Therapeutic strategies for the protection of renal oxygenation in experimental models of acute kidney injury
Almaç, E.

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CHAPTER 4

Based on:

L-NIL prevents renal microvascular hypoxia and increase of renal oxygen consumption after ischemia-reperfusion in rats
Legrand M*, Almac E*, Mik EG, Johannes T, Kandil A, Bezemer R, Payen D, Ince C

*M. Legrand and E. Almac contributed equally to this study.
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Abstract

Even though renal hypoxia is believed to play a pivotal role in the development of acute kidney injury, no study has specifically addressed the alterations in renal oxygenation in the early onset of renal ischemia-reperfusion (I/R). Renal oxygenation depends on a balance between oxygen supply and consumption, with the nitric oxide (NO) as a major regulator of microvascular oxygen supply and oxygen consumption. The aim of this study was to investigate whether I/R induces inducible NO synthase (iNOS)-dependent early changes in renal oxygenation and the potential benefit of iNOS inhibitors on such alterations. Anesthetized Sprague-Dawley rats underwent a 30-min suprarenal aortic clamping with or without either the nonselective NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) or the selective iNOS inhibitor L-N6-(1-iminoethyl)lysine hydrochloride (L-NIL). Cortical (CμPO₂) and outer medullary (MμPO₂) microvascular oxygen pressure (μPO₂), renal oxygen delivery (DO₂ren), renal oxygen consumption (VO₂ren), and renal oxygen extraction (O₂ER) were measured by oxygen-dependent quenching phosphorescence techniques throughout 2 h of reperfusion. During reperfusion renal arterial resistance and oxygen shunting increased, whereas renal blood flow, CμPO₂, and MμPO₂ (−70, −42, and −42%, respectively, P < 0.05), VO₂ren, and DO₂ren (−70%, P < 0.0001, and −28%, P < 0.05) dropped. Whereas L-NAME further decreased DO₂ren, VO₂ren, CμPO₂, and MμPO₂ and deteriorated renal function, L-NIL partially prevented the drop of DO₂ren and μPO₂, increased O₂ER, restored VO₂ren and metabolic efficiency, and prevented deterioration of renal function. Our results demonstrate that renal I/R induces early iNOS-dependent microvascular hypoxia in disrupting the balance between microvascular oxygen supply and VO₂ren, whereas endothelial NO synthase activity is compulsory for the maintenance of this balance. L-NIL can prevent ischemic-induced renal microvascular hypoxia.
Introduction

Acute renal failure (ARF) is a major contributor to morbidity [24] and mortality [3] among hospitalized patients. Recent evidence shows that renal ischemia-reperfusion (I/R), as occurs during vascular surgery and shock, not only affects the renal tubules but in addition damages the renal microcirculation [8, 29, 34], preventing proper tissue reperfusion [4, 6]. The decrease in microvascular blood flow can then decrease renal oxygen supply and induce renal hypoxia. Dysoxia will originate from an imbalance between oxygen supply and oxygen requirements, which may turn out to be an important contributor to the pathogenesis of ARF after I/R [27]. The damaging effects of dysoxia can then be maximized by the decrease in the inability to handle oxygen at the mitochondrial level. However, this theory remains mainly speculative since no studies have specifically addressed the question whether alterations in renal oxygen pathways occur in the early onset of renal I/R and data regarding renal microvascular oxygen supply and oxygen consumption in this setting are lacking.

Many regulatory factors contribute to the homeostasis of oxygen supply and consumption of the kidney [27]. Of these, nitric oxide (NO) is of prime importance because of its central regulatory role in intrarenal microcirculation (12) and \( \dot{V}O_{2\text{ren}} \) regulation [1,25]. NO synthesis is predominantly mediated by two enzymes: endothelial NO synthase (eNOS) and inducible NO synthase (iNOS). Whereas eNOS is present in endothelial cells of vasa recta, in inner medullary collecting duct and glomeruli, iNOS expression is upregulated in vascular smooth muscle cells, renal tubular cells, and in monocytes, macrophages, and neutrophils in inflammatory states such as I/R injury [12]. It has been demonstrated in several other organs system such as the myocardium and brain that I/R injury results in increased NO generation [17]. It is thought that this excess NO is iNOS derived and that it contributes to microcirculatory derangement [9]. This would occur via endothelial-leukocyte activation, peroxynitrite formation [10,17] with a potential oxygen radical-dependent renal vasoconstriction [2,28], and secondary inhibition of eNOS. Indeed, increase in iNOS-derived NO is suspected to inhibit eNOS activity and decrease eNOS-derived NO release, disrupting the paracrine counteracting vasodilatory effect of NO on vasoconstrictors at the microcirculatory level [17]. Furthermore, eNOS-derived NO, in contrast, has been shown to prevent platelet aggregation and leukocyte adhesion [26,32]. For instance, Jones et al. demonstrated that I/R injury in the mouse myocardium is enhanced in eNOS-deficient mice compared with wild-type [20a]. Therefore, eNOS-derived NO may participate in sustaining blood supply to the tissue after renal I/R injury. On the other hand, NO also has a direct influence on renal oxygen metabolism as shown the increase in
oxygen consumption and decrease in renal metabolism efficiency after infusion of a nonselective NOS inhibitor in healthy dogs [25]. Inhibition of mitochondrial respiration at the cytochrome oxidase level might be involved in this regulating function [11,16]. However, the role of iNOS on \( \dot{V}O_{2\text{ren}} \) after ischemia-reperfusion is unknown.

Clinically applicable therapy directly aimed at protecting or curing the kidney from such injury remains lacking. Our focus, therefore, is on microcirculation and oxygenation-targeted protective therapies. The dual action of NO on microcirculation and oxygen metabolism make it a potential central player in matching oxygen supply to oxygen consumption during renal I/R. In this study, using a newly developed phosphorescence technique allowing measurement of microvascular PO\(_2\) and \( \dot{V}O_{2\text{ren}} \) in vivo, we investigated whether alteration in renal microcirculatory oxygenation and oxygen handling occurs during renal I/R and the role of NO in such alterations. In doing so, we tested the following hypotheses: 1) I/R induces renal microvascular hypoxia in the cortex and the outer medulla, 2) the renal I/R-induced microvascular hypoxia is iNOS dependent, and 3) I/R induces an iNOS-dependent decrease in \( \dot{V}O_{2\text{ren}} \). We expect that this study will provide new insights into I/R-related ARF in identifying the central role of NO in renal oxygen transport pathways alterations during I/R. Furthermore, we expect to provide evidence for a protective role of the selective iNOS inhibitors L-NIL during renal I/R.

Materials and methods

Animals

All experiments in this study were approved and reviewed by the Animal Research Committee of the Academic Medical Center at the University of Amsterdam. Care and handling of the animals were in accordance with the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. Experiments were performed on 38 Sprague-Dawley rats (Harlan, Horst, The Netherlands) with body weight of 325 ± 6 g.

Surgical Preparation

The rats were anesthetized with an intraperitoneal injection of a mixture of 100 mg/kg ketamine (Nimatek®; Eurovet, Bladel, the Netherlands), 0.5 mg/kg medetomidine (Domitor; Pfizer, New York, NY), and 0.05 mg/kg atropine-sulfate (Centrafarm, Etten-Leur, the Netherlands). After tracheotomy, the animals were mechanically ventilated with a FiO\(_2\) of 0.4. Body temperature was maintained at
37±0.5°C during the entire experiment by external warming. The ventilator settings were adjusted to maintain end-tidal PCO₂ between 30 and 35 mmHg and arterial PCO₂ between 35 and 40 mmHg.

Vessels were cannulated with polyethylene catheters (outer diameter=0.9 mm; Braun, Melsungen, Germany) for drug and fluid administration and hemodynamic monitoring. A catheter in the right carotid artery was connected to a pressure transducer to monitor mean arterial blood pressure (MAP) and heart rate. The right femoral artery was cannulated for blood sampling. The right femoral vein was cannulated for continuous infusion of Ringer’s lactate (15mL/kg/h; Baxter, Utrecht, The Netherlands) and ketamine (50mg/kg/h; Nimatek®, Eurovet, Bladel, The Netherlands). The left kidney was exposed, decapsulated, and immobilized in a Lucite kidney cup (K. Effenberger, Pfaffingen, Germany) via a 4 cm incision in the left flank. Renal vessels were carefully separated under preservation of nerves and adrenal gland. A perivascular ultrasonic transient time flow probe was placed around the left renal artery (type 0.7 RB; Transonic Systems Inc., Ithaca, NY, USA) and connected to a flow meter (T206; Transonic Systems Inc.) to continuously measure renal blood flow (RBF). An estimation of the renal vascular resistance (RVR) was made as RVR [dynes.sec.cm⁻⁵] = (MAP/RBF) ×80. The left ureter was isolated, ligated and cannulated with a polyethylene catheter for urine collection.

After the surgical protocol (approximately 60 minutes) one optical fiber was placed 1 mm above the decapsulated kidney and another optical fiber 1 mm above the renal vein to measure oxygenation in the renal microvasculature and renal vein, respectively, using phosphorimetry [20]. A small piece of aluminum foil was placed on the dorsal site of the renal vein to prevent contribution of underlying tissue to the phosphorescence signal in the venous oxygenation measurement. Oxyphor G2 (a two-layer glutamate dendrimer of tetra-(4-carboxy-phenyl) benzoporphyrin; Oxygen Enterprises Ltd, Philadelphia, PA, USA) was subsequently infused (6 mg/kg IV over 5 min) followed by a 30 minute stabilization period. A short description of phosphorimetry is given below and a more detailed description of the technology has been provided elsewhere [20].

The experiment was terminated by infusion of 1 ml of 3 M potassium chloride. Finally, the kidney was removed and weighed, and correct placement of the catheters was checked postmortem.

**Experimental Protocol**

The rats were divided into five groups (Figure 1): 1) a time-control group (n = 7). 2) An I/R group (n = 7); animals were subjected to renal ischemia for 30 min by
suprarenal aortic occlusion with a custom-made vascular occluder followed by 2 h of reperfusion. The vascular occluder was placed on the aorta above the renal arteries and beneath the mesenteric artery; 3) an I/R-treated group with \( N^\omega \)-nitro-L-arginine methyl ester (L-NAME) \((n = 7)\): a nonspecific inhibitor of NOS bolus was intravenously infused \((10 \text{ mg/kg iv})\) 15 min before ischemia; 4) an I/R-treated group with \( L-N^6-(1\text{-iminoethyl})\)lysine hydrochloride (L-NIL) \((n = 7)\): a specific inhibitor of iNOS \((5)\), intravenously infused \((3 \text{ mg/kg iv bolus} 15 \text{ min before ischemia, followed by} 0.3 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1})\); and 5) a time-control group treated L-NAME \((n = 4)\).

**Blood variables**

Arterial blood samples \((0.5 \text{ ml})\) were taken from the carotid artery at three time points: 1) before aortic occlusion, \((\text{baseline, } t_0)\); 2) 15 min after reperfusion \((\text{initial reperfusion phase, } t_1)\); and 3) 120 min after reperfusion \((\text{late reperfusion phase, } t_2)\). The blood samples were replaced by the same volume of HES130/0.4 \((\text{Voluven, 6\% HES 130/0.4; Fresenius Kabi Nederland, Schelle, Belgium})\). The samples were used for determination of blood-gas values \((\text{ABL505 blood-gas analyzer; Radiometer, Copenhagen, Denmark})\), as well as for determination of the hemoglobin concentration, hemoglobin oxygen saturation, and sodium and potassium concentrations \((\text{OSM 3; Radiometer})\).

**FIGURE 1.** Schematic representation of the experimental protocol. US, urine sample. Time points were baseline \((t_0)\), 15 min after reperfusion \((\text{initial reperfusion phase, } t_1)\), and 120 min after reperfusion \((\text{late reperfusion phase, } t_2)\).

**Renal microvascular and venous oxygenation**

Microvascular oxygen tension in the renal cortex \((C_{\text{µO}_2})\), outer medulla \((M_{\text{µO}_2})\), and renal venous oxygen tension \((P_{\text{rO}_2})\) were measured by oxygen-dependent
quenching of phosphorescence lifetimes of the systemically infused albumin-targeted (and therefore circulation-confined) phosphorescent dye Oxyphor G2 (14,35). Oxyphor G2 (a two-layer glutamate dendrimer of tetra-(4-carboxy-phenyl) benzoporphyrin) has two excitation peaks ($\lambda_{\text{excitation1}}$ = 440 nm, $\lambda_{\text{excitation2}}$ = 632 nm) and one emission peak ($\lambda_{\text{emission}}$ = 800 nm). These optical properties allow (near) simultaneous lifetime measurements in microcirculation of the kidney cortex and the outer medulla due to different optical penetration depths of the excitation light. For the measurement of renal venous PO$_2$ ($P_{rv}O_2$), a mono-wavelength phosphorimeter was used. Oxygen measurements based on phosphorescence lifetime techniques rely on the principle that phosphorescence can be quenched by energy transfer to oxygen resulting in shortening of the phosphorescence lifetime. A linear relationship between reciprocal phosphorescence lifetime and oxygen tension (given by the Stern-Volmer relation) allows quantitative measurement of PO$_2$.

**Calculation of oxygenation parameters**

The equations used for calculation of the oxygenation parameters are listed in Table 1. The renal venous O$_2$ saturation ($S_{rv}O_2$) was calculated by using Hill’s equation with $P_{50} = 37$ Torr (4.9 kPa) and Hill coefficient = 2.7. [Hb], hemoglobin concentration; $S_{aO2}$, arterial O$_2$ saturation; $P_{aO2}$, arterial PO$_2$; $P_{rv}O_2$, renal venous PO$_2$; RBF, renal blood flow; MAP, mean arterial blood pressure.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Abbreviation</th>
<th>Equation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial O$_2$ content</td>
<td>$Ca_{O2}$</td>
<td>$1.31 \times [Hb] \times S_{aO2} + 0.003 \times P_{aO2}$</td>
<td>ml O$_2$/ml blood</td>
</tr>
<tr>
<td>Venous O$_2$ content</td>
<td>$Cv_{O2}$</td>
<td>$1.31 \times [Hb] \times S_{rv}O_2 + 0.003 \times P_{rv}O_2$</td>
<td>ml O$_2$/ml blood</td>
</tr>
<tr>
<td>Renal O$_2$ delivery</td>
<td>$DO_{2ren}$</td>
<td>$\text{RBF} \times C_{aO2}$</td>
<td>ml O$_2$·min·g$^{-1}$</td>
</tr>
<tr>
<td>Renal O$_2$ consumption</td>
<td>$VO_{2ren}$</td>
<td>$\text{RBF} \times (C_{aO2} - C_{vO2})$</td>
<td>ml O$_2$·min·g$^{-1}$</td>
</tr>
<tr>
<td>Renal O$_2$ extraction</td>
<td>$O_{2ER_{ren}}$</td>
<td>$(VO_{2ren}/DO_{2ren}) \times 100$</td>
<td>%</td>
</tr>
<tr>
<td>Microvascular O$_2$(re)distribution</td>
<td>$\Delta \mu PO_2$</td>
<td>$C_{\mu PO_2} - M_{\mu PO_2}$</td>
<td>mmHg</td>
</tr>
<tr>
<td>Renal vascular resistance</td>
<td>RVR</td>
<td>$(\text{MAP/RBF}) \times 100 \times 80$</td>
<td>dyn·s·cm$^{-5}$</td>
</tr>
</tbody>
</table>

**TABLE 1.** Calculation of oxygenation parameters.

**Renal function**

For analysis of urine volume, creatinine concentration, and sodium (Na$^+$) concentration at the end of the protocol, urine samples from the left ureter were
collected for 10 min. Creatinine clearance rate ($CL_{\text{crea}}$) per gram of renal tissue was calculated with standard formula: $CL_{\text{crea}}$ (mL/min/g) = (U×V)/P, where U is the urine creatinine concentration, V is the urine volume per unit time, and P is the plasma creatinine concentration. Renal sodium reabsorption ($T_{\text{Na}^+}$, [mmol/min]) was calculated as $T_{\text{Na}^+} = (P_{\text{Na}^+} \times CCR) - (U_{\text{Na}^+} \times V)$, where $U_{\text{Na}^+}$ is the urine sodium concentration and $P_{\text{Na}^+}$ is the plasma sodium concentration. The renal oxygen consumption efficiency for sodium transport ($VO_2/T_{\text{Na}^+}$) was assessed as the ratio of the renal $VO_2$ over the total amount of sodium reabsorbed ($T_{\text{Na}^+}$, [mmol/min]).

**iNOS and MPO Immunohistochemistry**

Kidney tissues were fixed in 10% formalin and embedded in paraffin. Kidney sections (5 μm) were deparaffinized with xylene and rehydrated with decreasing percentages of ethanol and finally with water. Antigen retrieval was accomplished by microwaving slides in citrate buffer (pH 6.0) for 10 min. Slides were left to cool for 20 min at room temperature and then rinsed with distilled water. Surroundings of the sections were marked with a PAP pen. The endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ for 10 min at room temperature and later rinsed with distilled water and PBS. Blocking reagent (TA-125-UB, Lab Vision, Fremont, CA) was applied to each slide followed by 5 min incubation at room temperature in a humid chamber. Kidney sections were incubated for overnight at 4°C with rabbit anti-mouse iNOS (iNOS rabbit Pab Neomarker, RB-1605-P, Fremont, CA) and incubated for 45 min at room temperature with anti-myeloperoxidase (MPO) antibodies (MPO rabbit RB-373-A Thermo Fisher Scientific). Antibodies were diluted in a large volume of UltrAb Diluent (Lab Vision, TA-125-UD) at 1:100. The sections were washed in PBS three times for 5 min each time and then incubated for 30 min at room temperature with biotinylated goat anti-rabbit antibodies (Lab Vision, TR-125-BN) [13]. After slides were washed in PBS, the streptavidin peroxidase label reagent (Lab Vision, TS-125-HR) was applied for 30 min at room temperature in a humid chamber. The colored product was developed by incubation with AEC (Lab Vision, TA-007-HAC). The slides were counterstained with hematoxylin and mounted in glycerol gelatin after being washed in distilled water. Both the intensity and the distribution of specific iNOS and MPO staining were scored. For each sample, an histological score (HSCORE) value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [HSCORE = $S_i \times P_i (i+1)$, where $i$ is the intensity score and $P_i$ is the corresponding percentage of the cells] [33]. We evaluated MPO reaction in the glomerulus from 30 selected glomeruli and in peritubular areas. We scored 1 if leukocytes could be seen in the glomerulus and 0 if not.
Statistical Analysis

Values are reported as means ± SE. The decay curves of phosphorescent intensity were analyzed by use of software programmed in LabVIEW 6.1 (National Instruments, Austin, TX). Statistical analysis was performed with GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). Two-way ANOVA analysis for repeated measurements was used for intergroup and intragroup comparisons; post hoc analyses were used with the Bonferroni posttest when $P < 0.05$. Intergroup comparisons for Clear$_{crea}$ and EF$_{Na^+}$ were analyzed by the unpaired Student's $t$-test. For all analyses, $P < 0.05$ was considered significant. Three time points were used for statistical analysis: baseline ($t_0$), 15 min after reperfusion (initial reperfusion phase, $t_1$), and 120 min after reperfusion (late reperfusion phase, $t_2$). Renal vascular resistances (RVR) were plotted vs. VO$_{2renal}$ and were analyzed and quantified by the Spearman correlation test.

Results

Systemic hemodynamic parameters

Systemic hemodynamic variables are presented in Table 2. The baseline values measured in each group were found to be similar. Whereas mean arterial pressure (MAP) remained stable throughout the entire experiment in the control group, MAP decreased (i.e., $-16\%$) in the I/R group during the reperfusion period. Treatment with L-NAME resulted in an initial significant increase in MAP with respect of baseline values during the initial reperfusion phase at $t_1$ followed by a progressive reduction in MAP at $t_2$. L-NIL treatment restored MAP to baseline values. Administration of L-NAME in control rats induced an increase in MAP that returned to baseline at $t_2$.

Renal hemodynamic parameters

RBF decreased slightly between $t_0$ and $t_2$ (i.e., $-21\%$) in the control group, whereas in the I/R group a significant decrease was found (i.e., $-70\%$) together with a significant increase in RVR (i.e., $+192\%$). L-NAME significantly increased the drop of RBF and the RVR, whereas L-NIL restored the RBF and RVR to control group and baseline values. Treatment with L-NAME in nonischemic rats induced a sustained decrease in RBF, with increase in RVR (Table 2).
Mean arterial pressure, mmHg

<table>
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<th></th>
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<th>t₂</th>
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<tr>
<td>Control group</td>
<td>101 ± 5</td>
<td>103 ± 5</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>I/R group</td>
<td>100 ± 3</td>
<td>114 ± 7</td>
<td>84 ± 10*</td>
</tr>
<tr>
<td>L-NAME group</td>
<td>107 ± 4</td>
<td>151 ± 4††‡</td>
<td>72 ± 4†*</td>
</tr>
<tr>
<td>L-NIL group</td>
<td>106 ± 4</td>
<td>142 ± 4††‡</td>
<td>91 ± 6</td>
</tr>
<tr>
<td>L-NAME control group</td>
<td>103 ± 6</td>
<td>157 ± 4††‡</td>
<td>102 ± 7</td>
</tr>
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Renal blood flow, ml/min

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<tbody>
<tr>
<td>Control group</td>
<td>4.0 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>3.2 ± 0.4†</td>
</tr>
<tr>
<td>I/R group</td>
<td>4.1 ± 0.2</td>
<td>2.3 ± 0.2†*</td>
<td>1.2 ± 0.1†*</td>
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<tr>
<td>L-NAME group</td>
<td>4.4 ± 0.3</td>
<td>2.2 ± 0.2†*</td>
<td>0.8 ± 0.1††‡</td>
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<tr>
<td>L-NIL group</td>
<td>4.5 ± 0.2</td>
<td>3.2 ± 0.3††‡</td>
<td>3.4 ± 0.2††‡</td>
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<tr>
<td>L-NAME control group</td>
<td>4.5 ± 0.1</td>
<td>2.9 ± 0.3</td>
<td>2.4 ± 0.2††‡</td>
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Renal vascular resistances, dyn·s·cm⁻⁵

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<tr>
<td>Control group</td>
<td>2,079 ± 180</td>
<td>2,415 ± 247</td>
<td>2,872 ± 345†</td>
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<tr>
<td>I/R group</td>
<td>2,008 ± 162</td>
<td>4,067 ± 437†</td>
<td>5,659 ± 269†*</td>
</tr>
<tr>
<td>L-NAME group</td>
<td>1,947 ± 141</td>
<td>5,914 ± 534†*</td>
<td>8,381 ± 1,683†*</td>
</tr>
<tr>
<td>L-NIL group</td>
<td>1,924 ± 110</td>
<td>3,769 ± 357†</td>
<td>2,191 ± 147††‡</td>
</tr>
<tr>
<td>L-NAME control group</td>
<td>1,846 ± 106</td>
<td>4,543 ± 622</td>
<td>3,354 ± 508‡</td>
</tr>
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</table>

**TABLE 2.** Presentation of hemodynamic parameters at 3 time points: t₀, t₁, and t₂. I/R, ischemia-reperfusion; L-NAME, Nω-nitro-L-arginine methyl ester; L-NIL, L-N⁶-(1-iminoethyl)lysine hydrochloride. * P < 0.05 vs. control; † P < 0.05 vs. t₀; ‡ P < 0.05% vs. I/R.

Renal oxygen supply, consumption, and extraction

At t₀, VO₂ren, DO₂ren, and O₂ER did not differ between groups (Table 3). DO₂ren and VO₂ren both decreased after I/R at t₂ (i.e., −70%, P < 0.0001, and −28%, P > 0.05, respectively). O₂ER in the I/R group increased between t₀ and t₂ in the same proportion as in the control group (~27%). Interestingly, VO₂ren and O₂ER both increased throughout the experiment in the control group. Whereas L-NAME further
decreased \( \text{DO}_{2\text{ren}} \), L-NIL restored \( \text{DO}_{2\text{ren}} \) to the control group level (i.e., \( P < 0.01 \) vs. I/R) and significantly increased \( \text{VO}_{2\text{ren}} \) above baseline values (\( P < 0.05 \)), above group control values (\( P < 0.05 \) at \( t_2 \)) and I/R group values (\( P < 0.001 \) at \( t_2 \)). However, when the \( \text{VO}_{2\text{ren}} \) was normalized by the \( \text{Na}^+ \) reabsorption, the \( \text{VO}_{2\text{ren}} \) remained stable in the control and L-NIL groups, whereas it increased in the I/R- and L-NAME-treated groups. Both nonselective and selective NOS inhibition further increased the \( \text{O}_2\text{ER} \) (\( P < 0.05 \) vs. \( t_0 \) for L-NAME, \( P < 0.0001 \) vs. \( t_0 \) and \( P < 0.05 \) vs. I/R for L-NIL, Figure 2). Treatment with L-NAME in control rats was associated with a decrease in \( \text{DO}_{2\text{ren}} \) but an increase of \( \text{VO}_{2\text{ren}} \) and \( \text{O}_2\text{ER} \). A significant correlation was found between postischemic RVR and \( \text{VO}_{2\text{ren}} \) (Figure 2), suggesting a role in decrease of microvascular oxygen supply in the decrease of \( \text{VO}_{2\text{ren}} \), reinforced by the correlation found between \( \text{DO}_{2\text{ren}} \) and \( \text{VO}_{2\text{ren}} \) at \( t_2 \).

<table>
<thead>
<tr>
<th>( \text{DO}_{2\text{ren}} ), \text{ml} \cdot \text{min} \cdot \text{g}^{-1} )</th>
<th>( t_0 )</th>
<th>( t_1 )</th>
<th>( t_2 )</th>
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<tbody>
<tr>
<td>control group</td>
<td>0.80 ± 0.09</td>
<td>0.68 ± 0.09</td>
<td>0.63 ± 0.1†</td>
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<tr>
<td>I/R group</td>
<td>0.72 ± 0.04</td>
<td>0.39 ± 0.03*†</td>
<td>0.20 ± 0.02*†</td>
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<td>L-NAME group</td>
<td>0.70 ± 0.09</td>
<td>0.35 ± 0.06*†</td>
<td>0.10 ± 0.01*†</td>
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<tr>
<td>L-NIL group</td>
<td>0.77 ± 0.07</td>
<td>0.52 ± 0.06†</td>
<td>0.54 ± 0.04†‡</td>
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<tr>
<td>L-NAME control group</td>
<td>0.69 ± 0.02</td>
<td>0.46 ± 0.07</td>
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<table>
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<tr>
<th>( \text{VO}_{2\text{ren}} ), \text{ml} \cdot \text{min} \cdot \text{g}^{-1} )</th>
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<th>( t_1 )</th>
<th>( t_2 )</th>
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<tbody>
<tr>
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<td>0.27 ± 0.04†</td>
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<tr>
<td>I/R group</td>
<td>0.15 ± 0.05</td>
<td>0.09 ± 0.02*</td>
<td>0.10 ± 0.01*</td>
</tr>
<tr>
<td>L-NAME group</td>
<td>0.12 ± 0.02</td>
<td>0.11 ± 0.03</td>
<td>0.07 ± 0.01*</td>
</tr>
<tr>
<td>L-NIL group</td>
<td>0.13 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.37 ± 0.04*†‡</td>
</tr>
<tr>
<td>L-NAME control group</td>
<td>0.13 ± 0.01</td>
<td>0.34 ± 0.12‡</td>
<td>0.40 ± 0.07†‡</td>
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</table>

<table>
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<th>( \text{O}_2\text{ER} ), %</th>
<th>( t_0 )</th>
<th>( t_1 )</th>
<th>( t_2 )</th>
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<tbody>
<tr>
<td>control group</td>
<td>19 ± 2</td>
<td>30 ± 4</td>
<td>45 ± 7†</td>
</tr>
<tr>
<td>I/R group</td>
<td>21 ± 3</td>
<td>21 ± 5</td>
<td>50 ± 7†</td>
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<tr>
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<td>40 ± 12</td>
<td>67 ± 6*†</td>
</tr>
<tr>
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<td>26 ± 3</td>
<td>69 ± 3*†‡</td>
</tr>
<tr>
<td>L-NAME control group</td>
<td>12 ± 1*‡</td>
<td>45 ± 9*‡</td>
<td>68 ± 9*‡</td>
</tr>
</tbody>
</table>

**TABLE 3.** Presentation of renal oxygenation parameters at 3 time points
### TABLE 3. Presentation of renal oxygenation parameters at 3 time points: $t_0$, $t_1$, and $t_2$. $CμPO_2$ and $MμPO_2$, cortical and outer medullary microvascular oxygen pressure, respectively. * $P < 0.05\%$ vs. control; † $P < 0.05\%$ vs. baseline; ‡ $P < 0.05\%$ vs. I/R.

**Renal microvascular oxygenation**

During the ischemic period, both cortical ($CμPO_2$) and outer medullary ($MμPO_2$) microvascular $PO_2$ decreased dramatically. Immediately after reperfusion both
parameters returned to baseline values, followed by a slow significant decrease over time between $t_1$ and $t_2$. L-NAME further increased the drop in $\mu$PO$_2$ with respect to the I/R group, whereas $\mu$PO$_2$ was partially restored with L-NIL (see Figures 3 and 4). L-NAME in control rats induced a decrease in $C_\mu$PO$_2$ and $M_\mu$PO$_2$ comparable to that in the I/R group.

**FIGURE 2.** Left: correlation between renal vascular resistance (RVR, expressed in dyn·s·cm$^{-5}$) and renal oxygen consumption ($\dot{V}O_{2ren}$, expressed in ml O$_2$·min·g$^{-1}$). Right: correlation between $\dot{V}O_{2ren}$ (expressed in ml O$_2$·min·g$^{-1}$) and renal oxygen delivery ($DO_{2ren}$, expressed in ml O$_2$·min·g$^{-1}$) at $t_2$.

**FIGURE 3.** Typical example of improvement in microvascular oxygen pressure ($\mu$PO$_2$) after L-$N^6$-(1-iminoethyl)lysine hydrochloride (L-NIL) infusion throughout the reperfusion (bottom) compared with without treatment (top).
Redistribution of oxygen between cortex and outer medulla occurred during reperfusion as demonstrated by changes in $\Delta \mu \text{PO}_2 (C_{\mu \text{PO}_2} - M_{\mu \text{PO}_2})$ (Figure 5). $\Delta \mu \text{PO}_2$ decreased over time in the I/R group ($P < 0.05$ between $t_0$ and $t_1$). The drop in $\Delta \mu \text{PO}_2$ was significant at $t_2$ in the L-NAME group ($P < 0.01$), because of a predominant decrease of $C_{\mu \text{PO}_2}$, whereas $\Delta \mu \text{PO}_2$ was similar in the L-NIL and control group, without redistribution occurring over time.

Furthermore, we found evidence for intrarenal oxygen shunting during reperfusion in the I/R group as suggested by the $P_{\text{rO}_2}$ values being higher or almost equal to the $\mu \text{PO}_2$ (Figure 5). In the L-NIL group, in contrast, no evidence for intrarenal oxygen shunting was found.

**FIGURE 4.** Changes in cortical ($C_{\mu \text{PO}_2}$) and outer medullary ($M_{\mu \text{PO}_2}$) $\mu \text{PO}_2$ (changes in % between $t_0$ and $t_2$) $^*P < 0.05\%$ vs. control. $^\dagger P < 0.05\%$ vs. ischemia-reperfusion (I/R, IR). L-NAME, $N^\omega$-nitro-L-arginine methyl ester.

**FIGURE 5.** Evolution of intrarenal oxygen shunting expressed as the $\Delta [P_{\text{vO}_2_{\text{ren}}} - C_{\mu \text{PO}_2}]$, where $P_{\text{vO}_2_{\text{ren}}}$ is renal venous $\text{PO}_2$, in the 4 groups between $t_0$, $t_1$, and $t_2$ (expressed in mmHg). $^*P < 0.05$ vs. control. $^\ddagger P < 0.05$ vs. $t_0$. $^\dagger P < 0.05$ vs. I/R. Values are expressed in means ± SD.
Renal function

Because of anuria in two animals in the L-NAME group and failure of ureteral cannulation in one animal of the I/R group, urine samples could not be obtained from these animals. The Clear\textsubscript{crea} did not change over time in the control rats (Figure 6, left). In the I/R group the averaged Clear\textsubscript{crea} decreased \~30\% between $t_0$ and $t_2$ ($P = 0.054$). The drop in Clear\textsubscript{crea} was more extensive in the L-NAME group ($P < 0.05$ vs. baseline and vs. control). Treatment with L-NIL restored Clear\textsubscript{crea} to baseline value (Figure 6, left). EF Na\textsuperscript{+}, used as marker of tubular function (Figure 6, right), increased from $2.5 \pm 2$ to $13 \pm 4$\% between $t_0$ and $t_2$ ($P < 0.05$ vs. control and baseline) upon I/R. L-NAME increased EFNa\textsuperscript{+}, whereas L-NIL restored EFNa\textsuperscript{+} to the control group values ($P < 0.05$ vs. I/R).

The oxygen cost of reabsorptive work

Na\textsuperscript{+}-reabsorptive work constitutes a major part of VO\textsubscript{2ren} in the healthy kidney. The VO\textsubscript{2}/Na\textsuperscript{+} reabsorption provides an indication of how much oxygen is effectively used for normal reabsorptive work. In the L-NIL group VO\textsubscript{2}/Na\textsuperscript{+}-reabsorption is found to be similar to the control group (Figure 7), suggesting an efficient use of oxygen for accomplishing the reabsorptive work. In contrast, we found a trend of increase in the VO\textsubscript{2}/Na\textsuperscript{+} reabsorption in both the ischemic and the L-NAME groups, suggesting a diversion of oxygen consumption from Na\textsuperscript{+}reabsorption to other oxygen-consuming pathways.
**FIGURE 7.** Evolution of VO$_{2ren}$/Na$^+$transport (TNa$^+$; ml·min·mmol$^{-1}$) between $t_0$ and $t_2$ in the control, I/R, L-NAME, and L-NIL groups (*$P < 0.01$ vs. control and L-NIL groups).

### iNOS and MPO staining

MPO activity has been reported to reflect the activation of infiltrative and inflammatory cells (polymorphonuclear cells but also monocytes and macrophages) (38). We found severe MPO reaction the glomerulus 15 min after reperfusion compared with baseline (0.36 ± 0.03 vs. 0.01 ± 0.009, respectively, $P < 0.0001$), indicating activation of inflammatory cells in the ischemic kidney. On the other hand, MPO staining was similar in the peritubular areas at baseline and 15 min after reperfusion. There was also an endogenous strong iNOS expression in the whole kidney, especially in the cytoplasm of tubular cells both at baseline and 15 min after reperfusion (328 ± 10 and 325 ± 16, respectively; not significant) (Figure 8).

### Discussion

This study has shown for the first time that 1) I/R injury induces progressive microvascular hypoxia in the cortex and medulla following reperfusion, 2) these microcirculatory alterations are associated with a decrease in renal oxygen supply and an increase in intrarenal oxygen shunting, 3) these alterations in oxygen transport pathways were associated with a deterioration in renal function, 4) iNOS participates in such alterations, 5) additional inhibition of eNOS further compromised microvascular oxygenation and renal function highlighting the physiological role played by eNOS-derived NO at the microcirculatory level, and 6) selective iNOS inhibition increased VO$_{2ren}$ and extraction but restored the oxygen cost of Na$^+$ reabsorption after I/R underlining the pivotal role of iNOS-derived NO in regulating oxygen utilization in the ischemic kidney.
Our study has described ischemia-induced renal oxygen transport pathways alterations. Even though renal hypoxia is believed to play a critical role in ischemia-induced renal injury, to the best of our knowledge, renal oxygenation alterations have never been described in the early stage of renal I/R. In our model, a decrease in renal oxygen supply, a decrease in cortical and medullary $\mu$PO$_2$, an increase in renal shunting, along with a decrease in VO$_{2ren}$ occurred throughout the reperfusion phase. Alteration of microvascular oxygenation and oxygen utilization might therefore promote renal damage leading to renal dysfunction.

To determine the cause for the alterations of DO$_{2ren}$ and $\mu$PO$_2$ following I/R, one first needs to ask the question as to what extent changes in macrohemodynamics changes during the reperfusion could be responsible for the observed phenomena. The moderate decrease in MAP observed during reperfusion is an unlikely cause, since Brezis et al. [7] have previously shown that a moderate hypotension in healthy rats, similar to that found in our experiments, was associated with a significant increase in medullary PO$_2$ together with a decrease in cortical PO$_2$. This observation was suggested to be related to a decrease in medullary oxygen consumption due the reduction of Na$^+$ tubular reabsorption associated with the decrease in renal blood flow. Recently our group has demonstrated that reduction of renal blood flow by partial ligation of the renal artery (and thus reduction of renal
perfusion pressure) was not associated with changes in μPO₂ [20]. The significant increase in renal vascular resistances observed in our study strongly suggests that the alterations of oxygenation took place at the microcirculatory level and were not linked to macrohemodynamic changes. From these considerations it is unlikely that the moderate macrohemodynamic changes occurring after I/R could be the cause of the microcirculatory hypoxia found in the present study.

Besides the decrease in DO₂ren, I/R increased intrarenal oxygen shunting which could also account in part in the deterioration in renal μPO₂ found in the reperfusion phase. Indeed, blood in the renal vein comes from two sources: 1) blood from the renal capillaries and 2) blood shunted from precapillary renal arteries to adjacent vein owing the vasculature arrangement of the kidney. Demonstration of the oxygen pressure of the venous effluent being higher than the microcirculatory PO₂ has been accepted as evidence of the presence of oxygen shunting in previous studies (20, 37). The PᵥO₂ value being higher than the μPO₂ after reperfusion in the I/R group suggests that there was a shunt of well-oxygenated blood to the renal vein bypassing the capillaries.

This study provides new insights concerning the respective role of different NO synthesis and their contribution to renal oxygenation pathways and renal function in renal I/R. First, selective inhibition of iNOS using L-NIL was shown to prevent the decline in oxygen delivery, oxygen extraction and consumption, and decline in μPO₂ during reperfusion. The protective effect of iNOS inhibition on renal microvascular oxygenation was present as soon as 15 min after reperfusion. The intense iNOS expression at baseline suggests a posttranscriptional regulation [23]. However, postischemic iNOS expression could also originate from infiltrative cells as suggested by the intense MPO staining in this time frame [21, 22] (Figure 8). Secondly, and in contrast, additional inhibition of eNOS by the nonselective NOS inhibitor L-NAME clearly deteriorated renal oxygen delivery and microvascular oxygenation associated with glomerular and tubular dysfunction. Inhibition of iNOS expression or iNOS activity has previously been shown to reduce renal damage caused by I/R of the kidney. Chatterjee et al. [10] have shown that use of different selective iNOS inhibitors (i.e., L-NIL and AE-ITU) can reduce ischemic-induced kidney damage and prevent renal dysfunction. If reduction in nitrotyrosine and peroxynitrite formation could be one of protective pathway, mechanisms of such protection remain largely unknown. The present study provides important insights into the pathophysiology of ischemic-induced renal failure in underlining the deleterious role played by iNOS-derived NO on the renal oxygenation. Thus selective iNOS blockage may prevent ischemic-induced renal microvascular hypoxia by improving renal oxygen supply and decreasing oxygen shunting. Previous study from Ichihara et al. [19] showed that LPS-induced iNOS
activation impaired endothelium-dependent NO-induced vasodilation of afferent and efferent glomerular arterioles. Moreover, inhibition of iNOS has also been found to improve the microcirculation perfusion in skeletal muscle, as assessed by Zhang et al. [39] using intravital microscopic techniques. Taken together, these results suggest that iNOS induction plays a key pathophysiological role in ischemic-induced (micro)circulatory hypoxia and may participate to the organ dysfunction by extending renal microvascular hypoxia way after the initial ischemia has been actually resolved.

Both I/R and nonselective NOS inhibition increased $\dot{V}O_2_{ren}$ and decreased the metabolic oxygen efficiency. In contrast, selective iNOS inhibition increased the $\dot{V}O_2_{ren}$ but restored the metabolic oxygen efficiency to the control group values expressed as the ratio $\dot{V}O_2_{ren}/TNa^+$. Nonselective NOS inhibitors have already been shown to increase $\dot{V}O_2_{ren}$ in healthy dogs under physiological conditions [25]. Bateman and coworkers [4a] have shown an increase in local skeletal muscle tissue oxygen consumption in a rat sepsis model of cecal ligation and perforation. With our present study, we show that I/R increases $\dot{V}O_2_{ren}$ and that this depends on the iNOS activity. The increase in total oxygen consumption with selective iNOS inhibitors could be due to an increase in tissue renal oxygen supply, as suggested by the significant correlation between $DO_2_{ren}$ and $\dot{V}O_2_{ren}$ after reperfusion (Figure 6). The increase of glomerular filtration and/or Na$^+$ load leading to increase tubular reabsorption also participates in this increase. Therefore, after normalization to the renal Na$^+$ transport, the $\dot{V}O_2_{ren}$ was found to be similar to that of the control group, indicating a decrease of the oxygen cost for the metabolic work of the kidney after iNOS inhibition. Furthermore, eNOS-derived NO is able to regulate cellular respiration at the mitochondrial level by reversibly inhibiting the complex IV of the mitochondrial electron transport chain [9]. NO thereby competes with oxygen at the cytochrome oxidase level, resulting in decrease in oxygen utilization. Therefore, iNOS-derived NO during I/R is believed to inhibit eNOS activity, thus shutting down the mitochondrial respiration and increasing oxygen utilization at the cellular level [9]. Future research should aim at defining what is the destiny of the oxygen being used.

Whereas administration of selective iNOS inhibitors prior to a potential ischemic hit protected the kidneys from microvascular hypoxia, additional inhibition of eNOS by a nonselective NOS inhibitor further reduced $DO_2_{ren}$ and $\muPO_2$. eNOS-derived NO is well known for its role in vascular tone regulation by acting on the surrounding vascular smooth muscle cells to produce potent vasodilatation [23]. Furthermore, it could also act in a paracrine way preventing the endothelial injury in inhibiting platelet aggregation and preventing leukocyte activation [7]. The inability
of endothelial cells exposed to hypoxia to produce NO could then participate to the microvascular injury and the renal damage [18].

Our study has, however, some shortcomings. First of all, the 2-h measurement period postischemia, as applied here, might be considered too short for assessment of the actual impact of I/R injury on the kidney. However, the purpose of the study was first to focus on renal hemodynamics and oxygenation changes in the early stage of I/R. Furthermore, our results clearly demonstrate that initiation of renal dysfunction and (micro)vascular oxygenation changes occur within this time scale. Finally, we are fully aware than complete selectivity toward one NOS isoform cannot be completely achieved using pharmacological compound. Unfortunately, knockout mice were not suitable for the present model because of the size of the probes. However, L-NIL has been found to have ~28-fold higher selectivity for iNOS than constitutive NOS (i.e., eNOS+nNOS) [30]. Furthermore, Rusai et al. [31] have shown that selective inhibition of nNOS has no effect on renal injury, underlining its marginal role in the pathophysiology of renal I/R. Then, we used creatinine clearance to assess glomerular filtration rate. This method can lead to imprecision due to backleak phenomena and slight tubular creatinine secretion. Likewise, using an in vivo model may lead to a misleading interpretation of the Na⁺ reabsorptive work, by underestimating the effective tubular reabsorption and not taking into account the backleak phenomena. Finally, the design of the study does not allow to draw conclusions regarding the respective contribution of oxygen supply, modulation of mitochondrial activity and oxygen shunting on the beneficial effect of selective iNOS inhibition after I/R.

Conclusions

Our results demonstrate renal I/R induces iNOS-dependent early alterations in renal oxygenation, in compromising renal oxygen supply and microvascular oxygenation, whereas eNOS is shown to participate in maintaining oxygen supply and microvascular PO₂. Furthermore, I/R and iNOS activation dysregulate the VO₂ren in increasing the metabolic cost of Na⁺ reabsorption. This study showed that selective iNOS inhibition may partially prevent renal hypoxia in favoring the balance between renal oxygen supply and consumption. The protective effect of selective iNOS inhibitors on renal microcirculation may be a major aspect in the protection against I/R-induced renal dysfunction. Future research should aim at confirming these results in the clinical setting and finding out whether the increase in eNOS activity may result in microcirculatory PO₂ improvement.
References


