Increased apical targeting of renal ENaC subunits and decreased expression of 11βHSD2 in HgCl2-induced nephrotic syndrome in rats

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Nephrotic syndrome may develop as a result of primary diseases such as minimal-change disease or immune glomerulonephritis. Membranous glomerulonephritis (MGN) remains the most common cause of primary nephrotic syndrome in adults (8). A further reason for its importance is that ~25–50% of patients progress to end-stage kidney disease over 10 years (33). Thus MGN has a different and more progressive clinical course compared with the nonprogressive benign character of minimal-change nephrotic syndrome (33). HgCl2 has been known to induce a systemic autoimmune disease, including membranous nephropathy with IgG deposits. This nephropathy is responsible for the development of high-range proteinuria and full-blown nephrotic syndrome associated with generalized edema and ascites (11).

Our laboratory demonstrated in a previous study that puromycin aminonucleoside (PAN)-induced nephrotic syndrome was associated with increased abundance and apical targeting of epithelial sodium channel (ENaC) subunits, which was proposed to play a role in the development of sodium retention (20). However, it remains to be elucidated whether the changes in expression and plasma membrane targeting of ENaC subunits in PAN-induced minimal-change nephrotic syndrome (caused by podocyte injury, not immune complex mediated) is unique to the PAN model or may be a more general characteristic of nephrotic syndrome, including immune complex-mediated glomerulonephritis. Furthermore, the physiological role of changes in subcellular distribution of ENaC subunits remains largely undefined in the abnormal sodium retention and ascites formation during disease states, and further studies may aid our understanding of molecular mechanisms of sodium retention.

11β-Hydroxysteroid dehydrogenase type 2 (11βHSD2) specifically modulates access of aldosterone to the mineralocorticoid receptor (MR) in a physiological environment where there is a molar excess of cortisol, and cortisol and aldosterone have similar affinity for the MR (3). Thus 11βHSD2 normally protects the MR from the mineralocorticoid actions of cortisol by converting cortisol to cortisone, which has a low affinity for the MR, allowing aldosterone to be the major mineralocorticoid (17). This hypothesis has received considerable support from in vitro experiments (5, 24) and from the observation that individuals with mutations of the gene encoding 11βHSD2 display the syndrome of apparent mineralocorticoid excess, a condition characterized by sodium retention, hypertension, and hypokalemia with decreased plasma aldosterone levels (35).
Reduced activity of 11βHSD2 causes renal sodium retention, and there is growing evidence that such a downregulation of 11βHSD2 accounts, at least partly, for abnormal sodium retention in disease states such as liver cirrhosis (1, 14). If this mechanism is operative in nephrotic syndrome rats, collecting duct sodium reabsorption might well be stimulated. This is also an important subject to study.

The vasopressin-induced water permeability as well as the translocation of aquaporin-2 (AQP2) from the intracellular vesicle to the apical plasma membrane in the collecting duct principal cells is well characterized, and there is an extensive axial heterogeneity in the subcellular localization of AQP2 along the connecting tubule (CNT) and collecting duct in different physiological settings (9). Nephrotic syndrome is characterized by an extracellular fluid volume expansion due to renal sodium and water retention. Thus, future investigations need to focus on defining the subcellular and segmental localization of AQP2 in nephrotic syndrome, which may play a role in the increased extracellular fluid volume expansion.

The purpose of this study is therefore to investigate whether immune complex-mediated nephrotic syndrome induced by HgCl2 in Brown Norway rats is associated with altered regulation of ENaC subunit and 11βHSD2 as well as AQP2 to elucidate the underlying molecular mechanisms responsible for the increased sodium and water retention.

METHODS

Experimental Protocols

The animal protocols have been 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.

Protocol 1 (HgCl2 experiment). Male Brown Norway rats (Elevage Janvier, Le Genest-St.-Isle, France) were used to develop experimental nephrotic syndrome. HgCl2 nephropathy (n = 9) was induced in Brown Norway rats by repeated injections of HgCl2 (Sigma) at a dose of 1 mg/kg body wt, diluted to 1 mg/ml in 0.9% NaCl; subcutaneous injections into the abdominal skin were administered on days 0, 2, 4, 7, 9, and 11. Control rats (n = 7) received the vehicle alone (i.e., sterile 0.9% saline). They were maintained in individual cages on a standard rodent diet (Altromin 1324, Lage, Germany) and allowed free access to drinking water at all times. Control rats were offered the standard rodent diet (Altromin 1324, Lage, Germany) in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupetin, 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. 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 they were 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 followed by incubation with secondary anti-rabbit (P448, DAKO, Glostrup, Denmark), anti-mouse (P447, DAKO), or anti-sheep (1713–035-147, Jackson Laboratories, Bar Harbor, ME) horseradish peroxidase-conjugated antibodies. The labeling was visualized by an enhanced chemiluminescence system (ECL or ECL+plus) and exposure to photographic film (Hyperfilm ECL, Amersham). ECL films were scanned using an AGFA scanner (ARCUS II).

Immunohistochemistry

A perfusion needle was inserted in the abdominal aorta of isoflurane-anesthetized rats, and the vena cava 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 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 EDTA) for 10 min. After the sections were cooled, nonspecific binding was blocked with 50 mM N-HCl 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 for 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 Ig, 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% H2O2. Mayer’s hematoxylin was used for counterstaining, and after dehydration coverslips were mounted with hydrophobic medium (Eukitt, Kindler, Freiburg, Germany). Light microscopy was carried out with a Leica DMRE (Leica Microsystems).

Primary Antibodies

Rabbit polyclonal antibodies to the following renal sodium transporters were utilized (20): type 3 Na/H exchanger (NHE3), N-K-2Cl cotransporter (NKCC2), thiazide-sensitive Na-CI cotransporter (NCC), and ENaC subunits α-ENaC, β-ENaC, and γ-ENaC. The polyclonal antibodies used in the study detect the uncleaved forms of the ENaC subunits (22). In addition, we previously used characterized rabbit polyclonal antibodies to AQP2 (26) and pAQP2 (10). A sheep polyclonal antibodies used in the study detect the uncleaved forms of −ENaC, −ENaC, and −ENaC. The α-subunit was kindly provided by Dr. D. M. Fambrough, Johns Hopkins University Medical School (Baltimore, MD).

Statistical Analyses

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

Table 1. Changes in renal function

<table>
<thead>
<tr>
<th>Control (n = 7)</th>
<th>MGN (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>219 ± 2</td>
</tr>
<tr>
<td>UO, mlday</td>
<td>11.6 ± 0.8</td>
</tr>
<tr>
<td>PNa, mmol/l</td>
<td>361 ± 8</td>
</tr>
<tr>
<td>PNa, mmol/l</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>PCr, mmol/l</td>
<td>34.4 ± 2.0</td>
</tr>
<tr>
<td>CO2, ml/min</td>
<td>1.06 ± 0.11</td>
</tr>
<tr>
<td>UNa, mmol/mmol</td>
<td>135.9 ± 0.3</td>
</tr>
<tr>
<td>Pk</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>UNa × UO, mmol</td>
<td>1.71 ± 0.09</td>
</tr>
<tr>
<td>UNa × UO, mmol</td>
<td>33.8 ± 2.0</td>
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<tr>
<td>Sodium balance, mmol/day</td>
<td>0.17 ± 0.08</td>
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<tr>
<td>FEK, %</td>
<td>0.87 ± 0.09</td>
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<tr>
<td>FEK, %</td>
<td>42.1 ± 3.47</td>
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<tr>
<td>UrineNa/K</td>
<td>0.50 ± 0.02</td>
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<tr>
<td>Uosm, mosmol/kgH2O</td>
<td>1.556 ± 0.85</td>
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<tr>
<td>Posm, mosmol/kgH2O</td>
<td>302 ± 2.2</td>
</tr>
<tr>
<td>UPosm</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>T H2O, µl/min/kg</td>
<td>148 ± 6</td>
</tr>
<tr>
<td>Ascites, ml</td>
<td>Nondetectable</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/ml</td>
<td>452 ± 37</td>
</tr>
</tbody>
</table>

Values are means ± SE measured on the last day of experiments (day 13). MGN, membranous glomerulonephritis induced by HgCl2 injection; UO, urine output; PNa, plasma sodium; PCr, plasma creatinine; CO2, creatinine clearance; PNa, plasma sodium; Pk, plasma potassium; UNa × UO, rate of urinary sodium excretion; sodium balance, the difference between dietary sodium intake and urinary sodium excretion in 24 h; UNa×CO2, sodium clearance; FEK, fractional excretion of potassium into urine; FEK, fractional excretion of sodium into urine; Uosm, urine osmolality; PNa, plasma sodium; T H2O, solute-free water absorption. *P < 0.05, †P < 0.01 compared with control.

RESULTS

Decreased Urinary Sodium Excretion and Fractional Excretion of Sodium in HgCl2-Induced Nephropathy

All HgCl2 nephropathic rats developed severe nephrotic syndrome characterized by marked proteinuria, hypoalbuminemia, and moderate amounts of ascites. Urinary protein excretion reached a +4 level from 10 days in HgCl2 nephropathic rats, whereas controls exhibited 0 or +1. Consistent with this, the plasma albumin level was significantly decreased in the HgCl2 nephropathic rats. Plasma urea and creatinine levels were increased, whereas creatinine clearance was decreased in HgCl2 nephropathic rats. In contrast, the urine output was not significantly different between two groups. Importantly, 24-h urinary sodium excretion and fractional excretion of sodium were decreased in HgCl2 nephropathic rats. Accordingly, HgCl2 nephropathic rats displayed a positive sodium balance. The urinary osmolality was not significantly different between the two groups. However, urine/plasma osmolality ratio and the solute-free water reabsorption were decreased, indicating decreased urinary concentration.

The urinary sodium/potassium ratio is widely used for evaluating aldosterone activity at the distal nephron (4). The urinary sodium/potassium ratio was markedly decreased in HgCl2 nephropathic rats, indicating increased aldosterone action in the distal nephron (Table 1). Accordingly, fractional excretion of potassium was increased. Consistent with this, plasma aldosterone levels were increased in HgCl2 nephropathic rats (Table 1).

Altered Protein Expression of ENaC Subunits

Semiquantitative immunoblotting was carried out to investigate whether the abundance of ENaC subunits changes in rats with HgCl2-induced nephrotic syndrome. The protein abundance of α-ENaC was increased in the cortex/OSOM and ISOM (Fig. 1). The protein abundances of β-ENaC and γ-ENaC were decreased in the cortex/OSOM but increased in the ISOM (Fig. 1). The analyses of normalized band densities are shown in Table 2.

Increased Targeted Expression of ENaC Subunits to the Apical Plasma Membrane in HgCl2-Induced Nephropathy

In HgCl2 nephropathy, γ-ENaC labeling was predominantly localized to the apical domains with only marginal labeling of the cytoplasm. This was evident in almost all cross-sectional tubules of distal convoluted tubule (DCT2) (Fig. 2A), CNT (Fig. 2D), cortical collecting duct (CCD) (Fig. 2F), and outer medullary collecting duct (OMCD) (Fig. 2H). In contrast, α-ENaC labeling in kidneys of control animals showed predominantly cytoplasmic labeling (Fig. 2, A, C, E, G).

Immunohistochemical analysis revealed similar changes in the subcellular distribution of α-ENaC and β-ENaC in kidneys from HgCl2 nephropathic rats. There was a marked increase in apical α-ENaC immunolabeling in DCT2 (not shown), CNT (Fig. 3, A and B), and collecting duct (not shown). Immunoperoxidase labeling of β-ENaC also showed markedly increased apical targeting in DCT2 (not shown), CNT (Fig. 3, C and D), CCD (Fig. 3, E and F), and OMCD (Fig. 3, G and H) in kidneys from HgCl2 nephropathic rats compared with control rats.

Decreased Protein Expression of 11BHS2 in HgCl2- and PAN-Induced Nephropathy

The protein abundance of 11BHS2 was decreased in the cortex/OSOM (52 ± 3 vs. 100 ± 6%, P < 0.05, Fig. 4, A and...
nolabeling for the Na-K-ATPase in the proximal tubule and inner medulla (Table 3). Compared with these controls, immunolabeling of Na-K-ATPase in the cortical and medullary collecting duct had similar labeling intensity in kidneys from HgCl2 nephropathic and control rats (Fig. 7).

As shown in Table 3, densitometric analysis revealed a marked decrease in NHE3 abundance in the renal cortex and ISOM, and a marked decrease in NKCC2 abundance in the cortex and ISOM. Consistent with this, immunocytochemistry demonstrated that the labeling of NHE3 in the proximal tubule (Fig. 8, A and B) and thick ascending limb (Fig. 8, C and D) was reduced in rats with HgCl2-induced nephrotic syndrome compared with control rats. This is consistent with the observed decrease in NHE3 protein abundance in cortex/OSOM and ISOM by immunoblotting. Moreover, the expression of NCC was also markedly decreased in the cortex (Table 3).

**Decreased Protein Expression of AQP2 and p-AQP2**

Semiquantitative immunoblotting revealed a marked decrease in AQP2 abundance in the inner medulla and ISOM, whereas in the cortex/OSOM a smaller decrease was seen (Fig. 9). Moreover, semiquantitative immunoblotting with antibodies that selectively recognize phosphorylated AQP2 (p-AQP2), which is phosphorylated in the PKA phosphorylation consensus site (Ser256) (10), demonstrated that the abundance of p-AQP2 was also decreased in the inner medulla, ISOM, and cortex/OSOM. Linear regression analysis revealed that there were significant correlations between AQP2 and p-AQP2 expression levels in cortex/OSOM (r = 0.78, P < 0.01), ISOM (r = 0.91, P < 0.01), and inner medulla (r = 0.88, P < 0.01) in control and HgCl2 nephrotic syndrome rats.

**Increased Apical Membrane Targeting of AQP2 and p-AQP2 in the Inner Medullary Collecting Duct**

In addition to the changes in protein abundance of AQP2, potential changes in trafficking of AQP2 and p-AQP2 were also examined by immunoperoxidase microscopy. There was a prominent difference in the subcellular localization of AQP2 in kidneys from HgCl2-induced nephrotic syndrome and control rats in the inner medullary collecting duct (Fig. 10, A and B). In HgCl2-induced nephropathy, AQP2 labeling was mainly associated with the apical plasma membrane domains with only marginal labeling of cytoplasmic domains (Fig. 10B). In contrast, AQP2 labeling in kidneys of control rats showed labeling throughout the cytoplasm and less prominent apical

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**Table 2. Summary of ENaC immunoblotting results**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>MGN (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex/OSOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.13</td>
<td>1.55±0.16*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.07</td>
<td>0.69±0.04*</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>1.00±0.08</td>
<td>0.57±0.04*</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00±0.12</td>
<td>1.52±0.12*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00±0.05</td>
<td>1.30±0.06*</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>1.00±0.07</td>
<td>1.27±0.11*</td>
</tr>
</tbody>
</table>

Values are means ± SE. ENaC, epithelial sodium channel; OSOM, outer stripe of the outer medulla; ISOM, inner stripe of the outer medulla. *P < 0.05 compared with control.
plasma membrane labeling (Fig. 10A). Immunohistochemistry also revealed similar changes in the subcellular distribution of p-AQP2 in kidneys from HgCl₂-induced nephropathy rats. There was a marked increase in apical p-AQP2 immunolabeling from HgCl₂-induced nephropathy rats (Fig. 10D) compared with control rats (Fig. 10C).

**Axial Heterogeneity in Changes in AQP2 Subcellular Localization**

Figure 11 shows the localization of AQP2 in the CNT, CCD, and OMCD. In CNT, AQP2 was localized in both apical and basolateral plasma membrane domains as well as intracellular vesicles in kidneys from controls (Fig. 11A) and HgCl₂ nephrotic syndrome rats (Fig. 11B). In CCD principal cells, immunolabeling of AQP2 is mainly associated with apical plasma membrane domains and very little labeling was observed in the basal part of the cell and the basolateral plasma membrane in controls (Fig. 11C). In contrast, AQP2 labeling was present in both apical and basolateral plasma membrane as well as intracellular vesicles in the principal cells of CCD in HgCl₂ nephrotic syndrome rats (Fig. 11D). The immunolabeling of AQP2 in the inner stripe of the OMCD was associated with the apical plasma membrane and intracellular vesicles, with less labeling in the basolateral plasma membranes in the controls (Fig. 11E). In HgCl₂-induced nephropathy, AQP2 labeling was mainly associated with the apical plasma mem-
brane domains, with only marginal labeling of cytoplasmic domains (Fig. 11F).

DISCUSSION

Rats of the Brown Norway strain, exposed to relatively low doses of HgCl₂, develop autoimmune responses to various antigens (6). In the present study, HgCl₂-treated Brown Norway rats displayed heavy proteinuria, markedly decreased fractional excretion of sodium, and development of ascites, indicative of development of full-blown nephrotic syndrome. The results demonstrated that HgCl₂-induced nephrotic syndrome was associated with 1) sodium retention, decreased urinary sodium excretion, development of ascites, and increased plasma aldosterone level; 2) increased apical targeting of ENaC subunits in DCT2, CNT, and collecting duct segments; and 3) decreased protein abundance of 11βHSD2.

Increased Apical Targeting of ENaC Subunits in HgCl₂-Induced Nephrotic Syndrome

The most important finding is the striking increase in apical targeting of ENaC subunits observed as increased immunolabeling in apical plasma membrane domains in the sodium-retaining nephrotic syndrome rats, suggesting that this contributes significantly to the increased renal tubular sodium reabsorption. This was seen in the DCT, CNT, CCD,

Fig. 3. Immunoperoxidase microscopy of α-ENaC and β-ENaC. HgCl₂ nephropathic rats showed markedly increased apical labeling of α-ENaC in CNT compared with control rats (A and B). Immunolabeling of β-ENaC was also markedly increased and redistributed to the apical plasma membrane in the CNT (C and D), CCD (E and F), and OMCD (G and H) in HgCl₂ nephropathic rats.
and medullary collecting duct. These observations, therefore, strongly support the view that the renal sodium retention associated with HgCl$_2$-induced nephrotic syndrome is caused by increased sodium reabsorption in the distal nephron, including the CNT and DCT. These results are consistent with the results of our previous studies of nephrotic syndrome induced by PAN (20). Thus a straightforward interpretation of these observations would be that increased apical targeting of ENaC subunits plays a role in the development of sodium retention in HgCl$_2$ nephrotic syndrome as well, and it could be argued that this mechanism in fact could be a general characteristic of nephrotic syndrome, including both minimal-change disease and immune complex-mediated glomerulonephritis.

**Increased Abundance of α-ENaC in HgCl$_2$-Induced Nephrotic Syndrome**

In the present study, the protein abundance of α-ENaC was increased whereas expression of β-ENaC and γ-ENaC was decreased in the cortex/OSOM. The changes in expression in the cortex/OSOM are consistent with the previously described pattern seen in response to aldosterone infusion (22), and because plasma aldosterone levels were significantly increased in HgCl$_2$-induced nephrotic syndrome, the observed changes in the subcellular distribution and protein expression of ENaC subunits would be consistent with this view. Curiously, we find that the regulation of ENaC subunit abundance is different in the outer medulla relative to the cortex. The protein expression...
of all ENaC subunits was increased in ISOM from HgCl2-induced nephrotic rats. This observation is similar to the changes in ENaC protein expression in response to chronic vasopressin infusion rather than aldosterone (12). Plasma vasopressin levels are increased in PAN nephrotic syndrome in rats (2, 31) and are presumably increased in HgCl2-induced nephrotic syndrome as well. The underlying mechanism for the segmental dysregulation of ENaC subunits remains obscure. However, it can be speculated that differences in sensitivity may be caused by differences in receptor expression patterns of aldosterone (7, 32), vasopressin (28) and 11βHSD2 (7, 32). The localization of aldosterone receptors indicates that high-affinity aldosterone receptors are most densely located in the distal tubule and CCD (15), and immunolabeling studies for 11βHSD2 demonstrated that the expression is most prominent in the CNT and CCD, with decreased labeling in medullary collecting duct segment (7, 32). Thus segmental differences in receptor expression could explain increased sensitivity to changes in circulating aldosterone levels in the cortex relative to the medulla, which could be more influenced by plasma vasopressin. We cannot rule out that the increased trafficking may be in part independent of aldosterone activation by the classic mineralocorticoid receptor. Nielsen et al. (25) recently demonstrated that the increased targeting of ENaC but not expression of ENaC in response to sodium restriction was insensitive to co-treatment with the mineralocorticoid receptor blocker spironolactone. The nature of such a receptor and the potential role for increased ENaC trafficking in nephrotic syndrome remains to be established.
It has been suggested that the activity of 11βHSD2 can regulate sodium reabsorption in aldosterone-responsive renal tubules by glucocorticoid-induced activation of MR (17, 34). We also demonstrate that there is downregulation of 11βHSD2 protein expression in the kidney in HgCl₂ nephropathy as well as in PAN nephrosis. This finding is in line with recent studies in nephrotic patients (38) and in experimental nephrotic syndrome induced by PAN or adriamycin (37). The investigators observed a rise in the plasma corticosterone/11-dehydrocorticosterone ratio as well as in the urinary (tetrahydrocorticosterone + 5α-tetrahydrocorticosterone)/11-dehydro-tetrahydrocorticosterone ratio in nephrotic syndrome (37) and interpreted this change as a result of decreased activity of 11βHSD2. Here, we propose that this may occur via downregulated protein expression of 11βHSD2 in nephrotic syndrome. These findings suggest that downregulation of 11βHSD2 provides access of glucocorticoids to the MR, resulting in increased activity of MR and collecting duct sodium retention in rats with nephrotic syndrome.

The underlying mechanisms for the regulation of 11βHSD2 are still unclear. Reduced activity of 11βHSD2 in vivo may possibly be caused by the presence of inhibitors, such as in bile acids in liver cirrhosis (30) or glycyrrhetinic acid-like factor in hypertension (23). The decreased activity of 11βHSD2 has also been demonstrated in response to potassium and acid loading (27, 36), and in the present study the nephrotic syndrome rats revealed high plasma potassium levels. Thus it is possible that hyperkalemia and/or potentially associated acidosis may explain the decreased activity of 11βHSD2. Further studies are needed to address which mechanisms are controlling the downregulation of 11βHSD2 in nephrotic syndrome.

### Decreased Protein Expression of 11βHSD2 in HgCl₂-Induced Nephrotic Syndrome

Apical targeting of ENaC subunits and protein abundance of α-ENaC can be regulated by aldosterone (21, 22). Indeed, plasma aldosterone levels were significantly increased in HgCl₂ nephropathy, and thus it can be speculated that aldosterone stimulates sodium reabsorption in HgCl₂ nephropathy. However, substantial evidence argues against a major role for aldosterone in nephrotic syndrome: 1) ascites and edema occur in rats with nephrosis, attributable to rabbit anti-rat kidney serum injection, without elevated aldosterone levels (19); 2) inhibition of angiotensin-converting enzyme by captopril failed to induce natriuresis in rats with PAN nephrosis despite decreased aldosterone levels (29); and 3) sodium retention was observed only in the affected proteinuria kidney in unilateral rat models of PAN nephrosis (18). These observations suggest that aldosterone may not alone be involved in sodium retention and presumably in ENaC regulation in nephrotic syndrome.

### Table 3. Densitometric analysis of sodium transporter expression

<table>
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<tr>
<th>transporter</th>
<th>Control (n = 6)</th>
<th>MGN (n = 8)</th>
</tr>
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<tbody>
<tr>
<td>Cortex/OSOM</td>
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<tr>
<td>Na-K-ATPase α1-subunit</td>
<td>1.00±0.10</td>
<td>0.67±0.10*</td>
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<tr>
<td>NHE3</td>
<td>1.00±0.09</td>
<td>0.48±0.07*</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00±0.08</td>
<td>0.22±0.04*</td>
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<tr>
<td>NCC</td>
<td>1.00±0.23</td>
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<tr>
<td>ISOM</td>
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<td>Na-K-ATPase α1-subunit</td>
<td>1.00±0.03</td>
<td>0.51±0.04*</td>
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<td>Nα-K-ATPase α1-subunit</td>
<td>1.00±0.17</td>
<td>0.94±0.14</td>
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</table>

Values are means ± SE. NHE3, type 3 Na/H exchanger; NKCC2, Na-K-2Cl cotransporter; NCC, NaCl cotransporter. *P < 0.05 compared with control.
pears to be mainly attributed to the decreased expression of the proximal tubule and the thick ascending limb. In contrast to the reduced expression of multiple channels and transporters in the proximal tubule and thick ascending limb, we here demonstrate an 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 HgCl₂-induced nephrotic syndrome.

An overall decrease in sodium excretion and a concomitant expansion of extracellular fluid volume are seen in this experimental model despite a decrease in expression of apical and basolateral sodium transporters in nephron segments at a site proximal to the connecting tubule (i.e., NHE3, NKCC2, Na-K-ATPase, and NCC), consistent with the previous findings seen in PAN (20)- and adriamycin-induced nephrotic syndrome (16). These findings suggest that downregulation of sodium transporters in the proximal nephron is a feature of nephrotic syndrome per se rather than being an idiosyncrasy of any particular model, and this may play a compensatory role against the sodium retention observed in HgCl₂-induced nephrotic syndrome.

Fig. 7. Immunoperoxidase microscopy of Na-K-ATPase α₁-subunit in the cortex (A and B) and ISOM (C and D). Compared with controls, immunolabeling for the Na-K-ATPase in the proximal tubule (PT) and thick ascending limb (TAL) was weaker in HgCl₂ nephropathic rats (A and B). In the CCD, staining for Na-K-ATPase antibody was comparable between 2 groups (A and B). In ISOM, immunolabeling of Na-K-ATPase in the medullary TAL was decreased, but unchanged in the medullary collecting duct, compared with controls (C and D).

Fig. 8. Immunoperoxidase microscopy of NHE3 in the PT and TAL of control rats and HgCl₂ nephropathic rats. In controls, NHE3 labeling was seen in apical plasma membrane domains of proximal tubule cells in cortex (A and B) and TAL in the ISOM (C), whereas the basolateral plasma membranes were unlabeled. In HgCl₂ nephropathic rats, the labeling of NHE3 in the proximal tubule and TAL was reduced (B and D) compared with control rats.
Enhanced Apical Plasma Membrane Targeting of AQP2 and p-AQP2 in the Medullary Collecting Duct Despite Decreased Abundance

The decreased protein abundance of AQP2 in HgCl2 nephropathy is consistent with the results of previous studies in adriamycin (16)- and PAN-induced nephrosis (2). The finding that the collecting duct water transport pathways are downregulated is consistent with the generally held view that the maintenance of water retention is not dependent on increased water reabsorption in the collecting duct but rather depends on increased NaCl absorption along the nephron. We speculate that the downregulation of AQP2 expression could represent a physiologically appropriate response to extracellular volume expansion, perhaps similar to the suppression of AQP2 that accounts for the vasopressin escape phenomenon (13).

Shuttling of AQP2 water channels into and out of the plasma membrane via exocytosis and endocytosis is the major means by which water transport in the collecting duct is regulated by vasopressin on a short-term basis (26). There was a decrease in the intensity of AQP2 and p-AQP2 labeling in the medullary

Fig. 9. Semiquantitative immunoblotting of aquaporin-2 (AQP2; A) and p-AQP2 (B) from control and HgCl2 nephropathic rats. Densitometric analyses revealed that the protein expression of AQP2 and p-AQP2 was significantly reduced in the ISOM and inner medulla, whereas in the cortex/OSOM a smaller decrease was seen in HgCl2 nephropathic rats compared with controls. *P < 0.05.

Fig. 10. Immunoperoxidase microscopy of AQP2 and p-AQP2 in the inner medullary collecting duct (IMCD) from control and HgCl2 nephropathic rats. A and B: IMCD from control rats showed diffuse cytoplasmic labeling of AQP2 with less prominent apical plasma membrane labeling, whereas IMCD from HgCl2 nephropathic rats exhibited AQP2 labeling which was mainly localized to the apical plasma membrane domains with marginal labeling of cytoplasm. C and D: immunocytochemical analyses revealed similar changes in the subcellular distribution of p-AQP2 in kidney from HgCl2 nephropathic rats. There was a marked increase in apical p-AQP2 immunolabeling in HgCl2 nephropathic rats (D) compared with control rats (C).
collecting ducts from HgCl₂ nephrotic rats compared with controls. However, the residual AQP2 and p-AQP2 can be seen to be located chiefly in the apical region of the principal cells, suggesting that trafficking mechanisms that deliver AQP2 to the plasma membrane are increased, not impaired. Therefore, we speculate that the short-term and long-term regulation of AQP2 occurs, in part, by independent mechanisms. The increased apical targeting of AQP2 may help to maintain the urinary concentration despite the decreased AQP2 abundance in nephrotic syndrome. It is noteworthy that there is a significant correlation between AQP2 and p-AQP2 expression levels, and p-AQP2 subcellular distribution tracks total AQP2 fairly well in both control and HgCl₂ nephrotic syndrome rats. Thus the effect of HgCl₂ nephropathy may be due to changes in the expression of AQP2 rather than effects from PKA phosphorylation-dependent regulation.

Our results demonstrated that there is an axial heterogeneity in the changes in AQP2 subcellular localization in HgCl₂ nephropathy compared with controls. Little AQP2 labeling of the basolateral plasma membrane was observed in CCD principal cells in controls. In this segment, a major increase in AQP2 expression in the basolateral plasma membrane was observed in HgCl₂ nephropathy, whereas in the CNT AQP2 subcellular localization was not affected. These changes in segmental and subcellular localization of AQP2 fit the previously described pattern seen in response to vasopressin infusion (9). From these observations, we hypothesize that vasopressin levels are high in this model, as in other models of nephrotic syndrome (2, 31) and that the observed downregulation of abundance of AQP2 without impairment of trafficking could be due to a vasopressin escape phenomenon (13). The underlying signaling mechanisms and its physiological roles remain uncertain, but the axial heterogeneity strongly suggests that cell-specific mechanisms are involved.

Summary

The results demonstrate an increased apical targeting of ENaC subunits combined with diminished abundance of 11βHSD2 in the DCT2, connecting tubule, and collecting duct. This is likely to play a role in sodium retention associated with HgCl₂-induced nephrotic syndrome in rats. Although it cannot be ruled out that the changes in 11βHSD2 are secondary to hyperkalemia and/or potentially associated acidosis, the results raise the possibility that downregulation of 11βHSD2 may, in part, play a role in increased activity of MR and collecting duct.

Fig. 11. Immunoperoxidase microscopy of AQP2 in CNT, CCD, and OMCD. AQP2 was localized in both apical and basolateral plasma membrane domains as well as intracellular vesicles in CNT in kidneys from controls (A) and HgCl₂ nephrotic syndrome rats (B). Interestingly, in CCD principal cells immunolabeling of AQP2 is mainly associated with apical plasma membrane domains, and very little labeling was observed in the basolateral plasma membrane in controls (C). In contrast, AQP2 labeling was present in both apical and basolateral plasma membrane as well as intracellular vesicles in the principal cells of CCD in HgCl₂ nephrotic syndrome rats (D). Immunolabeling of AQP2 in the inner stripe of outer medullary collecting duct was associated with the apical plasma membrane and the intracellular vesicles, with less labeling in the basolateral plasma membranes in the controls (E). In HgCl₂-induced nephropathy, AQP2 labeling was mainly associated with the apical plasma membrane domains, with only marginal labeling of cytoplasmic domains (F).
sodium retention in rats with nephrotic syndrome. The decreased abundance of sodium transporters expressed in the nephron at a site proximal to the CNT (i.e., NHE3, NKCC2, and NCC) may play a compensatory role in promoting sodium excretion.

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