Resil a cyclophilin-like protein gene expression parallels changes in sodium excretion in experimental nephrosis and is positively modulated by atrial natriuretic peptide

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Nephrotic syndrome is associated with an expanded interstitial volume and edema due to water and sodium retention (1-3). So far, the mechanisms underlying these abnormalities have only partially been clarified. The retention of water and sodium may depend on hypoalbuminemia, secondary to urinary losses of proteins (4), leading to the accumulation of extracellular fluid in the interstitial compartment. The ensuing reduction in plasma volume would result in neurohormonal adaptations, including renin-angiotensin system activation, that are signals for the kidney to retain water and sodium (5,6). In some patients, however, plasma volume is normal or even increased (7-9) with no evidence of renin-angiotensin system activation (10,11). In such cases, the cause of sodium retention might be independent of systemic events and possibly resides in the kidney (12). Experimental evidence is now available to suggest that, indeed, intrarenal abnormalities may favor the formation of edema in nephrotic syndrome (13-15). More recently, studies have documented that the kidney in nephrotic syndrome does not respond to atrial natriuretic peptide (ANP). Actually, experimental animals (16-18) and humans (19) with nephrotic syndrome had markedly blunted diuretic and natriuretic responses to the systemic infusion of ANP despite increased endogenous plasma levels of the hormone. Several possible explanations for this phenomenon have been attempted and include decreased density and/or affinity of renal sympathetic nerve activity. However, none of these explanations is fully convincing because comparable renal ANP receptor density and affinity have been reported in nephrotic and control rats (20), the in vitro cGMP production was of similar magnitude in suspensions of medullary ducts from the two groups of animals (21), and renal denervation only partially reversed the blunted diuresis and natriuresis to ANP in nephrotic rats (17).

Recently, it has been documented that the gene expression of a cyclophilin-like protein (Cy-LP) homolog of the cytosolic binding protein for cyclosporin A is increased in sodium-retaining conditions (22). Therefore, this study was designed with the following
aims: (1) to establish whether the expression of the gene encoding for Cy-LP is enhanced in kidneys from rats with nephrotic syndrome and sodium retention; (2) to evaluate whether, in these animals, changes in Cy-LP gene expression parallel changes in urinary sodium excretion; (3) to investigate whether ANP infusion modulates the Cy-LP gene in nephrotic rats.

**METHODS**

Male Sprague-Dawley rats (Charles River Italia, Calco, Italy) weighing 250 to 275 g at the start of the study were used. The animals were housed in a constant-temperature room with a light-dark cycle (light:dark, 12:12), fed standard rat diet (Altromin-Rieper, Vandoes, Italy), and were given ad libitum access to deionized water.

Experimental nephrosis was induced by a single iv injection of adriamycin (ADR; 5 mg/kg; Adriblastina, Farmitalia Carlo Erba, Milan, Italy) through the tail vein of nonanesthetized rats, by the method previously described (23).

**Experimental Design**

To investigate whether the expression of the Cy-LP gene is modified in kidneys from rats with experimental nephrosis, two groups of animals given ADR (N = 12) or vehicle (N = 12) were studied. Twenty-four-hour urine samples were collected by the use of metabolic cages before and 21 days after ADR or vehicle injection, and urine flow rate, urinary Na, and protein excretion were measured. At the end of the observation period, the animals were euthanized by decapitation, the kidneys were removed, and the cortex and medulla homogenate were analyzed for the gene expression of Cy-LP. In addition, because it has been previously reported that Cy-LP mRNA is more abundantly expressed in liver and lung than in other organs (22), for comparison the lung expression of the same gene was also evaluated.

To further evaluate the relationship between Cy-LP gene expression and Na retention in nephrotic syndrome, a time course of the two parameters was performed in ADR-treated rats. Nephrotic animals were studied at days 7 (N = 4), 14 (N = 4), 21 (N = 4), and 28 (N = 4) after ADR injection. At these times, 24-h urinary protein excretion, Na intake, and urinary Na excretion were determined. Animals were then euthanized by decapitation, kidneys were removed, and medulla homogenate was processed for Cy-LP gene expression. An additional group of control rats (N = 4) was also considered for comparison.

To examine whether ANP modulated Cy-LP mRNA in the renal medulla, nephrotic or control rats underwent ANP or vehicle infusion 21 days after ADR or vehicle injection. After 24-h urine collection in metabolic cages for the determination of urinary protein and Na excretion, animals were anesthetized with thiopental sodium (50 mg/kg body wt ip) and were placed on a temperature-regulated table. Surgical preparation consisted of tracheostomy, catheterization of left femoral artery and vein, and placement of a large-bore polyethylene tube into the bladder through a small suprapubic incision for urine collection into preweighed polyethylene tube (16). Arterial blood pressure was continuously monitored throughout the experiment directly from the femoral artery by a polyethylene catheter (PE-50; Clay Adams, Parsippany, NJ) connected to a Statham pressure transducer and a carrier amplifier (Battaglia Rangoni, Bologna, Italy). Two milliliters of saline solution was infused into the left femoral vein as a priming load, followed by a constant infusion via a syringe pump at 2 mL/h. After a 30-min equilibration period, two 10-min baseline urine collection periods were performed. On completion, synthetic rat ANP (ANP 1–28; Bachem Feinchemikalien, Bubendorf, Switzerland) dissolved in phosphate-buffered saline (0.01 M; pH 7.4) was infused at the rate of 1 µg/kg·min during a 60-min experimental period (20). Urine samples were collected every 10 min. The same protocol was repeated in additional control (N = 4) and ADR-treated (N = 4) rats, except that vehicle instead of ANP was infused over a 60-min period. At the end of the experiment, animals were euthanized, the kidneys were removed, and the medulla homogenate was processed for the gene expression of Cy-LP.

**Oligonucleotide Preparation for Cy-LP**

Two oligonucleotides were synthesized with a 380 B automatic DNA synthesizer (Applied Biosystems) on the basis of the previously published sequence of rat Cy-LP cDNA (22), i.e., the sense orientation primer (oligo A: 5′-GACATCGAATTCACGTGGTTTTCGCAAAGT-3′) beginning at position +489 of the coding strand and the antisense orientation primer (oligo B: 5′-GACATCTTCGAGCTTCTACGTGGAATCAGTGC-3′) complementary to the rat Cy-LP cDNA sequence starting from the translational stop codon. Restriction sites EcoRI and PstI were inserted in oligo A and oligo B, respectively.

**PCR Cloning of Cy-LP cDNA**

Ten micrograms of total rat kidney RNA was reverse transcribed into cDNA with oligo B as primer. Briefly, a 40-ml reverse transcriptase reaction mixture containing 10 µg total rat kidney RNA, 1× avain molani virus (AMV) buffer (50 mM Tris HCl, pH 8.3; 60 mM KCl; 10 mM MgCl2; 1 mM dithiothreitol; 50 mg/mL actinomycin D), 10 U of RNasin (Promega), 0.5 mM dioxynucleoside triphosphates, 1 µg of oligo B, and 12.5 U of AMV reverse transcriptase (Boehringer Mannheim GmbH, Mannheim, Germany) was incubated, after annealing, at 42°C for 2 h. The reverse transcription mixture was then extracted...
with phenol/chloroform and by ethanol precipitation. A polymerase chain reaction (PCR) was performed at a final concentration of 1× PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2 mM MgCl₂), 50 μM dNTP, 0.1 μM sense and antisense oligos (oligo A and oligo B), and 2.5 U of Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT) in a total volume of 100 μL. The mixture was overlaid with mineral oil and then amplified with a Perkin-Elmer/Cetus thermal cycler. The PCR amplification profile involved denaturation at 94°C for 45 s, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min. The cycle was repeated 30 times. To confirm the authenticity of DNA fragment isolated, the PCR product was digested with EcoRI and Pst1 and inserted into a M13 mp18 vector. The authenticity of the cDNA fragment obtained was verified by DNA sequencing.

RNA Preparation and Analysis

Total cellular RNA was isolated from tissue homogenates by the guanidium isothiocyanate/cesium chloride procedure (24). For Northern blot analysis, 20 μg of RNA was loaded for each lane. RNA was electrophoresed in 1.2% agarose gel with 6% formaldehyde according to standard procedures (25) and was transferred to GeneScreen Plus (Dupont, NEN Research Products, Boston, MA) in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate; pH 7.0). The EcoRI-Pst1 cDNA fragment of rat Cy-LP was labeled with [α-³²P] deoxyctydine triphosphate by a random-primed method (26). Membranes were hybridized for 20 h at 60°C with a 10⁶ cpm/labeled probe in a solution containing 1 M NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, and 100 μg/mL of salmon sperm DNA. Filters were washed at 60°C in 1× SSC containing 1% sodium dodecyl sulfate for 1 h and were then exposed to x-ray film for autoradiography. Membranes were subsequently rehybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (27), taken as the internal standard of the equal loading of the samples on the membrane. The relative signal intensities were quantitated by a laser scanning densitometer.

Analytical

The sodium concentration in urine samples was determined by an ion-selective electrode (E4Atm; Electrolyte Analyzer, Beckman, Brea, CA). Urinary protein concentration was measured by the Coomassie blue dye binding technique (28). Sodium and protein excretion was calculated by standard formulas. Sodium balance was calculated as the difference between Na intake and urinary Na excretion over a 24-h observation period.

Statistical Analysis

Values are presented as means ± SD. Data were analyzed by t test or two-way analysis of variance as appropriate. Significance of difference between group mean, subjected to analysis of variance, was established by the Tukey-Cicchetti test for multiple comparisons (29). Statistical significance was defined as P < 0.05.

RESULTS

Cy-LP Gene Expression in Nephrotic Rats

All animals injected with ADR developed nephrotic syndrome. Twenty-one days after ADR administration, the urinary protein excretion was significantly higher than that in rats receiving vehicle (585 ± 189 vs 16 ± 4 mg/day; P < 0.01). At the same time, the urinary Na excretion was markedly lower in ADR-treated rats than in control animals (1.25 ± 0.15 vs 2.12 ± 0.27 mEq/day; P < 0.01). Northern blot analysis showed that both renal cortex and medulla from control rats constitutively expressed Cy-LP gene (Figure 1). Whereas in the renal cortex, the expression of the gene encoding for Cy-LP was comparable in the two groups of animals, a transcript of identical size but greater intensity was observed in the renal medulla of nephrotic as compared with control rats (Figure 1). Densitometric analysis of the autoradiographic signal confirmed that Cy-LP mRNA was up-regulated in the renal medulla of nephrotic rats, with a twofold increase over that in controls (Figure 2). By contrast, the optical density of the autoradiographic signal from the renal cortex was comparable in nephrotic and control rats. Similarly, comparable expression of the Cy-LP gene was documented in the lung from nephrotic and control rats (Figure 2).

Parallel Changes in Na Excretion and Cy-LP Gene in Nephrotic Rats

Table 1 shows the time course of Na intake and urinary Na excretion in ADR-treated rats over a 4-wk observation period. No significant difference was found for Na intake in nephrotic rats at the different times considered. By contrast, a progressive decline in the urinary Na excretion was observed starting 14 days after ADR injection and reaching a nadir at day 21. Na excretion values were on average reduced by 26 and 45%, respectively, as compared with pre-ADR injection values. Thereafter, urinary Na excretion normalized and returned to baseline values at day 28. As a result, the overall Na balance became positive at day 14, with a further increase at day 21. Thereafter, a tendency to normalize was documented.

Figure 3 depicts the time course of Cy-LP gene expression in the renal medulla from ADR-treated rats. Although no significant changes in Cy-LP mRNA expression were found 7 days after ADR in-
Effect of ANP on Cy-LP Gene Expression in Nephrotic and Control Rats

As shown in Table 2, the infusion of ANP to normal, anesthetized rats resulted in an 18-fold increase in the urinary Na excretion as compared with baseline values. By contrast, a blunted natriuretic response to ANP exposure was found in nephrotic rats 21 days after ADR injection, with a ninefold increase in Na excretion as compared with preinfusion values. No change in urinary Na excretion was found after ANP infusion to ADR-treated rats.

TABLE 1. Time course of UNaV, Na intake, and Na balance in ADR-treated rats

<table>
<thead>
<tr>
<th>Day</th>
<th>UNaV  (mEq/24 h)</th>
<th>Na Intake  (mEq/24 h)</th>
<th>Na Balance  (mEq/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.06 ± 0.27</td>
<td>2.21 ± 0.19</td>
<td>0.16 ± 0.13</td>
</tr>
<tr>
<td>7</td>
<td>2.11 ± 0.31</td>
<td>2.24 ± 0.27</td>
<td>0.14 ± 0.08</td>
</tr>
<tr>
<td>14</td>
<td>1.53 ± 0.13b</td>
<td>2.30 ± 0.18</td>
<td>0.78 ± 0.16</td>
</tr>
<tr>
<td>21</td>
<td>1.15 ± 0.09b</td>
<td>2.21 ± 0.19</td>
<td>1.07 ± 0.13b</td>
</tr>
<tr>
<td>28</td>
<td>1.86 ± 0.16</td>
<td>2.19 ± 0.16</td>
<td>0.33 ± 0.10</td>
</tr>
</tbody>
</table>

a Values are mean ± SD. Urinary Na excretion in control group, 2.08 ± 0.15 mEq/24 h.
b P < 0.01 versus days 0, 7, and 28.
TABLE 2. Diuretic and natriuretic response to ANP or vehicle in control and ADR-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>ADR Nephrosis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td><strong>Urine Output (µL/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.0 ± 1.6</td>
<td>8.6 ± 3.2</td>
<td>4.5 ± 0.6</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>ANP</td>
<td>7.7 ± 1.5</td>
<td>189.4 ± 38.4</td>
<td>4.8 ± 1.8</td>
<td>58.9 ± 23.0</td>
</tr>
<tr>
<td><strong>UNaV (µEq/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1.07 ± 0.12</td>
<td>1.05 ± 0.06</td>
<td>0.54 ± 0.19</td>
<td>0.55 ± 0.10</td>
</tr>
<tr>
<td>ANP</td>
<td>1.04 ± 0.08</td>
<td>19.33 ± 2.70</td>
<td>0.59 ± 0.13</td>
<td>5.38 ± 2.14</td>
</tr>
</tbody>
</table>

- Values are mean ± SD.
- P < 0.01 versus pre of the same group.
- P < 0.01 versus post of ADR nephrosis.

Figure 4 shows Cy-LP gene expression in the renal medulla of control and ADR-treated rats challenged with ANP or its vehicle. As indicated by densitometric analysis of the autoradiographic signals, in control rats, ANP infusion did not result in any further increase in the expression of Cy-LP mRNA in the renal medulla as compared with that in animals given vehicle alone. In ADR-treated rats receiving vehicle, Cy-LP gene expression was 1.7-fold higher than in control animals exposed to vehicle. A small but significant (P < 0.05) further increase in the expression of Cy-LP mRNA was found when nephrotic rats were infused with ANP. The optical density of the autoradiographic signals was significantly (P < 0.01) higher than that in control animals given ANP.

DISCUSSION

Edema of nephrotic patients is considered to be the consequence of an excessive sodium retention in response to underfilled circulation (1); however, the possibility of an intrinsic abnormality of the kidney (12) is now increasingly recognized. The key observation in support of this latter theory was that of Bernard and coworkers (13), who showed that in experimental nephrosis about 47% of the sodium passing beyond the superficial late distal tubule was reabsorbed, whereas in the control rats, only 8% was removed. Some years later, Ichikawa and coworkers (14) documented that, in another model, an unknown intrarenal mechanism causes avid sodium retention by the proteinuric kidney in the absence of reduced systemic plasma protein concentration. An innovative approach to factors, other than underfilled plasma compartment, implicated in sodium metabolism within the kidney has been provided by the study of Iwal and Inagami (22), who sought to isolate genes whose expression in the kidney is modulated by the status of sodium intake. By screening a rat kidney cDNA library, they isolated a clone whose corresponding gene expression was modulated by sodium depletion; it was more abundant in spontaneously hypertensive rats (22). Sequence analysis of this cDNA revealed a deduced protein product with extensive homology to cyclophilin, a protein that binds cyclosporin A intracellularly. Because of such homology, this protein product was called Cy-LP.

These data show that renal Cy-LP mRNA is upregulated in nephrotic kidney, consistent with the theoretical possibility that Cy-LP reduces sodium excretion and thus determines a condition of sodium retention (22). Actually, in our experimental conditions, the expression of the gene encoding for Cy-LP was comparable in the renal cortex of normal and nephrotic animals, whereas up-regulation of the gene was selectively observed in the renal medulla of nephrotic kidneys. Changes in medullary Cy-LP gene expression paralleled closely sodium excretion at dif-
ferent time intervals from the induction of the disease. Although the modulation of a gene in a disease condition characterized by renal sodium retention does not necessarily implicate that the gene is involved in or has a direct effect on body fluid homeostasis, it does provide evidence for the intimate relation of the gene with factors that modulate sodium metabolism. Therefore, it is of interest that three genes isolated from kidneys of spontaneously hypertensive and Dahl salt-sensitive rats are up-regulated as compared with genes from the corresponding normotensive strains (30). Consistent modulation in the expression of these genes was also observed by changing NaCl intake with the diet.

Even though more than one hormonal system is likely to contribute to the final development of edema (2), several experimental (16–18,20) and human (19,31) studies agree on the view that tubular insensitivity to ANP is a relevant factor. In 1987, the first formal evidence has been provided that the nephrotic syndrome is associated with a marked reduction in diuretic and natriuretic responses to ANP (16), and a subsequent study (20) showed that such abnormality was intrinsic to the proteinuric kidney. Several subsequent reports have attempted to address the nature of this abnormality (17,20,21,32). Evidence has also accumulated that ANP inhibits sodium reabsorption of the medullary segment of the collecting duct (15,33). We therefore designed additional experiments with the aim of disclosing a possible link between the up-regulation of Cy-LP in the renal medulla of nephrotic animals and the blunted diuretic and natriuretic responses to ANP. The diverse biologic actions of ANP result from specific high-affinity interactions of the peptide with the membrane-associated receptors of target organs (15,33). Our previous findings of a comparable ANP receptor density and affinity in the renal medulla of control and nephrotic rats would exclude the suggestion that the blunted diuretic and natriuretic responses to ANP in ADR nephrosis are due to changes in ANP binding (20). Upon binding to specific receptors, ANP stimulates the guanylate cyclase portion of this transmembrane glycoprotein, which results in the accumulation of intracellular cGMP, the putative second messenger of ANP action (33,34). Besides directly activating the ion channel, cGMP stimulates a protein kinase, which alters the phosphorylation state of intracellular proteins (34). Of interest to the purpose of this study is the apparent fact that protein kinases are involved in the signal transduction pathway, which leads to the induction of the c-fos gene, the prototype for a group of inducible genes that convert short-term transmembrane signals into long-term responses, requiring transcriptional regulation of target genes (35).

To find out whether Cy-LP could have been one of those target genes possibly regulated by protein kinase-mediated stimulation of its promoter, we have studied the effect of ANP infusion on renal medulla Cy-LP mRNA. Data that, after ANP infusion in normal rats, Cy-LP gene expression was almost comparable to that found after vehicle infusion tend to exclude the fact that the second messenger generated by the binding of ANP to its receptor modulates Cy-LP gene expression in the medulla of normal kidney. By repeating the same experiments in animals with experimental nephrosis, we found that in nephrotic animals the expression of renal medullary Cy-LP mRNA was already positively modulated. The infusion of ANP significantly increased the expression level of Cy-LP mRNA in the kidney.

A possible interpretation of these results is that up-regulation of medullary Cy-LP mRNA is the intra-renal mechanism that determines salt retention in nephrotic syndrome. Of course, one cannot exclude the idea that up-regulation of the Cy-LP gene is a simple consequence of the decreased sodium excretion. However, the latter possibility conflicts with data that after ANP infusion in nephrotics, further up-regulation of the Cy-LP gene is associated not with a decreased but rather with an increased sodium excretion (albeit not to normal levels). If one favors the view that Cy-LP participates in sodium retention, then the blunted response to the ANP of nephrotics can be secondary to the positive modulation of Cy-LP gene. Actually, the precise role of Cy-LP in sodium retention as well as its possible effect of antagonizing ANP will only be unraveled when the peptide is available for pharmacologic studies.

REFERENCES


