Increased expression and apical targeting of renal ENaC subunits in puromycin aminonucleoside-induced nephrotic syndrome in rats

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Nephrotic syndrome is one of the most extensively studied models of glomerulonephritis in rats. This experimental model mimics the minimal change disease glomerulopathy found in human pathology. The characteristic lesion in both the experimental model and human disease consists of vacuolation and flattened foot processes of podocytes (51). In addition to glomerular lesions, functional alterations of tubular transport have been demonstrated in PAN-treated animals. In vivo micropuncture studies in the unilateral model of PAN-induced nephrotic syndrome have shown that sodium reabsorption is specifically increased in the collecting duct and not in the proximal tubule and distal nephron (29). Micropuncture of the accessible distal convoluted tubule (DCT) revealed that the tubular sodium load was similar in PAN-treated kidney and control kidney of the same rat, and the final urine sodium excretion was threefold lower in urine collected from the PAN-treated kidney compared with that in the untreated control kidney in the same animal (29). Thus this study directly points to a role of increased sodium reabsorption in the collecting duct and, hence, it may be hypothesized that dysregulation of the key sodium channels and transporters in the collecting duct may be responsible for this. A few studies subsequently demonstrated that sodium retention in PAN nephrotic rats is correlated with increased Na-K-ATPase activity and expression in the cortical collecting duct (CCD) (13, 14), and a recent study demonstrated no major changes in the protein abundance of the epithelial sodium channel (ENaC) subunits in whole kidney (3).

In the collecting duct, electrogenic sodium from the lumen into the cells is mediated by ENaC located in the apical plasma membrane (17). This represents the rate-limiting step for sodium absorption and is characterized by inhibition with submicromolar concentrations of the diuretic amiloride (22). Gain-of-function mutations cause Liddle’s syndrome, an inherited form of salt retention causing hypertension (24, 47). Conversely, loss-of-function mutations cause pseudohypoaldosteronism type 1, characterized by salt wasting and hypertension (11). Thus ENaC has a critical role in the maintenance of sodium homeostasis. On the basolateral side, Na-K-ATPase actively transports sodium out of the cell into the extracellular interstitium and provides the driving force for sodium reabsorption.

In the rat kidney, the three homologous subunits (α, β, and γ) that constitute the functional ENaC protein (10) are detected in the late DCT (DCT2), connecting tubule (CNT), CCD, and...
outer medullary collecting duct (OMCD), and to a lesser extent, the inner medullary collecting duct (IMCD) (46). It has been shown that α-ENaC is mainly present at the apical domains of the principal cells, whereas β- and γ-ENaC are mainly associated with intracellular vesicles dispersed in the entire cytoplasm (23). The physiological significance of the heterogeneity in the subcellular localization of the three subunits has not been established. The α-subunit is functional when expressed alone in Xenopus laevis oocyte, but channel activity is highly increased by association with β- and γ-subunits (4). The generation of gene knockouts of the individual subunits in mice demonstrated that altered expression of any of the three subunits has significant effects on multimeric ENaC protein sodium transport capacity (5, 28, 38). Regulation of sodium reabsorption by ENaC mediated by hormones such as aldosterone and vasopressin is associated with characteristic alterations in the expression of the individual ENaC subunits (18, 37). Chronic aldosterone infusion in rats increases the protein abundance of α-ENaC. Moreover, aldosterone causes a mobility shift of γ-ENaC from an 85-kDa band to 70-kDa band (37). Chronic vasopressin infusion results in significantly increased abundances of all three ENaC subunits (18). The apical plasma membrane expression of ENaC can also be altered by changes in the trafficking of the channel subunits to the apical plasma membrane. Under sodium-replete conditions, when aldosterone levels are low ENaC immunostaining of CCD revealed diffuse labeling throughout the principal cells, consistent with localization in a vesicular pool (37). In contrast, sodium restriction or aldosterone infusion caused a dramatic redistribution of ENaC to the apical membrane (36, 37). These results suggest that not only protein abundance but also translocation of ENaC to the apical plasma membrane are under the tight control.

The purpose of this study was therefore to investigate directly whether PAN-induced nephrotic syndrome in rats is associated with altered regulation of ENaC subunit protein abundance and/or changes in the segmental and subcellular localization to elucidate the underlying molecular mechanisms responsible for the increased sodium retention. We performed semiquantitative immunoblotting, immunohistochemistry, and immunoelectron microscopy with the following specific purposes: 1) to examine whether there are changes in the abundance of ENaC subunits in rats with PAN-induced nephrotic syndrome; 2) to examine whether the apical membrane targeting of ENaC subunits is affected; 3) to examine whether there are changes in the abundances of other important renal sodium transporters as the Na-K-ATPase, the Na+/H+ exchanger type 3 (NHE3), the Na+/K+2Cl- cotransporter (BSC-1), and the thiadizole-sensitive Na+/Cl- cotransporter (TSC); and 4) to examine whether these changes are associated with changes in urinary sodium excretion.

METHODS

Experimental Protocols

Protocol 1: PAN-treated rats and control rats with free access to food and water. Experiments were performed using male Munich-Wistar rats (250–300 g; Møllegaard Breeding Centre). PAN nephrotic syndrome (n = 6) was induced by a single intravenous injection of PAN (180 mg/kg body wt; Sigma, St. Louis, MO) via the femoral vein. Control rats (n = 6) received the vehicle alone (i.e., sterile 0.9% saline). They were maintained on a standard rodent diet (Altromin 1324, Lage, Germany) and allowed free access to drinking water and food at all times. During the last 3 days, the rats were subsequently maintained in the metabolic cages to allow urine collections for the measurements of protein, Na+, K+, creatinine, and osmolality. The water and food intake and body weight were monitored. The sodium balance was calculated as the difference between dietary sodium intake and urinary sodium excretion in the 24-h observation period. The rats were killed for immunohistochemical and morphological studies 7 days after PAN treatment. The rats were anesthetized with halothane (Halocarbon Laboratories), and a large laparotomy was made. Blood was collected from the inferior vena cava and analyzed for Na+, K+, creatinine, albumin, osmolality, and plasma aldosterone concentration. The right kidney was rapidly removed, dissected into zones [cortex, inner stripe of outer medulla (ISOM), and inner medulla], and processed for immunoblotting as described below. The left kidney was fixed by retrograde perfusion as described below.

Protocol 2: PAN-treated rats and control rats with paired feeding. Another set of PAN-treated rats (n = 6) and control rats (n = 6) was made. This protocol was identical to protocol 1 except that PAN-treated and control rats were pair fed. The rats were maintained in metabolic cages, and daily 24-h urine output and water intake were measured during the entire experimental period. In the control group, rats were offered the amount of food corresponding to the mean intake of food that the PAN-treated rats consumed during the previous day.

Clearance Studies, Plasma Aldosterone, and Urine Protein Measurements

Clearance studies were performed over the last 24 h in protocol 1 and protocol 2. At the end of each protocol, under halothane anesthesia, 2 ml of blood were collected into a heparinized tube for determination of plasma electrolytes and osmolality before the rats were killed. The plasma concentrations of sodium, potassium, and creatinine and the urinary concentration of creatinine were determined (Vitros 950, Johnson & Johnson). The concentrations of urinary sodium and potassium were determined by standard flame photometry (Eppendorf FCM6341). The osmolality of urine and plasma was determined by freezing-point depression (Advanced Osmometer, model 3900, Advanced Instruments, Norwood, MA, and Osmomat 030-D, Gonotec, Berlin, Germany).

Plasma aldosterone concentrations were determined using a commercially available radioimmunoassay kit (Coat-A-Count, Diagnostic Products, Los Angeles, CA).

Protein levels in urine were determined using a qualitative assessment by use of Multistix (Bayer, Bayer Denmark, Lyngby, Denmark).

Semiquantitative Immunoblotting

The dissected renal cortex, ISOM, and inner medulla were homogenized (Ultra-Turrax T8 homogenizer, IKA Labortechnik, Staufen, Germany) 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 4,000 × g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria, and the supernatant was pipetted off and kept on ice. The total protein concentration was measured (Pierce BCA protein assay reagent kit, Pierce, Rockford, IL). All samples were adjusted with isolation solution to reach the same final protein concentrations and solubilized at 65°C for 15 min in SDS-containing sample buffer and then stored at −20°C. To confirm equal loading of protein, an initial gel was stained with Coomassie blue. SDS-PAGE was performed on 9 or 12% polyacrylamide gels. The proteins were transferred by gel electrophoresis (Bio-Rad Mini Protean II) onto nitrocellulose membranes (Hybond ECL, RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBS-T (80 mM Na2HPO4, 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. The sites of antibody-antigen reaction were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) with an enhanced chemiluminescence system (ECL or ECL+ Plus) and exposure to photographic film (Hyperfilm ECL, Amersham Pharmacia Biotech). The band densities were quantitated by scanning the films and normalizing the densitometric values. Results are presented as the relative abundances between the groups. ECL films with bands within the linear range were scanned using an AID LAS scanner (LASERFOCUS II). The labeling density was corrected by densitometry of Coomassie-stained gels run in parallel to the blotted gels.

Immunohistochemistry
A perfusion needle was inserted in the abdominal aorta of halothane-anesthetized rats, and the vena caval vein was clamped to establish a blood outlet. Blood was flushed from the kidneys with cold PBS (pH 7.4) for 15 s, before being switched to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed and sectioned into 2- to 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 being embedded in paraffin, 2-μm sections of the tissue were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark).

The sections were dewaxed with xylene and rehydrated with graded ethanol. Sections had endogenous peroxidase activity blocked with 0.5% H2O2 in absolute methanol for 10 min. In a microwave oven, the sections were boiled in target retrieval solution (1 mmol/l Tris, pH 9.0, with 0.5 mM EGTA) for 10 min. After cooling, nonspecific binding was blocked with 50 mM NH4Cl in PBS for 30 min followed by 3 × 10 min with PBS blocking buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated with primary antibody (diluted in PBS with 0.1% BSA and 0.3% Triton X-100) overnight at 4°C. The sections were washed 3 × 10 min with PBS wash-buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with HRP-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO) for 1 h at room temperature. After 3 × 10-min rinses with PBS wash-buffer, the sites with antibody-antigen reaction were visualized with a brown chromogen produced within 10 min by incubation with 0.05% 3,3'-diaminobenzidine tetrachloride (Kem-En Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2 O2. Mayer's hematoxylin was used for counterstaining and, after dehyration, coverslips were mounted with a hydrophilic mounting media containing antifading agent (Fluka, Burlington, VT) and mounted on glass slides with coverslips. The sections were then viewed with a Leica DMRE microscope (Leica Microsystems). Immunofluorescent labeling was performed on ultrathin Lowicryl HM20 sections. Immunolabeling was performed on ultrathin Lowicryl HM20 sections. Sections were pretreated with the saturated solution of NaOH in absolute ethanol (2–3 s), rinsed, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100. Sections were rinsed and incubated overnight at 4°C with α-ENaC antibody diluted in 0.05 M Tris, pH 7.4, containing 0.1% Triton X-100 with 0.2% milk (diluted 1:5). After being rinsed, sections were incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (1:50; GAR, EM10, BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate before examination in a Philips Morgagni electron microscope operating at 70 kV.

Primary Antibodies
Rabbit polyclonal antibodies to the following renal sodium transporters were used: NHE3 (20), Na-K-2Cl cotransporter (NKCC2, BSC-1) (32), TSC (33), and ENaC subunits α-ENaC, β-ENaC, and γ-ENaC (37). The antisera were affinity-purified against the immunizing peptides as previously described (32, 33). The specificity of the antibodies has been demonstrated by showing unique peptide-able bands on immunoblots and a specific labeling by immunohistochemistry. A mouse monoclonal antibody against the Na-K-ATPase α1-subunit was kindly provided by Dr. D. M. Fambrough, Johns Hopkins University Medical School.

Statistical Analyses
Values are presented as means ± SE. Comparisons between two groups were made by unpaired t-test. P values <0.05 were considered significant.

RESULTS
Urinary Sodium Excretion and Fractional Excretion of Sodium Were Decreased in PAN-Treated Rats

In both protocols 1 and 2, all PAN-treated rats developed severe nephrotic syndrome with marked proteinuria, hypoalbuminemia, and large amounts of ascites (Tables 1 and 2). Urinary protein excretion was measured in PAN-treated rats, and the urinary protein level was greater than that of control animals after 2 or 3 days and reached a +4 level within 4 to 5 days, while controls exhibited 0 or +1. Consistent with this, the plasma albumin level was significantly decreased in the PAN-treated rats (148.0 ± 0.3 vs. 338.7 ± 12.7 μmol/l in protocol 1 and 148.0 ± 2.0 vs. 345.6 ± 10.4 μmol/l in protocol 2, P < 0.01, respectively; Tables 1 and 2). Plasma creatinine level was increased, whereas its renal clearance was decreased (P < 0.01; Tables 1 and 2) in PAN-treated rats. In contrast, the urinary output was not significantly different between the two groups. The food intake of PAN-treated rats in protocol 1 was significantly lower than that of control rats (14.8 ± 1.6 vs. 23.5 ± 2.4 g/day, P < 0.05), whereas it was not different in protocol 2. Importantly, the 24-h urinary sodium excretion (0.33 ± 0.05 vs. 1.56 ± 0.18 mmol/l) and fractional excretion of sodium (FENa; 0.34 ± 0.07 vs. 0.69 ± 0.06%, P < 0.01) were decreased in PAN-treated rats (protocol 1; Table 1). Accordingly, urinary sodium/creatinine in the rat was decreased (8.7 ± 1.6 vs. 31.5 ± 3.2 mmol/mmol, P < 0.01). The decreased sodium excretion was not related to any decrease in food intake, because pair-fed (same food intake as control rats) and PAN-treated rats (protocol 2) exhibited sig-
increased urine osmolality, urine/plasma osmolality, and solute-free water reabsorption in PAN-treated rats. Consequently, PAN-treated rats displayed a positive sodium balance (Tables 1 and 2). Moreover, the urinary osmolality (634.7 ± 53 vs. 1,698.5 ± 177 mosmol/kgH₂O, P < 0.01), urine/plasma osmolality ratio (2.0 ± 0.2 vs. 5.7 ± 0.6, P < 0.01), and the solute-free water reabsorption (58.9 ± 5.7 vs. 177.0 ± 11.5 µl·min⁻¹·kg⁻¹ body wt⁻¹, P < 0.01) were decreased, indicating decreased urinary concentration (protocol 1). A reduced urinary concentrating ability was also observed in protocol 2 with decreased urine osmolality, urine/plasma osmolality, and solute-free water reabsorption (Table 2).

Table 2. Changes in renal function (protocol 2)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Puromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UO, µl/min</td>
<td>11.4 ± 0.9</td>
<td>11.5 ± 0.4</td>
</tr>
<tr>
<td>Albumin, µmol/l</td>
<td>345.6 ± 10.4</td>
<td>148.0 ± 2.0*</td>
</tr>
<tr>
<td>P-Cr, µmol/l</td>
<td>27.6 ± 1.2</td>
<td>53.0 ± 7.5*</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>1.33 ± 0.13</td>
<td>0.57 ± 0.06*</td>
</tr>
<tr>
<td>P-Na</td>
<td>138.3 ± 0.4</td>
<td>130.6 ± 0.8*</td>
</tr>
<tr>
<td>P-K</td>
<td>4.1 ± 0.1</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>U₀ × U₀, mmol</td>
<td>1.71 ± 0.21</td>
<td>0.24 ± 0.04*</td>
</tr>
<tr>
<td>U₀/Ccr, mmol/ml</td>
<td>32.2 ± 2.2</td>
<td>6.0 ± 0.8*</td>
</tr>
<tr>
<td>Sodium balance, mmol/day</td>
<td>-0.23 ± 0.2</td>
<td>0.85 ± 0.2*</td>
</tr>
<tr>
<td>FENa, %</td>
<td>0.65 ± 0.06</td>
<td>0.26 ± 0.07*</td>
</tr>
<tr>
<td>FEK, %</td>
<td>62.4 ± 3.0</td>
<td>96.7 ± 13.6*</td>
</tr>
<tr>
<td>U-Osm, mosmol/kgH₂O</td>
<td>1,451.6 ± 98</td>
<td>828.9 ± 65*</td>
</tr>
<tr>
<td>U/Osm</td>
<td>296.0 ± 0.7</td>
<td>316.0 ± 2.8*</td>
</tr>
<tr>
<td>T/H₂O₂, µl·min⁻¹·kg⁻¹</td>
<td>170.5 ± 14.2</td>
<td>66.4 ± 5.6*</td>
</tr>
<tr>
<td>Ascites, ml</td>
<td>Nondetectable</td>
<td>14.57 ± 1.73</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 6 each. These values are measured at the last day of experiments (day 7). *P < 0.01 compared with control.

Semiquantitative immunoblotting was carried out to investigate whether the abundance of ENaC subunits changes in rats

![Fig. 1. Semiquantitative immunoblots of kidney proteins prepared from cortex (A), inner stripe of outer medulla (ISOM; B), and inner medulla (C) from control and puromycin aminonucleoside (PAN)-treated rats in protocol 1. The abundance of the α-epithelial Na channel (ENaC) band at 85 kDa was increased in the ISOM and inner medulla, while unchanged in the cortex. The band is a composite of several thin bands believed to represent different glycosylated forms of α-ENaC. The abundance of the β-ENaC band at 85 kDa was similarly increased in the ISOM and inner medulla, while unchanged in the cortex. This band also presents as several bands likely to represent different glycosylated forms of β-ENaC. γ-ENaC is seen as a narrow band around 85 kDa and a broader band around 70 kDa. The 70-kDa band was significantly increased in the cortex and ISOM, while unchanged in the inner medulla. *P < 0.05.](http://ajprenal.physiology.org/Downloaded from http://ajprenal.physiology.org)
with PAN-induced nephrotic syndrome. In protocol 1, the protein abundance of α-ENaC and β-ENaC was increased in the ISOM and in the inner medulla but was not altered in the cortex (Fig. 1). The abundance of the 70-kDa form of γ-ENaC was increased in the cortex and ISOM but not in the inner medulla (Fig. 1). In contrast, the abundance of 85-kDa γ-ENaC was markedly increased in the inner medulla but not in the cortex and ISOM (Fig. 1). The analyses of normalized band densities are shown in Table 3.

In protocol 2, the major changes of ENaC abundances were very similar to those of protocol 1 (Table 4), indicating that altered abundance of ENaC subunit in protocol 1 was not affected by the lower food and sodium intake in PAN-treated rats compared with controls. In the pair-fed experiments (protocol 2), the abundance of α-ENaC was increased in the cortex, ISOM, and inner medulla (Fig. 2). β-ENaC protein abundance was increased in the ISOM and inner medulla but was maintained in the cortex (Fig. 2). The abundance of the 70-kDa form of γ-ENaC was increased in the cortex and ISOM but not in the inner medulla, whereas the 85-kDa band abundance of γ-ENaC was markedly increased in the inner medulla but not in the cortex and ISOM (Fig. 2). The analyses of normalized band densities are shown in Table 4. Thus, in contrast to the previous demonstration of uniform downregulation of aquaporins and specific sodium transporters in experimental nephrotic syndrome (2, 19), the expression of ENaC subunits was either increased or unchanged.

*Indications of Increased Targeting of ENaC Subunits to the Apical Plasma Membrane in PAN-Treated Rats*

Immunohistochemical analyses of γ- and β-ENaC (protocol 1). In addition to regulation of protein abundances of the ENaC subunits, ENaC is also regulated by intracellular trafficking. To investigate whether the trafficking of ENaC subunits is altered in PAN-induced nephrotic syndrome, we carried out immunoperoxidase microscopy and confocal laser immunofluorescence microscopy of β- and γ-ENaC subunits. Immunolabeling confirmed previous studies showing ENaC expression from the second half of the DCT2 to the medullary collecting duct, with the expression being most prominent in the CNT and CCD with decreasing labeling in medullary

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**Table 3. Summary of ENaC immunoblotting results (protocol 1)**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Puromycin</th>
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</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.11</td>
<td>1.18±0.11</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.14</td>
<td>0.91±0.14</td>
</tr>
<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.11</td>
<td>1.21±0.13</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.28</td>
<td>3.31±0.19*</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.24</td>
<td>1.78±0.10*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.25</td>
<td>3.70±0.79*</td>
</tr>
<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.07</td>
<td>1.09±0.09</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.04</td>
<td>1.48±0.11*</td>
</tr>
<tr>
<td>IM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.25</td>
<td>3.06±0.23*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.22</td>
<td>2.64±0.26*</td>
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<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.20</td>
<td>6.31±0.17*</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.17</td>
<td>1.06±0.17</td>
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</table>

Values are expressed as means ± SE. ENaC, epithelial sodium channel; ISOM, inner stripe of the outer medulla; IM, inner medulla. *P < 0.05 compared with control.

**Table 4. Summary of ENaC immunoblotting results (protocol 2)**

<table>
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<tr>
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<th>Control</th>
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<tr>
<td>α-ENaC</td>
<td>1.00±0.04</td>
<td>1.43±0.07*</td>
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<tr>
<td>β-ENaC</td>
<td>1.00±0.13</td>
<td>1.17±0.13</td>
</tr>
<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.19</td>
<td>0.68±0.21</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.29</td>
<td>2.40±0.39*</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.09</td>
<td>1.78±0.08*</td>
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<tr>
<td>β-ENaC</td>
<td>1.00±0.07</td>
<td>1.65±0.09*</td>
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<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.10</td>
<td>1.39±0.10*</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.18</td>
<td>3.04±0.30*</td>
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<tr>
<td>IM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.13</td>
<td>1.86±0.32*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.25</td>
<td>3.70±0.79*</td>
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<tr>
<td>γ-ENaC 85 kDa</td>
<td>1.00±0.31</td>
<td>4.36±0.42*</td>
</tr>
<tr>
<td>γ-ENaC 70 kDa</td>
<td>1.00±0.18</td>
<td>1.42±0.09</td>
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</table>

Values are expressed as means ± SE. *P < 0.05 compared with control.
collecting duct segments. In control rats, immunoperoxidase staining for the β- and γ-ENaC subunits showed diffuse cytoplasmic labeling throughout the DCT2, CNT, and collecting duct principal cells, consistent with the localization of ENaC in a vesicular pool (Fig. 3, A, C, E), as previously observed (23, 35).

In PAN-treated rats, γ-ENaC labeling was predominantly localized to the apical domains (i.e., plasma membrane and intracellular vesicles close to the membrane) and only marginal labeling of the cytoplasm corresponding to labeling of intracellular vesicle. This was evident in almost all cross-sectioned tubules of DCT2 (Fig. 3B), CNT (Fig. 3D), and CCD (Fig. 3F). In contrast, γ-ENaC labeling in kidneys of control animals showed predominant cytoplasmic labeling (Fig. 3, A, C, E).

The subcellular and segmental localization was further investigated by double-labeling immunofluorescence microscopy for γ-ENaC and calbindin-D28k [a marker for DCT/CNT segments previously used (12)]. To confirm that the tubule segments were CCD and not CNT, double labeling with γ-ENaC and calbindin-D28k was carried out and analyzed by laser-scanning confocal microscopy. With double labeling, the tubule segment with γ-ENaC labeling did not show any calbindin-D28k labeling, confirming the tubule is CCD. As shown in Fig. 3H, the γ-ENaC labeling was markedly increased in the apical plasma membrane domains of CCD cells (where calbindin-D28k labeling was not present) in kidneys from PAN-treated rats. Similar γ-ENaC labeling patterns were observed in DCT2 and CNT (not shown).

Immunoblotting analyses revealed an upregulation of γ-ENaC in outer and inner medulla (Tables 3 and 4). Consistent with this, immunohistochemical analyses of γ-ENaC revealed both increased abundance and markedly increased labeling of the apical plasma membrane domains of OMCD principal cells (Fig. 4, A and B) and of IMCD cells (Fig. 4, C).

![Fig. 3. Immunoperoxidase and immunofluorescence microscopy of γ-ENaC in the second half of distal convoluted tubule (DCT2), connecting tubule (CNT), and cortical collecting duct (CCD). Immunoperoxidase labeling of γ-ENaC is dispersed in the cytoplasm of principal cells of the DCT2 (A), CNT (C), and CCD (E) in control rats in protocol 1. In contrast, γ-ENaC labeling was seen predominantly localized to the apical plasma membrane domains and only marginal labeling of cytoplasm was observed in DCT2 (B), CNT (D), and CCD (F) in protocol 1. Representative confocal immunofluorescence images for γ-ENaC in the CCD were shown for protocol 1 (G, H) and for protocol 2 (I, J; pair-fed). With double labeling of γ-ENaC and calbindin-D28k, the tubule segment with γ-ENaC labeling did not show any calbindin-D28k labeling, confirming that the tubule is CCD. In both protocols, the labeling is markedly increased in the apical plasma membrane domains after PAN treatment (H, J), whereas control rats showed diffuse cytoplasmic labeling (G, I). Arrows indicate apical labeling.]
To investigate whether the trafficking of ENaC subunits in PAN-induced nephrotic syndrome was attributed to the lower food intake, and hence decreased sodium intake, in PAN-treated animals compared with controls (protocol 1 where rats had free access to food), we repeated the same immunoperoxidase and immunofluorescence labeling for β- and γ-ENaC subunits in sections from kidneys of PAN-treated and control rats that had the same daily food and sodium intake (protocol 2). The immunolabeling pattern was exactly the same in protocol 2 compared with protocol 1. Along with the increased plasma aldosterone level, there was a prominent increase in apical trafficking of β- and γ-ENaC in PAN-treated rats. Representative confocal immunofluorescence image for γ-ENaC in the CCD showed redistributed apical labeling in pair-fed, PAN-treated rats (Fig. 3J), identical to the labeling pattern observed in protocol 1 (Fig. 3H).

Immunohistochemical analyses of α-ENaC (protocol 2). Control experiments using immunohistochemical analysis of α-ENaC in kidneys from rats treated with a low-salt diet for 3 days revealed substantial increase in α-ENaC immunolabeling and increased apical labeling compared with the labeling in kidneys from rats on a normal high-sodium-containing diet, as previously shown (37) (not shown). Immunoperoxidase microscopy of sections from kidneys of PAN-treated and control rats revealed increased apical immunolabeling of α-ENaC in DCT2 (Fig. 6, A and B), CNT (Fig. 6, C and D), CCD (Fig. 6, E and F), and OMCD (Fig. 6, G and H) from PAN-treated rats. This is consistent with the increased α-ENaC protein abundance observed by immunoblotting (Table 4).

Immunoelectron microscopy of α-ENaC (protocol 2). Immunoelectron microscopy further demonstrated the increase in α-ENaC labeling in the collecting duct principal cells in kidneys from PAN-treated rats. Immunoelectron microscopy was performed on ultrathin Lowicryl HM20 sections from the cortex of control rats (Fig. 7A) and PAN-treated rats (Fig. 7B). In controls, weak immunogold labeling of α-ENaC was seen and was mainly associated with intracellular vesicles (arrows in Fig. 7A) and labeling in the apical plasma membrane was sparse (arrowhead in Fig. 7A). In contrast, the α-ENaC labeling of the CCD principal cells was markedly increased in PAN-treated rats (Fig. 7B). In particular, the increased labeling of the apical plasma membrane (arrowheads in Fig. 7B) strongly indicated that PAN-induced nephrotic syndrome is associated with increased apical labeling of α-ENaC; hence this may play
a role in the increased sodium reabsorption in the collecting duct previously reported.

**Decreased Protein Expression of the Major Renal Sodium Transporters**

We performed additional immunoblot analyses to examine whether there are changes in the protein abundance of other major sodium transporters in the kidneys from PAN-treated rats compared with control rats (protocol 1; Fig. 8) to investigate whether dysregulation of other sodium transporters is also involved in the sodium retention and decreased urinary sodium excretion.

The monoclonal antibody against the α1-subunit of Na-K-ATPase recognized a band of ~96 kDa (31). This isoform is expressed in all renal tubule segments. Semiquantitative immunoblotting revealed a decrease in Na-K-ATPase abundance in the cortex (51 ± 11 vs. 100 ± 10%, \(P < 0.05\)) and ISOM (51 ± 12 vs. 100 ± 7%, \(P < 0.05\)), while it remained unchanged in the inner medulla. Immunoperoxidase labeling for the α1-isoform of Na-K-ATPase antibody produced a strong signal in the distal nephron (thick ascending limb of Henle and DCT) in kidneys from control rats (Fig. 9, A and B). Compared with these controls, immunolabeling for the Na-K-ATPase in the proximal tubule and thick ascending limb was significantly reduced in PAN-treated rats. In contrast, the immunolabeling of Na-K-ATPase in the CCD seemed of similar labeling intensity in kidneys from PAN-treated and control rats. The labeling pattern in the OMCD and IMCD was also similar between PAN-treated and control rats (not shown).

Reduced expression of the thick ascending limb transporters, NHE3 and BSC-1, has previously been demonstrated in adriamycin-induced nephrotic syndrome (19). Consistent with this, we observed a significant downregulation of both transporters in PAN-induced nephrotic syndrome. As shown in Fig. 8, densitometric analysis revealed a marked decrease in NHE3 abundance in the renal cortex (36 ± 13 vs. 100 ± 12%, \(P < 0.05\)) and ISOM (35 ± 6 vs. 100 ± 10%, \(P < 0.05\)) and a marked decrease in BSC-1 abundance in the cortex (19 ± 14 vs. 100 ± 7%, \(P < 0.05\)) and ISOM (9 ± 17 vs. 100 ± 10%, \(P < 0.05\)). Moreover, the expression of the TSC or NCC,
which is mainly involved in apical sodium reabsorption in the DCT (32), was also markedly decreased in the cortex (20 ± 20 vs. 100 ± 16%, P < 0.05).

Thus, both Na-K-ATPase and ENaC subunits appeared to be expressed at control or at higher levels in collecting ducts, contrasting with the observed reduced expression of sodium transporters in the proximal tubule and distal nephron.

DISCUSSION

The results demonstrate that PAN-induced nephrotic syndrome is associated with 1) sodium retention, decreased urinary excretion of sodium, marked ascites, and increased plasma aldosterone level; 2) upregulation of protein abundance of specific ENaC subunits in cortex, outer medulla, and inner medulla; and 3) increased apical targeting of ENaC subunits in DCT2, CNT, and collecting duct segments observed as increased immunolabeling in apical plasma membrane domains.

The most important finding is the striking increase in targeting of ENaC subunits to the apical plasma membrane domain, raising the possibility that this contributes significantly to the increased renal tubular sodium reabsorption. In contrast, the protein levels of other major sodium transporters expressed in nephron segments at a site proximal to the CNT (i.e., NHE3, BSC-1, Na-K-ATPase, and TSC) were significantly reduced in these segments, consistent with the previous findings seen in the adriamycin-induced nephrotic syndrome (19). Indeed, we also demonstrated that there was no downregulation of the Na-K-ATPase expression in the collecting duct. Taken together, these observations therefore strongly support the view that the renal sodium retention associated with PAN-induced nephrotic syndrome is caused by increased sodium reabsorption in CNT and collecting duct (15). We here propose that this occurs via upregulated protein expression and increased apical targeting of ENaC subunits. Thus this is likely to represent a key molecular basis for the sodium retention associated with PAN-induced nephrotic syndrome combined with the previously demonstrated increase in collecting duct Na-K-ATPase activity and protein abundance (13, 14). Thus

![Fig. 6. Immunoperoxidase microscopy of α-ENaC in DCT2, CNT, CCD, and OMCD in protocol 2. Immunoperoxidase labeling of α-ENaC is restricted to a narrow zone in the apical part including the plasma membrane domains of the principal cells of the DCT2 (A), CNT (C), CCD (E), and OMCD (G) in control rats. PAN-treated rats showed markedly increased apical immunolabeling of α-ENaC in DCT2 (B), CNT (D), CCD (F), and OMCD (H).](image-url)
our studies extend a previous study demonstrating that the total kidney abundance of ENaC subunits was not downregulated but maintained (3). Moreover, the present results extend this study by demonstrating the segmental specific upregulation of all three ENaC subunits, and most importantly, provide evidence for a significantly increased apical targeting by immunoelectron microscopy. The latter is essential because it is important to document that the increased expression is at the site of function, i.e., in the plasma membrane. Finally, we also confirm that there is a uniform downregulation of all investigated sodium transporters in the thick ascending limb and proximal tubule. This together with observed increased expression and increased targeting of ENaC subunits in combination with the previous functional micropuncture studies strongly support the view that the sodium retention in the nephrotic syndrome may occur in the DCT2-CNT-collecting duct. The present data therefore strongly support the view that this is likely involving dysregulation of ENaC with increased expression and apical targeting.

Increased or Sustained Abundance of ENaC Subunits Expression in PAN-Treated Rats: Role of Aldosterone

We here demonstrate that the expression of specific ENaC subunits was increased in the cortex, outer medulla, and inner medulla from kidneys of rats with PAN-induced nephrotic syndrome. One factor in controlling ENaC expression is aldosterone (36, 37). Indeed, plasma aldosterone levels were significantly increased in both protocols. Aldosterone is known to stimulate sodium reabsorption in the distal nephron and collecting duct. Consistent with this, aldosterone treatment in rats with glucocorticoid supplement after adrenalectomy increases renal sodium reabsorption and decreases urinary sodium excretion (27). In the aldosterone-responsive epithelia, aldosterone increases the density of ENaC in apical membranes (1, 6), and, moreover, increased abundance by de novo synthesis of ENaC subunits (or reduced degradation) could contribute to the stimulatory effect of aldosterone (36). Recently, it was demonstrated that aldosterone stimulates sodium reabsorption by the kidney, in part, through its action to increase the abundance of the TSC in the DCTs and α-ENaC in the collecting duct principal cells. Moreover, aldosterone causes a mobility shift of γ-ENaC from an 85-kDa band to a 70-kDa band without a change in total γ-ENaC protein abundance (37). The appearance of the 70-kDa form of γ-ENaC in response to aldosterone is putatively due to a channel-activating proteolytic cleavage (48). In the present study, PAN treatment was associated with the increased protein abundances of α-ENaC and 70-kDa band of γ-ENaC in the kidney, and this is consistent with an effect of aldosterone.

**Fig. 7.** Immunoelectron microscopy of α-ENaC in the principal cells of the CCD in ultrathin Lowicryl HM20 sections (protocol 2). In control rats (A), immunogold labeling of α-ENaC revealed weak labeling of intracellular vesicles (arrows) with sparse labeling of the apical plasma membrane (arrowhead). In contrast, in PAN-treated rats (B), immunolabeling of α-ENaC was increased and mainly associated with the apical plasma membrane (arrowheads). L, lumen; M, mitochondria.
onstrated that the abundance of TSC is increased by aldosterone (33). Thus it is noteworthy that TSC abundance was decreased despite increased plasma aldosterone levels in PAN-treated rats. Similar findings were reported for rats with CCl4-induced liver cirrhosis where TSC abundance was decreased despite that plasma aldosterone levels were increased (21, 30). Further studies are therefore warranted for the regulation of TSC in the DCT; however, the observed downregulation speaks against the possibility that TSC may be involved in the development of sodium retention.

Increased or Sustained Abundance of ENaC Subunits in PAN-Treated Rats: Role of Vasopressin

PAN-induced nephrotic syndrome has been known to be associated with high plasma vasopressin levels (2, 42). Vasopressin is also a major regulator of sodium transport in the collecting duct. In vitro isolation and microperfusion studies of rat CCD demonstrated that vasopressin and cAMP analogs stimulate sodium reabsorption within minutes (25, 26). Moreover, in amphibian A6 cells, vasopressin and cAMP analogs increased the apical membrane density of active ENaC (34), and the ENaC stimulation by vasopressin has been confirmed by patch-clamp experiments performed on the apical membrane of isolated rat CCD (45). These results, therefore, suggest that vasopressin induces translocation of ENaC from the intracellular pool to the apical plasma membrane. Moreover, Ecceibarger et al. (18) demonstrated that chronic vasopressin infusion in naturally vasopressin-deficient Brattleboro rats resulted in significantly increased abundances of all three ENaC subunits, whereas 7-day water restriction in Sprague-Dawley rats induced significantly increased abundances of only β- and γ-ENaC. The increase in γ-ENaC (sum of both 85- and 70-kDa forms) and β-ENaC subunit in the present study therefore is consistent with an effect of vasopressin (18). Also, increased apical targeting of β- and γ-ENaC in the cortical and medullary collecting duct may partly be attributed to the increased plasma vasopressin concentration. It has been known that aldosterone and vasopressin appear to act synergistically with regard to sodium transport (26, 43). Thus the upregulation and increased apical targeting of ENaC subunits in PAN-induced nephrotic syndrome may be the combined effects of increased circulating aldosterone and vasopressin.

Alternative Explanations for Sodium Retention and Dysregulation of ENaC in PAN-Treated Rats

Substantial evidence suggests that increased plasma aldosterone or vasopressin levels may not alone be involved in the sodium retention in experimental nephrotic syndrome: 1) adrenalectomy does not prevent PAN-induced ascites and renal sodium retention (50); 2) captopril, which decreases ANG II production, hence reduces adrenal aldosterone production, fails to induce natriuresis (40); 3) the blockade of aldosterone receptor does not alter urinary sodium excretion (15); and 4) PAN treatment induces similar sodium retention and ascites in vasopressin-deficient Brattleboro rats (13). In addition, in the unilateral model of PAN nephrosis, sodium retention occurs in the proteinuric kidney but not in the unaffected kidney (29). Thus these observations suggest that intrarenal local factors (rather than or in addition to circulating hormones) may be importantly involved in the sodium retention and, presumably, in the ENaC regulation in nephrotic syndrome. In addition to the described regulatory mechanisms, ENaC is also subject to regulation by the tubular sodium load (16), intraluminal flow rate (44), intracellular pH (39), and ANG II (9, 41). Further studies

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**Fig. 8. Semiquantitative immunoblots of kidney proteins prepared from cortex (A), ISOM (B), and inner medulla (C) in protocol 1.** In the cortex, there was reduced abundance of Na-K-ATPase α1-subunit, Na+/H+ exchanger type 3 (NHE3), Na+/K+-2Cl− cotransporter (BSC-1), and thiazide-sensitive Na+/Cl− cotransporter (TSC) in the PAN-treated rats. In the ISOM, the protein expression of Na-K-ATPase α1-subunit, NHE3, and BSC-1 was decreased. In contrast, in the inner medulla the protein abundance of Na-K-ATPase α1-subunit was unchanged compared with controls. Open bars, control; filled bars, puromycin. *P < 0.05.
will be needed to address which mechanisms are controlling the upregulation of ENaC subunits in PAN nephrosis.

**Upregulation and Increased Trafficking of ENaC Subunits in the Medullary Collecting Duct**

The renal tubule site of salt retention in experimental nephrotic syndrome has been determined by micropuncture and clearance studies to be beyond the DCT (7, 29). Current evidence suggests that the CCD is a primary site of salt retention in nephrotic syndrome (13, 15). However, it is not known whether the OMCD and IMCD participate in the sodium retention as well. A major finding in the present study is that upregulation and increased apical targeting of ENaC subunits were not restricted to the CCD but also found in the distal tubule, CNT, and medullary collecting ducts. Thus, in addition to the increased sodium reabsorption in the CCD, the medullary collecting duct might participate in the sodium retention in the nephrotic syndrome.

**Decreased Abundances of NHE3, BSC-1, TSC, and Na-K-ATPase in PAN-Treated Rats**

The abundance of α1-subunit of the Na-K-ATPase was markedly decreased in the kidney cortex and outer medulla, while unexpectedly remained unchanged in the inner medulla in PAN-induced nephrotic syndrome. These results are consistent with the observations in the adriamycin-induced nephrotic syndrome (19, 20). Immunoperoxidase microscopy for the Na-K-ATPase in the renal cortex showed that there was a dramatic decrease of labeling in the proximal tubule and thick ascending limb, whereas labeling in the CCD appeared to be conserved. The decreased abundance of Na-K-ATPase in the cortex demonstrated in the immunoblotting therefore appears to be mainly attributed to the decreased expression of the proximal tubule and thick ascending limb. In contrast to the reduced expression of multiple channels and transporters in the proximal tubule and thick ascending limb (published previously and in part here), we here demonstrate an increased expression and increased apical targeting of ENaC subunits and confirm the absence of reduced expression of Na-K-ATPase in the collecting duct. Thus this is likely to cause, at least in part, the sodium retention associated with PAN-induced nephrotic syndrome.

The observation that the protein abundances of NHE3 and BSC-1 were reduced in PAN-treated rats supports the view that the epithelial transport of the proximal tubule and thick ascending limb is impaired. Furthermore, in the present study, the immunolabeling of Na-K-ATPase in the proximal tubule and thick ascending limb was reduced. This finding is consistent with a previous micropuncture study in PAN-perfused kidney, which showed suppressed sodium reabsorption in...

Fig. 9. Representative immunoperoxidase labeling of Na-K-ATPase α1-subunit in the cortex in protocol 1. Compared with controls, immunolabeling for Na-K-ATPase in the proximal tubule (PT) and thick ascending limb (TAL) was weaker in PAN-treated rats (A, B). In CCD, staining for Na-K-ATPase antibody was comparable between 2 groups (C, D).
proximal convoluted tubules and thick ascending limb (29). Recently, it was demonstrated that NHE3 protein expression was reduced in rats with PAN-induced nephrotic syndrome (8). However, the authors also demonstrated that the immunoreactivity of NHE3 detected by an antibody that specifically recognizes the nonmegalin associated (referred to as the transporter competent pool of NHE3) was higher in PAN-treated rats. This may suggest the enhanced shift of NHE3 from an inactive pool to an active pool, and the total sodium transporter capacity of the brush-border membranes was not changed in PAN-nephrotic rats compared with controls despite the overall reduction in NHE3 expression (8). Thus a straightforward interpretation of these results would be that the lack of changes in sodium transport in the proximal tubule speaks against that proximal tubule NHE3 is important for sodium retention in nephrotic syndrome. The decrease in NHE3 and BSC-1, as observed in this study, may balance the decrease in glomerular filtration rate (GFR) that was observed, thus resulting in long-term glomerulotubular balance. Conversely, the decrease in GFR may have been dependent on the decrease in NHE3 and BSC-1 as a result of activation of the tubuloglomerular feedback mechanism. Either way, there appear to be coordinated changes in GFR and transporter abundance in the premacula densa segments that would serve to stabilize delivery of Na+ and water to the postmacula densa region as demonstrated in micropuncture studies (29).

Summary

The results demonstrate that rats with PAN-induced nephrotic syndrome had increased or sustained abundances of ENaC subunit expression and that there was increased apical targeting of ENaC subunits in the DCT2, CNT, and collecting duct segments. In contrast, the protein abundance of other sodium transporters expressed in the nephron at a site proximal to the CNT (i.e., NHE3, BSC-1, and TSC) was significantly reduced. This suggests that increased abundance and apical targeting of ENaC subunits in the distal nephron and collecting duct are likely to play a role in the development of sodium retention associated with PAN-induced nephrotic syndrome in rats.

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UPREGULATION OF ENaC SUBUNITS IN PAN NEPHROSIS


