Renal oxygenation in acute renal ischemia-reperfusion injury

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There has recently been considerable interest in the hypothesis that renal hypoxia contributes to the pathogenesis of acute kidney injury (AKI), including that arising from ischemia-reperfusion injury (IRI) (12, 20, 39). Renal tissue hypoxia has been demonstrated in clinically relevant models of acute IRI (17, 22, 23, 33, 38). In addition, there is now good evidence showing that chronic renal hypoxia, imposed at least partly through vascular rarefaction, is associated with the development of chronic kidney disease after acute IRI (1–5). However, it remains to be determined whether widespread hypoxia during the acute reperfusion period is obligatory or whether hypoxia is limited to certain anatomic or cellular locations.

In the hospital setting, renal IRI can arise from multiple iatrogenic causes (12). For example, renal ischemia and reperfusion can arise during abdominal aortic surgery as a result of aortic cross clamping, in which case it is not only the kidney that experiences ischemia but also the gastrointestinal tract and lower body (14). IRI also arises in the course of renal transplantation (36) or partial nephrectomy (32), in which case ischemia is localized to the kidney. Previous studies have documented the development of hypoxia, in both the renal cortex and outer medulla, during the first hours of reperfusion after a period of aortic cross clamping in rats (22, 23) and pigs (38). These studies used techniques for simultaneous measurement of renal microvascular PO2 as well as renal O2 delivery (Do2) and O2 consumption (V˙O2) (22, 23, 38). A consistent finding from these studies was marked deficits in renal Do2 during reperfusion (40–70%) but only small decreases or even increases in renal V˙O2 (22, 23, 38). Thus, widespread renal hypoxia during reperfusion in clinically relevant models of aortic cross clamping appears to arise because renal Do2 is reduced more than is V˙O2, perhaps in part due to upregulation of the inducible form of nitric oxide synthase and downregulation/inhibition of the endothelial isoform (22, 23).

These observations contrast with the picture arising from studies of human kidney transplantation or animal models that mimic it by induction of ischemia limited to the kidney. For example, using blood oxygen-dependent (BOLD) MRI, Oostendorp and colleagues (31) were unable to detect hypoxia in the renal cortex or inner medulla 1 and 24 h after a period of application of a clamp to the renal pedicle of mice, although there was some evidence of hypoxia in the outer medulla, at least in the first hour of reperfusion. Similarly, Pohlmann and colleagues (33) recently used BOLD MRI to demonstrate hypoxia in the outer medulla during 100 min of reperfusion after the renal pedicle of rats was clamped for 45 min. However, they could not detect hypoxia in the cortex or inner medulla. The one exception we could find in the literature was a study by Hoff and colleagues (17), in which fluorescence optodes were used to demonstrate relative hypoxia in both the cortex and outer medulla of rats in the 2 h after the renal pedicle was clamped for 45 min. In clinical studies, Rosenberger and colleagues (36) found, in biopsies from human kidney transplant recipients, considerably less immunostaining for hypoxia-inducible factor (HIF)-1α in nonfunctioning kidneys than in functional kidneys in the first hour after reperfusion.

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Am J Physiol Renal Physiol 306: F1026–F1038, 2014.—Tissue hypoxia has been demonstrated in both the renal cortex and medulla, during the acute phase of reperfusion after ischemia induced by occlusion of the aorta upstream from the kidney. However, there are also recent clinical observations indicating relatively well preserved oxygenation in the nonfunctional transplanted kidney. To test whether severe acute kidney injury can occur in the absence of widespread renal tissue hypoxia, we measured cortical and inner medullary tissue PO2 as well as total renal O2 delivery (Do2) and O2 consumption (V˙O2) during the first 2 h of reperfusion after 60 min of occlusion of the renal artery in anesthetized rats. To perform this experiment, we used a new method for measuring kidney Do2 and V˙O2 that relies on implantation of fluorescence optodes in the femoral artery and renal vein. We were unable to detect reductions in renal cortical or inner medullary tissue PO2 during reperfusion after ischemia localized to the kidney. This is likely explained by the observation that V˙O2 (~57%) was reduced by at least as much as Do2 (~45%), due to a large reduction in glomerular filtration (~94%). However, localized tissue hypoxia, as evidenced by pimonidazole adduct immunohistochemistry, was detected in kidneys subjected to ischemia and reperfusion, particularly in, but not exclusive to, the outer medulla. Thus, cellular hypoxia, particularly in the outer medulla, may still be present during reperfusion even when reductions in tissue PO2 are not detected in the cortex or inner medulla.

Tissue hypoxia; renal oxygen delivery; renal oxygen consumption
The apparently disparate observations in models of aortic cross clamping (22, 23, 38) versus those from clinical renal transplantation (36, 37) and renal ischemia in mice (31) and rats (33) raise the possibility that the renal response to reperfusion after ischemia localized to the kidney might differ substantially from that arising from renal reperfusion after aortic cross clamping. Both scenarios are clinically relevant. Thus, the chief aim of the present study was to assess renal tissue oxygenation and renal DO₂ and VO₂ during reperfusion after a period of ischemia localized to the kidney.

To achieve our aim, we first devised a method, analogous to that developed by Can Ince and colleagues (18, 29), for real-time measurements of renal DO₂ and VO₂. The main difference between our approach and theirs was the use of fluorescence optodes for measurements of blood PO₂ rather than systemic injection of a phosphor. We validated our new method for measurements of renal DO₂ and VO₂ and used it along with standard methods for measurements of total renal blood flow (RBF) and tissue PO₂ (fluorescence optodes) and perfusion (laser-Doppler flowmetry) (11) to document tissue oxygenation and its determinants over the first 2 h of reperfusion in anesthetized rats. In separate experiments, we used pimonidazole adduct immunohistochemistry to assess hypoxia at the cellular level.

METHODS

General

Male Sprague-Dawley rats (n = 42, mean weight: 390 ± 13 g) obtained from the Monash University Animal Research Platform were housed in a room maintained at a temperature of 23–25°C and a 12:12-h light-dark cycle. Rats were allowed free access to water and standard laboratory rat chow. All procedures were approved in advance by the Animal Ethics Committee of the School of Biomedical Sciences, Monash University, as being in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

The aims of the present study were threefold. First, we aimed to determine the relationship between blood PO₂ measured by fluorescence optodes, hemoglobin saturation (SO₂) measured by pulse oximetry, and hemoglobin concentration estimated from capillary tube hematocrit compared with their corresponding measurements obtained by direct blood oximetry (protocol 1). Our second aim was to investigate whether a single hemoglobin-O₂ dissociation curve (Bohr curve) could be used to determine both arterial and renal venous blood hemoglobin saturation (and thus O₂ content) from measurements of arterial and renal venous PO₂ (protocol 2). Finally, we aimed to investigate kidney oxygenation during a 2-h reperfusion period after 60 min of renal artery occlusion. For this third aim, separate experimental protocols were applied to quantify renal tissue PO₂ and whole kidney DO₂ and VO₂ (protocol 3) and to qualitatively assess the presence of hypoxia at the cellular level using pimonidazole adduct immunohistochemistry (protocol 4).

Surgical Preparation (Applicable to Protocols 1 and 2)

Rats were anesthetized with pentobarbital sodium (60 mg/kg ip plus 10–15 mg·kg⁻¹·h⁻¹ iv to effect, Sigma-Aldrich, Castle Hill, NSW, Australia). A tracheotomy was performed to facilitate ventilation, and the tail artery was catheterized for measurements of arterial pressure and collection of arterial blood samples. The right femoral vein was then catheterized to allow the delivery of maintenance fluids (BSA, Sigma-Aldrich, in 154 mM NaCl). Hemoglobin saturation was measured via a pulse oximeter and foot sensor (Mouse Ox, Starr Life Sciences, Oakmont, PA). During the experiments, rats were either allowed to breathe spontaneously (protocol 1) or were artificially ventilated (protocol 2, Ugo Basile, model 7025, SDR Clinical Technology), as described below for each protocol.

Protocols

Protocol 1: methods for measurement of blood PO₂, SO₂, and hemoglobin content. Protocol 1 is shown in Fig. 1A (n = 11). Our aim was to compare values of arterial and venous PO₂ obtained using fluorescence optodes and hemoglobin saturation obtained via pulse oximetry with those obtained via direct blood oximetry. In addition to the procedures described above [see Surgical Preparation (Applicable to Protocols 1 and 2)], rats were also instrumented with fluorescent optodes (BF/OT/E, E-series PO₂/temperature sensor, Oxford Opttronix, Oxford, UK) in both the left femoral artery and vein, which were advanced to the bifurcation of the aorta and inferior vena cava, respectively, to provide measurements of PO₂ of arterial and venous blood, respectively. Additionally, the jugular vein was isolated, and a catheter filled with heparinized saline was inserted as far as the superior vena cava to allow mixed venous blood to be sampled. Throughout the experimental procedures, a maintenance solution of 2% (wt/vol) BSA was delivered at a rate of 2 ml·kg⁻¹·h⁻¹. After a 20-min equilibration period, gas mixtures of O₂ concentrations of 0%, 13%, 15%, 17%, 21%, 30%, and 100% were passed over the tracheal catheter. The order of presentation of the gas mixtures was randomized. Once the PO₂ in blood measured by the fluorescent optodes plateaued (~5–10 min), blood samples were collected for blood gas analysis (ABL 700 series, Radiometer, Copenhagen, Denmark). Arterial blood was also collected into a capillary tube for measurements of hematocrit.

Protocol 2: can a single Bohr curve be used for measurements of arterial and renal venous blood SO₂ from arterial and venous PO₂? Protocol 2 is shown in Fig. 1B (n = 6). Blood PO₂, PCO₂, SO₂, total hemoglobin concentration, hematocrit, actual base excess, standard base excess, and concentrations of K⁺, Ca²⁺, glucose, and lactate were compared between arterial and renal venous blood at various levels of inspired O₂ content. In addition to the surgical preparation described above, renal venous blood was collected from the left kidney via a catheter inserted in the right renal vein and advanced to the left renal vein. Total RBF was also measured throughout via a transit-time ultrasound flow probe (type 0.7 VB, Transonic Systems) placed around the left renal artery. Rats were artificially ventilated at a rate of 80 breaths/min, a tidal volume of 2.5 ml, and a positive end-expiratory pressure of 3 cmH₂O. Animals received 2% (wt/vol) BSA at a rate of 6 ml/h iv during surgery, which was maintained at 2 ml/h thereafter. After a 20-min equilibration period, inspired O₂ was varied randomly to 17%, 19%, 21% (room air), and 30%. At each level of inspired O₂ concentration, a period of 5–10 min was allowed for equilibrium to be reached before renal venous and tail arterial blood samples were collected for oximetry (ABL 700 series).

Protocol 3: kidney oxygenation during IRI. Protocol 3 is shown in Fig. 1C (n = 15). In protocol 3, we estimated arterial and renal venous O₂ content by 1) estimating the hemoglobin concentration from capillary tube hematocrit, 2) estimating O₂ dissolved in plasma from the PO₂ of femoral arterial and renal venous blood determined using fluorescence lifetime oximetry (9), and 3) estimating SO₂ from Bohr curves constructed from measurements of arterial PO₂ (fluorescence lifetime) and hemoglobin saturation (pulse oximetry) (see Calculations). The data required for construction of the Bohr curves were obtained during the equilibration period before the experiment itself had commenced. The experiment was divided into three experimental periods: control (30 min), a period in which the blood flow to the kidney was occluded (60 min), and a 2-h period of reperfusion of the kidney (n = 6). A separate group of rats served as sham controls (n = 9).

Rats were anesthetized with thiobutabarbital sodium [Inactin (155 mg/kg ip), Sigma, St. Louis, MO] and artificially ventilated. During
all surgical procedures, 2% (wt/vol) BSA was infused intravenously at a rate of 6 ml/h. The BSA infusion was then maintained at 2 ml/h during the equilibration period and experiment. Arterial PO2 was measured via a fluorescent optode inserted into the left femoral artery as described for protocol 1, and venous PO2 was measured by inserting a fluorescent optode into the left renal vein via the right renal vein. Mean arterial pressure (MAP) and heart rate were measured via the tail artery. The left kidney was placed in a stable micropuncture cup, and fluorescence optodes were inserted to measure cortical (2 mm below surface of kidney) and inner medullary (5 mm below the surface of the kidney) PO2. A surface laser-Doppler probe (DP26, surface probe, Moor Instruments, Devon, UK) was used for measurements of cortical perfusion, whereas a 26-gauge needle probe (DP4s, Moor Instruments) was used for measurements of inner medullary perfusion (5 mm below the cortical surface). Before data analysis, offset values of laser-Doppler flux, obtained at the completion of the experiment once rats were euthanized with an overdose of pentobarbital, were subtracted from flux values obtained during the experiment (15). Total RBF was measured in the left kidney via a transit-time ultrasound flow probe placed around the renal artery.

At the end of the surgical preparations, rats received bolus doses of 10 μCi [3H]inulin (50 μl, Perkin-Elmer Australia), heparin (20 IU in 20 μl, Pfizer Australia), and pancuronium bromide (2 mg/kg, Astra Zeneca) intravenously. A maintenance infusion of 2% (wt/vol) BSA delivered 676 nCi/h [3H]inulin, 20 IU/h heparin, and 0.1 mg/kg/h pancuronium bromide throughout the experiment. A 1-h equilibration period was allowed before experimental manipulations commenced. Glomerular filtration rate (GFR) was measured by the clearance of [3H]inulin (30). Throughout the protocol, all rats were artificially ventilated with 40% O2 at a ventilation rate of 80 breaths/min and a tidal volume of 3.5–4.5 ml.

To generate the data required to construct the Bohr curves, during the first 15 min of the 60-min equilibration period, rats were ventilated with the following four gas mixtures: 17% O2, 19% O2, 21% O2 (room air), and 30% O2 in random order. The resultant Bohr curve was later used to determine blood O2 content from the measured arterial and venous blood PO2.

### Calculations

Bohr curves were modeled based on Hill’s equation and analyzed using nonlinear regression in GraphPad Prism (29). Arterial PO2 obtained by fluorescence optodes was plotted against SO2 obtained via pulse oximetry. In protocol 3, because there was little variation in the Bohr curves between rats (see RESULTS), a single Hill equation, based on data from all animals, was used to calculate arterial and venous SO2 as follows:

\[
\text{SO2} = \frac{a \times (\text{PO2}^b)}{\text{PO2}^c + \text{PO2}}
\]

where PO2 is either arterial or venous PO2 measured in the experiment (in mmHg), a is the O2-binding capacity of hemoglobin (ml O2/g hemoglobin), b is the Hill coefficient, and c is the PO2 in the blood at which hemoglobin is 50% saturated (P50) at equilibrium (in mmHg).

The O2 content of arterial and venous blood was calculated as follows:

\[
\text{Total blood O2 content (ml O2/dl)} = (0.0139 \times [\text{Hb}]) \times \text{SO2} + (0.003 \times \text{PO2})
\]

[Fig. 1](http://ajprenal.physiology.org/) Schematic representation of the three experimental protocols. In protocol 1 (A), arterial and venous blood samples (0.3 ml) were collected for blood gas analysis and the determination of capillary tube hematocrit at the end of each 10-min gas challenge. The shaded boxes indicate when gas mixtures of varying O2 concentration (ranging from 0% to 100%, where n represents the number of O2 concentrations measured in a single experiment) were delivered in random order to spontaneously ventilated rats. In protocol 2 (B), the inspired O2 concentration was varied between 17%, 19%, 21% (room air), and 30% in random order (shaded boxes) and experiment. Arterial PO2 blood samples were collected for oximetry from the tail artery and renal vein. In protocol 3 (C) during the equilibration period, each rat was exposed to brief periods of ventilation with room air and 15%, 18%, and 30% O2 to allow the construction of a hemoglobin-O2 dissociation curve (Bohr curve). The left renal artery was occluded at the end of the control clearance period and then released after 60 min of ischemia (or sham ischemia). After a 10-min period to allow equilibration, measurements were made over four consecutive 30-min clearance periods. Sx, surgery; E, equilibration period; BC, Bohr curve; C, control period; U1–U5, urine collection periods; Temp, temperature.
where Hb is the blood hemoglobin concentration (in g/dl); SO2 is in %, and PO2 is in mmHg.

Kidney DO2 and VO2 could then be calculated as follows:

\[
\text{DO2} (\text{ml O}_2/\text{min}) = (\text{arterial O}_2 \text{ content}/100) \times \text{RBF}
\]

\[
\text{VO2} (\text{ml O}_2/\text{min}) = [(\text{arterial} \ - \ \text{venous O}_2 \text{ content})/100] \times \text{RBF}
\]

**Histological Assessment of Cellular Hypoxia and Tissue Damage (Protocol 4)**

Cellular hypoxia was assessed using the hypoxia-sensitive marker pimonidazole hydrochloride (Hydroxyprobe) on a separate cohort of rats (n = 5 sham control rats and 5 rats subjected to IRI) treated identically to those used in protocol 3. The hypoxia marker was administered via the jugular vein at a dose of 60 mg/kg, 60 min after reperfusion had commenced. At the end of the reperfusion period, 60 min after the administration of pimonidazole, the left kidney was perfusion fixed with 4% (wt/vol) paraformaldehyde (paraformaldehyde powder, no. 158127, Sigma-Aldrich) at a pressure of 150 mmHg. The perfused left kidney was then processed for pimonidazole adduct immunohistochemistry. Antigen retrieval was carried out by subjecting kidney sections (5 μm) to citrate buffer (Target Retrieval Solution, DAKO Australia) at 90°C for 30 min. Sections were then washed in Tris-buffered saline with Tween 20 (TSBT; 0.05 mol/l Tris-HCl, 0.15 mM NaCl, and 0.05% (vol/vol) Tween 20, DAKO Australia). Excessive tissue peroxidase activity was then quenched by an incubation for 10 min in 0.03% (vol/vol) H2O2 containing sodium azide (DAKO Australia), after which sections were washed twice in TSBT. Sections were then treated with protein block solution (Protein Block Serum-free, DAKO Australia) for 5 min and washed twice in TSBT. Sections were then treated with a mouse primary antibody against pimonidazole (1:3,000 dilution, Hydroxyprobe) for 1 h before an incubation with horseradish peroxidase conjugated with goat anti-mouse secondary antibody (DAKO Australia) for 30 min. Sections were then washed twice with TSBT before an incubation with 3-di-aminobenzidine (DAKO Australia). Sections were then counter-stained with hematoxylin. Two sections were analysed from each of the 10 kidneys. An additional section from each kidney was processed and stained with hematoxylin and eosin alone.

**Statistical Analyses**

Data are expressed as means ± SE. All statistical analyses were performed using the SYSTAT statistical package (version 10, Cranes Software, Chicago, IL) or GraphPad Prism (version 6.0b for Mac, GraphPad Software, La Jolla, California). Two-tailed P values of ≤0.05 were considered statistically significant.

For protocol 1, ordinary least-products regression analysis (27) was used to determine the relationships between PO2 measured by fluorescence optodes, SO2 measured by pulse oximetry, and hemoglobin content estimated from hematocrit determined by the capillary tube technique, and their values were determined by blood oximetry.

For protocol 2, all parameters measured by blood oximetry were subjected to repeated-measures ANOVA (28). The analysis tested the hypotheses that each measured variable (1) was dependent on the level of inspired O2, (2) differed in arterial compared with renal venous blood, and (3) depended on some interaction between these factors.

For protocol 3, baseline parameters and P30 values were analyzed using Student’s unpaired t-test to determine whether there were systematic differences between the two groups of rats during the control periods before ischemia or sham ischemia. Data collected across the course of the experiment were subjected to repeated-measures ANOVA (28) to determine whether the effects of ischemia differed from those of sham ischemia.

**RESULTS**

**Protocol 1: Methods for Measurements of Blood PO2, Saturation, and Hemoglobin Content**

Changes in inspired O2 content resulted in rapid changes in arterial and venous PO2 measured by fluorescence optodes and SO2 measured by pulse oximetry. These variables reached a new steady state within 30–60 s of the change in inspired O2 content. Measurements from fluorescence optodes, pulse oximetry, and capillary hematocrit were obtained at the same time point at which blood samples were collected for blood oximetry.

Measurements of PO2 of arterial and venous blood were combined for the purposes of this analysis (Fig. 2A). There was a strong relationship between PO2 measured by fluorescence optodes and blood oximetry (r² = 0.84, P < 0.001). There was some proportional bias in that PO2 measured by fluorescence optodes tended to be less than that measured by blood oximetry (slope = 0.78 with 95% confidence limits of 0.69–0.87). However, there was no significant fixed bias in that the 95% confidence limits of the x-intercept (5.1 mmHg) included zero (−1.2 to 11.4 mmHg). There was a strong linear relationship between the two methods for measurements of arterial hemoglobin saturation (r² = 0.90, P < 0.001; Fig. 2B). Although there was a slight fixed bias (x-intercept = 8.5% with 95% confidence limits of 0.6–16.4%), there was no significant proportional bias. Hematocrit varied linearly with hemoglobin concentration measured by blood oximetry (Fig. 2C). Thus, hemoglobin concentration could be determined from capillary hematocrit using the following relationship:

\[
\text{Hemoglobin concentration (mg/dl)} = \text{capillary hematocrit (%)}/3.08
\]

This relationship was applied in protocol 3. Because bias in methods for measurements of blood PO2 (fluorescence optodes) and hemoglobin saturation (pulse oximetry) was at most relatively small, and because the Bohr curves we applied in protocol 3 were derived from the combination of these methods, we made no attempt to correct measurements of blood PO2 or SO2 in protocol 3.

**Protocol 2: Can a Single Bohr Curve Be Used for Measurements of Arterial and Renal Venous Blood SO2 From Arterial and Venous PO2?**

Altering the inspired O2 content did not significantly affect arterial or renal venous blood pH or PCO2 (Fig. 3) or total hemoglobin and hematocrit (data not shown). As would be expected, arterial and renal venous PO2 and SO2 were reduced during hypoxia (17% and 19% O2) and increased during hyperoxia (30% O2; Fig. 3). Neither PCO2 nor pH varied significantly between arterial and renal venous blood. When values were averaged over the four levels of inspired O2, the concentrations of Cl− and lactate were 1.6 ± 0.2 and 0.3 ± 0.1 mmol/l greater, respectively, in arterial compared with renal venous blood. In contrast, the HCO3− concentration, actual base excess, standard base excess, Ca2+ concentration, and glucose concentration were greater in renal venous compared with arterial blood (data not shown).

The Bohr curve depicting the relationship between arterial and renal venous PO2 and SO2, measured by blood oximetry,
shows that points at the upper end of renal venous blood PO2 overlap those at the lower end of arterial blood PO2 (Fig. 4). Furthermore, the Hill equation calculated using the combined data from arterial and renal venous blood samples explained 98% of the variance in the data set.

Kidney DO2 and V˙O2 were calculated for later comparison with values obtained using our new method based on blood oximetry using fluorescence optodes (protocol 3). Neither DO2 (P = 0.15) nor V˙O2 (P = 0.07) varied significantly with inspired O2 content (Fig. 5). During ventilation with 30% O2, arterial and renal venous blood O2 content averaged 20.6 ± 0.9

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Fig. 2. Comparison of methods of measurements of blood PO2 (A) and hemoglobin saturation (SO2; B) and the relationship between capillary tube hematocrit and blood hemoglobin determined by direct blood oximetry (C) in protocol 1. In A, the open circles represent individual observations of PO2 from blood drawn from the jugular vein, whereas the closed circles represent observations of PO2 from blood drawn from the tail artery. In B and C, the different symbols represent observations in different rats. Lines of best fit were determined by the ordinary least-products method (27). Solid lines show the relationship when the intercept was not fixed. Dashed lines show the relationship forced through the origin. Within A–C, the Pearson product-moment correlation coefficient (r2) and the probability (P) that the slope of the relationship is zero are shown for the relationship when the intercept was not fixed.

Fig. 3. Direct oximetry of arterial and venous blood samples in protocol 2. Values are means ± SE; n = 6. Open columns represent data derived from arterial blood samples, whereas solid bars represent data derived from venous blood samples. P values represent the outcomes of two-way repeated-measures ANOVA, with the within-subjects factors of inspired oxygen content and the source of the blood sample (artery/vein). The analysis tested the hypotheses that each measured variable (1) was dependent on the level of inspired O2 (P oxy content), 2) differed in arterial compared with renal venous blood (P artery/vein), and 3) depended on some interaction between these factors (P interaction).
during the 1-h period of ischemia (data not shown).

Protocol 3: Kidney Oxygenation in Ischemia-Reperfusion

Baseline systemic hemodynamic and renal function parameters are shown in Table 1. There were no significant differences between the sham and IRI groups. Estimates of baseline renal Do2 (0.96 ± 0.12 ml O2/min across both groups) and VO2 (0.18 ± 0.03 ml O2/min across both groups) were somewhat greater than those determined by blood oximetry in protocol 2, and fractional O2 extraction (15.3 ± 1.3%) was somewhat less. This could be accounted for by the 53% greater RBF seen in protocol 3 than in protocol 2, since the calculated arterial O2 content was similar in both protocols 2 and 3.

Cortical and medullary PO2 fell rapidly upon application of a microvascular clamp on the renal artery, approaching 0 mmHg within 2.5 min. In contrast, cortical and medullary PO2 were well maintained during the period of sham ischemia. MAP, SO2, and arterial PO2 were not significantly altered during the 1-h period of ischemia (data not shown).

MAP gradually fell over the course of the experiment. The magnitude of this effect was similar in the two groups of rats, reaching −13.7 ± 2.3 mmHg (averaged across both groups) during the final clearance period (Fig. 6). After ischemia, arterial PO2 and SO2 slightly increased but remained relatively stable across the 2-h reperfusion period. Across the total reperfusion period, they were, on average, 5.6 ± 2.5 mmHg and 1.3 ± 0.5% greater, respectively, than during the control period. In contrast, after sham ischemia, arterial PO2 (−7.2 ± 0.9 mmHg) and SO2 (−2.2 ± 0.3%) fell slightly (Fig. 6). Neither renal venous PO2 nor SO2 were significantly different during the reperfusion period relative to the control period before ischemia. These variables also remained relatively stable across the course of the experiment in rats subjected to sham ischemia (Fig. 6 and Table 2).

RBF decreased progressively over the course of the experiment in the sham group, being 19 ± 4% less during the period 90–120 min after the end of sham ischemia than during the control period. In contrast, there was a considerable deficit in RBF commencing immediately after reperfusion, so that RBF was 41 ± 13% less during the period 90–120 min after reperfusion commenced than during the control period (Fig. 7 and Table 2). This was reflected in the significantly greater reduction in cortical blood flow in rats subjected to ischemia (−16 ± 4% during the period 90–120 min after reperfusion commenced compared with the control period) than those subjected to sham ischemia (−8 ± 2%). In contrast, medullary blood flow was little altered after ischemia (Fig. 7 and Table 2).

Renal DO2 was markedly reduced during the reperfusion period, so that it averaged 45 ± 12% less during the period 90–120 min after reperfusion than during the control period. After sham ischemia, DO2 progressively reduced, in line with the reduction in RBF (Fig. 8).

Table 1. Baseline hemodynamic and oxygenation variables before ischemia or sham ischemia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Ischemia-Reperfusion</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Number of animals/group</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>98.3 ± 3.4</td>
<td>98.3 ± 4.3</td>
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<td>Arterial PO2, mmHg</td>
<td>85.1 ± 5.4</td>
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<td>Arterial SO2, %</td>
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<td>Renal venous PO2, mmHg</td>
<td>48.9 ± 4.9</td>
<td>53.2 ± 2.9</td>
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<tr>
<td>Renal venous SO2, %</td>
<td>77.9 ± 3.0</td>
<td>81.1 ± 1.5</td>
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<td>RBF, ml/min</td>
<td>6.4 ± 0.8</td>
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<td>GFR, ml/min</td>
<td>1.3 ± 0.2</td>
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<td>Filtration fraction, %</td>
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<td>CBF, units</td>
<td>356 ± 12</td>
<td>370 ± 37</td>
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<td>Cortical PO2, mmHg</td>
<td>33.3 ± 4.5</td>
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<td>MBF, units</td>
<td>37 ± 10</td>
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<td>Medullary PO2, mmHg</td>
<td>28.4 ± 3.1</td>
<td>21.6 ± 8.8</td>
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<td>Do2, ml O2/min</td>
<td>1.07 ± 0.16</td>
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<td>VO2, ml O2/min</td>
<td>0.18 ± 0.05</td>
<td>0.18 ± 0.05</td>
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<td>Fractional O2 extraction, %</td>
<td>15.7 ± 2.3</td>
<td>14.8 ± 0.8</td>
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<td>Arterial blood O2 content, ml O2/dl</td>
<td>17.9 ± 0.5</td>
<td>18.1 ± 0.7</td>
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<td>Renal venous blood O2 content, ml O2/dl</td>
<td>14.9 ± 0.6</td>
<td>10.7 ± 2.7</td>
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</tbody>
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Data are means ± SE. MAP, mean arterial pressure; SO2, hemoglobin saturation; RBF, renal blood flow; GFR, glomerular filtration rate; CBF and MBF, cortical and medullary blood flow, respectively; DO2, renal O2 delivery; VO2, renal O2 consumption. P values are the outcomes of a Student’s unpaired t-test.

Fig. 4. Hemoglobin-O2 dissociation curve (Bohr curve) derived from blood samples collected in protocol 2. The solid circles show data derived from blood samples taken from the femoral artery, whereas the open triangles show data derived from blood samples taken from the renal vein. Bohr curves were modeled based on the Hill equation and analyzed using nonlinear regression in GraphPad Prism (29). The curve can be described by the following equation: \( y = (97.7 \times x^{0.2})(4.6^{0.2} + x^{0.2}) \), and \( r^2 = 0.98 \).

Fig. 5. Kidney O2 delivery (Do2; solid bars) and O2 consumption (Vo2; open bars) at various levels of inspired O2 content in protocol 2. Values are means ± SE; \( n = 6 \).

Fig. 6. Hemoglobin-O2 dissociation curve (Bohr curve) derived from blood samples taken from the renal vein. Bohr curves were modeled based on the Hill equation and analyzed using nonlinear regression in GraphPad Prism (29). The curve can be described by the following equation: \( y = (97.7 \times x^{0.2})(4.6^{0.2} + x^{0.2}) \), and \( r^2 = 0.98 \).
GFR and renal \( \dot{V}O_2 \) were markedly reduced during reperfusion (by \(-94 \pm 2\% \) and \(-57 \pm 8\% \), respectively, when averaged across the 2-h period of reperfusion). GFR was well maintained after sham ischemia (\(-3 \pm 11\% \) change), but \( \dot{V}O_2 \) was reduced by \( 25 \pm 5\% \) (Figs. 7 and 8 and Table 2). Fractional \( O_2 \) extraction remained relatively stable across the course of the experiment in both groups of rats.

Neither cortical nor medullary \( PO_2 \) were significantly reduced during the reperfusion period compared with the control period before ischemia. Indeed, the responses of these variables to ischemia-reperfusion were indistinguishable from those to sham ischemia (Fig. 9 and Table 2).

\( P_{50} \) values for Bohr curves generated before commencement of the experimental protocol did not differ significantly (\( P = 0.34 \)) between rats that were subsequently exposed to ischemia (28.3 ± 2.4 mmHg) or sham ischemia (24.4 ± 3.1 mmHg; Fig. 10).

Protocol 4: Histological Assessment of Cellular Hypoxia and Tissue Damage

Renal tissue from rats subjected to sham treatment appeared relatively normal (Fig. 11). The cortex of rats subjected to IRI displayed occasional tubular casts. Other tubular profiles appeared slightly dilated, but the brush border of proximal tubules appeared relatively normal. In the outer stripe of the medulla, about half of the tubular profiles were filled with casts. Many of the remaining tubular profiles were dilated with some flattening and irregularity of the brush border. In the inner stripe of the medulla, about half of the tubular profiles were filled with casts or had irregular thinning of the brush border. In the inner medulla, apart from the presence of tubular casts, morphology was relatively normal.

Little or no pimonidazole staining was found in the cortex and outer stripe of the outer medulla of kidneys exposed to sham ischemia (Fig. 11). Some positive staining was found in the tubular elements in the inner stripe of the outer medulla and inner medulla, in thin limbs of the loop of Henle but not in collecting ducts. Patchy positive staining was found in the cortex of kidneys exposed to IRI, chiefly in the brush border of proximal tubules. More consistent staining was found on the apical aspects of tubular elements in the outer and inner stripes of the outer medulla. In the outer stripe in particular, pimonidazole staining appeared to be largely restricted to the apical membranes of dilated tubules. In the inner medulla, both thin limbs of the loop of Henle and collecting ducts displayed consistent pimonidazole staining. Note also that the lumen of some tubular elements within the kidneys subjected to IRI were also found to contain pimonidazole staining. This luminal staining appeared to correspond to the presence of tubular casts, so is likely to be artifactual (Fig. 12).

Fig. 6. Responses of arterial pressure and arterial and renal venous blood oxygenation to ischemia-reperfusion. Values are means ± SE of differences (Δ) in each variable during the four consecutive 30-min periods after reperfusion commenced compared with the 30-min period before renal ischemia (solid bars) or sham ischemia (open bars). MAP, mean arterial pressure. Outcomes of repeated-measures ANOVA for these data sets are shown in Table 2.
Table 2. Outcomes of repeated-measures ANOVA for the data shown in Figs. 6–9

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>Time</th>
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<tbody>
<tr>
<td></td>
<td>P value</td>
<td>Degrees of freedom</td>
</tr>
<tr>
<td>Figure 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔMAP, mmHg</td>
<td>0.31</td>
<td>1,13</td>
</tr>
<tr>
<td>ΔArterial PO2, mmHg</td>
<td>0.02</td>
<td>1,12</td>
</tr>
<tr>
<td>ΔArterial SO2, %</td>
<td>0.005</td>
<td>1,12</td>
</tr>
<tr>
<td>ΔRenal venous PO2, mmHg</td>
<td>0.11</td>
<td>1,11</td>
</tr>
<tr>
<td>ΔRenal venous SO2, %</td>
<td>0.20</td>
<td>1,11</td>
</tr>
<tr>
<td>Figure 7</td>
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<tr>
<td>%ΔRBF</td>
<td>0.05</td>
<td>1,13</td>
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<tr>
<td>%ΔCBF</td>
<td>0.02</td>
<td>1,13</td>
</tr>
<tr>
<td>%ΔMBF</td>
<td>0.29</td>
<td>1,12</td>
</tr>
<tr>
<td>%ΔGFR</td>
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<td>1,12</td>
</tr>
<tr>
<td>Figure 8</td>
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<td>%ΔDo2</td>
<td>0.09</td>
<td>1,11</td>
</tr>
<tr>
<td>%ΔV02</td>
<td>0.05</td>
<td>1,10</td>
</tr>
<tr>
<td>ΔFractional O2 extraction, %</td>
<td>0.9</td>
<td>1,9</td>
</tr>
<tr>
<td>Figure 9</td>
<td></td>
<td></td>
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<tr>
<td>ΔCortical PO2</td>
<td>0.69</td>
<td>1,12</td>
</tr>
<tr>
<td>ΔMedullary PO2</td>
<td>0.07</td>
<td>1,12</td>
</tr>
</tbody>
</table>

P values are the outcomes of repeated-measures ANOVA with the between-subjects factor of “group” (ischemia or sham ischemia) and the within-subjects factor of “time” (clearance periods after ischemia or sham ischemia). The Greenhouse-Geisser correction (25) was applied to P values arising from the within-subjects factor to protect against the risk of type 1 error arising from the repeated-measures design. G-G E is estimate of E derived by the Greenhouse-Geisser method. Δ, absolute change; %Δ, percent change.

DISCUSSION

Previous studies have documented reduced renal tissue PO2 in both the renal medulla and/or cortex during reperfusion after a period of aortic cross clamping (22, 23, 38). In this context, our present findings of relatively well-maintained cortical and inner medullary PO2, as measured by fluorescence optodes, during reperfusion after a period of occlusion of the renal artery may appear somewhat surprising. However, just as renal hypoxia observed after aortic cross clamping can be explained by the presence of a greater deficit in renal Do2 than V02 (22, 23, 38), the relative preservation of kidney tissue PO2 in our present experimental protocol can be explained by the fact that the deficit in renal V02 (~60%) matched or exceeded that of DO2 (~45%) during reperfusion. These observations reinforce the notion that kidney oxygenation is governed by the balance between O2 delivery and demand (8, 12). Nevertheless, pimonidazole adduct immunohistochemistry provided evidence of cellular hypoxia during reperfusion, localized particularly on the apical membranes of dilated tubules in the outer medulla. Thus, even in the absence of a detectable deficit in renal DO2 relative to V02, and thus widespread tissue hypoxia, localized hypoxia may drive development of AKI (20) and its subsequent progression to chronic kidney disease or end-stage renal failure (1).

How can we explain our inability to detect reduced renal tissue PO2 in the face of evidence of localized hypoxia at the cellular level detected by pimonidazole adduct immunohistochemistry? One might argue that our inability to detect significant reductions in tissue PO2 during reperfusion might simply be a type II statistical error. We cannot exclude this possibility. However, we are reassured by the fact that the fluorescence optode technique we used provides a measure of tissue PO2 averaged over a relatively large area of tissue. Consequently, it is associated with less variability and greater sensitivity to changes in tissue PO2 than the “gold standard” Clark micro-electrode (10, 25). Another potential explanation arises from the fact that we measured tissue PO2 in the cortex and inner medulla, but not in the outer medulla, yet the most prominent staining for pimonidazole adducts was observed in the outer medulla. This latter observation accords with those from studies using BOLD MRI, in which hypoxia was found to be localized specifically to the renal outer medulla in IRI (31, 33). It is also noteworthy that our present observations in rats of the absence of widespread tissue hypoxia as detected by fluorescence optodes fit well with the limited information from studies of kidney oxygenation after renal transplantation in humans. In the immediate postoperative period, HIF-1α immunostaining (presumably reflecting tissue hypoxia) was greater in biopsies from functional than nonfunctional kidneys (36). Similarly, using BOLD MRI in patients during the first 4 mo after renal transplantation, Sadowski et al. (37) provided evidence for greater oxygenation of the renal medulla in patients undergoing acute rejection than in the medulla of normally functioning transplanted kidneys, despite poorer medullary perfusion. Both Rosenberger et al. and Sadowski et al. proposed that the lesser hypoxia in nonfunctional than functional transplanted kidneys could be explained by the relative lack of V02 for Na+ reabsorption, due to failure of glomerular filtration. Our present observations provide direct evidence to support this proposition, since our experimental model, in which only the renal artery was clamped, was associated with a marked deficit in GFR (~94%) and V02 (~57%) during reperfusion, but RBF (~41%) and DO2 (~45%) were less affected. In accord with our findings, recent observations indicate that renal perfusion is relatively better maintained in experimental models of IRI in which only the renal artery is occluded than in models in which both the renal artery and vein are clamped (26).

How do these findings bear on the proposition that hypoxia drives the development and progression of kidney disease induced by renal ischemia-reperfusion? Basile and colleagues (2, 3, 6) as well as others (21, 42) have shown that renal IRI results in long-term damage to the kidney and the eventual
renal disease, because it provides a mechanistic link between postglomerular peritubular microvascular insufficiency and the inflammation and fibrosis that develop during the progression of chronic kidney disease, which, in turn, exacerbates hypoxia. Our observation of positive staining for pimonidazole, particularly localized to the apical aspects of dilated tubules in the outer medulla, confirms that hypoxia in the outer medulla is a characteristic feature of renal IRI and so could potentially contribute to the development of AKI (17, 31, 33).

An important morphological characteristic of the kidney during reperfusion is the presence of tubular casts. These casts displayed intense pimonidazole staining. Rather than reflecting tissue hypoxia, pimonidazole staining in the blocked lumen of tubules is likely an artifact. The remaining patent tubules, particularly in the outer medulla, often appeared dilated, with considerable damage to the brush border. The fact that pimonidazole staining was most intense at the apical aspect of these tubular epithelial cells may be significant. Presumably,
such dilated tubules have a relatively large demand for O₂ because they bear the burden of the remaining filtered load of Na⁺H⁺. This may explain their susceptibility to development of localized hypoxia.

We must apply some additional caveats to our conclusions. The first caveat relates to the fact that our present findings regarding renal hemodynamics are at odds with those of several recent studies that have documented reductions in medullary perfusion, but not total RBF or cortical perfusion, during reperfusion in rats after 45 min of warm ischemia (for a review, see Ref. 35). In the present study, we observed reductions in total RBF and cortical perfusion but not medullary perfusion. These differences may reflect the greater severity of AKI in our present model, in which we used a 60-min period of warm ischemia compared with these previous studies. One must also recognize an important limitation of laser-Doppler flowmetry in richly perfused tissues such as the kidney. In such tissues, laser-Doppler flux largely provides a measure of erythrocyte velocity rather than perfusion per se (7). Thus, we cannot exclude the possibility of reduced medullary perfusion during reperfusion in our experiments, mediated by reductions in the number of perfused vasa recta rather than reduced erythrocyte velocity. Second, for technical reasons, we were unable to measure Na⁺H⁺ clearance in our experiments, and thus could not assess changes in total Na⁺ reabsorption. Nevertheless, it seems reasonable to conclude that the major cause of the 57% reduction in renal VO₂ during reperfusion was reduced Na⁺ reabsorption as a consequence of the 94% reduction in GFR. Finally, we must show extreme caution in attempting to extend conclusions based on a rodent model of warm IRI to clinical situations (16). This point was recently brought into focus by the findings of Parekh and colleagues (32), who provided evidence that the human kidney is remarkably tolerant to the effects of ischemia induced by clamping the renal pedicle for periods of 30–60 min.

Previous studies of kidney oxygenation using aortic cross clamping have consistently shown the presence of widespread reductions in renal tissue PO₂ during the acute phase of renal IRI. In rats, Legrand and colleagues (22, 23) placed a vascular occluder on the aorta upstream from the renal arteries but downstream from the mesenteric artery. Thus, their model was one of ischemia and reperfusion of the kidneys, urogenital system, and hindlimbs. In pigs, Siegmund et al. (38) placed a vascular occluder on the aorta between the celiac trunk and superior mesenteric artery. Thus, their model was one of ischemia and reperfusion of the kidneys, gut, urogenital system, and hindlimbs. In contrast, in our present study, in which only the renal artery was occluded, reduced renal tissue PO₂ could not be detected. On one level, the disparity of our present findings with those from these previous studies is explicable in...
terms of effects on renal $\dot{V}O_2$, which was better maintained after the aorta was clamped than after occlusion of only the renal artery. However, collectively, these observations might suggest that ischemia and reperfusion of organs remote to the kidney could influence renal oxygenation during reperfusion, by enhancing renal VO$_2$. Such a proposition is consistent with the finding of Legrand et al. (22), that inhibition of the inducible isoform of nitric oxide synthase improved cortical and medullary oxygenation and enhanced the metabolic efficiency of tubular Na$^+$ reabsorption during reperfusion after release of a clamp on the aorta. They also found little evidence for the activation of endogenous renal inducible nitric oxide synthase during reperfusion but good evidence for activation of polymorphonuclear cells, monocytes, and macrophages. Thus, we speculate that the renal dysoxia observed after aortic cross clamp could at least partly be due to effects on nonrenal tissue, which, in turn, activate inflammatory cascades within the kidney itself. This hypothesis merits testing.

In the course of this study, we developed and validated a new method for measurements of renal DO$_2$ and VO$_2$. The
ability of O₂ to quench the phosphorescence induced by excited from the decay curve for phosphorescence, because of the clinically for measurements of blood gas status (19). We (10, 11, 24, 25) and others (17, Mik et al. (29). Their technique requires intravenous injection of a water-soluble near-infrared phosphor. PO₂ is then calculated from the decay curve for phosphorescence, because of the ability of O₂ to quench the phosphorescence induced by excitation of the phosphor. Our approach was to use a commercially available fiber optic probe with an immobilized platinum-based fluorophore situated at the sensor tip. The fluorophore is excited by a pulse of light, and because the lifetime of the fluorescence is inversely proportional to the concentration of O₂, PO₂ can be derived. We (10, 11, 24, 25) and others (17, 41) have previously used this approach for measurements of kidney tissue oxygenation. We are not aware of previous studies in which this approach has been used to assess organ DO₂ and VO₂, although fiber optic devices have been used clinically for measurements of blood gas status (19). We showed relatively good agreement between PO₂ measured by fluorescence optodes and direct blood oximetry, both for arterial and venous blood (protocol 1). We also showed good agreement between arterial SO₂ measured by the Mouse Ox pulse oximeter and direct blood oximetry. Thus, measurement of arterial PO₂ by fluorescence optodes, arterial SO₂ by pulse oximetry, and estimation of hemoglobin concentration from capillary tube hematocrit allowed us to calculate arterial blood O₂ content. Combined with measurement of RBF by transit-time ultrasound flowmetry, we were able to estimate renal DO₂. To estimate renal VO₂, we had to first establish that the pH and PCO₂ of blood during transit across the renal circulation did not change appreciably, so that a single hemoglobin-O₂ dissociation curve could be used to calculate SO₂ from PO₂ in arterial and renal venous blood (protocol 2). Having established this, we could then generate hemoglobin-O₂ dissociation relationships for arterial blood, during the equilibration period of protocol 3, by briefly exposing rats to gas mixtures with an O₂ content less than that used during the experiment itself (i.e., <40% O₂). These relationships were established in all but three rats studied in protocol 3, but because there was little between-rat variation, we used a single relationship determined from the data from all animals to calculate renal venous SO₂ from renal venous PO₂. This also allowed us to include, in our final analysis, the results of the three animals in which Bohr curves could not be constructed because of equipment failure during the equilibration period.

In conclusion, our present study, using a new method for simultaneous assessment of renal tissue PO₂ and whole kidney DO₂ and VO₂, indicates that failure of glomerular filtration in AKI caused by renal IRI injury provides some protection against the development of global renal tissue hypoxia by limiting renal tubular VO₂. Our observations may provide at least a partial explanation for the findings of Rosenberger and colleagues (36) of greater HIF-1α immunostaining in renal biopsies from patients with functional transplanted kidneys relative to those with nonfunctional grafts. This conclusion accords with the notion of “acute renal success” first suggested >35 yr ago by Thurau and Boylan (40). Their concept has remained controversial, in part because of studies showing that the metabolic cost of Na⁺ reabsorption is increased in various forms of AKI (22, 23, 34, 38). Regardless, even in the absence of widespread reductions in renal tissue PO₂ in renal IRI, localized hypoxia can be detected, particularly in, although not restricted to, the apical aspects of dilated tubules in the outer medulla. Such localized hypoxia may represent an important stimulus for the progression of AKI.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
REFERENCES


