Diminished NO generation by injured endothelium and loss of macula densa nNOS may contribute to sustained acute kidney injury after ischemia-reperfusion

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Submitted 7 September 2008; accepted in final form 22 October 2008

Kwon O, Hong S, Ramesh G. Diminished NO generation by injured endothelium and loss of macula densa nNOS may contribute to sustained acute kidney injury after ischemia-reperfusion. Am J Physiol Renal Physiol 296: F25–F33, 2009. First published October 29, 2008; doi:10.1152/ajprenal.90531.2008.—In postischemic acute kidney injury (AKI) or acute renal failure, a dissipation of glomerular filtration pressure is associated with an altered renal vascular tone and reactivity, as well as a loss of vascular autoregulation. To test the hypothesis that renal nitric oxide (NO) generation reflects endothelial damage in the kidney after ischemia-reperfusion, we quantified the urinary NO levels and identified the site of its generation in postischemic AKI. Subjects were 50 recipients of cadaveric renal allografts: 15 with sustained AKI and 35 with recovering renal function. Urine and blood samples were obtained after transplant, and intraoperative allograft biopsies were performed to examine NO synthases (NOSs) in the kidney. In the sustained AKI group, urinary nitrite and nitrate excretion (in μmol/g urine creatinine) was lower (12.3 ± 1.8 and 10.0 ± 1.4 on postoperative days 0 and 3) than in the recovery group [20.0 ± 3.6 and 35.1 ± 5.3 (P < 0.005 vs. sustained AKI on days 0 and 3) on postoperative days 0 and 3]. Endothelial NOS expression diminished from the peritubular capillaries of 6 of 7 subjects in the sustained AKI group but from only 6 of 16 subjects in the recovery group. No differences were observed in the inducible NOS staining pattern between the two groups. Neuronal NOS staining was rarely observed in the macula densa of subjects but was prominent in control tissues. These findings suggest that a diminished NO generation by injured endothelium and loss of macula densa neuronal NOS could impair the vasodilatory ability of the renal vasculature and contribute to the reduction in the glomerular filtration rate in postischemic AKI.

Acute renal failure; vasculature; nitric oxide synthase; confocal microscopy

ACUTE KIDNEY INJURY (AKI) or acute renal failure (ARF) occurs in 5% of all hospitalized patients and is associated with 25 to over 90% of mortality in patients (3, 10, 11, 20, 24, 29, 30, 44, 45, 48). A large cohort analysis has shown that AKI is associated with an odds ratio of death of 5.5 (29). Ischemia may lead to the destruction of tubular epithelial cells through vascular congestion or the “no reflow” phenomenon. In the current study, we quantified the production of NO from freshly transplanted cadaveric renal allografts and identified the site of its generation to characterize NOS and NO generation in the kidney after ischemia-reperfusion. This model has been used to characterize the damage to tubular structure and function, as well as the dissipation of glomerular filtration pressure in the kidney after ischemia-reperfusion, by immunohistochemical analysis and serial physiology study (2, 26, 27).

Nitric oxide (NO) has been shown to have several functions in the kidney, depending on its concentration, site of release, and duration of action (19). For example, NO generated by inducible NO synthase (iNOS) has been shown to have cytotoxic effects on renal tubular epithelial cells (35). In contrast, an increased expression of endothelial NOS (eNOS), which leads to the enhanced production of endothelium-derived NO, may ameliorate ischemic and toxic renal injury by mediating vasodilation, inhibiting leukocyte adhesion, and reducing platelet aggregation (19). Goligorsky et al. (19) proposed the key role of endothelial dysfunction in acute renal ischemia, suggesting that the defective production of endothelial NO may eventually lead to the destruction of tubular epithelial cells through vascular congestion or the “no reflow” phenomenon.

In the current study, we quantified the production of NO from freshly transplanted cadaveric renal allografts and identified the site of its generation to characterize NOS and NO generation in the kidney after ischemia-reperfusion.

METHODS

Subjects

The study group comprised 46 consecutive consenting recipients of cadaveric renal allografts at Indiana University Medical Center and 4...
recipients of cadaveric allografts at Stanford University Medical Center who did not subsequently have an episode of acute rejection or other medical or surgical complications during the first two posttransplant weeks. Among these 50 subjects, 23 subjects, 7 with sustained AKI and 16 recovery subjects, consented to intraoperative graft biopsy.

The control group for urine sampling comprised 10 living donors of renal allografts, and the control group for blood sampling consisted of 5 healthy volunteers. One patient undergoing laparoscopic nephrectomy for renal cell carcinoma at Indiana University Medical Center and four living related donors of healthy kidneys at Stanford University served as tissue controls.

Informed consent was obtained from each patient for this study that was previously approved by the Indiana University Purdue University in Indianapolis and Clarian Institutional Review Boards and the Committee for Research in Human Subjects at Stanford University. Patient age ranged from 23 to 70 yr. The age of the donors varied between 8 and 59 yr.

Protocol

In 23 recipients of cadaveric renal allografts, a wedge or needle biopsy of the allograft was performed ~ 1 h after the completion of the vascular anastomosis and the restoration of reperfusion of the transplanted kidney. To measure the renal NO generation, urine and blood samples were obtained 2 h and 3 days after the surgery. Graft function was monitored daily by collecting 24 h urine samples for creatinine clearance (CrCl) following surgery. Fifteen recipients of cadaveric renal allografts had severely depressed renal function during the first posttransplant week, defined as CrCl < 25 ml/min on postoperative day 7; these patients were designated the “sustained AKI” group. The remaining 35 recipients of cadaveric allografts exhibited recovery of graft function during the first week, defined as CrCl ≥ 25 ml/min on postoperative day 7; these patients were designated the “recovery” group (Fig. 1). The immunosuppressive regimen comprised corticosteroid, mycophenolate mofetil, and calcineurin inhibitor in all subjects. Calcineurin inhibitor was started on postoperative day 1 for the same goal blood levels in all subjects.

Control urine was obtained from 10 donors of living kidney transplants before kidney donation surgery. Control blood samples were obtained from five healthy volunteers. A control tissue sample was obtained from a grossly normal part of the kidney immediately after an arterial clamp was applied to the renal artery during laparoscopic nephrectomy for renal cell cancer. The time required to dissect the structures around the kidney as a part of the procedure before biopsy was 65 min. The control did not have a history of nephrologic diseases or systemic conditions affecting the kidney. The serum creatinine of the control was 0.8 mg/dl. The control tissues from living donor kidneys were taken by needle biopsy at the time of transplantation, immediately before an arterial clamp was applied to the kidney. These controls were proven to be healthy kidneys by an extensive pretransplant donor evaluation.

Measurement of NO

Fresh urine samples were collected through a bladder catheter. Urine samples were processed on ice or at 4°C within an hour following the collection. Fifty milliliters of each urine sample was centrifuged at 2,000 g for 10 min to remove the cellular components. The supernatants were stored in aliquots at −20°C before the measurement of nitrate and nitrite (NOx). NOx concentrations in urine and serum samples were measured by an assay using Griess reagent (Calbiochem), following the manufacturer’s instructions (33). This assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by the spectrophotometric quantitation of nitrite levels using Griess Reagent to quantitate NOx in the sample. Each sample was assayed in triplicate. The urinary NOx level was expressed in micromoles per gram urine creatinine. The four subjects in the sustained AKI group who produced < 20 ml urine per day were excluded from the calculations since their urinary NOx may not represent a NO generation in the kidney due to poor urine flow. All the other subjects produced more than 600 ml of urine a day and were analyzed for urinary NOx excretion. Intra-assay and interassay coefficient variations were below 19.6 and 16.5% on average, respectively. The quartile distributions of urinary NOx levels were illustrated using box plots for the two groups on the day of transplantation and on postoperative day 3 and for the controls (Fig. 2).

Immunohistochemistry

Antibodies. Monoclonal mouse antibodies to human eNOS (eNOS/ NOS type III), mouse iNOS (iNOS/NOS type II; 93% identical to human iNOS in the sequence containing epitopes for antibody), and human nNOS (nNOS/NOS type I) were obtained from BD Transduction Laboratories (Lexington, KY) and used at dilutions of 1:100, 1:25, and 1:25, respectively. The antibody to mouse iNOS has been shown to recognize human iNOS (24). Texas red-conjugated phalloidin, which stains for filamentous actin (F-actin), was purchased from Molecular Probes (Eugene, OR) and used at a dilution of 1:200. Dual staining with Texas red-conjugated phalloidin was used to identify the proximal and distal nephron segments and the vascular structures of interest.

Tissue preparation. Each tissue sample from the cortex, occasionally with the outer medulla, of renal allografts was immediately dropped into 10 ml 2% paraformaldehyde, 0.075 M lysine, and 0.01 M periodate fixative on ice for 30 min. The sample was washed three times for at least 10 min each with ice-cold phosphate buffered saline (PBS) consisting of (in mM) 2.7 KCl, 1.5 KH2PO4, 137 NaCl, and 8 NaH2PO4 (pH 7.4). Each sample was cryoprotected by incubating it for at least 48 h at 4°C in a 50-m1 conical tube containing 40 ml of 2.5 M sucrose in PBS. The sample was immersed in OCT cryoembedding compound (Miles), frozen in liquid N2, and stored at −80°C.

Immunofluorescence staining. The frozen tissue block was sectioned using a Leica CM 3050 cryotome (Leica, Nussloch, Germany). Six micrometer-thick sections were transferred onto ProbeOn Plus glass slides (Fisher Scientific). The frozen sections were extracted for 10 min at room temperature with cytoskeleton buffer, consisting of 50 mM NaCl, 300 mM sucrose, 10 mM piparzone-2,3-bis-(2-ethanesulfonic acid) (pH 6.8), 3 mM MgCl2, 0.5% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The slides were washed twice for 10
Each with PBS at room temperature and incubated for 2 h at room temperature in blocking solution (PBS containing 20% normal goat serum (NGS), 0.2% bovine serum albumin (BSA), 50 mM NH4Cl, 25 mM glycine, and 25 mM lysine) in a humidified chamber. After the incubation in the blocking solution, the slides were washed twice for 10 min each at room temperature with PBS containing 0.2% BSA between steps. After the final wash, the slides were overlain with glass coverslips in PBS containing 16.7% Mowiol (Calbiochem), 33% glycerol, and 0.1% paraphenylene diamine and viewed using the MRC-1024 confocal microscope (Bio-Rad, Hercules, CA) with a ×60 water objective lens.

Assessment of NOSs in kidney tissues. To evaluate the NOS expression in kidney tissues, reflecting sites of NO generation, serial images were taken at 0.2-μm intervals throughout the 6-μm depth of tissue sections using confocal microscopy. Three-dimensional (3-D) reconstructions of these images were generated with Metamorph software (Universal Imaging, West Chester, PA) (note: supplemental videos may be found with the online version of this article). Semi-quantitative scoring of eNOS staining was used to assess the degree of damage to eNOS in vascular endothelial cells. Each 3-D image was scored as 0 (intact or mild damage), indicating eNOS staining in the dominant portion of the endothelial cells (e.g., Fig. 3 and 4). The scores of the images of each vascular structure (artery, arteriole, and peritubular capillary) were averaged to determine the degree of damage in each case. The antibody to nNOS detected this protein in macula densa, the segment of a distal tubule adjacent to an arteriole next to the vascular pole of a glomerulus (Fig. 5). The antibody to iNOS was used to detect its expression in the tissue sections (Fig. 6). The analyses of images were performed in a blinded fashion to eliminate bias.

Statistical Analysis

All measurements are expressed as means ± SE. The significance of differences in the physiological and clinical findings between the two groups of patients was determined using Student’s unpaired t-test.
The statistical significance of the differences in serum and urine NOx levels between the two groups on different postoperative days and the controls and the scoring of eNOS expression among the two groups and the control was evaluated by one-way ANOVA with Dunnett’s post hoc multiple comparisons test. In addition, a statistical analysis was performed for correlations among total ischemic time, donor age, CrCl, and urinary NOx levels on days 0 and 3 and the degree of damage to eNOS of renal graft tissues in all subjects using nonparametric Spearman’s test. All statistical analyses were performed using SPSS 11.5 software.

RESULTS

Clinical Features

The clinical characteristics of the allografts and patient population are summarized in Table 1. The sex and age distribution of patients were similar in the sustained AKI and recovery groups. The total and warm ischemic times and donor age in the two groups also did not differ significantly. The causes of end-stage renal disease and antihypertensive medications, which patients were taking before the transplant operation, were also similar in the sustained AKI and recovery groups as shown in Table 2.

Renal Function

The subjects were classified into the sustained AKI or recovery groups according to graft function on postoperative day 7. Our analysis showed that this grouping of subjects already existed on the day of transplantation (Fig. 1). The subjects destined to exhibit sustained ARF displayed a CrCl on day 0 of only 4.5 ± 1.4 ml/min, whereas those destined to recover had a day 0 CrCl of 26.8 ± 2.4 ml/min (P < 0.00005). The urine flow rate was significantly lower in the sustained AKI group (1.1 ± 0.4 ml/min) than in the recovery group (5.8 ± 0.8 ml/min) on the day of transplantation (P < 0.00005). Among the 15 patients in the sustained AKI group, eight required dialysis during the first posttransplant week, whereas none of the 35 patients in the recovery group required dialysis treatment.

Urinary NO Excretion

Urinary NOx levels (in μmol/g urine creatinine) on the day of transplantation and postoperative day 3 were 12.3 ± 1.8 and
10.0 ± 1.4 in recipients of a cadaveric allograft destined to have sustained AKI, compared with 50.5 ± 9.2 in controls (P < 0.05) as shown in Fig. 2. Those destined to have the recovery of graft function also tended to have lower urinary NOx levels on the day of transplantation (20.0 ± 3.6). However, on posttransplantation day 3, urinary NOx levels in this group increased to 35.1 ± 5.3 and the values were significantly higher than those in the recipients destined to have sustained AKI (10.0 ± 1.4, P < 0.005). In addition, urinary NOx levels on posttransplant day 3 showed a significant correlation with CrCl on the day, with correlation coefficient 0.57698 and P = 0.00055.

Circulating NOx levels did not differ significantly among the different groups, being 6.2 ± 0.6 and 7.3 ± 0.7 μM in the sustained AKI and recovery groups on the day of transplantation, 9.1 ± 3.4 and 6.1 ± 0.8 μM on postoperative day 3, and 7.9 ± 0.9 μM in the healthy controls. Taken together, the urinary NOx levels may reflect NO generation in the kidney and were lower in the sustained AKI group than in the recovery group.

**Expression of NOSs**

The characteristics of eNOS, nNOS, and iNOS staining in the kidney tissues varied among individuals even in the same

Table 1. Characteristics of patient population

<table>
<thead>
<tr>
<th></th>
<th>Cadaveric Allograft</th>
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<tbody>
<tr>
<td></td>
<td>Sustained AKI</td>
<td>Recovery</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>Female/Male</td>
<td>4/11</td>
<td>12/23</td>
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<tr>
<td>Age, yr</td>
<td>47.1±3.0</td>
<td>46.0±2.0</td>
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<tr>
<td>Total ischemic time, min</td>
<td>1695.7±97.7</td>
<td>1608.9±55.8</td>
</tr>
<tr>
<td>Warm ischemic time, min</td>
<td>35.6±2.5</td>
<td>37.7±1.2</td>
</tr>
<tr>
<td>Donor age, yr</td>
<td>41.7±4.3</td>
<td>33.3±2.4</td>
</tr>
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</table>

Values are means ± SE. AKI, acute kidney injury.

Table 2. Causes of ESRD and antihypertensive medications before transplantation

<table>
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<tr>
<th></th>
<th>Sustained AKI</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>Causes of ESRD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>2 (13)</td>
<td>5 (14)</td>
</tr>
<tr>
<td>DM</td>
<td>4 (27)</td>
<td>13 (37)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>5 (33)</td>
<td>9 (26)</td>
</tr>
<tr>
<td>Renal hypoplasia</td>
<td>1 (7)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>PKD</td>
<td>1 (7)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>Obstructive uropathy</td>
<td>1 (7)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEI</td>
<td>3 (20)</td>
<td>11 (31)</td>
</tr>
<tr>
<td>ARB</td>
<td>1 (7)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>CCB</td>
<td>5 (33)</td>
<td>12 (34)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>1 (7)</td>
<td>19 (54)</td>
</tr>
<tr>
<td>Other vasodilator</td>
<td>1 (7)</td>
<td>7 (20)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate percentage of subjects in each group. ESRD, end-stage renal disease; DM, diabetes mellitus; PKD, polycystic kidney disease; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker.
functional group. However, the heterogeneity of staining within the same subject was less pronounced than among individual subjects.

**Arterial eNOS expression.** We detected arteries in only two recipients destined to have sustained AKI, both of which had severe damage to eNOS (score of 2). Of the seven in the recovery group with arteries, five had a score of 0 (intact or minimal damage) for eNOS, whereas the remaining two had scores of 0.3 and 1. The four control tissues with arteries had scores of 0.8, 0.5, 0, and 1 (Fig. 7, and Table 3).

**Arteriolar eNOS expression.** Only four of the seven recipients destined to have sustained AKI contained arteriole(s) in the tissue samples, all of which showed scores of ≤0.5 for eNOS expression in the arteriolar endothelium. Tissue samples from 15 of the 16 recipients destined to show recovery of graft function contained arteriole(s), and 10 of these had a score of 0 (intact or mild damage) for eNOS expression. Only two subjects in the recovery group demonstrated scores >0.5. The three control tissues containing arteriole(s) had scores of 0.5, 0.7, and 0.7. All of subjects and controls scored 1. We suspected that there was no meaningful biological difference among the subject groups and controls even though there was a statistical difference detected (Figs. 3 and 7, and Table 3).

**Peritubular capillary eNOS expression.** Of the seven recipients destined to have sustained AKI, four had moderate to severe damage to eNOS in peritubular capillary endothelium, with a score of ≥1, whereas only one subject was scored 0 and the other two were scored 0.5. In contrast, 13 of the 16 subjects destined to have recovering renal graft function demonstrated intact or only mild damage to eNOS, with a score of ≤0.5, whereas six of these patients had a score of 0. Only one subject in this group had severe damage (score of 2) to eNOS, whereas the other two subjects had scores of 1 and 0.7. All five control tissues had a score of 0 (intact or mild damage). The degree of damage to peritubular capillary eNOS was significantly inversely correlated with CrCl on day 0, with correlation coefficient = −0.47103 and P = 0.03114 (Figs. 4 and 7, and Table 3).

**nNOS expression.** Of the 16 macula densa in three control kidney tissues, 9 (56%) stained positive for nNOS (Fig. 5). In contrast, we were unable to detect staining for nNOS in 27 of the 30 (90%) macula densa images from 11 recipients (3 in the sustained AKI group, and 8 in the recovery group) of cadaveric allografts. Macula densa was not detected in tissue samples from 12 subjects (4 in the sustained AKI group, and 8 in the recovery group). No difference in macula densa nNOS expression was noted between the sustained AKI and recovery groups.

**iNOS expression.** iNOS expression was observed in some cells of distal nephron segments and damaged proximal convoluted tubules in endothelial and smooth muscle cells of some arteries and arterioles and in some cells in the peritubular spaces and intravascular compartment. When compared with control tissues, iNOS expression was higher in recipients of cadaveric allografts, but we did not detect any differences between the sustained AKI and recovery groups (Fig. 6).

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### Table 3. Degree of damage to endothelial nitric oxide synthase in renal vasculature

<table>
<thead>
<tr>
<th></th>
<th>Sustained AKI</th>
<th>Recovery</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteries</td>
<td>2.0±0.0</td>
<td>0.2±0.1</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>2</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Arterioles</td>
<td>0.1±0.1</td>
<td>0.2±0.1</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Median</td>
<td>0</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>4</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Peritubular capillaries</td>
<td>1.1±0.3</td>
<td>0.4±0.1</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Median</td>
<td>1.3</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>6</td>
<td>16</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. recovery and control; †P < 0.05 vs. control.

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*Fig. 7. Dot plots show the degrees (0 to 2; mild to severe) of damage to eNOS in arteries (A), arterioles (B), and peritubular capillaries (C) of cadaveric renal allografts after ischemia-reperfusion in subject groups: sustained AKI, recovery, and control.*
DISCUSSION

We have quantified here the renal NO generation and examined the NOS expression in postischemic AKI, using freshly transplanted cadaveric renal allografts as a model. When compared with that in healthy controls, renal NO generation was significantly lower in recipients destined to have sustained AKI, at least until postoperative day 3. In contrast, renal NO generation in recipients with recovering graft function did not differ from control levels. Since urinary creatinine excretion is impaired in sustained AKI, the actual urinary NOx excretion per unit time may be even greater than expressed here in the text (μmol/g urine creatinine) in the recovery group compared with the sustained AKI group. The decreased urinary NOx levels in our subjects on days 0 and 3 might reflect the state of low protein intake postoperatively compared with the control. However, all subjects were postoperatively on the same diet, and, yet, urinary NOx levels in those destined to have sustained AKI were significantly lower, especially on day 3, compared with the recipients destined to have the recovery of the graft function. We quantified urinary NOx excretion to represent the renal NO generation as previously reported (42). Hemoglobin in the urine after transplant surgery could reduce urinary NO by binding NO. However, the degree of hematuria immediately after transplant surgery is more related to the surgical technique used for ureterovesical anastomosis, the submucosal tunnel versus seromuscular approach, than the graft function. In our study population, there was no difference in the surgical technique between the sustained AKI and recovery groups. Therefore, urinary NO excretion was used to represent an overall renal NO generation. NO could be derived from different parts of renal cortex and medulla (25). Our observations were made on the cortex and the outer medulla due to the technical limitation in human kidney biopsies. Nevertheless, the data demonstrated alterations in the expression of NOS isoforms at least in the cortex and the outer medulla of human kidneys after ischemia-reperfusion. To accurately compare the images of the NOS isoforms, we assessed the degree of damage to NOS on 3-D reconstructions of serial optical slices of the tissue sections captured by confocal microscopy. Immunohistochemical analyses of these 3-D video images demonstrated diminished eNOS expression in cadaveric allografts after ischemia-reperfusion compared with control tissues. In addition, we were unable to detect nNOS expression in almost all macula densa from cadaveric allografts, whereas most macula densae in control tissues showed positive staining for nNOS. iNOS was observed in multiple sites of the kidney and was more prevalent in tissue sections from cadaveric allografts than in controls.

Freshly transplanted cadaveric renal allografts were analyzed since they are known to be an optimal model for postischemic AKI/ATN in humans (2, 15, 26, 27, 46). During the transplant procedure, a previously healthy kidney sustains a period of ischemia before implantation. Typically, there are no confounding factors such as sepsis, multiorgan failure, or exposure to nephrotoxins after the initial measurable period of ischemia. Extrinsic factors, such as angiotensin II, endothelin-1, and other vasoconstrictor agonists, including thromboxane A2, prostaglandin H2, adenosine, platelet-activating factor, and leukotrienes, are thought to influence a reduced renal blood flow in ischemic kidneys (5, 12, 18, 28, 31, 37). For example, increased circulating endothelin-1 and plasma renin activities were observed in renal allograft recipients with sustained AKI (2). An adequate density of glomerular atrial natriuretic peptide receptors and enhanced cGMP generation have been reported in human cadaveric renal allografts with sustained postischemic injury, suggesting that constrictedafferent arterioles are unresponsive to the vasorelaxant action of endogenous atrial natriuretic peptide in this form of postischemic AKI (46).

In the kidney, NO is known to be involved in the regulation of renal plasma flow, glomerular filtration rate, renin secretion, water and sodium excretion, and the maintenance of renal structural integrity (6, 7, 16, 22, 25, 36). Three isoforms of NOS are expressed in the kidney, two of which are constitutive (nNOS and eNOS; Ca²⁺ dependent) and one inducible (iNOS) (24). The infusion of NOS inhibitors such as N⁶-nitro-L-arginine methyl ester or N⁶-monomethyl-L-arginine into the renal artery has been shown to induce a preferential rise in afferent arteriolar resistance and a reduction of the glomerular ultrafiltration coefficient (17). In rats, L-arginine supplementation has been reported to significantly enhance the expression of NO signaling proteins and improve the recovery phase of ischemic AKI (41). The transplantation of functional endothelial cells into the ischemic kidney has been shown to have a renoprotective effect (8). A hypothesis on the key role of endothelial dysfunction as the primary cause of “no-reflow phenomenon” in acute renal ischemia states that the defective production of NO by damaged endothelial cells results in the impaired vasorelaxation of renal resistance arteries, diapedesis of polymorphonuclear leukocytes and monocytes, and local procoagulant and proaggregant conditions. These vascular events are followed by tubular alterations (19). It had been previously reported that a loss of NO production by glomerular eNOS in conjunction with an increased NO production by interstitial iNOS, together with the formation of reactive oxygen species and nitrotyrosine, is involved in the pathogenesis of human chronic renal transplant failure (1). Our finding that diminished eNOS expression and NO generation occur in the kidney after ischemia-reperfusion suggests a contributory role of endothelial dysfunction in the pathophysiology of postischemic AKI/ATN in humans. In contrast, the inhibition of iNOS has been reported to reduce the renal dysfunction and injury associated with ischemia-reperfusion of the kidney (12, 35). Our results suggest that iNOS expression increases in various sites of the kidney after ischemia-reperfusion compared with controls but that there is little difference between recovering patients and those destined to have sustained AKI in our tissue samples. While it is not possible in human subjects to accurately quantify how much renal NO is derived from each NOS isoform, previous reports have suggested that in rats, most of renal NOS is calcium dependent (16, 23). Together with these findings, our results may suggest that constitutive NOS (eNOS and nNOS) may be the major source of NO determining the difference between the different functional groups after ischemia-reperfusion. Macula densa cells are activated in response to the distal tubular flow rate. When the flow is too high, vasoconstriction of the afferent arterioles occurs, a process known as tubuloglomerular feedback (36). However, the signaling between the macula densa cells and the afferent arteriole, following the sensing of NaCl load at the macula densa site, has been difficult to determine, although intracellular calcium and adenosine A1 receptor have been regarded as possible links (39, 40,
ISCHEMIA INDUCES ALTERATION IN NOS AND NO IN THE KIDNEY

43). In addition, it has been suggested that prostaglandins and NO may be part of the mediator chain (38). nNOS is largely expressed in the macula densa cells, and its acute blockade has been shown to increase tubuloglomerular feedback sensitivity without affecting arterial blood pressure (36). In this study, we detected almost no nNOS expression in cadaveric allografts, suggesting that a diminished NO formation in the macula densa may contribute to the loss of the autoregulatory ability of the renal vasculature observed after ischemia-reperfusion. It is possible that NOS activity could be affected by posttranslational mechanisms. Assessing the activity of each NOS isoform at the cellular level by detecting NO generation in vivo is needed as a next step of study to further clarify the sites of NO generation.

In summary, we have observed a diminished urinary NOx excretion in cadaveric allografts destined to have sustained AKI. Damage to vascular eNOS and macula densa nNOS and increased iNOS expression were detected in cadaveric renal allografts after ischemia-reperfusion. The diminished eNOS expression was more pronounced in subjects destined to have sustained AKI. These results suggest that vascular endothelial damage, occurring after ischemia-reperfusion, could cause an impaired vasodilatory ability of the renal vasculature due to a decreased endothelial NO generation and may contribute to the reduction in glomerular filtration in recipients of cadaveric renal allografts. In addition, a diminished nNOS expression in macula densa may also play a role in the deranged vasoconstrictor regulation in postischemic AKI/ATN.

ACKNOWLEDGMENTS

We gratefully acknowledge the help of Scott Herring and Sharon Henson in recruiting subjects and thank Drs. Mark Pescovitz, Martin L. Milgrom, Ronald S. Filo, and Jonathan Fridell for allowing us to obtain tissue samples intraoperatively. We also thank Dr. Bryan D. Myers at Stanford University for providing some tissue samples and Dr. Katrina J. Kelly for retrieving subject information. This study was supported by the Department of Medicine of the Indiana University School of Medicine.

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