Original Article

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Association of Proteinuria with Urinary Concentration Defect in Puromycin Aminonucleoside Nephrosis

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Background: Puromycin aminonucleoside (PA) can induce nephrotic syndrome in rats, and proteinuria is an important mediator of tubulointerstitial injury in glomerulopathy. We assumed that glomerular proteinuria may affect tubular function, such as urinary concentration, and investigated whether a urinary concentration defect is associated with proteinuria in puromycin aminonucleoside nephrosis (PAN). We also investigated the defect response to enalapril.
Methods: Glomerular proteinuria was induced by a single intraperitoneal injection of PA (150 mg/kg BW) in male Sprague-Dawley rats. In a half of these rats, enalapril (35 mg/kg BW) was administered daily in a food mixture for two weeks. After the animal experiment, kidneys were harvested for immunoblot analysis and histopathologic examination.
Results: Compared with the control group, PA-treated rats had severe proteinuria, polyuria, and a lower urine osmolality. PA treatment induced remarkable tubulointerstitial injury and significant reductions in protein abundances of aquaporin-1 and Na-K-2Cl co-transporter type 2 (NKCC2). Proteinuria significantly correlated with osteopontin expression in the kidney and inversely correlated with renal expression of aquaporin-1, aquaporin-2, and NKCC2. The degree of tubulointerstitial injury significantly correlated with proteinuria, urine output, and osteopontin expression and inversely correlated with urine osmolality and renal expression of aquaporin-1, aquaporin-2, and NKCC2. No significant differences in parameters were found between PA-treated rats with and without enalapril.
Conclusion: In PAN, glomerular proteinuria was associated with tubulointerstitial injury and water diuresis. Downregulation of aquaporin-1 and NKCC2 can impair countercurrent multiplication and cause a urinary concentration defect in PAN.

Key Words: Enalapril, Proteinuria, Puromycin aminonucleoside, Tubulointerstitial injury, Urinary concentration

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INTRODUCTION

Puromycin aminonucleoside nephrosis (PAN) is a representative animal model of nephrotic syndrome and has been used to induce glomerular proteinuria[1)]. Electronic microscopic findings suggest that, in PAN, glomerular epithelial cell lesions produce severe proteinuria[2)]. This initial pathology accompanies glomerular hemodynamic alteration, leading to intraglomerular hypertension and progression to glomerulosclerosis. Proteinuria and glomerular pathologies can be ameliorated by angiotensin converting enzyme (ACE) inhibition[3)].

Angiotensin II can stimulate aquaporin-2 trafficking in the collecting duct via angiotensin II receptor type 1[4)]. Adult rats treated neonatally with enalapril showed a urinary concentrating defect in association with a decrease in inner medullary aquaporin-2 (AQP2) expression[5)]. Thus, urinary concentration may be modified by administration of angiotensin receptor blockers or ACE inhibitors.

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In addition to glomerular lesions, acute tubulointerstitial nephritis was induced by a single intraperitoneal injection of puromycin aminonucleoside (PA)[6]. This was characterized by tubulointerstitial infiltration of mononuclear cells and led to irreversible injury such as tubular atrophy and interstitial fibrosis. Tubulointerstitial changes can be relieved by ACE inhibition[7].

Proteinuria is an important marker of renal progression because of its role in tubulointerstitial injury[8]. When proximal tubular epithelial cells are exposed to a large amount of protein, many inflammatory mediators are activated during the process of protein uptake and produce tubular injury and interstitial inflammation[9,10]. However, tubular injury and inflammation are not limited to the proximal tubule but extend to the distal nephron segments including the medullary collecting duct[11].

Henle's loop and the collecting duct, in which aquaporin-1 (AQP1), AQP-2, and Na-K-2Cl cotransporter type 2 (NKCC2) are localized, are the major renal tubules for urine concentration. In association with downregulation of AQP1 and AQP2 water channels, a urinary concentration defect was previously reported in rats with PAN[12,13]. However, the role of Henle's loop was not addressed. The presents study was undertaken to assess the associations between proteinuria and markers of urinary concentration defect in PAN. Furthermore, we tested whether these changes were affected by ACE inhibition.

MATERIAL AND METHODS

1. Animal experiment

Specific pathogen-free male Sprague-Dawley rats (Orient Bio Inc., Seongnam, Korea) weighing 100-120 g were used for induction of PAN. Rats were divided into 3 groups: vehicle-treated controls (n=4), PA-treated rats (n=4), and PA+enalapril-treated rats (n=4).

All rats were fed 15 g of rodent chow per 180 g body weight daily and had free access to drinking water. For PA-treated rats, PA (Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline (vehicle) and used at a concentration of 10 mg/mL. These rats were given a single intraperitoneal injection of PA, 150 mg/kg BW[14]; vehicle-treated controls were given an intraperitoneal injection of the same volume of 0.9% saline. In PA+enalapril-treated rats, enalapril (Sigma, #E6888) was orally ingested in a food mixture at a daily dose of 35 mg/kg BW.

Animals were housed individually in metabolic cages to provide a daily fixed amount of food and to obtain 24-hour urine collection. The animal experiment was performed over two weeks, and kidneys were harvested for histopathologic examination and immunoblot analysis. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University.

2. Plasma and urine data

Biochemical values from plasma and urine were measured with an autoanalyzer (ADVIA 1650; Bayer Healthcare Ltd., Tarrytown, NY, USA). Urine osmolality was determined by an Advanced 2020 Osmometer (Advanced Instruments, Norwood, MA, USA) using the freezing-sedimentation method.

3. Immunoblot analysis

The right kidneys were rapidly removed and homogenized in 10 mL of ice-cold isolation solution using a tissue homogenizer. After homogenization, the total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Sigma) and adjusted to 2 μg/μL with isolation solution. The samples were stabilized by adding Laemmli sample buffer and heating to 60℃ for 15 min.

Initially, “loading gel” analysis was performed on each sample set. Five micrograms of protein from each sample were loaded into an individual lane and electrophoresed on 12% polyacrylamide-SDS mini-gels using a Mini PROTEAN III electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). These electrophoresis samples were stained with Coomassie blue dye (G-250, Bio-Rad; 0.025% solution in 4.5% methanol and 1% acetic acid). Selected bands from these gels were scanned (GS-700 Imaging Densitometry, Bio-Rad) to determine the density (Molecular Analyst version 1.5, Bio-Rad), and relative amounts of protein were loaded in each lane. Finally, protein concentrations were “corrected” to reflect these measurements.

For immunoblotting, 20 μg of protein from each sample were loaded into each lane and electrophoresed on polyacrylamide-SDS mini-gels. The proteins were transferred

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electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad). After blocking with 5% skim milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 30 min, the membranes were probed overnight at 4℃ with the respective primary antibodies. For probing blots, both antibodies were diluted in a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dL sodium azide, 50 mg/dL Tween 20, and 0.1 g/dL bovine serum albumin (pH 7.5). The primary antibody to osteopontin was murine monoclonal MPIIIB10 (purchased from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA); primary antibodies to AQP1, AQP2, and NKCC2 were kindly shared by Dr. Mark Knepper at National Institutes of Health. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce, Rockford, IL, USA) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed using an enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech). Densitometric values were normalized to the mean of the control group in a given experiment, which was defined as 100%.

4. Renal histopathology

Renal tissues were fixed in 10% neutral-buffered formalin and were embedded in paraffin. Four-micron paraffin sections were stained with periodic acid-Schiff (PAS). Tubulointerstitial injury was defined as tubular dilation, tubular atrophy, sloughing of tubular epithelial cells, or thickening of the tubular basement membrane and was scored on a scale of 0 to 4. On this scale, 0, no tubulointerstitial injury; 1, <25% of the tubulointerstitium injured; 2, 25 to 50% of the tubulointerstitium injured; 3, 51 to 75% of the tubulointerstitium injured; 4, >75% of the tubulointerstitium injured[15].

5. Statistics

Values are presented as mean ± SE. Statistical analysis was performed by Kruskal-Wallis followed by the Mann-Whitney *U*-test for post hoc comparisons. Correlations between variables of interest were analyzed by linear regression. P values less than 0.05 were considered statistically significant.

Table 1. Urine and plasma data

	Control (n=4)	PA (n=4)	PA+E (n=4)
Urine			
Volume (mL/d/100 g BW)	3.0 ± 0.2	6.0 ± 0.9*	9.7 ± 1.1*
Osmolality (mOsm/kg H_2O)	1,997 ± 54	799 ± 162*	585 ± 68*
Sodium (μmol/d/100 g BW)	652 ± 56	733 ± 98	657 ± 62
Potassium (μmol/d/100 g BW)	486 ± 22	423 ± 1	448 ± 11
Chloride (μmol/d/100 g BW)	1,127 ± 81	1,174 ± 113	1,091 ± 57
Protein (mg/d/100 g BW)	3.0 ± 1.0	147 ± 68*	186 ± 90*
Plasma			
Sodium (mmol/L)	149 ± 1	147 ± 1	145 ± 2
Potassium (mmol/L)	4.1 ± 0.1	4.7 ± 0.6	4.2 ± 0.1
Chloride (mmol/L)	100 ± 1	100 ± 1	99 ± 2
Protein (g/dL)	5.8 ± 0.0	5.6 ± 0.3	5.0 ± 0.3
Albumin (g/dL)	3.7 ± 0.1	3.3 ± 0.2	2.9 ± 0.3
Urea nitrogen (mg/dL)	15 ± 1	14 ± 1	20 ± 2
Creatinine (mg/dL)	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
Creatinine clearance (mL/min100 g BW)	0.32 ± 0.2	0.34 ± 0.3	0.30 ± 0.3

Data are mean ± SE. PA, puromycin aminonucleoside; E, enalapril.
Statistical analysis was performed by Kruskal-Wallis followed by the Mann-Whitney *U*-test for post hoc comparisons.
*p<0.05 vs. control by Mann-Whitney *U*-test.

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RESULTS

1. Urinary concentration defect

Table 1 shows urine and plasma data obtained on the final day of the animal experiment. Urine values were adjusted for body weight because the final body weight was different among groups (p<0.05): vehicle-treated controls, 236 ± 4 g; PA-treated rats, 188 ± 10 g; and PA+enalapril-treated rats, 216 ± 4 g.

Severe proteinuria was induced by a single intraperitoneal injection of PA but was not significantly affected by enalapril co-administration. Urine output increased in PA-treated and PA+enalapril-treated rats compared with vehicle-treated controls (p<0.05). Consistent with this, PA-treated and PA+enalapril-treated rats had lower urine osmolality than controls (p<0.05). Thus, urinary concentration defects were induced in PA-treated rats. However, plasma electrolytes, protein, albumin, blood urea nitrogen, and creatinine values demonstrated no significant differences between the groups.

2. Renal expression of osteopontin, AQP1, AQP2, and NKCC2

Figure 1 shows the results of immunoblot analysis for osteopontin, AQP1, AQP2, and NKCC2. We found no significant differences in osteopontin among the groups. However, AQP1 was decreased by PA treatment (p<0.05), but AQP2 was not. PA-treated rats had a lower NKCC2 abundance than controls (p<0.05).

3. Tubulointerstitial injury

Tubulointerstitial injury was estimated from PAS-stained renal tissues. In both cortex and medulla, a remarkable tubulointerstitial injury was induced by PA treatment and was not significantly affected by enalapril co-administration (Fig. 2).

4. Associations of urinary concentration defect with proteinuria, renal osteopontin, AQP1, AQP2, NKCC2, and tubulointerstitial injury

When the groups were assessed together, we found a positive correlation between proteinuria and urine output and an inverse correlation between proteinuria and urine osmolality (Fig. 3). As expected, AQP1, AQP2, and NKCC2 positively correlated with urine osmolality (Fig. 4). Interestingly, proteinuria was inversely associated with AQP1, AQP2,

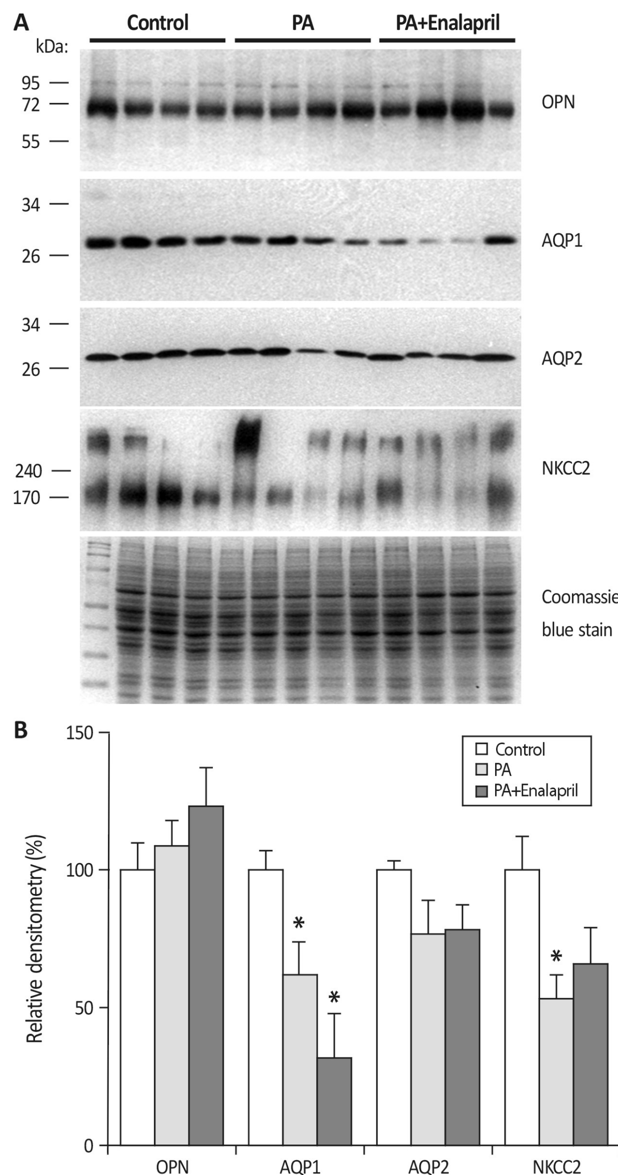

Fig. 1. Effects of puromycin aminonucleoside (PA) with and without enalapril administration on protein abundances of osteopontin (OPN), aquaporin-1 (AQP1), aquaporin-2 (AQP2), and Na-K-2Cl cotransporter type 2 (NKCC2) in whole kidney homogenate. Immunoblots, in which each lane was loaded with a protein sample from a different rat, reacted with respective specific antibodies as shown (**A**). Densitometric analyses reveal significant decrease in renal AQP1 and NKCC2 in PA-treated rats compared to controls (**B**). Bar graphs represent mean±SE. *, p<0.05 versus control.

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and NKCC2 (Fig. 5). Furthermore, renal expression of osteopontin positively correlated with proteinuria and inversely correlated with plasma albumin concentration (Fig. 6).

The tubulointerstitial injury score was positively associated with urine output and inversely correlated with urine osmolality (Fig. 7). Notably, this score was positively correlated with proteinuria and negatively associated with AQP1, AQP2, and NKCC2 expression (Fig. 8).

DISCUSSION

In this study, polyuria and severe proteinuria were induced by a single administration of PA in Sprague-Dawley rats. The urinary concentration defect was associated with tubulointerstitial injury and reduced expression of AQP1 and NKCC2 in the kidney. Furthermore, all measured parameters of urinary concentration correlated well with proteinuria.

In PAN, proteinuria is caused by pathology of glomerular epithelial cells[2] and can induce tubulointerstitial injury during endocytosis of filtered protein[6]. Thus, the proximal tubule is the major site of glomerular proteinuria-induced injury[10]. However, this study suggests that the tubulointerstitial injury extends to Henle's loop because AQP1 and NKCC2 are localized in the thin descending limb and the thick ascending limb, respectively[16].

In the kidney, Henle's loop and the collecting duct collaborate to concentrate urine. Glomerular filtered water is reabsorbed in the proximal tubule and the thin descending limb of Henle's loop via AQP1 water channels; NaCl is reabsorbed by the water-impermeable thick ascending limb of

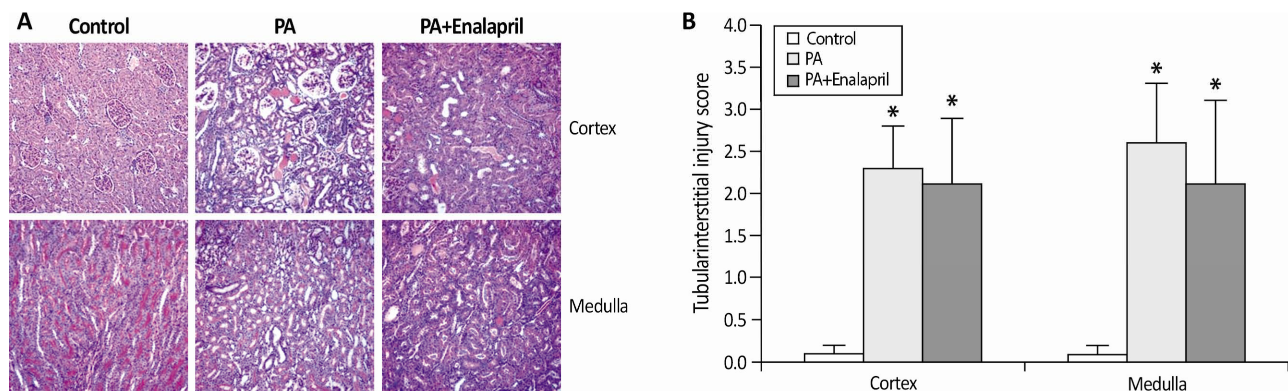

Fig. 2. Renal tubulointerstitial injury in puromycin aminonucleoside (PA)-treated rats with and without enalapril administration. Periodic acid-Schiff stain shows remarkable tubulointerstitial injury in PA-treated rat kidneys (**A**, ×200), and their tubulointerstitial injury was higher than that of controls in both cortex and medulla (**B**). Bar graphs represent mean±SE. *, p<0.05 versus control by Mann Whitney *U*-test.

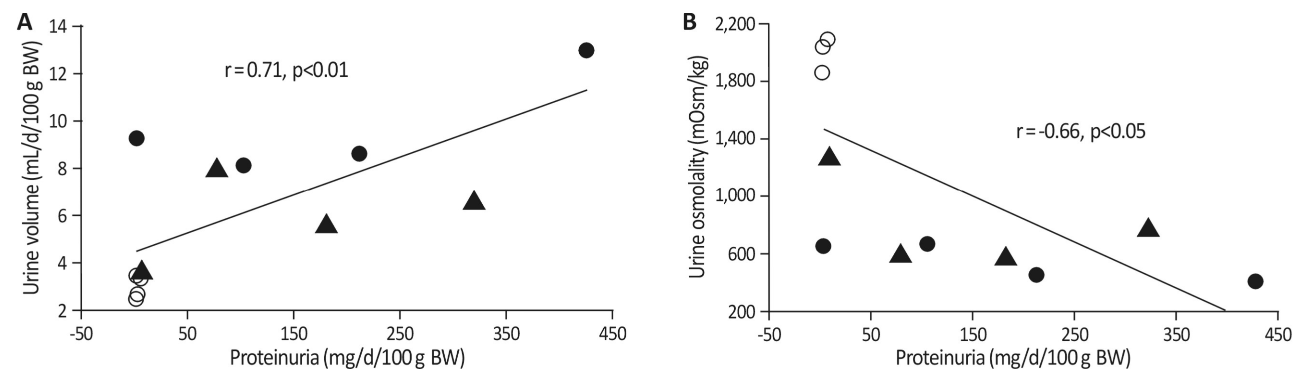

Fig. 3. Relationship between proteinuria and urinary concentration defect. Linear regression analyses reveal a positive correlation between proteinuria and daily urine volume (**A**) and an inverse correlation between proteinuria and urine osmolality (**B**). Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.

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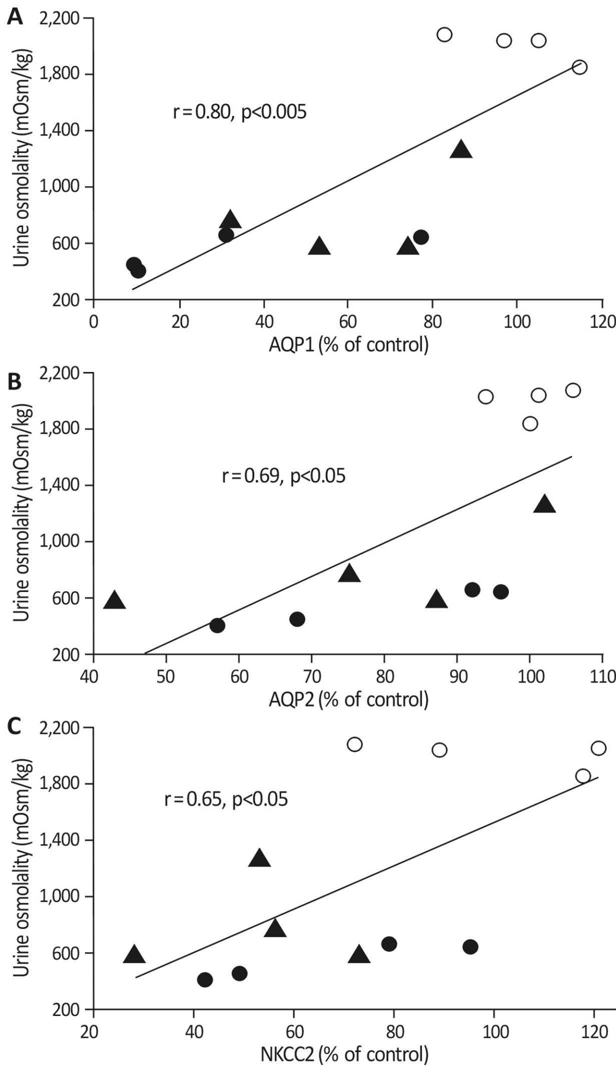


Fig. 4. Relationship between renal transporters and urinary concentration. The protein abundances of AQP1 (**A**), AQP2 (**B**), and NKCC2 (**C**) inversely correlate with urine osmolality. Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.

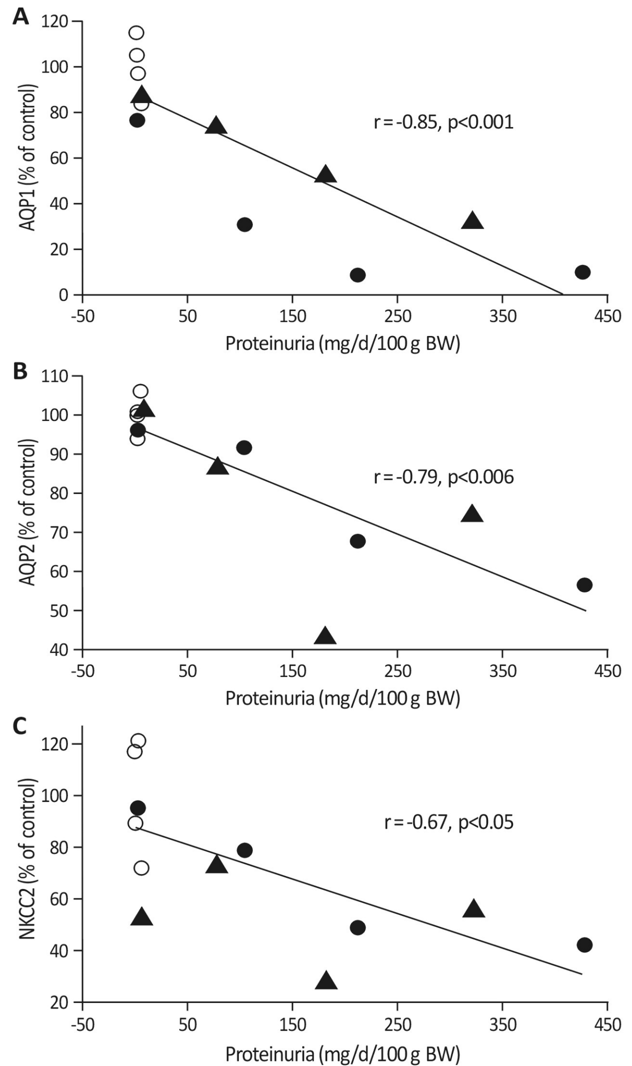
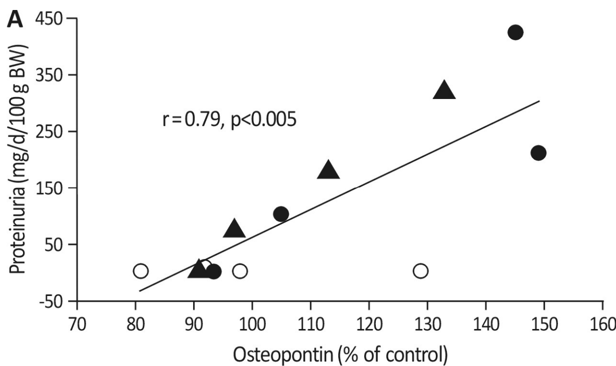


Fig. 5. Relationship between proteinuria and renal transporters. Linear regression analyses reveal inverse correlations between proteinuria and AQP1 (**A**), between proteinuria and AQP2 (**B**), and between proteinuria and NKCC2 (**C**). Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.



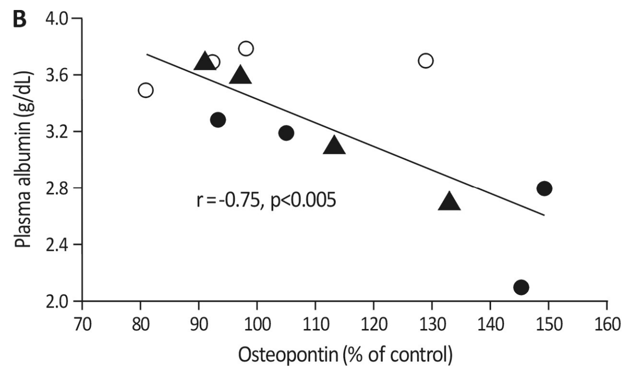


Fig. 6. Relationship between osteopontin expression and degree of proteinuria. Linear regression analyses reveal a positive correlation between osteopontin and proteinuria (**A**) and an inverse correlation between osteopontin and plasma albumin (**B**). Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.

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Henle's loop via the NKCC2 sodium transporter. This countercurrent multiplication establishes a corticomedullary concentration gradient, and medullary interstitial hypertonicity facilitates osmotic water absorption along the collecting duct[17]. NKCC2 is a sodium transporter that physiologically contributes to regulating water balance. Thus, AQP1, AQP2, and NKCC2 are critical components of urinary concentration and were investigated in this study.

We showed that AQP1 and NKCC2 were downregulated in rats with PAN, suggestive of disturbed countercurrent multiplication and resulting in water diuresis. However, we found no significant alteration of AQP2 in PA-treated rat kidneys. Previous studies have shown that AQP2 protein was downregulated in medullary collecting duct cells[12,13]. We used whole kidney homogenates for immunoblot analysis, and immunohistochemistry was not performed.

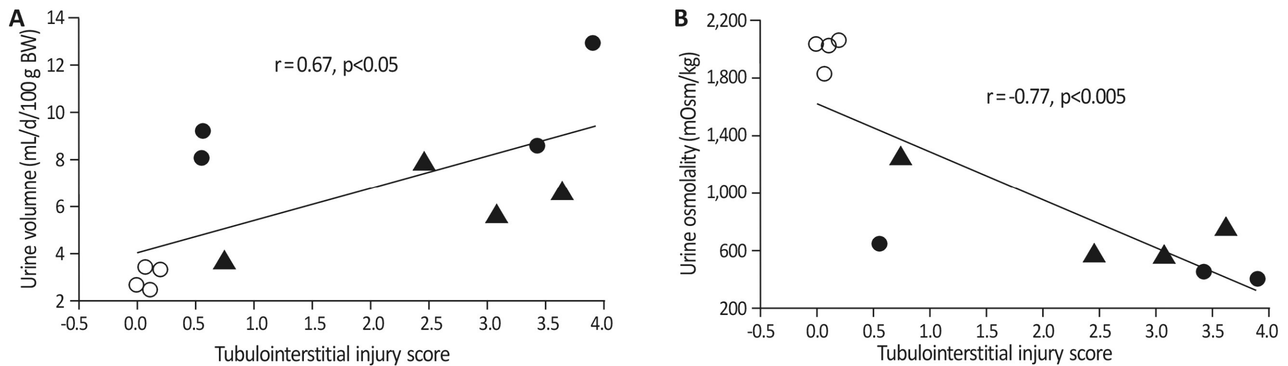

Fig. 7. Relationship between tubulointerstitial injury and urinary concentration. Linear regression analyses reveal a positive correlation between tubulointerstitial injury and daily urine volume (**A**) and an inverse correlation between tubulointerstitial injury and urine osmolality (**B**). Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.

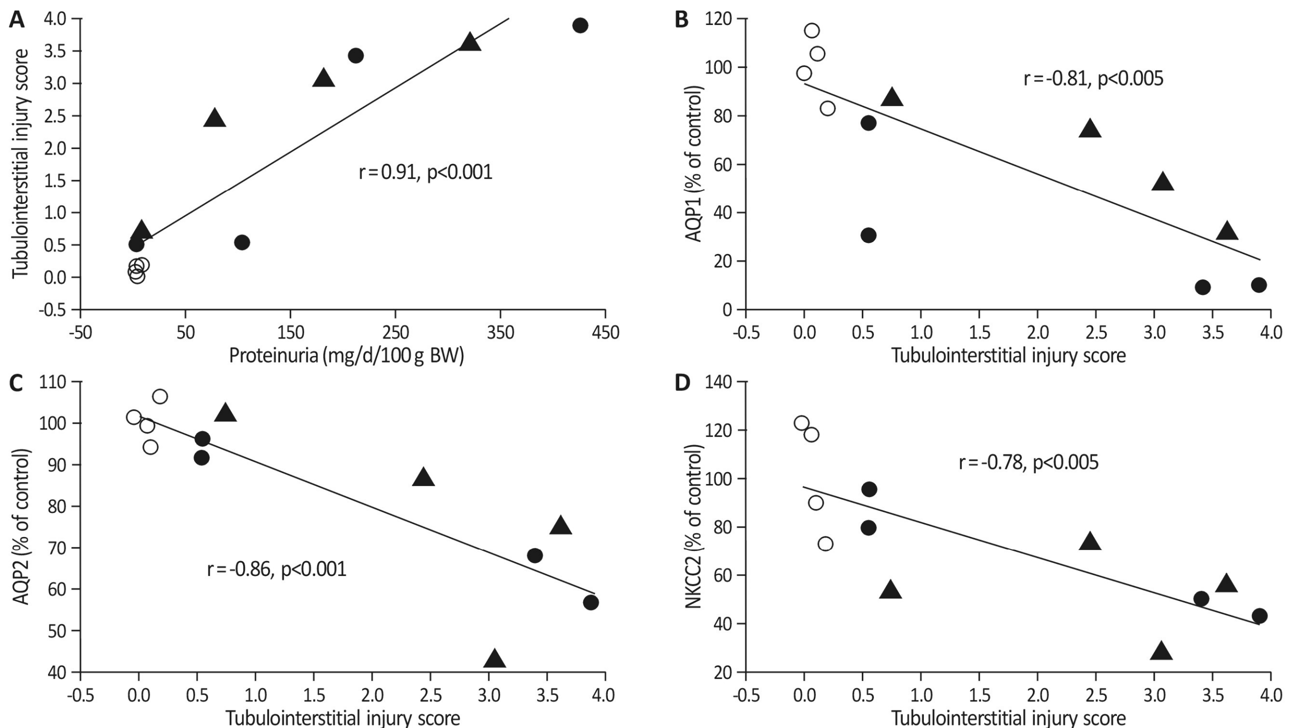

Fig. 8. Association of proteinuria with tubular markers. Proteinuria positively correlates with tubulointerstitial injury (**A**), and the tubulointerstitial injury score inversely correlates with AQP1 (**B**), AQP2 (**C**), and NKCC2 (**D**). Open circle (○), control; filled triangle (▲), PA; closed circle (●), PA+Enalapril.

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Consistent with the urinary concentration defect, renal tubulointerstitial injury was induced by PA administration. Tubular lesions were not limited to the cortex; these lesions extended to the medulla. The tubulointerstitial injury score correlated well with not only urine parameters (urine volume and osmolality), but also transport protein (AQP1, AQP2, and NKCC2) levels.

As expected, renal tubulointerstitial injury was significantly associated with proteinuria. PA administration might have simultaneously caused both glomerular and tubulointerstitial injury, but previous studies suggest that glomerular proteinuria secondarily induces tubulointerstitial injury[8-11]. We demonstrated that glomerular proteinuria is related to tubular defects and water diuresis.

Previous research has suggested that osteopontin is an early marker of tubular injury in experimental nephrotic syndrome[15]. Osteopontin mRNA was increased in glomeruli of rats with PAN[14]. Consistent with these findings, we found a significant association between proteinuria and osteopontin expression in the kidney. One possibility is that altered osteopontin expression in Henle's loop and the collecting duct are involved in urinary concentration defect. Previous studies have shown that renal damage increases osteopontin expression in all renal tubules and glomeruli[18,19].

We found no beneficial effects of enalapril on PAN. Neither proteinuria nor urinary concentration defect was significantly ameliorated by enalapril co-administration in PA-treated rats. On the contrary, enalapril co-administration demonstrated tendencies of increasing urine output and decreasing urine osmolality, although statistical significance was not reached. This result supports the stimulatory effect of angiotensin II on AQP2 in the collecting duct[20].

CONCLUSION

In summary, glomerular proteinuria in PAN was associated with tubulointerstitial injury and urinary concentration defect. We found downregulation of AQP1 and NKCC2, suggestive of impaired countercurrent multiplication. Altered expression of osteopontin, aquaporin-1, aquaporin-2, and NKCC2, leading to water diuresis, might be involved in the tubular defects in PAN.

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Disclosure

The authors declare no conflict of interest.

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