Oat1/3 restoration protects against renal damage after ischemic AKI

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The organic anion transport system of the renal proximal tubule plays a crucial role in the excretion of a variety of potentially toxic compounds (20, 35). This system consists of organic anion exchangers located at the basolateral membrane that represent the rate-determining step of elimination and the efflux step through the apical membrane (7, 10). The classic basolateral organic anion exchanger is the terminal step in the tertiary active transport system, which is dependent on an inward-directed Na+ gradient that drives the uptake of α-ketoglutarate, which is then exchanged for organic anions (14, 36). Oat1 and Oat3 are components of the basolateral polyspecific transporter for organic anions (18, 32), which were functionally described some time ago (36). In summary, the classic renal basolateral polyspecific uptake transporter for organic anions is represented by Oat1 and Oat3 (8, 29).

In human renal allografts, the clearance of the prototypical organic anion PAH was reduced for at least 7 days after transplantation (6). Based on the latter observation, we performed a study that showed downregulation of both Oat1 and Oat3 during reperfusion after ischemic acute kidney injury (iAKI) (28). This was subsequently confirmed by independent groups (9, 19). We also demonstrated that PGE2 leads to downregulation of the expression of both Oat1 and Oat3 in the rat NRK-52E proximal tubular cell line after long-term exposure (up to 72 h) (23). PGE2 levels are increased in the kidney cortex after acute renal ischemia (34), as well as during chronic renal ischemia (33). In a rat model of iAKI, we showed that low-dose indomethacin abolishes ischemia-reperfusion (I/R)-induced downregulation of both Oat1 and Oat3, which is reflected by an abrogated downregulation of organic anion secretory transport (PAH) as well as the renal clearance of endogenous organic anions (PGE2) (27).

Most notably, low-dose indomethacin not only rescued organic anion transport but also had a significantly beneficial effect on renal outcome [as determined by glomerular filtration rate (GFR)] without negatively affecting renal perfusion (RPF) (27). This finding was in accordance with those of some earlier studies that provided evidence that impairment of PGE2 formation has beneficial effects on renal outcomes after iAKI (12, 13). The same study also revealed that a possible explanation for the beneficial effect of low-dose indomethacin on renal outcome is its effect on renal organic anion transport (27). To elucidate whether this is really the case, we administered probenecid (a classic competitive inhibitor of renal organic anion transport) with low-dose indomethacin. Probenecid, an organic anion, is known to competitively block the uptake and enrichment of indomethacin into the proximal tubular cells (5).

When intracellular indomethacin uptake is abrogated using simultaneous probenecid application, we hypothesize that any inhibitory effect on cyclooxygenases (COXs) within the proximal tubuli should then be blocked. This prostaglandin will again be unrestricly generated by COX within the proximal tubular cells, leading to the downregulation of Oat1 and Oat3 along with an untreated I/R injury. Due to the combination of...
indomethacin and probenecid, downregulation of organic anion transport should recur and, if organic anion transport downregulation is involved in renal loss of function, it should be confirmed by an impaired renal outcome after iAKI.

MATERIALS AND METHODS

Materials

Tramadol (Tramal) was from Grünenthal (Aachen, Germany), xylacil hydrochloride (Rompun) was from Bayer (Leverkusen, Germany), and ketamine (Ketanest) was from Pharmacia and Upjohn (Erlangen, Germany). If not indicated otherwise, all substances were further diluted in 0.9% NaCl (wt/vol). If not stated otherwise, chemicals were from Sigma (St. Louis, MO).

In Vivo Experimental Procedure

The care of animals and experimental procedures performed in this study were in accordance with the German law for animal protection.

Experiments were performed as published recently (27, 28), where I/R injury was induced by bilateral clamping of renal arteries for 45 min in rats. Female Sprague-Dawley rats (200–250 g body wt) were obtained from Charles River (Kisslegg, Germany). After a period of at least 24 h in cages within a temperature-controlled room with 14:10-h light-dark cycle and standard food with free access to tap water, anesthesia was performed by intraperitoneal application of xylacil hydrochloride (10 mg/kg body wt) and ketamine (100 mg/kg body wt). All operative procedures were performed on thermoregulated heating boards to maintain body temperature at 37.0°C. Postoperative pain relief was ensured by subcutaneously administered tramadol (0.05 mg/kg body wt), and postoperative dehydration was prevented by subcutaneous administration of additional 1.0 ml 0.9% NaCl. Animals were divided into the following subgroups.

Clamped group (bilateral clamping and supplementation with saline). Both kidneys were prepared carefully by a bilateral flank incision. Renal arteries were dissected and temporarily ligated on both sides to start clamping with microclips simultaneously.

Sham group (sham operation and supplementation with saline). An identical procedure was performed analogous to what is described for the clamping group, except that no clamping of renal arteries was performed.

Clamped group (respective sham group) receiving low-dose indomethacin. Indomethacin was administered at 1 mg/kg ip 10 min before the end of the clamping (or after sham operation) period to ensure immediate delivery into the kidney at the beginning of reperfusion and to exclude possible renal effects of indomethacin already during ischemia.

Clamped group (respective sham group) receiving probenecid. Probenecid was given at 50 mg/kg ip 10 min after the end of the clamping (or after sham operation) period to exclude possible renal effects of probenecid already during ischemia.

Clamped group (respective sham group) receiving low-dose indomethacin and probenecid. Indomethacin and probenecid were both given as described above for the single application.

Control group (untreated animals). Animals with no previous treatment were investigated. These animals reflect a stable state after 30 min of infusion, urine was collected for 20 min and blood samples were drawn subsequently. Inulin concentrations of urine and plasma samples were determined by fluorescence spectrometry (1420 Victor 2 Multilabel Counter, Wallac). PAH concentrations were measured by photospectrometry at 550 nm (Dynatech Lab, Guernsey, UK) after primary dilution of urine (1:101) and serum (1:11) with 0.33% perchloric acid and centrifugation for 10 min at 12,000 rpm, followed by addition of 1% sodium nitrate (NaNO3), 5% ammonium amidosulfonate (H2NSO4NH4), and 32% HCl-0.1% N-(1-naphthyl)-ethylen diammonium dichloride (NNAD), which functions as a diazo-coupling reactant, which were added 1:2 to the postdilution supernatant. The coupling reaction was stopped after 10 min by loading ethanol (C2H5OH abs.) just before the photospectroscopic PAH measurement.

Calculations of inulin clearance and PAH net secretion (PNS) were performed according to the following equations: inulin clearance = (IU × VU)/(tU + t) and PNS = ([PAHU × VU]/H11003)/(GFR × PAHP), where IU is inulin concentration in urine; PAHU is PAH concentration in urine; IP is inulin concentration in plasma; PAHP is PAH concentration in plasma; VU is urine volume; and t is time of measurement.

Correction of PAH clearance for renal extraction was done in accordance to what we have published recently (2, 3). Corrected PAH clearance as a measure for renal perfusion is calculated as follows: corrPAH clearance = ([VU × (PAHU − PAHP)]/[PAHP]) × [1 − (0.9 × (PNScorrsham group))/PNSsham group]), with 0.9 being the extraction ratio set in untreated controls.

The amount of corrected PAH clearance was determined 24 h after iAKI in each group of intervention.

Organ Preparation and Tissue Harvesting

After drawing of blood samples, both kidneys were perfused under pressure-controlled conditions (100 mmHg) with ice-cold Krebs buffer [118 mM NaCl, 25 mM NaHCO3, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 11 mM glucose, 1.5 mM CaCl2(2H2O)] for 20 s. Subsequently, samples of renal cortex were snap-frozen in liquid nitrogen and stored at −80°C.

Real-Time RT-PCR

In brief, RT-PCR was performed according to the iQ SYBR-Green Supermix RT-PCR system protocol (Bio-Rad, Hercules, CA). PCR amplification protocol and primers were used as recently described (24, 25). Quantification was performed using the ΔΔCt method using β-actin as a reference gene, and expression in control cells was normalized to 1.

PGE2 Transport

PGE2 in the supernatant was determined by a competitive ELISA technique using a Correlate-EIA PGE2 Enzyme Immunoassay Kit from Assay Designs (Ann Arbor, MI) as described (24, 25). PGE2 clearance was calculated with the formula described above.

Protein Immunoblotting

For Western blot analysis, frozen kidney cortex was homogenized using a stainless steel mortar cooled by liquid nitrogen, dissolved in ultrasonic disperser UW 70 (Bandelin Electronic, Berlin, Germany). Total protein was measured in samples using the Bradford method (4). Samples of protein (5–40 μg) were analyzed by Western blotting with the respective antibodies. Rabbit Oat1 polyclonal antibody (diluted 1:500) and rabbit Oat3 polyclonal antibody (diluted 1:500) were from Alpha Diagnostic (San Antonio, TX). Blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Dako, Hamburg, Germany) and were developed using a chemiluminescence kit (ECL Plus) following the manufacturer’s instruction.
**Translational Physiology**

Oat 1/3 RESTORATION IMPROVES RENAL OUTCOME AFTER iAKI

A

![Graph A](image)

1mg/kg indomethacin: 
50mg/kg probenecid: 

B

![Graph B](image)

1mg/kg indomethacin: 
50mg/kg probenecid: 

Ca

![Graph Ca](image)

1mg/kg indomethacin: 
50mg/kg probenecid: 

Db

![Graph Db](image)

1mg/kg indomethacin: 
50mg/kg probenecid: 

**References**

AJP-Renal Physiol • doi:10.1152/ajprenal.00160.2014 • www.ajprenal.org
Fig. 2. A: effect of low-dose indomethacin and/or probenecid on the renal net secretion (PNS) of PAH 24 h after iAKI. Renal net secretion of PAH (PNS) was determined as described in MATERIALS AND METHODS \( \{PNS = \frac{[PAH_\text{in}] - V_\text{ref}}{\text{GFR} \times [PAH_\text{in}]} \} \). The amount of renal PAH net secretion was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. Renal net secretion of PAH was determined in each group 24 h after iAKI. n, Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group \((P < 0.05)\). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle \((P < 0.05)\). B: effect of low-dose indomethacin and/or probenecid on the renal transport of an organic anion of endogenous origin \(\text{PGE}_2\) 24 h after iAKI. Renal clearance of \(\text{PGE}_2\) was determined as described in MATERIALS AND METHODS \(\{\text{PGE}_2\text{ clearance} = \frac{(\text{PGE}_2\text{U} \times V_\text{ref})}{(\text{PGE}_2\text{P} \times \text{GFR})} \} \). The amount of renal \(\text{PGE}_2\) clearance was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. n, Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group \((P < 0.05)\). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle \((P < 0.05)\).

Detection of Invading Monocytes in the Renal Cortex

Immunofluorescence detection of invading ED-1-positive cells was done as previously described in detail (21). In brief, cryostat sections (5 μm) were fixed in PBS buffer with 4% paraformaldehyde at a temperature of 4°C for 10 min. After a rinsing with PBS buffer, sections were blocked with 50 mM NH₄Cl for another 10 min, followed by another rinsing in PBS. Additionally, sections were incubated with 0.1% Triton X-100 in PBS buffer for 10 min. Finally, they were blocked with 10% donkey serum in 0.1% Triton X-100 in PBS buffer for 1 h. Subsequently, the anti-rat macrophage antibody ED-1 (CD68 antibody, Acris BM 4000, Herford, Germany) was incubated 1:400 in 10% donkey serum in PBS buffer, followed by donkey anti-mouse Cy3-conjugated secondary antibody (1:500, Di-noval 715-165-151, Hamburg, Germany) in 10% donkey serum for 1 h. After a last rinse in PBS and H₂O, analysis of renal cortex was performed using an epifluorescence microscope (Nikon Eclipse TE 2000-S). Final analysis was performed by manual counting of the number of ED-1-positive cells in one randomly defined visual field of renal cortex.

Morphometric Analysis

Periodic acid-Schiff (PAS)-stained cryosections of the left kidney were analyzed in a blinded manner by a nephropathologist (M. Büttner-Herold). For semiquantification, a score of 0–4 was used: **
of inulin was determined as a measure of GFR as described in MATERIALS AND METHODS. Determination of the glomerular filtration rate (GFR) was done as shown elsewhere (3) and as described in MATERIALS AND METHODS. Corrected PAH clearance as a measure for renal perfusion was calculated as follows: \[ \text{corrPAH clearance} = \frac{V_U \times (\text{PAH}_{\text{in}} - \text{PAH}_{\text{out}})}{[(\text{PAH}_{\text{in}} - \text{PAH}_{\text{out}}) \times t]} \]. The amount of corrected PAH clearance was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. Renal net secretion of PAH was determined in each group 24 h after iAKI. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. Renal net secretion of PAH was determined in each group 24 h after iAKI. Probenecid abolishes the effect of low-dose indomethacin on Oat1/3 expression.

**RESULTS**

Probenecid abolishes the effect of low-dose indomethacin on Oat1/3 expression.

Since probenecid competitively inhibits indomethacin uptake into the proximal tubule, this finding strongly suggests that indomethacin acts within the proximal tubuli. Thus, with respect to the

**Data Analysis**

Data are presented as means ± SE. The n value is given in the text or in the figures. For all experiments, n equals the number of rats or the number of experiments (RT-PCR, Western blotting) with tissue or tissue extractions from distinctive rats. Statistical significance was determined by ANOVA as appropriate. Data from sham-operated animals were tested against untreated controls, and data from clamped animals were tested against sham-operated animals. Differences were considered statistically significant when \( P < 0.05 \).

**Caspase-3 Activity Assay**

Caspase-3 activity was measured using a fluorescence kit according to the manufacturer’s instructions (Clontech Laboratories, Heidelberg, Germany) with slight modifications (24). Rat kidney sections from the deep cortex/outer medulla were washed once with PBS buffer (4°C) and incubated with 150 µl cell lysis buffer for 10 min on ice, homogenized thoroughly, and again incubated for another 20 min on ice. Then, the lysate was centrifuged at 16,000 g for 10 min at 4°C. Sixty microliters of the supernatant was incubated with 50 µmol/l DEVD-AFC (Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin; final concentration) for 60 min at 37°C, and fluorescence of the cleaved product, AFC, was measured at 400-nm excitation and 505-nm emission wavelengths using a multiwell-multilabel counter (VICTOR 2, Wallac, Turku, Finland). Cleaved AFC was quantified by a calibration curve using known AFC concentrations. As a control, kidney extracts were incubated as described above but in the presence of caspase-3 inhibitor zDEVD-CHO. No activity could be found under these conditions. Protein content was determined with a bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard.

**Detection of Nitric Oxide Generation**

Nitrate and nitrite (NOx) levels in the plasma of the animals of respective groups were determined to measure the generation of nitric oxide (NO). Detection of NOx was performed using the nitrate/nitrite colorimetric assay kit obtained from Cayman Chemical (Ann Arbor, MI) in a 96-well plate according to the manufacturer’s protocol and as described (1).

**Detection of Nitric Oxide Generation**

Nitrate and nitrite (NOx) levels in the plasma of the animals of respective groups were determined to measure the generation of nitric oxide (NO). Detection of NOx was performed using the nitrate/nitrite colorimetric assay kit obtained from Cayman Chemical (Ann Arbor, MI). The amount of renal inulin clearance was determined by 10.220.33.2 on June 23, 2017 http://ajprenal.physiology.org/ Downloaded from

**Fig. 3.** A: effect of low-dose indomethacin and/or probenecid on the corrected clearance of PAH 24 h after iAKI. Correction of PAH clearance for renal extraction was done as shown elsewhere (3) and as described in MATERIALS AND METHODS. Corrected PAH clearance as a measure for renal perfusion was calculated as follows: \[ \text{corrPAH clearance} = \frac{V_U \times (\text{PAH}_{\text{in}} - \text{PAH}_{\text{out}})}{[(\text{PAH}_{\text{in}} - \text{PAH}_{\text{out}}) \times t]} \]. The amount of corrected PAH clearance was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. Renal net secretion of PAH was determined in each group 24 h after iAKI. n, Shown below respective bars.*Statistically significant difference between clamped group of each drug intervention and the corresponding sham group \( P < 0.05 \). §§Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle \( P < 0.05 \). B: effect of low-dose indomethacin and/or probenecid on the glomerular filtration rate (GFR) 24 h after iAKI. Renal clearance of inulin was determined as a measure of GFR as described in MATERIALS AND METHODS \[ \text{GFR} = \frac{I_U \times V_U}{[I_{\text{in}} \times V_U]} \]. The amount of renal inulin clearance was measured in each group of intervention 24 h after iAKI to determine GFR. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. n, Shown below respective bars.*Statistically significant difference between clamp group of each drug intervention and the corresponding sham group \( P < 0.05 \). §§Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle \( P < 0.05 \).
regulation of Oat1/3 by prostaglandins, these findings demonstrate that local proximal tubular COXs are of major importance in this context. This regulatory pattern is also present at the level of the respective proteins (Fig. 1, C and D). This finding is in good agreement with our own previously published data showing that the amounts of Oat1 and Oat3 at the protein level reflect the amount of the respective mRNA (26, 27).

**Probenecid Abolishes the Effect of Low-Dose Indomethacin on Organic Anion Transport**

Expression of the rate-limiting transporters Oat1 and Oat3 correlates with the level of organic anion secretory transport (as determined by PNS) (27, 28). The application of 50 mg/kg probenecid slightly reduced PNS compared with the untreated control animals (control 0 h) but was without effect compared with the sham-treated controls (Fig. 2A). In clamped animals, application of probenecid completely abolished the beneficial effect of low-dose indomethacin, leading to impaired PNS in the range seen in untreated clamped animals. In clamped animals treated with indomethacin, probenecid diminished PNS to values below those in clamped animals. We currently do not have an explanation for the latter phenomenon. However, in principle, PNS reflects the probenecid-induced effect on Oat1/3 mRNA and protein expression.

PGE2, an organic anion of endogenous origin, is a well-known substrate for organic anion transporters (17). We have demonstrated that PGE2 clearance is downregulated after iAKI and elucidated that this effect is due to impaired secretory tubular transport (27). Probenecid alone had no effect on PGE2 clearance in sham-treated or clamped animals (Fig. 2B). However, the application of probenecid completely abolished the beneficial effect of indomethacin in clamped animals, leading to impaired clearance of PGE2 to the level detected in clamped animals. Probenecid did not influence the effect of indomethacin in sham-treated animals. Thus the effect of probenecid on Oat1/3 expression and organic anion secretion (as determined by PNS) is reflected in the renal clearance of PGE2, an organic anion of endogenous origin.

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Fig. 4. A: effect of low-dose indomethacin and/or probenecid on a tubular damage score 24 h after iAKI. A well-established tubular damage score (ATN score) was determined 24 h after iAKI as described in MATERIALS AND METHODS. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. n, Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group (P < 0.05). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle (P < 0.05). B: representative cryosections demonstrating histopathological damage 24 h after iAKI with low-dose indomethacin and/or probenecid administration. Periodic acid-Schiff staining of cryosections was performed as described in MATERIALS AND METHODS. Representative tissue sections of the indicated groups 24 h following iAKI are depicted. Indomethacin and/or probenecid was administered as described.
Probenecid Abolishes the Beneficial Effect of Low-Dose Indomethacin on Renal Functional Outcome

As we did not sample renal venous probes in this study, we used the PNS ratio to calculate an estimate value for PAH in the renal vein as introduced before (3). Then, we calculated PAH clearance corrected for PAH extraction ratio (2, 3) as a measure of renal perfusion. Renal perfusion is downregulated after iAKI to a similar extent as shown elsewhere (2, 3). Probenecid alone had no effect on renal perfusion in sham-treated or clamped animals (Fig. 3A). However, the application of probenecid completely abolished the beneficial effect of caspase-3 activity [counts / μg x 30min].

\[ \begin{array}{c|c|c|c|c}
 & \text{control 0h} & \text{sham 24h} & \text{clamp 24h} \\
1\text{mg/kg indomethacin} & \cdot & + & - & + & - \\
50\text{mg/kg probenecid} & - & - & + & + & + \\
\end{array} \]

\[ \begin{array}{c|c|c|c|c}
 & \text{control 0h} & \text{sham 24h} & \text{clamp 24h} \\
1\text{mg/kg indomethacin} & - & + & + & + & + \\
50\text{mg/kg probenecid} & - & - & + & + & + \\
\end{array} \]

\[ \begin{array}{c|c|c|c|c|c|c|c|c|c}
 & \text{control 0h} & \text{sham 24h} & \text{clamp 24h} \\
1\text{mg/kg indomethacin} & - & + & + & + & + \\
50\text{mg/kg probenecid} & - & - & + & + & + \\
\end{array} \]

Fig. 5. A: effect of low-dose indomethacin and/or probenecid regarding induction of apoptosis in renal cortex 24 h after iAKI. Renal apoptosis was determined using caspase-3 activity as described in MATERIALS AND METHODS. Apoptosis induction was determined in each group 24 h after iAKI by the respective caspase-3 activity. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. \( n \), Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group (\( P < 0.05 \)). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle (\( P < 0.05 \)).

B: effect of low-dose indomethacin and/or probenecid on induction of induced nitric oxide synthase (iNOS) in renal cortex after AKI. Total RNA was generated from kidney cortex. The amount of iNOS mRNA expression in renal cortex 24 h after iAKI was normalized to the respective β-actin signal. Indomethacin and/or probenecid was administered as described in MATERIALS AND METHODS. \( n \), Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group (\( P < 0.05 \)). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle (\( P < 0.05 \)).

C: effect of low-dose indomethacin and/or probenecid on nitric oxide (NO) generation 24 h after renal ischemia-reperfusion injury. Nitrate/nitrite (NOx) plasma concentrations reflecting NO generation are given. Nitrate/nitrite was determined with a colorimetric assay as described in MATERIALS AND METHODS. Indomethacin (1 mg/kg body wt) or vehicle was administered intraperitoneally (ip) 10 min before finish of the operative procedure [sham or bilateral renal ischemia (45 min)]. Probenecid (50 mg/kg body wt) or vehicle was administered additionally 10 min after the operative procedure. iNOS mRNA expression was determined 24 h after iAKI. \( n \), Shown below respective bars. *Statistically significant difference between clamped group of each drug intervention and the corresponding sham group (\( P < 0.05 \)). §Statistically significant difference between clamped group plus the respective drug intervention and the clamped group with vehicle (\( P < 0.05 \)).
indomethacin in clamped animals, leading to impaired renal perfusion to the level detected in clamped animals. Probenecid did not influence the effect of indomethacin in sham-treated animals.

In a recent study, we showed that low-dose indomethacin (1 mg/kg) improves renal outcome as determined by the GFR (27). In this study, probenecid was administered in addition to low-dose indomethacin to investigate whether an improved renal outcome is due to improved organic anion transport. The use of 50 mg/kg probenecid had no effect in sham-operated animals when administered alone or in combination with low-dose indomethacin (Fig. 3B). Moreover, probenecid had no effect on the GFR 24 h after I/R injury. Most notably, however, probenecid completely abolished the beneficial effect of indomethacin in clamped animals, thereby reestablishing renal functional impairment at the level seen in untreated ischemic animals.

**Probenecid Abolishes the Beneficial Effect of Low-Dose Indomethacin on Renal Morphology**

PAS staining of renal cryosections revealed morphological tissue damage that was assessed as described. As expected, iAKI induced morphological damage (Fig. 4A). Low-dose indomethacin mitigated postischemic morphological alterations and renal damage to a small extent. This improvement in renal morphology failed when probenecid was also administered. Probenecid alone had no effect on morphological changes. In sham-treated animals, neither substance alone nor their combination had any effect on renal damage. As shown in Fig. 4B, 1 mg/kg indomethacin mitigated brush-border and tubular dilation losses in animals after renal I/R injury. The addition of probenecid impaired the beneficial effect induced by indomethacin. The use of probenecid alone had no effect.

**Probenecid Abolishes the Beneficial Effect of Low-Dose Indomethacin on Markers of Renal Injury**

As indicated in Fig. 5A, neither low-dose indomethacin nor probenecid had any effect on caspase-3 activity in sham-treated rats. In iAKI, the increased caspase-3 activity was completely abolished by the use of low-dose indomethacin. Probenecid alone did not affect caspase-3 activity after I/R injury; however, it completely abolished the effect of low-dose indomethacin on caspase-3 activity. Therefore, probenecid completely inhibits the beneficial effect of low-dose indomethacin on apoptosis.

We investigated inducible nitric oxide synthase (iNOS) mRNA levels in the deep cortex and the outer medulla (Fig. 5B). Neither low-dose indomethacin nor probenecid had any effect in sham-treated rats. The amount of iNOS mRNA was increased after I/R injury, which was totally abolished by the use of low-dose indomethacin. Probenecid alone did not affect iNOS after iAKI; however, it completely abolished the effect of indomethacin on iNOS. Similar to what was seen for apoptosis, probenecid completely inhibits the beneficial effect of low-dose indomethacin on iNOS induction.

Both nitrite and nitrate represent established plasma markers of renal NO generation after I/R injury. iAKI resulted in a significant increase in plasma NOx compared with that in sham-operated animals (Fig. 5C). Low-dose indomethacin completely abolished NOx generation due to I/R injury, which was prevented by the addition of probenecid. Probenecid alone had no effect on NOx in the plasma of either ischemic or non-ischemic rats. In iAKI, the increased nitrite and nitrate levels were completely abolished by the use of low-dose indomethacin.

**Probenecid Abolishes the Beneficial Effect of Low-Dose Indomethacin on Inflammation**

Probenecid completely abrogated the beneficial effect of low-dose indomethacin on inflammation. Probenecid completely inhibited the beneficial effect of low-dose indomethacin on monocyte chemoattractant protein-1 (MCP-1) mRNA induction (Fig. 6A). Low-dose indomethacin abolished the increased MCP-1 mRNA expression in clamped animals. Probenecid alone did not affect MCP-1 mRNA expression in sham-operated animals; however, probenecid abrogated the beneficial effect of indomethacin on MCP-1 mRNA expression. MCP-1 RNA was generated from kidney cortex. Data are given as MCP-1 induction where mRNA expression was determined 24 h after iAKI by qPCR. Indomethacin (50 mg/kg body wt) or vehicle was administered ip 10 min before finish of the operative procedure [sham or bilateral renal ischemia (45 min)]. Probenecid (50 mg/kg body wt) or vehicle was administered additionally 10 min after the operative procedure. MCP-1 mRNA expression was determined 24 h after iAKI. The amount of MCP1 mRNA signal was normalized to the respective β-actin signal. The difference in MCP-1 expression between the respective clamped group and the corresponding sham group are depicted. Differences were calculated after a priori pairing of the PCR signal values. *Statistically significant difference between the clamped group and the corresponding sham group (P < 0.05); n = 6 for every bar.

**Probenecid Abolishes the Beneficial Effect of Low-Dose Indomethacin on Cytoprotection**

Probenecid completely inhibited the beneficial effect of low-dose indomethacin on caspase-3 activity. Therefore, probenecid completely inhibits the beneficial effect of low-dose indomethacin on apoptosis. Probenecid completely inhibited the beneficial effect of low-dose indomethacin on apoptosis. Probenecid completely inhibited the beneficial effect of low-dose indomethacin on apoptosis.
sham-operated animals. Similarly, indomethacin had no effect in sham-treated animals. Noteworthy, the NOx levels correlate well with cortical iNOS mRNA expression, a finding that is in good agreement with the hypothesis that iNOS is the major source of NOx after I/R injury.

Neither Low-Dose Indomethacin Nor Probenecid Affects Renal Inflammation Parameters

Since inflammatory processes are thought to be involved in renal damage after I/R injury, we investigated the local generation of monocyte chemoattractant protein-1 (MCP-1) and, as a consequence of MCP-1 generation, the invasion of ED1+ cells. Consequently, we detected a twofold increase in MCP-1 mRNA levels in the deep cortex and outer medulla region of the kidneys 24 h after ischemia (Fig. 6A; expressed as “ischemia-induced induction”). Low-dose indomethacin (1 mg/kg) or probenecid (50 mg/kg) did not affect the ischemia-induced induction of MCP-1 expression. However, low-dose indomethacin combined with probenecid led to a threefold increase in MCP-1 mRNA.

We detected the invasion of ED1+ cells in tissue cryosections from the cortex and outer medulla 24 h after I/R injury. Ischemia induced ED1+ cell invasion (Fig. 6B; “ischemia-induced invasion”) in the respective areas. However, neither low-dose indomethacin nor probenecid had any effect on the latter invasion. The same results were observed when a combination of both was used. Thus, the observed difference in MCP-1 induction correlates with increased ED1+ cell recruitment in the respective treatment groups.

DISCUSSION

In the present study, we addressed a question that emerged following our recently published data in a rodent model of iAKI (23, 27, 28). Is the beneficial effect on renal outcome induced by low-dose indomethacin due to its anti-inflammatory potency or its effect on renal organic anion transport? Investigation of the time response of organic anion transporter expression and renal function after renal I/R injury demonstrated a stable maximum detrimental effect 6-24 h after iAKI (28). Therefore, we focused on a reperfusion interval of 24 h in the present study. The effect of ischemia observed 24 h after reperfusion is in good agreement with previously published data (27, 28), which indicates good reliability and reproducibility of this particular in vivo model of iAKI. Low-dose indomethacin was administered intraperitoneally 10 min before the end of ischemia as a single dose of 1 mg/kg body wt, which has no detrimental effect on renal perfusion, as we demonstrated previously (27).

Probenecid was administered through the same route 10 min after ischemia to prevent the inhibition of indomethacin distribution due to its well-known competitive effects on renal organic anion uptake transport. Probenecid competitively inhibits renal organic anion transport (5). Its plasma half-life in rats is generally ~4 h (11), but since probenecid and its metabolites are at least partially excreted via the kidneys, a prolonged half-life can be assumed, but a relevant competitive effect on organic anion transport at the time of PNS measurement (24 h after application) is expected to be negligible. This finding is in good accordance with the fact that neither PNS nor PGE2 clearance in sham animals treated with probenecid (alone or in combination with low-dose indomethacin) differed from these parameters in sham-treated animals.

Probenecid alone had no effect on the expression of rOat1, rOat3, PNS, or PGE2 in posts ischemic animals. However, it completely prevented these already described beneficial effects of low-dose indomethacin after I/R injury (27). This finding is in agreement with the hypothesis that indomethacin uptake after iAKI is inhibited by probenecid, whereby proximal tubular COX activity is regained (Fig. 7). As a consequence, proximal tubular PGE2 generation in iAKI might reoccur and lead to PGE2-induced downregulation of rOat1 and rOat3 (23, 24, 27). However, it is important to note that probenecid completely abolishes the functional improvement of low-dose indomethacin after IR injury (27), as indicated by corrected PAH clearance (renal perfusion) and GFR (renal filtration). This was reflected in principle by morphology patterns, as shown by PAS staining and acute tubular necrosis scoring. In contrast when probenecid is combined with low-dose indomethacin, the inductive effect on rOat1/3 is abrogated and the substrate excretion of the transporters deteriorates simultaneously. We consider this evidence that the downregulation of organic anion secretion after I/R injury may be causally involved in iAKI, perhaps by the accumulation of toxic endogenous organic anion metabolites within the renal tissue.

Since indomethacin-induced improvement of general renal function (as determined by the GFR and renal perfusion) and kidney tissue morphology is abolished by probenecid, we hypothesized that this effect should also be observed in an inverse pattern for cellular parameters of ischemic damage in
the kidney, e.g., expression of iNOS or apoptosis. Apoptosis has been reported to occur within the kidney cortex following iAKI, most dominantly in the renal epithelial cells (30). The maximal deleterious effects are observed in the deep cortex and outer medulla. To investigate established markers of renal damage that differ from the GFR, we investigated the activity of caspase-3 in lysates of the latter kidney sections. Upregulation of iNOS is a hallmark in renal I/R injury (16), and excess NO generated by iNOS is thought to play a major role in post-I/R injury (15, 16). In fact, we found that this is the case since the amount of iNOS mRNA and caspase-3 activity act similarly to general renal function. Of note, NO levels in the serum samples correlated with the renal iNOS mRNA levels, a fact which supports the hypothesis that plasma NO elevation in iAKI results primarily from renal iNOS activity (15, 16). In short, the parameters involved in renal cellular injury behaved in an inverse pattern to that of renal function or morphology. We consider these data supportive of what was mentioned above with respect to the functional parameters: impaired expression of Oat1/3 due to renal I/R might be mechanistically involved in the development of renal damage.

It is well known that the synthesis of MCP-1 is induced by nuclear factor-κB after renal I/R injury, leading to monocyte/macrophage invasion (31). Since MCP-1 is known to attract monocytes/macrophages and lead to their tissue invasion after macrophage invasion (31), we detected the invasion of ED1+ cells in tissue cryosections from the cortex and outer medulla 24 h after I/R injury. There was a slight increase in both parameters in iAKI that was not affected by low-dose indomethacin, probenecid, or a combination of both. Thus, in this particular model, there was only a minor induction of inflammatory events that were not additionally affected by low-dose indomethacin or probenecid-induced inhibition of indomethacin action.

Moreover, detection of the proinflammatory cytokines TNF-α and INF-γ in the plasma using a cytometric bead array (BD Biosciences) showed neither an increase in clamped animals nor a variation in any other experimental group (data not shown). Values of the respective parameters were found at minimal detection levels of the assay. In comparison, in an established mouse model of sepsis (22), we found cytokine levels near 250 pg/ml (TNF-α) and 900 pg/ml (INF-γ) in septic animals vs. values close to the detection limit in the untreated controls (Raspé C, Bucher M, and Sauvant C, unpublished data). Thus we consider this evidence that neither a relevant local nor a global (extrarenal) inflammatory response dominates in our particular model system of renal I/R injury.

In summary, we conclude the following: local inflammatory events do not play a major role in the renal damage of iAKI in our particular model system, making it a valuable tool for investigating the mechanisms of renal damage after I/R injury that are not due to classic inflammatory pathways. Moreover, we conclude that the respective concentration and application of low-dose indomethacin is below the level required to inhibit these minor local inflammatory events, which is in accordance with the hypothesis that the beneficial effects induced by indomethacin are not due to its anti-inflammatory potency (27). In contrast, we believe that the beneficial effect of indomethacin on renal outcome after I/R injury is due to the restoration of the organic anion secretory capacity of the kidneys. This claim is supported by the observation that probenecid in the presence of low-dose indomethacin abrogates the upregulation of organic anion transporter expression, the benefits of substrate handling, and the improvement of global renal function (filtration, perfusion). Further studies are required to confirm this hypothesis. If this hypothesis proves true, it will represent a new mechanistic model of the induction of renal damage after I/R injury.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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F208 OAT 1/3 RESTORATION IMPROVES RENAL OUTCOME AFTER iAKI


