Regulation of Renal Sodium Transporters during Severe Inflammation

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Sepsis is the most common cause of acute renal failure (ARF) in the intensive care unit (1). ARF is present in 20% of patients with severe sepsis and 50% of patients with septic shock (2) and is clinically defined as a deterioration of GFR and tubular function. Little is known about the pathophysiology of endotoxemic tubular dysfunction with failure in urine concentration and increased fractional sodium excretion.

Urine concentration requires establishment and maintenance of a hypertonic medullary interstitium, which depends on the NaCl reabsorption (3). The Na⁺/H⁺-exchanger (NHE3) and the functional unit of ROMK and Na⁺-K⁺-2Cl⁻ co-transporter (NKCC2) are key components that are responsible for sodium reabsorption by the thick ascending limb (3,4). ROMK, also located in the cortical collecting duct (CCD), is responsible for recycling K⁺ across the apical membrane, which is critical for continuous sodium uptake by NKCC2 (5–8). In the collecting duct, the vasopressin-regulated epithelial sodium channel (ENaC), composed of three homologous subunits (9,10), accounts for the fine adjustment of the sodium reabsorption. The Na⁺/K⁺-ATPase in the basolateral membrane is essential for the efficient sodium reabsorption along the whole nephron.

Findings that endotoxemia diminishes expression of renal V₂ receptors and aquaporin-2 (11) directed our interest toward the regulation of tubular sodium transporters during experimental sepsis. We hypothesized that endotoxemia downregulates tubular sodium transporters. On the basis of our previous findings that proinflammatory cytokines downregulate several vasoconstrictive receptors in renal tissue (12–15), we assumed that cytokines affect the expression of renal sodium transporters.

To test our hypotheses, we performed in vivo experiments with (1) mice that were administered an injection of LPS as a model for severe experimental Gram-negative sepsis; (2) mice that were administered injections of cytokines TNF-α, IL-1β, or IFN-γ; (3) knockout mice that had a deficiency for TNF-α, IL-1 receptor-1 (IL-1R1), or IFN-γ and were administered an injection of LPS; (4) mice that did or did not have glucocorticoid pretreatment, which attenuates LPS-induced cytokine production (16–18) and were administered an injection of LPS; (5) mice that were administered an injection of low-dosage LPS using as a model of inflammation without systemic hypotension and renal ischemia; (6) mice with renal artery clipping to reduce renocortical flux to 40% of controls to imitate LPS-induced renal hypoperfusion (19); and (7) mice with renal ischemia-reperfusion injury by complete renal artery occlusion followed by reperfusion. In addition, we investigated the regulation of renal
sodium transporters during inflammatory conditions in vitro using murine CCD cells.

**Materials and Methods**

**Animals**

All animal experiments were performed according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male mice (20 to 25 g) with deficiencies for TNF-α (B6.129S6-Tnfr1<sup>−/−</sup>), IL-1R1 (B6.129S7-Il1r1<sup>−/−</sup>), IFN-γ (B6.129S7-Ifng<sup>−/−</sup>) and their wild-type strains B6129SF2/J and C57BL/6J, respectively, were purchased from the Jackson Laboratory (Bar Harbor, ME).

**Experimental Inflammation**

Wild-type mice received NaCl (control) or LPS (Escherichia coli, serotype 0111:B4; Sigma, St. Louis, MO; 10 mg/kg) intraperitoneally and were killed 6, 12, and 24 h (<i>n</i> = 6 per group) after LPS injection and after determination of hemodynamic and renal parameters. Wild-type and cytokine knockout mice received NaCl (control) or LPS 10 mg/kg intraperitoneally (<i>n</i> = 6 per group) and were killed 12 h after injection, the point of time with the strongest effect. Wild-type mice were additionally treated with NaCl (control), TNF-α, IL-1β, IFN-γ (1 μg/μL; PeproTech, London, UK), or the combination of TNF-α plus IL-1β or low-dosage LPS (1 and 5 mg/kg) intraperitoneally and were killed 12 h after injection (<i>n</i> = 6 per group). In addition, wild-type mice (<i>n</i> = 6 per group) that received a single high dosage of dexamethasone alone (10 mg/kg, intraperitoneally) and dexamethasone 2 h before LPS injection were investigated. The dosage of LPS, cytokines, and dexamethasone was chosen from data that were taken from the literature (12,15,20–24).

**Renal Hypoperfusion**

For induction of renal hypoperfusion, left renal arteries of mice (<i>n</i> = 6 per group) were clipped with 0.11-mm ID silver clips, and mice were killed 6, 12, and 24 h thereafter. The internal diameter of the renal clip was adjusted to reduce the renocortical flux to approximately 40% of the prestenotic level to ensure comparability with septic conditions (19).

**Renal Ischemia-Reperfusion Injury**

For induction of renal ischemia-reperfusion injury, renal arteries of mice (<i>n</i> = 6 per group) were totally occluded for 30 min with microaneurysm clamps followed by reperfusion. In sham controls, left renal arteries were only touched with forceps.

**Measurement of Mean Arterial Pressure and Renal and Blood Parameters**

Mice were anesthetized with Sevoflurane, using a Trajan 808 (Dräger, Lübeck, Germany) 6, 12, or 24 h after injection. The right femoral artery was cannulated for continuous monitoring of mean arterial pressure (MAP) and heart rate (Siemens SC 9000, Munich, Germany). The left femoral vein was cannulated for maintenance infusion and the bladder for collecting urine. FITC-inulin (0.5%; Sigma) was added to the infusion for determination of GFR. Plasma and urine FITC-inulin concentrations were measured after an equilibration period of 30 min every 30 min for 2 h and averaged for calculation of inulin clearance. FITC-inulin in plasma and urine samples was measured using a spectrofluorometer (Shimadzu, Columbia, MD). Needle laser Doppler probes (Transonic, Ithaca, NY) were inserted into the renal cortex connected to laser Doppler flowmeters (BLF 21), and renocortical tissue perfusion was taken from the literature (12,15,20–24).

### Table 1. Primer set for sodium transporter analysis by real-time PCR<sup>a</sup>

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROMK</td>
<td>TCA GAC CAA TAT AAA CTT</td>
</tr>
<tr>
<td></td>
<td>TGG GAA AGA GTT TCT GCGG</td>
</tr>
<tr>
<td>NKCC2</td>
<td>ATC TTT GCT TTT GCC AAT GC</td>
</tr>
<tr>
<td></td>
<td>ATG TCA TTG GTG GGA TCC AC</td>
</tr>
<tr>
<td>ENaC-α</td>
<td>TAC GCG ACA ACA ATC CCC AAG TGG</td>
</tr>
<tr>
<td></td>
<td>ATG GAA GAC ATC CAG AGA TTG GAG</td>
</tr>
<tr>
<td>ENaC-β</td>
<td>CAT AAT CCT AGC TTG CCT TTG TGG A</td>
</tr>
<tr>
<td></td>
<td>CAG TTG CCA TAA TCA GGG TAG AAG A</td>
</tr>
<tr>
<td>ENaC-γ</td>
<td>TGC AAG CAA TCC TGT AGC TTT AAG</td>
</tr>
<tr>
<td></td>
<td>GAA GCC TCA GAT GGC CAC TGT</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/K&lt;sup&gt;+&lt;/sup&gt;-ATPase α&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CTC CAG CAA CAG GAC CCG GCG</td>
</tr>
<tr>
<td></td>
<td>GAT CTC AGC GGC CCT TGC AGG</td>
</tr>
<tr>
<td>NHE3</td>
<td>GTA GTT GTG TGC CAG GTT CTC</td>
</tr>
<tr>
<td></td>
<td>GGA ACA GAG GGC GAG GAG CAT</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGA CCT TCA ACA CCC CAG CC</td>
</tr>
<tr>
<td></td>
<td>ACA GCA CTG TGT TGG CAT AG</td>
</tr>
</tbody>
</table>

<sup>a</sup>ENaC, epithelial sodium channel; NHE3, Na<sup>+</sup>/H<sup>+</sup>-exchanger, NKCC2, Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>−</sup> co-transporter; ROMK, renal outer medullary potassium channel.
measured before and 6, 12, and 24 h after renal artery clipping. Measurement of blood parameters was performed on a Rapidpoint 405 (Bayer, Leverkusen, Germany). Urinary sodium concentration was measured by standard flame photometry (Eppendorf, Hamburg, Germany) adapted to small samples (25).

Cell Culture
Murine CCD cells (M1 cell line) that expressed ROMK, ENaC, and Na⁺/K⁺-ATPase transporters (25) were a gift from Dr. R. Schreiber (Department of Physiology, University of Regensburg). Cells were incubated for 12 h with (1) serum-free medium (control); (2) IL-1β (100 ng/ml); (3) TNF-α (100 ng/ml); (4) IFN-γ (100 ng/ml); and (5) a mixture of TNF-α, IL-1β, and IFN-γ (100 ng/ml each). Murine recombinant cytokines were used for the experiments from PeproTech, and the dosage was chosen as published data (12–15).

mRNA Extraction and Real-Time PCR Analysis
Total RNA from tissues and cells was extracted and reverse-transcribed, and real-time PCR was carried out using the LightCycler system (Roche, Basel, Switzerland) as described (15). Each primer set (Table 1) was checked using a BLAST search to ascertain that the sequences were unique for each transporter. β-Actin was used as reference gene.

Protein Preparation
Kidneys were homogenized in ice-cold homogenization buffer in the presence of protease inhibitors followed by centrifugation with 500 × g for 15 min at 4°C. The resultant supernatant was centrifuged at 20,000 × g for 30 min at 4°C. The resultant supernatant was used for determination of tissue cytokine concentration, and the resultant pellet was reconstituted in blotting buffer and used for Western blotting.

Tissue Concentrations of Cytokines
Tissue concentrations of TNF-α, IL-1β, and IFN-γ were determined using ELISA kits (R&D Systems, Minneapolis, MN) and set into proportion to total protein.

Western Blot
Protein samples (40 µg) were electrophoretically separated on a 10% polyacrylamide gel and transferred to nitrocellulose membrane, which was blocked overnight in 5% nonfat dry milk diluted in Tris-buffered saline with 0.1% Tween-20, and then incubated for 1 h at room temperature with rabbit polyclonal antibodies against ROMK (Alomone, Jerusalem, Israel; 1:500), NHE3, ENaC (Chemicon, Temecula, CA; 1:500 each), Na⁺/K⁺-ATPase α1 (Upstate, Chicago, IL; 1:1000), and β-actin (Sigma; 1:5000). After washing, the membrane was incubated for 2 h with secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000; ENaC 1:1000, β-actin 1:5000) and subjected to a chemiluminescence detection system. Quantitative assessment of bands was performed densitometrically.

Statistical Analyses
Data were analyzed by ANOVA with multiple comparisons followed by the t test with Bonferroni adjustment. P < 0.05 was considered significant.

Results
Hemodynamic and Blood Parameters
MAP was 74.3 ± 1.6 mmHg in the control group and decreased to 68 and 64% of control 12 and 24 h after LPS injection, respectively. The heart rate of septic mice was significantly higher after LPS injection compared with the control group. Hemoglobin and hematocrit values showed no differences between control and septic groups, whereas plasma sodium increased 12 and 24 h after LPS administration.

IL-1β also led to tachycardia and arterial hypotension with hypernatremia. Injection of only dexamethasone did not influence hemodynamic and blood parameters in wild-type mice 6, 12, or 24 h after injection compared with control (data not shown). Treatment of LPS plus dexamethasone attenuated LPS-induced arterial hypotension in comparison with sole LPS treatment after 12 and 24 h (Table 2).

Table 2. Effect of LPS (10 mg/kg intraperitoneally) without or with dexamethasone (10 mg/kg intraperitoneally) and IL-1β (1 µg/g intraperitoneally) on hemodynamic, blood, and renal parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>LPS 6 h</th>
<th>LPS 12 h</th>
<th>LPS 24 h</th>
<th>LPS + Dexamethasone 6 h</th>
<th>LPS + Dexamethasone 12 h</th>
<th>LPS + Dexamethasone 24 h</th>
<th>IL-1β 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>20.0 ± 0.6</td>
<td>20.1 ± 0.2</td>
<td>19.4 ± 0.4</td>
<td>19.6 ± 0.2</td>
<td>19.9 ± 0.3</td>
<td>19.7 ± 0.2</td>
<td>19.7 ± 0.1</td>
<td>19.9 ± 0.3</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>74 ± 1</td>
<td>61 ± 2^b</td>
<td>50 ± 2^b</td>
<td>47 ± 3^b</td>
<td>64 ± 3^c</td>
<td>62 ± 2^c</td>
<td>60 ± 2^c</td>
<td>54 ± 3^c</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>486 ± 11</td>
<td>527 ± 7^b</td>
<td>587 ± 12^b</td>
<td>625 ± 13^b</td>
<td>507 ± 10</td>
<td>576 ± 12^b</td>
<td>610 ± 12^b</td>
<td>589 ± 12^b</td>
</tr>
<tr>
<td>Plasma Na⁺ (mmol/L)</td>
<td>144.5 ± 0.7</td>
<td>145.1 ± 0.7</td>
<td>150.5 ± 1.0</td>
<td>151.7 ± 0.9</td>
<td>145.5 ± 0.9</td>
<td>149.9 ± 0.7</td>
<td>150.7 ± 1.3</td>
<td>150.1 ± 1.1</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.1 ± 0.5</td>
<td>13.1 ± 0.3</td>
<td>12.9 ± 0.2</td>
<td>13.1 ± 0.2</td>
<td>13.4 ± 0.4</td>
<td>13.3 ± 0.3</td>
<td>13.1 ± 0.2</td>
<td>13.2 ± 0.4</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>37.1 ± 0.8</td>
<td>36.1 ± 0.5</td>
<td>36.3 ± 1.3</td>
<td>36.2 ± 1.2</td>
<td>36.3 ± 1.0</td>
<td>37.3 ± 1.1</td>
<td>36.1 ± 1.1</td>
<td>36.6 ± 1.3</td>
</tr>
<tr>
<td>Urine flow (µl/min)</td>
<td>0.62 ± 0.03</td>
<td>0.51 ± 0.03^b</td>
<td>0.32 ± 0.02^b</td>
<td>0.30 ± 0.03^b</td>
<td>0.58 ± 0.04</td>
<td>0.44 ± 0.03^b</td>
<td>0.44 ± 0.02^c</td>
<td>0.35 ± 0.06^b</td>
</tr>
<tr>
<td>GFR (µl/min per g body wt)</td>
<td>10.3 ± 0.4</td>
<td>7.1 ± 2^b</td>
<td>2.7 ± 0.2^b</td>
<td>1.5 ± 0.1^b</td>
<td>8.8 ± 0.2^c</td>
<td>3.8 ± 0.3^c</td>
<td>3.4 ± 0.6^c</td>
<td>3.0 ± 0.6^c</td>
</tr>
<tr>
<td>Urinary Na⁺ excretion (mmol/min per g body wt)</td>
<td>12.1 ± 0.5</td>
<td>10.1 ± 0.6</td>
<td>8.5 ± 1.0^b</td>
<td>8.0 ± 1.1^b</td>
<td>11.0 ± 0.7</td>
<td>8.6 ± 0.8^b</td>
<td>10.2 ± 0.6^b</td>
<td>9.2 ± 0.6^b</td>
</tr>
<tr>
<td>Fractional Na⁺ excretion (%)</td>
<td>0.88 ± 0.11</td>
<td>0.93 ± 0.15</td>
<td>2.10 ± 0.20^b</td>
<td>3.76 ± 0.27^b</td>
<td>0.86 ± 0.17</td>
<td>1.39 ± 0.14^b</td>
<td>2.01 ± 0.21^b</td>
<td>1.96 ± 0.14^b</td>
</tr>
<tr>
<td>Tubular Na⁺ reabsorption (µmol/min)</td>
<td>27.6 ± 0.5</td>
<td>21.3 ± 0.5^b</td>
<td>8.1 ± 0.5^b</td>
<td>4.2 ± 0.6^b</td>
<td>25.2 ± 0.5^c</td>
<td>12.2 ± 0.5^c</td>
<td>10.1 ± 0.6^c</td>
<td>9.3 ± 0.7^b</td>
</tr>
</tbody>
</table>

^aData are means ± SEM, n = 6. MAP, mean arterial pressure.
^bP < 0.05 versus control.
^cP < 0.05 versus respective LPS.
Renal Parameters

GFR was lower in mice that were administered injections of LPS, and urine flow decreased to 52 and 48% of control 12 and 24 h after injection, respectively. Urinary sodium excretion decreased significantly and fractional sodium excretion was 2.4 and 4.3 times higher in LPS-treated mice compared with control 12 and 24 h after LPS treatment, respectively. Tubular sodium reabsorption in the mice that were administered injections of LPS was considerably lower than in the control group, decreasing to 29 and 15% of control 12 and 24 h after sepsis induction, respectively (Table 2).

Twelve hours after injection of IL-1β, renal function of these mice was restricted comparable to mice that were administered injections of LPS. Injection of only dexamethasone showed no relevant changes in functional renal parameters in wild-type mice 6, 12, and 24 h after injection compared with controls (data not shown). Treatment of mice that were administered injections of LPS with dexamethasone attenuated renal injury as indicated by a higher GFR, a higher tubular sodium reabsorption, and a reduced fractional sodium excretion compared with sole LPS injection.

Effect of LPS on Renal Sodium Transporter Expression

ROMK mRNA was downregulated to 5, 5, and 54% of control and NKCC2 mRNA decreased to 41, 18, and 33% of control 6, 12, and 24 h after LPS injection, respectively. ENaC mRNA levels showed a concomitant fall of all three subunits to approximately 75, approximately 55, and approximately 80% of control 6, 12, and 24 h after LPS administration, respectively. In accordance, Na+/K+-ATPase and NHE3 mRNA decreased to approximately 40% of control level 6, 12, and 24 h after LPS injection, respectively (Figure 1).

Figure 2 demonstrates protein expression of ROMK, NHE3, Na+/K+-ATPase, and ENaC as determined by Western blot analysis. The decline of ROMK mRNA abundance was paralleled by a decrease of ROMK protein. NHE3 protein fell to 69 and 41% of control 12 and 24 h after sepsis induction, respectively. Western blot of Na+/K+-ATPase and ENaC-α and -β decreased to approximately 60% of control as well as ENaC-γ to approximately 50% of control 12 and 24 h after sepsis induction, respectively.

Figure 1. Effect of LPS (10 mg/kg), dexamethasone (10 mg/kg), and the combination of both on renal outer medullary potassium channel (ROMK), Na+/K+-2Cl− co-transporter (NKCC2), epithelial sodium channel (ENaC), Na+/K+-ATPase α1, and Na+/H+-exchanger (NHE3) mRNA in the kidney 6, 12, and 24 h after intraperitoneal injection. Values are related to signals that were obtained for β-actin mRNA and given as percentage of vehicle control. Data are means ± SEM of six mice per group. *P < 0.05 versus control; #P < 0.05 versus LPS.
Effect of Cytokines on Renal Sodium Transporter Expression

For further characterization of the mechanisms along which tubular sodium transporters could be downregulated during endotoxemia, renal gene expression in mice that were administered injections of TNF-α, IL-1β, and IFN-γ was examined, because these proinflammatory cytokines are known to be abundantly generated mediators during sepsis (26). mRNA of all tubular sodium transporters was strongly depressed after injection of TNF-α or IL-1β and decreased especially after the combination of both to mRNA levels that were comparable with those after LPS injection. IFN-γ also downregulated ROMK, ENaC-γ, Na+/K+-ATPase, and NHE3 mRNA (Figure 3).

Effect of LPS on Sodium Transporter Expression in Cytokine Knockout Mice

To investigate whether LPS-induced downregulation of renal sodium transporter expression could be inhibited by omission of single cytokine effects, we measured mRNA levels of sodium transporters in LPS-treated knockout mice with a deficiency for TNF-α, IL-1R1, or IFN-γ and compared the effect with the impact in their wild-type control strain. Cytokine-knockout and wild-type mice that were administered injections of NaCl revealed comparable sodium transporter mRNA levels (data not shown). Twelve hours after LPS injection, mRNA levels of all tubular sodium transporters were depressed and the downregulative effect of LPS on renal sodium transporters could not be diminished in any knockout species (Figure 4).

Effect of Dexamethasone on Sodium Transporter Expression in LPS-Treated Mice

Because absence of a single cytokine effect had no effect on LPS-induced downregulation of renal sodium transporters, mice were additionally treated with dexamethasone (10 mg/kg, intraperitoneally). As shown in Table 3, concomitant treatment with dexamethasone markedly attenuated renal tissue cytokine concentration after LPS injection. Therefore, we used dexamethasone treatment as a model to investigate the impact of a diminished action of more than one single cytokine on sodium transporter gene expression.

Sole dexamethasone administration showed no differences in the expression of renal sodium transporters compared with control level (Figure 1). Mice that were treated with LPS plus dexamethasone still revealed a significant downregulation of
sodium transporter gene expression; however, concomitant injection of dexamethasone substantially attenuated the LPS-induced suppression of NHE3, ROMK, NKCC2, ENaC, and Na\(^+\)/K\(^+\)-ATPase gene expression and tended to result in abolishing the suppressive effect of LPS on renal sodium transporters 24 h after LPS injection (Figure 1). Also on the protein level, the positive effect of additional dexamethasone treatment on sodium transporter membrane protein is markedly noticeable in comparison with sole LPS injection (Figure 5).

### Table 3. Effect of LPS (10 mg/kg intraperitoneally) without or with dexamethasone (10 mg/kg intraperitoneally) on renal tissue cytokine concentrations

<table>
<thead>
<tr>
<th>Cytokine (pg/mg protein)</th>
<th>Control LPS</th>
<th>LPS + Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
<td>12 h</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>ND</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>IL-1(\beta)</td>
<td>1.8 ± 0.3</td>
<td>72.1 ± 4.4(^b)</td>
</tr>
<tr>
<td>IFN-(\gamma)</td>
<td>ND</td>
<td>184 ± 13</td>
</tr>
</tbody>
</table>

\(^a\)Data are means ± SEM, \(n = 6\). ND, not detectable.

\(^b\) \(P < 0.05\) versus control.

\(^c\) \(P < 0.05\) versus respective LPS.

### Effect of Low-Dosage LPS on Hemodynamic/Renal Parameters and on Sodium Transporter Expression

Mice were administered injections of low-dosage LPS as a model for inflammation without arterial hypotension and renal ischemia. Twelve hours after injection of both 1 and 5 mg/kg LPS, renal tissue cytokine concentrations increased but mice remained normotensive. However, low-dosage LPS was sufficient enough to decrease renal parameters and expression of renal sodium transporters (Figure 6, Table 4).

### Effect of Renal Hypoperfusion and Ischemia-Reperfusion on Sodium Transporter Expression and Tissue Cytokine Concentration

Clipping of the left renal artery resulted in a renal hypoperfusion with decreased renocortical tissue perfusion to approximately 40% of control level, which is comparable to values of septic animals (19). Renal hypoperfusion did not change gene expression of tubular sodium transporters compared with sham-operated animals 6, 12, and 24 h after clipping. In contrast, ischemia-reperfusion significantly decreased mRNA levels of renal sodium transporters. Neither renal clipping nor ischemia-reperfusion affected expression of \(\beta\)-actin. Renal tissue concentrations of IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) were not influenced after renal hypoperfusion but were elevated after renal ischemia-reperfusion compared with sham-treated mice 6, 12, and 24 h after operation (Figure 7).

### Effect of Cytokines on Sodium Transporter Expression in CCD Cells

As hypothesized and already demonstrated \textit{in vivo}, the proinflammatory cytokines IL-1\(\beta\), TNF-\(\alpha\), and IFN-\(\gamma\) downregulated expression of ROMK, ENaC, and Na\(^+\)/K\(^+\)-ATPase in murine CCD cells. This underlines our assumption that cytokines mediate the downregulative effect of LPS on renal sodium transporters also in the absence of ischemia (Figure 8). LPS, cytokines, and dexamethasone had no effect on expression of \(\beta\)-actin gene or protein.

### Discussion

The pathophysiology of sepsis-associated renal tubular dysfunction with impaired urinary concentration and increased fractional sodium excretion is only poorly understood. Adequate tubular function with the ability of potent urinary con-
centration and sodium reabsorption depends on the functional expression of several tubular transporters, such as ROMK, NKCC2, ENaC, Na⁺/K⁺-ATPase α₁, and NHE3 (27). Therefore, we aimed to characterize the regulation of these transporters during severe experimental inflammation.

As an in vivo model for experimental inflammation, we used the intraperitoneal injection of LPS, an established, easy-to-apply maneuver with a low dispersion and mortality rates like the peritonitis models of sepsis (28). However, limitations such as overwhelming, short-lived LPS reactions are not rejectable (28). In our study, LPS treatment caused a time-dependent pronounced arterial hypotension that was associated with

![Figure 6](image_url)

**Figure 6.** Effect of LPS (1, 5, and 10 mg/kg) on mean arterial pressure (MAP) and tubular sodium reabsorption (A); the expression of ROMK, NKCC2, ENaC, Na⁺/K⁺-ATPase α₁, and NHE3 mRNA in the kidney (B); and cytokine concentration in renal tissue (pg/mg protein; C) 12 h after intraperitoneal injection. Values of MAP and tubular sodium reabsorption are related to data of control mice and given as percentage of vehicle control. Values of sodium transporters are related to signals that were obtained for β-actin mRNA and given as percentage of vehicle control. Data are means ± SEM of six mice per group. *P < 0.05 versus control.

Table 4. Effect of low-dosage LPS (1 and 5 mg/kg intraperitoneally) on hemodynamic, blood, and renal parameters 12 h after injection

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>LPS 1 mg/kg</th>
<th>LPS 5 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>20.1 ± 0.3</td>
<td>20.2 ± 0.4</td>
<td>20.0 ± 0.2</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>492 ± 10</td>
<td>490 ± 18</td>
<td>513 ± 16</td>
</tr>
<tr>
<td>Plasma Na⁺ (mmol/L)</td>
<td>144.9 ± 0.4</td>
<td>148.9 ± 0.7b</td>
<td>149.7 ± 1.3b</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.3 ± 0.4</td>
<td>13.2 ± 0.2</td>
<td>13.1 ± 0.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>37.0 ± 0.6</td>
<td>37.0 ± 1.2</td>
<td>36.3 ± 1.2</td>
</tr>
<tr>
<td>Urine flow (µl/min)</td>
<td>0.63 ± 0.04</td>
<td>0.43 ± 0.03b</td>
<td>0.36 ± 0.03b</td>
</tr>
<tr>
<td>GFR (µl/min per g body wt)</td>
<td>10.1 ± 0.3</td>
<td>3.6 ± 0.3b</td>
<td>3.0 ± 0.2b</td>
</tr>
<tr>
<td>Urinary Na⁺ excretion (nmol/min per g body wt)</td>
<td>12.3 ± 0.3</td>
<td>8.6 ± 0.4b</td>
<td>8.1 ± 0.4b</td>
</tr>
<tr>
<td>Fractional Na⁺ excretion (%)</td>
<td>0.84 ± 0.13</td>
<td>1.61 ± 0.10b</td>
<td>1.88 ± 0.19b</td>
</tr>
</tbody>
</table>

Data are means ± SEM, n = 6.

bP < 0.05 versus control.
tachycardia and ARF with reduced GFR, indicating the validity of our sepsis model. Fractional sodium excretion and tubular sodium reabsorption, both indicators of tubular function in this study, decreased after LPS injection, indicating tubular damage, which is in accordance with previous findings (29,30). Noteworthy, it has been shown that LPS infusion had an immediate antinatriuretic effect in the first hours after injection, leading subsequently to a marked increase in fractional sodium excretion, suggesting a delayed LPS-induced impairment in tubular sodium reabsorption and therefore confirming our results (31).

In this study, depressed renal tubular function is accompanied by a marked downregulation of ROMK, NKCC2, ENaC, Na+/K+-ATPase, and NHE3, denoting a possible causal linkage between impairment of renal sodium reabsorption and expression of these transporters. This hypothesis is strengthened by studies that described the coexistence of renal tubular dysfunction and downregulation of tubular sodium transporters, especially NHE3, Na+/K+-ATPase, and NKCC2 after renal ischemia-reperfusion (32–35). Because hypernatremia was observed in mice that were treated with LPS, one could assume that high sodium plasma levels influence expression of sodium transporters. However, in a previous study, hypernatremia did not affect expression of tubular sodium transporters (36).

We were further interested in the mechanisms and pathways along which septic conditions lead to downregulation of tubular sodium transporters. The hallmark of sepsis is the potent induction of proinflammatory cytokines such as TNF-α, IL-1β, or IFN-γ (26). Therefore, we were interested in the effect of these mediators on functional renal and hemodynamic parameters and on the expression of renal sodium transporters. Similar to LPS, injection of IL-1β caused a significant depression of hemodynamic and renal function. In addition, we found that IL-1β, TNF-α, and especially the combination of both extensively downregulated expression of ROMK, NKCC2, ENaC, Na+/K+-ATPase, and NHE3, whereas IFN-γ only partially inhibited expression of these transporters. We conclude that cytokines possibly mediate LPS-induced downregulation of tubular sodium transporters.

To test this hypothesis further, we performed experiments with knockout mice that had a deficiency for TNF-α, IL-1R1, or IFN-γ. However, it turned out that the absence of single-cytokine effects does not prevent LPS-induced downregulation of tubular sodium transporters, suggesting that LPS-induced inhibition of sodium transporter gene expression cannot be attributed to single cytokines likely because of the overlapping interactions of several proinflammatory cytokines. These experimental in vivo results are in accordance with results of clinical trials that reported no beneficial effects of anti–single-cytokine strategies in patients with sepsis (37–39).
Therefore, we performed experiments with mice that were administered injections of glucocorticoid to reduce LPS-induced upregulation of several proinflammatory cytokines. Indeed, we found that expanded inhibition of the induction of proinflammatory cytokines by glucocorticoid treatment attenuated LPS-induced downregulation of all sodium transporters. In accordance with previous data (40), glucocorticoid treatment alleviated the effect of LPS on renal function. These results emphasize the possible impact of proinflammatory cytokines in the pathogenesis of sepsis-induced ARF with tubular dysfunction and downregulation of renal sodium transporters.

Because renal ischemia is potentially present in septic ARF with cardiovascular depression (19,28,30,31) and because downregulation of several tubular sodium transporters has been demonstrated after renal ischemia-reperfusion injury (32–35), one could assume that the effects that were observed in this study may be due to renal ischemia that resulted from systemic hypotension. To clarify the role of systemic hypotension and renal ischemia in the regulation of renal sodium transporter expression, we performed several additional experiments.

First, we investigated the effect of inflammation in the absence of hypotension and renal ischemia on the expression of renal sodium transporters. We used low-dosage LPS, which caused an increase in proinflammatory cytokines in renal tissue without systemic hypotension. Mice that were administered injections of low-dosage LPS showed impaired renal function and expression of renal sodium transporters, supporting our hypothesis that proinflammatory cytokines also in the absence of renal ischemia account for downregulation of renal sodium transporters under septic conditions. This is in line with previous trials that reported alleviated renal parameters and elevated TNF-α concentration under normotensive conditions after low-dosage LPS treatment, which could be diminished by supplementary injection of TNF neutralizers (22–24). For completeness, it should be mentioned that in a previous study, immunoblotting of rat kidney homogenates revealed an increased expression of NKCC2 protein after low-dosage LPS injection. However, this must not conflict with our results, because in this trial, mice showed antinatriuresis to the point of kidney extraction, leading subsequently to an increase in fractional sodium excretion, which indicates a delayed LPS-induced impact on renal function and sodium transporters in that study (31).

Second, we determined the impact of renal hypoperfusion on the expression of renal sodium transporters. We chose the renal artery clipping model, which decreases renocortical tissue perfusion to 40% of controls and is comparable to values of septic animals (19). In this model of renal hypoperfusion, expression of renal sodium transporters was not influenced compared with sham-clipped mice. In addition, renal tissue cytokine con-

Figure 8. Effect of proinflammatory cytokines (IL-1β, TNF-α, and IFN-γ, each 100 ng/ml) on the expression of ROMK, ENaC, and Na⁺/K⁺-ATPase mRNA in cortical collecting duct cells 12 h after exposition. Values are related to signals that were obtained for β-actin mRNA and given as percentage of vehicle control. Data are means ± SEM of six wells per group. *P < 0.05 versus control.
centrations were unchanged after renal clipping in our and previous investigations (41,42). We conclude that renal hypoperfusion plays a minor role in mediating the downregulation of tubular sodium transporters during severe inflammation.

Third, we investigated the effect of renal ischemia-reperfusion on tubular sodium transporter expression. As already demonstrated by others (32–35), also in this study, renal ischemia-reperfusion led to decreased expression of renal sodium transporters, suggesting that ischemia is involved in the regulation of renal sodium transporter expression. However, the ischemia-reperfusion model is limited for imitating sepsis-induced renal ischemia, because ischemia-reperfusion means a complete renal artery occlusion followed by reperfusion, which contrasts to the prolonged renal hypoperfusion under septic circumstances (19). It must be clearly stated that in a complex in vitro model of severe inflammation, one cannot completely exclude the possible presence and influence of renal hypoperfusion followed by reperfusion on the expression of renal transporters.

In the end, to analyze the specific and independent impact of proinflammatory cytokines on the expression of renal sodium transporters in a model that completely excluded a possible hypotensive and ischemic effect, we performed in vitro experiments with murine CCD cells that expressed ROMK, ENaC, and Na\(^{+}/K\(^{+}\)-ATPase transporters (43–46). Proinflammatory cytokines suppressed the expression of ROMK, ENaC, and Na\(^{+}/K\(^{+}\)-ATPase, underlining our assumption that cytokines primarily account for the LPS-induced alteration on renal sodium transporters. This is in accordance with previous findings of cytokine-mediated inhibition of sodium reabsorption (47–49) and decreased expression of the Na\(^{+}/K\(^{+}\)-ATPase (50,51) in CCD cells.

**Conclusion**

Our data demonstrate that tubular sodium transporters are downregulated during severe experimental sepsis and suggest that proinflammatory cytokines are relevant in mediating regulation of renal sodium transporters during sepsis. Because of overlapping actions of different proinflammatory cytokines, downregulation of sodium transporters cannot be attributed to single cytokines, providing an explanation for why anti–single-cytokine strategies did not improve the outcome of patients with sepsis (37–39). In addition, our findings contribute to the explanation and understanding of the beneficial effects of glucocorticoids on the outcome of patients with sepsis (52–55).

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**Disclosures**

None.

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