

Renal ischemia in the rat stimulates glomerular nitric oxide synthesis

JOSÉ M. VALDIVIELSO,¹ CARLOS CRESPO,² JOSÉ R. ALONSO,²
CARLOS MARTÍNEZ-SALGADO,¹ NELIDA ELENO,¹ MIGUEL ARÉVALO,³
FERNANDO PÉREZ-BARRIOCANAL,¹ AND JOSÉ M. LÓPEZ-NOVOA¹

¹Instituto Reina Sofía de Investigación Nefrológica, Departamento de Fisiología y Farmacología,
²Departamento de Biología Celular y Patología, and ³Departamento de Anatomía Humana
e Histología, Universidad de Salamanca, 37007 Salamanca, Spain

Received 23 March 2000; accepted in final form 17 October 2000

Valdivielso, José M., Carlos Crespo, José R. Alonso, Carlos Martínez-Salgado, Nelida Eleno, Miguel Arévalo, Fernando Pérez-Barriocanal, and José M. López-Novoa. Renal ischemia in the rat stimulates glomerular nitric oxide synthesis. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R771–R779, 2001.—Renal ischemia in humans and in experimental animals is associated with a complex and possibly interrelated series of events. In this study, we have investigated the glomerular nitric oxide (NO) production after renal ischemia. Unilateral or bilateral renal ischemia was induced in Wistar rats by clamping one or both renal arteries. NO production was assessed by measuring glomerular production of nitrite, a stable end product of NO catabolism, and NO-dependent glomerular cGMP production and by assessing the glomerular NADPH diaphorase (ND) activity, an enzymatic activity that colocalizes with NO-synthesis activity. Furthermore, we determined the isoform of NO synthase (NOS) implicated in NO synthesis by Western blot and immunohistochemistry. Glomeruli from rats with bilateral ischemia showed elevated glomerular nitrite and cGMP production. Besides, glomeruli from this group of rats showed an increased ND activity, whereas glomeruli from the ischemic and nonischemic rats with unilateral ischemia did not show this increase in nitrite, cGMP, and ND activity. In addition, glomeruli from ischemic kidneys showed an increased expression of endothelial NOS without changes in the inducible isoform. Addition of L-NAME in the drinking water induced a higher increase in the severity of the functional and structural damage in rats with bilateral ischemia than in rats with unilateral ischemia and in sham-operated animals. We can conclude that after renal ischemia, there is an increased glomerular NO synthesis subsequent to an activation of endothelial NOS that plays a protective role in the renal damage induced by ischemia and reperfusion.

nitric oxide synthase

RENAL ISCHEMIA IS RESPONSIBLE for acute tubular necrosis observed during kidney transplantation. Kidney is also susceptible to suffer ischemia in other situations such as aortic abdominal surgery or severe hypotension. Renal ischemia in humans and in experimental ani-

mals is associated with a complex and possibly interrelated series of events involving tubular obstruction, passive backflow of filtrate and preglomerular vasoconstriction (9), and a fall in glomerular filtration rate (GFR) and renal blood flow (RBF) (12, 23).

Nitric oxide (NO) is an endogenous vasodilator with an important role in the pathophysiology of many renal diseases (11). It has been suggested that the vasoconstriction observed in the postischemic acute renal failure (ARF) phase is associated with a loss in the ability of endothelium to synthesize vasodilators, mainly NO. These conclusions come from the lack of response to NO-dependent vasodilators as a result of ischemic endothelial injury (6, 13). Recently, several papers have suggested that NO is involved in the maintenance of RBF, urinary flow, and GFR in rats with ARF (5, 8, 25). Noiri et al. (18) reported an increased inducible NOS (iNOS)-derived NO production in ischemic kidneys and that inhibition of iNOS expression decreased the tubular damage. In addition, we have reported that the administration of an NO donor ameliorated the renal damage induced by ischemia and reperfusion (10). Now it is well documented both in experimental and clinical studies that the NO-mediated modulation of vascular tone represents a local but highly effective and sensitive system for the control of organ blood flow, playing a major role in the intraglomerular dynamics regulation (27). Marked alterations in glomerular hemodynamics occur after ischemic ARF, but to date, most attention has been devoted to the study of total kidney NO production and tubular iNOS expression (13). The aim of this study was to assess the effect of renal ischemia on the glomerular synthesis of NO. This purpose has been afforded by measuring glomerular production of nitrite, a stable end product of NO catabolism, by measuring NO-dependent glomerular cGMP production, and by assessing the glomerular NADPH-diaphorase (ND) activity, an enzymatic activity that colocalizes with NO-synthesis activity. Furthermore, we aimed to determine the isoform of NOS implicated

Address for reprint requests and other correspondence: J. M. López-Novoa, Departamento de Fisiología y Farmacología, Edificio Departamental, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain (E-mail: jmlnovoa@gugu.usal.es).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

in NO synthesis by Western blot and immunohistochemistry.

In addition, most studies on NO production in renal ischemia have been performed in the model of unilateral ischemia (UI) and contralateral nephrectomy, a model in which both ischemia and uremia are associated. Thus we studied a model with bilateral ischemia (BI) and uremia and another model in which only a kidney is subjected to ischemia. In this latter model, the other kidney is able to excrete most of the substances the ischemic kidney cannot excrete. In addition, this model also allows one to study two kidneys, one ischemic and the other nonischemic, subjected to a similar environmental milieu.

MATERIALS AND METHODS

Animals. The experiments have been carried out in female Wistar (250 g) rats (IFFA-Credo, Barcelona, Spain). Animals were fed standard rat chow (Panlab, Madrid, Spain) and water ad libitum. A timer permitted light between 0800 and 2000. Temperature was controlled to $20 \pm 1^\circ\text{C}$. Animals were used in experimental protocols according to the regulations of the Conseil de l'Europe and the Spanish government.

Surgical technique. Rats were anesthetized with ketamine hydrochloride (4 mg/kg im) and diazepam (3 mg/kg ip). Ischemia was induced by clamping one (UI) or both (BI) renal arteries for 1 h. Afterwards, 24 h of reperfusion were allowed in all groups. Experimental groups were as follows: sham-operated rats (Sham), Sham receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of nitro-L-arginine methyl ester (L-NAME; Sigma Aldrich Química, Madrid, Spain) in drinking water the day before and the day after the surgical procedure (Sham-NAME), UI rats [in these rats, the left kidney is ischemic (UI), whereas the right kidney is nonischemic (UNI)], UI rats receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of L-NAME in drinking water the day before and the day after the surgical procedure (UI-NAME), BI rats (in these rats, both kidneys are subjected to ischemia), and BI rats receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of L-NAME in drinking water the day before and the day after the surgical procedure (BI-NAME). In the groups of rats drinking L-NAME, basal values were obtained 24 h before starting the treatment (48 h before renal ischemia).

Renal-function studies. Rats were weighed and placed in metabolic cages with free access to food and water. The day before starting the treatment and the day after inducing the ischemia, urine was collected into graduated cylinders containing 100 μl of 0.1% sodium azide (to minimize the bacterial contamination) and 1 ml of mineral oil (to avoid evaporation). A blood sample (150 μl) was also collected from the caudal vein. Creatinine in plasma and urine was determined by a modification of Jaffé reaction (3).

Morphological studies. Animals were anesthetized with ketamine hydrochloride (4 mg/kg im) and diazepam (3 mg/kg ip), and kidneys were removed after tying the renal pedicle and then cut by a sagittal section in two halves, which were then fixed by immersion in buffered 4% formaldehyde for 1 day. After the pieces were dehydrated, they were embedded in paraffin, cut in 4- μm sections, mounted on glass slides, and counterstained with periodic acid-Schiff staining for light-microscopy analysis. Morphological changes were analyzed blindly by a histologist (M. Arévalo).

Glomerular isolation and incubation. Animals were anesthetized with ketamine hydrochloride (4 mg/kg im) and diazepam (3 mg/kg ip), and the kidneys were perfused in situ with ice-cold isotonic saline through the abdominal aorta. Glomer-

uli were obtained by mechanical sieving as previously described (19). In rats with UI, glomeruli were obtained from the ischemic and nonischemic kidney separately. After the isolation, the final preparation consisted of glomeruli without Bowman's capsule and without afferent or efferent arterioles. Tubular contamination was always $<5\%$. Glomeruli isolation and all subsequent procedures required specific cautions to minimize contamination by bacteria or lipopolysaccharide (LPS). Such measures included using pyrogen-free disposable and endotoxin-free RPMI 1640 culture medium.

Glomeruli were plated out in 4×6 well plates and incubated as previously described (20) for 24 h at 37°C in sterile conditions. It was assayed the "in vitro" effect of two NO synthesis inhibitors, L-NAME and aminoguanidine (Sigma Aldrich; both at final concentration of 10^{-4} M in the wells).

NO_2^- assay. Nitrite concentration was determined in the supernatant of glomerular incubation by a modification of the Griess reaction, as previously described (20). Briefly, 500 μl of sample were mixed with 250 μl of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% orthophosphoric acid; Sigma Aldrich) and incubated for 15 min at room temperature. Absorbance was measured at 560 nm. Standard NO_2^- calibration was done by using sodium nitrite.

Glomerular cGMP production. Isolated glomeruli were suspended in ice-cold Tris-glucose buffer containing 2.5 mM CaCl_2 . The incubation was performed in the presence of 10 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich). The glomeruli suspension was placed in a shaking water bath at 37°C . In some tubes, 10^{-4} M L-NAME (final concentration) was added. The incubation stopped after 15 min by adding 2 ml of ice-cold buffer and centrifuging for 30 s in a microfuge at 1,000 g. Supernatants were aspirated and replaced by 1 ml of absolute ethanol. The ethanol extraction of intracellular cGMP was performed twice. Ethanol extracts from each sample were pooled and evaporated in a stream of nitrogen. Dried samples were resuspended, and cGMP was assayed with a commercial kit (DuPont NEN Research products, Bad Homburg, Germany). The recovery of cGMP during the extraction procedure was determined by adding [^3H]cGMP (2,000–3,000 counts/min) to the sample. The percentage of recovery was always $>89\%$. In preliminary experiments, we observed that the inter- and intra-assay coefficients of variation were 11.9% and 8.4%, respectively.

ND histochemistry + iNOS immunohistochemistry. Animals were deeply anesthetized with ketamine hydrochloride (50 mg/kg ip) and perfused through the ascending aorta with 100 ml of isotonic saline solution (0.9% NaCl in water) followed by a fixative solution made of 4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, kidneys were postfixed for 4 h in the same fixative solution. Tissue was cryoprotected with 30% sucrose. After cryoprotection, 30- μm sections were cut on a Leyca cryostat, collected in gelatin-coated slides, and washed carefully in PB and in 0.1 M Tris·HCl buffer, pH 8.0.

After sections were washed, they were processed for ND histochemistry as described elsewhere (1, 2). Briefly, sections were incubated for 60–90 min at 37°C in an incubation solution made up of 1 mM reduced β -NADPH (Sigma Aldrich), 0.3 mM nitro blue tetrazolium (Sigma Aldrich), and 0.08% Triton X-100 in 0.1 M Tris·HCl buffer, pH 8.0. The course of the reaction was controlled under the microscope. When the histochemical reaction was concluded, sections were washed in 0.1 M Tris·HCl buffer, pH 8.0. The following controls of the histochemical reaction specificity were carried out: 1) incubation without the substrate β -NADPH, 2) incubation without the chromogen nitro blue tetrazolium,

3) denaturation of the enzyme activity by heating the tissue at 84°C for 5 min, 4) overfixation of tissue (2 wk in 10% formalin), and 5) substitution in the incubation medium of the substrate NADPH by NADP (Sigma Aldrich). In all cases, no residual reactivity was observed. When the histochemical reaction was concluded, sections were dehydrated in a graded ethanol series, cleared with xylene, and placed on coverslips with Entellan (Merck, Darmstadt, Germany).

In the immunohistochemistry for iNOS, sections were incubated with primary antibody (anti-mac-NOS monoclonal antibody, Affinity, Nottingham, UK) diluted 1:4,000 in PB (48 h at 4°C). The sections were washed in PB and incubated with biotinylated anti-mouse IgG (Vectastain ABC kit, Vector Laboratories) diluted 1:250 for 3 h at 20°C and then in Vectastain ABC reagent diluted 1:250 for 2 h. Tissue-bound peroxidase was visualized using 0.05% 3,3'-diaminobenzidine and 0.003% hydrogen peroxide in 0.1 M Tris·HCl buffer (pH 7.6) for 5–10 min under visual control.

For the double labeling, sections previously processed for iNOS immunohistochemistry were stained for the detection of ND activity following the same procedure described above. After the process of single or double labeling, sections were mounted on gelatin-coated slides, dehydrated in graded ethanol series, and mounted with Entellan. Specific controls for the ND histochemistry were carried out as described previously (1, 2). The specificity of the immunostaining was controlled by omitting the specific antibody in the first incubation step. Additionally, interference by endogenous peroxidase was ruled out by staining some sections with chromogen and hydrogen peroxide. No residual immunoreaction was found. As a positive control, we used glomeruli from rats pretreated with a single dose of LPS (20 mg/kg ip) 7 h before the experiment.

Western blot. To obtain a protein-enriched fraction from isolated glomeruli, homogenization procedure was omitted and substituted by an incubation of 15 min at 4°C in 20 mM Tris, pH 8.0, containing 140 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, and 1–2 mM phenylmethylsulfonyl fluoride. Methods to obtain the glomerular protein and to perform Western blots have been previously described (24). The primary antibodies used were a polyclonal antibody anti-macrophage-type iNOS (dilution 1:1,000) and monoclonal anti-endothelial-type constitutive NOS (NOS III; dilution 1:2,500; both from Transduction Laboratories, Lexington, KY). Secondary antibodies used were goat anti-rabbit-horse-radish peroxidase (HRP) (dilution 1:10,000) and goat anti-mouse-HRP (dilution 1:30,000; both from Bio-Rad Laboratories, Madrid, Spain). A lysate of rat alveolar macrophages incubated with phorbol 12-myristate 13-acetate (1 μM) + LPS (10 μg/ml) was processed parallel with glomerular lysate and used as control for a positive reaction with iNOS in Western blots. Expression of iNOS in glomeruli and cortex of animals treated with LPS (single doses of 20 mg/kg, 7 h before the experiment) was used as a positive control. The radiograph was digitalized (scanner model UM6552 Trust) with the program Adobe Photoshop 3.0 in a Power Macintosh G3 Computer. Then, optical density of the bands were measured with the program MacBAS V2.2.

Statistical analysis. Results are presented as means ± SE. Statistical analysis of the data was carried out with one-way analysis of variance for repeated measurements.

RESULTS

Renal function. Data on renal function are shown in Table 1. Basal period corresponds to the values obtained before starting the treatment or the surgical

Table 1. Creatinine clearance and plasma creatinine in rats with unilateral or bilateral renal ischemia

	Basal	Experimental
<i>Creatinine clearance, ml·min⁻¹·kg body wt⁻¹</i>		
Sham (n = 8)	0.610 ± 0.066	0.579 ± 0.024
Sham-NAME (n = 10)	0.656 ± 0.056	0.459 ± 0.027*
BI (n = 8)	0.722 ± 0.045	0.144 ± 0.034*
BI-NAME (n = 8)	0.695 ± 0.030	0.032 ± 0.007*†
UI (n = 16)	0.687 ± 0.032	0.341 ± 0.049*
UI-NAME (n = 8)	0.668 ± 0.077	0.283 ± 0.046*
<i>Plasma creatinine, mg/l</i>		
Sham (n = 8)	8.092 ± 0.371	7.005 ± 0.708
Sham-NAME (n = 10)	9.222 ± 0.401	10.694 ± 0.808
BI (n = 8)	8.600 ± 0.310	28.597 ± 5.188*
BI-NAME (n = 8)	6.963 ± 1.221	35.325 ± 3.314*
UI (n = 16)	8.347 ± 0.387	13.321 ± 0.624*
UI-NAME (n = 8)	7.428 ± 0.586	15.744 ± 1.933*

Data are means ± SE. Statistical significance: **P* < 0.01 vs. basal; †*P* < 0.01 vs. bilateral ischemic (BI; paired Student's *t*-test). Groups are Sham, rats sham operated; BI, rats with bilateral ischemia; UI, rats with unilateral ischemia; NAME, groups of rats treated with nitro-L-arginine methyl ester in drinking water.

procedure. The experimental period corresponds to the values obtained 24 h after inducing ischemia. Renal ischemia induced a significant decrease in creatinine clearance both in BI (80%) and UI (50%) groups. This decrease was higher in the BI group. Sham rats did not show changes in creatinine clearance. Treatment with L-NAME in the drinking water induced a 30% decrease in creatinine clearance in Sham rats, a higher decrease (58%) in UI rats, and a much higher percentage (95%) in BI rats (Table 1).

The changes in plasma creatinine were compatible with those of creatinine clearance (Table 1). After unilateral or bilateral renal ischemia, a significant increase in plasma creatinine levels was observed, being significantly higher in the BI group (333%) vs. the UI group (160%). Treatment with L-NAME increased plasma creatinine levels in ischemic groups but did not modify them in the Sham group. The effect of L-NAME, increasing plasma creatinine levels, was much higher in BI (507%) than in the UI (212%) group.

Morphological damage. Light-microscopy examination revealed several degrees of tubular alterations in the kidneys from the BI group and in the ischemic kidney from the UI group. Most of the tubular structures were partially damaged, but some of them showed cellular necrosis and total atrophy. Moreover, tubular lumens were frequently filled with hyaline casts or heterogeneous cellular debris (Fig. 1B). Treatment with L-NAME increased the tubular damage, being higher in the BI (Fig. 1D) than in the UI (Fig. 1C) group. Almost all the tubules showed structural alterations with the lumen collapsed, loss of the cellular border, or even total cellular atrophy. Hyaline casts could also be observed in the tubular lumen. Glomeruli capillaries appeared collapsed and with the lumen obstructed. Kidneys from Sham rats and nonischemic kidneys from the UI group did not show any morpho-

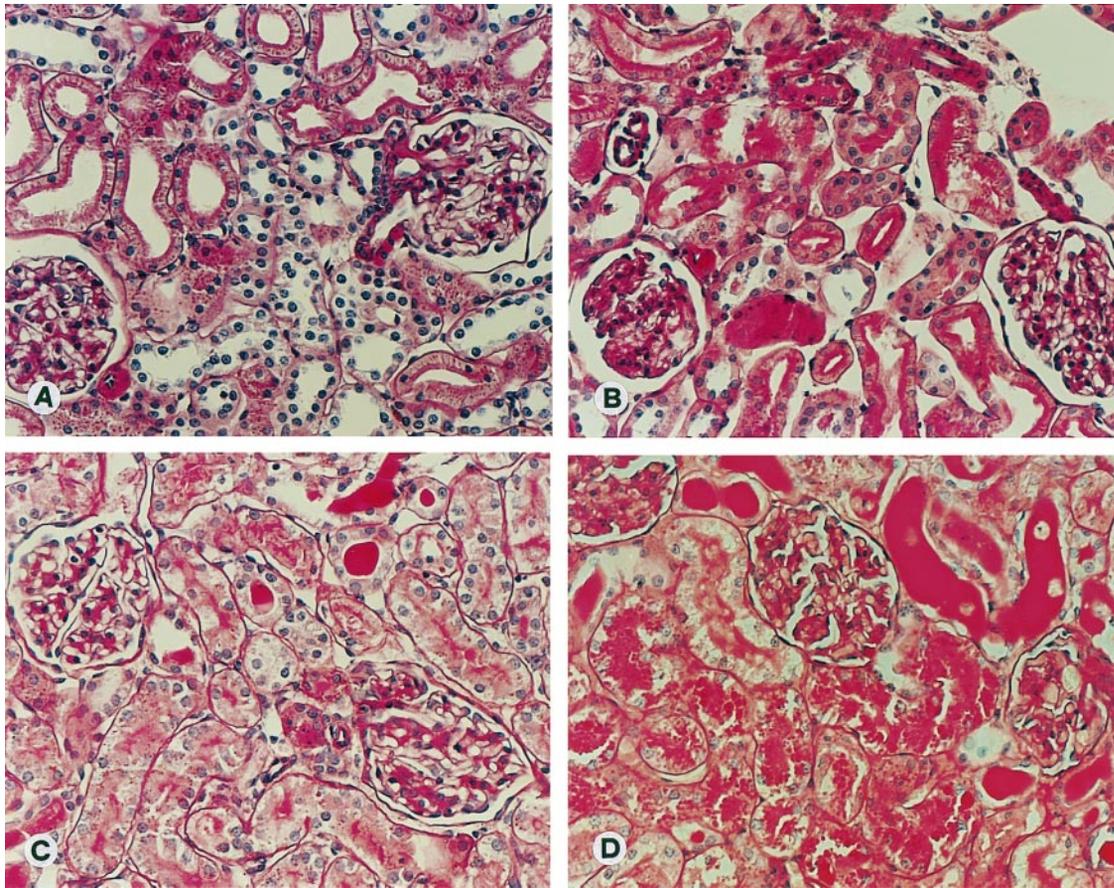


Fig. 1. Representative light micrographs of renal cortex from the Sham group (A), rats with bilateral ischemia (B), ischemic kidney from rats with unilateral ischemia and nitro-L-arginine methyl ester (L-NAME) in the drinking water (C), and rats with bilateral ischemia and L-NAME in the drinking water (D). Periodic acid-Schiff staining ($\times 275$).

logical alteration. Treatment with L-NAME did not modify the normal morphological structures in these groups (Fig. 1A).

Electron-microscopy analysis confirmed these results, as is shown in Fig. 2. Control group had normal renal structure (Fig. 2B). Rats in the BI group (Fig. 2D) showed cellular damage mainly in proximal tubules seen as partial loss of brush-border microvilli, increased cytoplasm vacuolization and lysosomes, and widened spaces in the basolateral interdigitations. Despite these alterations, most of the tubular cells were viable. However, in some tubules, total cell necrosis was also seen (data not shown). This damage is markedly increased in rats with BI treated with L-NAME (Fig. 2F) in this group; most of the proximal tubules were necrotic as shown in the figure. Glomeruli do not seem to have any ultrastructural alterations (Fig. 2, A, C, and E).

Glomerular nitrite production. Data on glomerular nitrite production from ischemic rats are shown in Fig. 3. Glomeruli obtained from rats with BI produced significantly higher amounts of nitrite than glomeruli from Sham rats (Sham: 760 ± 39 ; BI: $1,403 \pm 93$ pmol/1,000 glomeruli, $P < 0.01$). Nitrite production by glomeruli from both the ischemic and nonischemic kid-

neys from rats with UI was not significantly different from glomeruli of Sham rats (Sham: 760 ± 39 ; UI: 902 ± 47 ; UNI: 477 ± 34 pmol/1,000 glomeruli). However, glomeruli from the ischemic kidney produced significantly higher amounts of nitrite than glomeruli from the nonischemic kidney ($P < 0.01$). Incubation with 10^{-4} M L-NAME decreased the glomerular nitrite production in BI values similar to those from the Sham group (Sham: 760 ± 39 ; BI + L-NAME: 856 ± 119 pmol/1,000 glomeruli). Neither L-NAME nor aminoguanidine had a significant effect when added to glomeruli from ischemic and nonischemic kidneys from rats with UI (Sham: 760 ± 39 ; UI + L-NAME: 782 ± 32 ; UNI + L-NAME: 594 ± 17).

Glomerular cGMP production. Data on glomerular cGMP production are shown in Fig. 4. Glomeruli both from rats with bilateral and UI showed increased levels of cGMP production with respect to glomeruli from the Sham group (Sham: 0.94 ± 0.08 ; BI: 3.17 ± 0.05 , $P < 0.01$ vs. Sham; UI: 1.79 ± 0.18 , $P < 0.01$ vs. Sham; UNI: 0.96 ± 0.09 fmol/1,000 glomeruli). This increase was partially reversed by incubating the glomeruli with L-NAME (10^{-4} M; BI: 3.17 ± 0.05 ; BI + NAME: 2.08 ± 0.17 fmol/1,000 glomeruli, $P < 0.01$).

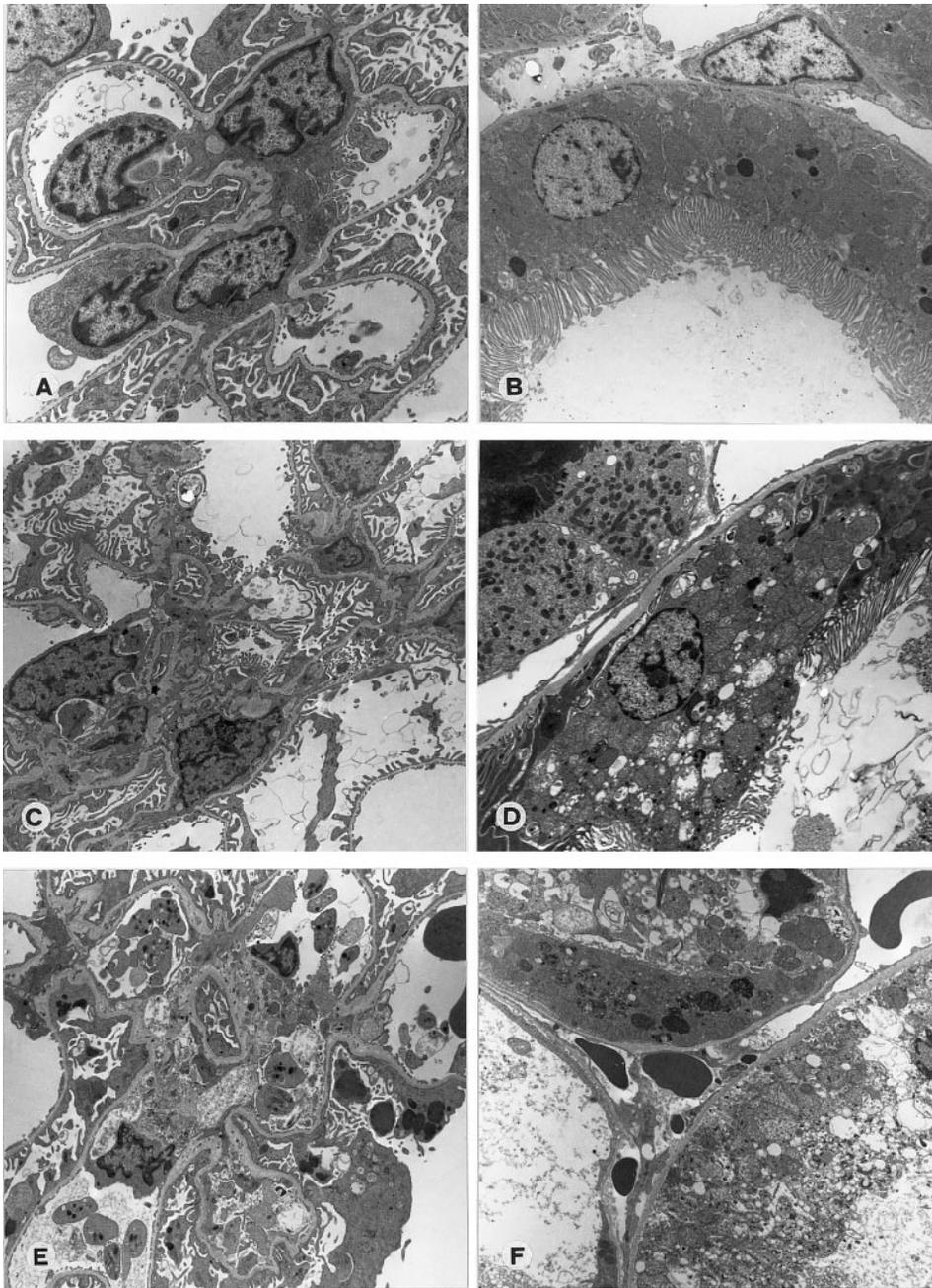


Fig. 2. Representative electronic micrographs. *A*: glomerulus from Sham group ($\times 3,900$). *B*: tubuli from Sham group ($\times 3,360$). *C*: glomerulus from a rat with bilateral ischemia ($\times 3,360$). *D*: tubuli from a rat with bilateral ischemia ($\times 3,360$). *E*: glomerulus from a rat with bilateral ischemia and L-NAME in the drinking water ($\times 3,360$). *F*: tubuli from a rat with bilateral ischemia and L-NAME in the drinking water ($\times 3,360$).

ND histochemistry. Figure 5 shows representative images of ND staining in the groups studied. A significantly higher number of cells with positive ND activity was observed in glomeruli from BI rats (Fig. 5*B*) compared with the Sham rats (Fig. 5*A*).

Western blot analysis. Western blot results are shown in Fig. 6. As can be seen in the figure, one slight band corresponding to NOS III appears in glomeruli from Sham animals. In glomeruli from BI and UI rats, a clear band in the weight of NOS III is shown (Fig. 6*A*) corresponding to an increase of 39% in the BI group and 27% in the UI group. No band was observed in the UNI group. Also, no bands were observed in Western blot performed with iNOS antibody in any experimental group (Fig. 6*B*). However, the rats treated with LPS

show a clear band, demonstrating that it is possible to detect glomerular iNOS.

Double labeling. Results of double labeling are shown in Fig. 7. No immunoreactivity for iNOS was detected inside the glomeruli in either Sham rats (Fig. 7*A*) or in BI rats (Fig. 7*B*), although in these animals, ND activity was present in the kidney.

DISCUSSION

The first purpose of this study was to assess the effect of ischemia on glomerular NO production. Glomeruli from rats with BI showed a higher basal nitrite production than glomeruli from control animals. In the conditions of the incubation medium, NO was trans-

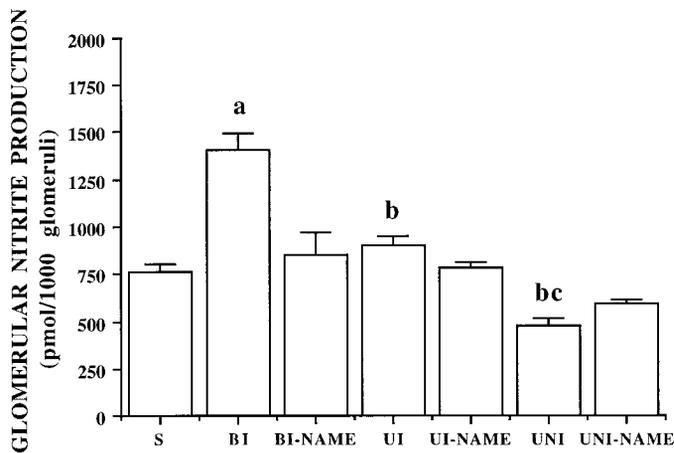


Fig. 3. Glomerular nitrite production. Groups are: S, glomeruli from Sham rats; BI, glomeruli from bilateral-ischemic rats; BI-NAME, glomeruli from bilateral-ischemic rats receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of L-NAME in drinking water; UI, glomeruli from the ischemic kidney of unilateral ischemia rats; UI-NAME, glomeruli from UI rats receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of L-NAME in drinking water; UNI, glomeruli from the nonischemic kidney of UI rats; UNI-NAME, glomeruli from the nonischemic kidney of UI rats receiving $1 \text{ mg} \cdot \text{day}^{-1} \cdot \text{rat}^{-1}$ of L-NAME in drinking water. Data are means \pm SE of 10 experiments. Statistical significance: ^a $P < 0.01$ vs. S group; ^b $P < 0.01$ vs. BI group; ^c $P < 0.01$ vs. UI group.

formed to nitrite and then to nitrate, with a constant ratio between the concentrations of nitrite and nitrate. Thus, in the absence of bacterial contamination, nitrite production is an indirect assessment of NO production (16). Furthermore, the addition of any of two inhibitors of NO synthesis reduced the glomerular nitrite production from rats with BI to similar values to the ones of Sham rats, confirming that this increased nitrite production reflected increased glomerular NO synthesis. These results also agree with those of Rivas-Cabañero and colleagues (20, 21), who showed an increased glomerular NO production in rats with gentamicin-induced ARF, and with those of Conger et al. (7), which showed an increased NO production in rats with norepinephrine-induced ARF.

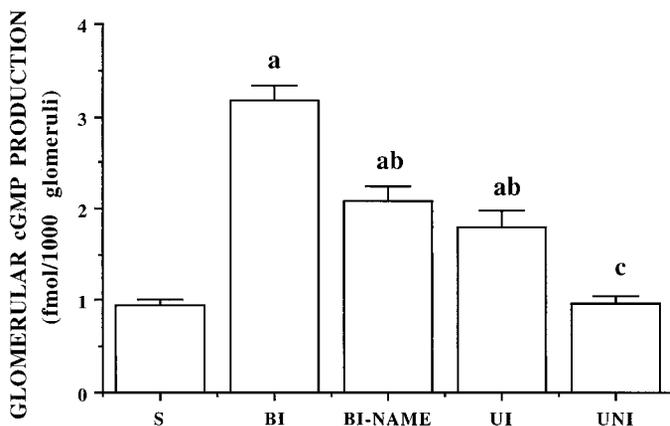


Fig. 4. Glomerular cGMP production. Data are means \pm SE of 10 experiments. Statistical significance: ^a $P < 0.01$ vs. S group; ^b $P < 0.05$ vs. BI group; ^c $P < 0.01$ vs. BI group.

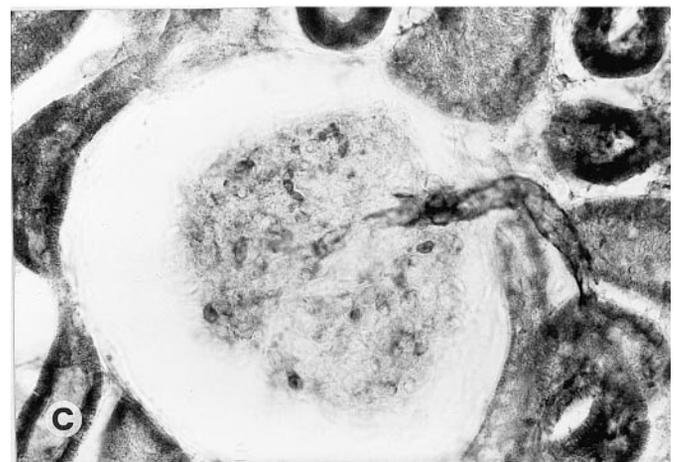
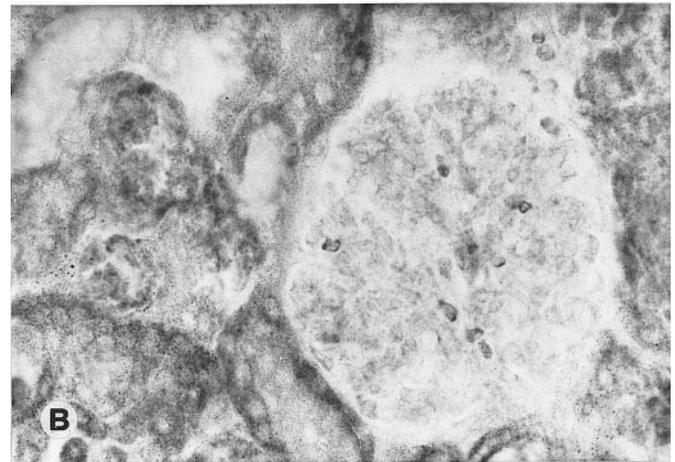
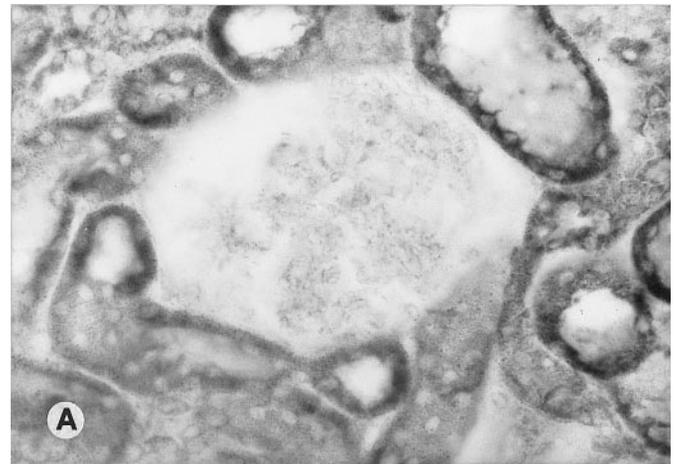


Fig. 5. Representative images from NADPH-diaphorase histochemistry in a glomeruli from Sham group (A), rat with bilateral ischemia (B), positive control: rat treated with lipopolysaccharide (C; 20 mg/kg ip).

A further assessment of glomerular NO synthesis in rats with renal ischemia was done by evaluating the ND activity. A very wide colocalization between ND and NOS was reported in neural and nonneural tissue; thus ND is considered as a histochemical marker for NOS (22). ND histochemistry showed many cells with positive ND activity in glomeruli from rats with BI,

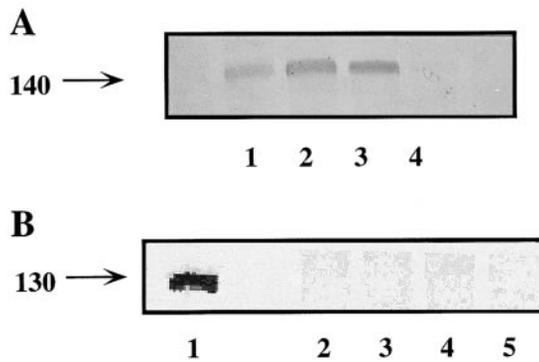


Fig. 6. A: Western blot for constitutive nitric oxide synthase (NOS III). Lane 1: glomeruli from Sham rats. Lane 2: glomeruli from rats with BI. Lane 3: glomeruli from the ischemic kidney of rats with UI. Lane 4: glomeruli from the nonischemic kidney of rats with UI. B: Western blot for inducible NOS (iNOS). Lane 1: glomeruli from a rat treated with lipopolysaccharide (20 mg/kg ip). Lane 2: glomeruli from Sham rats. Lane 3: glomeruli from rats with BI. Lane 4: glomeruli from the ischemic kidney of rats with UI. Lane 5: glomeruli from the nonischemic kidney of rats with UI.

whereas only a few ND positive cells were observed in glomeruli from control rats. These results provide further evidence that BI treatment induces an increase in NOS activity.

A third way for assessing the increase in NO production was to measure a biological response to NO, as is cGMP production. This approach showed results in agreement with those obtained with nitrite production and ND activity: glomeruli from rats with BI showed a higher cGMP production than glomeruli from Sham rats.

These results, obtained from different technical procedures, demonstrate that BI induces an increase in glomerular NO synthesis and release. However, glomeruli from ischemic kidney of UI rats, and thus without uremia, did not show an increase in nitrite production compared with glomeruli from Sham rats. In addition, the NO synthesis inhibitor L-NAME had no significant effect in glomerular nitrite production in glomeruli from ischemic kidneys of UI rats. These results suggest that ischemia alone (without uremia) is not able to increase glomerular NO synthesis.

The next purpose of this study was to assess what isoform of NOS is involved in this increased NO synthesis. The absence of a band in the range for iNOS molecular weight and the increase of intensity of a band in the molecular weight of NOS III suggest that ischemia stimulates NOS III but not iNOS expression. This lack of iNOS induction is also shown in the results of double labeling by ND and iNOS immunohistochemistry. The possible lack of affinity of the antibody was discarded by using glomeruli from rats treated with LPS, in which a clear band could be observed. Thus we can deduce that ischemia alone is able to increase NOS III levels but not NO production or ND activities. These data are in agreement with the recent report of Kakoki et al. (13) demonstrating that during renal ischemia, total renal calcium-dependent NOS activity was decreased, but the expression of NOS III was

increased. This low-NOS III activity was restored by treatment with tetrahydrobiopterin, a cofactor that stabilizes NOS III in its dimeric form, which is an active form (26). Our results are in apparent contradiction with those of Noiri et al. (18) that reported that iNOS-derived NO increased in ischemic kidney and in isolated tubules from ischemic kidneys. However, it should be noted that our study has been performed in isolated glomeruli; presumably iNOS regulation is different in epithelial tubular cells and in glomerular cells.

Although published results suggest that NO plays a pivotal role in ischemic renal failure, most of the studies show only indirect evidences obtained after stimulation or inhibition of NO synthesis (4–6). Our study performed direct measurements of NO production (nitrites and cGMP production), NOS activity (ND), and NOS isoforms protein expression by Western blot. All these results demonstrate that in bilateral renal ischemia, there is an increased glomerular synthesis of NO that is based on an increased expression of NOS III.

The last aim of the study was to assess the role of the increased glomerular NO synthesis in renal ischemia. This was tested by assessing the effect of NO synthesis inhibition with L-NAME on kidney structure and func-

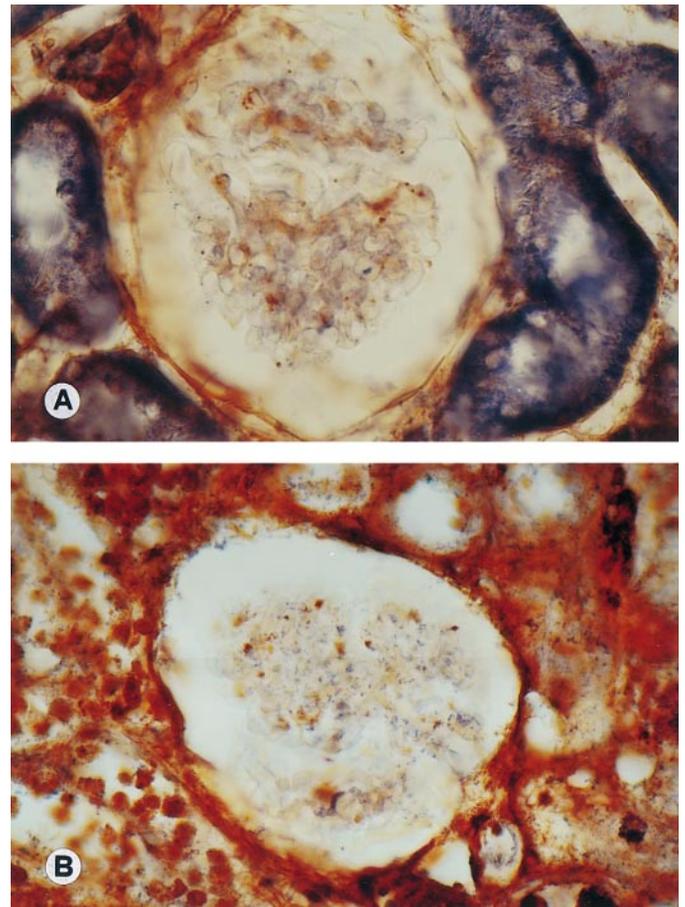


Fig. 7. Representative image from double-labeling studies (NADPH-diaphorase hystochemistry + iNOS immunohistochemistry). A: Sham group. B: rats with BI.

tion after renal ischemia. Our results show that the effect of L-NAME in creatinine clearance is greater in rats with BI than in all the other groups. In addition, the morphological damage observed in ischemic kidneys is aggravated by addition of L-NAME in drinking water, but the impairment was more marked in the kidneys of rats with BI than in those from rats with UI. All these data suggest a protective role for NO in the bilateral renal ischemia. Something similar has been reported for gentamicin-induced renal failure (25). A further proof of the beneficial role of NO in ischemic renal failure is the fact that the administration of exogenous NO donors had been shown to be unequivocally beneficial against ischemia-induced renal failure and tubular necrosis (4, 10).

In glomeruli from nonischemic kidneys of UI rats, there are normal nitrite and cGMP productions but no expression of NOS III or iNOS proteins. The source of basal nitrite production is probably an NOS-independent reaction because it is not inhibited by L-NAME. In our hands, there is always a basal NOS-independent nitrite production (24) that can be explained by other enzymatic activities, such as arginase. Recently, an alternative nonenzymatic pathway for the generation of NO has been described through the reaction of hydrogen peroxide with arginine (17). This pathway would be important in cases of ischemia and reperfusion.

Another possible cause of the increase in the NO production after renal ischemia may be the changes in renal perfusion pressure. However, we have shown in previous studies that after renal ischemia and reperfusion, there are no significant changes in blood pressure (14, 15).

It can be concluded that rats with BI, but not with UI, show an increased NO production. This increased NO synthesis is associated with an increase in NOS III levels. Inhibition of NO synthesis aggravates the morphological damage and the decrease in GFR induced by ischemia. Taken together, these results suggest a role for increased renal NO synthesis in protecting the kidney from the damage induced by ischemia and counteracting the vasoconstriction that characterizes renal ischemia.

Perspectives

Increased glomerular NO synthesis after renal ischemia seems to be a protective mechanism that counteracts vasoconstrictor and inflammatory phenomena occurring during the reperfusion period. These phenomena play a major role to impair the recovery of renal function after ischemia or after renal transplant. Increased NO release occurs when renal ischemia and uremia occur simultaneously. Thus the precise knowledge of the mechanisms of increased NO synthesis and the role of NO on these responses could allow for pharmacological modulation of this phenomenon and thus to try to reinforce the protective effects of NO on renal function.

REFERENCES

1. **Alonso JR, Arévalo R, García-Ojeda EE, Porteros A, Briñón JG, and Aijón J.** NADPH-diaphorase active and calbindin D-28k-immunoreactive neurons and fibers in the olfactory bulb of the hedgehog (*Erinaceus europaeus*). *J Comp Neurol* 351: 307–327, 1995.
2. **Alonso JR, Arévalo R, Porteros A, Briñón JG, Lara J, and Aijón J.** Calbindin D-28k and NADPH coexistence in the magnocellular neurosecretory nuclei. *Neuroreport* 3: 249–252, 1992.
3. **Bonsnes RW and Tausski HA.** The colorimetric determination of creatinine of the Jaffe reaction. *J Biol Chem* 158: 581–584, 1945.
4. **Caramelo C, Espinosa G, Manzarbeitia F, Cernadas MR, Pérez-Tejerizo G, Tan D, Mosquera JR, Digiuni E, Monton M, Mills I, Hernando L, Casado S, and López-Farre A.** Role of endothelium-related mechanisms in the pathophysiology of renal ischemia/reperfusion in normal rabbits. *Circ Res* 79: 1031–1038, 1996.
5. **Chintala MS, Chiu PJS, Vemulapalli S, Watkins RW, and Sybertz EJ.** Inhibition of endothelial derived relaxing factor aggravates ischemic acute renal failure in anesthetized rats. *Naunyn Schmiedebergs Arch Pharmacol* 348: 305–310, 1993.
6. **Conger JD, Robinette JB, and Schrier RW.** Smooth muscle calcium and endothelium derived relaxing factor in the abnormal vascular response of acute renal failure. *J Clin Invest* 82: 532–537, 1998.
7. **Conger JD, Robinette JB, Villar A, Raj L, and Shultz P.** Increased nitric oxide activity despite lack of response to endothelium-dependent vasodilators in postischemic acute renal failure in rats. *J Clin Invest* 96: 631–638, 1995.
8. **Cristol JP, Thiemermann C, Mitchell JA, Walder C, and Vane JR.** Support of renal blood flow after ischemic-reperfusion injury by endogenous formation of nitric oxide and of cyclooxygenase vasodilator metabolites. *Br J Pharmacol* 109: 188–194, 1993.
9. **Finn WF.** Enhanced recovery from postischemic acute renal failure. Micropuncture studies in the rat. *Circ Res* 46: 440–448, 1980.
10. **García-Criado FJ, Eleno N, Santos-Benito F, Valdunciel JJ, Reverte M, Lozano-Sánchez FS, Ludeña MD, Gómez-Alonso A, and López-Novoa JM.** Protective effect of exogenous nitric oxide on the renal function and inflammatory response in a model of ischemia-reperfusion. *Transplantation* 66: 982–990, 1998.
11. **Hill-Kapturczak N, Kapturczak MH, Malinski T, and Groos P.** Nitric oxide and nitric oxide synthase in the kidney: potential roles in normal renal function and in renal dysfunction. *Endothelium* 3: 253–299, 1995.
12. **Honda N and Hishida A.** Pathophysiology of experimental nonoliguric acute renal failure. *Kidney Int* 43: 513–521, 1993.
13. **Kakoki M, Hirata Y, Hayakawa H, Suzuki E, Nagata D, Tojo A, Nishimatsu H, Nakanishi N, Hattori Y, Kikuchi K, Nagano T, and Omata M.** Effects of tetrahydrobiopterin on endothelial dysfunction in rats with ischemic acute renal failure. *J Am Soc Nephrol* 11: 301–309, 2000.
14. **López-Farré A, Bernabeu F, Gómez-Garre D, Ramón y Cajal S, Braquet P, and López-Novoa JM.** Platelet-activating factor antagonists treatment protects against postischemic acute renal failure in rats. *J Pharmacol Exp Ther* 253: 328–333, 1990.
15. **López-Farré A, Gómez-Garre D, Bernabeu F, and López-Novoa JM.** A role for endothelin in the maintenance of post-ischaemic renal failure in the rat. *J Physiol (Lond)* 444: 513–522, 1991.
16. **Marletta MA, Yoon PS, Iyengar R, Leaf CD, and Wishnok JS.** Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. *Biochem J* 27: 8707–8711, 1988.
17. **Nagase S, Takemura K, Ueda A, Hirayama A, Aoyagi K, Kondoh M, and Kayama A.** A novel nonenzymatic pathway for the generation of nitric oxide by the reaction of hydrogen peroxide and D or L-arginine. *Biochem Biophys Res Commun* 233: 150–153, 1997.
18. **Noiri E, Peresleni T, Miller F, and Goligorsky MS.** In vivo targeting of inducible NO synthase with oligodeoxynucleotides

- protects rat kidney against ischemia. *J Clin Invest* 97: 2377–2383, 1996.
19. **Olivera A and López-Novoa JM.** Effects of adenosine and adenosine analogues on cAMP accumulation in cultured rat mesangial cells and isolated glomeruli. *Br J Pharmacol* 107: 341–346, 1992.
 20. **Rivas-Cabañero L, Montero A, and López-Novoa JM.** Glomerular nitric oxide synthesis in gentamicin-induced renal failure. *Eur J Pharmacol* 270: 119–121, 1994.
 21. **Rivas-Cabañero L, Valdivielso JM, and López-Novoa JM.** Increased glomerular nitric oxide synthesis in ischemic acute renal failure in the rat. *Ren Fail* 17: 479–481, 1995.
 22. **Schmidt HH, Gagne GD, Nakane M, Pollock JS, Miller MF, and Murad F.** Mapping of neural nitric oxide synthase in the rat suggests frequent co-localization with NADPH diaphorase but not with soluble guanylyl cyclase, and novel paraneural functions for nitrinergic signal transduction. *J Histochem Cytochem* 40: 1439–1456, 1992.
 23. **Stein JH, Lifschitz MD, and Barnes LD.** Current concepts on the pathophysiology of acute renal failure. *Am J Physiol Renal Fluid Electrolyte Physiol* 234: F171–F181, 1978.
 24. **Valdivielso JM, Pérez-Barriocanal F, García-Estañ J, and López-Novoa JM.** Role of nitric oxide in the early renal hemodynamic response after unilateral nephrectomy. *Am J Physiol Regulatory Integrative Comp Physiol* 276: R1718–R1723, 1999.
 25. **Valdivielso JM, Rivas Cabañero L, Pérez-Barriocanal F, and López-Novoa JM.** Effect of nitric oxide synthesis modification on renal function in gentamicin-induced nephrotoxicity. *Environ Toxicol Pharmacol* 3: 123–128, 1997.
 26. **Wever RMF, van Dam T, van Rijn HJ, de Groot F, and Rabelink TJ.** Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase. *Biochem Biophys Res Commun* 237: 340–344, 1997.
 27. **Zatz R and de Nucci G.** Effects of acute nitric oxide inhibition on rat glomerular microcirculation. *Am J Physiol Renal Fluid Electrolyte Physiol* 261: F360–F363, 1991.

