COX-2 inhibition attenuates endotoxin-induced downregulation of organic anion transporters in the rat renal cortex

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Renal excretion of organic anions such as para-aminohippurate is reduced during severe sepsis and following ischemia/reperfusion injury. In order to better define the pathophysiology of sepsis-associated renal tubular dysfunction we measured the effect of lipopolysaccharide on renocortical organic anion transporter (OAT) expression in the rat. Prostaglandin E2 (PGE_2) downregulates OATs *in vitro*, therefore, we also evaluated the effect of the cyclooxygenase (COX)-2 inhibitor parecoxib on this process. Endotoxemia caused a time- and dose-dependent decrease of OAT1 and OAT3 expression that paralleled increased renocortical COX-2 expression and PGE_2 formation. Pretreatment with parecoxib decreased endotoxin-stimulated PGE_2 formation. Parecoxib attenuated OAT1 and OAT3 gene repression in the rat kidney following endotoxin treatment and during ischemia/reperfusion-induced acute renal injury. COX-2 inhibition improved the creatinine clearance in lipopolysaccharide-treated rats but not after ischemia/reperfusion-induced acute renal injury. The decreased clearance of para-aminohippurate in rats following endotoxin- or ischemia/reperfusion-induced renal injury was improved by parecoxib. Our findings show that COX-2 derived prostanoids downregulate OATs during lipopolysaccharide-induced acute renal injury.

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Sepsis and septic shock are important risk factors for acute renal failure (ARF), which is defined as the abrupt decline in glomerular filtration rate and tubular function.[1] The mortality rate of sepsis-related ARF is still high at 75%.[2] Therefore, the understanding of the pathogenesis of sepsis-related ARF is of critical importance. Several *in vivo* and *in vitro* studies have suggested that the reduction of glomerular filtration rate in sepsis is secondary to altered glomerular hemodynamics.[3] However, the pathophysiology of sepsis-associated renal tubular dysfunction with altered renal handling of drugs has been poorly explained.

The kidney excretes a large variety of drugs. Two principle processes are responsible for their renal elimination: glomerular filtration and proximal tubular secretion.[4] The tubular secretion of organic anions, for example, needs cellular transport mechanisms. Organic anion transporters (OATs) are necessary for the uptake of organic anions from the peritubular plasma across the basolateral membrane into the proximal tubule cell in exchange for α-ketoglutarate.[5] Among the several OATs, the transporters OAT1 and OAT3 have been proposed to be responsible for this step.[6] In the past years, it became apparent that several chemically unrelated endogenous and exogenous compounds, like *para*-aminohippurate (PAH), cAMP, diuretics, and antibiotics are transported such as the OAT system.[6] In addition, prostaglandin E2 (PGE_2) is secreted into the urine at substantial rates by the OAT system of renal proximal tubules, likely by OAT1 and OAT3.[6,7] Recently, it has been demonstrated that PGE_2 downregulates the expression OAT1 and OAT3 *in vitro*.[8] Moreover, it has been found that OATs are downregulated during ischemia/reperfusion (I/R)-induced ARF and ureteral obstruction,[9–11] conditions under which renal cyclooxygensase (COX)-2 expression is increased.[12,13] Therefore, one may assume that the downregulation of OATs during ARF could be because of an increased formation of COX-2 derived PGE_2.

As renal COX-2 expression is increased in response to lipopolysaccharide (LPS),[14] which is an experimental approach commonly used in examining the pathogenesis of sepsis, and because administration of LPS alters the renal handling of several drugs,[15–22] we investigated in this study

the effect of LPS on the expression of OAT1 and OAT3 in the rat renal cortex. As we found that the LPS-induced downregulation of OATs is paralleled by an increased expression of renocortical *COX-2* gene, we hypothesized that the downregulation of OATs during endotoxemia could be because of an enhanced formation of COX-2 derived PGE_2 in the rat renal cortex. Therefore, we studied the effect of the COX-2 inhibitor parecoxib on LPS-induced downregulation of renocortical OATs and in addition on I/R-induced downregulation of OATs.

RESULTS

Effect of LPS on OAT1 and OAT3 expression

Injection of LPS for 3 h did not alter mRNA expression of OAT1 and OAT3. However, treatment with LPS for 6 and 12 h decreased OAT1 and OAT3 mRNA expression in the rat renal cortex. OAT1 mRNA was downregulated to 56 and 42% of control levels 6 and 12 h after LPS injection, respectively. OAT3 mRNA levels decreased to 44 and 30% of control levels 6 and 12 h after LPS administration, respectively (Figure 1a). Injection of increasing doses of LPS (1, 3, and 10 mg/kg) caused a dose-dependent downregulation of OAT1 mRNA abundance to 67, 50, and 44% of control levels 12 h after LPS injection, respectively. OAT3 mRNA abundance also decreased dose dependent to 55, 40, and 29% of control values, respectively (Figure 1b). We further investigated the expression of OAT1 and OAT3 protein in basolateral membranes from the renal cortex of vehicle and LPS-treated animals and found that the expression of OAT1 and OAT3 protein was decreased in animals treated for 12 h with LPS (Figure 1c). OAT1 immunoreactivity was detected in proximal tubules of vehicle-treated rats and was clearly decreased in rats treated with LPS (Figure 2a–d).

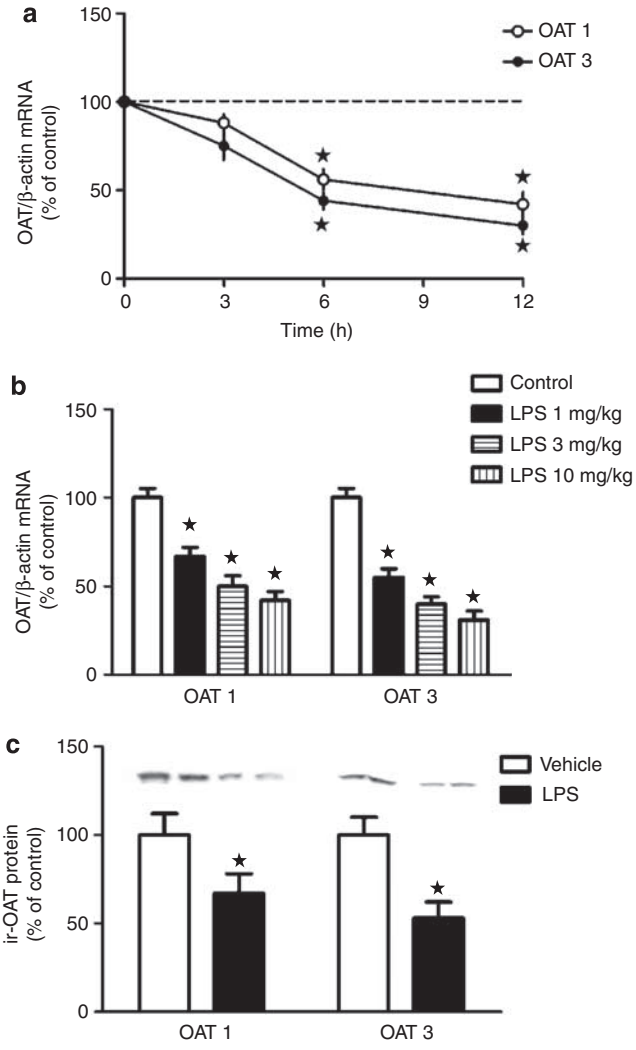

Figure 1 | Effect of lipopolysacharide (LPS) on organic anion transporters (OATs). (**a**) Time-dependent effect of LPS (10 mg/kg) on renocortical OAT1 and OAT3 mRNA expression related to β-actin mRNA expression. (**b**) Dose-dependent effect of LPS on renocortical OAT1 and OAT3 mRNA expression related to β-actin mRNA expression. (**c**) Effect of LPS (10 mg/kg for 12 h) on OAT1 and OAT3 protein expression in basolateral membranes from the renal cortex. Insets show representative immunoblots for OAT1 and OAT3. Values are mean ± s.e.m. for six rats. $^*P < 0.05$ vs control.

Time- and dose-dependent effect of LPS on COX-1 and COX-2 expression and on renocortical PGE_2 formation

We further evaluated the effect of LPS on renocortical PGE_2 formation and on the expression of the two COX-isoforms, because it has been suggested that PGE_2 downregulates OATs *in vitro*.[8] Intravenous injection of LPS did not influence renocortical *COX-1* gene expression (Figure 3a–c). Treatment with LPS (10 mg/kg) for 3, 6, and 12 h increased COX-2 mRNA expression in the rat renal cortex 3.1-, 3.8-, and 3.4-fold, respectively (Figure 3a). Injection of increasing doses of LPS (1, 3, and 10 mg/kg) caused a dose-dependent upregulation of COX-2 mRNA abundance to 190, 249, and 379% of control levels 12 h after LPS injection, respectively (Figure 3b). Twelve hours after LPS injection, renocortical COX-2 protein expression was increased about twofold of control levels (Figure 3c). The increase in *COX-2* gene expression was paralleled by an increase in renocortical PGE_2 concentration to 188% of baseline values (Figure 3d).

Effect of parecoxib on LPS-induced renocortical PGE_2 concentration

To evaluate the role of COX-2 derived PGE_2 for the downregulation of OATs during LPS treatment, we further investigated the effect of the COX-2 inhibitor parecoxib (20 mg/kg; i.p.). Parecoxib treatment decreased basal PGE_2 tissue concentration to about 76% of control values and pretreatment with parecoxib for 1 h inhibited the LPS-induced rise in renocortical PGE_2 formation (Figure 3d).

Effect of parecoxib on LPS-induced downregulation of OAT1 and OAT3 expression

Sole parecoxib treatment did not influence the expression of OAT1 and OAT3 (Figure 4a–d). In animals treated for 12 h with LPS in combination with parecoxib, mRNA expression of OAT1 substantially increased from 33 to 70% compared to treatment with LPS alone (Figure 4a). Furthermore, the

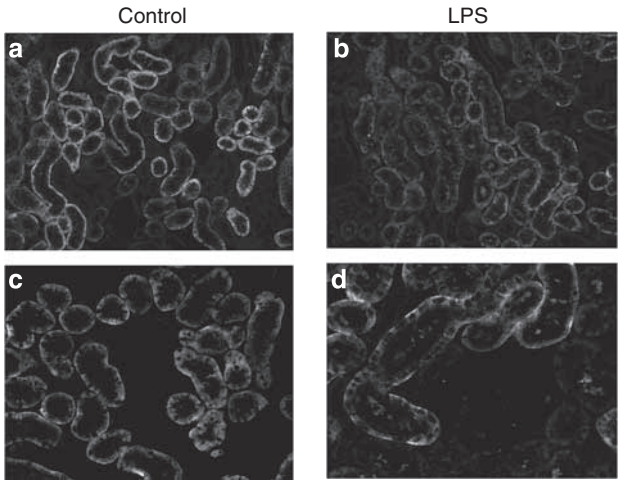

Figure 2 | Immunohistochemistry of OAT1 in the renal cortex. Vehicle (**a**, **c**) and LPS-treated rats (**b**, **d**). These figures are representative of typical samples from three rats. **a**, **b** ×200. **c**, **d** ×400.

combination of LPS with parecoxib attenuated the LPS-induced downregulation of OAT3 mRNA abundance from 43 to 70% (Figure 4b). Consistent with the mRNA expression levels, the decreased immunoreactivity of OAT1 and OAT3 in LPS-treated rats was markedly attenuated in response to additional parecoxib treatment (Figure 4c and d).

Renal and hemodynamic parameters of rats

Mean arterial pressure (MAP) decreased 12 h after LPS injection from 92 ± 4 to 47 ± 7 mm Hg. Sole parecoxib did not alter MAP, but attenuated the LPS-induced fall in MAP to about 72 ± 6 mm Hg. Plasma urea levels and plasma creatinine levels increased 3.4- and 2.8-fold after injection of LPS, respectively. Parecoxib did not alter plasma creatinine levels, but attenuated the LPS-induced rise in plasma creatinine levels (Figure 5a). LPS injection decreased creatinine clearance from 0.85 ± 0.07 to 0.36 ± 0.04 ml/min. Parecoxib did not alter creatinine clearance, but attenuated the LPS-induced decrease in creatinine clearance (Figure 5b). PAH clearance was decreased 12 h after LPS injection. Parecoxib did not alter PAH clearance, but attenuated the

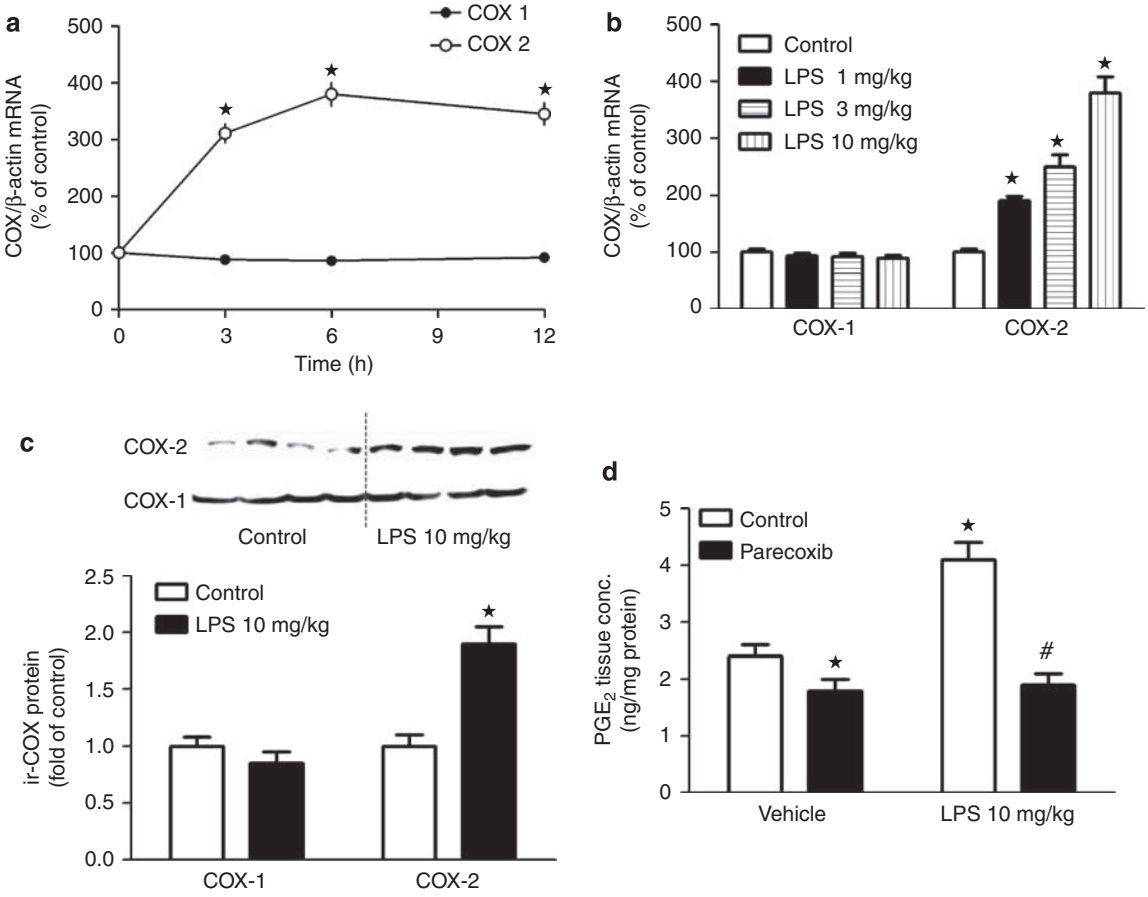

Figure 3 | Time- and dose-dependent effects of lipopolysacharide (LPS) on cyclooxygenase (*COX*) gene expression and effect of parecoxib on renocortical prostaglandin (PG) E_2 concentration. (**a**) Time- (LPS 10 mg/kg for 0, 3, 6, and 12 h) and (**b**) dose-dependent effect of LPS (1, 3, and 10 mg/kg for 12 h) on renocortical COX-1 and COX-2 mRNA expression related to β-actin mRNA expression. (**c**) Renocortical COX-1 and COX-2 protein expression in rats treated with LPS (10 mg/kg) for 12 h. (**d**) Renocortical prostaglandin E_2 (PGE_2) concentration in rats treated with LPS (10 mg/kg), parecoxib (20 mg/kg) or the combination of LPS with parecoxib for 12 h. Inset shows representative immunoblots for COX-1 and COX-2. Values are mean ± s.e.m. for six rats. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs LPS.

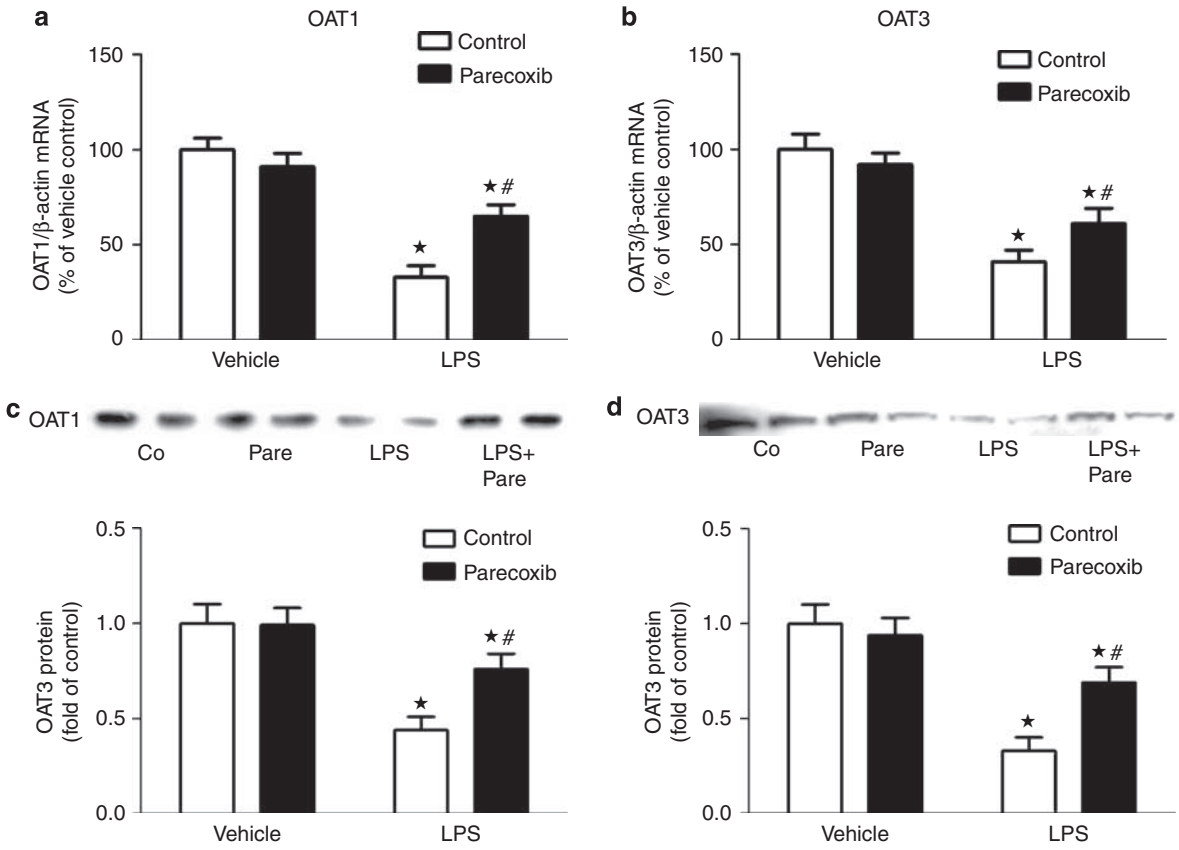

Figure 4 | Effect of parecoxib on lipopolysacharide (LPS)-induced (10 mg/kg) downregulation of renocortical organic anion transporters (OATs) 12 h after injection of LPS. (**a**) Renocortical OAT1 and (**b**) OAT3 mRNA expression related to β-actin mRNA expression. (**c**) Renocortical OAT1 and (**d**) OAT3 protein expression. Insets show representative immunoblots for OAT1 and OAT3 (Co = Control; Pare = Parecoxib). Values are mean ± s.e.m. for six rats. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs LPS.

LPS-induced fall in PAH clearance (Figure 5c). We further calculated tubular PAH secretion, which was not altered by sole treatment with parecoxib. However, parecoxib attenuated the LPS-induced fall in PAH net secretion (Figure 5d).

Effect of parecoxib on I/R-induced downregulation of OAT1 and OAT3 expression and on renal parameters

MAP was not altered by I/R nor by additional or sole treatment with parecoxib after 12 h compared to the sham group. Ischemia for 30 min and reperfusion for 12 h decreased renocortical OAT1 and OAT3 expression. Sole parecoxib treatment did not influence the expression of OAT1 and OAT3 (Figure 6a–c), but attenuated the I/R-induced downregulation of OATs. The expression of OAT1 increased from 35 to 65% and the expression of OAT3 from 22 to 47% (Figure 6a–c). I/R clearly increased renocortical COX-2 protein expression as well as renocortical PGE_2 tissue concentration (Figure 6d). Parecoxib did not influence plasma creatinine levels and did not alter the I/R-induced rise in plasma creatinine levels (Figure 7a). Parecoxib did not influence basal creatinine clearance and did not alter the I/R-induced decrease in creatinine clearance (Figure 7b). Parecoxib did not influence basal PAH clearance, but attenuated the I/R-induced fall in PAH clearance (Figure 7c). Parecoxib did not change basal tubular PAH secretion, but attenuated the I/R-induced fall in tubular PAH secretion (Figure 7d).

DISCUSSION

In this study, we aimed to characterize the regulation of renocortical OATs during severe experimental inflammation. A bolus of 10 mg/kg LPS in our *in vivo* model caused a pronounced arterial hypotension associated with increased plasma creatinine levels, reduced creatinine clearance and reduced PAH clearance, indicating the validity of our model of severe experimental sepsis.[23,24] In line with a previous observation, we found that injection of LPS does not alter renocortical COX-1 expression, but that LPS increases renocortical COX-2 mRNA and protein expression,[14] suggesting that the increase of COX-2 in the renal cortex is a rapid process that is because of enhanced *de novo* synthesis of COX-2 protein rather than to impaired degradation. We further found that the increase in COX-2 is dose dependent and that the increase in renocortical COX-2 expression leads to an enhanced formation of renocortical PGE_2. This finding fits with previous studies reporting a dose-dependent increase in renal tissue cytokine formation,[25–27] which are well-known stimuli for COX-2 expression during inflammation.[28]

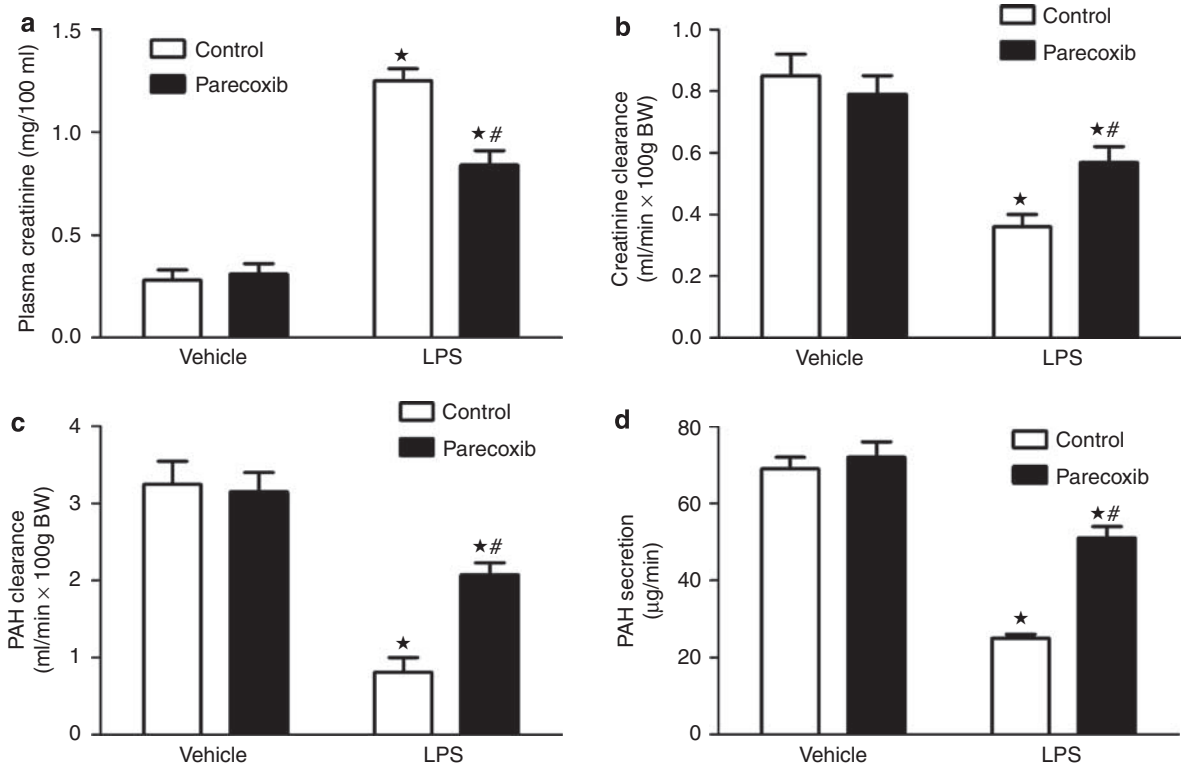

Figure 5 | Effect of parecoxib (20 mg/kg), lipopolysacharide (LPS) (10 mg/kg) or their combination on plasma creatinine levels, creatinine clearance, *p*-aminohippurate (PAH) clearance and PAH secretion. (**a**) Plasma levels of creatinine, (**b**) creatinine clearance, (**c**) PAH clearance, and (**d**) PAH secretion were determined 12 h after LPS injection. Values are mean ± s.e.m. for six rats. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs LPS.

We now found that LPS-induced ARF caused a time- and dose-dependent downregulation of *OAT1* and *OAT3* gene expression. Recently, it has been reported that PGE_2 regulates *OAT* gene expression *in vitro*. It has been shown that PGE_2 inhibits its own renal transport by downregulation of *OAT1* and *OAT3* gene expression *in vitro*.[8] In addition, it has been found that I/R downregulates the renal expression of OAT1 and OAT3.[9,10] As COX-2 expression is increased under endotoxemia and I/R,[12] one might suggest that the downregulation of OATs during ARF could be because of an enhanced formation of COX-2 derived PGE_2. We attempted to prove such a causal link between COX-2 expression and OAT1 and OAT3 expression by the use of the COX-2 inhibitor parecoxib. Parecoxib clearly attenuated the LPS-induced increase in renocortical PGE_2 tissue concentration, suggesting that the LPS-induced rise in PGE_2 concentration is COX-2 dependent. We now found that COX-2 inhibition attenuates the LPS-induced downregulation of *OAT1* and *OAT3* gene expression, suggesting that COX-2 derived PGE_2 formation is of importance for the LPS-induced downregulation of OATs.

To prove the functional consequence for the attenuated downregulation of *OAT1* and *OAT3* gene expression, we further investigated the renal clearance of PAH. Within the kidney, the organic anion PAH undergoes glomerular filtration and tubular secretion.[29] With regard to the basolateral uptake of PAH from the blood into proximal tubule cells, especially OAT1 and OAT3 are of major importance and therefore for the tubular secretion of PAH.[29] Confirming previous observations, COX-2 inhibition *per se* did not alter plasma creatinine concentration, creatinine clearance, and PAH clearance.[30,31] In line with data obtained for indomethacin, a nonselective COX inhibitor, we found that the LPS-induced decrease in PAH clearance was ameliorated by COX-2 inhibition, suggesting that the uptake of PAH and therefore the secretion of PAH is improved.[17] However, inhibition of COX-2 also ameliorated the LPS-induced fall in creatinine clearance, probably because of the attenuation of LPS-induced hypotension.[24] As the clearance of PAH depends on glomerular filtration rate and tubular secretion, the amelioration of PAH clearance by COX-2 inhibition could be because of the attenuation of *OAT* gene expression or because of the amelioration of glomerular filtration rate.

To further specify the impact of COX-2 derived prostanoids for the downregulation of OATs and for the renal excretion of PAH, we further studied the effect of parecoxib on *OAT* gene expression and PAH clearance under I/R. In line with previous reports, we found that I/R downregulates *OAT1* and *OAT3* gene expression and that this downregulation is paralleled by a decrease in PAH clearance.[9,10] We now found that COX-2 inhibition attenuates the downregulation of *OAT1* and *OAT3* gene expression in response to ischemia, suggesting that COX-2 derived prostanoids are of

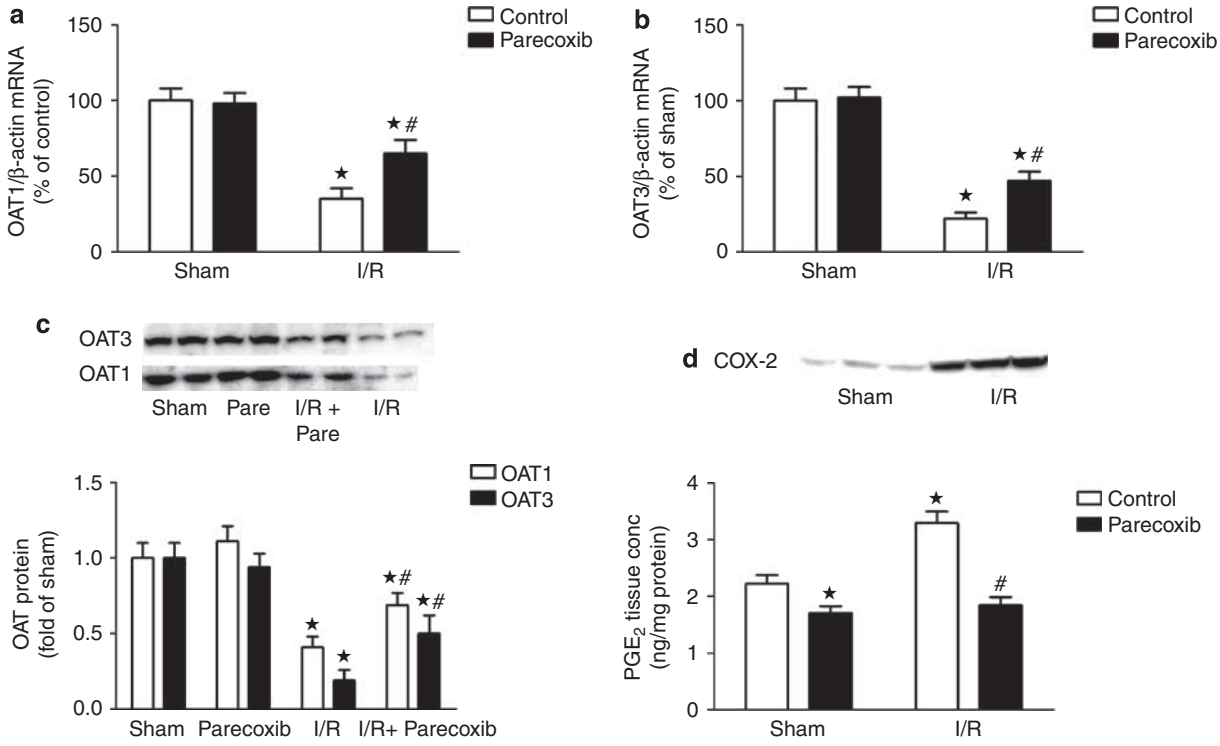

Figure 6 | Effect of parecoxib (20 mg/kg) on ischemia/reperfusion (I/R)-induced downregulation of renocortical organic anion transporters (OATs). (**a**) Renocortical OAT1 and (**b**) OAT3 mRNA expression related to β-actin mRNA expression 12 h after ischemia for 30 min. (**c**) Relative levels of renocortical OAT1 and OAT3 protein expression and representative immunoblots for renocortical OAT1 and OAT3 protein (Co = Control; Pare = Parecoxib). (**d**) Representative immunoblots for renocortical COX-2 protein expression and renocortical PGE_2 tissue concentration 12 h after ischemia for 30 min. Values are mean ± s.e.m. for six rats. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs I/R.

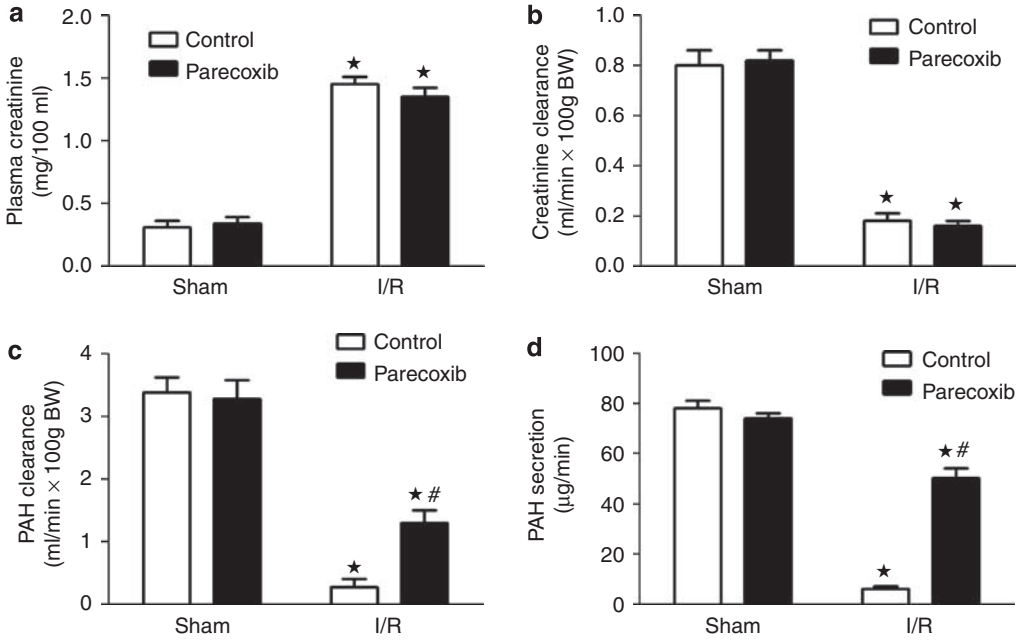

Figure 7 | Effect of parecoxib (20 mg/kg) and ischemia/reperfusion (I/R) on plasma creatinine levels, creatinine clearance, *p*-aminohippurate (PAH) clearance and PAH secretion. (**a**) Plasma levels of creatinine, (**b**) creatinine clearance, (**c**) PAH clearance, and (**d**) of PAH secretion were determined 12 h after ischemia for 30 min. Values are mean ± s.e.m. for six rats. $^{*}P<0.05$ vs control. $^{\#}P<0.05$ vs I/R.

importance for this downregulation under I/R. To prove this assumption, we investigated the effect of I/R on renocortical COX-2 protein expression and on renocortical PGE_2 concentration and found that COX-2 protein as well as PGE_2 tissue concentration were increased under I/R. This finding fits very well with other studies reporting of an

increased COX-2 expression under I/R.[12,32–34] In line with a previous report, COX-2 inhibition did not ameliorate creatinine clearance in response to I/R in our study.[31] We also found that MAP was not altered after I/R.[35] The unchanged MAP under I/R may be therefore a good explanation, why additional parecoxib treatment did not alter the decrease in creatinine clearance under I/R. However, the COX-2 inhibitor parecoxib attenuated the decrease in PAH clearance. As parecoxib did not alter creatinine clearance during I/R, the amelioration of PAH clearance by parecoxib under I/R could be because of the attenuation of the downregulation of *OAT* gene expression.

COX-2 inhibition did not completely abolish the downregulation of OATs during LPS-induced and I/R-induced ARF. Therefore, additional mechanisms have to be involved into the downregulation of OATs. As cAMP has been shown to increase the expression of at least human *OAT3* gene and because adenylyl cyclases expression and therefore cAMP synthesis is decreased in response to LPS and I/R,[36–38] a decreased synthesis of cAMP might be a possible additional mechanism that could be responsible for the downregulation of renal OATs.

In conclusion, we found that LPS-induced renocortical *COX-2* gene expression and PGE2 formation is paralleled by a downregulation of *OAT1* and *OAT3* gene expression. Inhibition of COX-2 attenuated the LPS-induced decrease in *OAT1* and *OAT3* gene expression. Further, COX-2 inhibition attenuated the downregulation of renocortical OAT1 and OAT3 expression because of I/R. Moreover, COX-2 inhibition ameliorated LPS-induced renal dysfunction and attenuated LPS and ischemia-induced decrease of PAH transport. Therefore, this study contributes to our understanding about the mechanism of regulation and the pathophysiological implications of OATs under ARF. However, because COX-2 inhibition does not completely attenuate the downregulation of OATs during LPS-induced and I/R-induced ARF, additional mechanisms must be involved in the downregulation of renal OATs.

MATERIALS AND METHODS

Experimental animals

All animal experiments were performed according to *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Male Sprague–Dawley rats (200–225 g) were obtained from Charles River (Sulzfeld, Germany). Rats received isotonic NaCl-solution (control) or LPS (*Escherichia coli*; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany; 10 mg/kg) intravenously and were killed 3, 6, or 12 h ($n=6$ per group) after LPS injection. In addition, rats ($n=6$ per group) treated with parecoxib (20 mg/kg; i.p.) alone or parecoxib 1 h before LPS injection were investigated. The doses of LPS and of parecoxib were chosen from the literature.[31,39] Rats were anesthetized with sevoflurane, using a Trajan 808 (Dräger, Lübeck, Germany). For induction of renal I/R injury, renal arteries of rats ($n=6$ per group) were totally occluded for 30 min with microaneurysm clamps followed by reperfusion for 12 h. In sham controls, renal arteries were only touched with a forceps. The right femoral artery was cannulated for continuous monitoring of MAP (Siemens SC 9000, Munich, Germany). The left femoral vein was cannulated for maintenance infusion and the bladder for collecting urine. Clearance of PAH, plasma levels of creatinine, and creatinine clearance were determined as described previously.[40,41]

Real-time PCR analysis

Real-time PCR was performed in a LightCycler (Roche, Mannheim, Germany) as described previously.[42] The primer sets were chosen from the literature.[10] For each sample, the ratio of the amount of mRNA to β-actin mRNA was calculated.

Protein preparation and Western blot

Protein preparation and immunoblotting were performed as described previously.[43] Antibodies against OAT1 and OAT3 (Alpha Diagnostics, San Antonio, TX, USA; 1:500) and COX-1 and COX-2 (Cayman Chemical, Ann Arbor, MI, USA; 1:1,000) were used. The preparation of basolateral membranes was performed as described in the literature by others.[11] OAT1 and OAT3 were detected as approximately 60- and 110-kDa bands, respectively, as previously reported.[10,44–46]

Immunohistochemistry

The kidneys were perfusion fixed with 4% paraformaldehyde and processed as described.[47] Immunolabeling was performed on 5-µm paraffin sections. Sections were incubated with a commercial available antibody against OAT1 (Alpha Diagnostics; 1:200) overnight at 4 °C. After several washing steps and blocking with phenylhydrazine, the sections were incubated with a rhodamine (tetramethyl rhodamine isothiocyanate)-conjugated fluorescent antibody (Dianova, Hamburg, Germany) for 2 h and mounted with glycergel (DakoCytomation, Glostrup, Denmark).

Determination of tissue PGE_2 levels

Renocortical tissue levels of PGE_2 were assayed as described previously.[48]

Statistical analyses

Data were analyzed by analysis of variance with multiple comparisons followed by the *t*-test with Bonferroni adjustment. $P<0.05$ was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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