Role of nitric oxide in the early renal hemodynamic response after unilateral nephrectomy

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Valdivielso, José M., Fernando Pérez-Barrioncano, Joaquin García-Estan, and José M. López-Novoa. Role of nitric oxide in the early renal hemodynamic response after unilateral nephrectomy. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1718–R1723, 1999.—We evaluated the involvement of nitric oxide (NO) in the early hemodynamic response to uninephrectomy (UNX) in rats. Animals were uninephrectomized, and 48 h after removal of the kidney, the effect of infusing Nω-nitro-L-arginine methyl ester (L-NAME) on renal function was studied. Glomeruli were isolated, and glomerular nitrite and cGMP productions were measured. In addition, endothelial constitutive NO synthase (NOS III) and inducible NO synthase (iNOS) were assessed by Western blot and by measuring the conversion of arginine to citrulline. UNX animals showed an increase in renal plasma flow that was inhibited by L-NAME in a higher proportion than in sham-operated (SO) animals. No differences were observed in systemic NO-dependent vascular tone, since mean arterial pressures showed similar increments in SO and UNX rats. Glomeruli from UNX animals showed an increase in glomerular nitrite production that was blunted by L-NAME addition. Also, cGMP levels were increased in glomeruli from UNX animals, and this increase was inhibited by L-NAME. Western blot analysis showed no differences in NOS III but a higher iNOS amount in glomeruli from UNX than in those from SO rats. No significant differences between UNX and SO rats were found in calcium-dependent NOS enzymatic activity in the renal cortex. However, calcium-independent enzymatic activity was markedly higher in the renal cortex of UNX than in those from SO animals. In conclusion, glomeruli from rats 48 h after UNX had a greater production of NO than those from SO animals. This increased glomerular NO production is based on an increase in the iNOS isoform. Increased glomerular NO synthesis seems to play a role in the decreased renal vascular resistance observed after unilateral nephrectomy in rats.

Reduced renal mass; renal blood flow

Reduction in renal mass is associated with several structural and functional adaptations including compensatory renal growth and hemodynamic changes (13). This hemodynamic adaptation consists of renal vasodilatation with increased blood flow through the remnant kidney (10). The mediators of the renal hemodynamic adaptations have not been definitively identified. Several recent studies have demonstrated that the endothelium plays a critical role in the modulation of renal vascular tone (27). Endothelium-derived nitric oxide (NO) is a major regulatory factor of renal vascular resistance (RVR) and renal blood flow (RBF) (3, 17, 25). In addition, it has been demonstrated that the vascular endothelium is capable of modulating the tone of the underlying smooth muscle in response to local changes in shear stress, pressure, and other mechanical factors (6, 15). This study assesses whether increased renal NO production may contribute to the hemodynamic adaptations observed after renal mass reduction. In addition, we assessed whether changes in NO synthase (NOS) isoforms are involved in this adaptation.

Materials and Methods

Animals

Male Wistar rats of ~250 g, born and raised in the animal facilities of the Salamanca and Murcia Universities, were used in the present study. All the experimental protocols were performed according to the guidelines for the ethical treatment of the animals, as specified by the European Union and the Ministerio de Agricultura, Pesca y Alimentación de España.

Animals were anesthetized with ketamine and placed on a heated table. A subcostal incision was made, and after ligation of renal pedicle, the left kidney was removed (UNX group). Afterwards, the incision was sutured, and the animals were maintained in sterile conditions until glomerular isolation or clearance studies were performed. A group of rats undergoing the same surgical procedures without removal of the kidney was used as control sham-operated (SO) group.

In Vivo Studies

Ten SO and 10 UNX animals (48 h after nephrectomy) were anesthetized with pentobarbital sodium (50 mg/kg body wt) and placed on a heated animal board. Rectal temperature was monitored with a thermometer and maintained at 37.5°C. Animals were surgically prepared for clearance studies by inserting PE-50 polyethylene catheters in the femoral artery and vein and in the right ureter. The femoral artery was connected to a pressure transducer and a recorder (Beckman R511A) for the continuous recording of mean arterial pressure (MAP). Urine was collected from the right ureter into weighed plastic vials containing 0.5 ml of water-stabilized mineral oil. An isotonic saline infusion containing [methoxy-14C]inulin and 3H-labeled p-aminohippuric acid (PAH) was started at 3 ml/h through the venous catheter to allow for clearance determinations. After 30 min of equilibration, two basal 30-min urine collections were performed, and a blood sample (150 μl) was taken at the beginning and end of each clearance period. Packed cell volume was determined by the microcapillary method. After the two basal periods, Nω-nitro-L-arginine methyl ester (L-NAME; 50 mg·h−1·kg body wt−1)
was infused during three consecutive 30-min clearance periods.

\(^3\text{H}\) and \(^{14}\text{C}\) activities were measured in blood and urine samples using a two-channel liquid scintillation counter (Beta IV, Kontron Instruments). Inulin and PAH clearances were calculated according to standard formulas.

**In Vitro Studies**

Glomerular isolation and incubation. Glomerular nitrite production was assayed in glomeruli obtained 48 h after left kidney removal. Animals were anesthetized with ketamine, and the kidneys were perfused “in situ” with ice-cold isotonic saline through the abdominal aorta. Glomeruli were obtained by mechanical sieving as previously described (22). After the isolation, the final preparation consisted of glomeruli without Bowman’s capsule and without afferent or efferent arterioles. Tubular contamination was always <5%. Glomerular isolation and all subsequent procedures required specific precautions to minimize contamination by bacteria or lipopolysaccharide (LPS). Such measures included using pyrogen-free disposable materials and endotoxin-free RPMI 1640 culture medium. Glomeruli were plated out in 4 × 6-well plates and incubated for 2 h at 37°C in sterile conditions. Glomerular suspensions were divided, and in one-half of the wells, the NO synthesis inhibitor L-NAME (final concentration 10\(^{-4}\) M) was added from the beginning of the incubation. Protein concentration was measured according to the Bradford method after glomerular lysis and homogenization.

Nitrite assay. Nitrite concentration was determined as previously described in the supernatant of glomerular incubation by a modification of Griess reaction (11). Briefly, 500 µl of sample were mixed with 250 µl of Griess reagent [1% sulfanilamide and 0.1% N-1-naphthyl ethylenediamine dihydrochloride in 2.5% o-phosphoric acid (Sigma)] and incubated for 15 min at room temperature. Absorbance was measured at 560 nm. Standard nitrite calibration was done using sodium nitrite.

Glomerular cGMP production. Isolated glomeruli from SO and UNX rats were suspended in ice-cold Tris glucose buffer containing 2.5 mM CaCl\(_2\). The incubation was performed in the presence of 10 mM IBMX (Sigma). The glomerular suspension was divided, and in one-half of the tubes, L-NAME (10\(^{-4}\) M final concentration) was added from the beginning of the incubation. The incubation was stopped 15 min later 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 are dissolved, and cGMP was assayed with a commercial kit (Du Pont-New England Nuclear Research Products, Bad Homburg, Germany). The recovery of cGMP during the extraction procedure, determined as the percentage of recovery of \[^3\text{H}\]cGMP added to the sample (2,000-3,000 cpm), was always >89%. In preliminary experiments, we observed that the inter- and intra-assay coefficients of variation were 11.9 and 8.4%, respectively.

Western blot. Glomeruli were lysed by incubation for 15 min in 20 mM Tris, pH 8.0, containing 140 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40, and the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride (PMSF), 500 µg/ml aprotinin, and 50 µg/ml soybean trypsin inhibitor. The mixture was spun down (10 min at 5,000 rpm) at 4°C. Debris was then discarded, and the supernatant was kept as glomeruli lysate. Aliquots were used for protein determination according to Bradford’s method (5), and 100 µg of protein were mixed (1:1) with buffer sample (10% 2-mercaptoethanol, 0.05% bromphenol blue, 20% glycerol, and 125 mM Tris, pH 6.8). Proteins were then separated in a 10% SDS polyacrylamide gel with a buffer containing 190 mM glycine and 25 mM Tris (pH 8.3). Electrophoresed proteins were transferred to a polyvinylidene difluoride membrane for 90 min by 500 mA with 190 mM glycine in 20 mM Tris base buffer, pH 8.5. Nonspecific binding blockade was performed overnight in the incubation buffer (20 mM Tris containing 150 mM NaCl and 0.1% Tween 20) to which 3% BSA was added. The mixture was then quickly rinsed with the same incubation buffer without BSA, and the membrane was incubated for 1 h in the same buffer solution containing 1:500 antimacrophage inducible NOS (NOS) antisemur (Transduction Laboratories, Lexington, KY) or 1:2,500 antiserum anti-endothelial constitutive NOS (NOS III) (Transduction Laboratories). Afterwards, the membrane was incubated for 30 min in the same buffer containing 1:30,000 peroxidase-labeled anti-mouse IgG. The membrane was washed four times for 10 min each with the incubation buffer.

NOS activity. NOS activity was assayed by measuring the conversion of L-[\(^3\text{H}\)]arginine to L-[\(^3\text{H}\)]citrulline, as previously described (9). In brief, rats were killed by cervical dislocation. The kidneys were rapidly removed, and the cortex was isolated, weighed, and homogenized (4°C) in 5 ml of a buffer containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 12 mM mercaptoethanol, 10 µg/ml leupeptin, 10 µg/ml PMSF, 10 µg/ml soybean trypsin inhibitor, and 2 µg/ml aprotinin, pH 7.5. The homogenate (20 µl) was incubated (37°C, 20 min) in the presence of a mixture containing calmodulin (100 nM), NADPH (1 mM), tetrahydrobiopterin (30 µM), L-arginine (10 µM), L-[\(^3\text{H}\)]arginine (3 pmol), and CaCl\(_2\) (2.5 mM). The reaction was then stopped by addition of 1 ml of a stop buffer (4°C) containing HEPES (20 mM), EDTA (2 mM), and EGTA (2 mM). Finally, the mixture was applied to a 1 ml Dowex AG 50W-X8 (Na\(^{+}\) form) column, and the L-[\(^3\text{H}\)]citrulline was eluted with 2 ml of stop buffer. The radioactivity in the eluate was measured by liquid scintillation counting. All the assays were run in duplicate. The homogenates were assayed as described (total activity) in the presence of L-NAME (0.1 mM) and in the absence of calcium.

**Statistical Analysis**

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

**RESULTS**

**In Vivo Studies**

Clearance studies were completed in 10 UNX and 10 SO animals. Two days after UNX or sham operation, the weight of the rats was similar in both groups (251.8 ± 9.4 g in SO rats; 246.0 ± 7.1 g in UNX animals). Basal MAP was higher in UNX than in SO animals (Fig. 1). Systemic administration of L-NAME resulted in an acute pressor response, both in SO and UNX rats. The maximal increase in MAP was similar in both groups of rats (25–30 mmHg) (Fig. 1). Two days after UNX or SO, right kidney weight was significantly higher in UNX animals (1.03 ± 0.07 g) than in SO rats (0.88 ± 0.06 g, P < 0.01). Thus data on renal function are expressed per gram of kidney weight. Figure 2 shows glomerular filtration rate (GFR) of the right
kidney in both SO and UNX rats before and after L-NAME infusion. GFR decreased with the L-NAME infusion in both SO and UNX rats in a similar proportion. RPF for the right kidney is shown in Fig. 3. L-NAME induced a significant decrease in this parameter in both groups of animals, but the decrease was significantly higher in UNX rats (3.8 ± 0.2 ml/min) than in SO rats (1.8 ± 0.2 ml/min). RVR for the right kidney is shown in Fig. 4. L-NAME increases RVR in both groups of animals, but the increase was markedly higher in UNX rats (253%) than in SO rats (130%).

In Vitro Studies

Protein content was similar in glomeruli from UNX rats (166.3 ± 5.9 mg protein/1,000 glomeruli) than in those from SO rats (166.6 ± 21.1 mg protein/1,000 glomeruli). Thus results of glomerular nitrite and cGMP production are expressed per 1,000 glomeruli. The results of glomerular nitrite production are shown in Table 1. Forty-eight hours after nephrectomy, glomeruli from UNX rats produced higher amounts of nitrite than glomeruli from SO rats. Addition of L-NAME in the culture media abolished the increase in nitrite production.

Glomeruli from UNX rats had a significantly higher cGMP production than those from SO animals. This
been already reported by Griffin et al. (12), thus despite differences in the functional renal mass has are infused (26). The similarity in pressor response than in several papers in which similar doses ofL-NAME rats (25–30 mmHg) and of the same order of magnitude maximal increase in MAP was similar in both groups of acute pressor response, both in SO and UNX rats. The thus calcium-independent activity was marked NOS enzymatic activity in the kidney was significantly lower in UNX than in SO rats. However, calcium-independent enzymatic activity was markedly higher in the kidney of UNX than in those from SO animals. Thus calcium-independent NOS activity in SO rats was about one-half of calcium-dependent activity, whereas after UNX, calcium-independent activity was four times that of calcium-dependent activity.

**DISCUSSION**

Systemic administration of L-NAME resulted in an acute pressor response, both in SO and UNX rats. The maximal increase in MAP was similar in both groups of rats (25–30 mmHg) and of the same order of magnitude than in several papers in which similar doses of L-NAME are infused (26). The similarity in pressor response despite differences in the functional renal mass has been already reported by Griffin et al. (12), thus higher production was also blunted by addition of L-NAME (Table 1).

Figure 5 shows the results of the Western blot analysis for iNOS. We observed that glomeruli obtained 48 h after nephrectomy have more iNOS protein than those obtained from SO rats. Quantification of six different Western blots reveals that iNOS amounts in glomeruli from UNX rats increased by 163 ± 13% compared with SO animals (P < 0.001). No significant changes in NOS III were observed in glomeruli from UNX rats compared with SO animals (Fig. 6).

Data from calcium-dependent and calcium-independent NOS enzymatic activity in the kidneys of SO and UNX rats are shown in Table 2. Calcium-dependent NOS enzymatic activity in the kidney was significantly lower in UNX than in SO rats. However, calcium-independent enzymatic activity was markedly higher in the kidney of UNX than in those from SO animals. Thus calcium-independent NOS activity in SO rats was about one-half of calcium-dependent activity, whereas after UNX, calcium-independent activity was four times that of calcium-dependent activity.

**Table 1. Glomerular nitrite production in sham-operated rats and in rats 48 h after uninephrectomy**

<table>
<thead>
<tr>
<th>Nitrite, fmol/1000 Glomeruli</th>
<th>cGMP, fmol/1000 Glomeruli</th>
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<tbody>
<tr>
<td><strong>Basal</strong></td>
<td><strong>+ L-NAME</strong></td>
</tr>
<tr>
<td>Sham operated</td>
<td>168 ± 10</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>383 ± 74†</td>
</tr>
</tbody>
</table>

Values are means ± SE of at least 6 experiments, each by triplicate. L-NAME, N⁵-nitro-L-arginine methyl ester. Statistical significance: *P < 0.01 vs. basal period; †P < 0.01 vs. sham-operated rats.

**Fig. 5. Effect of unilateral nephrectomy on glomerular inducible nitric oxide synthase (iNOS) protein expression.** Representative Western blot analysis for iNOS III. Similar results were obtained in 3 separate experiments. Densitometric analysis of 140-kDa band reveals that optical density is similar (P > 0.1) in glomeruli from uninephrectomized rats (UNX) and in those from sham-operated animals (sham).

**Suggesting that in both groups of animals the tonic release of NO plays a similar role in the control of systemic vascular resistance.**

Studies in several species have indicated that the tonic release of NO contributes to the maintenance of RBF and RVR (3, 4, 17). The effect of L-NAME on renal vasculature is not only due to the autoregulatory process, because the increase in RVR was higher than the increase in MAP. RBF per kidney was markedly higher in UNX than in SO rats. L-NAME induced a decrease in RBF in both groups, with the decrease higher in UNX rats (~7.5 ml/min) than in SO animals (~2.5 ml/min). In addition, whereas in SO rats L-NAME induced a twofold increase in RVR, in UNX rats RVR increased more than four times. The present study thus shows that NO blockade resulted in a larger response of the renal resistance vessels to L-NAME in UNX than in SO rats. It can be reasoned that if increased NO is a major mediator of the compensatory hemodynamic adaptation after UNX, the blockade of its synthesis should lead to greater effects in animals with renal mass reduction, that is, the result observed in the present experiments. These results do not agree with those of Griffin et al. (12), showing similar changes in RBF after N⁵-monomethyl-L-arginine infusion. However, there is a major difference between both studies, because our study was performed 48 h after uninephrectomy, a time at which functional adaptive mechanisms are in full operation but structural changes have not yet occurred. In contrast, the study of Griffin et al. (12) was performed 3–4 wk after uninephrectomy, where structural changes consequent to compensatory hypertrophy have already occurred and functional adaptation is probably less important. Our study is in agreement with that of Weissstuch et al. (29), demonstrating

**Table 2. Renal cortical nitric oxide synthase enzymatic activity**

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<tr>
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<th>Ca²⁺ Dependent, pmol·min⁻¹·mg Protein⁻¹</th>
<th>Ca²⁺ Independent, pmol·min⁻¹·mg Protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operated</td>
<td>57.82 ± 6.65</td>
<td>27.29 ± 3.69</td>
</tr>
<tr>
<td>Unilateral nephrectomy</td>
<td>22.74 ± 8.44*</td>
<td>84.81 ± 10.19*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 11 experiments each. Statistical significance: *P < 0.05 vs. sham-operated rats.
that blockade of NO synthesis prevents the increase in RPF after uninephrectomy.

After L-NAME infusion, GFR was similarly reduced in UNX and SO rats. In addition, UNX animals showed a reduced filtration fraction compared with SO animals, thus suggesting that the reduced RVR shown by SO animals is predominantly based on a reduced efferent arteriolar resistance. In addition, after NO blockade, filtration fraction markedly increased in UNX rats, whereas the increase in SO rats was less marked. This can be explained as a disproportionate efferent arteriolar vasconstriction after NO blockade, with increased intraglomerular pressure. This effect of NO blockade on efferent arteriolar contraction in normal rats has been previously reported by Baumann et al. (2), Bayliss et al. (3), Chen and Sanders (7), Raij and Bayliss (23), and Zatz and de Nucci (31). Because this effect was more marked in UNX than in SO rats, this again suggests that renal NO synthesis is increased after uninephrectomy.

The increased renal synthesis of NO after uninephrectomy is further confirmed by the higher glomerular production of nitrites (a stable end product of NO) in UNX than in SO rats. This increase was completely blocked by L-NAME. In addition, glomeruli from UNX rats showed an increased cGMP production, which can be also inhibited by incubation with L-NAME, giving further support to the hypothesis of an increased glomerular NO production. Wight et al. (30) observed an increased urinary cGMP excretion after UNX, an increase that was blocked by L-NAME. In addition, Schlondorff and Weber (24) reported in UNX rats an increase in the particulate form of guanylate cyclase, that is, the isoform activated by NO. This indicates that increased cGMP excretion (suggesting increased renal NO production) after UNX in rats is mediated by an increased NO generation.

NO synthesis, and thus NOS activity, can be modulated by changes in perfusion pressure, shear stress, and other mechanical factors (6, 15). After UNX there is an increase in MAP and RBF, and presumably an increase in shear stress, thus suggesting that NO synthesis is increased as a consequence of these changes.

There are at least three NOS isoforms, two major constitutive forms (neuronal and endothelial isoforms) and one inducible isoform (iNOS) expressed in virtually all nucleated cells subjected to immunologic and certain nonimmunologic stimuli (16). iNOS is constitutively expressed in several parts of the kidney, including the glomeruli (16). In addition, it has been observed that surgery induces an increase in iNOS expression in rats (18). We have observed that after UNX there is a higher iNOS expression than in SO rats. The regulation of iNOS expression and activity in response to inflammatory cytokines and LPS has received considerable attention in a variety of cell types and tissues, including the kidney (16). In contrast, little is known about the homeostatic regulation of iNOS expression in tissues as the kidney, which constitutively express this isoenzyme. However, the fact that iNOS expression is modified in the kidney with changes in sodium intake indicates that nonimmune regulation of iNOS contributes to the normal function of the kidney (20). In the present study, iNOS expression, assessed by Western blot and enzymatic activity measurement (calcium-independent NOS activity), is markedly activated 48 h after unilateral nephrectomy. Tyrosine kinases appear to be involved in posttranslational modification of iNOS and may play a role in modulating the functional activity of this enzyme. It should be noted that after uninephrectomy there is an increased synthesis of growth factors, some of them activating tyrosine kinases (14). In addition, shear stress, which is presumably increased after unilateral nephrectomy, activates tyrosine kinases in endothelial cells (1, 8), and the 5'-control region of the human iNOS gene contains a shear stress response element (21). Thus it seems plausible that increased shear stress after uninephrectomy could activate iNOS expression.

In addition, local effects exerted by growth factors in the kidney could also be one mechanism to control iNOS expression after uninephrectomy. It has been reported that transforming growth factor (TGF)-β1 expression is markedly depressed after uninephrectomy in rats (19). TGF-β1 appears to negatively modulate renal iNOS expression in vivo, because TGF-β1 knockout mice exhibit enhanced iNOS expression in kidney (28).

In conclusion, glomeruli from rats 48 h after uninephrectomy had a greater production of NO than those from SO animals. This increased glomerular NO production is based on an increase of the macrophage-type iNOS. Increased glomerular NO synthesis seems to play a role in the decreased RVR observed after unilateral nephrectomy in rats.

Perspective

Renal hemodynamic compensatory hypertrophy could play a dual role after renal mass reduction. First, it can compensate renal mass reduction by increasing renal function, thus avoiding a marked reduction in renal function. However, renal hyperfunction seems to be also involved in the genesis of chronic renal failure after renal mass reduction, a damage that seems to be mediated by increased glomerular pressure. Thus the precise knowledge of the role of NO in this adaptive hemodynamic response could allow us to pharmacologically modulate this phenomenon, and thus to try to reinforce the positive response and to block the negative one.

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