Rapid and segmental specific dysregulation of AQP2, S256-pAQP2 and renal sodium transporters in rats with LPS-induced endotoxaemia

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Abstract

Background. Acute renal failure (ARF) is a frequent complication of sepsis. Characteristics of ARF in sepsis are impaired urinary concentration, increased natriuresis and decreased glomerular filtration rate (GFR), in which inducible nitric oxide synthase (iNOS) has been revealed to play a role.

Aims. We aimed to investigate renal water and sodium excretion and in parallel the segmental regulation of renal AQP2 and major sodium transporters in rats with acute

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LPS-induced endotoxaemia. Next, we aimed to examine the changes of iNOS expression and activated macrophage infiltration in the kidney and the effects of iNOS inhibition on AQP2 and NKCC2 expression in LPS rats.

Methods. Rats were treated with LPS (i.p.) or with LPS + iNOS inhibitor L-NIL, and 6 h later kidneys were subjected to semiquantitative immunoblotting and immunohistochemistry.

Results. Polyuria and increased natriuresis were seen 6 h after LPS injection alongside downregulation of both AQP2 and S256-phosphorylated AQP2 in CTX/OSOM and ISOM but not in inner medulla (IM). Thick ascending limb sodium transporters NHE3 and NKCC2 were downregulated in ISOM and NaP2 was decreased in CTX/OSOM, whereas NCC and ENaC were not consistently downregulated. Immunolabelling intensity of iNOS was increased in vascular structures and transitional epithelium, and an infiltration of activated macrophages was seen in CTX and ISOM. L-NIL co-treatment prevented the downregulation of NKCC2 but not AQP2 in LPS rats.

Conclusions. Early downregulation of AQP2 and sodium transporters takes place segmentally in the kidney after LPS administration. In addition, an infiltration of activated macrophages and increased iNOS expression may play a role in the urinary concentrating defect in acute LPS-induced endotoxaemia.

Keywords: acute renal failure; aquaporin; iNOS; LPS; urine concentration

Introduction

Acute renal failure (ARF) is a frequent complication of many diseases and an important independent risk factor for lethal outcome [1,2]. In experimental studies with LPS injections, glomerular filtration rate (GFR) has been shown to decline in the absence of changes in mean arterial pressure (MAP) as long as rats are unanaesthetized [3] or if a low dose of LPS is given [4]. Decreases in RBF are, however, frequently observed in the model [5–7]. The mechanism involved is thought to be paradoxical renal vasoconstriction in spite of a general systemic hyperdynamic state [8]. The described observations render the possibility that an intrinsic mechanism in the kidney participates in the reduction in GFR, for instance the onset of the tubuloglomerular feedback mechanism, which is known to take place when NaCl delivery to the macula densa is increased [9].

ARF is associated with a urinary sodium-losing condition, where dysregulation of major renal sodium transporters has been demonstrated to play a critical role in increased natriuresis, e.g. ischaemia and reperfusion injury and ureteral obstruction [10,11]. Previously we demonstrated that α-MSH treatment of rats with bilateral ischaemia-induced ARF significantly reduced the downregulation of AQP2 and major renal sodium transporter levels and that this was paralleled by functional improvement [10]. Since α-MSH is a potent anti-inflammatory agent that inhibits neutrophil migration and production of neutrophil chemokines and nitric oxide (NO) [12,13], it is likely that expression levels of renal sodium transporters, and hence urinary sodium excretion could be, at least in part, regulated by inflammatory conditions.

Increased fractional excretion of water has been described previously in the LPS model [3] and a role for AQP2 dysregulation could be considered [14]. Although AQP2 protein abundance and trafficking are regulated mainly by vasopressin, other regulators can also become involved, including inflammatory mediators [15–18]. Tubular segment-specific regulation, phosphorylation and trafficking of AQP2 in LPS-induced endotoxaemia have to our knowledge not previously been studied in detail.

During experimental endotoxaemia, inhibition of the inducible form of nitric oxide synthase (iNOS) has been demonstrated to be protective on the GFR [19]. NO has known natriuretic and diuretic effects [20], which from studies of TAL ion transport are presumed to involve inhibition of NKCC2 [21]. But both the involvement of iNOS in these actions and tissue localization of the protein are subjects of debate, as reviewed [22]. It has been shown recently that in an LPS model, increased NO levels in plasma are derived only from parenchyma and not from haematopoietic cells [23] although macrophages, when activated, are known to express iNOS [24]. However, macrophages also have abundant levels of superoxide which readily reacts with NO to form peroxynitrite, a far more toxic substance than NO [24]. These products might then be differentially implicated in the pathogenesis of septic ARF.

The aims of the present study were therefore to focus on early phases of experimentally induced septic ARF and to examine (1) segment-specific changes of AQP2 expression as well as phosphorylation and trafficking of AQP2; (2) segment-specific changes of protein expression, and subcellular localization of major renal sodium transporters; (3) expression of iNOS and infiltration of activated macrophages (i.e. iNOS expressing) and (4) the effects of iNOS inhibition on the regulation of AQP2 and NKCC2 protein expression in the kidney after LPS treatment. This will help to determine whether tubular dysfunction occurs at an early time point and could be the initial change leading to ARF in sepsis.

Subjects and methods

Experimental protocols

Experiments were performed using male 230–250 g Wistar-Hannover rats (Taconic Europe, Eiby, Denmark). 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 licensees for use of experimental animals issued by the Danish Ministry of Justice. During the entire experiment, there was a 12-h artificial light/dark cycle and a temperature of 21 ± 2°C.

Protocol 1: a time course experiment to collect functional data for 24 h after an intraperitoneal (IP) LPS bolus injection

Rats were placed in metabolic cages 3 days before the start of the experiment for acclimatization. On the day of the experiment, the rats were injected IP with either 300 µl saline (control) or LPS (Escherichia Coli serotype 0127 B8, L3129, Sigma-Aldrich, St. Louis, Missouri, USA) (2.5 mg/kg) in 300 µl of saline during light isoflurane anaesthesia. All rats also received an injection of buprenorphin (Temgesic, Schering-Plough, Brussels, Belgium) subcutaneously for pain relief. Twelve-hour urine samples were collected at the time of the IP injections (time 0), 3-h urine samples were collected for the following 12 h and finally 12-h urine samples were collected at the end of the experiment. Rats were killed and blood
was collected 24 h after the IP injections. During the experiment, they had free access to water, but in order to avoid differences in food intake they were not fed until 12 h after the IP injections, where they were given 10 g of food (normal rat chow Altromin #1320, Chr. Petersen A/S, Ringsted).

Protocol 2: LPS injections followed by kidney preparation at a relevant time point found in protocol 1

Acclimatization and IP injections were carried out as described in the time course experiment. Three-hour urine samples were collected at time 0 and again 3 and 6 h after the IP injections. At 6 h, during isoflurane anaesthesia, body temperatures were measured rectally (technic db-3B dri-block, Buch & Holm A/S, DK-2730 Herlev, Denmark), rats were killed, blood was collected and kidneys were either prepared for immunoblotting (eight rats in each group) or perfusion fixed for immunohistochemistry (four rats in each group). A pilot study had shown that water intake was not significantly different between control and LPS groups, but LPS rats did not eat. For this reason, rats had free access to water, but food was not given for the 6-h duration of the experiment.

Protocol 3: effects of N6-(1-iminoethyl)lysine (L-NIL) on AQP2 and NKCC2 regulation

Four groups of rats were injected with either saline, L-NIL (Alexis Biochemicals, San Diego, California, USA) and saline, saline and LPS or L-NIL and LPS according to protocols previously described by Schwartz et al. [19]. The rats then followed the same procedure as described in protocol 2.

Inhalation anaesthesia and sampling of blood and tissue

As previously described [25], all rats were anaesthetized with isoflurane and a large laparotomy was made. Blood was collected from the inferior vena cava. Kidneys were either rapidly removed, dissected into regions [cortex/outer stripe of outer medulla, inner stripe of outer medulla (ISOM) and inner medulla (IM)] and processed for immunoblotting described below, or perfusion fixed as described below.

Primary antibodies

Affinity-purified polyclonal rabbit anti-rat aquaporin-2 (H7661) was developed against the same sequence that we have used for LL127AP [26,27] and has recently been characterized [28]. An antibody previously characterized against the phosphorylated AQP2 (p-AQP2; phosphorylated in the PKA-phosphorylation consensus site Ser-256) [29] was used. Commericially available rabbit polyclonal antibodies against INOS (Transduction Laboratories, BD Biosciences, San José, California, USA and Santa Cruz, Santa Cruz, California, USA) and CD-68 (Serotec, Raleigh, North Carolina, USA) were purchased and used. The NaP2 antibody was developed and characterized previously [30]. Rabbit polyclonal antibodies to the following renal sodium transporters were used: the type 3 Na/H exchanger (NHE3); the Na-K-2Cl cotransporter (NKCC2, BSC-1); the thiazide-sensitive Na-Cl cotransporter (NCC, TSC); and ENaC subunits β-ENaC and γ-ENaC [31–33]. The antisera were affinity purified against the immunizing peptides as previously described [34,35]. 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 1-subunit was kindly provided by Dr. D. M. Fambrough (Johns Hopkins University Medical School, Baltimore, MD).

Semiquantitative immunoblotting

Methods routinely used in our laboratory were used as previously described [25] and PVDF membranes (Millipore, Billerica, MA 01821, USA) were used, on which antibodies were visualized with immunofluorescence-conjugated secondary antibodies (Alexa 680 goat anti-rabbit, Invitrogen, Carlsbad, California, USA) and scanned (Li-Cor, Odyssey infrared imager, Lincoln, Nebraska, USA). The blots were analysed using rolling ball background subtraction and subsequent band density quantification using Image J (NIH Image, National Institute of Standards and Technology, Gaithersburg, MD, USA). Results are presented as densities relative to the control group.

Immunohistochemistry

Kidneys were fixed by retrograde perfusion via the abdominal aorta with 4% paraformaldehyde in PBS. Immunolabelling was performed on sections from paraffin-embedded preparation (2 μm thickness) using methods described previously in detail [25,37].

Statistical analyses

Values are presented as means ± standard error of the mean. Two group comparisons were made by the unpaired t-test. P-values < 0.05 were considered significant. Comparisons between four groups were made by ANOVA followed by Tukey’s multiple comparisons test. Multiple comparisons tests were only applied when a significant difference (P < 0.05) was determined in the ANOVA.

Results

Time course experiment showed significant changes in urine flow rate and urinary sodium excretion at 6 h after LPS injection (protocols 1 and 2)

LPS rats had a higher urine flow rate and urine sodium-to-creatinine ratio 6 h after injections (Figure 1) compared to control rats at the same time point, with no difference in urine osmolality between the groups. At 9 h, urine osmolality was lower in LPS rats compared to controls with no significant difference in urine output or the ratio of sodium to creatinine (Figure 1). From this study, the time point of 6 h after LPS injection was chosen as the relevant point for all further experiments because of being the earliest time point where sodium excretion and urine flow were observed to be higher in the LPS group. In protocol 2, findings from protocol 1 were repeated (Figure 1) at the 6-h time point where the rats were killed.

Increased urine output and sodium excretion occurred alongside changes in AQP2 and sodium transporter expression (protocol 2)

At the end of the experiment, 6 h after IP injections, LPS rats appeared sick. By observation, movements and grooming were slight when compared to the control group. Moreover, they had a higher body temperature: 37.9 ± 0.1°C versus 36.7 ± 0.2°C in controls (P < 0.05).

Creatinine clearance was unchanged at 6 h (Table 1), indicating that GFR had not yet declined significantly in the LPS group at the time of immunoblotting analyses. Urinary excretion of both sodium and potassium was higher in the LPS group, as assessed by a higher rate of excretion, higher fractional excretion and lower plasma levels compared to controls (Table 1). Sodium loss appeared to be relatively higher than potassium loss in LPS rats compared to controls (Table 1), as assessed by a higher sodium-to-potassium ratio in urine, indicating low functional effect of aldosterone, although plasma aldosterone levels were significantly elevated in LPS rats (Table 1).

As the urinary concentration mechanisms appeared to be altered as assessed by the increased urine volume, we studied protein expression and trafficking of AQP2. Semiquantitative immunoblotting revealed decreased expression of the AQP2 protein in cortex and inner stripe of outer medulla (CTX/OSOM) in rats injected with LPS, whereas no difference in AQP2 protein expression was observed in the inner medulla (IM) (Figure 2). Protein expression of phosphorylated AQP2 (pAQP2, Ser 256) showed the same pattern and approximately the same levels of downregulation as
Renal AQP2 and sodium transporters in LPS-induced endotoxaemia

Fig. 1. Time course of urine output, urine osmolarity and Na/creatinine in protocols 1 and 2. In protocol 1, at 6 h after IP LPS injections, urine output and Na/creatinine were significantly increased in the LPS group \( (n = 4) \) compared to the control group \( (n = 4) \). Significant differences in Na/creatinine between the groups were also seen at 12 h and 24 h. Urine osmolality was lower in LPS rats at 9 h. From this experiment, 6 h was chosen for kidney preparation in protocol 2. The functional results from protocol 1 were reproduced in protocol 2 with increased urine output and Na/creatinine 6 h after LPS injections in the LPS group \( (n = 12) \) compared to controls \( (n = 12) \) with no difference in urine osmolality.

Table 1. Biochemical results from plasma and urine samples from rats in protocol 2

<table>
<thead>
<tr>
<th></th>
<th>Control ( (n = 12) )</th>
<th>LPS ( (n = 12) )</th>
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<tbody>
<tr>
<td>P-osmolality (mosm/kg(H_2)O)</td>
<td>304.00 ± 2.46</td>
<td>300.82 ± 2.38</td>
</tr>
<tr>
<td>P-Na (mM)</td>
<td>137.91 ± 0.55</td>
<td>135.64 ± 1.10*</td>
</tr>
<tr>
<td>P-K (mM)</td>
<td>4.45 ± 0.09</td>
<td>3.85 ± 0.10*</td>
</tr>
<tr>
<td>P-creatinine (µM)</td>
<td>16.27 ± 2.04</td>
<td>20.45 ± 1.14</td>
</tr>
<tr>
<td>P-urea (µM)</td>
<td>4.41 ± 0.31</td>
<td>6.83 ± 0.92*</td>
</tr>
<tr>
<td>P-albumin (µM)</td>
<td>375.91 ± 8.11</td>
<td>350.8 ± 13.29</td>
</tr>
<tr>
<td>P-aldosterone (pg/mL)</td>
<td>291.86 ± 52.04</td>
<td>602.88 ± 43.11*</td>
</tr>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>2.97 ± 0.34</td>
<td>2.76 ± 0.27</td>
</tr>
<tr>
<td>FeNa (%)</td>
<td>0.13 ± 0.02</td>
<td>0.61 ± 0.10*</td>
</tr>
<tr>
<td>FeK (%)</td>
<td>14.3 ± 1.9</td>
<td>32.9 ± 2.2*</td>
</tr>
<tr>
<td>U-Na X V (µmol/min)</td>
<td>0.45 ± 0.06</td>
<td>2.46 ± 0.49*</td>
</tr>
<tr>
<td>U-K X V (µmol/min)</td>
<td>1.62 ± 0.17</td>
<td>3.38 ± 0.27*</td>
</tr>
<tr>
<td>U-Na/U-K</td>
<td>0.28 ± 0.03</td>
<td>0.65 ± 0.10*</td>
</tr>
<tr>
<td>Uosm/Posm</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.2</td>
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P-Na, plasma sodium; P-K, plasma potassium; FeNa, fractional excretion of sodium; FeK, fractional excretion of potassium.

* \( P < 0.05 \).

the constitutive forms of AQP2, indicating no change in the degree of phosphorylation (Figure 2). Immunohistochemistry was performed to study the changes of subcellular localization of AQP2 and revealed more prominent AQP2 labelling along the apical plasma membrane of collecting duct principal cells in LPS rats (Figure 3).

Given the salt-losing state we observed, we looked into the regulation of sodium transporters. The main apical sodium transporter of the proximal tubule is the type 3 Na\(^+\)/H\(^+\) exchanger, NHE3. Expression of NHE3 in the proximal tubule was assessed by semiquantitative immunoblotting of homogenates from CTX/OSOM and showed no change in protein expression between the

Fig. 2. Segment-specific changes in protein expression of AQP2 and pAQP2. Semiquantitative immunoblotting of homogenized rat kidney tissue from the cortex and outer stripe of the outer medulla (CTX/OSOM), inner stripe of outer medulla (ISOM) and inner medulla (IM) obtained from control and LPS rats from protocol 2. (A) Representative immunoblot of samples from protocol 2; seven control and seven LPS samples incubated in an anti-AQP2 antibody. Bands are seen at \( \sim 27 \) kD and \( \sim 33–40 \) kD. (B) Densitometric analysis of immunoblot of samples from control (white) and LPS (black) rats shows a decrease in AQP2 expression in LPS rats in both CTX/OSOM and ISOM, but no change in AQP2 expression was seen in IM of LPS rats. (C) Representative immunoblot of samples from protocol 2; seven control and seven LPS samples incubated in a phospho-specific 256 Ser-pAQP2 antibody. (D) Densitometric analysis of immunoblot of samples from control (white) and LPS (black) rats shows a decrease in pAQP2 expression in LPS rats in both CTX/OSOM and ISOM, but no change in pAQP2 expression was seen in IM of LPS rats.
Altered expression of iNOS in the kidney and its role in the regulation of protein expression of renal AQP2 and NKCC2 were studied in LPS rats. A marked upregulation of iNOS appeared on immunoblots of kidney homogenates from all three kidney zones (Figure 8), and this was further demonstrated by immunoperoxidase labelling on kidney sections (Figure 9). Immunohistochemistry demonstrated that no iNOS labelling was observed in control rats (Figure 9A–C). In contrast, in LPS rats there was prominent iNOS labelling in the transitional epithelium of the calyx lining the papilla, ISOM and adjacent areas of CTX (Figure 9D–F); the first mentioned being in line with previous studies of mice challenged with high doses of LPS determining iNOS mRNA upregulation on the papilla surface 6 h after LPS administration [39] and iNOS protein upregulation on the papilla surface 8 h after LPS administration [23]. Our study also revealed subcellular localization of the protein in all kidney zones (Figure 9I).

Double labelling of iNOS and the macrophage-specific marker CD68 revealed iNOS expression in macrophages. iNOS-labelled macrophages (activated macrophages) were most abundantly present and most adjacent to kidney tubules in the ISOM, but were also seen in CTX located mostly to the glomeruli (Figure 9J–K), whereas iNOS-labelled macrophages were scarcely seen in the IM. Localization appeared to be more diffuse in the cytoplasm compared to the organized distribution observed in the transitional epithelium (Figure 9G–H). In contrast, CD68-positive cells without iNOS expression (non-activated macrophages) were most abundantly present in CTX and outer medulla and were also observed in the inner medulla with no clear difference between the groups as observed by light microscopy (Figure 9L). Moreover, we observed sporadic labelling of Bowman’s capsule of LPS rats and the endothelium of peritubular capillaries in all kidney zones of LPS rats. This localization of the iNOS protein in vascular structures fits well with known effects of iNOS on renal blood flow in an LPS model [40].
Fig. 4. Segment-specific changes in protein expression of sodium transporters in CTX and ISOM. Semiquantitative immunoblotting of homogenized rat kidney tissue from the cortex and outer stripe of the outer medulla (CTX/OSOM) and the inner stripe of outer medullar (ISOM) obtained from control and LPS rats from protocol 2 and micrograph showing immunolabelling of pNKCC2 on sections from control and LPS rats from protocol 2. (A) Representative immunoblots of CTX/OSOM samples from protocol 2; seven control and seven LPS samples incubated in a NaPi-2 antibody, NHE-3 antibody and NKCC2 antibody. (B) Densitometric analysis of immunoblots of CTX/OSOM samples from control (white) and LPS (black) rats shows marked downregulation of NaPi-2 and no changes in NHE3 and NKCC2 in LPS rats compared to controls. (C) Representative immunoblots of ISOM samples from protocol 2; seven control and seven LPS samples incubated in a NHE-3 antibody and NKCC2 antibody. (D) Densitometric analysis of immunoblots of ISOM samples from control (white) and LPS (black) rats shows decreased protein expression of NHE3 and NKCC2 in LPS rats compared to controls. (E) Representative micrographs showing immunoperoxidase labelling for pNKCC2 on kidney sections from control (I and III) and LPS (II and IV) rats. Micrographs are taken in kidney cortex (I and II) and inner stripe of outer medulla (III and IV) and pNKCC2 labelling in cortical and medullary thick ascending limbs is observed for assessment of protein expression. Micrographs show that pNKCC2 immunolabelling intensity appears unchanged in cortex of LPS rats compared to controls, whereas it is decreased in ISOM of LPS rats compared to controls. This was confirmed by blinded observation.

Effect of iNOS on AQP2 and NKCC2 abundances

As accumulation of macrophages and the most distinct changes in protein expression of AQP2 and especially sodium transporters appeared to be concomitant events taking place chiefly in the ISOM, we studied the effect of an iNOS inhibitor, L-NIL, on AQP2 and sodium transporter expression in the ISOM, which was administered in order to prevent production of both iNOS and peroxinitrite, a known cell toxin, in macrophages surrounding tubules in the ISOM. We observed a significant effect of L-NIL on NKCC2 protein abundance in the ISOM (Figure 10E and F), whereas AQP2 and pAQP2 were not significantly altered by L-NIL (Figure 10A–D).

Subcellular localization of AQP2 was studied and it was found that apical labelling compared to whole cell labelling appeared more prominent in LPS rats than other groups (control, L-NIL treated or L-NIL + LPS-treated group, Figure 10G). The difference in targeting between LPS and
L-NIL-LPS rats was confirmed by an observer blinded to the identity of the rat.

**Discussion**

The main findings of the present study were that the early effects of LPS-induced ARF include segment-specific downregulation of AQP2 and pAQP2 in cortex and outer medulla, NaPi2 in cortex and NKCC2 and NHE3 in outer medulla, coinciding with polyuria and natriuresis. Moreover, iNOS expression was significantly increased in response to LPS administration, and immunolabelling of the protein was seen in the transitional epithelium on the surface of the papilla and in the activated macrophages in the cortex and outer medulla of LPS rats. Importantly, administration of iNOS inhibitor, L-NIL, prevented the downregulation of NKCC2 expression in ISOM of LPS rats. These results suggest that a compound derived from iNOS, possibly in activated macrophages, may play a role for the segment-specific dysregulation of major renal sodium transporters in the early phase of LPS-induced ARF.

Segment-specific dysregulation of AQP2, but maintained apical targeting in the kidneys of rats with LPS-induced endotoxaemia

Regulation of AQP2 expression, phosphorylation of AQP2 and shuttling of AQP2 can be vasopressin dependent [29]
Fig. 6. Subcellular localization of ENaC subunits in kidney cortex. Representative micrographs showing immunoperoxidase labelling for β- and γ-ENaC on kidney sections from control (A and C) and LPS (B and D) rats. Pictures are taken in kidney cortex. In the control and the LPS group, labelling was observed in collecting duct principal cells primarily located to intracellular vesicles, and no obvious change in the subcellular distribution of ENaC subunits was observed.

Fig. 7. Segment-specific changes in protein expression of Na–K-ATPase. Semiquantitative immunoblotting of homogenized rat kidney tissue from the cortex and outer stripe of the outer medulla, (CTX/OSOM), inner stripe of outer medulla (ISOM) and inner medulla (IM) obtained from control and LPS rats from protocol 2. (A) Representative immunoblot of samples from protocol 2; seven control and seven LPS samples incubated in an anti-Na–K-ATPase antibody (α1-subunit). A band is seen at ∼ 100 kD. (B) Densitometric analysis of immunoblot of samples from control (white) and LPS (black) rats shows no significant change in protein expression in LPS rats in any of the three zones.

Fig. 8. Segment-specific changes in protein expression of iNOS. Semiquantitative immunoblotting of homogenized rat kidney tissue from the cortex and outer stripe of the outer medulla, (CTX/OSOM), inner stripe of outer medulla (ISOM) and inner medulla (IM) obtained from control and LPS rats from protocol 2. (A) Representative immunoblot of samples from protocol 2; seven control and seven LPS samples incubated in an anti-iNOS antibody. A band is seen at ∼ 130 kD. (B) Densitometric analysis of immunoblot of samples from control (white) and LPS (black) rats shows an increase in iNOS expression in LPS rats in CTX/OSOM, ISOM and IM.

Protein could be considered, as a change in protein expression is generally considered a delayed, long-term means of decreasing water reabsorption. This observed downregulation of AQP2 expression and a potential increase in degradation could be related to activation of pro-inflammatory mechanisms.

Interestingly, AQP2 protein expression was decreased, whereas trafficking appeared maintained or even enhanced in the LPS group. Also, despite the downregulation of AQP2, a possibility for maintained water permeability of the collecting duct in LPS rats is conceivable since urine osmolality is unchanged compared to control rats. The enhanced targeting of AQP2 to the apical plasma membrane 6 h after LPS treatment is likely to represent a compensatory effect to the increased urinary water excretion. The signalling cascades mediating this remain undefined but it is likely that increased vasopressin signalling may play a role, and other signalling pathways influencing cellular cAMP levels may also participate.

Segment-specific dysregulation of major renal sodium transporters

The present study reveals early segment-specific downregulation of NaPi2 in the proximal tubule and NKCC2 and NHE3 in the ISOM. Hence, a potential increase of sodium and chloride delivery to the macula densa and thus an activation of TGF may influence the previously observed decreased GFR and RBF in experimental sepsis models [3–8]. The knowledge from a recent study [44] that sodium transporter mRNA levels continue to decline with time, could
NKCC2 levels will only influence GFR if downregulation is only seen in the ISOM, as the role of NKCC2 in TGF via its localization in the macula densa [49] will then not be impaired. In this study, we saw that exact pattern with sustained NKCC2 abundance in CTX/OSOM. The joint downregulation of NHE3 in the ISOM (i.e. medullary thick ascending limb) could also be considered as a moderator of NKCC2 function due to its effects on intracellular pH [50] and to the effect of intracellular pH on NKCC2 function [51]. This impaired function of the mTAL could play an important role in the increased FENa and FEH2O known from the model [3] due to reduced countercurrent urinary concentration.

The observed upregulation of α- and γ-ENaC is presumably a compensatory change, presumably via aldosterone, but there appears to be no shift in the γ subunit to the activated 70 kD form, and there is no visible change in shuttling of the ENaC subunits, although this would be expected because of the increased aldosterone levels [52]. This could indicate a too recent increase in aldosterone levels or, alternatively, a local resistance to aldosterone, impeding a distal compensation to the proximal downregulation of transporters. The decrease in protein expression of α- and γ-ENaC subunits and Na–K–ATPase observed in a previous study [44] appears to be a later response, as the present study did not show these changes in any zones.

Creatinine clearance was unchanged at the 6-h time point, which could indicate that GFR decline had not taken place at the time point of downregulation of proximal tubule sodium transporters. Thus, with regard to the time course of decline in proximal sodium transport versus the decline in GFR, the possibility remains that sodium transporter downregulation could be the initial change in this setting, and that decreased proximal sodium transport is partly responsible for the decline of GFR and RBF known from the model.

Expression of inducible nitric oxide synthase

NO is a chemical relaxing factor derived from endothelium, which has important roles for renal haemodynamics and renal sodium and water metabolism [53,54]. Due to the well-known effects of NO promoting natriuresis and diuresis e.g. to inhibit sodium reabsorption in TAL due to activity of eNOS [48], and to inhibit vasopressin-stimulated osmotic water permeability in isolated and perfused cortical collecting duct [55] but on the other hand to increase targeting of AQP2 [56], it is relevant to investigate the role of iNOS in these changes. Based on previous, elaborate studies, we can presume, simplistically, that the iNOS we observed in epithelial and endothelial cells produces NO, whereas the activated macrophages in our study primarily produce the more toxic peroxynitrite [23,24]. As parenchymal iNOS expression was observed in all kidney zones mostly in surface epithelium of the papilla and NO only travels a few cell widths [24], our studies do not directly indicate a role for NO in the changes in renal water and sodium handling. However, if iNOS has a role in these changes, it is then more likely due to an infiltration of activated macrophages as these appeared to be most abundantly present in the ISOM, where changes in transporter regulation were also most distinct. In contrast, inner medulla was almost completely spared from activated epithelial and endothelial cells produces NO, whereas the activated macrophages in our study primarily produce the more toxic peroxynitrite [23,24]. As parenchymal iNOS expression was observed in all kidney zones mostly in surface epithelium of the papilla and NO only travels a few cell widths [24], our studies do not directly indicate a role for NO in the changes in renal water and sodium handling. However, if iNOS has a role in these changes, it is then more likely due to an infiltration of activated macrophages as these appeared to be most abundantly present in the ISOM, where changes in transporter regulation were also most distinct. In contrast, inner medulla was almost completely spared from activated nephron cortex (A, D, G, J). In the LPS group, iNOS labelling was observed in the inner medulla (E, H, K) where co-localization with CD-68 is seen. CD68 positive cells without iNOS expression were observed in all kidney zones of control and LPS rats. Picture (L) is taken in ISOM of the LPS rat. In the inner medulla (C, F, I), no iNOS labelling is visible in controls (C). In the LPS group, iNOS labelling is observed in the surface transitional epithelium all around the papilla (F) whereas iNOS expressing macrophages were hardly seen in the inner medulla. Subcellular localization appeared different in epithelial cells (I) compared to macrophages (G and H). In the transitional epithelium, labelling is seen mostly in proximity to the surface membrane, whereas in macrophages, iNOS labelling is observed more diffusely in the cytoplasm.

Protein expression of NaPi2 is known to decrease in acute renal injury, for example uni- and bilateral ureteral obstruction [11,45]; the latter was later shown to be partially mediated by angiotensin II [46] and authors suggest that ANP could be supplementarily responsible for downregulation of the protein, as levels of ANP are high [47]. ANP has been shown to influence phosphate excretion [48] and could be considered in the LPS model.

NKCC2 was downregulated 6 h after the LPS challenge in this study. The post-translational regulation of NKCC2 is also an important point of interest when determining the acute influence on tubular sodium transport. The present finding that levels of phosphorylated NKCC2 are lower in LPS rats further substantiates that NKCC2 plays a part in the tubular dysfunction in sepsis. One could hypothesize that explain the steady decline also seen in GFR in the LPS model [3].

Fig. 9. Tissue localization and subcellular localization of iNOS. Representative micrographs showing immunoperoxidase labelling for iNOS on kidney sections from control (A–C) and LPS (D–I) rats and immunoperoxidase labelling for iNOS (brown colour) and (strept)avidin labelling for CD-68 (blue colour) on kidney sections from LPS rats (J, K). In kidney cortex (A, D, G, J), no labelling is visible in the control group (A). In the LPS group, iNOS labelling is observed occasionally in Bowmans capsule (not shown) and (G) co-localization with CD-68 is seen in cells mainly localized to the glomerulus (J). In inner stripe of outer medulla (B, E, H, K), no labelling is visible in the control group (B). In the LPS group, iNOS labelling is observed in the endothelium of the outer medullary capillary net (E) and in interstitial cells (H) where co-localization with CD-68 is seen (K). CD68 positive cells without iNOS expression were observed in all kidney zones of control and LPS rats. Picture (L) is taken in ISOM of the LPS rat. In the inner medulla (C, F, I), no iNOS labelling is visible in controls (C). In the LPS group, iNOS labelling is observed in the surface transitional epithelium all around the papilla (F) whereas iNOS expressing macrophages were hardly seen in the inner medulla. Subcellular localization appeared different in epithelial cells (I) compared to macrophages (G and H). In the transitional epithelium, labelling is seen mostly in proximity to the surface membrane, whereas in macrophages, iNOS labelling is observed more diffusely in the cytoplasm.
Fig. 10. Effect of L-NIL treatment on AQP2, pAQP2 and NKCC2 in ISOM. Semiquantitative immunoblotting of AQP2 (A), pAQP2 (C) and NKCC2 (E) in homogenized rat kidney tissue from the inner stripe of outer medullar (ISOM) and representative micrographs showing immunoperoxidase labelling for AQP2 on kidney sections obtained from control, L-NIL, LPS and L-NIL-LPS rats from protocol 3. (A and B) Densitometric analysis of AQP2 in immunoblot of samples from control (white, n = 6), L-NIL (dark grey, n = 6), LPS (stripes, n = 6) and L-NIL-LPS (light grey, n = 6) rats shows a decrease in AQP2 expression of LPS rats compared to controls (ctrl 1 ± 0.09, L-NIL 0.93 ± 0.09, LPS 0.59 ± 0.05, L-NIL-LPS 0.73 ± 0.12, P = 0.02). (C and D) Densitometric analysis of pAQP2 in immunoblot shows no significant alterations of pAQP2 between ctrl and LPS or L-NIL and LPS groups. (E and F) Densitometric analysis of NKCC2 in immunoblot of samples shows a significant increase in NKCC2 in L-NIL-LPS rats compared to LPS rats. (G) Representative micrographs showing immunoperoxidase labelling for AQP2 on kidney sections from control (I), L-NIL (II), LPS (III) and L-NIL-LPS (IV) rats. Micrographs are taken in the ISOM and AQP2 labelling in collecting duct principal cells was studied for subcellular localization. In ISOM of LPS rats (III), a more prominent labelling intensity of the apical membrane is observed that in the same segments of control, L-NIL and L-NIL-LPS rats (I, II and IV), alongside lesser labelling intensity of intracellular vesicles.
macrophages and also from AQP2 protein downregulation. Therefore, there is a potential link between the presence of inflammatory cells (e.g. activated macrophages) with the production of proinflammatory mediators and peroxynitrite and the regulation of AQP2 and sodium transporters NHE3 and NKCC2. This was further examined by administration of iNOS inhibitor, L-NIL. By giving an iNOS inhibitor, the downregulation of NKCC2 was prevented, whereas changes in AQP2 expression were not significant in L-NIL-LPS rats compared to LPS rats. This could be a subject of interest as the sodium-losing state we observed presently might be an initial trigger of the impairment of GFR and RBF.

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Conflict of interest statement. None declared.

References

Methods.

Male Han:SPRD rats with PKD (Cy/+ rats treated with rapamycin (0.2 mg/kg/day IP) or vehicle from 1 to 12 months of age. Mean trough levels of rapamycin (ng/mL) were 6.6 ± 0.1 at 8 weeks of age. Twelve-month-old littermates (+/+) were used as normal controls.

Results. Twelve-month-old male Cy/+ rats treated with the vehicle had a more than doubling of kidney volume, severe chronic renal failure, severe hypertension and increased heart weight compared to normal littermate controls (+/+). After rapamycin treatment, 12-month-old Cy/+ rats had markedly improved kidney volume, renal function, blood pressure and heart weight not statistically different from controls. Rapamycin reduced the cyst volume density.