Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity and hormones in single nephron segments from nephrotic rats

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SUMMARY

1. In the nephrotic syndrome the kidneys retain salt and water, which leads to oedema formation. The site of this sodium retention has been localized in the cortical collecting tubule by micropuncture studies. Whether or not this phenomenon is an intrinsic renal problem or is the consequence of changes in hormonal activities is still a matter of discussion.

2. Using the model of puromycin aminonucleoside-induced nephrotic syndrome in the rat, we measured Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in nephron segments from control and nephrotic rats and investigated the regulatory role of aldosterone and endogenous ouabain-displacing factor.

3. Nephrotic animals had a twofold increase in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in the cortical collecting tubule only (control versus nephrotic: 1065 ± 68 versus 2081 ± 274 pmol h\textsuperscript{-1} mm\textsuperscript{-1}, P = 0.036), which was not modified by adrenalectomy and was independent of the kidney content of endogenous ouabain-displacing factor. Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in the cortical collecting tubule correlated with the sodium balance in both control and nephrotic rats.

4. The data are consistent with the view that sodium retention in this model of the nephrotic syndrome is a primary event, i.e. an increase in sodium transport throughout the cortical collecting tubule expressed as a twofold increase in Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity which is no longer under hormonal regulation (aldosterone and endogenous ouabain-displacing factor).

INTRODUCTION

The nephrotic syndrome is associated with a disorder of volume regulation characterized by an avid salt retention [1, 2] leading to the development of oedema. Two theories have been proposed [2]. In the classical 'underfill' theory oedema is considered to be secondary to salt retention resulting from renal hypoperfusion. According to this theory the primary event is the decrease in plasma volume due to the diminution of plasma oncotic pressure resulting from hypoalbuminaemia. This causes transfer of fluid and electrolytes from the plasma to the interstitial space 'underfilling' the blood compartment and resulting in reduction of renal blood flow, stimulation of the renin-aldosterone system, diminution of urinary sodium elimination and finally extracellular fluid volume expansion. This mechanism applies mainly to the nephrotic syndrome associated with minimal change disease observed in children. By contrast, in most adults with the nephrotic syndrome due to minimal change disease or other glomerular lesions such as membranous or proliferative nephropathies, an initial plasma volume expansion is suggested by a normal renal blood flow, a low plasma renin activity, a low aldosterone level and the absence of stimulation of the sympathetic system which characterizes an 'overflow' status. Therefore the primary event responsible for the sodium retention should be a renal intrinsic excretory defect which leads to extracellular fluid volume expansion [3, 4].

Puromycin aminonucleoside (PAN)-induced nephrotic syndrome is a good experimental model of the overflow type. Micropuncture studies using this model [5] localized the sodium retention to the cortical collecting tubule (CCT). However, the mechanisms of this abnormal
sodium retention are not yet fully understood. Although alterations in the aldosterone system and the natriuretic hormones could contribute to the perturbation of the sodium homoeostasis in this setting, primary dysfunction of the epithelial renal cells may play an important role [6].

Na⁺,K⁺-ATPase plays a major role in active cation transport across the cell membrane, particularly in kidney cells [7–9]. Because changes in the Na⁺,K⁺-ATPase activity reflect alterations in transcellular sodium transport, we measured this enzyme activity in different nephron segments in PAN–induced nephrotic syndrome in rats [1,10]. To evaluate the participation of adrenal steroids in the regulation of renal Na⁺,K⁺-ATPase activity [11,12] in this disease model, the aldosterone system was stimulated by a low sodium intake or suppressed by adrenalectomy (ADX).

There is considerable evidence that a humoral natriuretic factor which displays ouabain-like properties may be an endogenous inhibitor of renal Na⁺,K⁺-ATPase and through this exerts its natriuretic effect [13,14]. To look for possible alterations of this natriuretic system in the nephrotic syndrome we extracted endogenous ouabain-displacing factor (EODF) from kidney tissue and measured it by a receptor-binding assay [15].

The aim of the present study of the nephrotic syndrome was: (1) to confirm biochemically the site of increased sodium retention along the nephron by measuring Na⁺,K⁺-ATPase activity, and (2) to evaluate whether or not Na⁺,K⁺-ATPase activity remains under hormonal regulation (aldosterone and EODF).

METHODS
Experimental design
Eleven experiments were performed in male Wistar rats (Physiopathology Institute, Bern, Switzerland). Rats entered the study with a body weight ranging from 100 to 140 g. They were kept in individual metabolic cages for 12 days, including a 4-day control period and an 8-day experimental period. Twenty-four hour urine samples were collected for measuring proteinuria and sodium excretion. Sodium balance was expressed as sodium excretion in per cent of sodium intake or in fractional and absolute terms for animals from group III (ADX) who had free access to salt. Animals were divided into three groups as described below.

Physiopathological model
The nephrotic syndrome was induced with one single intraperitoneal injection of PAN; Sigma; 15 mg/100 g body weight diluted in 1.0 ml of a 0.9% sodium chloride solution [1,10,16]. Control animals received a single intraperitoneal injection of the same vehicle.

Group I (sodium intake: 3 mmol/day). Animals received 3 mmol of NaCl a day added to their 30 ml of distilled drinking water, and were on a salt-deficient diet. The rats that were unable to drink this total amount of fluid were discarded. Six animals were made nephrotic as described above. Rats belonging to the control group \( (n = 6) \) were treated exactly the same way but they did not receive PAN. Development of the nephrotic syndrome was ensured by measurements of 24 h proteinuria and calculation of the sodium balance. On day 8 after injection the animals were killed.

Group II (sodium intake: 0.06 mmol/day). Animals were treated exactly like those in group I, except that their daily NaCl intake was 0.06 mmol. Animals were killed on day 8.

Group III (ADX). Twelve animals were adrenalectomized under light ether anaesthesia 6 days before entering the study. They had free access to a 0.9% (w/v) NaCl drinking solution and were fed a salt-deficient diet. The adequacy of ADX was assessed by the marked decrease in urinary potassium excretion observed 6 days after surgery. On day 6, half of the animals were injected with PAN to make them nephrotic and the other half were injected with vehicle. Six days later the animals were killed.

Chemical analysis
Urinary protein was determined by the method of Lowry et al. [17]. Sodium and potassium were measured by a flame spectrophotometer.

Isolation of nephron segments
On the experimental day, animals were anaesthetized with Nembutal (pentobarbitone, 50 mg/kg body weight intraperitoneally) for surgery. The left kidney was perfused in situ via the abdominal aorta with 4 ml of modified Hanks solution containing 137 mmol/l NaCl, 5 mmol/l KCl, 0.8 mmol/l MgSO₄, 0.33 mmol/l Na,HPO₄, 0.44 mmol/l KH₂PO₄, 1 mmol/l MgCl₂, 10 mmol/l Tris–HCl and 1 mmol/l CaCl₂, at pH 7.4, followed by an identical solution containing, in addition, collagenase (1.8 mg/ml, 0.87 unit/mg, Clostridium histolyticum; Serva, Heidelberg, F.R.G.) and BSA (2 mg/ml; Sigma, St Louis, MO, U.S.A.). The kidney was immediately removed at the end of perfusion. Small pieces of kidney tissue were cut along the corticomedullary axis and were incubated for 30 min at 30°C in the same buffer containing collagenase (0.4 mg/ml) and BSA (0.25 mg/ml). Segments of nephron were then dissected under a stereomicroscope in cold microdissection buffer in which collagenase and BSA were omitted and 0.25 mmol/l CaCl₂ was added. The cortical ascending limb (CALT) and cortical collecting tubule (CCT) were dissected out and characterized according to topographical and anatomical criteria. After dissection each segment of nephron was photographed to measure its length.

Measurement of Na⁺,K⁺-ATPase activity
Na⁺,K⁺-ATPase activity was measured in CAL and CCT segments after a two-step permeabilization–hypotonic shock procedure followed by congelation on dry ice, by the method of Doucet et al. [18] without modifica-
Determination of the number of cells per length unit of isolated segment

Enzyme activities are often referred to protein or DNA tissue content. For the single-nephron experiments, Na⁺,K⁺-ATPase activity is classically referred to the length of the tubule. To make sure that the DNA content per mm of tubule does not change in the pathological model compared with the normal rat, the number of cells per length unit was determined as described by Garg et al. [9]. Nephron segments were dissected in the same manner as for Na⁺,K⁺-ATPase determination and tubules were transferred to the hollow of a sunken bacteriological slide. Then they were incubated with Acridine Orange (20 μg/ml; Sigma) in the modified Hanks medium for 2 min, followed by washing with the modified Hanks medium alone. The dye stains nuclei which are then visualized by fluorescence microscopy. Pictures were taken using a black-and-white film. The number of nuclei per tubule length unit was determined by counting two to three areas of 0.1 mm of each tubule. The values of six to eight tubules were averaged. CAL and CCT segments from control (n=6) and nephrotic (n=6) rats in group I were used for this experiment.

Measurement of EODF in kidney extracts

The right kidney was removed to extract natriuretic material. The wet weight was recorded and the kidney was placed in 10 ml of double-distilled water adjusted to pH 3.4 with acetic acid. The solution was heated for 20 min at 65°C, and then the material was homogenized and centrifuged at 3000 rev./min for 25 min at 4°C. The supernatant was stored at -20°C until determination of EODF by a receptor-binding assay [15] using Na⁺,K⁺-ATPase prepared from the outer medulla of rabbit kidney as described by Jorgensen [19]. Before assay, the freeze-dried samples were extracted with methanol and desalted on a silica gel column with a mixture of chloroform and ethanol. The extracted fraction, containing no ammonium and less than 0.5 mmol/l potassium, possesses the natriuretic activity. The solution was incubated with [³H]ouabain for 60 min and the bound fraction was separated from the free ligand by filtration through a Millipore filter. The filters were washed with cold buffer solution, dissolved in Filter Count (Packard) and the radioactivity was counted. Standard curves were established using reference urine extracts and the results are given in nmol of the calculated active material content [15].

Statistics

All data are expressed as means ± SEM. Statistical analysis was by the Wilcoxon two-tailed test. When necessary analysis of variance (Kruskal–Wallis one-way non-parametric analysis of variance) were performed and correlation coefficients were determined.

RESULTS

Metabolic cage studies

Protein excretion. On the experimental day, the proteinuria of the nephrotic rats was significantly greater than that of the control animals (Fig. 1).

Sodium balance. Control rats from groups I and II excreted about 90% of their allocated sodium intake throughout the entire experimental period. Their paired nephrotic animals started to retain sodium after 4 days. On day 5 sodium retention reached 60% of the intake in rats from group I and 80% in those from group II. Rats from group III with free access to sodium went on negative balance immediately after ADX and then progressively returned to their basal sodium balance. During the 6-day experimental period, control ADX rats ingested 32.7 ± 3.7 and retained 10.5 ± 2.6 mmol of sodium, whereas nephrotic ADX rats ingested 28.1 ± 2.1 and retained 19.21 ± 1.9 mmol of sodium and developed oedema and ascites. On the experimental day the nephrotic animals in all three groups showed significant sodium retention compared with their paired controls (Fig. 1).

EODF

The tissue content of EODF corresponded to the sodium intake and/or the extracellular fluid volume in all the groups. However, there were no statistical differences in EODF activities among the control rats from the three groups (P=0.2). EODF activities in nephrotic rats were higher than those measured in control rats but did not reach statistical significance. EODF activity was not significantly different (P=0.07) among the nephrotic rats in the three groups (Table 1).

Na⁺,K⁺-ATPase activity

Group I. Preliminary experiments showed no difference in Na⁺,K⁺-ATPase activity in the proximal convoluted tubule (PCT) of nephrotic rats compared with that of the control animals (2900 ± 350 versus 2700 ± 230 pmol h⁻¹ mm⁻²; n=6). PAN-induced nephrotic syndrome did not alter Na⁺,K⁺-ATPase activity in the CAL segment. By contrast Na⁺,K⁺-ATPase activity was increased in the CCT in the nephrotic rats (Table 2).

Nephrotic rats and their controls exhibited an equal number of cells per mm tubule length in CAL and in CCT (Table 3). Na⁺,K⁺-ATPase activity in the CCT segment correlated with the amount of sodium retained during the 8-day experimental period (r=0.73; y=741 + 98x).

Group II. Animals on a low-salt intake had a higher basal Na⁺,K⁺-ATPase activity than that of group I rats. Here again Na⁺,K⁺-ATPase activity in the CCT was increased in the nephrotic rats. By contrast, there was no
Fig. 1. Twenty-four hour proteinuria (a) and sodium balance expressed as sodium excretion in per cent of sodium intake (b) in control (○) and nephrotic (●) rats. Day 0, before injection of PAN or vehicle; day 8 (group I and II) and day 6 (group III) before the rats were killed. Values are means with bars indicating SEM. Statistical significance: *P<0.05. Groups are defined in the Methods section.

Table 1. Kidney tissue content of EODF

<table>
<thead>
<tr>
<th>Group</th>
<th>EODF content (nmol/mg)</th>
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<tbody>
<tr>
<td></td>
<td>Group I (sodium intake: 3 mmol/day)</td>
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<tr>
<td>Control</td>
<td>1.566 ± 1.14</td>
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<tr>
<td>Nephrotic</td>
<td>1.986 ± 0.64</td>
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</table>

PAN has been used to induce the nephrotic syndrome in the rat [1, 10]. The efficacy of the procedure is demonstrated in this study for all the three groups by the development of proteinuria and salt retention over a 6-8 day period [1, 10, 16]. While the salt intake remained constant at the level defined by the protocol for the first two groups, ADX animals (group III) had a free salt intake to facilitate the maintenance of sodium balance in the absence of aldosterone. It is worth noting that nephrotic rats from group III reduced their salt intake 4-6 days after injection of PAN, when extracellular volume expansion appeared. By contrast, the ADX control rats maintained a stable salt intake of about 5 mmol/day throughout the study. The present data show, in this model of nephrotic syndrome, a significant increase in Na⁺,K⁺-ATPase activity [8, 20] occurring only in the CCT, which indicates this segment is the site of the sodium retention accompanying the nephrotic syndrome in agreement with micropuncture results [21] obtained in the same model. Both micropuncture studies and the present biochemical study failed to show any differences in sodium transport or in the activity of the sodium pump in other segments of the nephron, more specifically in the PCT, as ruled out by preliminary studies, and the CAL.

Since there was no significant change in the cell density in the CCT from nephrotic rats, the increase in Na⁺,K⁺-ATPase activity represents an increase in enzyme activity per cell [8] which correlates with the degree of salt retention. This indicates that in this model of the nephrotic syndrome sodium retention is due to an
Na⁺,K⁺-ATPase activity and hormones in nephrotic rats

Table 2. Na⁺,K⁺-ATPase activity in CAL and CCT segments

Values are means ± SEM (n = 6). Statistical analysis was by Wilcoxon's two-tailed test. Significant differences at P < 0.05 between control and nephrotic rats were seen only in the CCT for the three groups.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Na⁺,K⁺-ATPase activity (pmol h⁻¹ mm⁻¹)</th>
<th>P</th>
<th>CCT</th>
<th>Na⁺,K⁺-ATPase activity (pmol h⁻¹ mm⁻¹)</th>
<th>P</th>
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<td>Group I (sodium intake: 3 mmol/day)</td>
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</tr>
<tr>
<td>Control</td>
<td>5206 ± 476</td>
<td>1.000</td>
<td>1065 ± 68</td>
<td>0.0360</td>
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</tr>
<tr>
<td>Nephrotic</td>
<td>5236 ± 418</td>
<td></td>
<td>2081 ± 274</td>
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<tr>
<td>Group II (sodium intake: 0.06 mmol/day)</td>
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<tr>
<td>Control</td>
<td>5458 ± 395</td>
<td>0.1422</td>
<td>1544 ± 105</td>
<td>0.0360</td>
<td></td>
</tr>
<tr>
<td>Nephrotic</td>
<td>4592 ± 231</td>
<td></td>
<td>2840 ± 117</td>
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<td></td>
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<tr>
<td>Group III (ADX)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>4455 ± 376</td>
<td>0.0935</td>
<td>459 ± 71</td>
<td>0.0360</td>
<td></td>
</tr>
<tr>
<td>Nephrotic</td>
<td>3751 ± 307</td>
<td></td>
<td>1224 ± 103</td>
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</table>

Table 3. Number of cells per millimetre length in isolated nephron segments

Values are means ± SEM (n = 6). There were no statistically significant differences between the control and the nephrotic groups (control versus nephrotic: CAL, P = 0.5896; CCT, P = 0.8339; Wilcoxon's two-tailed test).

<table>
<thead>
<tr>
<th>Segment</th>
<th>No. of cells/mm length</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>CAL</td>
<td>283 ± 26</td>
</tr>
<tr>
<td>CCT</td>
<td>362 ± 32</td>
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increase in transcellular sodium transport that occurs selectively in the CCT.

Na⁺,K⁺-ATPase activity in the CCT is physiologically modulated both by hormonal factors and intracellular sodium concentration [7–9]. Among the hormones acting on Na⁺,K⁺-ATPase in this segment are mineralocorticoids and the putative EODF [22]. In our control ADX rats Na⁺,K⁺-ATPase activity is diminished by 70% in this segment, which is in agreement with prior reports [11,12]. However, ADX did not prevent the development of the nephrotic syndrome induced by PAN nor the two- to three-fold increase in Na⁺,K⁺-ATPase activity that was observed in nephrotic ADX rats as well as in those from groups I and II having normal or stimulated aldosterone secretion.

EODF is a putative hormone [13,14], the level of which has been reported to correlate directly with the extracellular fluid volume [22,23]. EODF is considered to be natriuretic as it inhibits Na⁺,K⁺-ATPase in distal segments of the nephron [4]. One may hypothesize that salt retention in the nephrotic syndrome is secondary to inadequate production of EODF resulting in a stimulation of Na⁺,K⁺-ATPase activity in the CCT and thereby of sodium retention. The present results are not in agreement with such a hypothesis, as nephrotic rats had the greatest tissue content of EODF together with the highest Na⁺,K⁺-ATPase activity in the CCT.

The present findings clearly indicate that the salt retention in this model of the nephrotic syndrome is the consequence of an increased reabsorption of sodium through the tubular cells of the CCT where the Na⁺,K⁺-ATPase activity is elevated and is no longer regulated by aldosterone nor responsive to EODF, although these two hormones mostly act on that segment. These findings favour the hypothesis of a primary intrarenal alteration in sodium transport [5,21] being responsible for the sodium retention that is characteristic of the nephrotic syndrome. Further work is necessary to elucidate the mechanisms by which Na⁺,K⁺-ATPase activity is stimulated.

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