Title: Oat1/3 Restoration Protects against Renal Damage after Ischemic AKI.

Running title: Oat 1/3 restoration improves renal outcome after iAKI.

Subject: Basic research on pathophysiology of renal disease and progression.


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Abbreviations: (i)AKI – (ischemic) acute kidney injury; GFR – glomerular filtration rate; RPF – renal plasma flow; I/R -ischemia/reperfusion; PAH - para-aminohippurate; PGE2 - Prostaglandin E2; RPF – renal plasma flow.
Abstract
Expression of proximal tubular organic anion transporters Oat1 and Oat3 is reduced by prostaglandin E2 (PGE2) after renal ischemia and reperfusion (I/R) injury. We hypothesized that impaired expression of Oat1/3 is decisively involved in the deterioration of renal function after I/R injury. Therefore, we administered probenecid, which blocks proximal tubular indomethacin uptake, to abolish the indomethacin mediated restoration of Oat1/3 regulation and its effect on renal functional and morphological outcome.

Ischemic AKI was induced in rats by bilateral clamping of renal arteries for 45 min with 24h follow up. Low-dose indomethacin (1mg/kg) was given i.p. at the end of ischemia. Probenecid (50mg/kg) was administered i.p. 20 min later. Indomethacin restored the expression of Oat1/3, PAH net secretion and PGE2 clearance. Additionally, indomethacin improved kidney function as measured by GFR, renal perfusion as determined by corrected PAH clearance and morphology, whereas in opposite it reduced renal cortical apoptosis and nitric oxide production. Notably, indomethacin did not affect inflammation parameters in the kidneys (e.g. MCP-1, ED1+-cells). On the other hand, probenecid blocked the indomethacin induced restoration of Oat1/3 and moreover abrogated all beneficial effects.

Our study indicates that the beneficial effect of low-dose indomethacin in iAKI is not due to its anti-inflammatory potency, but in contrast to its restoration of Oat1/3 expression and/or general renal function. Inhibition of proximal tubular indomethacin uptake abrogates the beneficial effect of indomethacin by resetting the PGE2 mediated Oat1/3 impairment, thus re-establishing renal damage. This provides evidence for a mechanistic effect of Oat1/3 in a new model of the induction of renal damage following ischemic AKI.

Keywords: Ischemia; reperfusion; cyclooxygenase (COX); ischemic acute kidney injury (iAKI); renal plasma flow (RPF); glomerular filtration rate (GFR), kidney cortex, monocytes/macrophages; renal organic anion transport
**Introduction**

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 classical 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 since substantial time. (36) In summary, the classical 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 paraaminohippurate (PAH) was reduced for at least 7 days after transplantation. (6) Based on the latter observation, we performed a study that showed down regulation 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 prostaglandin E2 (PGE2) leads to down regulation 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 down regulation of both Oat1 and Oat3, which is reflected by an abrogated down regulation of organic anion secretory transport (PAH) as well as the renal clearance of endogenous organic anions (PGE2). (27)
Most notably, low-dose indomethacin did not only rescue organic anion transport but also
had a significantly beneficial effect on renal outcome (as determined by 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 classical 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 (COX) within the
proximal tubuli should then be blocked. Thus, prostaglandin will again be unrestrictedly
generated by the COX within the proximal tubular cells, leading to the down regulation of
Oat1 and Oat3 along with an untreated I/R injury. Due to the combination of indomethacin
and probenecid, down regulation of organic anion transport should recur and, if organic anion
transport down regulation is involved in renal loss of function, it should be confirmed by
impaired renal outcome after iAKI.
Materials and Methods

**In vivo experimental procedure.** 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 to 250 g body weight) were obtained from Charles River (Kissleg, Germany). After a period of at least 24 hours in cages within a temperature-controlled room with 14 hour light-and 10 hour dark cycle and standard food with free access to tap water, anaesthesia was performed by intraperitoneal application of xylacin hydrochloride (10 mg/kg body weight) and ketamine (100 mg/kg body weight). All operative procedures were performed on thermoregulated heating boards to maintain body temperature at 37.0°C. Postoperative pain relief was assured by subcutaneously administered tramadol (0.05 mg/kg body weight) and postoperative dehydration was prevented by subcutaneous administration of additional 1.0 ml 0.9% NaCl. Animals were divided into the following subgroups.

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

- **Sham group (sham-operation and supplementation with saline).** Identical procedure was performed in analogy as described for clamping group, except that no clamping of renal arteries was performed.

- **Clamping group (resp. sham group) receiving low-dose indomethacin.** Indomethacin was administered at 1 mg/kg i.p. 10 min before the end of the clamping (or after sham operation) period, in order to assure immediate delivery into the kidney right at the beginning of reperfusion and to exclude possible renal effects of indomethacin already during ischemia.

- **Clamping group (resp. sham group) receiving probenecid.** Probenecid was given at 50 mg/kg i.p. 10 min after the end of the clamping (or after sham operation) period, in order to exclude possible renal effects of probenecid already during ischemia.
Clamping group (resp. 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 day 0.

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

Measurement of clearances of inulin (GFR) and PAH (RPF). Inulin- and PAH-clearances were determined as described recently.(27, 28) In brief, fluorescein-isothiocyanate-inulin (inulin) and para-aminohippuric acid (PAH) (each 2.5 mg/mL 0.9 % NaCl) were initially administered as a bolus injection, followed by constant infusion of both substances (inulin 5 mg/h, PAH 5 mg/h) using a Secura FT perfusor (B. Braun, Melsungen, Germany). When reaching a steady 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 Victor² Multilabel Counter, Wallace, USA). PAH concentrations were measured by photospectrometry at 550nm (Dynatech Lab, Guernsey, UK) after primary dilution of urine (1:101) and serum (1:11) with perchloric acid 0.33 % and centrifugation for 10 min at 12.000 rpm, followed by addition of sodiumnitrit (NaNO₂) 1 %, ammoniumamidosulfonate (H₂NSO₃NH₄) 5 %, HCl 32 %, N-(1-Naphthyl)-Ethylendiammoniumdichlorid (NNAD) 0.1 %, which functions as a diazo-coupling reactant, which were added 1:2 to the postdilution supernatant. Coupling reaction was stopped after 10 min by loading ethanol (C₂H₅OH abs.) just before the photospectrometric PAH measurement.

Calculations of inulin clearance and PAH net secretion (PNS) were performed according to the equations:

\[
inulin \text{ clearance} = \frac{(I_U \times V_U)}{(I_P \times t)};\]

\[
PNS = \left[\frac{(PAH_U \times V_U)}{t}\right] - \left[\text{GFR} \times PAH_P\right];\]
where I\textsubscript{U} is inulin concentration in urine; PAH\textsubscript{U} is PAH concentration in urine; I\textsubscript{P} is inulin concentration in plasma; PAH\textsubscript{P} is PAH concentration in plasma; V\textsubscript{U} 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 (3) (2). Corrected PAH clearance as a measure for renal perfusion is calculated as follows:

\[ \text{corrPAH clearance} = \left[ \frac{V\textsubscript{U} \times (PAH\textsubscript{U} - PAH\textsubscript{RV})}{(PAH\textsubscript{P} - PAH\textsubscript{RV}) \times t} \right]. \]

The amount of PAH in the renal vein is estimated also in accordance to (3) as

\[ PAH\textsubscript{P} \times [1 - (0.9 \times (\text{PNS I/R-group} / \text{PNS corresponding sham group})); \text{ with 0.9 being the extraction ratio set in untreated controls}. \]

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

**Organ preparation and tissue harvesting.** After drawing blood samples, both kidneys were perfused under pressure-controlled conditions (100 mm Hg) with ice-cold Krebs buffer (NaCl 118mM, NaHCO\textsubscript{3} 25mM, KCl 4.8mM, KH\textsubscript{2}PO\textsubscript{4} 1.2mM, MgSO\textsubscript{4} 1.2mM, Glucose 11mM, CaCl\textsubscript{2}(2H\textsubscript{2}O) 1.5mM) for 20 seconds. Subsequently, samples of renal cortex were snap-frozen in liquid nitrogen and stored at \textasciitilde80°C.

**Realtime-R(everse)T(ran)scriptase-PCR.** In brief, RT-rPCR was performed according to iQ SYBR-Green Supermix RT-PCR system protocol (Biorad, CA, USA). PCR amplification protocol and primers were used as recently described (1, 24, 25) Quantification was performed using the \( \Delta\Delta C_T \) method using \( \beta \)-actin as reference gene and expression in control cells was normalized to 1.

**PGE2-transport.** PGE\textsubscript{2} in the supernatant was determined by competitive ELISA technique using the Correlate-EIA\textsuperscript{TM} PGE\textsubscript{2} Enzyme Immunoassay Kit from Assay Designs (Ann Arbor, Michigan, USA) as described (24, 25) PGE2 clearance was calculated with the formula described above.
Detection of indomethacin. Indomethacin was detected by competitive ELISA technique using the Max Signal® indomethacin ELISA kit from Bioo Scientific (Austin, TX, USA) as described by the manufacturer.

Protein immunoblot. For Western blot analysis, frozen kidney cortex was homogenized using a stainless steel mortar cooled by liquid nitrogen, dissolved in lysis buffer containing 25 mmol/L Tris-HCl, 7 mmol/L reduced glutathione, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2 mol/L phenylmethylsulfonyl fluoride (PMSF), 1 μmol/L leupeptin, 1 μmol/L pepstatin, 1 μmol/L trans-epoxysuccinyl-L-leucylamido butane, and 1 mg/mL trypsin inhibitor, and further minced with an ultrasonic disperser UW 70 (Bandelin Electronic, Berlin, Germany). Total protein was measured in samples using the Bradford method. (4) Samples of protein (5 to 40 μg) were analyzed by Western blot 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, USA). 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 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were analyzed densitometrically using the Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA).

Detection of invading monocytes in 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 paraformaldehyde 4% at a temperature of 4°C for 10 min. After rinsing with PBS buffer sections were blocked with NH₄Cl 50mM for another 10 min, followed by another rinsing in PBS. Additionally sections were incubated with Triton X100 0.1% in PBS buffer for 10 min. Finally they were blocked with 10% donkey-serum in 0.1% TritonX100 0.1% in PBS buffer for 1h. Subsequently, the anti-rat macrophages antibody ED1 (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 (Dianova 715-165-151, Hamburg, Germany) in 10% donkey serum for 1h. After a last rinse in PBS and H\textsubscript{2}O, 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 ED1 positive cells in one randomly defined visual field of renal cortex.

**Morphometric analysis.** Periodic acid-Schiff (PAS) stained cryo-sections of the left kidney were analyzed in a blinded manner by a nephropathologist (M.B-H.). For semi-quantification a score of 0-4 was used: 0 = no abnormality; 1 = mild signs of tubular damage; 2 = moderate signs of tubular damage; 3 = severe signs of tubular damage; 4 = signs of tubular damage in all tubuli. Either the complete cortical area was scored using a 100fold magnification or the S3 segment was scored separately using a 200fold magnification. Each visual field was assigned the best fitting score. Score values of each case were added and the sum was divided by the number of visual fields evaluated to obtain the final score value.

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

**Caspase-3 activity assay.** Caspase-3 activity was measured using a fluorescence kit according to the manufacturer's instructions (Clontech Laboratories GmbH, Heidelberg, Germany) with slight modifications.(24) Rat kidney section from 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, homogenised thoroughly and again incubated for another 20 min on ice. Then the lysate was centrifuged at 16000g for 10 min at 4°C. 60 µl 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, 7-amino-4-
trifluoromethylcoumarin (AFC), was measured at 400 nm excitation and 505 nm emission
wavelength using a multiwell-multilabel counter (Victor², 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 bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard.

Data analysis. Data are presented as mean ± SEM. 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-Blot) 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.

Materials. Tramadol (Tramal®) was from Grünenthal (Aachen, Germany), xylacin
hydrochloride (Rompun®) was from Bayer (Leverkusen, Germany), and ketamine (Ketanest®)
was from Pharmacia & Upjohn (Erlangen, Germany). If not indicated otherwise, all
substances were further diluted in 0.9 % NaCl (w/v). If not stated otherwise, chemicals were
from Sigma (St. Louis, MO, USA).
Results

Probenecid abolishes the effect of low-dose indomethacin on Oat1/3 expression. Low-dose indomethacin, probenecid, and the combination of both had no effect on the amount of Oat1- mRNA in sham-treated animals as evaluated by quantitative polymerase chain reaction (qPCR) experiment findings (Fig. 1A). I/R injury strongly diminished the amount of Oat3 mRNA, a finding that differed from that in animals receiving low-dose indomethacin. In iAKI, probenecid alone had no effect, whereas it completely abrogated the beneficial effect of low-dose indomethacin on Oat3 mRNA. As shown in Figure 1B, the same was true for Oat3 mRNA.

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 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 (Figs. 1C, 1D). 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 PAH net secretion [PNS]).(27, 28) The application of 50 mg/kg probenecid slightly reduced PNS compared to the untreated control animals (control 0 h) but was without effect compared to 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 down regulated 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 by the renal clearance of PGE2, an organic anion of endogenous origin.

**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 (3) (2) as a measure for renal perfusion. Renal perfusion is down regulated after iAKI to a similar extent as shown in (3) (2). 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 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 glomerular filtration rate (GFR).(27) In this study, probenecid was administered in addition to low-dose indomethacin to investigate whether 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 re-establishing a 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 morphologic tissue damage that was assessed as described. As expected, iAKI induced morphological damage (Fig. 4A). Low-dose indomethacin mitigated post-ischemic morphologic 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 Figure 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 Figure 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 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 to 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 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 MCP-1 and, as a consequence of MCP-1 generation, the invasion of ED1+ cells. Consequently, we detected a 2-fold 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 3-fold 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 of MCP-1 induction correlates with increased ED1+ cell recruitment in the respective treatment groups.
In the present study, we addressed a question that emerged following our recently published data in a rodent model of iAKI. 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. 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, 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 weight, which has no detrimental effect on renal perfusion, as we demonstrated previously.

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. Its plasma half-life in rats is generally about 4 h, 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 post-ischemic animals. However, it completely prevented these already described beneficial effects of low-dose indomethacin after I/R injury. 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 down regulation 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 down regulation 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. Up regulation of inducible nitric oxide (NO) synthase (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 the 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 monocyte chemo attractant protein-1 (MCP-1) is induced by nuclear factor-κB after renal I/R injury, leading to monocyte/macrophage tissue invasion.\(^{(31)}\) Since MCP-1 is known to attract monocytes/macrophages and lead to their tissue invasion after ischemic injury,\(^{(31)}\) 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 pro inflammatory cytokines tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) in the plasma using a cytometric bead array (CBA; BD Biosciences, USA) 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 versus values close to the detection limit in the untreated controls (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 classical 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. 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 up regulation 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.
Disclosure

The authors state that there are no interests to disclose.
References


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FIGURE LEGENDS:

Figure 1A: Effect of low dose indomethacin and/or probenecid on the mRNA levels of Oat1 in renal cortex 24h after iAKI.

Total RNA was generated from kidney cortex. The amount of Oat1 mRNA expression in renal cortex 24 h after iAKI was normalized to the respective β-actin signal. Indomethacin and/or probenecid were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 1B: Effect of low dose indomethacin and/or probenecid on the mRNA levels of Oat3 in renal cortex 24h after iAKI.

Total RNA was generated from kidney cortex. The amount of Oat3 mRNA expression in renal cortex 24 h after iAKI was normalized to the respective β-actin signal. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 1C: Effect of low dose indomethacin and/or probenecid on the protein levels of Oat1 in renal cortex 24h after iAKI.

Protein was extracted from kidney cortex. The amount of Oat1 protein expression in renal cortex 24 h after iAKI was normalized to the respective β-actin signal. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical
significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Single typical blots are giving in the lower panel of the figure.

**Figure 1D: Effect of low dose indomethacin and/or probenecid on the protein levels of Oat3 in renal cortex 24h after iAKI.**

Protein was extracted from kidney cortex. The amount of Oat3 protein expression in renal cortex 24 h after iAKI was normalized to the respective ß-actin signal. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Single typical blots are giving in the lower panel of the figure.

**Figure 2A: Effect of low dose indomethacin and/or probenecid on the renal net secretion (PNS) of para-aminohippuric acid (PAH) 24h after iAKI.**

Renal net secretion of PAH (PNS) was determined as described in the methods section [PNS = [(PAH\text{U} x V\text{U}) / t] – [GFR x PAH\text{P}]]. The amount of renal PAH net secretion was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecide were administered as described in the methods section. Renal net secretion of PAH was determined in each group 24 h after iAKI. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. §
indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 2B: Effect of low dose indomethacin and/or probenecid on the renal transport of an organic anion of endogenous origin (PGE2) 24h after iAKI.

Renal clearance of PGE2 was determined as described in the methods section \[\text{PGE2 clearance} = \frac{(\text{PGE2}_U \times V_U)}{(\text{PGE2}_P \times t)}\]. The amount of renal PGE2 clearance was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 3A: Effect of low dose indomethacin and/or probenecid on the corrected clearance of para-aminohippuric acid (PAH) 24h after iAKI.

Correction of PAH clearance for renal extraction was done in accordance to (3) and to what is described in the methods section. Corrected PAH clearance as a measure for renal perfusion is calculated as follows: \[\text{corrPAH clearance} = \frac{V_U \times (\text{PAH}_U - \text{PAH}_{RV})}{(\text{PAH}_P - \text{PAH}_{RV}) \times t}\]. The amount of corrected PAH clearance was determined 24 h after iAKI in each group of intervention. Indomethacin and/or probenecid were administered as described in the methods section. Renal net secretion of PAH was determined in each group 24 h after iAKI.. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.
Figure 3B: Effect of low dose indomethacin and/or probenecid on the glomerular filtration rate [GFR] 24h after iAKI.

Renal clearance of inulin was determined as a measure of glomerular filtration rate as described in the methods section [GFR = (I_U x V_U) / (I_P x t)]. The amount of renal inulin clearance was measured in each group of intervention 24 h after iAKI to determine glomerular filtration rate. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 4A: Effect of low dose indomethacin and/or probenecid on a tubular damage score 24h after iAKI.

A well-established tubular damage score (ATN score) was determined 24 h after iAKI as described in the methods section. Indomethacin and/or probenecide were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 4B: Representative cryo-sections demonstrating histopathological damage 24h after iAKI with. low dose indomethacin and/or probenecid administration

PAS staining of cryo-sections was performed as described in methods. Representative tissue sections of the indicated groups 24 h following iAKI are depicted. Indomethacin and/or probenecide were administered as described.
Figure 5A: Effect of low dose indomethacin and/or probenecid regarding induction of apoptosis in renal cortex 24h after iAKI.

Renal apoptosis was determined using caspase-3 activity as described in the methods section. Apoptosis induction was determined in each group 24 h after iAKI by the respective caspase-3 activity. Indomethacin and/or probenecid were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 5B: Effect of low dose indomethacin and/or probenecid on induction of 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 ß-actin signal. Indomethacin and/or probenecid were administered as described in the methods section. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

Figure 5C: Effect of low dose indomethacin and/or probenecid on nitric oxide generation 24h after renal ischemia/reperfusion injury.

Nitrate/nitrite plasma concentrations reflecting NO generation are given. Nitrate/nitrite was determined with a colorimetric assay as described in the methods section. Indomethacin [1mg/kg bw] or vehicle were administered i.p. 10 min before finishing the operative procedure (sham or bilateral renal ischemia [45 min]). Probenecid [50 mg/kg bw] or vehicle
were administered additionally 10 min after the operative procedure. iNOS mRNA expression was determined 24 h after iAKI. * indicates statistical significant difference between clamp group of each drug intervention and the corresponding sham group. § indicates statistical significant difference between clamp group plus the respective drug intervention and the clamp group with vehicle. n is given bellow the respective bars.

**Figure 6A: Effect of low dose indomethacin and/or probenecid on induction of MCP-1 in renal cortex 24h after iAKI.**

Total 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 [1mg/kg bw] or vehicle were administered i.p. 10 min before finishing the operative procedure (sham or bilateral renal ischemia [45 min]). Probenecid [50 mg/kg bw] or vehicle were 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 ß-actin signal. The difference of MCP-1 expression between respective clamp group and the corresponding sham group are depicted. Differences were calculated after a priori pairing of the PCR signal values. The amount MCP-1 mRNA signal was normalized to the respective ß-actin signal. * indicates statistical significant difference between the clamp group with the corresponding sham group. n is 6 for every bar.

**Figure 6B: Effect of low dose indomethacin and/or probenecid on invasion of ED-1 positive cells (monocytes/macrophages) into renal cortex 24h after iAKI.**

ED-1 positive cells were detected by immunofluorescence signal of cryo-sections as described in the methods section. Macrophage/monocyte invasion is given as the difference of ED-1 positive cells per square, when the respective clamp group is compared with the
corresponding sham group. * indicates statistical significant difference from sham group animals. n is 6 for every bar.

**Figure 7:** Graph showing hypothesized mechanism of action of probenecid on indomethacin induced regulation of Oat1/3 expression after renal ischemia/reperfusion injury

Probenecid (Prob) competitively impairs the uptake of indomethacin (Indo) in the renal proximal tubular cells. As a cause, indomethacin cannot inhibit cyclooxygenase (COX) activity. Thus, prostaglandins (most probably PGE2) will be generated unhindered from arachnoidonic acid (AA). These prostaglandins activate a signalling cascade in an autocine manner via binding to prostaglandin receptors (EP) of proximal tubular cells, thus diminishing the expression of both basolateral organic anion transporters Oat1 and Oat3.
Fig. 1A

mRNA OAT1 / β-actin [control 0h set as 1]

1mg/kg indomethacin: - - - - - - - +
50mg/kg probenecid: - - + + - - + +
Fig. 1B

mRNA OAT3 / β-actin [control 0h set as 1]

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

control 0h  | sham 24h  | clamp 24h
-----------|----------|----------
 6          | 6 6 6 6  | 6 6 6 8  |
Fig. 1C-α

OAT1 / β-actin [control 0h set as 100%]

1mg/kg indomethacin: - + - + - + - +
50mg/kg probenecid: - - + + - - + +
Fig. 1C-β

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Fig. 1D-α

OAT3 / β-actin [control 0h set as 100%]

control 0h | sham 24h | clamp 24h

1mg/kg indomethacin: - + - + - + - +
50mg/kg probenecid: - - + + - - + +
Fig. 1D-β

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Fig. 2A

PAH net secretion [µg/min]

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<td>1mg/kg indomethacin:</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>50mg/kg probenecid:</td>
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<td>-</td>
<td>+</td>
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Number of samples: 6
Fig. 2B

PGE$_2$ clearance [ml/min]

Control 0h  | Sham 24h  | Clamp 24h

1mg/kg indomethacin:
-  | +  | -  | +  | -  | +  | -  | +

50mg/kg probenecid:
-  | -  | +  | +  | -  | -  | +  | +
Fig. 3A

![Bar graph showing corrected PAH clearance [ml/min] for different groups.](image)

- **control 0h**: 4.1 ml/min (11 subjects)
- **sham 24h**: 3.7 ml/min (8 subjects)
- **clamp 24h**: 2.3 ml/min (6 subjects)

1mg/kg indomethacin:
- control 0h: -
- sham 24h: +
- clamp 24h: -

50mg/kg probenecid:
- control 0h: -
- sham 24h: +
- clamp 24h: +
Fig. 4A

ATN score [units]

control 0h  sham 24h  clamp 24h

1mg/kg indomethacin:  -  +  -  +  -  +  -  +
50mg/kg probenecid:  -  -  +  +  -  -  +  +
Fig. 5A

Caspase-3 activity [counts/µg x 30min]

Control 0h: 6
Sham 24h: 6 6 5 5
Clamp 24h: 6 5 5 5

1mg/kg indomethacin: - + - + - + - +
50mg/kg probenecid: - - + + - - + +
Fig. 5B

Rel. iNOS mRNA normalized to untreated controls

- 1mg/kg indomethacin:
  - - + - + - + -

- 50mg/kg probenecid:
  - - + + - - + +
Fig. 5C

- NOx in plasma [µM]
- 0 20 40 60 80 100 120

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

- control 0h
- sham 24h
- clamp 24h
Fig. 6A

MCP-1 induction by ischemia and reperfusion
Fig. 6B

Δ ED1 signal per square unit

clamp + indo

clamp + prob

clamp + indo + prob

clamp