Increased expression but not targeting of ENaC in adrenalectomized rats with PAN-induced nephrotic syndrome

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1The Water and Salt Research Center and 2Institute of Anatomy, University of Aarhus, Aarhus C, Denmark; 3Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Korea; and 4Institute of Clinical Medicine, Aarhus University Hospital, Aarhus N, Denmark

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De Seigneux, Sophie, Soo Wan Kim, Sophie C Hemmingsen, Jørgen Frøkiær, and Søren Nielsen. Increased expression but not targeting of ENaC in adrenalectomized rats with PAN-induced nephrotic syndrome. 2005. Am J Physiol Renal Physiol 289:F208–F217, 2006. First published January 10, 2006; doi:10.1152/ajprenal.00399.2005.—Sodium retention is a hallmark of nephrotic syndrome (NS). The collecting duct (CCD) is increased in experimental nephrosis models (7, 12). The sodium epithelial channel (ENaC) is also increased in expression and apically targeted in the connecting tubule (CNT) and the collecting duct in puromycin aminonucleoside (PAN) nephrosis (23) under high aldosterone levels. Moreover, there is also an increase in the abundance of the 70-kDa band of the γ-subunit of the ENaC in PAN animals (23). Thus at the molecular level, sodium retention in the PAN nephrotic syndrome model is associated with upregulation and increased targeting of ENaC and increased activity of Na-K-ATPase in the collecting duct. This is coherent with the fact that treatment with amiloride, a drug known to inhibit ENaC activity, is successful in inhibiting edema formation in experimental nephrosis (9). However, the triggering events leading to these molecular observations are still unknown. It has been hypothesized that there is a primary kidney defect, based on observations in unilateral nephrotic kidney models, where only the kidney injected with PAN retains sodium (18). However, the importance of systemic factors, such as aldosterone, on sodium retention is still a matter of debate.

Systemic and local factors are known to regulate ENaC abundance, targeting, and activity as well as Na-K-ATPase activity. These include aldosterone (16, 25), vasopressin (11, 25, 26), and ANG II (4). Aldosterone is known to increase Na-K-ATPase activity in the CCD (16) and to increase ENaC redistribution to the apical membrane (25) of CNT and collecting duct principal cells and to promote the expression of a 70-kDa form of the γ-subunit of the Na-K-ATPase. In conclusion, PAN treatment in the absence of aldosterone induced sodium retention, increased ENaC expression, but did not change the subcellular distribution of ENaC. This indicates that the previously observed enhanced apical targeting of ENaC in PAN-induced NS (Kim SW, Wang W, Nielsen J, Praetorius J, Kwon TH, Knepper MA, Frøkiær J, and Nielsen S. Am J Physiol Renal Physiol 286: F922–F935, 2004), whereas the global expression of the α1-subunit of the Na-K-ATPase was unchanged. In conclusion, PAN treatment in the absence of aldosterone induced sodium retention, increased ENaC expression, but did not change the subcellular distribution of ENaC. This indicates that the previously observed enhanced apical targeting of ENaC in PAN-induced NS (Kim SW, Wang W, Nielsen J, Praetorius J, Kwon TH, Knepper MA, Frøkiær J, and Nielsen S. Am J Physiol Renal Physiol 286: F922–F935, 2004) is caused by aldosterone and that development of sodium retention can occur in the absence of aldosterone in NS.

epithelial sodium channel; sodium retention; aldosterone; AQP2; vasopressin

SODIUM RETENTION IS A CARDINAL feature of nephrotic syndrome. The cause of this is still incompletely understood. Micropuncture studies have designated the collecting duct as a major site for sodium retention in experimental nephrosis (18). Accordingly, the activity of the Na/K pump in the cortical collecting duct (CCD) is increased in experimental nephrosis models (7, 12). The sodium epithelial channel (ENaC) is also increased in expression and apically targeted in the connecting tubule (CNT) and the collecting duct in puromycin aminonucleoside (PAN) nephrosis (23) under high aldosterone levels. Moreover, there is also an increase in the abundance of the 70-kDa band of the γ-subunit of the ENaC in PAN animals (23). Thus at the molecular level, sodium retention in the PAN nephrotic syndrome model is associated with upregulation and increased targeting of ENaC and increased activity of Na-K-ATPase in the collecting duct. This is coherent with the fact that treatment with amiloride, a drug known to inhibit ENaC activity, is successful in inhibiting edema formation in experimental nephrosis (9). However, the triggering events leading to these molecular observations are still unknown. It has been hypothesized that there is a primary kidney defect, based on observations in unilateral nephrotic kidney models, where only the kidney injected with PAN retains sodium (18). However, the importance of systemic factors, such as aldosterone, on sodium retention is still a matter of debate.

Systemic and local factors are known to regulate ENaC abundance, targeting, and activity as well as Na-K-ATPase activity. These include aldosterone (16, 25), vasopressin (11, 25, 26), and ANG II (4). Aldosterone is known to increase Na-K-ATPase activity in the CCD (16) and to increase ENaC redistribution to the apical membrane (25) of CNT and collecting duct principal cells and to promote the expression of a 70-kDa form of the γ-subunit of the Na-K-ATPase, thought to be linked to activation of the channel by proteolytic cleavage (25, 31). It is therefore tempting to link this hormone with the findings observed in PAN nephrosis. Moreover, levels of vasopressin, ANG II, and aldosterone are increased in the initial phase of nephrosis (17, 23, 29), leading to the hypothesis that they may play significant roles in the development of sodium retention. As the time course and magnitude of sodium retention are indistinguishable in nephrotic Brattleboro rats (vasopressin deficient) compared with normal nephrotic rats (7), vasopressin is not considered to be a major factor in the development of sodium retention in nephrotic syndrome. We have also demonstrated that ANG II AT1 receptor blockade did not improve the sodium retention associated with PAN-induced nephrotic syndrome nor the time course of its development (de Seigneux S, Kim SW, Frøkiær J, and Nielsen S, unpublished observations). The situation is less clear for aldosterone. There have
been contradictory studies on the impact of aldosterone absence on the development of edema in nephrotic syndrome (6, 33). Furthermore, even though pharmacological blockade of aldosterone has been proven to be unsuccessful regarding sodium retention in experimental nephrosis (9), this cannot rule out the effect of aldosterone, as pharmacological mineralocorticoid receptor blockade has been demonstrated to be useless against ENaC trafficking (27) and would not avoid the action of aldosterone through the glucocorticoid receptor (15). Finally, the Na/K pump in the collecting duct of nephrotic rats is still activated in the absence of aldosterone (33). However, nothing is known about ENaC regulation in that condition. It is therefore not known whether the enhanced ENaC targeting observed in puromycin nephrosis is a primary dysregulation in nephrotic syndrome or is secondary to the high aldosterone levels observed in this condition.

We therefore studied the development of nephrotic syndrome in adrenalectomized rats, aiming at defining the role of aldosterone in sodium retention and ENaC targeting observed in this pathological condition. We used immunoblotting, immunohistochemistry, as well as renal function parameters in adrenalectomized and dexamethasone-treated control (ADX) and PAN (ADX-PAN) rats. The purposes of this work were 1) to observe whether nephrotic syndrome can develop in the absence of aldosterone; 2) to determine whether the ENaC targeting observed in PAN nephrosis is present in the absence of aldosterone; 3) to determine whether dysregulation of the other sodium transporters are influenced by the lack of aldosterone; and 4) to correlate the changes in sodium transporters with possible changes in sodium retention.

METHODS

Experimental protocol. Experiments were performed using male Hannover-Wistar rats (240–250 g, Taconic-Møllegard Breeding Centre). The animal protocol was approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus, according to the licenses for use of experimental animals issued by the Danish Ministry of Justice.

An adrenalectomy was performed by bilateral flank incisions, visualization, and isolation and removal of the adrenal gland. At the time of the adrenalectomy, osmotic minipumps were filled with dexamethasone (Sigma D 1756, Sigma, St. Louis, MO) dissolved in DMSO (Merck, Hohenbrunn, Germany) and diluted with isotonic saline. The pumps were equilibrated with normal saline for 4 h before insertion. Under these conditions, the pumps delivered 1.2 μg dexamethasone·100 g body wt⁻¹·day⁻¹ to rats subcutaneously (sc) for 14 days. This dose has been demonstrated to maintain normal body weight gain, glomerular filtration rate, and fasting plasma glucose and insulin in adrenalectomized rats (30). The rats were then given 6 days to adapt to the lack of aldosterone and were allowed free access to saline and salt-depleted food during this period to avoid volume depletion. All rats were provided dexamethasone supplementation during the study. After 6 days, the rats were subjected to a single intravenous injection of PAN (180 mg/kg body wt, Sigma, St. Louis, MO) dissolved in DMSO and injected into the inferior caval vein or to the same amount of vehicle injection (sterile isotonic saline, Merck, Hohenbrunn, Germany). The injection was realized under light isoflurane (Forane; Abbott Laboratories, Gentofte, Denmark) anesthesia.

We performed clearance studies over the last 24 h. 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 flame photometry (Eppendorf FCM6341). The osmolality of urine and plasma was determined by freezing-point depression (model 3900, Advanced Osmometer, Advanced Instruments, Norwood, MA, and Osmomat 030-D, Gonotec, Berlin, Germany).

Semiquantitative immunoblotting. The dissected renal cortex/OSOM, inner stripe of outer medulla (ISOM), and IM 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, 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. All samples were added to an isolation solution and solubilized at 65°C for 15 min in SDS-containing sample buffer and then stored at −20°C. The total protein concentration was measured (Bio-Rad protein assay reagent kit, Bio-Rad Laboratory). To confirm equal loading of protein, initial gels were 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 RPN3023D, Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBS-T (80 mM NaH₂PO₄, 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 an antibody-antigen reaction were visualized with horseradish peroxidase-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) with an enhanced chemiluminescence system (ECL or ECL+) and exposure to photographic film (Hyperfilm ECL, Amersham Pharmacia Biotech). The band densities were quantified 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 AGFA scanner (ARCUS II). The labeling density was corrected by densitometry of corresponding Coomassie-stained gels.

Immunohistochemistry. A perfusion needle was inserted into the abdominal aorta of isoflurane-anesthetized rats, and the inferior caval vein was cut to establish an outlet. Blood was flushed from the kidneys with cold PBS (pH 7.4) for 15 s, before a switch 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 1 additional hour, followed by 3 × 10-min washes with 0.1 M cacodylate buffer, pH 7.4. The tissue was

1 In pilot experiments using PAN-treated adrenalecetomized animals without dexamethasone supplementation, we observed very high mortality. Thus dexamethasone supplementation was required in our model.
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 s rinses with PBS wash buffer, 10 min with PBS blocking-buffer containing 1% nonfat dry milk, and 0.2% gelatin and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO) for 1 h followed by 10-min rinses with PBS wash buffer, 10 min with PBS wash buffer containing 0.1% 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 0.1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with horseradish peroxidase-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 an 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% H2O2. Mayer’s hematoxylin was used for counterstaining and after dehydration coverslips were mounted with hydrophilic medium (Eukitt, Kindler, Freiberg, Germany). Light microscopy was carried out with Leica DMRE (Leica Microsystems). All sections from the control and experimental group were processed and immunolabeled simultaneously.

**Primary antibodies.** Rabbit polyclonal antibodies to the following renal sodium transporters were used: the type 3 Na/H exchanger (NHE3); the Na-K-Cl cotransporter (NKCC2, BSC-1); the thiazide-sensitive Na-CI cotransporter (NCC, TSC); and ENaC subunits β-ENaC and γ-ENaC (21, 22, 25). The antisera were affinity purified against the immunizing peptides as previously described (19, 20). The specificity of the antibodies has been demonstrated by showing unique peptide-ablatable bands on immunoblots and a specific labeling by immunohistochemistry. A rabbit polyclonal antibody against the α-ENaC subunit for immunoblotting was a gift from Pr. B. C. Rossier (Dept. of Pharmacology, University of Lausanne, Lausanne, Switzerland). A mouse monoclonal antibody against the Na-K-ATPase α1-subunit was kindly provided by Dr. D. M. Fambrough (Johns Hopkins University Medical School, Baltimore, MD). We also used a previously characterized rabbit aquaporin-2 (AQP2) antibody (28).

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

### RESULTS

Aldosterone-deficient rats developed nephrotic syndrome. ADX-PAN rats developed nephrotic syndrome as they presented with ascites, hypoalbuminemia, and massive proteinuria (dipstick positive 4+ in ADX-PAN rats compared with 0–1+ in ADX rats, Uristix, Bayer, Leverkusen, Germany) at 5.5 days after having been injected with puromycin compared with ADX rats (Table 1). Plasma aldosterone was undetectable in both groups of animals and was used as a marker of successful adenectomy (Table 1).

Urine sodium and potassium excretion were altered in ADX-PAN-treated rats. The rats were followed in metabolic cages throughout the study. Nephrotic rats manifested ascites from day 4.5 and were killed on day 5.5 in the presence of significant ascites. The sodium balance was positive from day 4 and stayed positive in PAN-ADX rats (Fig. 1).

ADX-PAN rats consumed significantly more saline toward the end of the experiment, and therefore the amount of saline given to the ADX control rats was increased to the same extent. On the second to last day of the experiment, there was a higher consumption of saline in the ADX-PAN-treated group than in the ADX group together with a clear decrease in urinary sodium excretion, leading to rapid ascites formation in the ADX-PAN group. However, on the day of death, when the urine was collected and analyzed, sodium intake was not significantly different between the groups, allowing for comparison. At the time of death, the sodium-to-creatinine ratio as well as the fractional excretion of sodium (FeNa) were lower in

<table>
<thead>
<tr>
<th>Table 1. Urine and plasma data</th>
<th>ADX</th>
<th>ADX-PAN</th>
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<tbody>
<tr>
<td>ADX</td>
<td>ADX-PAN</td>
<td></td>
</tr>
<tr>
<td>n = 8</td>
<td>n = 11</td>
<td></td>
</tr>
<tr>
<td>Urinary output (V), μl/min</td>
<td>33.6 ± 4.9</td>
<td>6.6 ± 1.5†</td>
</tr>
<tr>
<td>PNa, μmol/l</td>
<td>307 ± 2</td>
<td>&lt;100‡</td>
</tr>
<tr>
<td>PCr, μmol/l</td>
<td>11.5 ± 1.9</td>
<td>21.5 ± 2.9*</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
<td>2.8 ± 0.9</td>
<td>0.9 ± 0.3‡</td>
</tr>
<tr>
<td>PNa, mmol/l</td>
<td>134 ± 2</td>
<td>135 ± 2</td>
</tr>
<tr>
<td>PK, mmol/l</td>
<td>5.3 ± 0.1</td>
<td>4.9 ± 0.1*</td>
</tr>
<tr>
<td>Uosm × V, μmol/min</td>
<td>6.27 ± 0.04</td>
<td>12.8 ± 0.02‡</td>
</tr>
<tr>
<td>UsCr/UcCr</td>
<td>217 ± 17</td>
<td>72 ± 9‡</td>
</tr>
<tr>
<td>FeNa, %</td>
<td>2.0 ± 0.4</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>FeK, %</td>
<td>9.2 ± 0.4</td>
<td>13.7 ± 1.4</td>
</tr>
<tr>
<td>T/H2O/Cr, × 100, %</td>
<td>0.7 ± 0.2</td>
<td>1.3 ± 0.2*</td>
</tr>
<tr>
<td>Uosm</td>
<td>5.3 ± 0.2</td>
<td>2.3 ± 0.3‡</td>
</tr>
<tr>
<td>Uosm/mosmol/kgH2O</td>
<td>509 ± 39</td>
<td>841 ± 65‡</td>
</tr>
<tr>
<td>PNa/mosmol/kgH2O</td>
<td>293 ± 3</td>
<td>309 ± 2‡</td>
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<tr>
<td>U/PNa</td>
<td>1.8 ± 0.2</td>
<td>2.7 ± 0.2‡</td>
</tr>
<tr>
<td>Aldosterone, nmol/l</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Ascites, ml</td>
<td>Undetectable</td>
<td>21.5 ± 4.3‡</td>
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</table>

Values are means ± SE measured on the last day of the experiment. No. of rats: ADX, adrenalectomized; ADX-PAN, adrenalectomized and treated with puromycin; NHE3, renal sodium transporters were used: the type 3 Na/H exchanger; V, urine output/min; PNa, plasma concentration of sodium; PCr, plasma concentration of creatinine; Ccr, creatinine clearance; PNa, plasma concentration of sodium; PK, plasma concentration of potassium; Uosm × V, rate of urinary sodium excretion; UsCr/UcCr, ratio between urinary sodium and creatinine concentration; FENa, fractional excretion of sodium in urine; FEK, fractional excretion of potassium in urine; T/H2O/Cr, ratio between free water clearance and Ccr; Uosm/UcCr, ratio between urinary sodium and potassium concentration; U/PNa, urine osmolality; PNa, plasma osmolality; U/PNa, ratio between urine and plasma osmolality. *P < 0.05 compared with ADX. †P < 0.01 compared with ADX.
ADX-PAN animals compared with ADX animals (Table 1), consistent with relative sodium retention and with previous observations in normal PAN rats (7, 23). Thus ADX-PAN rats were able to develop relative sodium retention compared with their control ADX rats. Urinary sodium-to-potassium ratio was also significantly lower in ADX-PAN rats compared with ADX rats.

**Urinary concentration was maintained in PAN-ADX rats.**

On the last day of the experiment, urine osmolality as well as the urine-to-plasma osmolality ratio were increased in ADX-PAN rats compared with ADX rats (Table 1), indicating increased urine concentration. This is in contrast to normal PAN-treated rats, which are characterized by a maintained urine output and decreased urine osmolality (14, 23). Thus the urinary concentration capacity was partly maintained in ADX-PAN rats.

**ENaC abundance was increased in PAN aldosterone-deficient rats and type 2 11β-hydroxysteroid dehydrogenase abundance was unchanged.** Semiquantitative immunoblotting was performed to investigate whether the abundance of ENaC subunits was changed in ADX-PAN rats in the cortex/OSOM (Fig. 2A) and ISOM (Fig. 2B). There was a significant increase in the abundance of α-ENaC in cortex/OSOM and ISOM assessed by immunoblotting (Fig. 2, A and B). Also, β-ENaC abundance was slightly increased in cortex/OSOM and ISOM (Fig. 2, A and B). An increase was also seen for γ-ENaC in cortex/OSOM and ISOM. Notably, the 70-kDa band of the γ-ENaC was absent in the cortex and ISOM in ADX as well as PAN-ADX rats (Fig. 2, A and B).

As type 2 11β-hydroxysteroid dehydrogenase (11βHSD2) is also known to be important for ENaC regulation, we performed immunoblotting for this enzyme. Immunoblotting of 11βHSD2 in membrane fractions from kidney cortex revealed low levels, with no difference between the two groups (100 ± 11% in ADX rats vs. 114 ± 10% in ADX-PAN rats, \( P = 0.33 \)).

**Absence of enhanced ENaC targeting in ADX-PAN rats.** ENaC is subject to complex regulation, including regulation of expression of subunit abundance and of subcellular trafficking. We performed immunoperoxidase microscopy to examine whether there are changes in ENaC targeting (seen as changes in labeling in the apical plasma membrane domains) in ADX-PAN rats. To distinguish between the segments, we used morphological and localization criteria. CNTs are located in the cortical labyrinth only and present with cells that are not as tall as cells from the distal convoluted tubule (DCT) but taller and with a more apical nucleus compared with collecting duct cells. CDDs were chosen in the medullary rays only to avoid confusion and present with an epithelium that is flat in shape. To ensure that the changes we observe could be ascertained to the segments we define, we also performed double labeling with calbindin and ENaC subunits for confirmation.

Immunohistochemistry revealed that ENaC labeling was seen in late DCT (DCT2) and segments distal to DCT2 until the inner medullary collecting duct (IMCD) in both ADX and ADX-PAN rats. The β- and γ-subunit were diffusely present in the cytoplasm of the principal cells. There was no evidence of enhanced β- or γ-ENaC apical targeting in the ADX-PAN rats compared with ADX rats. The absence of enhanced targeting and increased global labeling intensity was apparent in CNT (Fig. 3, A and B), CCD (Fig. 3, C and D), and outer medullary collecting duct (data not shown) of ADX-PAN rats compared with ADX rats. Moreover, there was no enhanced targeting of the α-ENaC subunit in ADX-PAN rats compared with ADX rats along the collecting duct either (Fig. 4), and in both groups the labeling was very weak, although labeling in kidney sections from ADX-PAN rats was slightly stronger.

**Abundance of sodium transporters in PAN-treated rats.** To investigate whether there are changes in the abundance of key sodium transporters that may be involved in the development of sodium retention in ADX-PAN rats, we performed immunoblotting and immunohistochemical analyses of NCC, NHE3, NKCC2, and Na-K-ATPase. The abundance of NHE3 and NKCC2 was significantly decreased in ADX-PAN rats in cortex/OSOM (Fig. 5A) and ISOM (Fig. 5B) compared with ADX rats. Immunoperoxidase labeling was performed to assess whether there were regional changes or differences in the subcellular localization (Fig. 6). Immunoperoxidase microscopy revealed a global decrease in the abundance of all sodium transporters. There was variability with regard to staining intensity between kidney zones and kidney tubules, as previously described in PAN rats (3, 13). Representative pictures are shown for NCC in the cortex (Fig. 6, A and B), NHE3 in proximal tubules (Fig. 6, C and D), and medullary thick ascending limb (Fig. 6, E and F). NKCC2 abundance was globally decreased in both the cortical (not shown) and medullary thick ascending limb (Fig. 6, G and H). Assessed by immunoblotting (Fig. 5, A–C), the global abundance of the α1-subunit of the Na/K pump was not significantly changed in the cortex/OSOM, ISOM, and IM of ADX-PAN rats compared with ADX rats.

**AQP2 abundance was unchanged in ADX-PAN rats.** To understand the basis for the increased urinary concentration observed in ADX-PAN rats, we performed immunoblotting aiming at quantifying the relative abundance of AQP2 in both groups. AQP2 abundance was slightly increased in the renal...
cortex of ADX-PAN rats, whereas it was unchanged in ISOM and IM (Fig. 7).

**AQP2 trafficking was enhanced in ADX-PAN rats.** We performed immunohistochemistry to analyze the subcellular localization of AQP2 in kidneys from ADX and PAN-ADX rats. There were no apparent changes in AQP2 labeling of apical plasma membrane domains in CNT and collecting duct principal cells in the kidney cortex (Fig. 8, A and B) and outer medulla (data not shown) between the two groups. In the IM, there was markedly enhanced AQP2 labeling of apical and basolateral plasma membrane domains of IMCD principal cells in kidneys from ADX-PAN rats compared with ADX rats (Fig. 8, E–H).

**DISCUSSION**

PAN-treated ADX rats develop nephrotic syndrome with sodium retention and ascites. PAN-treated ADX rats developed nephrotic syndrome in the absence of aldosterone as they presented with hypoalbuminemia, albuminuria, and ascites. This is consistent with data from previous work showing...
sodium retention in nephrotic ADX rats and with data obtained in a unilateral model of nephrotic syndrome showing sodium retention exclusively in the affected kidney (18, 33). Thus aldosterone is not required for the development of nephrotic syndrome. It should be emphasized that the present study does not allow for an evaluation of whether aldosterone may have a role in the severity of sodium retention in PAN nephrosis. Thus it remains possible that aldosterone may potentiate or worsen sodium retention in the context of normal nephrotic rats.

In the present study, rats were killed 5.5 days after PAN injection when sodium retention is already at its peak in PAN nephrotic syndrome (7). The rats presented with a significant decrease in urinary sodium excretion and a significant decrease in FeNa. Due to the altered behavior of ADX rats regarding food and water intake and due to the fact that ADX rats are losing salt in their basal state, we were not able to perform a direct comparison with normal PAN nephrotic rats. However, we have previously performed experiments using the same strain of rats, with the same dose of puromycin injected into normal rats (23). In both settings, rats developed sodium retention, supporting the view that aldosterone is not a prerequisite for sodium retention. However, FeNa was not decreased to the same extent in ADX-PAN rats compared with ADX rats as it was in normal PAN rats (i.e., with aldosterone) compared with their controls (23). Obviously, it cannot be excluded that this is caused by aldosterone (and hence ENaC targeting). However, these are indirect comparisons and should be interpreted with caution as the experiments were not performed in parallel, allowing for confounding factors.

Interestingly, urine osmolality was increased, which is in contrast to observations in PAN rats that have not been subjected to adrenalectomy (i.e., with aldosterone), where urine osmolality is reduced (compared with normal control rats) and there is a marked decrease in urinary sodium excretion (23). In the present study, the urinary sodium-to-potassium ratio was markedly decreased in the PAN-treated ADX rats. This indicated that despite the absence of aldosterone, there is sodium relative to potassium retention in PAN-ADX rats compared with ADX rats.

Increased expression but absence of changes in the subcellular distribution of ENaC subunits in kidneys from PAN-ADX compared with ADX rats. Immunoblotting revealed an increase in ENaC subunit expression in response to PAN treatment of ADX rats compared with non-PAN-treated ADX rats. We analyzed the subcellular distribution of the subunits to see whether there was an enhanced apical targeting of ENaC in the absence of aldosterone. Immunohistochemical analyses revealed that in PAN-ADX rats the subcellular distribution of ENaC subunits was similar to that observed in kidneys from control ADX rats. This is in contrast to the previously observed enhanced ENaC subunit targeting in DCT, CNT, and CCD of Fig. 4. Immunoperoxidase microscopy of α-ENaC in CCD from ADX (A) and ADX-PAN (B) rats. Normal (with intact adrenal gland) PAN-treated rat sample is shown as positive control for marked apical targeting (C).

Increased expression but absence of changes in the subcellular distribution of ENaC subunits in kidneys from PAN-ADX compared with ADX rats. Immunoblotting revealed an increase in ENaC subunit expression in response to PAN treatment of ADX rats compared with non-PAN-treated ADX rats. We analyzed the subcellular distribution of the subunits to see whether there was an enhanced apical targeting of ENaC in the absence of aldosterone. Immunohistochemical analyses revealed that in PAN-ADX rats the subcellular distribution of ENaC subunits was similar to that observed in kidneys from control ADX rats. This is in contrast to the previously observed enhanced ENaC subunit targeting in DCT, CNT, and CCD of Fig. 5. Semiquantitative immunoblotting of samples form cortex/OSOM (A), ISOM (B), and inner medulla (IM; C) for sodium transporters. The relative abundance between the 2 groups is presented in the bar graph. ADX, open bars; PAN-ADX, filled bars. Abundance of the Na+/H+ exchanger type 3 (NHE3) and the Na+/K+-2Cl cotransporter (NKCC2; BSC1) is shown in cortex/OSOM (A) and ISOM (B). The α1-subunit of the Na+/K+-ATPase is shown in cortex/OSOM, ISOM, and IM (A, B, and C, respectively). *P < 0.05.
kidneys from PAN-treated rats having intact adrenal glands (23). Moreover, the 70-kDa band of the \( \gamma \)-ENaC subunit was absent in kidneys from PAN-ADX rats in contrast to what was previously observed in PAN animals (23). The presence of this band has previously been shown to correlate highly with cleavage and activation of the channel in response to aldosterone (25, 31). Thus the present observations suggest that the previously observed enhanced membrane targeting and cleavage (i.e., appearance of the 70-kDa band) of ENaC in PAN rats (non-ADX) are secondary to the high aldosterone level and not due to a primary kidney defect.

As indicated above, there was an increase in the abundance of all three ENaC subunits in kidneys from PAN-ADX rats. Increased abundance of all ENaC subunits has previously been observed in response to chronic vasopressin administration in vivo (11). The functional role of the observed increase in abundance of ENaC subunits for the development of sodium retention is difficult to assess. The major increase appears to be in the cytoplasmic pool of ENaC. A relative increase in the abundance of ENaC in the apical plasma membrane would nevertheless be expected due to the global upregulation observed. However, this cannot be proven or quantified by the methods used.
Vogt and Favre (33) have demonstrated that in the same setting (adrenalectomized rats) the Na-K-ATPase was activated in the collecting duct of kidneys from PAN-induced nephrotic rats in the absence of aldosterone and that it was proportionally even further activated in adrenalectomized rats treated with PAN compared with nonadrenalectomized rats treated with PAN. This could be compensating partly for the absence of increase in ENaC targeting. Thus the present results and the results of Vogt and Favre (33) (including functional data) indicate that the collecting duct still tends to reabsorb sodium and secrete potassium in the absence of aldosterone in puromycin-treated nephrotic animals. Increased activity of the Na/K pump and an increased amount of ENaC subunits may, in part, explain the reduced F_{\text{Na}} as well as the decreased urinary sodium-to-potassium ratio. The stimulus for this maintained activity of the Na-K-ATPase and increase in ENaC abundance in the absence of aldosterone is not clear (33). Vasopressin was demonstrated not to be responsible for this activation in normal PAN rats (7, 8). However, it cannot be excluded that in the absence of aldosterone vasopressin may, in part, stimulate the Na-K-ATPase and increase ENaC abundance. Other factors may also play important roles because inhibition of vasopressin action has only been demonstrated to affect sodium excretion in the acute setting in vivo (2). Moreover, results from experiments with unilateral PAN-induced nephropathy (13, 18) suggested that local factors are directly involved in the development of sodium retention or alter, to some extent, the sensitivity of the kidney to systemic hormones. Among local
factors, the role of 11βHSD2 has also been debated for the development of sodium retention in nephrotic syndrome. This enzyme oxidizes physiological glucocorticosteroids (corticosterone in rats) to impede their action on the mineralocorticoid receptor and therefore to increase their selectivity for the glucocorticoid receptor. Downregulation of this enzyme is described in experimental nephrosis (21). However, this enzyme may not account for the persistence of sodium reabsorption relative to potassium observed in the present study. Indeed, by performing an adrenalectomy the source of physiological mineralocorticoids as well as glucocorticosteroids was removed. Glucocorticoids were replaced by dexamethasone, which has high affinity for the glucocorticoid receptor (21 compared with 1 for cortisol) and a low affinity for the mineralocorticoid receptor (0.0094 compared with 1 for aldosterone) (10). Dexamethasone will therefore not react significantly with the mineralocorticoid receptor. Moreover, 11βHSD2 is unable to oxidize dexamethasone and is therefore unlikely to play a major role in the observed enhanced sodium relative to potassium retention (10). Regulation of 11βHSD2 may also have an effect on sodium retention in non-ADX rats but appear not to be essential for sodium retention. More studies are needed to establish whether puromycin treatment results in an increased sensitivity of kidneys to systemic hormonal changes and which local factors might influence sodium retention.

Urinary concentration was increased in PAN rats. It is well established that rats with PAN-induced nephrotic syndrome have a urinary concentration defect consistent with severe downregulation of AQP2 and AQP3 (1, 13). In the present study, ADX-PAN rats had reduced urine output with increased urine osmolality compared with control ADX rats and also increased the urine-to-serum osmolality ratio. This is in contrast to the observations in normal PAN rats (13, 23). Moreover, it has been demonstrated that expression of AQP2 and AQP3 is markedly decreased in nephrotic models (14, 23). Because plasma vasopressin levels have been shown to be elevated in experimental nephrotic syndrome (29), it is thought that there is some degree of escape from the effect of vasopressin in nephrotic syndrome. In the present studies where PAN rats were adrenalectomized, the rats did not have reduced AQP2 expression, and the targeting of AQP2 appears maintained and even increased in IMCD. We hypothesize that in the absence of aldosterone, the vasopressin resistance known to be a feature of nephrotic syndrome is less severe. The role of other hormones such as oxytocin cannot be ruled out either. The importance of dexamethasone supplementation in the partial correction of urinary concentration mechanisms can also be evoked. Dexamethasone was used to substitute for glucocorticoids after adrenalectomy. Glucocorticoids (dexamethasone) have been reported to be important in urinary concentration mechanisms (5). We substituted a similar dose in both groups, so the difference between groups cannot be accounted for by the supplementation. As we supplemented with a physiological dose of glucocorticosteroids, and as no depletion in glucocorticosteroid is known in nephrotic rats, dexamethasone supplementation would probably not play a major role in the improved urinary concentration in ADX-PAN rats compared with what is observed in normal PAN rats. Altogether, maintained expression and increased targeting of AQP2 are consistent with the observed increase in urinary concentration in puromycin-treated ADX rats.

Interestingly, we also observed apparent basolateral AQP2 staining in the medullary collecting duct of ADX-PAN rats. In normal PAN rats, as described in other type of nephrosis as well (14, 21), apical targeting is observed in the IMCD with scant basolateral staining visible only in the IMCD near the tip of the medulla and clearly less pronounced than in ADX-PAN rats. This apparent increase in basolateral labeling in ADX-PAN rats might be due to a higher proportion of apical targeting of the cytoplasmic aquaporins, leading to a visual increase in basolateral staining as described with vasopressin (24). It could also be due to basolateral trafficking, as has been described in response to acute vasopressin treatment of a medulla already exposed to vasopressin (32).

Dysregulation of sodium transporters. Na-K-ATPase protein abundance, known to be globally decreased in the cortex and medulla of non-nephrectomized nephrotic rats as a reflection mainly of proximal tubules and thick ascending limbs (2, 13, 23), was unchanged in ADX-PAN rats compared with ADX rats. It is, however, surprising as the apical sodium transporters such as NHE3, NKCC2, and NCC were down-regulated in the cortex and ISOM of our rats. The cause of downregulation of sodium transporters in nephrotic syndrome remains incompletely understood. One probable hypothesis is that the decrease in the sodium transporters observed is due to the decrease in glomerular filtration rate that is observed in PAN-treated rats. By a glomerulotubular balance mechanism, this would lead to a proportional decrease in sodium reabsorption in the proximal segments of the kidneys and could therefore explain the downregulation observed. This is supported by renal micropuncture studies in PAN-treated rats, where proximal sodium reabsorption is proportional to the filtered sodium (18). Another hypothesis is that the decrease in sodium transporter expression is partially dependent on mechanical factors due to the presence of protein casts (3, 13, 23). The latter would also provide an explanation for the tubule-to-tubule variation in the downregulation. Finally, this downregulation could be a type of compensating mechanism proximal to a hyperactive collecting duct (23). This could also be linked to vasopressin resistance as described earlier for AQP2, at least in the case of NKCC2 and Na-K-ATPase in thick ascending limb segment, as they are both regulated by vasopressin. Interestingly, in the absence of aldosterone the downregulation in PAN is not as severe, as also observed for AQP2.

In conclusion, ADX-PAN-treated rats developed significant sodium retention despite the absence of aldosterone. ENaC expression was enhanced in ADX nephrotic rats compared with ADX rats, whereas there were no detectable changes in ENaC targeting, strongly indicating that the enhanced ENaC targeting in non-nephrectomized PAN-treated rats is caused by aldosterone. The role of the increase in the abundance of ENaC subunits in sodium retention is unclear, and the results also strongly support the view that in the absence of enhanced ENaC targeting, other factors, presumably in the collecting duct, play a significant role in the development of sodium retention.

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