TRANSPORTERS

Nitric oxide differentially regulates renal ATP-binding cassette transporters during endotoxemia

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Abstract Nitric oxide (NO) is an important regulator of renal transport processes. In the present study, we investigated the role of NO, produced by inducible NO synthase (iNOS), in the regulation of renal ATP-binding cassette (ABC) transporters in vivo during endotoxemia. Wistar–Hannover rats were injected with lipopolysaccharide (LPS$^+$) alone or in combination with the iNOS inhibitor, aminoguanidine. Controls received detoxified LPS (LPS$^-$). After LPS$^+$, proximal tubular damage and a reduction in renal function were observed. Furthermore, iNOS mRNA and protein, and the amount of NO metabolites in plasma and urine, increased compared to the LPS$^-$ group. Coadministration with aminoguanidine resulted in an attenuation of iNOS induction and reduction of renal damage. Gene expression of 20 ABC transporters was determined. After LPS$^+$, a clear up-regulation in Abca1, Abcb1/P-glycoprotein (P-gp), Abcb11/bile salt export pump (Bsep), and Abcc2/multidrug resistance protein (Mrp2) was found, whereas Abcc8 was down-regulated. Up-regulation of Abcc2/Mrp2 was accompanied by enhanced calcein excretion. Aminoguanidine attenuated the effects on transporter expression. Our data indicate that NO, produced locally by renal iNOS, regulates the expression of ABC transporters in vivo. Furthermore, we showed, for the first time, expression and subcellular localization of Abcb11/Bsep in rat kidney.

Keywords Renal drug transport · iNOS · Lipopolysaccharide · Aminoguanidine · Nephrotoxicity

Abbreviations

ABC	ATP-binding cassette
AKI	acute kidney injury
BSA	bovine serum albumin
Bsep	bile salt export pump
BUN	blood urea nitrogen
calcein-AM	calcein-acetoxymethylester
ET	endothelin
FE_{Na}	fractional sodium excretion
FR	fractional reabsorption
GFR	glomerular filtration rate
HBSS	Hank's balanced salt solution
IL	interleukin
LPS	lipopolysaccharide
M-MLV	Moloney murine leukemia virus
Mrp	multidrug resistance protein
NF-κB	nuclear factor kappa B
NOx	NO metabolites
NOS	nitric oxide synthase
P-gp	P-glycoprotein
RPP	renal perfusion pressure

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RQ-PCR	quantitative real-time polymerase chain reaction
TBS	Tris-buffered saline
TNF-α	tumor necrosis factor-α

Introduction

The free radical nitric oxide (NO) has been shown to play an important role in various physiological processes in the kidney, including salt and fluid reabsorption, renal hemodynamics, renin secretion, and tubuloglomerular feedback [1]. Endogenous NO is enzymatically produced from conversion of the amino acid L-arginine to L-citrulline, a reaction that is catalyzed by the enzyme NO synthase (NOS). This enzyme exists in three isoforms; the neuronal (nNOS) and endothelial, both of which are Ca^{2+}- and calmodulin-dependent constitutive isoforms, and an inducible (iNOS) isoform [2]. Mediators associated with sepsis, such as endotoxin and the proinflammatory cytokines, interleukin-1β (IL-1β), IL-2, and tumor necrosis factor-α (TNF-α), have been shown to induce iNOS, thereby increasing production of NO. Sustained, high-output generation of NO by iNOS can cause cell death and tissue damage through lipid peroxidation, DNA damage, and proapoptotic effects [3]. This is the result of NO itself and the oxidant peroxynitrite, which is generated after reaction of NO with superoxide anions [4].

In contrast to many other organs, iNOS is constitutively expressed in the kidney, in particular in the medulla and in proximal tubules [5]. Many in vivo animal studies showed the importance of iNOS as a contributor to the pathophysiology of acute kidney injury (AKI). For example, it has been shown that selective chemical inhibitors of iNOS [6, 7], NO scavengers [8], iNOS gene knockout mice [9], or antisense oligodeoxynucleotides to iNOS [10] resulted in less renal tubular injury and improved structural and functional outcome. This renoprotective effect was partly due to the rescue of tubular epithelial cells from injury by NO [3].

Using killifish renal proximal tubules, we found previously that NO has a regulatory role in the transport activity of the ATP-binding cassette (ABC) transporter multidrug resistance protein 2 (Abcc2/Mrp2) via an intracellular signaling pathway in response to the action of several nephrotoxic chemicals in vitro [11]. This pathway involved at least endothelin (ET) release, binding to the basolateral ET$_B$ receptor, and activation of NOS, soluble guanylyl cyclase, and protein kinase C [12]. A similar regulatory pathway was found for the ABC transporter P-glycoprotein (Abcb1/P-gp) in rat brain capillaries [13]. Both studies indicate that the activation of NOS is most likely Ca^{2+}-independent and we concluded that the production of NO is a result of the activation of iNOS.

The proximal tubule is responsible for the excretory transport of xenobiotics, xenobiotic metabolites, and waste products of metabolism from blood into urine. Consequently, the proximal tubule is also an important target for toxic effects and is often the first site of damage in AKI [14]. Luminal transport proteins, like the ABC transporters ABCB1/P-gp, ABCC2/MRP2, and ABCC4/MRP4, are important efflux transporters in the kidney [15] and, therefore, play a role in the detoxification of tubule cells. The observed effects of NO on Abcc2/Mrp2 in killifish proximal tubule may be part of the normal progression of cellular events that occur during AKI.

In the present study, we hypothesized that iNOS has a role in the regulation of renal ABC transporters during nephrotoxicity in vivo. An endotoxemia model was used in which rats were exposed to lipopolysaccharide (LPS), and the gene expression of 20 ABC transporters was evaluated. In addition, we examined the effect of the iNOS inhibitor, aminoguanidine, to determine whether the change in expression of the renal efflux transporters is caused directly by NO generated by iNOS.

Materials and methods

Animals and experimental design

Specified pathogen-free male Wistar–Hannover rats (240–295 g; purchased from Harlan, Zeist, The Netherlands) were kept under routine laboratory conditions at the Central Animal Laboratory of the Radboud University Nijmegen Medical Centre. This strain was used because they do not develop hydronephrosis spontaneously like normal Wistar rats, which in turn could have a down-regulating effect on iNOS expression [16]. The local Animal Care Committee approved all experimental procedures.

Different pilot studies have been conducted to determine the route of administration, intravenously or intraperitoneally (IP), concentration of the endotoxin LPS (LPS$^+$, *Escherichia coli* 0127:B8, Sigma-Aldrich, Zwijndrecht, The Netherlands) and aminoguanidine (Sigma-Aldrich), and the time point of coadministration in this strain. LPS and aminoguanidine were dissolved in sterile Hank's balanced salt solution (HBSS, Gibco, Paisly, UK) supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES, Gibco), and set to pH 7.4 with NaOH.

Thirty rats were, stratified to body weight, injected IP with 5 mg/kg LPS$^+$ and were killed after 3, 6, 12, 24, or 48 h ($n=6$ for each experimental group). Fifteen rats served as controls and received 10 mg/kg detoxified LPS (LPS$^-$, $n=3$ per group), in which the biologically active component, lipid A, essential for the activation of NOS, is lacking

[17]. Although the control group is small, standard errors appeared to be satisfactory to allow a reduction and refinement of animals in this group. Twelve rats were injected IP with both 5 mg/kg LPS[+] and, after 1 h, 100 mg/kg of the iNOS inhibitor aminoguanidine (Sigma-Aldrich), and were killed 6 and 12 h after LPS[+] ($n=6$ per group). For the isolated perfused kidney experiments, six rats received LPS[+], four received LPS[−], and four were untreated and served as controls, and were killed after 12 h as described under the "Isolated kidney perfusion" section.

To collect urine before and after the injection, rats were housed in metabolic cages, washed with 2% chlorohexidine (Genfarma, Zaandam, The Netherlands) to prevent bacterial growth. Urine volumes were recorded and samples were snap-frozen in liquid nitrogen and stored at −80°C until assayed. During the experiment, body weight and body temperature were determined. At indicated time points after LPS injection, the rats were anesthetized with 5% isoflurane and heparin (125 IU/100 g) was injected via the penile vein to minimize blood clotting. The left kidney was removed, divided in four pieces, and snap-frozen in liquid nitrogen for immunohistology or fixed in Bouin's solution for routine histology. Arterial blood was obtained by aortic puncture and the rats were killed by exsanguination. The right renal vein was punctured and, subsequently, the kidney was perfused with HBSS–HEPES for 2 min at 7.5 ml/min (Minipuls 2, Gilson, Villiers-le-Bel, France). After perfusion, the kidney was removed, divided, and snap-frozen in liquid nitrogen for quantitative real-time polymerase chain reaction (RQ-PCR) and Western blotting. Thrombocyte and leukocyte counts were determined in blood as inflammatory parameters, and the concentrations of creatinine, urea, protein, glucose, and sodium were analyzed in blood and urine by routine clinical chemistry. The activity of alkaline phosphatase, a proximal tubule injury marker, was determined in 26-fold diluted urine samples, as described before [18].

Determination of mRNA expression

RNA was isolated using a Mikro-dismembrator U (Sartorius B. Braun Biotech, Melsungen, Germany). To prevent RNA degradation, we washed the metal cylinders with 0.5 M NaOH. Perfused kidneys were pulverized (2,000 rpm, 30 s) and transferred in ice-cold TRIzol reagent (Invitrogen, Breda, The Netherlands), and then RNA was isolated as described previously [19]. RQ-PCR on cDNA was performed according to the TaqMan® protocol in optical tubes using either the ABI PRISM 7700 single reporter Sequence Detection System ($n=6$, Applied Biosystems, Zwijndrecht, The Netherlands) or the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card Sequence Detection System (three pooled samples from

nine different rats, Applied Biosystems) according to the manufacturer's instructions. All experiments were performed in triplicate. Different rat genes were amplified with a predeveloped Gene Expression Assay, provided by Applied Biosystems.

Western blotting

Total membrane and cytosolic fractions from perfused kidneys were isolated using a Mikro-dismembrator U (Sartorius BBI Systems GmbH, Melsungen, Germany; 2,000 rpm for 30 s) and subjected to Western blotting as described previously [20]. Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as standard. Samples were separated on a 6 or 12% sodium dodecylsulfate polyacrylamide gel and transferred to Hybond-C pure nitrocellulose membrane (Amersham, Buckinghamshire, UK). The membrane was incubated overnight at 4°C with antibodies against NOSII/iNOS (1:1,000, according to Vos et al. [21]), ABCB1/P-gp (1:200, C219, DakoCytomation, Dako, Denmark), Abcc2/ Mrp2 (1:250, M$_2$III-6, Alexis Biochemicals, San Diego, CA, USA), ABCC4/MRP4 (1:1,000, as described by van Aubel et al. [22]), Abcb11/bile salt export pump (Bsep) (1:1,000, kindly provided by Dr. Bruno Stieger, University Hospital Zürich, Switzerland), or β-actin (1:10,000, Sigma) in Tris-buffered saline supplemented with 0.1% Tween-20 and 1% nonfat dried milk.

Nitrate and nitrite determination

The total amount of the stable NO metabolites (NOx), nitrate and nitrite, were determined as a measure of NO radicals production, using the Griess reaction, according to Moshage et al. [23]. Heparinized plasma was diluted 1:4 and urine samples were diluted 1:40 or 1:80 with distilled water. Each sample was determined in triplicate. Calibration curves were made with sodium nitrite and sodium nitrate in distilled water. The nitrate recovery was 90–100% over the entire range tested (0–50 μmol/l). The coefficient of variation between the different experiments was less than 5%.

Histology and immunohistology

Kidneys fixed in Bouin's solution were dehydrated, embedded in paraplast (Amsterstad, Amsterdam, The Netherlands), and cut into 3-μm sections, which were stained with periodic acid-Schiff for routine histology. For immunohistologic studies, serial tissue sections of 1.5 μm were cut from frozen kidneys using a cryostat as described previously [24]. The slices were incubated for 60 min at room

temperature with antibodies against either NOSII/iNOS (1:200, according to Vos et al. [21]) or ABCB1/P-gp (1:50, C219, DakoCytomation) in PBS supplemented with 1% BSA. For the antibodies peroxynitrite (1:500, Sigma) and Abcb11/Bsep (1:1,000, as described by Huls et al. [24]), cryostat slices of 4 µm were used, fixed in acetone for 5 min. Endogenous peroxidase was blocked by incubation with 5% H_2O_2 for 5 min, and nonspecific protein binding was blocked with 10% normal goat serum (Vector, Burlingame, CA, USA) in PBS for 10 min at room temperature. Slices were incubated with the primary antibody in PBS 1% BSA overnight at 4°C. Primary antibody was detected using the Vectastain Elite peroxidase ABC kit (Vector) with 3,3′-diaminobenzidine as substrate. Finally, slices were counterstained with hematoxylin, dehydrated, and mounted in mounting medium. Sections

were examined with a confocal laser-scanning microscope (Leica lasertechnik GmbH, Heidelberg, Germany).

Isolated kidney perfusion

Twelve hours after the administration of LPS+ ($n=6$) or LPS− ($n=4$), the transport activity of Abcc2/Mrp2 was determined by monitoring the urinary excretion of calcein in an isolated perfused rat kidney, as described in detail previously [25]. Briefly, rats were anesthetized IP with pentobarbital (6 mg/100 g), and furosemide was injected IP (1 mg/100 g) to prevent deterioration of the distal nephron.
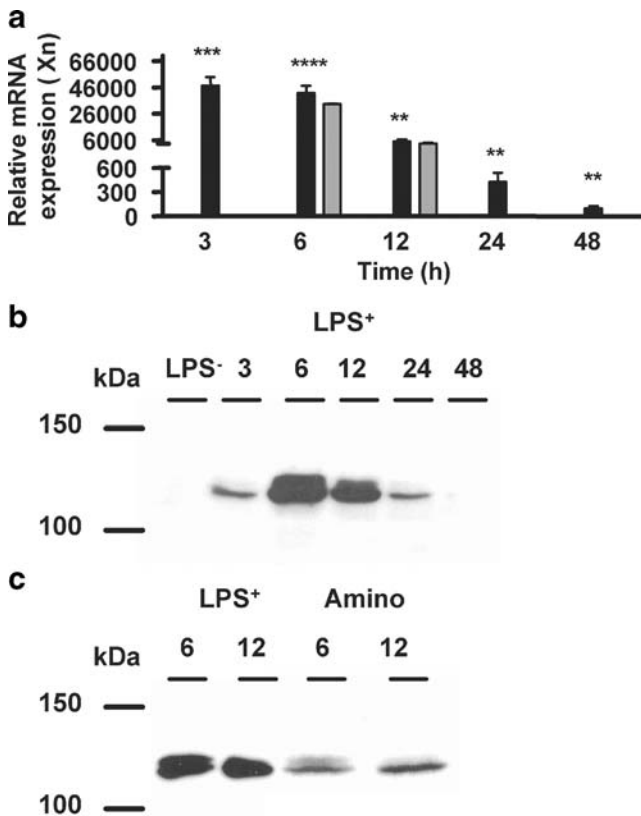

Fig. 1 iNOS mRNA and protein. a iNOS mRNA expression in rat kidney at different time points after LPS+ was determined with RQ-PCR without (closed bars, $n=6$) or with coadministration of aminoguanidine (gray bars, $n=6$). The iNOS mRNA expression was normalized for the GAPDH C_T value (16.06±0.04, $n=15$), and ΔC_T values for the LPS− were set to 1. Data are expressed as mean±SE. Significantly different compared to the LPS− (double asterisks $P<0.01$, triple asterisks $P<0.001$, quadruple asterisks $P<0.0001$). Cytosolic fractions of rat kidney were isolated and expression of iNOS was determined by Western blotting. Representative images ($n=4$) show maximal iNOS protein expression 6 h after LPS+ administration (b), and coadministration with aminoguanidine reversed this induction (c)
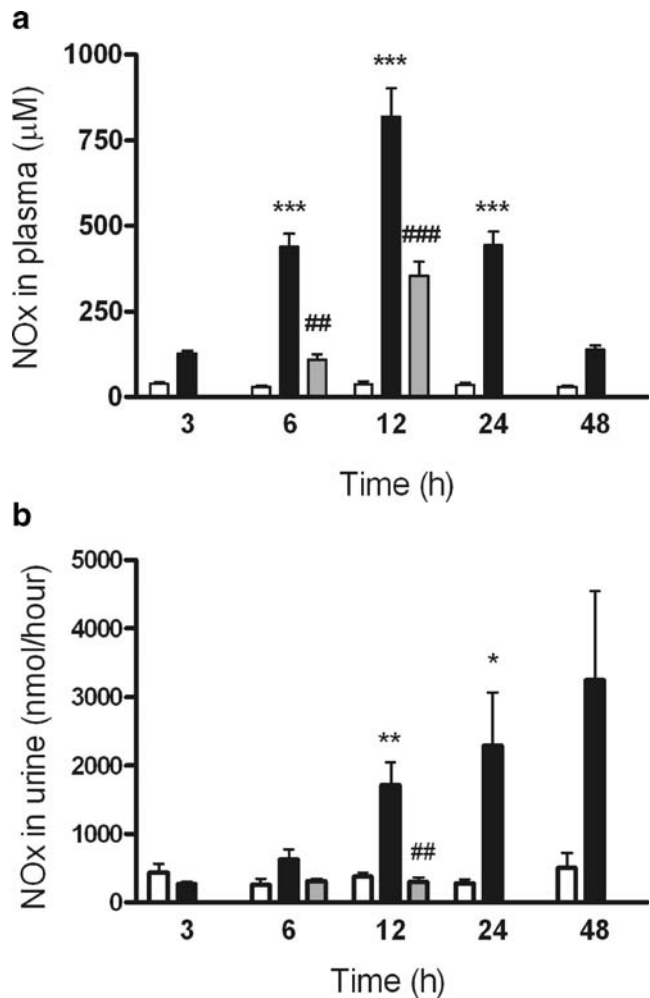

Fig. 2 NOx in plasma and urine. NOx levels in plasma and urine were measured at various times after LPS− (open bars, $n=3$) or LPS+ (closed bars, $n=6$) treatment or LPS+ together with aminoguanidine (gray bars, $n=6$). a Blood samples show a peak concentration at 12 h and coadministration with aminoguanidine reversed this increase at both 6 and 12 h. b The urinary excretion of NOx increased 12 h after LPS+ treatment, and aminoguanidine returned this increase to baseline levels. Data are expressed as mean±SE. Significantly different compared to the LPS− (single asterisks $P<0.05$, double asterisks $P<0.01$, triple asterisks $P<0.001$) or LPS+ (double sharp signs $P<0.01$, triple sharp signs $P<0.001$)

Heparin (125 IU/100 g) was injected in the spleen. The kidney was excised and placed in a fluid bath with a constant temperature of 37.5°C and rats were killed by exsanguination. Vitamin B12 was used for the determination of the glomerular filtration rate (GFR) and calcein-acetoxymethylester was used as a source for the fluorescent substrate calcein [25].

Statistical analysis

Values are given as mean ± SE. Differences between the experimental groups were tested using one-way ANOVA with Bonferroni's correction or two-way ANOVA corrected for repeated measurements. A two-sided P value < 0.05 was considered significant.
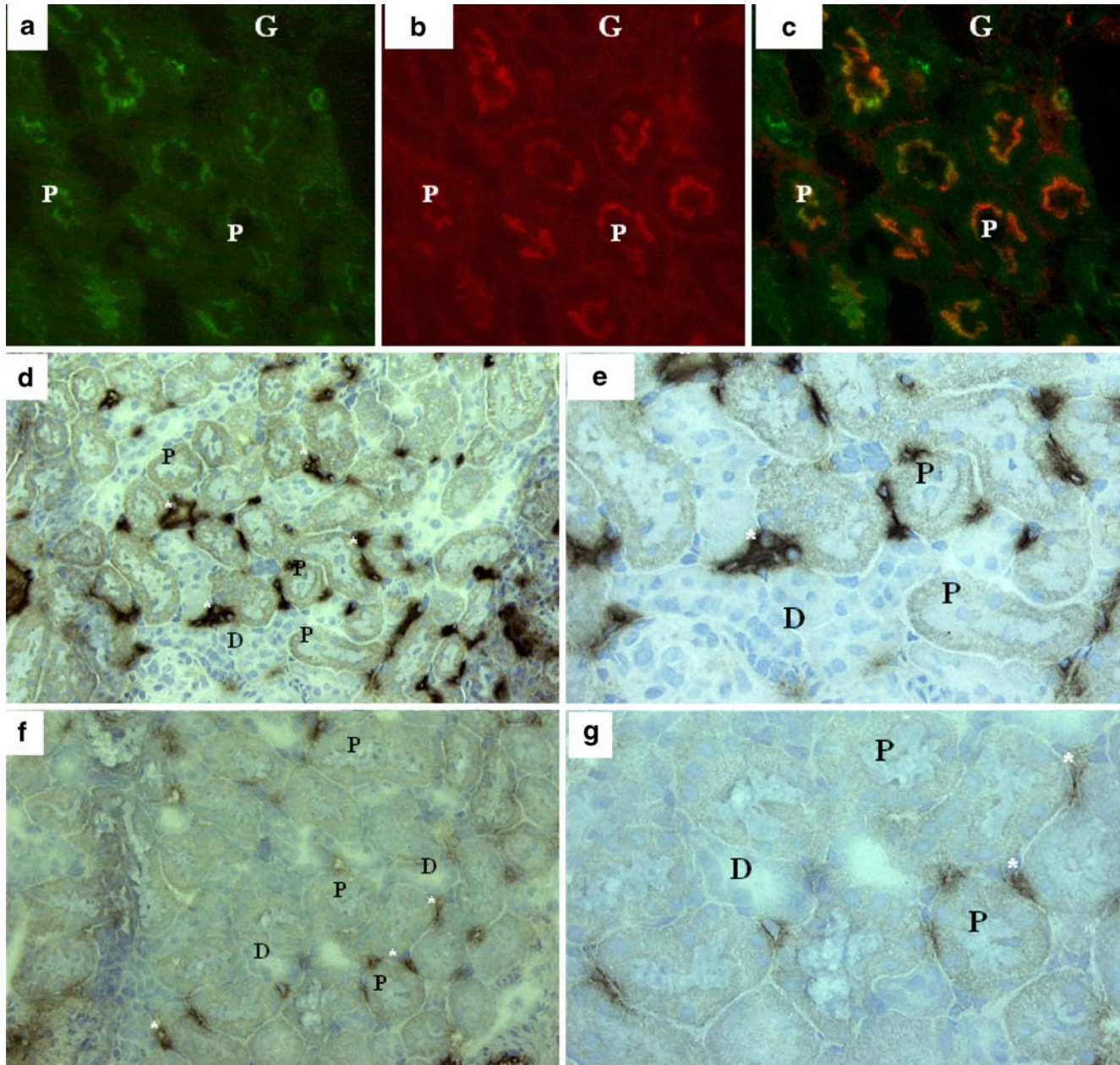

Fig. 3 Immunofluorescence microscopic analysis of iNOS and peroxynitrite in the kidney. a–c Representative immunohistochemical images show that iNOS (green staining) is expressed in the vicinity of the apical membrane (P-gp counterstaining, red) in all segments of the proximal tubule 12 h after LPS[+] treatment. d, e Formation of 3-nitrotyrosine-protein adducts, a reliable biomarker of peroxynitrite generation, were visible in the cortex in peritubular capillaries and proximal tubules 12 h after LPS[+] treatment. f, g Coadministration with aminoguanidine resulted in attenuated formation of 3-nitrotyrosine-protein adducts at 12 h. G glomerulus, asterisks peritubular staining, P proximal tubule, D distal tubule. Original magnification d, f ×200 and a–c, e, g ×400

Results

Signs of inflammation

A few hours after LPS$^+$ administration, inflammatory symptoms appeared, like diarrhea and piloerection. Body temperature was elevated after 24 h and the loss in body weight increased from −3% (t=3 h), due to normal circadian rhythm, to 12% (t=48 h), compared to the controls (Table a in the supplementary material 1). Furthermore, administration of LPS$^+$ resulted in the expected leucopenia (t=3 h) followed by leukocytosis (t=48 h) and thrombocytopenia, with a maximum at 48 h. Coadministration of LPS$^+$ with aminoguanidine did not affect the inflammatory parameters, except for a greater loss in body weight 12 h after LPS administration.

iNOS induction after LPS treatment

RQ-PCR was used to determine the iNOS mRNA levels in kidney samples. The relative expression of iNOS in LPS$^-$ rats was normalized for the average cycle threshold (C_T) value for the housekeeping gene, GAPDH (C_T=16.06± 0.04, n=15), and set to 1. GAPDH expression remained stable during endotoxemia (C_T=16.22±0.09, n=42). Furthermore, no circadian rhythm for the different genes was detected in control animals.

Analysis of three separate experiments revealed a more than 40,000 times induction in iNOS mRNA 3 h after LPS$^+$ compared to controls (Fig. 1a), which decreased with time, but was still 100 times higher than control values 48 h after LPS$^+$. Treatment with both LPS$^+$ and aminoguanidine slightly, but not significantly, reversed iNOS mRNA induction

Table 1 Renal functional parameters

Kidney function	Time (h)	Controls (LPS$^-$)	LPS$^+$	LPS$^+$ and aminoguanidine
Total urine volume (ml)	3	1.6±0.24	1.7±0.27	
	6	2.4±0.65	4.45±0.98	7.3±0.76
	12	3.2±0.57	10.0±0.70[a]	9.4±0.65[a]
	24	11.5±0.25	13.7±2.11	
	48	23.5±3.47	18.0±1.57	
BUN (mmol/l)	3	6.6±0.20	9.9±0.74	
	6	4.6±0.72	14.0±1.48[b]	12.1±0.82[b]
	12	6.3±0.24	12.3±1.38[a]	15.4±1.27[b]
	24	7.0±0.58	10.6±0.58	
	48	6.5±0.10	9.1±0.38	
Blood creatinine (mmol/l)	3	0.035±0.002	0.049±0.002[b]	
	6	0.039±0.002	0.053±0.002[b]	0.043±0.019[d]
	12	0.037±0.002	0.048±0.002[c]	0.036±0.032[e]
	24	0.038±0.001	0.051±0.001[a]	
	48	0.042±0.001	0.052±0.001	
Glucose excretion (μmol/h)	3	0.290±0.045	0.481±0.341	
	6	0.333±0.086	0.420±0.100	0.478±0.051
	12	0.425±0.106	0.537±0.054	0.332±0.080
	24	0.254±0.006	0.984±0.039[c]	
	48	0.325±0.037	0.606±0.128	
Creatinine clearance (ml/min)	3	1.33±0.12	0.78±0.19	
	6	1.06±0.15	0.87±0.09	0.93±0.35
	12	1.01±0.05	0.90±0.03	1.01±0.13
	24	1.19±0.02	0.91±0.20	
	48	1.00±0.07	1.08±0.37	
FE$_{Na}$ (%)	3	0.816±0.004	0.365±0.073	
	6	1.046±0.145	0.358±0.094[b]	1.423±0.310[e]
	12	1.094±0.097	0.458±0.075[a]	1.610±0.282[e]
	24	1.070±0.024	0.429±0.065[c]	
	48	1.033±0.077	0.121±0.024[a]	

Data are expressed as mean±SE, n=6 for each time point for both LPS$^+$ and LPS$^+$ +aminoguanidine and n=3 for LPS$^-$

FE$_{Na}$ fractional sodium excretion

[a] Significantly different compared to the control group; $P<0.01$
[b] Significantly different compared to the control group; $P<0.001$
[c] Significantly different compared to the control group; $P<0.05$
[d] Significantly different compared to the LPS$^+$ group; $P<0.01$
[e] Significantly different compared to the LPS$^+$ group; $P<0.001$

compared to LPS$^+$ alone (Fig. 1a). Maximal iNOS protein expression was observed in cytosolic fractions 6 h after LPS$^+$, and returned to LPS$^-$ level at 48 h (Fig. 1b). In contrast to its effect on iNOS mRNA expression, aminoguanidine reversed the induction in protein expression (Fig. 1c).

NOx levels in plasma and urine were measured using the Griess assay. An increase in NOx concentration in plasma was already visible 3 h after LPS$^+$, with a peak concentration at 12 h. Coadministration with aminoguanidine reversed the increase in NOx significantly at both 6 and 12 h (Fig. 2a). The urinary excretion of NOx increased 12 h after LPS$^+$, and was still elevated 48 h later. Aminoguanidine returned this increase to baseline levels at both time points (Fig. 2b).

Renal localization of iNOS and formation of 3-nitrotyrosine-protein adducts, a reliable biomarker of peroxynitrite generation [26], were determined by immunohistology. There was no iNOS staining visible in kidney sections of rats treated with LPS$^-$. Positive iNOS staining was seen in both experimental groups after LPS$^+$, in inflammatory cells, and in proximal tubule cells. Forty-eight hours after LPS$^+$, iNOS staining was no longer detectable. Figure 3 shows

representative immunohistochemical images with iNOS expression in all segments of the proximal tubule (3A+C). After colocalization with Abcb1/P-gp (3B), iNOS expression in the vicinity of the apical membrane was visible (3A+C). 3-Nitrotyrosine-protein adducts were predominantly present in the cortex in peritubular capillaries and proximal tubules after LPS$^+$ (Fig. 3d,e). Coadministration with aminoguanidine attenuated the formation of adducts in the cortex (Fig. 3f,g).

Aminoguanidine restores the slightly impaired kidney function and loss of brush border membrane

Kidney function was slightly impaired after LPS$^+$ as indicated by increased blood urea nitrogen (BUN), an increase in plasma creatinine, and an increased urinary glucose excretion (Table 1). Furthermore, the urine/plasma creatinine ratio was decreased (data not shown) as was the creatinine clearance, although not significantly.

Histological examination after LPS$^+$ revealed damage to the proximal tubules, compared to LPS$^-$ (Fig. 4a,b). Loss of brush border membranes and formation of vacuoles

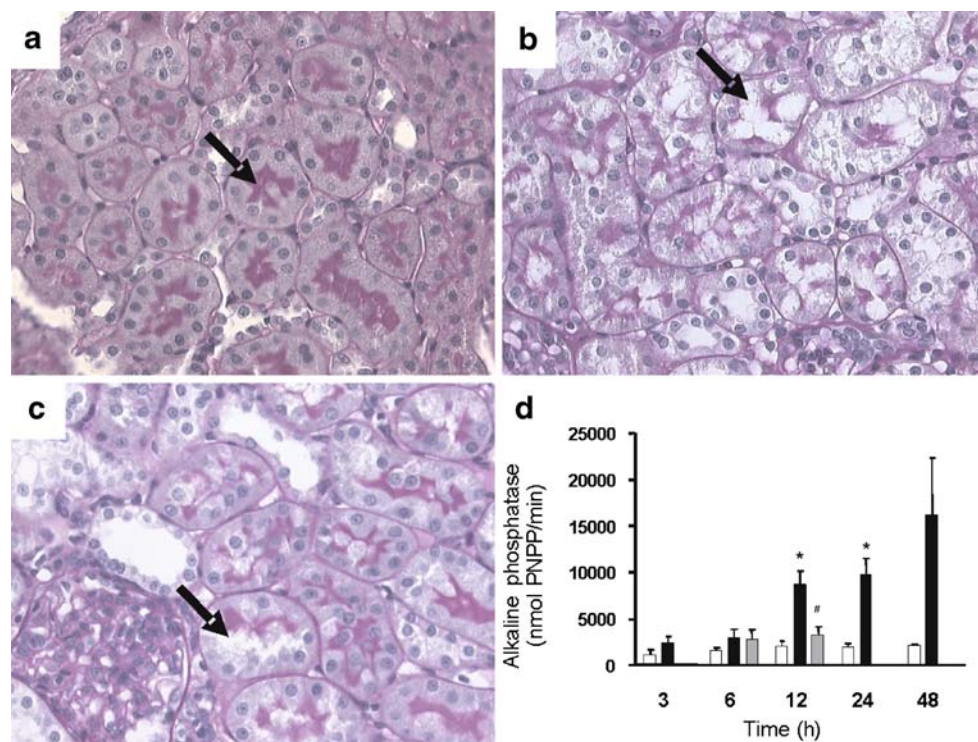**Fig. 4** Proximal tubular damage during endotoxemia. Histological examination after LPS$^+$ revealed damage to the proximal tubules compared to LPS$^-$. **a** LPS$^-$-treated rat with intact brush border membrane in proximal tubules. **b** Twelve hours after LPS$^+$, damage to the proximal tubules is observed with loss of brush border membranes and formation of vacuoles. **c** Treatment with both LPS$^+$ and aminoguanidine resulted in less damage to the proximal tubule. Kidneys of LPS$^+$-treated rats killed after 24 or 48 h also showed signs of proximal tubule damage (data not shown). Proximal tubules are indicated by *arrows*. Original magnifications (**a–c**) ×400. **d** Histological data are supported by an increase in the activity of alkaline phosphatase, marker for proximal tubule damage, in urine samples 12 h after LPS$^+$ administration (*closed bars*, $n=6$), compared to controls (*open bars*, $n=3$). Coadministration with aminoguanidine (*gray bars*, $n=6$) reduced this proximal tubule damage. Data are expressed as mean±SE. Significantly different compared to the LPS$^-$ (*double asterisks* $P<0.01$) or LPS$^+$ (*sharp sign* $P<0.05$)

were observed. Aminoguanidine reduced LPS[+]-induced damage to the proximal tubule (Fig. 4c). The activity of alkaline phosphatase in urine samples was assayed to get an indication of proximal tubule injury. Twelve hours after LPS[+], the activity of alkaline phosphatase was increased in urine samples and was still significantly increased after 48 h (Fig. 4d). Furthermore, the expression of kidney injury molecule 1 (Kim-1 [27]) was strongly up-regulated, 400 times more than control values, 6 and 12 h after LPS[+] (Table b in the supplementary material 1). Except for

BUN, aminoguanidine improved all parameters of renal function, as shown in Table 1 and Fig. 4. No differences in urinary protein excretion were found and, remarkably, the fractional excretion of filtered sodium was decreased (Table 1).

Furthermore, the mRNA expression of different biotransformation enzymes [28], enzymes involved in the NO pathway [2] or known to be activated during oxidative stress [29, 30] was determined. Twelve and 24 h after LPS[+] treatment, expression of the metabolizing enzymes gluta-

Table 2 Gene expression of efflux transporters during endotoxemia

Gene symbol	Protein synonym	Assay IDs	Delta C_T values[a]							
			LPS[−] (controls)	LPS[+] $t=3$ h	LPS[+] $t=6$ h	LPS[+] $t=12$ h	LPS[+] $t=24$ h	LPS[+] $t=48$ h	LPS[+] + amino $t=6$ h	LPS[+] + amino $t=12$ h
Abca1	Abca 1	Rn00710172_m1	7.1±0.31	5.1±0.16	5.8±0.22	7.3±0.55	7.3±0.35	6.8±0.23	6.1±0.23	8.0±0.12
Abca2	Abca 2	Rn00577821_m1	3.5±0.40	3.5±0.35	4.1±0.15	4.5±0.38	4.3±0.35	3.6±0.06	4.4±0.38	4.4±0.12
Abca5	Abca 5	Rn00597824_m1	9.8±0.06	9.8±0.35	9.8±0.10	9.5±0.12	10.0±0.23	10.2±0.21	9.5±0.25	9.7±0.35
Abcb1a	P_gp	Rn00591394_m1	8.4±0.01	Fig. 5a						
Abcb1b	P_gp	Rn00561753_m1	10.8±0.04	Fig. 5b						
Abcb4	Abcb 4	Rn00562185_m1	10.7±0.62	12.3±0.16	12.5±0.21	12.8±0.15	10.9±0.15	7.3±0.42	13.2±0.46	12.4±0.24
Abcb6	Abcb 6	Rn00589801_m1	5.5±0.35	5.9±0.26	6.4±0.25	6.5±0.52	5.9±0.38	5.4±0.14	6.4±0.25	6.2±0.32
Abcb9	Abcb 9	Rn00573757_m1	8.1±0.20	8.5±0.38	9.3±0.29	8.8±0.17	8.6±0.10	8.7±0.17	9.2±0.42	8.9±0.15
Abcb11	Bsep	Rn00582179_m1	10.5±0.40	8.6±0.37	10.3±0.24	11.9±0.21	8.7±0.17	5.4±0.49	9.9±0.21	9.7±0.21
Abcc1	Mrp1	Rn00574093_m1	5.9±0.25	6.2±0.26	6.1±0.25	6.1±0.25	6.3±0.21	5.9±0.23	6.4±0.40	6.2±0.15
Abcc2	Mrp2	Rn00563231_m1	6.7±0.31	Fig. 5c						
Abcc3	Mrp3	Rn00589786_m1	8.4±0.15	8.7±0.12	8.8±0.508	8.6±0.06	8.0±0.35	8.2±0.12	8.8±0.32	8.7±0.23
Abcc4	Mrp4	Rn01465699_q1	6.6±0.06	Fig. 5d						
Abcc5	Mrp5	Rn00588341_m1	7.1±0.01	7.9±0.03	7.6±0.15	6.4±0.06	6.5±0.50	6.5±0.38	7.6±0.45	6.4±0.36
Abcc6	Mrp6	Rn00578778_m1	7.0±0.14	7.0±0.01	8.4±0.21	8.4±0.12	7.8±0.05	6.8±0.35	8.6±0.07	8.3±0.14
Abcc8	Sur1	Rn00564778_m1	11.5±0.40	11.4±0.21	11.4±0.26	13.4±0.15	14.8±0.15	13.2±0.25	11.7±0.23	13.7±0.31
Abcc9	Sur2	Rn00564842_m1	6.8±0.01	7.7±0.26	8.4±0.31	8.1±0.15	7.2±0.32	6.8±0.40	8.0±0.35	8.2±0.32
Abcg1	Abcg1	Rn00585262_m1	6.8±0.15	7.8±0.25	6.8±0.25	7.1±0.40	6.8±0.31	6.7±0.21	6.4±0.31	7.3±0.36
Abcg2	Abcg2	Rn00710585_m1	4.1±0.24	4.5±0.07	4.6±0.21	5.0±0.06	4.5±0.14	4.5±0.07	4.9±0.42	5.2±0.07
Abcg5	Abcg5	Rn00587092_m1	18.8±0.29	[b]						

[a] Data of nine pooled samples, measured in triplicate, are expressed as mean ± SD
[b] Low expressed transport protein: C_T value is not considered as valid

thione S-transferases (GST-α2 and GST-α5) and uridine diphosphate-glucuronosyltransferases (UDPGT1a6) was down-regulated (Table b in the supplementary material 1, at least a 2 times lower expression). The arginine metabolic enzymes, argininosuccinate synthetase and arginase 2, were not changed after LPS$^+$. In contrast, arginase 1 mRNA expression was reduced. The gene expression of the ET$_B$ receptor, which gets activated as part of the NO-dependent signaling pathway regulating Abcb1/P-gp and Abcc2/Mrp2 [13, 31], did not change during endotoxemia. Three and 6 h after LPS$^+$ the mRNA expressions of super oxide dismutase 2, heam-oxygenase 1, and nuclear factor κB (NF-κB) were up-regulated (i.e., at least a 2 times higher expression). Coadministration of aminoguanidine had no effect on the gene expression of all these proteins. In contrast, amino-guanidine reversed the down-regulation of nNOS gene expression during endotoxemia (Table b in the supplementary material 1).

LPS$^+$ treatment affects ABC drug transporter expression in the kidney

The mRNA expression of 20 renal ABC transporters was profiled. Abcb1a/P-gp, Abcb1b/P-gp, Abcc2/Mrp2 and Abcc4/Mrp4 expression was determined with the ABI PRISM 7700 single reporter sequence detection system. In rodents, Abcb1/P-gp exists as two functional isoforms,

which are encoded by two different genes: *Abcb1a* and *Abcb1b* [32]. The other ABC transporters were assessed with the ABI/PRISM 7900HT Gene Expression Micro Fluidic Card (Table 2). Their expression was normalized for the average C_T value for GAPDH, as described for iNOS. Other housekeeping genes, like glucuronidase and hypo-xanthine guanine phosphoribosyl transferase 1, gave comparable values to GAPDH (data not shown). Abcb1b/Pg-p and Abcc2/Mrp2 were clearly up-regulated 3 h after LPS$^+$ and were still elevated 12 and 24 h later (Fig. 5b,c). Furthermore, Abcb1a/P-gp and Abcc4/Mrp4 were slightly, but not significantly, increased 12 h after LPS$^+$ treatment (Fig. 5a,d). Table 2 presents a list of the expression of other transporter genes during endotoxemia. Abca1 showed a slight up-regulation 3 h after LPS$^+$, but returned to basal levels within 12 h. Furthermore, Abcb11 was up-regulated, but only significantly after 48 h. A down-regulation was observed for Abcb4 and Abcc8 (at least a 2 times lower expression). The expression of the Abcg genes was not affected by endotoxemia.

Coadministration with aminoguanidine blocked the induction in mRNA expression of both Abcb1b/P-gp and Abcc2/Mrp2 at 6 and 12 h, respectively (Fig. 5b,c). Furthermore, aminoguanidine reduced the increased protein expression of both transporters 12 h after LPS$^+$ treatment (Fig. 6b,d), indicating a regulatory role for NO in the expression of these efflux transporters. Abcc4/Mrp4 protein

Fig. 5 mRNA expression of different renal ABC transporters. Gene expression of Abcb1/P-gp (a) and Abcb1b/P-gp (b), Abcc2/Mrp2 (c), and Abcc4/Mrp4 (d) were determined in rat kidney at different time points after LPS$^-$ (*open bars*, n=3) or LPS$^+$ (*closed bars*, n=6) treatment or LPS$^+$ together with aminoguanidine (*gray bars*, n=6). ΔC_T values for the LPS$^-$, given in Table 2, were set to 1. Data are expressed as mean±SE. Significantly different compared to the LPS$^-$ (*single asterisks* $P<0.05$, *double asterisks* $P<0.01$, *triple asterisks* $P<0.001$) or LPS$^+$ (*sharp signs* $P<0.05$)
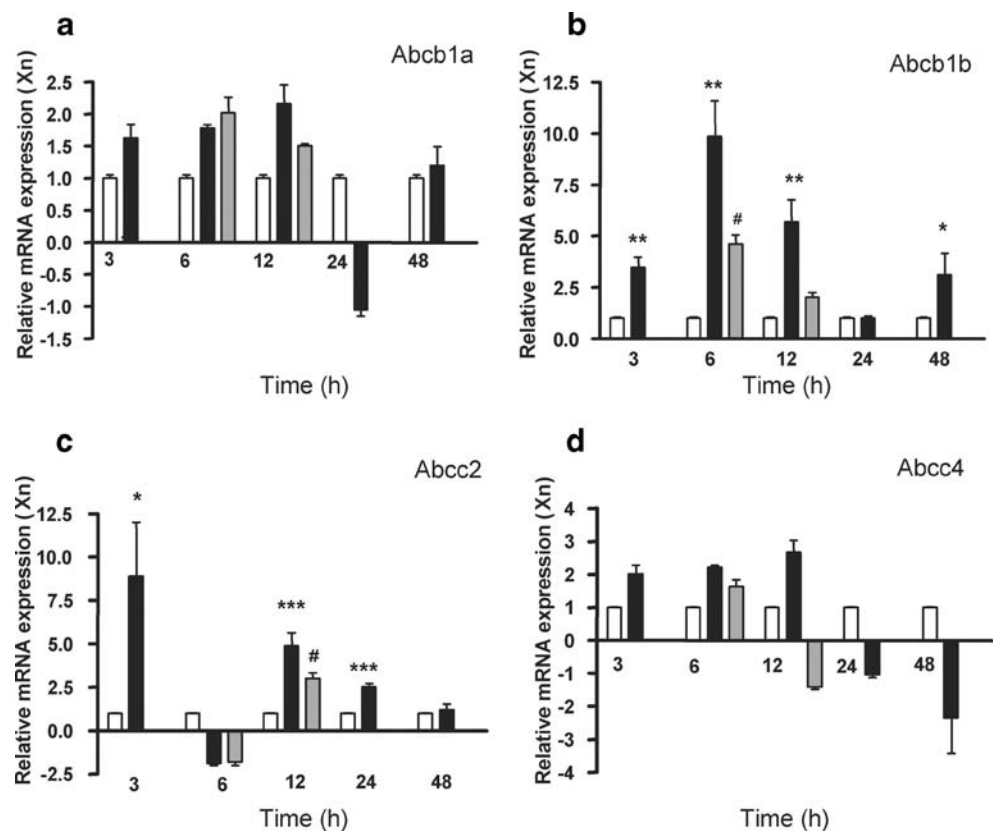
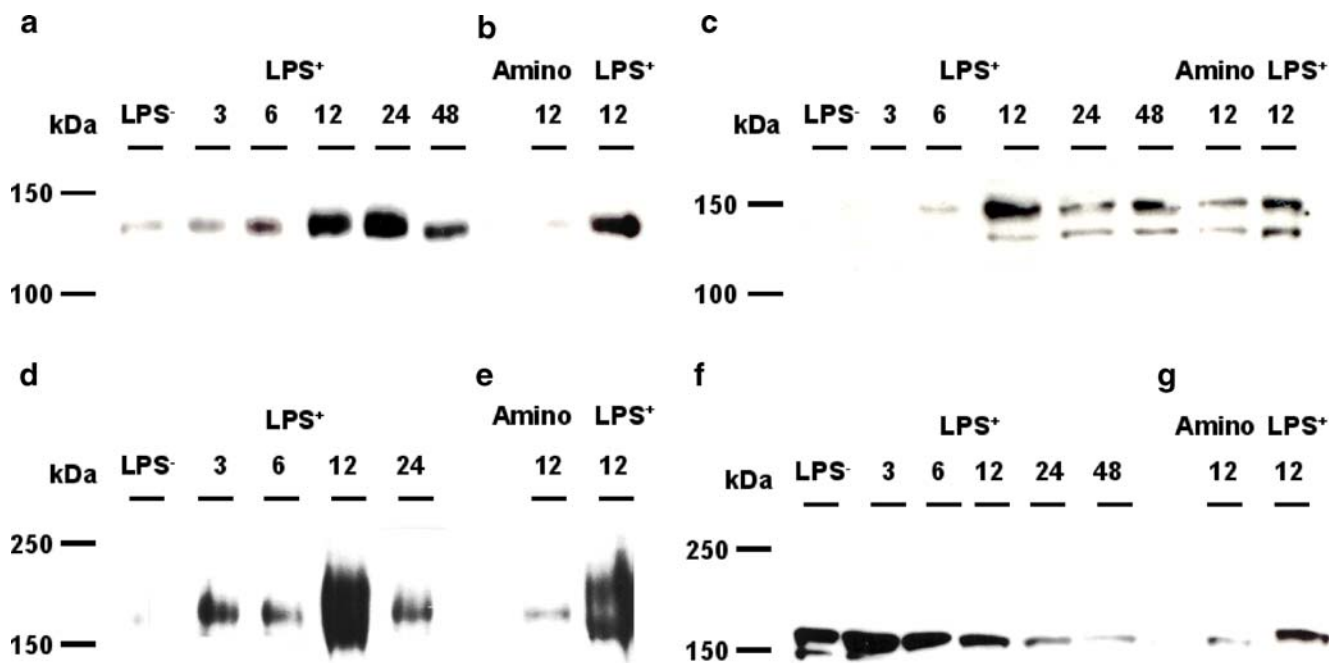Fig. 6 Protein expression of different renal ABC transporters. Total membrane fractions of rat kidney were isolated and expression of Abcb1/P-gp (a, b), Abcb11/Bsep (c), Abcc2/Mrp2 (d, e), and Abcc4/Mrp4 (f, g) was determined by Western blotting. Representative images (n=4) show maximal Abcb1/P-gp protein expression 12 and 24 h after LPS+ (a), and coadministration with aminoguanidine reversed this induction (b). The protein expression of Abcb11/Bsep was up-regulated 12 h after LPS+ treatment, which was reversed by aminoguanidine (c). Maximal Abcc2/Mrp2 protein expression was seen 12 h after LPS+ (d) and aminoguanidine reversed this induction (e). Abcb4/Mrp4 protein expression was down-regulated during endotoxemia (f), which was intensified by aminoguanidine (g)

levels, on the other hand, decreased even more after treatment with both LPS+ and aminoguanidine. Gene expression levels of other transporters were not affected by aminoguanidine (Table 2).

The expression patterns of Abcb1/P-gp, Abcb11, Abcc2/Mrp2, and Abcc4/Mrp4 were investigated further. Using total membrane fractions, we found an up-regulation of Abcb1/P-gp 6 h after LPS+, with maximal protein expression 12 and 24 h after LPS+ (Fig. 6a). Abcb11 is the major liver canalicular bile salt export pump (Bsep), but surprisingly, this transporter is also expressed at the apical membrane of mice renal proximal tubule, as we discovered recently [24]. In agreement with an increased mRNA expression, Abcb11/Bsep protein was up-regulated after LPS+ (Fig. 6c).

Although we are not completely certain whether the C219 antibody against Abcb1/P-gp also detects Abcb11/Bsep [33], our Western blot results showed expression of Abcb1/P-gp, with a molecular weight of 130 kDa, and it does not seem to cross-react with Bsep at 160 kDa.

By using immunohistochemistry, the expression and localization of Abcb11/Bsep was investigated in endotoxemic rat kidneys. In agreement with mouse kidney, positive staining was observed in the apical membranes of the proximal tubule 12 h after LPS+ treatment (Fig. 7). In addition, we observed staining in rat glomerular endothelium and peritubular capillaries.

Furthermore, a clear increase in Abcc2/Mrp2 protein was observed after LPS+ (Fig. 6d), but a down-regulation in

Fig. 7 Distribution of Abcb11/Bsep in rat kidney cortex. a, b Representative immunohistochemical images show apical staining in proximal tubules (indicated by arrows). In addition, glomerular and peritubular staining of Bsep in the cortex was found. G glomerulus, asterisks peritubular staining, P proximal tubule, D distal tubule. Original magnification in a ×200, and in b ×400
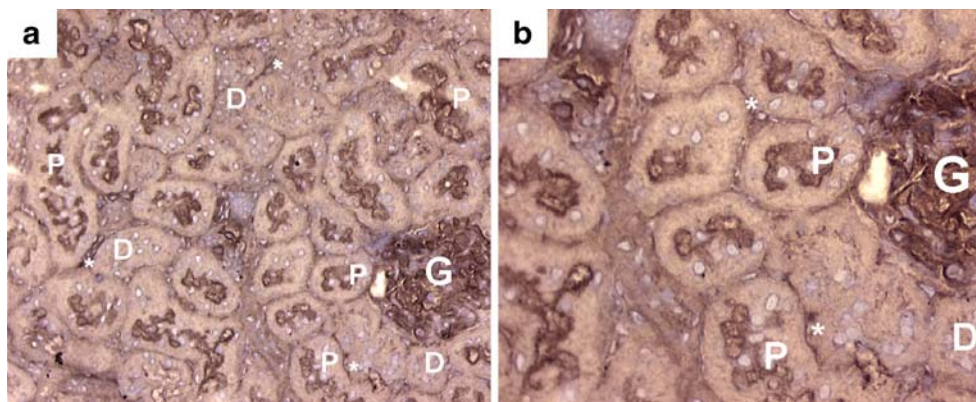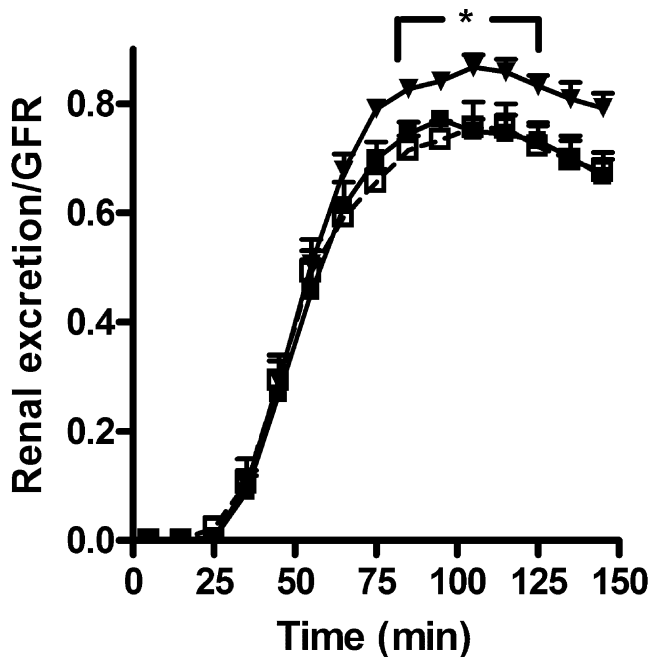
Fig. 8 Renal calcein excretion in isolated perfused rat kidneys. Secretion of the fluorescent calcein into the urine was measured after 12 h in untreated (*closed squares* control; *n*=4), LPS⁻ (*open squaresn*=4), or LPS⁺ (*trianglesn*=6) rats. Urinary excretion was corrected for GFR. Significantly different compared to the LPS⁻ (*asterisk P*<0.05)

Abcc4/Mrp4 was found (Fig. 6f). We measured the function of Abcc2/Mrp2 by monitoring the urinary excretion of calcein in an isolated perfused rat kidney 12 h after LPS⁺ or LPS⁻. A 16±3% increase in calcein excretion was observed after LPS⁺, as shown in Fig. 8. We do not expect calcein to be a Bsep substrate because Mrp2-deficient (TR−) rats clearly showed that calcein excretion was highly reduced [25], despite the presence of Bsep in these strains (unpublished data). Twenty-four hours after LPS⁺, Abcc2/Mrp2 activity was still elevated, although not significantly. In accordance, endotoxemia affected renal function as observed by diminished GFR and fractional reabsorption of water in isolated perfused rat kidneys (Table 3).

Discussion

In the present study, we investigated the role of NO, produced by iNOS, in the differential expression of renal ABC transporters in rats in vivo. A clear up-regulation of Abca1, Abcb1/P-gp, Abcb11/Bsep, and Abcc2/Mrp2 expression was found after LPS⁺. In addition, we observed a functional up-regulation of Abcc2/Mrp2 in perfused kidneys. This supports a detoxifying role of the efflux pump, which is in accordance with the up-regulation of Abcc2/Mrp2 protein in killifish renal tubules [34, 35], in rat kidney, and in MDCKII cells overexpressing human ABCC2/MRP2 after long-term exposure to the nephrotoxicant gentamicin [20], and of abcb1/P-gp in mouse kidneys after ischemic reperfusion injury [24]. Indeed, blocking Abcb1/P-gp during endotoxemia leads to further renal damage (Heemskerk, Wouterse, Russel, and Masereeuw, preliminary findings). The renal drug transporters are likely to be under direct influence of NO produced by iNOS, as coadministration of the iNOS inhibitor, aminoguanidine, attenuated the LPS-induced effects on transporter expression. Aminoguanidine treatment results in decreased NO production and, subsequently, reduced oxidative stress by decelerating the formation of peroxynitrite. Under these conditions, less toxic metabolites may be produced, explaining the restoration in transporter expression and reversal of proximal tubular damage.

In contrast to our findings, Cherrington et al. [36] reported that coadministration of aminoguanidine failed to change the expression of different hepatic drug transporters during endotoxemia. This discrepancy might be due to organ differences and the fact that Cherrington et al. pretreated the animals with the NOS inhibitor, whereas we gave aminoguanidine 1 h after LPS⁺. The lack of an effect of aminoguanidine on the expression of different biotransformation enzymes, enzymes involved in the iNOS pathway and during oxidative stress, supports a direct function of NO itself in ABC transporter regulation.

Table 3 Functional parameters of isolated perfused rat kidneys in control rats (no additional treatment) and after exposure to LPS⁻ or LPS⁺ for 12 h

Parameter	Controls (*n*=4)	LPS⁻ (*n*=4)	LPS⁺ (*n*=6)
GFR (μl/min)	350±25	380±17	290±20[a]
Diuresis (μl/min)	18.6±1.8	21.1±1.3	17.4±1.0
FR$_{water}$ (%)	94.7±0.2	94.5±0.3	92.7±0.6[a]
RPP (mmHg)[b]	88.4±1.5	84.5±1.7	88.7±1.0

Data are expressed as mean ± SE of four to six perfused kidney experiments over the period 25–115 min. Untreated rats served as controls for this model. Kidneys were perfused for 150 min as described in the "Materials and methods" section

[a] Significantly different from perfused control kidney (P<0.05)

[b] Constant flow during the experiments was 18±0.5 ml/min

FR fractional reabsorption, *RPP* renal perfusion pressure

The role of two up-regulated efflux pumps, Abca1 and Abcb11/Bsep, in the kidney is unknown. In humans, ABCA1 is widely expressed and found to be the causative gene in Tangier disease, a disorder of cholesterol transport between tissues and the liver, mediated by binding of the cholesterol onto high-density lipoprotein particles [37]. ABCA1 controls the extrusion of membrane phospholipid and cholesterol toward specific extracellular acceptors; however, the exact role of the protein in this process is not known. Moreover, its expression and function in the kidney requires further investigation. The presence of Abcb11/Bsep, the major canalicular bile salt export pump, was recently demonstrated by us in mouse kidney [24], and here we show a clear apical expression in rat kidney proximal tubules for the first time as well. Hence, the function of Abcb11/Bsep in the kidney still needs to be determined. In addition to their up-regulation in the kidney, we found a down-regulation of Abcb1/P-gp, Abcc2/Mrp2, and Abcb11/Bsep in the liver (data not shown). This is in agreement with previous findings for the hepatic expression of the transporters during inflammation [36, 38–40], obstructive jaundice [41], and cholestasis [42]. An induction of the transporters in the kidney during liver failure may function as a compensatory elimination mechanism to reduce enhanced levels of circulating bile salts caused by endotoxin-induced cholestasis [39] and/or to protect proximal tubular cells against the higher exposure via primary urine.

A differential regulation was found for Abcc4. Although this transporter is expressed in the same nephron segments as Abcb1/P-gp and Abcc2/Mrp2, the apically expressed efflux pump clearly has a distinct regulation. In killifish, we previously found renal proximal tubules exposed to nephrotoxicants or ET-1 that Abcc2/Mrp2-mediated transport and Mrp2 expression in the luminal membrane were increased after 24 h [34], whereas the expression of Abcc4/Mrp4 remained unchanged [35]. Furthermore, Abcc4/Mrp4 protein levels in rat kidney were decreased during cholestasis in contrast to the Abcc2/Mrp2 levels [43]. No changes in Abcc4/Mrp4 mRNA were found, suggesting a posttranscriptional regulation of transporter expression, including phosphorylation and protein routing. A down-regulation of ABC transporters, as observed for Abcc8/Sur1 and initially also for Abcb4, may be explained by the different functions of these ABC members in the kidney.

LPS itself may affect transporter expression after binding to the Toll-like receptor 4 and a second, not yet identified, factor through activation of NOS-dependent pathways, as was found for Abcb1/P-gp in rat brain capillaries [44]. Furthermore, systemic TNF-α, released during endotoxemia, may regulate efflux pumps, including ABC transporters [45, 46]. This cytokine might act through NOS activation [44], but also through a pathway that does not involve iNOS-derived NO during endotoxin-associated renal failure [47]. Furthermore, regulation of iNOS after exposure to LPS occurs at the level of gene transcription and may require activation of the NF-κB pathway [48]. It has been shown previously that an up-regulation of Abcb1/P-gp in renal failure, i.e., after ischemic–reperfusion damage [24] or exposure to cadmium [49], might be signaled through an NF-κB-dependent pathway. Consistent with these findings, we observed a clear induction (sevenfold) of NF-κB expression 3 h after LPS+ as well.

Selective inhibition of renal iNOS has important implications for the treatment of septic acute kidney disease. Changes in the expression levels of renal drug transporters alters the pharmacokinetics of many clinically important drugs [50] and has implications for the prediction of overall drug disposition during sepsis. For example, glucocorticoids are substrates for Abcb1/Pg-p and are often prescribed for their anti-inflammatory properties in septic patients. An up-regulation of Abcb1/Pg-p, as observed in our study, might have consequences for therapeutic levels of these substrates [51].

In conclusion, after LPS+, a clear up-regulation in Abca1, Abcb1/P-gp, Abcb11/Bsep, and Abcc2/Mrp2 was found, whereas Abcc8/Sur1 was down-regulated. Abcb4 and Abcc4/Mrp4 show a differential pattern during the course of endotoxemia. The renal drug transporters are likely to be under direct influence of NO produced by iNOS, as coadministration of the iNOS inhibitor, aminoguanidine, attenuated the LPS-induced effects on their expression. Apparently, an up-regulation of important efflux pumps diminishes the renal accumulation of toxic compounds and attenuates further proximal tubular damage during endotoxemia.

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