dot-Blotting**

The whole kidney homogenate sample was applied directly onto the nitrocellulose membrane using a microfiltration apparatus (Bio-Dot microfiltration apparatus; Bio-Rad, Richmond, CA). When the entire sample was filtered through the membrane and two washes were completed, the membrane was removed from the apparatus. After blocking with 5 g/dl nonfat dry milk for 30 min, the membrane was probed with the respective antibodies following the procedure described above. The dot density was analyzed by scanning densitometry.

**Immunocytochemistry**

Kidneys from experimental and control rats were fixed by vascular perfusion. The rats were anesthetized with Inactin (thiobutalbarbital, 100 mg/kg body wt, intraperitoneally), and a laparotomy was performed. The abdominal aorta was cannulated below the renal arteries, and the kidneys were perfusion-fixed at a pressure of 140 mmHg for approximately 3 min. The fixative contained periodate-lysine-parafformaldehyde (PLP) with 2% paraformaldehyde and 0.1% glutaraldehyde (28). After perfusion fixation, the kidneys were post-fixed in the fixative for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen, approximately 0.8 mm thick. The tissue blocks were infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen, essentially as described previously (29). The frozen tissue blocks were cryosectioned for immunohistochemistry.

Affinity-purified polyclonal anti-aquaporin-2, anti-aquaporin-3, anti-aquaporin-4, and anti-aquaporin-1 were used. Cryosections of thickness approximately 0.8 mm were obtained with a Reichert Ultratcut S Cryo-ultramicrotome and placed on gelatin-coated glass slides. After preincubation with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) or 0.1% skimmed milk and 0.05 M glycine for 5 min, the sections were incubated overnight at 4°C with the primary antibody diluted in PBS with 0.3% Triton X-100 and with 0.1% BSA or 0.1% skimmed milk. The labeling was visualized by incubation for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody (P448 1:100; Dako, Glostrup, Denmark), followed by incubation with diaminobenzidine for 10 min. Sections were counterstained with Meier counterstain. The following controls were performed. (1) The primary antibody was substituted with nonimmune rabbit IgG purified on a protein A column. (2) Incubations were carried out without use of primary antibody, or without primary and secondary antibody. All controls revealed a complete absence of labeling.

**Statistical Analyses**

Quantitative data are presented as mean ± SEM. Statistical comparisons were accomplished by unpaired t test (when variances were the same) or by the Mann-Whitney rank sum test (when variances were significantly different between groups). P values <0.05 were considered statistically significant.

**Results**

**Effect of Ischemia on Cellular Levels of Aquaporins and Na-K-2Cl Cotransporter**

Rats were sacrificed 18 h after the 45-min clamp of the left kidney and right nephrectomy. Sham-operated control rats had the right nephrectomy but the left kidney remained untouched. The urine output for the 18-h period after the surgery was 0.27 ± 0.06 ml/h in the rats with ischemic left kidneys (“experimental rats”) and 0.12 ± 0.02 ml/h in the rats with intact left kidneys (“control rats”) (P < 0.05). Thus, as expected, the experimental rats developed a significant increase in water excretion. The corresponding water intakes were 0.29 ± 0.08 ml/h in experimental rats and 0.19 ± 0.05 ml/h in control rats (NS). Animal weights at the time of the surgery were 234 ± 4 g for experimental rats and 238 ± 6 g for control rats. Eighteen hours after surgery, the serum creatinine concentration was increased in the experimental rats (experimental rats, 1.53 ± 0.54 mg/dl versus control rats, 0.51 ± 0.14 mg/dl) and creatinine clearance was decreased (experimental rats, 0.11 ± 0.06 ml/min versus control rats, 0.81 ± 0.47 ml/min). The urine osmolality was reduced in the experimental rats (571 ± 83 mosmol/kg H₂O versus 1279 ± 155 mosmol/kg H₂O), and plasma osmolality was unchanged (experimental rats, 286 ± 5.4 mosmol/kg H₂O versus control rats, 287 ± 1.9 mosmol/kg H₂O).

Figure 1 shows semiquantitative immunoblots for aquaporin-2, aquaporin-3, aquaporin-1, and the Na-K-2Cl cotransporter in the left kidney of the control and the experimental rats described in the previous paragraph. Aquaporin-4 was not sufficiently abundant in whole kidney homogenates to assess its expression level using immunoblotting, and was instead examined by immunocytochemistry, below. Coomassie-stained polyacrylamide gels were run in parallel to assure equality of loading in each lane (see Materials and Methods). As shown in Figure 1, there was a significant decrease in aquaporin-2 protein abundance in the ischemic kidneys relative to the controls (normalized band densities: control 100 ± 15%, ischemic 45 ± 9%; P < 0.01). There was also a significant decrease in aquaporin-3 in the ischemic kidneys relative to the controls (band densities: control 100 ± 17%, ischemic 37 ± 5%; P < 0.05). The decrease in aquaporin-3 protein abundance in the ischemic kidneys was confirmed by dot blotting (data not shown). In contrast, there was no significant change in the expression levels of aquaporin-1 (band densities: control 100 ± 8%, ischemic 84 ± 2%; P = 0.20) and the Na-K-2Cl cotransporter (band densities: control 100 ± 13%, ischemic 92 ± 10%; P = 0.66). Thus, the increase in urine flow seen in this model is associated with a suppression of cellular levels of the collecting duct aquaporins, aquaporin-2 and aquaporin-3.

As shown in Figure 1, the right kidney was removed at the time of clamping. To test whether the observations are dependent on the nephrectomy, we repeated the protocol without carrying out the nephrectomy. Figure 2 shows...
these results. In this protocol, the untouched right kidney was
the control for the clamped contralateral left kidney. As before,
the clamping period was 45 min and animals were killed after
18 h for analysis of the relative amounts of each of the
transporters in the kidneys. In the 18-h period after surgery,
water intake was $0.32 \pm 0.07$ ml/h, urine output from both
kidneys was $0.19 \pm 0.03$ ml/h, and urine osmolality was
1382 $\pm$ 182 mosmol/kg H$_2$O. Animal weights were 237 $\pm$ 2 g
at the time of surgery. Eighteen hours after surgery, the serum
creatinine concentration was $0.91 \pm 0.03$ mg/dl, and the cal-
culated creatinine clearance for both kidneys was $0.39 \pm 0.03$
ml/min. Again, there was a decrease in the levels of aqua-
opin-2 (band densities: control $100 \pm 14\%$, ischemic $40 \pm
5\%$; $P < 0.01$) and aquaporin-3 (band densities: control $100 \pm
25\%$, ischemic $27 \pm 6\%$; $P < 0.01$) in the ischemic kidneys.
However, in this protocol, the renal levels of aquaporin-1 and
the Na-K-2Cl cotransporter were also found to be diminished
(aquaporin-1 band densities: control $100 \pm 9\%$, ischemic $34 \pm
5\%$, $P < 0.001$; and Na-K-2Cl cotransporter band densities:
control $100 \pm 16\%$, ischemic $19 \pm 4\%$, $P < 0.001$). Thus, as
with the first protocol, aquaporin-2 and -3 protein abundances
were decreased. However, the two protocols give contrasting
responses with regard to aquaporin-1 and Na-K-2Cl cotrans-
porter protein abundance, suggesting that nephrectomy may
alter the response of the kidney to ischemia with regard to synthesis or degradation of the transporter proteins.

**Time Course**

We carried out experiments to assess aquaporin and Na-K-2Cl cotransporter protein abundance at additional time points, i.e., 36 and 72 h, after the 45-min clamp of the left renal pedicle. In these experiments, right nephrectomy was carried out in both experimental rats and control rats, and semiquantitative immunoblots were run to compare transporter abundance in the post-ischemic left kidneys with the control left kidneys. Figure 3 shows a compendium of urinary flow rates measured in these experiments with values plotted at the midpoint of urinary collection periods. As can be seen, water excretion was elevated throughout the period of observation. Figures 4 through 7 show the changes in aquaporins and Na-K-2Cl cotransporter protein abundance seen at various time points after the ischemic insult. As shown in Figure 4, aquaporin-2 levels were significantly decreased early in the time course, but returned toward normal at the 72-h time point. Aquaporin-3 protein abundance (Figure 5) also decreased rapidly, but in contrast to aquaporin-2, remained decreased throughout the period of observation. The renal level of aquaporin-1, the aquaporin expressed in the proximal tubule, fell significantly, although the fall occurred beyond the point at which significant increases in water flow occurred (Figure 6). Finally, the abundance of the Na-K-2Cl cotransporter protein, which is expressed in the thick ascending limb of Henle’s loop, did not decrease significantly during the time course (Figure 7).

**Figure 3.** Water excretion measurements in time course studies. Water excretion rates in experimental (post-ischemic) rats were normalized by values from control rats. Data shown are a compendium of all water excretion measurements made in time course studies. The values are plotted at the midpoint of urine collections. The 9-h value was from the set of rats killed 18 h after left renal pedicle clamping. The 24-h value was from the set of rats killed 24 h after clamping. The 36- and 54-h time point values were from the set of rats killed 72 h after clamping. *P < 0.05 versus the control values. Absolute urine flow rates for control rats: 9 h, 0.12 ± 0.02 ml/h; 24 h, 0.11 ± 0.01 ml/h; 36 h, 0.19 ± 0.01 ml/h; 54 h, 0.13 ± 0.02 ml/h. Absolute urine flow rates for rats with ischemic left kidney: 9 h, 0.27 ± 0.06 ml/h; 24 h, 0.22 ± 0.02 ml/h; 36 h, 0.58 ± 0.08 ml/h; 54 h, 0.32 ± 0.06 ml/h. Water intake rates for control rats: 9 h, 0.19 ± 0.05 ml/h; 24 h, 0.41 ± 0.03 ml/h; 36 h, 0.72 ± 0.05 ml/h; 54 h, 0.85 ± 0.05 ml/h. Water intake rates for rats with ischemic left kidney: 9 h, 0.29 ± 0.08 ml/h; 24 h, 0.34 ± 0.05 ml/h; 36 h, 1.19 ± 0.05 ml/h; 54 h, 1.10 ± 0.05 ml/h.

**Figure 4.** Time course of aquaporin-2 protein abundance changes after 45-min clamping of left renal pedicle. The data plotted are results from densitometry of bands from ischemic kidneys (normalized by dividing by control values from the same blot, mean ± SEM). Immunoblots were run using kidneys obtained at different time points after the release of the clamp using separate controls for each time point. The 18-h point represents data from Figure 1. Blots for 36- and 72-h time points are not shown. Asterisks indicate that the normalized band density was significantly decreased (*P < 0.05) relative to the left kidneys of control rats.

**Figure 5.** Time course of aquaporin-3 protein abundance changes after 45-min clamping of left renal pedicle. For details, see legend of Figure 4.
Immunocytochemistry

Figure 8 shows immunoperoxidase immunocytochemistry for aquaporin-2 (A and B), aquaporin-3 (C and D), aquaporin-4 (E and F), in collecting ducts of 36-h post-ischemic kidneys (A, C, and E), and control kidneys (B, D, and F) prepared with the same protocol as used for rats presented in Figure 1, i.e., with right nephrectomy carried out for both the experimental and control rats. As shown in Figure 8A, there was a marked reduction in aquaporin-2 labeling in the post-ischemic kidney (compare with control, shown in Figure 8B). However, there was substantial aquaporin-2 labeling of the apical plasma membrane domain suggesting that aquaporin-2 trafficking is not impaired in the post-ischemic kidneys. Aquaporin-3 labeling was also decreased in post-ischemic kidneys consistent with immunoblotting results (compare Figure 8C with control shown in Figure 8D). As shown in Figure 8E, in contrast to aquaporin-2 and aquaporin-3, there was only a marginal reduction in aquaporin-4 labeling density in the inner medullary collecting ducts of post-ischemic kidney.

Figure 9 shows immunoperoxidase immunocytochemistry for aquaporin-1 in the renal inner medulla. In this region, aquaporin-1 is expressed in the thin descending limbs of Henle’s loops. The left panel shows a section from a post-ischemic kidney and the right panel shows a control kidney from a rat that had undergone right nephrectomy but not clamping of the left kidney. As can be appreciated, there was no major decline in aquaporin-1 protein abundance in the thin descending limb.

Differential Centrifugation of Aquaporin-2

To address whether the increase in urine flow seen in the post-ischemic animals is associated with failure of trafficking of aquaporin-2 to the plasma membrane, we carried out differential centrifugation of whole kidney homogenates (Figure 10). Previous studies have demonstrated that the 17,000 × g fraction is enriched in collecting duct plasma membranes and the 200,000 × g fraction contains primarily intracellular vesicles (25,27). Thus, if immunoblots are run with both of these membrane fractions, the ratio of band densities (17,000 × g/200,000 × g) provides an index of trafficking into the plasma membrane. As shown in Figure 10, this ratio was increased rather than decreased at the 18-h time point. In addition, the ratio was not significantly different between post-ischemic kidneys and control kidneys at 36 and 72 h. Thus, as also indicated by immunocytochemical localization experiments (Figure 8), we found no evidence that the increase in urine flow seen in the post-ischemic kidneys was due to a failure of trafficking of aquaporin-2 to the plasma membrane.

Discussion

The two major conclusions from this study are: (1) collecting duct cells are significantly affected by renal ischemia; and (2) the increased urine flow associated with acute renal injury is due at least in part to reduced renal levels of the collecting duct water channels, aquaporin-2 and aquaporin-3.

Collecting Duct Cells Are Significantly Affected by Renal Ischemia

It is generally accepted that the two major sites of renal damage resulting from ischemia are the proximal tubule and the thick ascending limb of Henle’s loop (2), the two major sites of ATP utilization for sodium transport (30). Rates of sodium transport and ATP utilization are relatively low in the collecting duct (30) and one would expect that collecting ducts should be relatively invulnerable to ischemic injury. Nevertheless, we observed in this study a marked decline in the abundances of both aquaporin-2 and aquaporin-3, indicative of substantial impairment of the molecular apparatus for transepithelial water transport in the collecting duct. Although the mechanism of the reduced abundance of aquaporins was not examined, both direct as well as indirect effects of ischemia on the collecting duct cells are possible. Indirect effects could include responses to altered systemic homeostasis secondary to
Figure 8. Immunocytochemical labeling of collecting duct aquaporins. Immunoperoxidase immunocytochemistry in thin sections of inner medullas of left kidneys from Sprague Dawley rats 36 h after the left kidney was clamped for 45 min and the right kidney was removed (A, C, and E "post-ischemic kidneys") and for the corresponding time point in control rats in which the right kidney was removed but the left kidney was untouched (B, D, and F "control kidneys"). (A and B) Aquaporin-2. Note substantial reduction in collecting duct aquaporin-2 labeling in the post-ischemic kidney. (C and D) Aquaporin-3. Note marked reduction in collecting duct aquaporin-3 labeling in the post-ischemic kidney. (E and F) Aquaporin-4. Note that there is no major reduction in collecting duct aquaporin-4 labeling in the post-ischemic kidney.
acute renal failure, or responses to autcoids or reactive species released from other affected cells, or responses to altered flow or composition of tubule fluid delivered to the collecting duct as a result of upstream nephron damage. However, an indirect effect due to disordered systemic homeostasis appears to be unlikely because the suppression of aquaporin levels was seen in the experiment in which the right kidney remained intact and therefore prevented most systemic effects of acute renal injury in the left kidney. Thus, we believe that the most likely explanation for the suppression of aquaporin-2 and -3 levels in the kidney is either ischemic damage to the collecting duct cells, secondary effects of diffusible mediators, or altered flow or composition of tubule fluid delivered to the collecting duct. This study did not address whether the observed changes in aquaporin levels in the post-ischemic kidneys was due to altered protein synthesis (e.g., due to decreased transcription, decreased mRNA stability, or decreased translation) or to altered protein degradation.

That local intrarenal factors may play a role with respect to dysregulation of aquaporin-2 was demonstrated in another pathophysiologic model using experimentally induced unilateral ureteral obstruction (31). In response to 24 h of ureteral obstruction, aquaporin-2 levels were markedly reduced in the obstructed kidney compared with the unobstructed contralateral kidney or compared with kidneys from sham-operated control animals (31). In addition, it was shown that targeting of aquaporin-2 to the apical plasma membrane was maintained despite the severe downregulation of aquaporin-2 levels. Thus, this is very similar to the reduced abundance and maintained targeting observed in the post-ischemic kidneys in this study, and raises the possibility that common signal transduction pathways may be affected in the two settings.

The Increased Urine Flow Seen with Moderate Acute Renal Injury Is Associated with Reduced Acute Renal Levels of Aquaporin-2 and Aquaporin-3

Water excretion is dependent on a balance between the GFR and the net rate of water absorption along the renal tubule. Severe acute renal failure is generally associated with oliguria, due to a decline in GFR that cannot be compensated for via changes in tubular water reabsorption. Luminal casts may contribute to the oliguria by decreasing GFR and causing tubular fluid backleak as a result of tubular obstruction (32). However, mild to moderate ischemic acute renal failure, as well as the recovery phase of severe acute renal failure, is frequently associated with increased urine flow, presumably due to suppression of tubular water reabsorption, which exceeds the reduction in GFR. One important factor in this decrease in tubular water reabsorption, particularly in the recovery phase of severe acute renal failure, is the osmotic diuresis that may accompany the elimination of urea and other species that accumulate in the blood due to the renal failure. In this study, we have identified another factor that is likely to contribute to decreased renal tubular water reabsorption in mild to moderate acute renal failure, i.e., a decrease in the abundance of aquaporins-2 and -3, water channels that are responsible in large part for the ability of water to penetrate the collecting duct epithelium and return water to the bloodstream in response to vasopressin.

The chief model used in this study was 45-min clamping of the left kidney followed by right nephrectomy. The major findings using this model are summarized in Figures 4 to 7. The model produced substantial increases in water excretion in the post-ischemic kidney at all time points (Figure 3). As illustrated in Figures 4 and 5, the increase in water excretion at
early time points after clamping was associated with marked decreases in the levels of the collecting duct aquaporins (aquaporin-2 and -3). Aquaporin-4 was not sufficiently abundant in kidney homogenates to assess its renal abundance using immunoblotting. However, immunocytochemistry suggested that it was not greatly decreased at least in the inner medulla. The substantial decline in aquaporin-2 and -3 protein abundance would be predictive of a significant impairment in collecting duct water permeability and in the ability of the collecting duct fluid to osmotically equilibrate with the hypertonic medullary interstitium. At later time points, the levels of these two aquaporins displayed an upward trend, suggesting that the collecting ducts were on a time course toward recovery. That a reduction in aquaporin-2 has a major significance for urinary concentration ability has been shown in previous studies of inherited nephrogenic diabetes insipidus (33), inherited central diabetes insipidus (24), and in acquired nephrogenic diabetes insipidus (16,19,20,31). However, until recently the role of basolateral aquaporins has not been well established.

Although we did not use a model of oliguric acute renal failure in this study, we would anticipate that a similar suppression of aquaporin-2 and -3 protein abundance would also be seen in oliguric models, a view that is borne out by our preliminary observations using a rat model of bilateral ischemic damage due to clamping of both renal arteries (T.-H. Kwon, S. Nielsen, M. A. Knepper, and P. Fernández-Llama, unpublished observations). The decline in urine flow in such models is undoubtedly due to the associated decrease in glomerular filtration, which would overwhelm any decrease in collecting duct water permeability. Previous studies using a bilateral clamping model revealed both oliguric and polyuric phases, both associated with decreased urinary osmolality (21), presumably due in part to decreased collecting duct water permeability.

The urinary concentrating process depends on the coordinated function of the loop of Henle and the collecting ducts (34). The thick ascending limb of the loop of Henle powers the countercurrent multiplier process responsible for generation of a corticomedullary osmolality gradient, while the collecting ducts, under the control of vasopressin, allow variable degrees of osmotic equilibration, resulting in variable water excretion and a reciprocal relationship between urinary flow and urinary osmolality. Therefore, hypothetically, increased urine flow with moderate post-ischemic renal injury could be associated with a failure of either the thick ascending limbs or collecting ducts, or both. Figure 7 shows that there was no substantial decline in the abundance of the thick ascending limb Na-K-2Cl cotransporter in post-ischemic model, in contrast to what was found with the aquaporins. This observation does not rule out the possibility that the increased urine flow could have resulted in part from a decline in countercurrent multiplication due to other effects in the thick ascending limb. It should be emphasized that Na-K-2Cl cotransporter protein abundance did decline in post-ischemic kidneys in rats in which contralateral nephrectomy was not carried out. At this point, it is unclear why the two models differ with regard to effects on Na-K-2Cl cotransporter protein abundance. Possibly, uremia (present in nephrectomized rats but not in non-nephrectomized rats) was a
contributing factor. Recent studies using a bilateral clamping model of ischemic acute renal failure with marked azotemia also showed a lack of suppression of Na-K-2Cl cotransporter protein abundance (T.-H. Kwon, S. Nielsen, M. A. Knepper, and P. Fernández-Llama, unpublished results), supporting the view that the decline in thick ascending limb Na-K-2Cl protein abundance may be blunted in the uremic state. Alternatively, hemodynamic differences in the post-ischemic kidneys of the two models may have contributed to differences in Na-K-2Cl cotransporter levels.

Figure 6 shows that aquaporin-1, expressed in the proximal tubule, exhibited a decrease in abundance. Changes in fluid transport in the proximal tubule can have significant effects on the urinary concentrating mechanism by altering flow rates of tubule fluid delivery to the thick ascending limbs and collecting ducts. Therefore, damage to the proximal tubule, demonstrated in this study by a decline in aquaporin-1 protein abundance, could have contributed to the increased urine flow. However, the decline in aquaporin-1 protein abundance was not seen at the 18-h time point, when significant increases in urine flow had already developed in the post-ischemic kidney. Aquaporin-1 is extremely abundant both in the proximal tubule and descending thin limbs (9,35). Recent studies have confirmed that aquaporin-1 plays a vital role in the countercurrent multiplier mechanism by allowing efficient osmotic water equilibration. Mice with an aquaporin-1 gene knockout were polyuric, and prolonged dehydration was fatal because of inability to concentrate urine significantly above plasma osmolality (36). Importantly, it was subsequently shown that the osmotic water permeability of the proximal tubule (37) and descending thin limb (38) was reduced more than 80%. Therefore, damage to the proximal tubule demonstrated in this study by a sharp decline in aquaporin-1 protein abundance (and perhaps reduced aquaporin-1 in the descending thin limb) could have contributed to the increased urine flow during the later stages of the post-ischemic time course. Immunocytochemistry of medullary thin sections from ischemic and control kidneys, however, suggests that there was little or no decline in the aquaporin-1 protein abundance in thin descending limb at the 36-h time point, raising the possibility that the major reduction in aquaporin-1 is in the proximal tubule.

Aquaporin-2 Trafficking

Aquaporin-2, the vasopressin-regulated water channel expressed in the apical plasma membrane of collecting duct cells, is regulated in two ways (6): (1) short-term regulation via vasopressin-induced trafficking of aquaporin-2 to the plasma membrane; and (2) long-term regulation of aquaporin-2 protein abundance. In this study, aquaporin-2 underwent a change in abundance in response to ischemic injury that appeared to be inversely correlated with urine flow. Immunocytochemical observations confirmed the decline in cellular aquaporin-2 levels, but did not show any change in the distribution of aquaporin-2 in the collecting duct cells, suggesting that there is no impairment of aquaporin-2 trafficking. Lack of impairment of trafficking was confirmed by differential centrifugation experiments. A decline in aquaporin-2 level with intact trafficking to the plasma membrane was previously seen in rats with unilateral ureteral obstruction (31), in rats with puromycin aminonucleoside-induced nephrotic syndrome (14), and in rats with adriamycin-induced nephrotic syndrome (12).

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References


