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Renal and systemic nitric oxide synthesis in rats with renal mass reduction

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Renal and systemic nitric oxide synthesis in rats with renal mass reduction. In rats undergoing renal mass reduction (RMR) oral supplementation with the nitric oxide (NO) precursor L-arginine increases glomerular filtration rate and ameliorates signs of glomerular injury, suggesting that chronic renal failure in the rats is a condition of low NO formation in the kidney. On the contrary, data are available that in the systemic circulation of uremics, both rats and human beings, NO is formed in excessive amounts and may contribute to platelet dysfunction and bleeding tendency, well-known complications of uremia. The present study was designed to clarify the pathophysiology of renal and systemic NO synthesis in uremia. We showed that renal *ex vivo* NO generation, measured as the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline, was lower than normal in RMR rats, seven days after surgery, and progressively worsened with time in close correlation with signs of renal injury. Consistent with these results, urinary excretion of the stable NO metabolites, NO_2^-/NO_3^-, significantly decreased in rats with RMR. To go deeper into the cellular origin and biochemical nature of this abnormality we used two histochemical approaches that could locate either NO synthase (NOS) catalytic activity (NADPH-diaphorase) or NOS isoenzyme expression (immunoperoxidase). NADPH-diaphorase documented a progressive loss of renal NOS activity in RMR rats that co-localized with a strong progressive decrease of inducible NOS isoenzyme (iNOS) immunostaining. At variance with iNOS, endothelial cell NOS (ecNOS) staining was rather comparable in RMR and control kidneys. At variance to the kidney, in the systemic circulation of RMR rats the synthesis of NO increased as reflected by higher than normal plasma NO_2^-/NO_3^- concentrations. High systemic NO likely derives from vessels as documented by the increased NOS activity and higher expression of both iNOS and ecNOS in the aorta of RMR rats. Up-regulation of systemic NO synthesis might be an early defense mechanism against hypertension of uremia. On the other hand, more NO available to circulating cells may sustain the bleeding tendency, a well-known complication of uremia.

Nitric oxide (NO), an L-arginine derivative, is implicated in neuronal transmission, immune response and vasodilation, beside acting as a platelet function modulator [1, 2]. The conversion of L-arginine to L-citrulline that leads to the formation of NO is catalyzed by a family of NO synthase (NOS) enzymes that exist in at least three distinct isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (ecNOS). All three isoforms have been detected in the kidney [3, 4]. Immunohistochemical [5] and reverse transcription-polymerase chain reaction (RT-PCR) techniques [6] have located ecNOS in the glomerulus and afferent and efferent arterioles. Immunohistochemistry [7, 8] and *in situ* hybridization [8] show that the major site of nNOS in the kidney is the macula densa. Both isoforms are crucially involved in renal pathophysiology and local release of NO serves to control renal blood flow and modulates the activity of tubuloglomerular feedback [9–11]. The glomerular mesangium and afferent arteriole, as well as various segments of the nephron, express the inducible isoform of NOS (iNOS) [12–14], under basal conditions and after immune activation [12, 13]. The steady state amount of iNOS mRNA and protein in normal rat kidney is highest in tubuli of the outer medulla, and *in vitro* studies on rat renal homogenates showed that the specific activity of NO synthase in the medulla was three times that in the cortex [15].

The renal vascular bed produces large amounts of NO whose basal production maintains renal blood flow (RBF) and glomerular filtration rate (GFR) [16, 17]. The kidney seems more sensitive to acute inhibition of NO synthesis than other organs in that the intravenous infusion of the NO synthesis inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME), at a dose that did not modify systemic blood pressure, reduced RBF and GFR in normal rats [16].

Rats undergoing extensive renal mass reduction (RMR) develop systemic hypertension and exhibit a decrease in both GFR and RBF, despite an initial adaptive increase in single-nephron GFR and plasma flow per nephron [18]. Animals with RMR also develop severe proteinuria and structural changes in the kidney, including glomerulosclerosis, which eventually lead to renal insufficiency [18, 19]. Reyes and coworkers observed that giving the NO precursor L-arginine to RMR rats increased GFR and effective renal plasma flow (ERPF), reduced proteinuria and preserved renal morphology [20]. These findings were confirmed by another study, which found that oral supplementation of L-arginine at a much lower dose had a protective effect on urinary protein excretion and renal function in RMR rats [21]. It thus appears that chronic renal failure in the rats is a condition of low NO formation in the kidney and that exogenous correction of the defect markedly limits disease progression.

On the contrary, data are available that in the systemic circulation of uremics, either in experimental animals or humans, NO is formed in excessive amounts. Therefore, rats with RMR [22]–like human beings [23]– have a prolonged bleeding time that

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returns completely to normal when the animals are given N-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of NO synthase [24]. In a recent study [25] plasma from uremic patients induced NO synthesis in cultured endothelial cells much more than plasma from healthy subjects.

The present study was designed to clarify the pathophysiology of NO synthesis in uremia and was aimed at unraveling the apparent conflict of less NO being formed in the kidney in the face of enhanced systemic availability.

METHODS

Chemicals

[^{3}H] L-arginine (56.4 Ci/mmol) was purchased from New England Nuclear, (Boston, MA, USA). Tumor necrosis factor α (TNFα, specific activity 6.1 $\times$ 10^7 U/mg) and interleukin 1 β (IL-1β, specific activity 5 $\times$ 10^7 U/mg) were a gift from BASF KNOLL (Ludwigshafen, Germany); interferon γ (INFγ) was obtained from Institut Roussel Uclaf (Paris, France). Dowex AG 50 WX-8 was from Bio Rad (Richmond, CA, USA). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and diaminobenzidine tablets were from Merck (Darmstadt, Germany), Nω-nitro-L-arginine, β-NADPH, nitroblue tetrazolium, Triton X-100, diphenyliodonium (DPI) and all other chemicals were from Sigma Chemicals (St. Louis, MO, USA). A low-nitrate diet was prepared from commercial standard diet (Rieper, Bolzano, Italy) by water nitrate extraction. Briefly, the pulverized diet was resuspended in distilled water at 37°C and whipped, then was filtered and dried up. Since water-soluble vitamins and minerals were lost by water extraction, the low-nitrate diet was supplemented with minerals, vitamins, choline, DL-methionine, L-arginine, in amounts recommended by the American Institute of Nutrition.

Experimental design

Male Sprague-Dawley rats (Charles River Italia, Calco, Italy; $N = 37$) underwent right nephrectomy and ligation of two or three branches of the left renal artery according to Olson et al [26]. Thirty-seven rats were sham-operated and served as controls (CTR). Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publ. No. 85-23, 1985).

To evaluate renal NO synthesis three groups of RMR rats and three groups of control rats were studied at seven days ($N = 5$), 30 days ($N = 6$) and 90 days ($N = 6$) after surgery. Animals were sacrificed by ether anesthesia, the kidneys were removed and decapsulated. A specimen of renal tissue was embedded in paraffin for evaluation of glomerulosclerosis and tubulointerstitial damage, and the remaining tissue was minced into a fine paste and homogenized by sieving (stainless steel sieve, 140 mesh; Giuliani, Turin, Italy) to obtain a total renal tissue preparation consisting of a mixture of glomeruli, tubuli and single cells.

Portions of renal tissue (about 1.5 mg protein) from control and RMR rats were incubated in Hank's buffer (Tris 27.5 mmol/liter, Na_2HPO_4 0.7 mmol/liter, KH_2PO_4 0.74 mmol/liter, KCl 5.4 mmol/liter, NaCl 136 mmol/liter, dextrose 9.6 mmol/liter, $CaCl_2$ 1.87 mmol/liter and $MgSO_4$ 0.8 mmol/liter) supplemented with L-arginine (10 μmol/liter) and [^{3}H] L-arginine (0.5 μCi) for eight hours at 37°C in a water bath under slow shaking. Renal NO synthesis was evaluated by measuring the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline and the data were corrected for the protein content of each sample.

Portions of Hank's buffer containing [^{3}H] L-arginine were also incubated for eight hours and used as blanks. To verify whether [^{3}H] L-citrulline production actually reflected NO synthase activity, samples of renal tissue from control rats ($N = 3$) were incubated for eight hours with both [^{3}H] L-arginine and Nω-nitro-L-arginine (L-NNA, 300 μmol/liter), a specific inhibitor of NO synthesis. As positive controls, portions of control renal tissue ($N = 3$) were incubated with a cocktail of cytokines (TNFα 1000 U/ml, IL-1β 10 U/ml, INFγ 200 U/ml) for 24 hours in the presence of [^{3}H] L-arginine and excess L-arginine (100 μmol/liter) [27].

To obtain an indirect *in vivo* index of renal NO synthesis, 24-hour urinary excretion of nitrites/nitrates (NO_2^-/NO_3^-), the stable NO metabolites, was measured in additional control ($N = 8$) and RMR rats ($N = 8$) studied longitudinally 7, 30 and 90 days after the surgical procedure. For each time point, to minimize dietary NO_3^- intake, rats were fed a low NO_3^- diet (< 8 nmol/g) and given distilled water to drink, starting three days before the 24-hour urine collection [28]. Metabolic cages were cleaned with a germicidal soap to prevent NO_2^- and NO_3^- formation by bacteria [28]. At the same time points plasma samples were collected from RMR and CTR rats to measure plasma NO_2^-/NO_3^- concentrations as an indicator of systemic NO synthesis. At 90 days animals were sacrificed and portion of renal tissue was paraffin-embedded for histological evaluation.

To evaluate any differences in activity and localization of renal and systemic NO synthase isoenzymes in RMR and CTR rats, three additional groups of RMR and CTR rats ($N = 4$ for each group) were sacrificed at 7, 30 and 90 days after surgery. The catalytic activity of NOS in the kidney and thoracic aorta was assessed by isoenzyme-independent enzymatic oxidation of nitroblue tetrazolium in the presence of NADPH (NADPH-diaphorase). Expression of the endothelial (ecNOS) and inducible (iNOS) isoforms of NO synthase was evaluated on the same tissues by immunoperoxidase with specific antibodies. A portion of tissue was paraffin-embedded for histological evaluation.

Renal function, measured as serum creatinine, urinary protein excretion and bleeding time were assayed in all RMR and CTR rats.

Determination of [^{3}H] L-citrulline formation from [^{3}H] L-arginine

Incubations were stopped by adding one volume of ice-cold 15% trichloroacetic acid (TCA). TCA-treated samples were centrifuged at 10,000 $\times$ g to precipitate proteins. The supernatant was extracted five times with one volume of water-saturated ether, vacuum lyophilized and resuspended in 2 ml HEPES, pH 5.5, and applied to 2 ml wet bed volumes of Dowex AG 50 WX-8 (100 to 200 mesh, Li^+ form), followed by 2 ml of water. [^{3}H] L-citrulline was quantitated by liquid scintillation counting in the 4-ml column effluent and identified as described [29].

Urinary and plasma nitrites/nitrates

NO_2^-/NO_3^- were measured semi-automatically using a High Performance Liquid Chromatograph (model 421A; Beckman Instruments Inc., Berkeley, CA, USA) coupled with a 163 Variable Wavelength detector (Beckman Instruments Inc.) and a

Shimadzu C-R3A Chromatopac Recorder-Integrator (Kjoto, Japan). Briefly, urine and plasma samples were treated with zinc sulfate (60 μmol/liter, final concentration) and centrifuged to eliminate proteins. Supernatants were eluted into a Dowex AG 50 WX-8 column followed by a cadmium column which catalyzes the reduction of nitrate to nitrite (eluent borate buffer, pH = 8.5). The post-column eluate reacted with Griess reagent [5% H_3PO_4, 1% sulphanylic acid, 0.1% of N-(1-naphthyl)-ethylenediamine, vol:vol:vol] to form a purple azo dye, and the color was analyzed with a UV-VIS detector at $\lambda = 504$ nm. The absorbance peak area was measured and the NO_2^-/NO_3^- concentration in the sample was calculated by extrapolation from a standard nitrate curve. Values were corrected for recovery which averaged 80% as determined by addition of known amounts of standard nitrate to an additional portion of each sample [30].

Tissue preparation for histochemistry

Animals were anesthetized by diethyl ether inhalation; left kidneys were perfused through the descending aorta for five minutes with PBS to wash out the blood, then for 10 minutes with 4% p-formaldehyde in PBS. Biopsy slices 2 mm thick were cut perpendicularly to the major axis of the kidney and fixation was completed by immersion in p-formaldehyde solution overnight. The thoracic aorta was surgically dissected and fixed by immersion in 4% p-formaldehyde in PBS overnight at 4°C. A portion of each sample was processed for conventional paraffin inclusion, and the remainder was treated with 10% sucrose in PBS for cryoprotection, then frozen in liquid nitrogen.

NADPH-diaphorase

Frozen sections 3 μm thick were cut on a cryostat (HM500-O; Microm, Zeiss Oberkochen, Germany). Sections were air-dried, then washed in PBS 0.05 M pH 7.4 for five minutes at room temperature and permeabilized by immersion in 0.3% Triton X-100/PBS 0.01 M, pH 7.2 at 4°C for 30 minutes [31]. NADPH-diaphorase reaction was performed by incubating slides with 1 mM βNADPH/0.2 mM nitroblue tetrazolium/100 mM Tris-HCl buffer pH 8.0 containing 0.2% Triton X-100 for one hour at 37°C [32]. The reaction was stopped by rinsing sections in PBS 0.05 M pH 7.4.

In all experiments the reproducibility of the reaction was followed on a control tissue section. Negative controls were run without NADPH or in the presence of the NO synthesis inhibitor DPI [15, 33]; reactivity was totally NADPH-dependent and was abolished by DPI.

Slides were observed on a DM/RB microscope (Leitz, Leica, Milan, Italy) by a pathologist blind to the nature of the experiment.

Immunoperoxidase

Three-μm paraffin sections from renal and aortic tissue were processed for light microscopy immunohistochemistry using an avidin-biotin horseradish peroxidase complex technique (ABC method, ABC-Elite; Vector Laboratories, Burlingame, CA, USA). Rabbit polyclonal antibody directed against mouse macrophage inducible NOS (Transduction Laboratories, Exeter, UK) and mouse monoclonal antibody against human endothelial NOS (Transduction Laboratories) were used. Both antibodies recognize rat NOS antigens [34, and Transduction Laboratories catalog]. Briefly, the sections were dewaxed, rehydrated and incubated for one hour with 0.3% H_2O_2 in methanol to quench endogenous peroxidase. Tissue was permeabilized in 0.1% Triton X-100 in PBS 0.01 M, pH 7.2, for 30 minutes and aspecificities were blocked by 30 minutes incubation with non-immune sera (goat serum for anti-iNOS, horse serum for anti-ecNOS). All the above steps were carried out at room temperature. Slides were then incubated overnight at 4°C in a moist chamber with the primary antibody (anti-iNOS 1:25, anti-ecNOS 1:150) in PBS/1% bovine serum albumin (Miles, Bayer, Milan, Italy), followed by the secondary antibody (biotinylated goat-anti-rabbit IgG, or biotinylated horse-anti-mouse IgG), ABC solution, and finally developed with diaminobenzidine as described. The sections were then counterstained with Harris hematoxylin (Biooptica, Milan, Italy). Negative controls were obtained by omitting the primary antibody on a second section present on all the slides.

For the detection of ecNOS on renal tissue, as the above method did not clearly separate any signal from the background (data not shown), an amplification system was employed. The sections were processed as described above, but before the ABC solution they were incubated with a monoclonal antibody anti-biotin (dilution 1:30; DAKO, Milan, Italy) for 30 minutes at room temperature, followed by another incubation with biotinylated antibody. Methyl green was employed to counterstain the samples instead of hematoxylin.

The slides were observed under the light microscope.

Histology

For morphological evaluations 3-μm thick sections were cut from the paraffin blocks and stained with Masson's Trichrome and PAS. Glomerulosclerosis was semiquantitatively analyzed by light microscopy by the same pathologist who did the immunohistochemistry examinations. Briefly, 0 was attributed to normal glomeruli, 1+ to glomeruli with sclerosis 0 to 30%, 2+ to glomeruli with 30% to 60%, 3+ to glomeruli with 60% to 100%, and 4+ to completely sclerosed tufts. The numbers of glomeruli for each class were counted and the sclerosis score was calculated as a balanced average [35]. Tubulointerstitial damage and tubular casts were also evaluated [36].

Analytical

Serum creatinine was measured with the alkaline picrate method [37]. Urinary protein concentration was determined by the Coomassie blue G dye-binding assay with BSA as standard [37]. Protein content of renal tissue samples was measured with the method of Lowry et al [38]. Bleeding time was determined as previously described [24].

Statistical analysis

All results are expressed as mean ± SE. Data on NO production in renal tissue incubated with L-NNA or the cocktail of cytokines were analyzed by Student's t-test for unpaired data. Renal NO synthesis, proteinuria, serum creatinine concentration and bleeding time in control and RMR rats 7, 30 and 90 days after surgery were analyzed by the Kruskal-Wallis test. Data on plasma and urinary nitrites/nitrates were analyzed by between-within analysis of variance. Linear regression analysis was used to correlate NO synthesis with the degrees of proteinuria and glomerulosclerosis in RMR rats, and the Spearman correlation test was used to correlate renal NO synthesis with tubular damage. Statistical significance was defined as $P < 0.05$.

Table 1. Laboratory findings in RMR and CTR rats

Group	Day	Urinary protein[c] *mg/day*	Serum creatinine[c] *mg/dl*	Glomeruli with sclerotic changes[d] *% (range)*	Tubular damage[d]	Interstitial infiltrate[d]	Casts[d]	Bleeding time[c] *seconds*
RMR	7	47 ± 3[a]	0.97 ± 0.07	4 (0–13)	+/−	−	+/−	187 ± 5.42[a]
RMR	30	275 ± 33[ab]	1.5 ± 0.06[ab]	28 (0–71)	++	+/++	+	201 ± 11.7[a]
RMR	90	292 ± 40[ab]	1.7 ± 0.08[ab]	39 (8–66)	++/+++	++	+	281 ± 12.7[a]
CTR	7	22 ± 0.6	0.61 ± 0.01	0	−	−	−	95 ± 3.45
CTR	30	30 ± 1.1	0.62 ± 0.01	0	−	−	−	111 ± 1.71
CTR	90	24 ± 2.1	0.70 ± 0.01	0	−	−	−	93 ± 2.35

Data are mean ± SE.
[a] $P < 0.01$ vs. CTR at corresponding time
[b] $P < 0.05$ vs. RMR at day 7
[c] $N = 37$ (7 days after surgery), $N = 28$ (30 days after surgery), $N = 18$ (90 days after surgery), see experimental design
[d] $N = 9$ (7 days after surgery), $N = 10$ (30 days after surgery), $N = 18$ (90 days after surgery), see experimental design

RESULTS

In RMR rats urinary protein excretion was significantly higher than in control rats at each time point considered (Table 1). Proteinuria was already present seven days after surgery ($P < 0.01$) and further increased on days 30 and 90 ($P < 0.01$). During time rats with RMR developed progressive renal insufficiency as documented by serum creatinine values (Table 1). RMR rats sacrificed seven days after surgery as well as all CTR rats had no renal lesions. In RMR rats 28% and 39% of glomeruli respectively had sclerotic lesions on days 30 and 90. At the same times in RMR rats abnormalities also affected non-glomerular structures and included tubular atrophy, casts in the distal tubuli, interstitial inflammation and fibrosis (Table 1).

Seven days after renal ablation bleeding time values were significantly ($P < 0.01$) longer than in controls. By 30 and 90 days hemostatic changes were even more evident (Table 1).

NO synthesis in renal tissue from CTR and RMR rats

Normal renal tissue synthesized considerable amounts of NO *in vitro* as indicated by the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline. Generation of [^{3}H] L-citrulline in CTR renal tissue was almost completely abolished (1.77 ± 0.31 vs. 20.31 ± 1.65 pmol/mg protein, $N = 3$, $P < 0.05$ vs. vehicle) by the addition of 300 μmol/liter L-NNA, a specific NO synthesis inhibitor, indicating that [^{3}H] L-citrulline formation closely reflected renal NO synthesis. When normal renal tissue was preincubated with cytokines in the presence of excess L-arginine, NO synthesis was significantly ($P < 0.05$) increased (17.24 ± 1.48 nmol/mg protein, $N = 3$) compared to unstimulated tissue (7.31 ± 0.89 nmol/mg protein, $N = 3$), indicating the presence of inducible NO synthase enzyme.

As shown in Figure 1, NO release was significantly lower in renal tissue from RMR rats than control rats at each time point (7 days, 14.09 ± 2.85 vs. 22.43 ± 0.72, $N = 5$, $P < 0.05$; 30 days, 8.82 ± 1.24 vs. 17.19 ± 2.49, $N = 6$, $P < 0.05$; 90 days, 0.61 ± 0.25 vs. 17.33 ± 3.16, $N = 6$, $P < 0.01$; pmol/mg protein). In RMR rats renal NO synthesis paralleled renal disease progression and was almost undetectable 90 days after the surgical procedure ($P < 0.01$ vs. 7 days and 30 days). Renal NO synthesis negatively correlated with the degree of proteinuria ($r = 0.50$, $N = 17$, $P < 0.05$) as well as with glomerulosclerosis ($r = 0.72$, $N = 17$, $P < 0.05$) or tubular damage (rho = 0.76, $N = 17$, $P < 0.05$).

Consistently with *ex-vivo* data, urinary NO_2^-/NO_3^- excretion was significantly lower in RMR rats than in control rats throughout the experimental period (7 days, 822 ± 242 vs. 6155 ± 1531, $P < 0.05$; 30 days, 566 ± 369 vs. 4653 ± 1033, $P < 0.01$; 90 days, 479 ± 180 vs. 3211 ± 569, $P < 0.01$; nmol/24 hr, $N = 8$; Fig. 2). In CTR rats studied seven days after surgery, urinary NO_2^-/NO_3^- excretion was numerically but not significantly higher than at 30 and 90 days. Urinary excretion of NO_2^-/NO_3^- in normal rats of comparable age was 3750 ± 765 nmol/24 hr ($N = 4$), suggesting that the higher urinary NO_2 /NO_3^- in CTR animals seven days after surgery might be related to the sham operation.

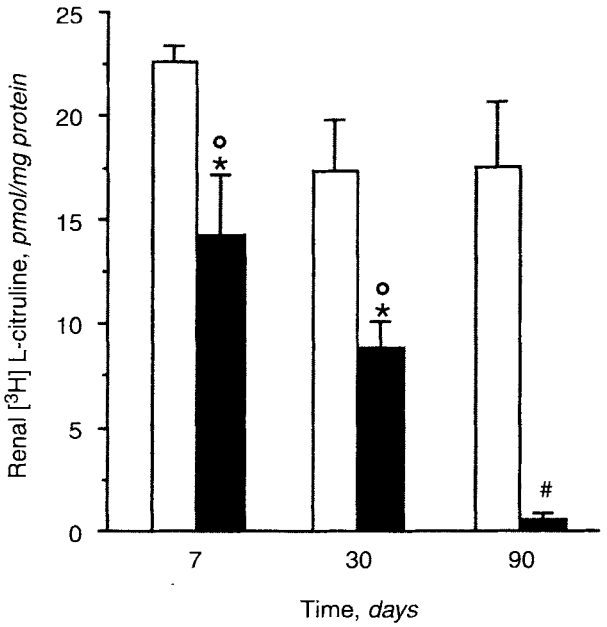


Fig. 1. Production of NO by renal tissue from RMR rats (■) seven days ($N = 5$), 30 days ($N = 6$) and 90 days ($N = 6$) after the surgical procedure, compared to age-matched sham-operated rats (CTR rats; □). Data are mean ± SE. *$P < 0.05$, #$P < 0.01$ versus control rats, °$P < 0.01$ versus RMR 90 days.

Location of NOS activity and NOS isoenzymes in CTR and RMR kidney

NADPH-diaphorase. In order to locate renal NO synthesis at cellular level we used the NADPH-diaphorase technique, which detects catalytic NOS activity irrespective of the enzyme isoform. NADPH-diaphorase staining in CTR rats was similar at all time

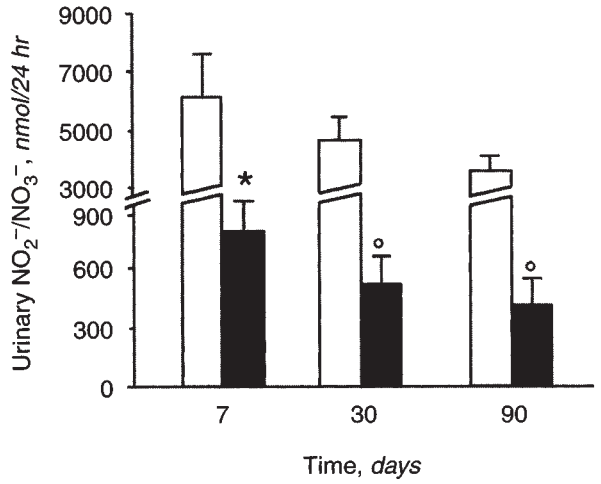


Fig. 2. Urinary excretion of nitrites/nitrates in (■) RMR rats seven days ($N = 8$), 30 days ($N = 8$) and 90 days ($N = 8$) after the surgical procedure, compared to age-matched sham-operated rats (CTR rats; □). Data are mean ± SE. *$P < 0.05$, °$P < 0.01$ versus control rats.

points examined after sham operation, except for a slightly higher intensity seven days after surgery (Fig. 3 A-C). Staining was stronger in the macula densa [3, 5, 8], while the glomerular tuft showed a weak positivity that seemed to draw the capillary network [10]. The cytoplasm of most cortical and outer medullary tubuli was intensely colored while the inner medulla was almost negative [15]. The vessels showed marked endothelial staining and frequently the arterial smooth muscle cells presented a well detectable signal too (Fig. 3 A-C) [3]. This pattern was comparable to that recently observed by McKee, Scavone and Nathanson in the human kidney [15].

Renal sections from RMR rats seven days after the surgical procedure showed the same staining distribution as in CTR rats, but a weaker intensity (Fig. 3D). After that the diaphorase staining pattern changed strikingly in RMR rats. By 30 days the decrease in NOS activity was focal and affected some portions in the cortex and outer medulla more than others. Areas of decreased staining frequently corresponded to areas of sclerosis where no signal could be found anywhere in tubuli and glomeruli. An exception was the macula densa where staining was substantially preserved compared to CTRs. The diaphorase signal was also detected in most interstitial capillaries and afferent and efferent arterioles (Fig. 3E).

At 90 days there was a further diffuse decrease in NOS activity, NADPH-diaphorase staining being barely detectable in most tubuli and glomeruli. Some areas along the vascular endothelium of interstitial vessels and afferent/efferent glomerular arterioles stained positively but weakly (Fig. 3F). In the rare morphologically preserved regions, low NADPH diaphorase activity was still present in glomeruli.

iNOS and ecNOS immunoperoxidase. Expression of the inducible isoform of NOS was similar in all control groups. The enzyme showed focal staining in a few glomerular cells in the tuft as well as in the epithelium of the Bowman capsule. In the cortex, staining was intense on collecting ducts as well as on few proximal tubuli, while most distal tubuli had a weak signal (Fig. 4B). Outer medullary tubuli stained strongly, while vessels, interstitial areas and inner medullary tubuli, except thick ascending limbs, were completely negative (data not shown).

RMR kidney sections presented a progressive decrease of iNOS antigen compared to CTR kidneys. Thus, at seven days iNOS staining had the same distribution as controls, though it was weaker. Like the diaphorase staining, 30 days after surgery iNOS immunoreactivity had markedly decreased in glomeruli as well as in most tubuli, particularly in areas affected by fibrosis. By 90 days the iNOS signal dropped further in glomeruli and tubuli with no sclerotic lesions, and no iNOS immunoreactivity at all was detectable in sclerotic regions (Fig. 4C).

In CTR rats a low but specific ecNOS signal was localized in glomerular capillary loops [5], particularly in the glomerular endothelium, and in the brush border of proximal tubuli (Fig. 5A). Staining was strong in the endothelium of the interlobular arteries, interstitial arterioles and capillaries [39]. In contrast to iNOS, ecNOS staining was rather comparable in RMR and control kidneys. However, in RMR rats the ecNOS signal was reduced in glomeruli affected by sclerosis, possibly because of loss of functional endothelium (Fig. 5C).

Omitting the primary antibody, against either iNOS or ecNOS did not produce any staining of adjacent sections in all experiments (Fig. 4A and Fig. 5 B, D).

Systemic NO synthetic pathway in CTR and RMR rats

Plasma NO_2^-/NO_3^- concentrations are given in Figure 6. In CTR animals, plasma NO_2^-/NO_3^- did not change with time (7 days, 23.88 ± 5.61; 30 days, 18.14 ± 2.30; 90 days, 20.37 ± 4.60; nmol/ml, $N = 8$). By contrast, in RMR rats plasma NO_2^-/NO_3^- gradually rose (7 days, 35.42 ± 4.70; 30 days, 34.25 ± 6.92; 90 days, 43.82 ± 8.51; nmol/ml, $N = 8$), to such a degree that at 90 days absolute values were significantly different from those in the corresponding CTR animals ($P < 0.05$, Fig. 6).

Thoracic aorta from CTR rats stained weakly for NADPH-diaphorase on endothelial cells with a few traces on the vessel wall (Fig. 7A) [40]. Endothelial staining appeared to be more intense in aorta sections from RMR on day 7 than in CTR rats and further increased at 30 and 90 days, suggesting higher NOS activity. Furthermore, at the latter time point a segmentary marked increase in the diaphorase signal was observed also in smooth muscle cells of RMR aortas, compared to CTR aortas (Fig. 7B).

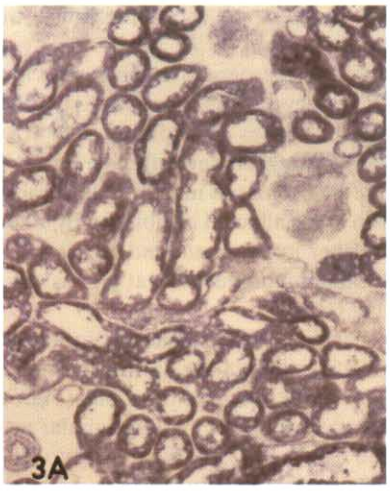
In CTR rats immunoperoxidase showed a strong ecNOS signal on the aortic endothelium (Fig. 7C) [39], whereas iNOS was found on smooth muscle cells of arterial wall and on the endothelium (Fig. 7E). Of note, endothelial iNOS staining was weaker than ecNOS.

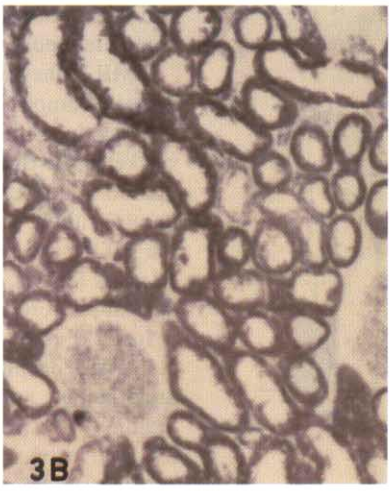
In line with the diaphorase results, ecNOS expression in RMR aortas was greater than in CTRs and increased with time (Fig. 7D). As for iNOS, there were no appreciable differences between CTR and RMR aortas at 7 and 30 days. By 90 days a sharp increase in the iNOS signal was observed in RMR aortas, in both endothelial and smooth muscle cells (Fig. 7F).

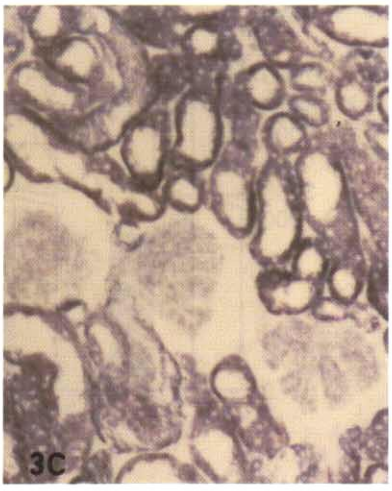
When the primary antibodies was omitted no staining was observed in adjacent sections in all experiments (Fig. 7 G, H).

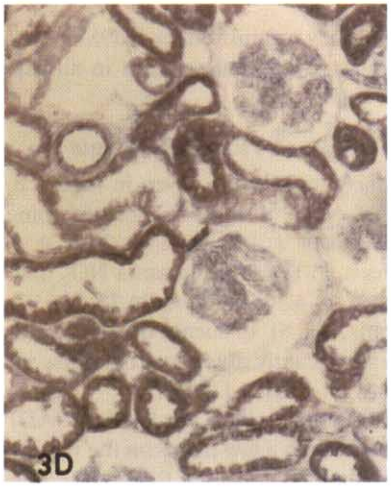
DISCUSSION

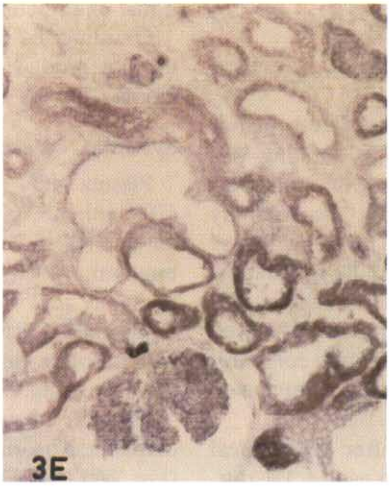
In the present report we have shown that in rats with RMR renal generation of NO was remarkably lower than normal. The abnormality was already evident seven days after surgery and progressively worsened with time, in close correlation with signs of

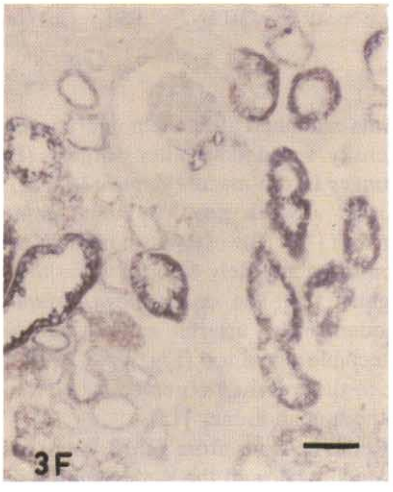


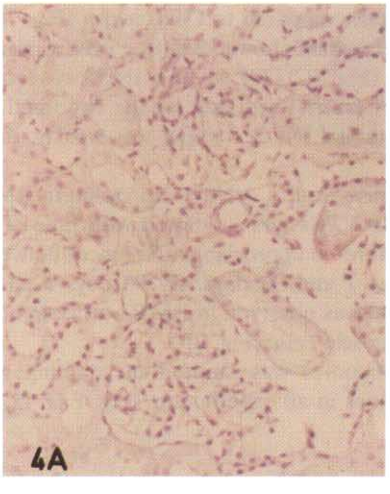


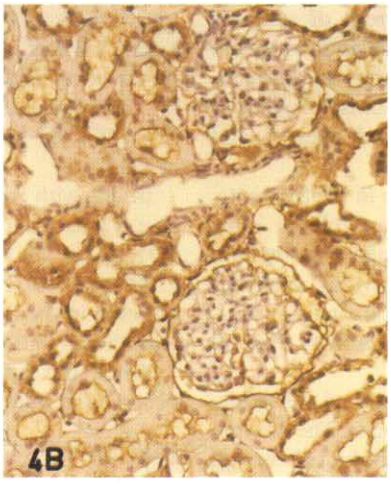


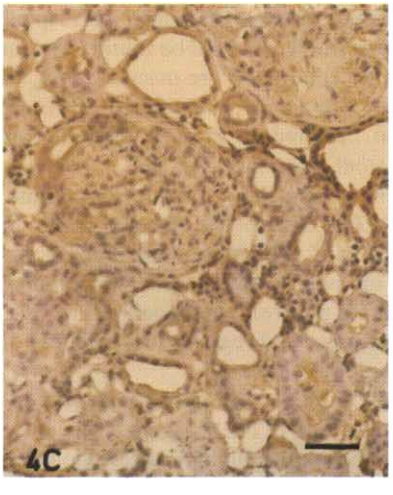












renal injury. Consistent with these results, NO_2^-/NO_3^- urinary excretion significantly decreased in rats with RMR compared to control animals. These data support previous reports [20, 21, 41] that progressive nephropathies were associated with a reduced capacity of the kidney to generate NO.

To go deeper into the cellular basis and the biochemical nature

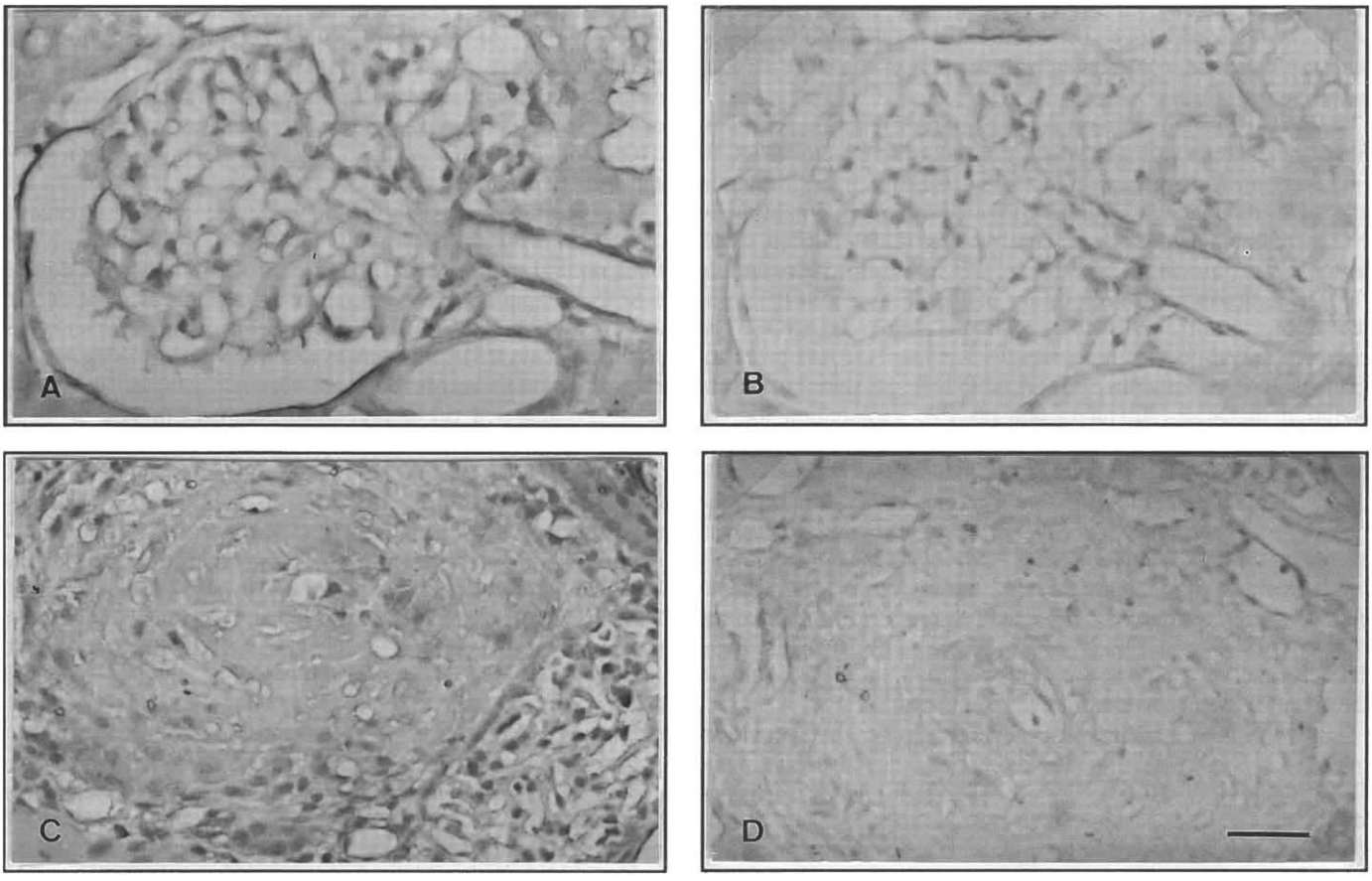


Fig. 5. ecNOS immunoperoxidase staining of CTR (*A*) and RMR (*C*) rats studied 90 days after surgery. Specific reactivity is present on glomerular capillary loops both in CTR and in RMR rats (methyl green counterstaining). No staining is seen when the primary antibody is omitted both in CTR (*B*) and in RMR (*D*) kidneys (magnification ×355, bar = 35 μm).

of this abnormality, we used two histochemical approaches, that could locate either NOS catalytic activity [5, 7, 15] or NOS isoenzyme expression [5, 7, 15, 39]. NADPH-diaphorase documented a progressive loss of renal NOS activity in RMR rats in all the structures examined including glomeruli (except macula densa

Fig. 3. NADPH-diaphorase staining of kidney cortex. Reactivity is strong on the macula densa and tubuli in CTR kidney (*A, B, C*: CTR rats studied 7, 30 and 90 days after surgery; *D, E, F*: RMR rats studied 7, 30 and 90 days after surgery). Seven days after surgery in RMR rats (D) staining is weaker. E. NADPH-diaphorase staining in kidney cortex of RMR rats studied at 30 days shows one glomerulus and a few normal tubuli surrounding a negative area. A similar negative area is shown in panel F (RMR rats on 90 days), where only the afferent and efferent arterioles of a sclerosed glomerulus and few tubuli are stained (magnification ×90; bar = 80 μm). Publication of this figure in color was made possible by a grant from Hoechst Marion Roussel, Frankfurt am Main, Germany.

Fig. 4. iNOS immunoperoxidase staining of CTR (*B*) and RMR (*C*) 90 days after surgery. Note the groups of tubuli strongly marked, and the focal glomerular staining in CTR rats as compared to the diffuse iNOS decrease in RMR (Harris hematoxylin counterstaining). *A*. No staining is seen with omission of the primary antibody (magnification ×180; bar = 40 μm). Publication of this figure in color was made possible by a grant from Hoechst Marion Roussel, Frankfurt am Main, Germany.

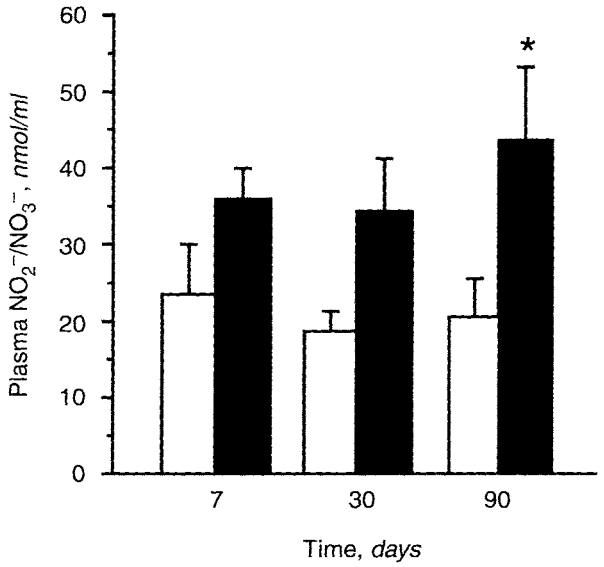


Fig. 6. Plasma concentration of nitrites/nitrates in RMR rats (■) seven days (*N* = 8), 30 days (*N* = 8) and 90 days (*N* = 8) after surgery, compared to age-matched sham-operated rats (CTR rats, □). Data are mean ± SE. *$P < 0.05$ versus control rats.

and afferent and efferent arterioles), proximal tubules and collecting ducts. A strong, progressive decrease of iNOS immunostaining was also observed, colocalizing with the loss of NADPH-diaphorase signal.

In glomeruli soon after surgical ablation of renal mass inflammatory mediators such as platelet-derived growth factor (PDGF) [42] and transforming growth factor β (TGFβ) [43] are formed in excessive amounts. PDGF and TGFβ are both potent inhibitors of NO synthesis and dose-dependently block IL-1β-induced iNOS mRNA in rat mesangial cells [44, 45]. It is therefore conceivable that an exaggerated concentration of PDGF and TGFβ in glomeruli of rats with RMR down-regulates glomerular iNOS. The recent observation that the potent vasoconstrictor and promitogenic peptide endothelin-1 (ET-1) inhibits IL-1β-stimulated transcription of iNOS [46] offers one possible explanation for the reduced expression of iNOS in the outer medulla of rats with RMR. Renal ET-1 gene expression and synthesis time-dependently increased in the RMR model and correlated with the progressive renal injury [47, 37]. Although the cellular origin of the increased renal synthesis of ET-1 is not yet clear, preliminary *in situ* hybridization studies in our laboratory in RMR rats [48] showed intense staining for ET-1 mRNA, mainly in the proximal tubular cells. One may speculate that in rats with RMR excessive ET-1 formed by the proximal tubular epithelium down-regulates iNOS in the outer medulla.

Unlike iNOS, the isoenzyme ecNOS is expressed in comparable amounts in RMR and control kidneys. This is not surprising, since PDGF and TGFβ do not affect ecNOS [2, 49] while ET-1 even stimulates it [50].

NO in the renal vasculature and macula densa appears to control renal blood flow and tubulo-glomerular feedback [9, 10, 51]. Deng and Baylis [52] found that chronic administration of a NO synthesis inhibitor to rats increased systemic and glomerular blood pressure, followed by glomerular damage. These data suggest that reduced renal NO synthetic capacity may contribute to starting or maintaining intraglomerular hypertension.

Besides its vasodilatory properties, NO inhibits mesangial cell proliferation [53] and extracellular matrix synthesis [54, 55]. Thus, dietary supplementation with L-arginine to rats with RMR limited the early phase of cell proliferation in the remnant glomerulus [56].

It has been proposed that NO production by iNOS in medullary thick ascending limbs serves to maintain and regulate medullary blood flow and oxygenation [57]. There is also evidence that NO, through the generation of cGMP, inhibits renal tubular Na^+,K^+-ATPase [58], resulting in diuresis and natriuresis [58, 59]. This mechanism could at least partly explain the antidiuretic and antinatriuretic actions of low doses of NOS inhibitors in the absence of any measurable effect on either RBF or GFR [60]. It is tempting to speculate that a decrease in tubular NO synthesis in RMR rats may cause salt retention and aggravate glomerular and systemic hypertension. This is supported by a recent report that a high salt diet exacerbated the blood pressure elevation and worsened both glomerulosclerosis and interstitial fibrosis in RMR rats [61].

Diminished renal NO formation could therefore play some role in the hemodynamic and non-hemodynamic abnormalities reported in the model of extreme renal ablation [18] that precede and eventually lead to the development of glomerulosclerosis and renal scarring. Present findings of a linear inverse correlation between the amount of NO formed by renal tissue and indexes of renal damage in rats with remnant kidney are consistent with this interpretation. Also relevant is the fact that administration of L-arginine to rats with subtotal nephrectomy limits renal disease progression, as reflected by less proteinuria and less structural damage [20]. Possibly L-arginine given to uremic rats enhances iNOS activity in the kidney, which is totally dependent on substrate availability [1, 2].

We found that unlike in the kidney, in the systemic circulation of RMR rats the synthesis of NO increased, as reflected by higher than normal plasma NO_2^-/NO_3^- concentrations. In addition, NADPH-diaphorase documented an appreciable increase of NOS activity in the aorta of uremic animals, suggesting that enhanced systemic NO in RMR rats derives at least in part from higher than normal synthetic activity in large vessels. Increased diaphorase staining paralleled the immunohistochemistry finding of higher ecNOS and iNOS expression in the aorta of RMR rats.

The enhanced ecNOS expression and larger amount of NO formed in large vessels of RMR rats can serve as a defense mechanism to limit systemic blood pressure elevation in this model. This possibility is supported by a recent study showing that L-NAME treatment aggravate arterial hypertension in rats with RMR [62]. A similar system may operate even in non-uremic models of hypertension [63], as documented by findings that NO synthesis (measured by conversion of radiolabeled L-arginine to L-citrulline) in aortic segments from spontaneously hypertensive rats is twice that in aorta from normotensive controls [64].

Causes of high vascular ecNOS expression in RMR, as in other experimental models of hypertension, very likely include changes in shear stress since endothelial cells appear to release NO in response to changes in blood flow [65], and ecNOS mRNA and the corresponding protein are induced in endothelial cells after exposure to fluid shear in a cone-and-plate apparatus [66]. A similar mechanism operates in hypertension where pulsatile stretch and distension of the arterial wall favor NO release, as indicated by recent findings that cyclic strain induces the expression of ecNOS transcripts and protein levels in cultured endothelial cells [67].

Besides ecNOS, in this study we found an increased expression of iNOS in systemic vessels of RMR rats that was not confined to the endothelium but was also detectable in smooth muscle. These results are consistent with a previous study showing that plasma from uremic humans stimulated NO synthesis (conceivably by induction of iNOS activity) more than normal plasma [25]. The stimulatory activity of uremic plasma was attributed to TNFα, a potent inducer of iNOS [29], that circulates in increased amounts in the plasma of patients with chronic renal failure either undialyzed [68] or on maintenance hemodialysis, [25, 68]. Both in uremic animals [69] and humans [70] plasma cGMP is considerably higher than normal and there is evidence that cGMP stimulates NO formation through induction of iNOS mRNA [71]. The stimulatory activity is associated with enhanced TNFα mRNA expression and can be substantially prevented by antibodies that neutralize TNFα [71]. It is therefore possible that excessive formation of cGMP in uremia is responsible for iNOS induction in systemic vessels, an effect that appears to be mediated by TNFα.

Besides its vasoactive properties, NO inhibits platelet aggregation *in vitro* [1, 2] and platelet adhesion to cultured endothelial cells [1, 2]. The *in vivo* counterpart of this activity is the prolongation of skin bleeding time observed in healthy volunteers given NO by inhalation [72]. Since uremic animals and humans do have

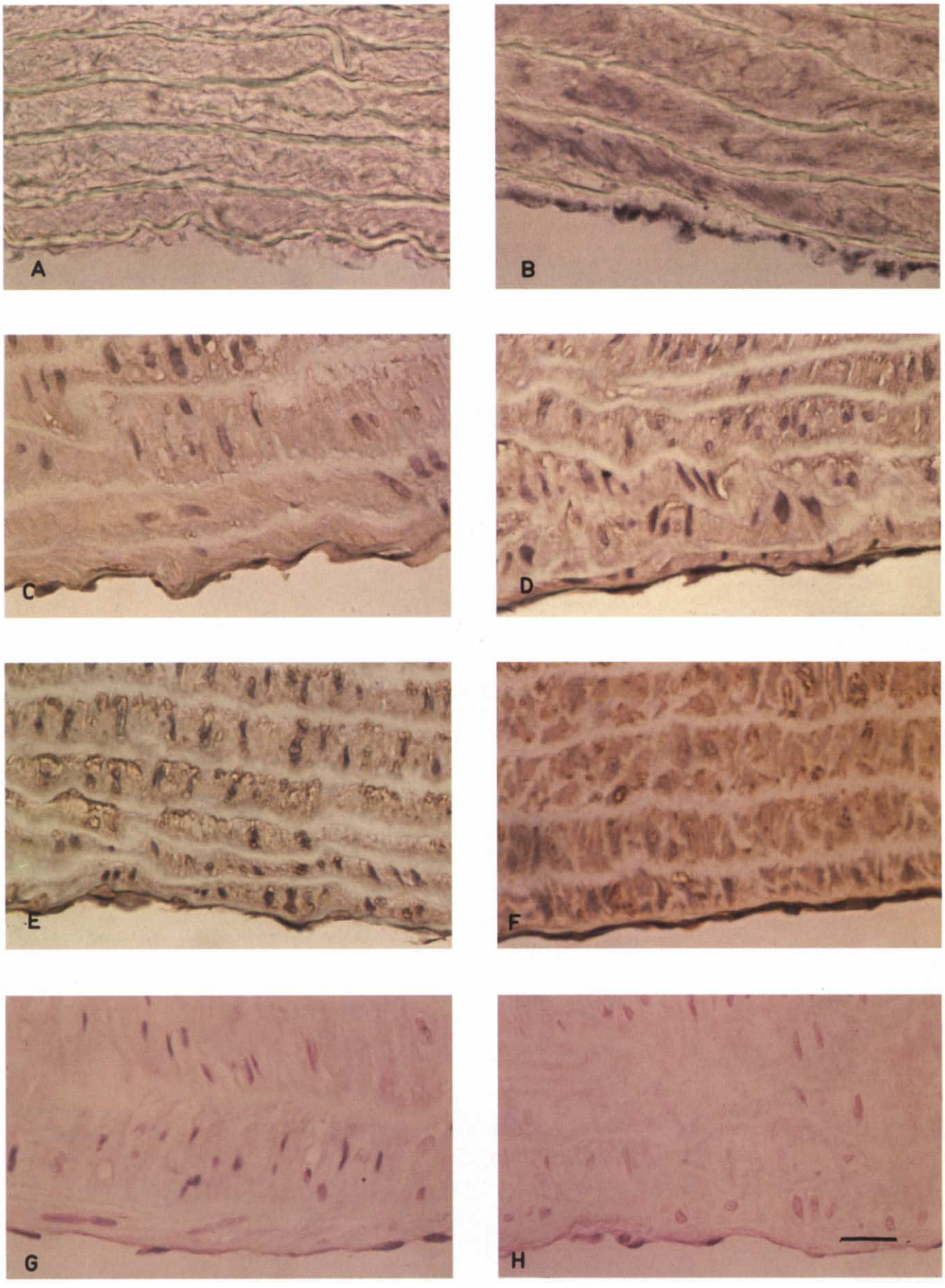


Fig. 7. Localization of NOS isoforms in the thoracic aortas. Sections from CTR and RMR rats studied 90 days after surgery are presented in panels *A, C, E* and panels *B, D, F*, respectively. *A* and *B*. Histochemical staining for NOS enzymatic activity by the NADPH-diaphorase method. *C* and *D*. Immunoperoxidase with a specific antibody against ecNOS. *E* and *F*. Immunoperoxidase with a specific antibody against iNOS. NOS activity as well as ecNOS and iNOS staining are markedly increased on arterial endothelium in the RMR aortas. In RMR aortas vascular smooth muscle cell iNOS signal is focally increased too. No staining is seen when primary antibodies are omitted (panel G, ecNOS; panel H, iNOS; magnification ×535; bar = 20 μm). Publication of this figure in color was made possible by a grant from Hoechst Marion Roussel, Frankfurt am Main, Germany.

a prolonged bleeding time [22, 23] that is normalized in animals by the NO antagonist L-NMMA [24], it seems logical to postulate that excessive systemic NO formation may play a role in the abnormal primary hemostasis of uremia.

In summary, in the present paper we present an integrated view of the potential role of the L-arginine-NO pathway in the physiology and pathology of the uremic condition.

In the kidney, with the progressive development of renal insufficiency, less and less NO is formed locally, apparently as a consequence of a time-dependent progressive reduction in the amount of the iNOS molecule expressed in the kidney. In this setting a marked reduction of NO, in the face of continuous local generation of vasoconstrictor and promitogenic substances, may well contribute to intraglomerular capillary hypertension and cell proliferation.

By contrast, uremia appears to involve an abnormally large amount of NO being released into the systemic circulation, and our present study indicates consistently that this is a consequence of excessive generation of the gas by systemic vessels due to upregulation of both ecNOS and iNOS. While the increase in vascular ecNOS occurs early in response to progressive renal function deterioration, up-regulation of iNOS can only be detected in the advanced phases of the disease and is not confined to the smooth muscle cells, but also involves the vascular endothelium. We suggest that up-regulation of NO-forming enzymes might be an early defense mechanism against hypertension of uremia. On the other hand, more NO available to circulating cells may sustain the bleeding tendency, a well-known complication of uremia in experimental animals [22] and humans [23].

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