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IL-1 receptor blockade alleviates endotoxin-mediated impairment of renal drug excretory functions in rats

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THE ENDOTOXINS RELEASED FROM the cell wall of gram-negative microorganisms play an important role in the initiation and maintenance of the severe inflammatory response of organisms during sepsis. These lipopolysaccharides bind to toll-like receptors (TLR) in tissues, especially at the surface of macrophages, and trigger a nuclear factor-κB-directed immune response. This response is based on the increased production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and nitric oxide (NO) released by inducible nitric oxide synthase (iNOS), which subsequently distort the morphology and function of several organs (6). Untoward acute injury can be seen especially in the kidney (acute kidney injury; AKI) and typically includes reduced blood flow, with a decreased glomerular filtration rate (GFR). Consequent ischemia of tubular segments leads to an alteration of transport functions, with reduced urinary concentration ability and eventually to acute tubular necrosis. The urinary excretion of endo- and xenobiotics is further mitigated by profound changes in the expression of transporting proteins in tubules (2, 5, 18, 26, 27). In this situation, the pharmacological modulation of the regulatory mediator pathways, such as the inhibition of iNOS or activation of the protective heme oxygenase (HMOX)-CO pathway, may significantly restore GFR and tubular excretory functions (20, 35) and consequently affect the pharmacokinetics of drugs administered in therapy for sepsis (55). However, the influence of immune response-modifying agents on the drug-excretory capacity of the renal tubule during such a status has not been described as yet.

The drugs which have a significant immunosuppressive effect, which are routinely administered to patients during septic shock, are glucocorticoids (29). The rationale for their use is to support the insufficient endogenous secretion of cortisol, to enable the maintenance of the vascular reactivity to catecholamines, and consequently, blood pressure. However, corticosteroids in applied dosages also possess a strong genomic effect, based on the suppression of NF-κB and their...
Influence of both the corticosteroid dexamethasone and anakinra showed a preventive effect on endotoxin-induced down-regulation of some crucial drug transporters in the liver (16). Corresponding data about a renal effect are missing. Although later studies were unable to find a positive effect of a recombinant human IL-1 receptor antagonist on septic acute renal failure (52), the synthetic IL-1 receptor antagonist anakinra, a recombinant human IL-1 receptor antagonist on septic acute renal survival, as well as for the prevention of acute tubular injury during endotoxemia or sepsis, may be the more specific anti-inflammatory cytokine approach using IL-1 receptor blockade (4, 11–13, 36). In addition, corticosteroids are known for their ability to induce drug transporters in the liver during endotoxemia (6a). Taken together, these drugs may have strong potential to modify renal excretion of therapeutics and endobiotics during sepsis.

An alternative therapeutic strategy for the improvement of survival, as well as for the prevention of acute tubular injury during endotoxemia or sepsis, may be the more specific anti-inflammatory cytokine approach using IL-1 receptor blockade (4, 11–13, 36). In addition, corticosteroids are known for their ability to induce drug transporters in the liver during endotoxemia (6a). Taken together, these drugs may have strong potential to modify renal excretion of therapeutics and endobiotics during sepsis.

Materials and Methods

Chemicals. Azithromycin (AZT) was used in its original formulation for parenteral administration (Sumamed, Pliva-Lachema, Brno, Czech Republic); dexamethasone was purchased from Sigma (St. Louis, MO); ANA was used in its original formulation for parenteral administration (Kineret, Biovitrum, Stockholm, Sweden), and LPS (from Salmonella enterica serotype typhimurium) was obtained from Sigma-Aldrich (St. Louis, MO).

Animal models. All procedures were approved by the Animal Care Committee at Charles University in Prague, Faculty of Medicine in Hradec Kralove, Male Wistar rats (280–320 g; purchased from Konarovicz, Czech Republic) were kept under temperature- and light-controlled conditions, on a 12:12-h light-dark cycle. Throughout the study, the rats were fed a commercial food diet and had free access to water. The rats were divided into the following four groups (n = 12 for each): 1) control group, which received only saline at designated time points; 2) LPS-treated group, which was injected with 4 mg/kg body wt of LPS intraperitoneally (ip); 3) DEX+LPS-treated group, which received 10 mg/kg body wt of dexamethasone ip 1 h before LPS administration; and 4) ANA+LPS-treated group, which received 10 mg/kg body wt of anakinra ip 1 h before and 4 h after LPS treatment. The regimens were selected on the basis of previous studies (6a, 16). The administration of a steroid before LPS in rodents is commonly used because of the reduced receptor-binding capacity for glucocorticoids under septic conditions in animals (9, 44). Anakinra was used with the same regimen as recommended previously (16) only we have added the dose 5 h after initial administration due to the short half-life of the agent in rats. After the application of LPS or corresponding saline, all the animals were housed in metabolic cages for 10 h, where water consumption was monitored and urine was collected. Thereafter, the animals were anesthetized ip with pentobarbital sodium (50 mg/kg). Six animals from each group were euthanized by exsanguination to obtain plasma for biochemical analysis and the liver and kidneys for expression analysis; samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis. The other six animals from each group underwent an in vivo clearance study.

In vivo clearance study. Rats were under pentobarbital sodium anesthesia (50 mg/kg ip). The right jugular vein was used for drug administration and the continuous infusion of physiological saline (2 ml/h), the left carotid artery for blood sampling, the common bile duct for bile collection, and the urinary bladder for urine collection were cannulated. Thereafter, the rats received a single bolus of AZT (20 mg/kg). Blood samples (~0.3 ml) were taken at designated time intervals (0, 4, 10, 30, 60, 120, 180, and 240 min) after the injection of AZT. Bile and urine samples were collected in preweighed tubes at 30-min intervals throughout the experiment. Blood was centrifuged at 2,000 g for 5 min at 4°C to obtain plasma. Organs were snap-frozen in liquid nitrogen and, together with plasma, bile, and urine samples, kept at −80°C until assayed.

Analytic procedures. The concentrations of AZT, citrulline/arginine, and nitrates/nitrates were determined by previously described HPLC methods (14). Biochemical analyses of serum and urine were performed on a Cobas Integra 800 and Cobas Mira Plus (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Bile acids were determined from serum, urine, and bile using a commercial kit (Diazyme Europe) according to the manufacturer’s instructions. Fractional excretion was calculated according to the following formulas: FEA = 100 × (urine sodium × plasma creatinine)/(plasma sodium × urine creatinine); FEurea = 100 × (urine urea × plasma creatinine)/(plasma urea × urine creatinine).

Pharmacokinetic analysis. Data were analyzed with Kinetic and MS Excel software. Pharmacokinetic parameters of intravenously administered AZT were calculated according to standard noncompartmental techniques (14). The area under the plasma concentration-time curve (AUC0–∞) was calculated from the sum of the AUC0-Tlast, estimated by the log-linear trapezoidal rule from time 0 to the time of the last measured concentration (Tlast), and the AUC was extrapolated to infinity (AUC0–∞) and calculated as the last measured concentration divided by the elimination constant (K10), estimated on the basis of linear regression analysis. The half-life (T1/2) was calculated by dividing ln(2) by K10. Total clearance (CLtot) was calculated as CLtot = Dose/AUC0–∞. The apparent volume of distribution (Vd) was calculated as Vd = CLtot × AUMC/AUC0–∞, where AUMC is the area under the first moment plasma concentration vs. time curve. The biliary (CLB) and renal (CLR) clearances were calculated as CLB = XBile/AUC0-Tlast and CLR = XUrine/AUC0-Tlast, where XBile and Xurine were the amounts of AZT excreted to bile and urine, respectively, during the evaluated period, and Tlast was 240 min. Tubular secretory clearance (CLn) of AZT was calculated as the difference between CLR (AZT renal clearance) − CLch (creatinine clearance).

Histological examination. The kidneys were fixed in 10% neutral buffered formalin solution and thereafter embedded in paraffin and stained with hematoxylin-eosin. Pictures were taken using an Olympus BX51 microscope with an Olympus DP71 camera. Glomerular and tubular damage was evaluated by the same specialist.

Western blot analysis. One hundred milligrams of kidney or liver tissue was minced with scissors in 0.5 ml RIPA buffer (Sigma) in the presence of protease inhibitors, homogenized with ULTRA-TURRAX (2 × 15 s), and centrifuged (3,000 rpm for 10 min, 4°C). Protein concentrations were measured with a BCA Protein Assay Kit (ThermoScientific), using bovine serum albumin as a standard. Proteins (100 μg) were separated on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking in Tris-buffered saline, supplemented with 0.05% of Tween 20 and 5% of nonfat dry milk, the membranes were incubated for 1 h with appropriate primary antibodies (Table 1). Blots were then incubated for 1 h in horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Prague), and the membranes were visualized with enhanced chemiluminescence reagents (ThermoPierce). The band intensities were detected using ScanMaker i900 (UMAX) and QuantityOne imaging software (Bio-Rad). Equal protein loading was confirmed by immunodetection of GAPDH (kidneys) or β-actin (liver).
Table 1. Antibodies used in Western blotting

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Catalog no.</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Detected Molecular Weight, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oat1</td>
<td>Sigma</td>
<td>SAB2102179</td>
<td>1:1,000</td>
<td>1:2,000</td>
<td>62</td>
</tr>
<tr>
<td>Oat2</td>
<td>Sigma</td>
<td>AV42708</td>
<td>1:2,500</td>
<td>1:3,000</td>
<td>60</td>
</tr>
<tr>
<td>Oat3</td>
<td>Sigma</td>
<td>SAB2102179</td>
<td>1:1,000</td>
<td>1:2,000</td>
<td>62</td>
</tr>
<tr>
<td>P-gp</td>
<td>Sigma</td>
<td>P7965</td>
<td>1:500</td>
<td>1:1,000</td>
<td>145</td>
</tr>
<tr>
<td>Mrp2</td>
<td>Alexis</td>
<td>ALX 801-037</td>
<td>1:500</td>
<td>1:1,000</td>
<td>190</td>
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<tr>
<td>β-Actin</td>
<td>Sigma</td>
<td>A 5316</td>
<td>1:5,000</td>
<td>1:8,000</td>
<td>42</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Sigma</td>
<td>G 8795</td>
<td>1:5,000</td>
<td>1:8,000</td>
<td>37</td>
</tr>
<tr>
<td>HMOX1</td>
<td>Sigma</td>
<td>H4535</td>
<td>1:1,000</td>
<td>1:3,000</td>
<td>32</td>
</tr>
<tr>
<td>iNOS</td>
<td>BD Bioscience</td>
<td>ALX 801-037</td>
<td>1:500</td>
<td>1:1,000</td>
<td>130</td>
</tr>
</tbody>
</table>

Antibodies were obtained from GE Healthcare Life Sciences: ECL mouse IgG, horseradish peroxidase (HRP)-linked anti-mouse and anti-rabbit secondary linked F (ab')2 fragment (catalog no. NA931) and ECL rabbit IgG, HRP-linked F (ab')2 fragment (catalog no. NA 934). HMOX1, heme oxygenase-1; iNOS, inducible nitric oxide synthase.

Real-time qRT-PCR. Real-time PCR was performed on a 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Total RNA was isolated from liver and kidney tissue samples using TRIzol reagent (Sigma-Aldrich) and converted into cDNA by a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Thirty nanograms of cDNA was loaded into one reaction. All experiments were performed in triplicate. Genes were amplified using TaqMan Fast Universal PCR Master Mix and predesigned TaqMan Gene Expression Assay kits (Table 2). All data were normalized to GAPDH, and differences in gene expression were calculated from detected ΔCt values, as described previously (22).

Detection of HMOX1 activity. Twenty microliters of tissue sonicate was incubated for 15 min at 37°C in septum-sealed, CO-free 2-ml vials, containing 20 μl of 50 μM methemalbumin as a substrate in the absence (blank) or presence of 20 μl of 4.5 mM NADPH. Blank reaction vials contained 0.1 M potassium phosphate (pH 7.4) in place of NADPH. The amount of CO generated by the reaction and released into the vial headspace was quantified by gas chromatography. HMOX activity was calculated by the number of picomoles of CO produced per milligram of protein per hour.

Data analysis. We detected maintenance of mRNA expression of transporting proteins (see below). Dexamethasone and prednisolone each significantly increased levels of CO, with a consequent rise in CO production (Fig. 1), while the fractional excretion of urea was restored, which suggests its positive influence on organic anion transport.

RESULTS

Effect of immunosuppressive therapy on LPS-mediated AKI. The administration of LPS raised serum parameters typical of AKI, urea and creatinine (Table 3). The inclusion of corresponding urine analyses showed a significant decrease in creatinine clearance and an increase in microalbuminuria, the indicators of impaired glomerular filtration (GFR), which was combined with altered tubular functions, as demonstrated by changes in the fractional excretion of sodium and urea (Fig. 1). Urine production in LPS animals remained unchanged, presumably due to opposite changes between GFR and sodium fractional excretion. Pretreatment with either drug prevented the development of glomerular impairment in particular (Fig. 1, C and D). Tubular functions were mainly influenced by dexamethasone. The drug further increased an LPS-induced rise in the fractional excretion of sodium, with a consequent decrease in urine production (Fig. 1), while the fractional excretion of urea was restored, which suggests its positive influence on organic anion transport.

Biochemical analyses of serum also confirmed the cholestatic potential of LPS, as indicated by increased levels of bilirubin, bile acids, and activities of ALP (Tables 3 and 4). Both agents protected against the development of all these alterations. Moreover, kinetic parameters of bile acid elimination (Table 4) demonstrated the restoration of their impaired biliary clearance, suggesting significant modification of responsible transporting proteins (see below). Dexamethasone also prevented impairment of urinary clearance of bile acids and predictably raised the level of glycemia.
Histological evaluation showed normal architecture of renal parenchyma in control animals. LPS administration produced cellular vacuolization and impairment of apical membrane integrity in renal tubular cells, without microscopic changes in glomeruli (Fig. 2B). Despite variable results from tubular functional parameters (Fig. 1, E and F), both drugs were able to attenuate such pathological changes, as exemplified in Fig. 2, C and D.

Immunosuppressive agents modulated LPS-induced changes in renal elimination of AZT. To evaluate the function of drug-transporting proteins, we measured pharmacokinetic parameters of biliary and urinary elimination of AZT, a typical substrate for multidrug transporters Mdr1 and Mrp2 (45) (Table 5). Untreated endotoxemic animals showed significant decreases in both biliary and renal excretion and clearance of the antibiotic, which was associated with prolongation of its half-life. In the case of the liver, the change could only be attributed to the consequence of reduced active biliary secretion. Analysis of urinary parameters suggested that reduced GFR is responsible for the alteration in particular. Pretreatment with either drug did not show any consistent effect on AZT biliary clearance, but both raised renal clearance through an

Table 3. Selected serum biochemical parameters related to endotoxemic liver and kidney impairment

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>LPS</th>
<th>DEX-LPS</th>
<th>ANA-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugated bilirubin, µM</td>
<td>BD</td>
<td>3.0 ± 1.6</td>
<td>BD</td>
<td>BD</td>
</tr>
<tr>
<td>Total bilirubin, µM</td>
<td>2.5 ± 0.5</td>
<td>9.3 ± 8.3*</td>
<td>3.0 ± 0.6†</td>
<td>3.8 ± 1.7†</td>
</tr>
<tr>
<td>AST, µkat/l</td>
<td>2.2 ± 0.4</td>
<td>4.5 ± 1.0</td>
<td>4.1 ± 1.1</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>ALT, µkat/l</td>
<td>0.6 ± 0.03</td>
<td>2.8 ± 0.6**</td>
<td>0.7 ± 0.05††</td>
<td>1.4 ± 0.3††</td>
</tr>
<tr>
<td>ALP, µkat/l</td>
<td>1.7 ± 0.1</td>
<td>5.6 ± 1.7***</td>
<td>2.5 ± 0.4†††</td>
<td>3.3 ± 0.6†††</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>8.1 ± 1.3</td>
<td>5.6 ± 1.2</td>
<td>11 ± 3.2††</td>
<td>8.3 ± 3.3</td>
</tr>
<tr>
<td>Urea, mM</td>
<td>6.8 ± 0.7</td>
<td>21 ± 8.0***</td>
<td>11 ± 4.6↑↑</td>
<td>13 ± 4.6↑↑</td>
</tr>
<tr>
<td>Creatinine, µM</td>
<td>30 ± 6.5</td>
<td>61 ± 16.1***</td>
<td>47 ± 11↑↑</td>
<td>44 ± 9.7↑↑</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6/group. Ctrl, control; LPS, endotoxemic rats; DEX, dexamethasone-pretreated rats; ANA, anakinra-pretreated rats; BD, below detection limit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control values. †P < 0.05, ††P < 0.01, †††P < 0.001, significantly different from LPS values.
increase in GFR, which may contribute to shortening of $T_{1/2}$. Interestingly, the tendency for the reduction of Vd after LPS, most probably due to tissue hypoperfusion, becomes significant during coadministration with dexamethasone. The effect may reflect the reduced distribution of AZT into tissues, as a consequence of restored blood vessel permeability after the administration of an immunosuppressant during endotoxemia. In agreement, total clearance of AZT remained limited in the dexamethasone group, despite an increase in renal clearance.

**LPS-induced changes in renal drug transporters were differentially changed by applied immunosuppressants.** Important renal transporters, which are responsible for the elimination of AZT and other organic anions and drugs used in the treatment of sepsis, were determined. LPS treatment significantly upregulated mRNA levels of transporters at the apical membrane (Mrp2, Mrp4, Mdr1b, and Asbt) and downregulated basolateral Oat and Oct uptake transporters, as well as the multidrug and toxin extrusion protein 1 (Mate1) apical transporter, compared with controls (Table 6). The administration of either drug prevented changes in apical membrane transporters, where dexamethasone also showed a protective effect on Asbt and Bsep. However, both only had a minimal effect on the transcription of basolateral molecules. In addition, we also analyzed mRNA expression of genes crucial for AZT pharmacokinetics in samples from the in vivo clearance study, i.e., 14 h after LPS challenge (Table 7). The administration of both agents demonstrated similar effect as at 10 h.

With respect to the anionic nature of AZT, we further analyzed the protein expression of Oat1-3, Mrp2, and P-gp (Fig. 3). Using crude membrane fractions, we confirmed the transcriptional downregulation of Oat1 and the upregulation of Mrp2 in untreated LPS rats. Oat2 and Oat3 proteins were posttranscriptionally upregulated by endotoxins. The administered drugs had diverse effects on transporters; while dexamethasone prevented Mrp2 induction, anakinra restored Oat1 and Oat2 levels.

To confirm the reliability of our model, we also evaluated the influence of LPS on mRNA expression of corresponding hepatic transporters. The majority of the changes observed in our study, i.e., the upregulation of Mdr1b and the downregulation of other bile acid or drug transporters in untreated LPS rats (Table 8), complies with former reports (6a, 16, 17). Only dexamethasone was able to partially alleviate changes in Ntcp, Bsep, and Mdr1b gene transcription, while anakinra accentuated those in Mrp4 and Mdr1b (Table 8).

**Effect of immunosuppressive therapy on LPS-mediated changes in regulatory molecules.** We evaluated the effect of both agents on two principal pathways regulating renal damage during endotoxemia, CO and NO. LPS treatment significantly enhanced HMOX1 expression and CO production in both the liver and kidneys (Fig. 4). Dexamethasone, as well as anakinra, returned both indicators to the control level. Similarly, untreated LPS showed a marked induction of iNOS in both organs, followed by an increase in NO plasma concentrations (Fig. 5). Only dexamethasone was able to significantly reduce the tissue content of the iNOS protein, which corresponded to
arginine ratio in serum and attenuated the LPS-mediated in-
trinsics renal impairment, especially by preserving glomerular
receptor blockade, by anakinra, may both alleviate endoto-
receptor blockade or IL-1RA during endotoxemia/sepsis, also
resembling an endotoxemia insult (42). In agreement, both
compounds reduced renal IL-6 mRNA, confirming their anti-
role in the development of AKI, and even sole administration
of IL-1

improved by anakinra to a similar extent as to that with the use
of dexamethasone. In fact, Berry et al. (4) recently suggested that
IL-1β-mediated neutrophil recruitment may play a key role in
the development of AKI, and even sole administration of IL-1β
can produce a systemic and kidney response closely
resembling an endotoxemia insult (42). In agreement, both
compounds reduced renal IL-6 mRNA, confirming their anti-
inflammatory influence, but predictably anakinra was unable to
change TNF-α expression. On the other hand, contradictory
results, with the absence of any beneficial effect of the IL-1β
receptor blockade or IL-1RA during endotoxemia/sepsis, also

DISCUSSION
The main finding of the present study is that nonselective
immunosuppression by a corticosteroid or the selective IL-1
receptor blockade, by anakinra, may both alleviate endotox-
amic renal impairment, especially by preserving glomerular
filtration. On the other hand, the endotoxemic depression of
renal tubular functions, including drug excretory capacity,
was only partially influenced by the agents. While diverse effects
were detected in LPS-downregulated basolateral transporters,
the prevention of LPS-mediated upregulation was seen in the
apical excretory Mrp2, which, however, had no effect on the
excretion of its substrate, AZT. Impairment of tubular excre-
tory functions of endobiotics, namely urea and bile acids, was
favorably influenced by dexamethasone.

The positive modulation of some signs of AKI during
endotoxemia, especially the restoration of GFR, has already
been described for corticosteroids and is mainly ascribed to the
downregulation of iNOS (25, 40, 47, 48). We have verified this
mechanism for dexamethasone with the reduction of iNOS,
NO plasma levels and the citrulline/arginine ratio, which is the
indicator of arginine breakdown by NOS (31). Importantly,
unlike in other forms of AKI, such as ischemic or cisplatin
induced (1), only the IL-1 receptor blockade in our study
showed that crucial functional parameters of LPS-imposed AKI
were significantly related to this pathway, and could be
improved by anakinra to a similar extent as to that with the use
of dexamethasone. In fact, Berry et al. (4) recently suggested that
IL-1β-mediated neutrophil recruitment may play a key role in
the development of AKI, and even sole administration of IL-1β
can produce a systemic and kidney response closely
resembling an endotoxemia insult (42). In agreement, both
compounds reduced renal IL-6 mRNA, confirming their anti-

Table 5. Pharmacokinetic parameters of intravenously administered azithromycin (20 mg/kg)

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>LPS</th>
<th>DEX-LPS</th>
<th>ANA-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vdss, l/kg</td>
<td>9.4 ± 4.1</td>
<td>6.9 ± 2.4</td>
<td>3.8 ± 1.6†</td>
<td>6.2 ± 2.8</td>
</tr>
<tr>
<td>AUC0-∞, mg·l⁻¹·min⁻¹</td>
<td>377 ± 80</td>
<td>552 ± 153</td>
<td>627 ± 187</td>
<td>360 ± 225</td>
</tr>
<tr>
<td>t1/2, min</td>
<td>148 ± 62</td>
<td>232 ± 76*</td>
<td>120 ± 62†</td>
<td>89 ± 32‡</td>
</tr>
<tr>
<td>Xurine, mg/240 min</td>
<td>0.15 ± 0.03</td>
<td>0.08 ± 0.03**</td>
<td>0.11 ± 0.03†</td>
<td>0.1 ± 0.04</td>
</tr>
<tr>
<td>Xurine, mg/240 min</td>
<td>21.1 ± 3.6</td>
<td>14 ± 3.7*</td>
<td>13 ± 3.6*</td>
<td>20 ± 6.9</td>
</tr>
<tr>
<td>CLBile, ml/min</td>
<td>0.5 ± 0.08</td>
<td>0.2 ± 0.1*</td>
<td>0.2 ± 0.06*</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>CLBile, ml/min</td>
<td>3.9 ± 1.2</td>
<td>1.4 ± 0.9**</td>
<td>2.4 ± 0.7†</td>
<td>2.8 ± 1.3‡</td>
</tr>
<tr>
<td>CLCR, ml/min</td>
<td>1.7 ± 0.3</td>
<td>0.9 ± 0.5**</td>
<td>1.4 ± 0.2†</td>
<td>1.7 ± 0.4†</td>
</tr>
<tr>
<td>CLR - CLcr</td>
<td>2.3 ± 1.0</td>
<td>0.5 ± 0.5**</td>
<td>1.0 ± 0.6*</td>
<td>1.1 ± 1.0*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 6/group. AUC area under the curve; t1/2, distribution-phase half-life; Xurile, biliary excretion; Xurine, urinary excretion; CLtot, total clearance; Vdss, steady-state volume of distribution; CLBile, biliary clearance; CLR, renal clearance; CLCR, creatinine clearance; CLRS, tubular secretory clearance of azithromycin. *P < 0.05, **P < 0.01, significantly different from control values. †P < 0.05, ††P < 0.01, significantly different from LPS values.

a change in NO2 levels. However, total NO in plasma was
decreased by both agents. The only substrate for NOS is
arginine, which is converted to NO and citrulline. As a result,
lowered plasma levels of arginine and an enhanced concentra-
tion of citrulline may be found in sepsis, which also complies
with our results (Fig. 5F). Arginine deficiency in such condi-
tions may contribute to a number of negative effects, such as
endothelial and T cell dysfunction (34). Importantly, the ad-
ministration of both agents in our study restored the citrulline/arginine ratio in serum and attenuated the LPS-mediated in-
duction of TNF-α and IL-6 mRNA in the kidneys (Table 6).

Table 6. Gene expression of renal drug transporters and cytokines measured in kidneys

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ctrl</th>
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<th>DEX-LPS</th>
<th>ANA-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abcc2 (Mrp2)</td>
<td>100 ± 20</td>
<td>266 ± 298*</td>
<td>137 ± 35†</td>
<td>91 ± 28†</td>
</tr>
<tr>
<td>Abcc4 (Mrp4)</td>
<td>100 ± 8</td>
<td>180 ± 56*</td>
<td>161 ± 57</td>
<td>156 ± 42</td>
</tr>
<tr>
<td>Abcb1a (Mdr1a)</td>
<td>100 ± 23</td>
<td>58 ± 23**</td>
<td>63 ± 28**</td>
<td>46 ± 13**</td>
</tr>
<tr>
<td>Abcb1b (Mdr1b)</td>
<td>100 ± 64</td>
<td>184 ± 109**</td>
<td>83 ± 35**</td>
<td>97 ± 48***</td>
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<tr>
<td>Abcb11 (Bsep)</td>
<td>100 ± 71</td>
<td>50 ± 20</td>
<td>502 ± 71***†††</td>
<td>112 ± 52</td>
</tr>
<tr>
<td>Slc10a2 (Asbt)</td>
<td>100 ± 27</td>
<td>161 ± 55*</td>
<td>43 ± 30***†††</td>
<td>172 ± 90</td>
</tr>
<tr>
<td>Slc22a6 (Oat1)</td>
<td>100 ± 17</td>
<td>41 ± 19***</td>
<td>43 ± 7***</td>
<td>77 ± 29**†</td>
</tr>
<tr>
<td>Slc22a7 (Oat2)</td>
<td>100 ± 52</td>
<td>18 ± 17**</td>
<td>45 ± 32**</td>
<td>36 ± 21**</td>
</tr>
<tr>
<td>Slc22a8 (Oat3)</td>
<td>100 ± 23</td>
<td>39 ± 10**</td>
<td>43 ± 16**</td>
<td>69 ± 38*</td>
</tr>
<tr>
<td>Slc22a11 (Oct1)</td>
<td>100 ± 27</td>
<td>77 ± 33</td>
<td>45 ± 16*</td>
<td>86 ± 33</td>
</tr>
<tr>
<td>Slc22a12 (Oct2)</td>
<td>100 ± 30</td>
<td>51 ± 19*</td>
<td>45 ± 16*</td>
<td>69 ± 16</td>
</tr>
<tr>
<td>Slc22a3 (Oct3)</td>
<td>100 ± 27</td>
<td>46 ± 25*</td>
<td>70 ± 25</td>
<td>81 ± 38</td>
</tr>
<tr>
<td>Slc47a1 (Mate1)</td>
<td>100 ± 45</td>
<td>40 ± 17**</td>
<td>44 ± 23**</td>
<td>43 ± 13**</td>
</tr>
<tr>
<td>Slc47a2 (Mate2)</td>
<td>100 ± 36</td>
<td>82 ± 18</td>
<td>97 ± 34</td>
<td>96 ± 13</td>
</tr>
<tr>
<td>Il-6 (IL-6)</td>
<td>100 ± 40</td>
<td>15,141 ± 7,085***</td>
<td>1,188 ± 770†††</td>
<td>8,335 ± 2,913***†††</td>
</tr>
<tr>
<td>Tnf (Tnf-α)</td>
<td>100 ± 69</td>
<td>673 ± 210***</td>
<td>345 ± 243***</td>
<td>694 ± 125***</td>
</tr>
</tbody>
</table>

Values are means ± SD presented as % of control values (measured in control saline-treated animals); n = 6/group. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control values. †P < 0.05, ††P < 0.01, †††P < 0.001, significantly different from LPS values.

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Tracz et al. (46) demonstrated that the LPS challenge in HMOX1−/− mice led to a greater activation of NF-κB in the liver and an elevation of serum cytokines and chemokines, while Vanova et al. (50) showed significant alleviation in LPS-induced hepatic damage by CO inhalation. On the other hand, Poole et al. (39) detected improved hemodynamic and renal hallmarks of endotoxemic AKI, after the administration of the HMOX1 inhibitor zinc protoporphyrin. The authors ascribed the effect to local NO release, which may occur in decreasing concentrations of CO. However, both drugs in our study prevented the LPS-mediated induction of CO production, with a parallel reduction of NO plasma levels. Upon consideration that negative regulation was described between NO and CO during sepsis (15), we suggest that changes in CO release in our study reflected the severity of inflammation during endotoxemia, rather than causal protective mechanisms.

Endotoxemia, as well as sepsis, has a critical negative impact on transported mediated drug elimination in all excretory organs (17). The new information provided by our study is the influence of both evaluated agents on renal drug-transporting proteins during endotoxemia. Previous studies described downregulation of Oat1, Oat3, or Oct1-3 basolateral transporters (21, 23), and inconsistent induction (Mdr1, Mrp2, or Bsep) (18–20), maintenance (Mdr1, and Mrp2) (5, 55), or even downregulation (Mdr1) (2) of apical tubular proteins in LPS animals. Importantly, in these studies such modifications were associated with corresponding changes in the kinetics of sub-

### Table 7. Changes in mRNA expression of selected genes in rats after 4-h clearance study with azithromycin (14 h after administration of LPS)

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>LPS</th>
<th>DEX-LPS</th>
<th>ANA-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc22a6 (Oat1)</td>
<td>100 ± 33</td>
<td>43 ± 27**</td>
<td>18 ± 6***</td>
<td>63 ± 24*</td>
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<tr>
<td>Slc22a7 (Oat2)</td>
<td>100 ± 64</td>
<td>28 ± 20*</td>
<td>84 ± 34</td>
<td>48 ± 26</td>
</tr>
<tr>
<td>Slc22a8 (Oat3)</td>
<td>100 ± 45</td>
<td>49 ± 31*</td>
<td>9 ± 6***‡</td>
<td>79 ± 29</td>
</tr>
<tr>
<td>Slc10a2 (Asbt)</td>
<td>100 ± 34</td>
<td>290 ± 106*†</td>
<td>19 ± 14+++</td>
<td>116 ± 32+++</td>
</tr>
<tr>
<td>Abcc2 (Mrp2)</td>
<td>100 ± 22</td>
<td>124 ± 90*</td>
<td>63 ± 27††</td>
<td>78 ± 24</td>
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</tbody>
</table>

Values are means ± SD presented as % of control values (measured in control saline-treated animals; n = 6/group. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control values. †P < 0.05, ††P < 0.001, significantly different from LPS values. ‡P < 0.05, ‡‡P < 0.01, significantly different from corresponding values measured after 10 h from LPS administration.

exist (37, 52). Whether these discrepancies are a matter of appropriate dosage, or of pharmacological differences between IL-1RA and anakinra, requires further characterization. Knowing the short half-life of anakinra in rats, and based on a previous study on the prevention of liver impairment (16), we therefore selected a repeat dose administration after 5 h, which produced a reliable effect.

CO, another gaseous product significantly involved in cell signaling, could also be very important for the pathogenesis of AKI. Unlike NO, the role of CO released from hemoglobin by inducible HMOX1 is mainly considered nephroprotective (35).
strates for these transporters. Our data from the LPS group is mainly consistent with these observations. The only discrepancy was in the up- instead of downregulation of the Oat3 transporter, and currently there is no explanation for this effect. Concerning renal tubular apical proteins, we support the transcriptional induction of multidrug transporters during endotoxemia (20), which was formerly ascribed to the inducing effect of cumulating anionic substrates, such as bile acids (18). However, a similar reduction of plasma bile acid by both agents, and differential changes in multidrug protein contents, suggest the existence of another mechanism. Indeed, dexamethasone is a well-known enzyme inducer, and through stimulation of the PXR receptor (38), it is also able to increase the expression of Mdr1 and Mrp2 in excretory organs, including the kidneys of healthy animals (32, 33). However, its administration in our study led to the downregulation of Mrp2, which points to a prevailing regulation of these transporters during endotoxemia by inflammatory mediators, which were effectively suppressed by the agent. In agreement, Heemskerk et al. (19) showed that the upregulation of Mdr1 is also NF-κB dependent, which is the transcription factor suppressed by corticosteroids (7). In contrast, anakinra changed Oat1 and Oat2 protein, which signifies the regulatory role of the IL-1 receptor in their posttranscriptional regulation.

The function of renal drug transporters was evaluated by the measurement of the clearance parameters of azithromycin, which is the substrate for Mdr1 and Mrp2 (45). The CLR/CLCR ratio of 2.6 showed a marked secretion of the drug in the renal tubuli of control animals, and its significant decrease in LPS animals, despite the transcriptional upregulation of Mrp2 and

<table>
<thead>
<tr>
<th>Slc10a1 (Ntcp)</th>
<th>Ctrl</th>
<th>LPS</th>
<th>DEX-LPS</th>
<th>ANA-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ± 15</td>
<td>4 ± 2***</td>
<td>22 ± 8***††</td>
<td>9 ± 4***</td>
<td></td>
</tr>
<tr>
<td>Abcb11 (Bsep)</td>
<td>100 ± 32</td>
<td>20 ± 5***</td>
<td>62 ± 39††</td>
<td>22 ± 3***</td>
</tr>
<tr>
<td>Slc22a2 (Oat1a2)</td>
<td>100 ± 46</td>
<td>2 ± 1***</td>
<td>18 ± 16***</td>
<td>3 ± 3***</td>
</tr>
<tr>
<td>Abcc2 (Mrp2)</td>
<td>100 ± 34</td>
<td>2 ± 1***</td>
<td>17 ± 18***</td>
<td>2 ± 1***</td>
</tr>
<tr>
<td>Abcc3 (Mrp3)</td>
<td>100 ± 42</td>
<td>52 ± 28***</td>
<td>26 ± 10***</td>
<td>44 ± 12***</td>
</tr>
<tr>
<td>Abcc4 (Mrp4)</td>
<td>100 ± 16</td>
<td>115 ± 20</td>
<td>153 ± 25††</td>
<td>170 ± 37***††</td>
</tr>
<tr>
<td>Abcb1b (Mrp1)</td>
<td>100 ± 72</td>
<td>1,017 ± 780***</td>
<td>337 ± 265†</td>
<td>1,409 ± 415***†††</td>
</tr>
</tbody>
</table>

Values are means ± SD presented as % of control values (measured in control saline-treated animals); n = 6/group. *P < 0.05, **P < 0.01, significantly different from control values. †P < 0.05, ††P < 0.01, †††P < 0.001, significantly different from LPS values.

Fig. 4. Heme oxygenase-1 (HOMX1) activity (A and B) and protein expression (C and D) in the kidneys and liver of control (Ctrl), LPS-administered (LPS), and DEX- or ANA- pretreated LPS rats. Values are means ± SD (n = 6). *P < 0.05, †P < 0.01, ††P < 0.001, compared with controls. †P < 0.05, ††P < 0.01, †††P < 0.001, compared with LPS.
the maintenance of Mdr1 protein expressions, respectively. Such a discrepancy suggests that both transporters are not the only proteins responsible for azithromycin tubular secretion. Decreased basolateral uptake by Oat1 and increased apical reuptake by Oat2, the major renal transporters for organic anions, may indeed indicate the involvement of these molecules in AZT elimination (51). In support, both immunosuppressants were unable to increase tubular secretion of the antibiotic, because of diverse effects on the transporters. Although the relationship of AZT to transporters from the solute carrier family, SLC22, awaits further clarification, our data may have further implications. First, the comparison of mRNA and protein contents showed that during endotoxemia, transcriptional regulation prevails in renal Oat1, Mrp2, and Mdr1, while posttranscriptional modification is important for Oat2 and Oat3 and that these processes are dependent on IL-1 receptor stimulation. Second, corticosteroids or IL-1RA administration under such circumstances differentially modulate individual transporters and may modify the kinetics of their substrates, where drugs commonly administered in sepsis also belong, such as penicillins or cephalosporines (51).

Comparison of renal clearances of AZT and creatinine demonstrated that both therapies modified kinetics of AZT especially by changes in GFR. Although creatinine clearance is not considered as an optimal marker for GFR due to its minor tubular secretion and reabsorption (49), reported values and degree of its reduction after LPS administration in our study are in agreement with former data reported with inulin (49, 54). Together with restoration of creatinine clearance in our endotoxemic animals administered with either immunosuppressant, despite uninfluenced expression of all transporters mediating creatinine secretion (Oct2/3, Mate1/2, and Oat2/3) (28), it suggests sufficient sensitivity of this marker for estimation of GFR in our settings. This assumption is further supported by corresponding changes in microalbuminuria, the marker of glomerular impairment. On the other hand, persistence of increased serum creatinine levels after administration of either immunosuppressant to endotoxemic animals despite normalization of creatinine clearances may reflect restoration of decreased creatinine production during sepsis (8), and/or incomplete attenuation of impaired kidney perfusion.

We, herein, also demonstrated that both compounds increased the biliary elimination of bile acids and reduced their levels, as well as other indicators (bilirubin, ALP) of cholestasis in plasma. In addition, we discovered that dexamethasone reduced the expression of the renal tubular bile acid uptake
transporter Asbt and increased the efflux, Bsep, with a consequent increase in bile acid renal clearance to control levels. Although the extent of renal bile acid elimination is much smaller than hepatic elimination, the increase in bile acid elimination after the administration of dexamethasone is interesting, because the drug has been recently described as a promotor of cholestasis, due to the inhibition of the transcripational activity of the farsenoid-X receptor (30), which is the bile acid sensor. Transcriptional upregulation of the farsenoid-X receptor target gene Bsep in the kidneys and liver by dexamethasone and its absence after the administration of anakinra, signify the involvement of another, as yet unknown, regulatory pathway. This consideration is also supported by the lack of the anakinra effect on the other tubular excretory functions, whereas dexamethasone restored the fractional excretion of urea, the change attributable to the upregulation of urea transporters (41), and raised the fractional excretion of sodium, which is well known during corticosteroid therapy and reflects their stimulatory effect on sodium transportation in tubuli (43).

Although we are aware of the limitations of the tested animal model of sepsis for the generalization of obtained results, reported similarities allow us to conclude that our results support the use of the presented immunosuppressants for the prevention of AKI during sepsis and for the partial compensation of the deficit in renal drug elimination under these circumstances, especially with the restoration of GFR. However, marked variability in modulation of tubular secretion of AZT by both agents in our study suggests that significant augmentation of active tubular secretion of drugs can be expected in some individuals in sepsis administered with immunosuppressants, what may contribute to high interindividual variability in the elimination of coadministered agents. In addition, we have demonstrated the involvement of the IL-1 pathway in the regulation of drug excretory mechanisms during endotoxemic AKI, and also presented the complex influence of corticosteroids, the agents currently used in this indication. Our data also point towards the involvement of transporters, other than multirdrug-resistance transporters, in the urinary elimination of AZT.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

F398 IL-1 RECEPTOR BLOCKADE ALLEVIATES TOXIC RENAL IMPAIRMENT


