Role of Uroguanylin, a Peptide with Natriuretic Activity, in Rats with Experimental Nephrotic Syndrome

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Uroguanylin induces natriuresis and diuresis in vivo as well as in vitro and is found mainly in the intestine and the kidney. However, the roles of uroguanylin in nephrotic syndrome, which is associated with sodium and water retention, have not been determined. Therefore, changes in the urine and plasma concentration of immunoreactive uroguanylin (ir-uroguanylin) and its mRNA expression in the kidney and intestine were examined using rats with puromycin aminonucleoside (PAN)-induced nephrosis. Male Sprague-Dawley rats were separated into control and nephrotic groups, and then the urinary excretion of sodium, protein, and ir-uroguanylin was examined over time. The plasma levels and renal and intestinal mRNA expression of uroguanylin at the periods of sodium retention and remarkable natriuresis also were evaluated. The sequential changes of urinary ir-uroguanylin excretion in the nephrotic group were similar to those of urinary sodium excretion. When the urinary excretion of ir-uroguanylin and sodium peaked, the plasma level of ir-uroguanylin also increased compared with that of the control group. Uroguanylin mRNA expression in the kidney increased during the period of sodium retention and then decreased during the period of remarkable natriuresis. Uroguanylin mRNA expression in the small intestines of control and nephrotic rats were identical. However, in a unilateral PAN-induced proteinuria, uroguanylin expression significantly increased in the PAN-perfused kidney compared with that in the opposite kidney. Considering the natriuretic effect of uroguanylin, these results suggested that uroguanylin plays an important role as a natriuretic factor in nephrotic syndrome via both the circulation and the kidney itself.


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Uroguanylin is a small peptide that activates transmembrane guanylate cyclase (GC) C, influences cellular function via intracellular cyclic guanosine monophosphate (cGMP) (1), and induces the urinary excretion of sodium and water in isolated perfused rat kidneys (2) and in mice in vivo (3). Uroguanylin and guanylin are major hormones in one family of cGMP agonists, whereas atriopeptins (atrial natriuretic peptide [ANP], brain natriuretic peptide, and C-type natriuretic peptide) are a separate and distinct family of peptides that act on different GC (GC-A, -B) to signal via cGMP. We reported that uroguanylin excretion into the urine is increased in humans and rats on a high-salt diet compared with those on a low-salt diet (4,5), indicating that uroguanylin functions as an intestinal natriuretic peptide. Moreover, uroguanylin levels are increased in the circulation of patients with renal disease and congestive heart failure (6,7). Forte et al. (8) suggested that uroguanylin participates in the physiologic maintenance of the sodium balance. However, how uroguanylin functions in the pathophysiologic mechanism of nephrotic syndrome, a condition associated with sodium and water retention, remains obscure. The present study examined serial changes in the urine and plasma concentration of immunoreactive uroguanylin (ir-uroguanylin) and the renal expression of uroguanylin in rats with nephrosis induced by puromycin aminonucleoside (PAN). We also compared uroguanylin expression between a PAN-perfused kidney and its normal counterpart in the same milieu using a unilateral model of PAN-induced nephrosis.

Materials and Methods

PAN-Induced Nephrosis

Male Sprague-Dawley rats that weighed 160 to 180 g were housed in a temperature- and light-controlled environment with a 12:12-h light/ dark cycle and then placed in individual metabolic cages 1 d before starting the experiments. The animals were randomized into control and nephrotic groups; the latter received a single intraperitoneal injection of PAN (Sigma, St. Louis, MO) at a dose of 150 mg/kg body wt, dissolved in saline (day 1). Control rats received an injection of an equivalent volume of vehicle. Nephrotic rats were allowed free access to standard rat chow (Nihon CLEA, Tokyo, Japan) and water throughout the study, whereas pair-fed control animals had free access to water but were fed with the mean daily intake of the corresponding nephrotic rats. Urinary sodium, protein, and creatinine were measured in 24-h urine samples by indirect potentiometry using an automatic analyzer with ion-selective electrodes, by colorimetric reactions using pyrogallol red, and by the enzymatic assay, respectively. On days 4, 7, and 8, we killed seven animals from each group by cervical dislocation for blood sampling and mRNA studies. We avoided differences in the circadian...
expression of uroguanylin by standardizing the experimental schedule for both groups.

**Unilateral PAN-Induced Proteinuria**

Unilateral proteinuria was induced by selective perfusion of the left kidney using 15 mg of PAN in 1.5 ml of saline as detailed by Hoyer et al. (9). Seven days later, anesthesia was induced by an intraperitoneal injection of 50 mg/kg body wt pentobarbital, and the right external jugular vein was cannulated. The ureters were cannulated with polyethylene no. 10 tubing, and urine from each kidney was collected. Normal saline was infused at a rate of 0.05 ml/min. Urinary sodium, protein, and creatinine were measured as described above, and then the bilateral kidneys were resected for mRNA studies. All experiments described above proceeded according to the regulations established by the Animal Research Committee of Miyazaki Medical College.

**Uroguanylin RIA**

The uroguanylin RIA for plasma and urine proceeded as described by Fukae et al. (5). This RIA specifically recognizes uroguanylin and prouroguanylin, both of which contain reduced and S-carboxymethylated (RCM) forms.

**Characterization of Ir-Uroguanylin in Urine**

Urine was obtained from three control and nephrotic rats. Each urine sample was treated by the same method to gain the RCM peptides and separately applied to Sep-Pak C-18 columns (Waters Associates, Milford, MA), which were preequilibrated with 0.1% trifluoroacetic acid (TFA)-H$_2$O. The columns were washed with 15% acetonitril (CH$_3$CN)-0.1% TFA-H$_2$O, eluted with 60% CH$_3$CN-0.1% TFA-H$_2$O, and lyophilized. The lyophilized samples were reconstructed with H$_2$O and subjected to reverse-phase HPLC (RP-HPLC) on TSK ODS SIL 120 A column (4.6 × 150 mm). A 10 to 60% linear gradient of CH$_3$CN in 0.1% TFA-H$_2$O was run for 60 min at a flow rate of 1.0 ml/min. Corresponding 1-min samples were collected and lyophilized. The samples were reconstructed with RIA buffer and subjected to the RIA. Synthetic rat RCM uroguanylin and prouroguanylin were chromatographed under the same conditions.

**ANP Measurement**

Blood samples taken from seven rats per group on days 4, 7, and 8 were also measured for ANP immunoactivity with a commercially available kit (Peninsula Laboratories, Belmont, CA).

**Real-Time Reverse Transcriptase–PCR**

Total RNA (5 μg) that was extracted using the Total RNA Isolation Reagent (Invitrogen, Carlsbad, CA) was reverse-transcribed using SuperScript reverse transcriptase (Invitrogen) into cDNA. Rat uroguanylin mRNA levels were measured by Real-Time Quantitative PCR (Prism 7700 Sequence Detector; Applied Biosystems, Foster City, CA) as described by the manufacturer, and the following oligonucleotide probes were labeled with 6-carboxylfluorescein as the reporter and 6-carboxytetramethyl-rhodamine as the quencher: ATCCACCCGACGCAGGGACAGTTT (nucleotides 264 to 287) for uroguanylin and TGACGCAGGTCCATGCACAGTCC (nucleotides 548 to 577) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Complementary DNA were amplified using the following pairs of oligonucleotides: TCCCCGTGGGATGTTGCTACAAC (nucleotides 209 to 228, forward primer) and AGCCCGATCCAGGGCAACATTT (nucleotides 343 to 352, reverse primer) for uroguanylin and TCCTGCACCAACCACTGTCTCTC (nucleotides 475 to 496, forward primer) and CACAGCCTTGCCAGCACCAGT (nucleotides 655 to 675, reverse primer) for GAPDH. Levels of uroguanylin mRNA were normalized to those of internal control GAPDH mRNA. All PCR products were verified once by sequencing, and all reactions proceeded in duplicate.

**Statistical Analyses**

Results are expressed as means ± SEM. Differences between two groups were evaluated using $t$ test. Statistical significance was established at $P < 0.05$.

**Results**

**PAN-Induced Nephrosis and Urinary Uroguanylin**

Figure 1A shows that the time course of PAN-induced nephrosis consisted of three periods that were based on variations in urinary sodium excretion. During the first period between 2 and 6 d after the PAN injection, urinary sodium excretion...
significantly decreased. This phenomenon was not related to decreases in food intake, because urinary sodium excretion in pair-fed control rats was significantly higher during the same period. Urinary sodium excretion abruptly increased during the second period from 8 to 9 d after the PAN injection and returned to the same levels as the pair-fed control during the third period on day 10. Proteinuria increased on day 4, which was 2 d after the reduction in urinary sodium excretion, and reached a plateau on day 8 (Figure 1C). Urinary ir-uroguanylin excretion started to increase in the nephrotic group on day 4, rapidly increased on day 7, peaked on day 8, and then gradually decreased (Figure 1B). Changes in body weights of control or nephrotic rats were as follows: control (n = 6) versus nephrotic (n = 6) rats, day 4, 214.3 ± 1.2 versus 219.3 ± 2.1 g (P = 0.053); day 7, 220.7 ± 0.7 versus 246.8 ± 6.2 g (P = 0.02).

Characterization of Ir-Uroguanylin in Urine
Uroguanylin molecules in control and nephrotic urine were analyzed by RP-HPLC coupled with RIA. Representative RP-HPLC profiles of molecules in the urine are shown in Figure 2. In nephrotic urine, two major immunoreactive peaks were found, the main one at elution position of rat RCM uroguanylin and the minor rest at that of prouroguanylin.

Uroguanylin mRNA Expression of Kidney and Intestine
We also evaluated uroguanylin expression in the kidney and intestine on days 4 and 7 or 8 as representative periods of sodium retention and remarkable natriuresis, respectively. Uroguanylin mRNA levels increased in the kidneys of the nephrotic group compared with the control group on day 4 (179.7 ± 41.8 versus 100 ± 12.4%; n = 7; P < 0.05) but decreased on days 7 and 8 (35.4 ± 7.2 versus 100 ± 23.2%; n = 7; P < 0.01; Figure 3A). Intestinal levels did not significantly differ between the groups (Figure 3B).

Plasma Ir-Uroguanylin and ANP
Plasma levels of ir-uroguanylin in the nephrotic group were significantly higher than those in the control group on days 4 and 7, respectively.
and 7 or 8 (1887.4 \pm 122.2 \text{ versus } 936.5 \pm 63.9 \text{ fmol/ml } [n = 7; P < 0.0001]; 3441.9 \pm 594.4 \text{ versus } 1084.3 \pm 43.8 \text{ fmol/ml } [n = 7; P = 0.0019], respectively; Figure 4A). Plasma levels of ANP in the nephrotic group did not differ from the control group on day 4 (124.7 \pm 18.0 \text{ versus } 136.9 \pm 28.4 \text{ pg/ml } [n = 7; P = 0.36] but significantly increased on days 7 and 8 (240.0 \pm 31.1 \text{ versus } 102.7 \pm 41.9 \text{ pg/ml } [n = 7; P = 0.01; Figure 4B).

Unilateral PAN-Induced Proteinuria

We studied an intrarenal mechanism of uroguanylin to be distinguished from the effects of alterations in the systemic or circulating factors (ANP and plasma uroguanylin) by using the model of unilateral PAN-induced proteinuria. Uroguanylin mRNA expression increased in the unilaterally PAN-perfused kidney compared with the nonperfused counterpart (164.6 \pm 21.1 \text{ versus } 100 \pm 14.7\%; [n = 5; P < 0.05; Figure 5A). The excretion of ir-uroguanylin increased in the PAN-perfused kidney compared with the opposite kidney (18.05 \pm 2.43 \text{ versus } 5.68 \pm 0.83 \text{ nmol/mmol } [n = 5; P = 0.0013; Figure 5B).

**Discussion**

Various peptides and chemical mediators involved in nephrotic syndrome have generally been evaluated using the model of PAN-induced nephrosis. In addition to proteinuria, urinary sodium excretion in PAN-induced nephrotic rats significantly differs from that of controls (10). The time course of PAN-induced nephrosis consists of sodium retention and remarkable natriuresis followed by natriuresis that is comparable to controls. Uroguanylin has a natriuretic activity, the mRNA for which is expressed in many tissues of the body as well as the kidney and intestinal tract (11). The present study established how uroguanylin levels vary and how uroguanylin mRNA is expressed in experimental nephrosis.

The pathogenesis of sodium retention in nephrotic syndrome remains somewhat obscure. Ichikawa et al. (12) reported the importance of intrarenal mechanisms in sodium retention in the unilateral PAN-induced nephrosis model. Investigators have proposed that a reduced GFR and glomerular coefficient, decreased tubule sensitivity to ANP through cGMP phosphodiesterase activation, and increased tubular reabsorption through Na\(^+\)/K\(^+\) ATPase activation are associated with sodium retention (10,13–17). The present study found that uroguanylin mRNA levels in the nephrotic kidney increased

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**Figure 4.** Changes in plasma ir-uroguanylin and atrial natriuretic peptide in control and nephrotic groups on day 4 and on days 7 and 8. Columns, mean ± SEM from five animals. *P < 0.05, t test.

**Figure 5.** Renal uroguanylin mRNA expression and urinary excretion of ir-uroguanylin in unilateral PAN nephrosis. (A) Renal uroguanylin mRNA expression determined by quantitative RT-PCR in PAN-perfused and nonperfused kidneys. (B) Urinary excretion of protein, sodium, and ir-uroguanylin in PAN-perfused and nonperfused kidneys. Values are means ± SEM from five animals. *P < 0.05, t test.
during the period of sodium retention but decreased during the natriuretic phase. Thus, the values for urinary sodium excretion were inversely correlated with uroguanylin mRNA expression levels in the kidney. Furthermore, we identified the same relationship in unilateral PAN nephrosis, in which model fractional sodium excretion was lower in the proteinuric than in the intact kidney (12). The effects of nephrosis on the kidney can be evaluated directly in this model, as the influence of systemic hemodynamics and the humoral environment can be excluded. Therefore, our findings indicate that uroguanylin functions as an intrarenal response to sodium retention.

Remarkable natriuresis in PAN-induced nephrosis has not been addressed in depth. We found that the urinary ir-uroguanylin excretion of nephrotic rats reached a maximum (approximately more than eightfold control levels) on day 8, when sodium excretion was significantly higher than that of controls. Plasma levels of ir-uroguanylin in the nephrotic group also increased on days 7 and 8, in contrast to decreased uroguanylin expression in the kidney. Therefore, the increased urinary ir-uroguanylin excretion after day 7 or 8 seems to be derived mainly from the increased plasma uroguanylin. Several laboratories, including ours, have shown that 10-kD prouroguanylin is the main molecular form of ir-uroguanylin in plasma (4,18), and it is a molecule smaller than albumin (66 kD) (19), the main protein in the urine of the PAN-induced nephrotic rats (20). Filtered loads of low-molecular-weight proteins, in general, do not increase despite an increase in the permeability of the glomerular barrier, because the glomerular sieving coefficient values for small proteins are high even under normal conditions (21). Decreased reabsorption in the tubules therefore may be the reason for the higher uroguanylin excretion in the nephrotic group. Indeed, the result of characterization of ir-uroguanylin in urine of the nephrotic group showed two immunoreactive peaks indicating its bioactive form and precursor. However, the former was more detected and the total amount of uroguanylin bioactivity was increased as shown in Figure 2. Because uroguanylin has another cGMP-independent pathway unlike ANP (22) and downregulates the message for the Na+/K+ ATPase γ-subunit in mice (23), uroguanylin may be a candidate of natriuretic factor for nephrotic animals. However, ANP was previously suspected to be a causative factor for sodium retention in nephrotic syndrome. At present, the nephrotic state is considered as a condition of ANP resistance. The models with experimental nephrosis are characterized by no change or an increase in the plasma concentration of ANP, with reference to the volume status. Each plasma ANP level at the time as urinary sodium excretion significantly increased compared with controls. Movement of urinary sodium excretion inversely correlated with uroguanylin mRNA expression levels in the kidney, suggesting that urinary sodium excretion regulates uroguanylin mRNA expression in the kidney. In conclusion, uroguanylin might participate in directing renal sodium transport in the nephrotic kidney as an endocrine and as a paracrine/autocrine factor.

In summary, we studied levels of uroguanylin in PAN-induced nephrosis, which is associated with sodium and water retention. Urinary ir-uroguanylin excretion and plasma levels of ir-uroguanylin in the nephrotic group peaked at the same time as urinary sodium excretion significantly increased compared with controls. Movement of urinary sodium excretion inversely correlated with uroguanylin mRNA expression levels in the kidney, suggesting that urinary sodium excretion regulates uroguanylin mRNA expression in the kidney. In conclusion, uroguanylin might participate in directing renal sodium transport in the nephrotic kidney as an endocrine and as a paracrine/autocrine factor.

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