Changes of Atrial Natriuretic Peptide System in Rats with Puromycin Aminonucleoside-Induced Nephrotic Syndrome

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Sodium retention is a hallmark of nephrotic syndrome. We investigated whether sodium retention is associated with changes of natriuretic peptide system at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndrome. At day 7 after PAN (puromycin aminonucleoside) injection, the urinary excretion of sodium was decreased, along with the development of ascites and positive sodium balance. The plasma and urinary ANP (atrial natriuretic peptide) immunoreactivities were increased. ANP mRNA expression was increased in the heart and kidney, whereas that of NPR (natriuretic peptide receptor)-A and NPR-C mRNA was decreased in the kidney. The expression of NEP was decreased in the kidney. At day 14, urinary excretion of sodium did not differ from the control. The plasma ANP level and heart ANP mRNA expression returned to their control values. The expression of ANP mRNA in the kidney was increased in association with increased urinary ANP immunoreactivities. The expression of NPR-A in the kidney became normal, whereas that of NPR-C kept decreased. The expression of NEP (neutral endopeptidase) remained decreased. These findings suggest that the increased renal ANP synthesis in association with decreased metabolism via NEP and NPR-C may play a compensatory role against the development of sodium retention in nephrotic syndrome. The decreased of NPR-A expression in the kidney may contribute to the ANP resistance at day 7. The subsequent recovery of NPR-A expression may play a role in promoting sodium excretion in later stage (at day 14).

Key Words: Nephrotic syndrome, Natriuretic peptide, Puromycin aminonucleoside

INTRODUCTION

Nephrotic syndrome is a common manifestation of renal disease and is associated with avid sodium retention, leading to the development of edema and ascites (Vande Walle et al, 2001). However, the cause of this syndrome is still incompletely understood. We have demonstrated that puromycin aminonucleoside (PAN)-induced nephrotic syndrome is associated with upregulation of protein abundance of specific ENaC subunits, and increased apical targeting of ENaC subunits in connecting tubule and collecting duct segments (Kim et al, 2004). These observations suggest that the sodium retention is at least in part caused by increased tubular sodium reabsorption in PAN-induced nephrotic syndrome.

On the other hand, positive sodium balance may not prevail in the long run in sodium retentive diseases, suggesting that compensatory mechanisms work against the sodium retention at the later stage. The urinary sodium excretion may change according to the different stages of nephrotic syndrome (Deschenes et al, 2000) and liver cirrhosis (Kim et al, 2005; Kim et al, 2006). The time course of PAN-induced nephrosis consists of urinary sodium retention, followed by natriuresis that is comparable to control (Deschenes et al, 2000). PAN-treated rats display a positive sodium balance during initial sodium retaining period of decreased sodium excretion. During maintenance or compensatory period, urinary sodium excretion increases progressively, so that sodium balance becomes negative. These findings may suggest that there are dynamic changes in sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndrome. The underlying mechanism for the temporal changes in renal tubular sodium reabsorption and sodium retention remains undefined. However, it can be speculated that the rise in extracellular fluid volume at later stages of nephrotic syndrome exceeds the threshold level and triggers enhanced activity of atrial natriuretic peptide (ANP) and/or recovery of tubular hypo-responsiveness, thereby, may contributing to the natriuresis against further sodium retention.

Higher ANP levels occur in the setting of sodium retention, indicating that ascitic patients with nephrotic syndrome exhibit reduced natriuretic response to this peptide. Indeed, a marked renal resistance to natriuretic action of ANP has been demonstrated in humans and experimental

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ABBREVIATIONS: PAN, puromycin aminonucleoside; ANP, atrial natriuretic peptide; NPR, natriuretic peptide receptor; NEP, neutral endopeptidase; OSOM, outer stripe of outer medulla; ENaC, epithelial sodium channel.
animals (Peterson et al., 1988; Perico et al., 1989). This phenomenon has been attributed to accelerated degradation of second messenger, cGMP (Valentin et al., 1992). However, systemic changes of ANP clearance and natriuretic peptide receptor as a possible cause of renal resistance at two different stages have never been investigated in nephrotic syndrome. Given the pivotal role of renal ANP system played in regulation of renal sodium handling as well as renal and systemic hemodynamics, it is conceivable that the changes of renal ANP system may contribute to the pathogenesis of sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndrome.

It should be emphasized that the two time periods are two in a continuum of changes during the initial development of sodium retention and the later stages with partial or full compensation. The underlying mechanism for the temporal changes may causally associated with the dynamic changes of ANP system in the kidney. The purpose of this study was therefore to investigate whether PAN induced nephrotic syndrome in rats is associated with altered regulation of ANP/natriuretic peptide receptor in the kidney and to elucidate the underlying molecular mechanisms responsible for the increased sodium retention.

METHODS

Animals

The animal study was approved by the Ethics Committee of Chonnam National University Medical School. Animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Sprague-Dawley male rats, weighing 160 to 180 g, were used. PAN nephritic syndrome (n=8) was induced by a single intravenous injection of PAN (180 mg/kg body weight; Sigma, St. Louis, MO) via the femoral vein. Control rats (n=8) received the vehicle alone (i.e., sterile 0.9% saline). They were maintained on a standard rodent diet and allowed free access to drinking water and food that the PAN rats consumed during previous day. The animals (Peterson et al., 1988; Perico et al., 1989). This phenomenon has been attributed to accelerated degradation of second messenger, cGMP (Valentin et al., 1992). However, systemic changes of ANP clearance and natriuretic peptide receptor as a possible cause of renal resistance at two different stages have never been investigated in nephrotic syndrome. Given the pivotal role of renal ANP system played in regulation of renal sodium handling as well as renal and systemic hemodynamics, it is conceivable that the changes of renal ANP system may contribute to the pathogenesis of sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndrome.

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Real-time PCR

cDNA was made by reverse transcribing 5 μg of total RNA using oligo (dT) priming and superscript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. cDNA was quantified using Smart Cycler II System (Cepheid, Sunnyvale, CA), and SYBR Green was used for detection. Each PCR reaction contained the following final concentrations: 10 μM forward primer, 10 μM reverse primer, 2X SYBR Green Premix Ex Taq (TAKARA BIO INC, Seta 3-4-1, Japan), 0.5 μl of cDNA and H2O to bring the final volume to 20 μl. Relative levels of mRNA were examined by real-time PCR, using a Rotor-Gen2 Smart Cycler System (Corbett research, Mortlake, New South Wales, Australia) according to the manufacturer’s directions. Table 1 shows the nucleotide sequence of primers. The real-time reverse transcription-PCR was performed according to the following steps: 1) 95°C for 5 min; 2) 95°C for 20 s; 3) 58 to 60°C for 20 s (optimized for each primer pair); 4) 72°C for 30 s; and 5) 85°C for 6 s to detect SYBR Green (nonspecific products melt at <85°C, therefore, are not detected). Steps 2 ~ 5 were repeated for an additional 64 cycles, while temperature was increased from 60 to 95°C at the end of the last cycle to produce a melt curve. The ratio of each gene and GAPDH level (relative gene expression number) was calculated by subtracting the threshold cycle number of the target gene from that of GAPDH and raising 2 to the power of this difference (Livak & Schmittgen, 2001).

Tissue preparation and radioimmunooassy of ANP

For the determination of immunoreactive-ANP, the plasma and urine were extracted with Sep-Pak C18 cartridges (Waters; Milford, MA, USA). The adsorbed peptide was eluted with 60% acetonitril in 0.1% trifluoroacetic acid. The

Isolation of total RNA

Heart and kidney was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in distilled water. The RNA concentration was determined by the absorbance at 260 nm (Ultraspec 2000; Pharmacia Biotech, Cambridge, UK).

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Table 1. Primers used in the polymerase chain reaction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>GAPDH sense</td>
<td>ATCAAAATGGGTGATCTGGTGGCTGTG</td>
</tr>
<tr>
<td></td>
<td>antisense:GAGGTTTCTCCAGGCGCCATGTCAG</td>
</tr>
<tr>
<td>ANP sense</td>
<td>CATGGGCTCTCTTCTCATCA</td>
</tr>
<tr>
<td></td>
<td>antisense:GAGGCGCATGATCCTA</td>
</tr>
<tr>
<td>NPR-A sense</td>
<td>GGTGATGATGACCCCTCATCT</td>
</tr>
<tr>
<td></td>
<td>antisense:TTCCTGGAACCTCCTAC</td>
</tr>
<tr>
<td>NPR-C sense</td>
<td>CGAGGGCTTGGTGGCTATTGC</td>
</tr>
<tr>
<td></td>
<td>antisense:CGAGGACCGCGGGCGGGGGGGGGGGGGGGGGGGG</td>
</tr>
</tbody>
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GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANP, atrial natriuretic peptide; NPR-A, natriuretic peptide receptor-A; NPR-C, natriuretic peptide receptor-C.
eluates were dried under vacuum using a Speed-Vac evaporator (Savant; Farmingdale, NY, USA). The lyophilized samples were reconstituted with phosphate buffer (pH 7.4) containing 50 mM NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100, and 0.01% sodium azide. After incubation with anti-ANP antibody (Phoenix; Belmont, CA, USA) for 24 h at 4°C, 125I-ANP (specific activity, 1,420 Ci/mmol) was added to each reaction mixture and incubated for another 24 h at 4°C. The separation of the free form from the bound form was achieved by the addition of secondary antibody, and the radioactive pellet was counted in a gamma counter (Packard; Meriden, CT, USA). Nonspecific binding was <1.0%. The 50% intercept was at 202.8±15.2 pg/tube (n=8). The intra- and interassay coefficients of variation were 3.6±0.6 (n=8) and 8.6±2.6% (n=8), respectively.

**Semiquantitative immunoblotting**

The dissected Cortex/OSOM was homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, with pH 7.2. The homogenates were centrifuged at 1,000 g for 15 min at 4°C to remove unbroken whole cells, nuclei and mitochondria. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Bio-Rad Mini Protein II, Bio-Rad, Hercules, CA, USA) onto nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBST (80 mM Na 2HPO4, 20 mM NaH2PO4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with primary antibodies, followed by incubation with secondary anti-rabbit (P448, Dako, Glostrup, Denmark) horseradish peroxidase-conjugated antibodies. The labeling was visualized by an enhanced chemiluminescence system. Affinity-purified rabbit polyclonal antibodies to neutral endopeptidase (NEP; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were commercially obtained.

**Immunohistochemistry**

For perfusion fixation, a perfusion needle was inserted into the abdominal aorta, and the vena cava was cut to establish an outlet. Blood was flushed from the kidney with cold phosphated buffered saline (PBS, pH 7.4) for 15 sec, before switching to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed and sectioned into 2–3 mm transverse sections and immersion fixed for additionally 1 h, followed by 3×10 min washes with 0.1 M cacodylate buffer of pH 7.4. The tissue was dehydrated in graded ethanol and left overnight in xylene. After embedding in paraffin, 2 μm sections were made on a rotary microtome (Leica Microsystems A/S; Herlev, Denmark).

Immunolabeling was performed on sections from paraffin-embedded preparations (2 μm thickness) using methods described previously (Kim et al, 2005; Kim et al, 2006). Affinity-purified rabbit polyclonal antibodies were used against the neutral endopeptidase (NEP; Santa Cruz; CA, USA). The microscopy was carried out using light microscope (Olympus, Tokyo, Japan).

**Statistical analysis**

Results are expressed as mean±SEM. The statistical significance of differences between two groups was determined by using the unpaired student t-test. p values <0.05 were considered significant.

### RESULTS

**Renal function**

Table 2 shows renal functional parameters. At day 7, PAN-treated rats developed ascites, as indicated by visible pools of fluid in the lateral abdominal gutters. Plasma creatinine level was increased, and its clearance was decreased. Total and fractional excretion of sodium decreased, along with a positive sodium balance. At day 14, PAN-treated rats still developed significant ascites. Plasma creatinine clearance was increased, and its plasma level was decreased. Total and fractional excretion of sodium was normal with sodium balance unchanged.

#### Tissue and urinary levels of ANP

Fig. 1 shows plasma and urine ANP levels. At day 7, the plasma and urinary levels of ANP were significantly increased. At day 14, urinary ANP excretion was increased in PAN rats compared with controls, while the plasma ANP level was comparable between two groups.

<table>
<thead>
<tr>
<th>Table 2. Changes in renal function</th>
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<tr>
<td><strong>Day 7</strong></td>
</tr>
<tr>
<td><strong>Control</strong> (n=8)</td>
</tr>
<tr>
<td>Body weight (g)</td>
</tr>
<tr>
<td>Pcr (mg/dL)</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
</tr>
<tr>
<td>UoV (mEq/day)</td>
</tr>
<tr>
<td>FENa (%)</td>
</tr>
<tr>
<td>Na balance (mmol/day)</td>
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</table>

Values are expressed as mean±SEM. These values were obtainedat the last day of experiments. P-Cr, plasma creatinine; Ccr, creatinine clearance; UoV, rate of urinary sodium excretion; FENa, fractional excretion of sodium into urine; Na balance, the difference between dietary sodium intake and urinary sodium excretion. *p<0.05, when compared with corresponding control group.
**ANP mRNA expression in the heart and kidney**

Fig. 2 shows the expression of ANP in the heart and kidney. At day 7, the mRNA expression of cardiac ANP was significantly increased in the PAN-induced nephrotic syndrome. At day 14, the mRNA expression of ANP was not changed. At day 7 and 14, the mRNA expression of renal ANP was significantly increased in the rats with PAN-induced nephrotic syndrome compared with controls.

**mRNA expression of natriuretic peptide receptor in the kidney**

Fig. 3 shows the expression of NPR-A and NPR-C in the...
Fig. 3. Expression of NPR-A and NPR-C mRNA in the kidney. Columns show real-time PCR data representing control and PAN-treated rats. At day 7, the mRNA expression of NPR-A and NPR-C expression was decreased. At day 14, the mRNA expression of NPR-C was decreased compared with control, while the NPR-A expression was not changed (mean±SEM, n=8 each). *p<0.05 vs. control.

Fig. 4. (A) Immunoblotting of NEP was decreased in PAN-treated rats compared with control. (B) Immunoperoxidase microscopy of NEP labeling was associated with apical plasma membrane of the proximal tubule in control rats. Immunoperoxidase microscopy demonstrated decreased NEP immunolabeling in the proximal tubule in PAN-treated rats (mean±SEM, n=8 each). *p<0.05 vs. control. Magnification: ×200.

Expression of NEP

Fig. 4 shows the expression of NEP in the kidney. The protein expression of NEP in the kidney was markedly de-

kidney. At day 7, the mRNA expression of NPR-A and NPR-C expression was decreased. At day 14, the mRNA expression of NPR-C was decreased compared with control, while the NPR-A expression was not changed.
increased in PAN rats (day 7 and day 14) compared with controls. Immunohistochemistry revealed marked down-regulation of NEP in the proximal tubule of PAN-induced nephrotic syndrome.

**DISCUSSION**

In the present study, PAN-treated rats showed decreased fractional excretion of sodium and positive sodium balance at day 7. The sodium retention and ascites formation may causally be related with an increased tubular reabsorption of sodium. However, there are dynamic changes in sodium retention at different stages (i.e., a sodium retaining stage and a compensatory stage) of nephrotic syndrome. At day 14, there was no evidence of positive sodium balance despite the marked ascites.

The PAN-induced nephrosis is also characterized by increases or no changes in the plasma concentration of ANP, corresponding to the volume status according to the different stages of nephrotic syndrome. An excessive retention of sodium and water may occur in the sodium retaining stages, in which an expansion of extracellular fluid volume would account for the increase of plasma ANP. The increase of plasma ANP levels was accompanied by increases of cardiac tissue ANP mRNA in the sodium retaining stage. On the contrary, the plasma ANP levels and its expression in the heart were normalized in the compensatory stage.

On the other hand, Singer et al. (1987) showed that a natriuresis does not correlate well with plasma ANP levels after acute intravenous saline infusion. A substantial increase in circulating ANP for several days by chronic intravenous ANP infusion produces no changes or trivial increases in urinary sodium excretion (Drewett & Garbers, 1994). An atrial-distended, cardiac-denervated dog did not show a natriuresis (Goetz et al., 1986). These observations suggest that renal natriuretic peptide system, rather than circulating ANP, plays a role in renal handling of sodium homeostasis. In the present study, ANP mRNA expression was found to be increased in the kidney from PAN-induced nephrotic syndrome. Accordingly, urinary immunoreactivity of ANP was increased as well, which may play a compensatory role against the development of sodium retention.

Binding of ANP to NPR-C results in internalization of the receptor-ligand complex followed by hydrolytic degradation of ANP. Additionally, the widely distributed ectoenzyme NEP cleaves the ANP Cys7-Phe8 bond close to the disulphide bond, breaking the ring structure and making ANP biologically inactive (Wilkins et al., 1997). In the present study, mRNA expression of NPR-C was decreased, and the protein expression of NEP was markedly decreased, mainly in the proximal convoluted tubules of PAN rats. Consequently, the urinary excretion of ANP was significantly higher at both stages than in control. Reduction of the clearance of ANP results in an augmentation of its tissue levels. Consequently, not only increased synthesis of ANP but also downregulation of NEP and NPR-C plays a compensatory role against the development of sodium retention.

There is an evidence to indicated that the distal nephron has decreased sensitivity to ANP in nephrotic syndrome. In humans, studies using various forms of volume expansion showed insufficient natriuretic response despite the elevation of plasma ANP (Peterson et al., 1988; Wolff et al., 1989). ANP resistance can be demonstrated specifically in the affected kidney of experimental unilateral nephrosis (Perico et al., 1989), and isolated kidneys of nephrotic rats (Perico et al., 1993). It could result from diminished ANP binding to its receptors, impaired cell signaling after binding, or enhanced phosphodiesterase activity. Among the subtypes of NPR thus far known, NPR-A which mediates the biological effects of ANP through generation of cGMP (Parkes et al., 1988) has been shown to be down-regulated in the kidney at the sodium retaining stage. The effect of local ANP may be dissipated when the expression of NPR-A is decreased.

It is interesting to note that the rats with nephrotic syndrome apparently exhibited dynamic changes in sodium retention as well as NPR-A expression. At day 7, nephrotic syndrome animals showed sodium retention and decreased excretion of sodium, despite the increased synthesis of ANP in the kidney and urine ANP immunoreactivity. These find- ings can be explained in part by decreased NPR-A expression. On the other hand, at day 14, the animals did not show any changes in urinary sodium excretion despite the marked ascites. The recovery of NPR-A expression in the kidney permits the natriuresis along with increased local ANP immunoreactivity. The increased renal synthesis of ANP in association with decreased metabolism of ANP via NEP and NPR-C may play a compensatory role against the sodium retention, promoting sodium excretion at later stage of nephrotic syndrome.

**ACKNOWLEDGEMENTS**

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-00E0306).

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