Nitric oxide-induced regulation of renal organic cation transport after renal ischemia-reperfusion injury

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Nitric oxide-induced regulation of renal organic cation transport after renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 301: F997–F1004, 2011. First published August 10, 2011; doi:10.1152/ajprenal.00264.2011.—Renal organic cation transporters are downregulated by nitric oxide (NO) in rat endotoxemia. NO generated by inducible NO synthase (iNOS) is substantially increased in the renal cortex after renal ischemia-reperfusion (I/R) injury. Therefore, we investigated the effects of iNOS-specific NO inhibition on the expression of the organic cation transporters rOct1 and rOct2 (Slc22a1 and Slc22a2, respectively) after I/R injury both in vivo and in vitro. In vivo, Nω-(1-iminoethyl)-l-lysine (l-NIL) completely inhibited NO generation after I/R injury. Moreover, l-NIL abolished the ischemia-induced downregulation of rOct1 and rOct2 as determined by qPCR and Western blotting. Functional evidence was obtained by measuring the fractional excretion (FE) of the endogenous organic cation serotonin. Concordant with the expression of the rate-limiting organic cation transporter, the FE of serotonin decreased after I/R injury and was totally abolished by l-NIL. In vitro, ischemia downregulated both rOct1 and rOct2, which were also abolished by l-NIL; the same was true for the uptake of the organic cation MPP. We showed that renal I/R injury downregulates rOct1 and rOct2, which is most probably mediated via NO. In principle, this may be an autocrine effect of proximal tubular epithelial cells. We conclude that rOct1, or rOct1 and rOct2 limit the rate of the renal excretion of serotonin. l-NIL; nitric oxide; serotonin; AKI; iNOS

THE EPITHELIAL CELLS OF THE PROXIMAL TUBULI IN THE KIDNEYS EFFECTIVELY ELIMINATE NUMEROUS ORGANIC ANIONS AND CATIONS, INCLUDING ENDOGENOUS SUBSTANCES, METABOLIC WASTES, AND XENOBIOTICS AND THEIR METABOLITES VIA RENAL EXCRETION. THIS IS ACCOMPLISHED BY TRANSPORT PROCESSES MEDITATED BY PLASMA MEMBRANE TRANSPORTERS. WITH RESPECT TO ORGANIC CATIONS, SUCH TRANSPORTER SYSTEMS ARE MAINLY MEDIATED VIA THE BASAL LATERAL UPTAKE TRANSPORTERS OCT1 (SLC22A1), OCT2 (SLC22A2), AND OCT3 (SLC22A3), WHEREAS THE APICAL EFFLUX STEP IS MEDIATED LARGELY BY THE ATP-BINDING CASSETTE (ABC) TRANSPORTER P-GLYCOPROTEIN (P-GP; ABCC1) (13).

After renal ischemia-reperfusion (I/R) injury, nitric oxide (NO) is produced in renal tissue in deleterious amounts. This increase in NO production is due to a substantial increase in the NO-producing enzyme inducible NO synthase (iNOS), which is well known to take place after renal ischemia and subsequent reperfusion (4). We recently reported that this happens if I/R injury is simulated in vitro in rat proximal tubular cells (NRK-52E) in culture (23).

In an in vivo rat model of LPS-induced endotoxemia, Heemskerk et al. (6) show that the amounts of rOct1 and rOct2 are diminished due to NO generated by iNOS. As there is some evidence that the renal excretory transport of organic cationic substances is reduced after renal I/R injury (17), we therefore hypothesized that this may be also due to a similar mechanism also involving NO generated after ischemia.

Concordant with the detoxifying role of pGP in rat kidneys, its upregulation is described after renal I/R injury (8), and during endotoxemia (5). This pGP upregulation is most likely under the direct influence of NO produced by iNOS, at least in the case of endotoxemia (6). This means that the diminishment of organic cation clearance after the I/R injury cannot be due to pGP. Moreover, the rate-limiting step of organic cation excretion is determined by proximal tubular influx carriers, in particular the Octs, rather than by apically located transporters (e.g., Octn2, MATE1, and MATE2) or pGP as mentioned above (6) (16).

The influx of a huge variety of organic cations is mediated by the OCTs. Until now, three isoforms have been identified: Oct1 (Slc22a1), Oct2 (Slc22a2), and Oct3 (Slc22a3). In rat kidneys, rOct1 and rOct2 equally contribute to organic cation uptake, whereas Oct2 is the principal isoform in humans (12). Oct3 expression is reported to be relatively low in the kidneys (28), leading to the assumption that its role in organic anion excretion is negligible (6). Furthermore, basolaterally expressed Octn1 is also known to be of minor importance in organic cation flux (31).

Therefore, in the present study, we investigated whether renal I/R injury downregulates the rate-limiting organic cation transporters rOct1 and rOct2 (and rOct3), and thus the rate of NO. To determine this, renal cortical expression of rat Octs and organic cation transport were investigated in a well-established in vivo model of ischemic acute kidney injury (iAKI) after 24 h (26). We investigated the same phenomenon in a recently established proximal tubular in vitro model system of I/R injury in parallel (23).

MATERIALS AND METHODS

In Vivo Experimental Procedure

I/R injury was induced in rats by bilateral clamping of the renal arteries for 45 min followed by a reperfusion period of 24 h as described previously (26). Female Sprague-Dawley (SD) rats (200–250 g body wt) were obtained from Charles River Wiga (Kisslegg, Germany). Anesthesia was performed by intraperitoneal application of xylazine 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°C. Postoperative pain relief was ensured by subcutaneous application of tramadol (0.05 mg/kg body wt); postoperative dehydration was prevented by subcutaneous administration of an additional 1.0 ml of 0.9% NaCl. Animals were divided into the subgroups described below.

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

Sham group (sham operation and supplementation with saline). The same procedure as that for the clamping group was performed, except that the renal arteries were not clamped.

Clamping group (respectively sham group) receiving l-NIL. iNOS was specifically inhibited by 3 mg/kg l-NIL given intraperitoneally 10 min before the end of clamping (or the sham operation) period to ensure immediate delivery into the kidneys right at the beginning of reperfusion and exclude preconditioning pretreatment effects before ischemia.

Control group (untreated animals). Animals with no previous treatment were investigated. These animals reflect day 0.

Renal function was determined by measurement of inulin clearance reflecting glomerular filtration rate in vivo after the respective experimental procedures by detection of the FITC-inulin (Sigma-Aldrich, St. Louis, MO) concentration in plasma and urine samples that were taken 24 h after the I/R injury, as described previously in detail (26). Inulin clearance was calculated as follows: inulin clearance = (inulinP × VU)/(inulinU × t), where inulinP is inulin concentration in plasma; and t is time of measurement.

In Vitro Experimental Procedure

Cell culture. NRK-52E cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). NRK-52E cells were cultured in DMEM enriched with 26 mmol/l NaHCO3 and 5% (vol/vol) fetal calf serum. Cells were maintained in culture at 37°C in a humidified 5% CO2 atmosphere, O2 fraction below 1% conditions for 2 h and then cultivated under standard cell culture conditions (2 ml/culture dish) representing a reperfusion period for 48 h. The cells were exposed to hypoxic conditions using a hermetic chamber filled with 95% N2 and 5% CO2 (see acidosis). During filling, the O2 fraction was measured and filling was continued until the O2 fraction in the chamber decreased below 1%. An O2 fraction <1% represents a Po2 <7.6 mmHg, which is well within the range of measured values in clamped kidneys (20) and well below the critical Po2 for oxygen consumption in the kidney cortex (14). The O2 fraction was stable throughout the 2-h incubation period. Acidosis occurs during renal ischemia in vivo, and a pH of 6.6 is described in renal tissue (7). The stability of pH was ensured by using a bicarbonate-MES-buffered Ringer solution in a 5% CO2 atmosphere. Correct pH was ensured by measurement before and after the incubation period. Aglycemia mimics the absence of glucose due to consumption without redelivery in ischemia and was applied by using a glucose-free buffer as mentioned above.

In vitro ischemia. [3H]methyl-4-phenylpyridinium (MPP+) uptake into suspended NRK-52E cells was measured as described previously (19). Confluent cells were detached, collected by centrifugation at 1,000 g for 10 min, and suspended at 37°C in PBS containing 0.5 mM MgCl2 and 1 mM CaCl2 (transport PBS). Cells were incubated for 10 s at 37°C in transport PBS containing 0.1 μM [3H]MPP with or without 2 mM nonradioactive MPP. [3H]MPP (3.1 TBq/mmol) was obtained from Biotrend (Cologne, Germany). Uptake was stopped by the addition of ice-cold transport PBS containing 100 μM quinine (stop solution). Cells were washed three times with ice-cold stop solution, solubilized with 4 M guanidine thiocyanate, and analyzed for radioactivity.

In Vitro I/R model. Anti-rat Oct2 polyclonal antibody (host: rabbit; dilution 1:1,000) and affinity purified using the antigenic peptide. The antibody was prepared, purified, and characterized as described previously (11). Anti-rat Oct2 polyclonal antibody (host: rabbit; dilution 1:1,000) and anti-rat Oct3 polyclonal (host: rabbit; dilution 1:2,000) were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Blots were subsequently incubated with horseradish per-
Differences were considered statistically significant at \(P < 0.05\). Any presented Western blot data of protein expression were corrected for the respective loading control (β-actin as reference protein).

Real-time RT-PCR. RNA from kidney cortices was extracted using a Qiagen RNA Isolation Kit (Qiagen, Hilden, Germany). The RNA concentration was determined, and cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Benicia, CA) according to the manufacturer’s instructions. Oligonucleotide primers and PCR protocols were as described previously (15). In brief, RT-PCR was performed according to the iQ SYBR Green Supermix RT-PCR system protocol (Bio-Rad Laboratories). Initial denaturation was performed for 3 min at 95°C. PCR amplification was performed for 45 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s. The final elongation step was 72°C for 10 min. For rOct1, the primers were 5'-tgcggcgaagctttcctcgt-3' (sense) and 5'-tcaaggtacggcagcaac-3' (antisense), resulting in a 151-bp RT-PCR product. For rOct2, the primers were 5'-atgacctctgtctcta-3' (sense) and 5'-cctgattctgact-3' (antisense), resulting in a 161-bp RT-PCR product. For rOct3, the primers were 5'-aatcagccagaaacctct-3' (sense) and 5'-atatcaccagcgcactgt-3' (antisense), resulting in a 153-bp RT-PCR product. For β-actin, the primers were 5'-tctacaatgagctgcgtgtg-3' (sense) and 5'-ttccagctggtcttggtc-3' (antisense), resulting in a 129-bp RT-PCR product as described previously (23). The RT-PCR products were tested for the correct size by agarose gel electrophoresis and melting point analysis. The products were quantified using the \(\Delta\Delta C_T\) method with β-actin as a reference gene. Expression in sham animals was normalized to 1.

**Materials**

Tramadol (Tramal) was from Grünenthal (Aachen, Germany), xylazine hydrochloride (Randun) was from Bayer (Leverkusen, Germany), and ketamine (Ketanest) was from Pharmacia and Upjohn (Bridgewater, NJ). If not indicated otherwise, all substances were diluted in 0.9% NaCl (wt/vol). If not stated otherwise, chemicals were from Sigma-Aldrich.

**Data Analysis**

Data are presented as means ± SE. The \(n\) values are given in the text and figures. For all experiments, \(n\) equals the number of rats or experiments (RT-PCR, Western blotting) with tissue or tissue extractions from distinctive rats. Statistical significance was determined by unpaired Student’s \(t\)-tests or ANOVA where appropriate. Data from sham-operated animals were tested against untreated controls, and data from clamped animals were tested against sham-operated ones. Differences were considered statistically significant at \(P < 0.05\).

**RESULTS**

**Effect of \(N^\text{6}-(1-\text{Iminooethyl})-\text{l-lysine}\) on NO Levels and Renal Function After I/R Injury In Vivo**

The amount of nitrate/nitrite in plasma was determined using a well-accepted measure of NO generation after iAKI caused by renal I/R injury (2). As shown in Fig. 1, renal ischemia followed by reperfusion clearly increased NO, whereas this was not the case after the specific inhibition of iNOS by administering 3 mg/kg \(N^\text{6}-(1-\text{Iminooethyl})-\text{l-lysine}\) (l-NIL) at the end of the ischemic period. Thus l-NIL totally abolishes NO generation by iNOS inhibition after renal I/R injury in vivo.

![Fig. 1. Effect of inducible nitric oxide synthase (iNOS) inhibition by \(N^\text{6}-(1-\text{Iminooethyl})-\text{l-lysine}\) (l-NIL) on NO generation reflected in nitrate/nitrite plasma concentration after ischemia-reperfusion (I/R) injury in vivo. Shown is a bar graph of nitrate/nitrite plasma concentrations reflecting NO generation in the control group (0 h), sham group, clamping (clamp) group, and sham and clamping groups after l-NIL application (3 mg/kg body wt) in Sprague-Dawley rats in vivo 24 h after the respective procedure. Nitrate/nitrite was determined using a colorimetric assay as described in MATERIALS AND METHODS. *Statistically significant difference between sham-operated and clamped animals (\(P < 0.05\)).](999)

Inulin clearance was determined as a measure of renal function. Inulin clearance was 0.58 ± 0.077 ml/min (\(n = 11\)) in untreated controls and 0.57 ± 0.040 ml/min (\(n = 9\)) in sham-operated animals, indicating that surgical intervention had no effect on glomerular filtration rate. These values agree well with previously published data (26), indicating that this model is reproducible and reliable. As expected, iAKI caused by I/R injury decreased inulin clearance to 0.19 ± 0.023 ml/min (\(n = 11\)). In sham treated animals, after the administration of 3 mg/kg l-NIL dihydrochloride, inulin clearance increased significantly to 0.83 ± 0.058 ml/min (\(n = 5\)). This was also the case in animals after I/R injury receiving l-NIL. Treatment with l-NIL led to an inulin clearance of 0.44 ± 0.049 ml/min, which indicates that l-NIL has a beneficial effect on renal function after I/R injury as described previously (2).

**Effect of l-NIL on Oct1, Oct2, and Oct3 Expression in Rat Renal Cortex After I/R Injury In Vivo**

Since NO increased after I/R injury and l-NIL abolished this increase, it seems reasonable to investigate rOct1, rOct2, and rOct3 expression in the rat renal cortex under these circumstances. As indicated in Fig. 2, the amounts rOct1 and rOct2, but not rOct3 were reduced after ischemia and 24-h reperfusion in vivo. The application of l-NIL (3 mg/kg after ischemia) totally abrogated the downregulation of both rOct1 and rOct2. In sham-treated animals, l-NIL had no effect on the amount of either transporter. Levels of rOct3 were not affected by either ischemia and subsequent reperfusion or l-NIL.

Next, we investigated whether the amounts of rOct1 and/or rOct2 mRNA in this cortical specimen are also reduced after I/R injury. Since there was no detectable effect on rOct3 protein levels, we did not investigate its mRNA regulation. As
indicated in Fig. 3, rOct1 and rOct2 mRNA levels were reduced after ischemia and 24-h reperfusion. The application of l-NIL (3 mg/kg after ischemia) completely abrogated the downregulation of both rOct1 and rOct2 mRNA. In sham-treated animals, l-NIL had no effect on the mRNA levels of either transporter. As mentioned previously, rOct3 is thought to be of minor importance for renal organic cation excretion. This is highlighted by the fact that rOct3 mRNA was markedly lower in the rat kidney cortex than both rOct1 (ΔC_t = 10) and rOct2 (ΔC_t = 12).
Effect of L-NIL on Fractional Excretion of Serotonin in Rats After I/R Injury In Vivo

To functionally investigate the effects of L-NIL on proximal tubular organic cation excretory transport, we investigated the renal fractional excretion of the endogenous cationic metabolite serotonin. Serotonin is a known substrate of Oct1 in rodents and is probably also transported by Oct2 (13). As shown in Fig. 4, I/R injury substantially diminished the fractional excretion of serotonin. The application of L-NIL improved the fractional excretion of serotonin to the levels of the control and sham animals. In sham animals, L-NIL had no effect on the fractional excretion of serotonin.

Effect of L-NIL on Oct1, Oct2, and Oct3 Expression After I/R Injury In Vitro

A large extent of iNOS expression takes place in proximal tubular cells after ischemia (3). Moreover, we previously showed that iNOS expression is elevated during reperfusion after model ischemia in our in vitro proximal tubular cell model (23). Therefore, we investigated whether proximal tubular cells are able to mediate the downregulation of rOcts in an autocrine fashion, at least in principle. The expression of rOcts was investigated by real-time RT-PCR after a 2-h model ischemia and 48-h reperfusion as alterations of key parameters following I/R damage in this specific conditions of our model I/R injury reflect those of the in vivo system (23, 24). The expression of both rOct1 and rOct2 diminished substantially after model I/R injury applied to isolated proximal tubular cells (Fig. 5). Parallel to the in vivo situation, the application of L-NIL (30 μM) abolished this effect. This indicates that the I/R injury-induced downregulation of rOct1 and rOct2 by NO may in fact be an autocrine mechanism of the proximal tubule.

Effect of L-NIL on Organic Cation Transport After I/R Injury In Vitro

Next, we tested whether the regulation mentioned above is also present at the functional level and is therefore most likely present at the protein level as well. For this purpose, we investigated the basolateral uptake of the prototypical organic cation MPP into proximal tubular NRK-52E cells after control treatment or model I/R injury. As presented in Fig. 6, ischemia and 48-h reperfusion led to a substantial reduction of organic cation uptake in vitro. Moreover, L-NIL application after I/R injury completely restored MPP uptake up to control values.
L-NIL alone had no effect on organic cation uptake in control cells. Therefore, the in vitro data accurately reflect the in vivo situation and indicate that the observed effect may be of an autocrine fashion in principle.

DISCUSSION

The effect of ischemia on renal function observed after 24-h reperfusion is corroborated by previously published data (27), (26), indicating that this in vivo model system of ischemic acute kidney injury is reliable and reproducible. Investigations of the time response of organic cation transporter expression (data not shown) and renal function after renal ischemia (27) reveal a maximum detrimental effect 24 h after renal I/R injury. A single dose of L-NIL (3 mg/kg body wt) was administered intraperitoneally at the end of ischemia. The upregulation of iNOS is a hallmark of renal I/R injury (4); excess NO generated by iNOS is thought to play a pivotal role in this process. Thus the beneficial effect of L-NIL on renal function after ischemia and subsequent reperfusion is most likely due to the impairment of deleterious amounts of iNOS-induced NO.

As indicated by qPCR and Western blotting, L-NIL prevents the I/R-induced downregulation of both rOct1 (Slc22a1) and rOct2 (Slc22a2) in vivo. This agrees well with the working hypothesis developed in detail in the introduction, indicating that I/R-induced NO leads to the downregulation of rOct1 (Slc22a1) and rOct2 (Slc22a2). Thus L-NIL should also improve the secretion of the endogenous organic cation transporter substrate serotonin, since it is a known substrate of Oct1 and is probably also transported by Oct2 in rodents; this is indeed clearly indicated by measurements of the fractional excretion of serotonin. Thus the regulation of rOct1 (Slc22a1) and rOct2 (Slc22a2) expression is functionally relevant with respect to the renal excretion of organic cations of endogenous origin. In vitro, L-NIL also prevents impaired rOct1 (Slc22a1) and rOct2 (Slc22a2) expression as indicated by qPCR; the latter is functionally reflected by an impaired uptake of the organic cation MPP, a well-known substrate for Oct1, Oct2, and Oct3 of various species, which is also prevented by L-NIL. This indicates that the same regulatory pattern holds true at the protein level in vitro; this is further indicated by in vivo data showing identical regulatory patterns at the mRNA and protein levels. Therefore, the in vivo data 1) accurately reflect the in vivo situation and 2) indicate that the observed effect may be of an autocrine fashion in principle.

The detailed mechanism by which NO reduces rOct1 (Slc22a1) and rOct2(Slc22a2) expression after I/R injury remains unclear. It is known from in vitro studies that rOct1-mediated transport is enhanced by PKC, PKA, and endogenous tyrosine kinases (18) and is inhibited by cGMP (25). The
activation of iNOS and subsequent NO release are known to induce cGMP production, which then inhibits rOct1-mediated transport. However, this assumption only holds true if cGMP regulates Oct1 in the long term as well, which is the case for MRP2 (22). This must to be investigated in detail in future studies, as this is highly speculative.

Oct1/2 double-knockout mice revealed that in rodents, Oct1 (Slc22a1) and Oct2 (Slc22a2) together are essential for the renal secretion of many small organic cations (10). Changes in the expression of renal drug transporters alter the pharmacokinetics of many drugs (29) and have implications in the prediction of overall drug deposition and excretion (21). Thus reduced tubular uptake of organic cationic drugs may cause increased drug toxicity due to its accumulation. On the other hand, reduced drug uptake via Ochts may decrease the local therapeutic efficacy within the kidneys themselves or diminish metabolic drug activation as well as metabolization in the kidneys or urinary tract. However, in conjunction with the fact that pGP is upregulated in rat kidneys after renal I/R injury (8) (as well as during endotoxemia) (5), this may represent a protective mechanism against the accumulation of potentially toxic metabolites. Reduced tubular uptake together with increased functional expression of efflux pumps could potentially lead to a decreased amount of respective compounds in the proximal tubular epithelium itself. The upregulation of pGP is most likely under the direct influence of NO produced by iNOS, at least in the case of endotoxemia (6). As a cause, this means that the diminishment of organic cation clearance after I/R-injury cannot be due to pGP. Moreover, it has been shown that the rate-limiting step of organic cation excretion is determined by proximal tubular influx carriers rather than pGP (6, 16) or other apically located transporters (e.g., OCTN2, MATE1, and MATE2). In conjunction with our findings, this suggests that the renal clearance of serotonin is predominantly determined by the activities of rOct1 (Slc22a1) and rOct2 (Slc22a2) and not by the apical efflux step. To our knowledge, this is the first study that indicates rate-limiting roles of rOct1 (Slc22a1) or rOct1 (Slc22a1) plus rOct2 (Slc22a2) for renal serotonin clearance and fractional excretion.

Recently, Heemskerk et al. (6) found similar regulatory effects on the expression of rOct1 (Slc22a1) and rOct2 (Slc22a2), and organic cation secretion after model endotoxemia induced by LPS using 100 mg/kg aminoguanidine (an iNOS inhibitor). However, in contrast to renal I/R-injury (i.e., the present study), the inhibition of NOS activity in endotoxemia did not increase rOct1 (Slc22a1) and rOct2 (Slc22a2) mRNA, indicating that NO regulates renal cation transporters at the posttranscriptional level after endotoxemia. Thus, although NO is involved in rOct1 (Slc22a1) and rOct2 (Slc22a2) regulation after both endotoxemia and renal ischemia, the mechanisms leading to their downregulation appear to be different under both circumstances.

Serotonin is known to exert harmful effects such as the induction of platelet shape change, platelet aggregation, vascular smooth muscle cell proliferation, and vasoconstriction (exhibited in atheromatous coronary vessels); it is also known to be elevated in peripheral vascular diseases (9). As Ochts are described to be downregulated in diabetes (30), it is intriguing to speculate about the coherence of diabetes-induced renal organic cation transporter downregulation leading to increased circulating serotonin, which might subsequently be involved in the development of diabetes-associated vascular disease.

In summary, we showed that renal I/R injury downregulates rOct1 (Slc22a1) and rOct2 (Slc22a2), which are most probably mediated by NO. In principle, this may be an autocrine effect of proximal tubular epithelial cells. Furthermore, we showed that rOct1 (Slc22a1), or rOct1 (Slc22a1) and rOct2 (Slc22a2) limit the rate of the renal excretion of serotonin.

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DISCLOSURES

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

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