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Decreased Nitric Oxide Synthesis in Rats with Chronic Renal Failure

The present study was aimed at investigating whether an altered role of nitric oxide (NO) is involved in chronic renal failure (CRF). Rats were subjected to 5/6 nephrectomy and kept for 6 weeks to induce CRF. On the experimental day, after measurement of arterial pressure under anesthesia, the arterial blood was collected, and thoracic aorta and kidney were rapidly taken. NO metabolites (NOx) were determined in the plasma, urine, aorta and kidney. The expression of NO synthase (NOS) isozymes was determined in the kidney and aorta by Western blot analysis. The expression of NOS mRNA in the glomeruli was also determined by RT-PCR. There were significant increases in arterial pressure and serum creatinine levels in CRF. Urine NOx levels were decreased in CRF, whereas plasma NOx levels were not altered. Aorta and kidney tissue NOx levels were also decreased in CRF. The expression of endothelial constitutive (ec) and inducible (i) isoforms of NOS proteins was decreased in the kidney and aorta in CRF. Accordingly, the expression of ecNOS and iNOS mRNA was decreased in the glomeruli in CRF. In conclusion, NO synthesis is decreased in the kidney and vasculature of CRF rats.

Key Words: *Kidney Failure, Chronic; Nitric Oxide; Nitric Oxide Synthase*

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INTRODUCTION

The importance of nitric oxide (NO) in the regulation of blood pressure and renal function has been well established. The synthesis of NO is catalyzed by a family of NO synthases (NOS) that are either constitutive or inducible (1). In the kidney, both endothelial constitutive and inducible isoforms of NOS are located in the vasculature and various segments of the nephron (2-5). The local release of NO controls renal hemodynamics (6, 7) and modulates the activity of tubuloglomerular feedback (8). Therefore, an altered renal production of NO may be involved in the renal disease progression.

However, NO metabolism has not been completely understood in chronic renal failure (CRF). Several studies have shown that in the systemic circulation of uremics, either in experimental animals or humans, an excessive amount of NO is formed (9, 10). On the contrary, Vaziri et al. (11) showed a significant reduction in urinary excretion of NO metabolites and decreased expression of NOS proteins in the thoracic aorta and remnant kidney in rats with CRF.

The present study was aimed at further investigating whether and to what extent NO system is altered in CRF. NO metabolites were measured in the plasma and urine, and the expression of NOS isoforms were determined in the kidney and aorta in rats with experimental CRF.

MATERIALS AND METHODS

Animal preparation

All experiments were performed using male Sprague-Dawley rats weighing 200 to 250 g. The animals were fed on standard rat chow and water ad libitum. To induce CRF, the animals were subjected to a right nephrectomy and partial infarction of left kidney by ligation of two segmental renal arteries (five-sixths nephrectomy) under ketamine anesthesia (50 mg/kg, i.p.). They were examined 6 weeks later. A group of sham-operated rats served as control.

Experimental protocols

On the experiment day, under thiopental anesthesia (50 mg/kg, i.p.), polyethylene catheters were inserted in the right femoral artery to measure blood pressure. The urinary bladder was exposed through a small lower midline abdominal incision and was cannulated with PE 50 tubing for urine collection. After urine collection, the arterial blood was collected to determine hemoglobin,

hematocrit, blood urea nitrogen (BUN), creatinine and NOx. The kidneys and thoracic aortae were also taken and immediately frozen in liquid nitrogen. They were stored at -70℃ until used.

Colorimetric assay of nitrites

Plasma and urine concentrations of nitrites and nitrates (NOx), the stable intermediary products of NO, were determined with a colorimetric NO assay kit (Oxford Biochemedical Research Inc., Oxford, MI, U.S.A.). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrite with Griess reagent, 80 μL 3-[N-Morpholino]propanesulfonic acid (MOPS) (50 mmol/L)/EDTA (1 mmol/L) buffer and 5 μL tissue samples were added to wells. Nitrate reductase (0.01 U) and 10 μL NADH (2 mmol/L) were added to the reaction mixture, and the plate was shaken for 20 min at room temperature. Color reagents, sulfanilamide, and N-(1-Naphthyl) ethylenediamine dihydrochloride were added, and the absorbance values at 540 nm were read in a microtiter plate reader (Bio-Rad model 3550). NOx concentration was estimated from a standard curve, which was constructed with the use of standard reagents included in the assay kit.

Protein preparation and Western blot analysis

The kidneys and thoracic aortae were homogenized with Polytron homogenizer at 3,000 rpm in a solution containing 250 mmol/L sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 50 mmol/L potassium phosphate buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two consecutive low speed spins (3,000 g, 5 min; 10,000 g, 10 min). The supernatant was then centrifuged at 100,000 g for 60 min. The supernatant was used for blotting of iNOS, and the pellet was resuspended for protein blotting of ecNOS. The protein concentration of the homogenate was determined by the method of Bradford (12).

Protein samples were electrophoretically size-separated with a discontinuous system consisting of 7.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. High-range molecular weight markers (BioRad; Hercules, CA, U.S.A.) were loaded as size standard. An equivalent amount of total tissue protein (100 μg) was loaded on each lane. After separation, the proteins were electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for one hr, and incubated with a 1:2,000 dilution of monoclonal mouse anti-iNOS and anti-ecNOS antibodies (Transduction Laboratories; Lexington, KY, U.S.A.) in 2% nonfat milk/TBST for one hour at room temperature. The membranes were then incubated with a horseradish peroxidase-labeled goat anti-mouse IgG (1:1,000) or goat anti-rabbit IgG in 2% nonfat milk in TBST for 2 hr. The bound antibody was detected by enhanced chemiluminescence on X-ray film or hyperfilm (Amersham; Little Chalfont, Buckinghamshire, England). The membranes were stripped between incubations with different antibodies in a Tris-buffered solution containing 2% sodium dodecyl sulfate and 100 mmol/L β-mercaptoethanol at 50℃.

Analysis of NOS mRNA expression by RT-PCR

The glomerulus was isolated by graded sieve methods (13). The kidney was decapsulated and the cortex was filtered through standard sieves (250, 150, 125, and 75 μM) consecutively. Glomeruli on 75 mm sieve were collected by centrifugation (1,000 g for 15 min at 4℃). Total RNA was isolated from the aorta according to the protocols of Ultraspec™ RNA isolation system (Biotecx Laboratories; Houston, TX, U.S.A.). RNA concentration was determined by the absorbance read at 260 nm (Ultraspec 2000, Pharmacia Biotech, Cambridge, England). For the RT step, 1 mg total RNA was incubated with 200 U of reverse transcriptase (Gibco BRL; Grand Island, NY, U.S.A.), Rnasin (10 U), dNTP mix (10 mmol/L), DTT (0.1 mol/L), $MgCl_2$ (25 mmol/L), oligo (dT) (0.5 μg/μL), and reaction buffer [200 mmol/L Tris-HCl (pH 8.4), 500 mmol/L KCl] in a final volume of 20 μL.

PCR cycles were performed in a DNA thermal cycler (M.J. Research, Watertown, MA, U.S.A.) with the following profile: denaturation 1 min at 94℃, annealing 1 min at 58℃ for iNOS primers and 1 min extension step at 72℃; denaturation 45 sec at 94℃, annealing 45 sec at 62℃ for ecNOS primers and 1.5 min extension step at 72℃; denaturation 45 sec at 94℃, annealing 45 sec at 56℃ for β-actin primers and 1.5 min extension step at 72℃. The last cycle was ended with 5 min of elongation at 72℃.

iNOS, ecNOS and β-actin primers were prepared as previously reported (3, 14, 15). The iNOS cDNA was amplified using primers (sense primer: 5'-TGTTCCAC-CAGGAGATGTTG-3': and antisense primer: 5'-CTCC-TGCCCACTGAGTTCGTC-3'), allowing the amplification of 576 bp fragments. The ecNOS cDNA was amplified using primers (sense primer: 5'-ACGGAGCAGCA-AATCCAC-3': and antisense primer: 5'-CAGGCTGCA-GTCTTTGATC-3'), allowing the amplification of 819 bp fragments. The β-actin cDNA was amplified using primers (sense primer: 5'-GACTACCTCATGAAGATCCTG-ACC-3': antisense primer: 5'-TGATCTTCATGGTGCT-

AGGAGCC-3'), allowing the amplification of 423 bp fragments. The PCR contained 20 pmole of each primer, 250 μmol/L dNTP mix, 1.5 mmol/L $MgCl_2$, 40 mmol/L KCl reaction buffer [50 mmol/L Tris-HCl (pH 8.3)], and 1U of *Taq* polymerase in a final volume of 20 μL.

The PCR products were size fractionated by 1.5% agarose gel electrophoresis, and visualized under UV light with ethidium bromide staining. NOS and β-actin cDNA were quantified by IMAGER™ & 1D MAIN (Bioneer, Korea). The NOS cDNA was normalized by comparison with β-actin cDNA.

Statistical analysis

Results are expressed as means±SEM. The statistical significance of differences between the groups was determined by unpaired t-test.

RESULTS

NO metabolites

The initial body weight was comparable between CRF and control groups (225±6 vs. 228±7 g). However, it was significantly lower in CRF at the conclusion of the study. Table 1 shows various functional data in control and CRF. The arterial blood pressure was significantly increased in CRF. Serum creatinine levels were significantly increased and severe proteinuria was noted. Hemoglobin as well as hematocrit was decreased.

No significant differences were noted in plasma NOx levels between CRF and control groups (Fig. 1). However, urine NOx levels were significantly lower in CRF (Fig. 1). NOx levels in the aorta and kidney were also decreased in CRF (Fig. 2).

NOS expression in the kidney and aorta

Two isoforms of NOS (ecNOS and iNOS) were determined in the kidney and thoracic aorta by Western blot

Table 1. General and laboratory parameters

	Control	CRF
Body weight (g)	485±6	429±10*
MAP (mmHg)	94.5±7.8	152.1±11.3†
Hemoglobin (g/dL)	14.4±3.1	10.9±0.8†
Hematocrit (%)	43.7±0.7	33.7±0.8†
Serum creatinine (mg/dL)	0.40±0.1	0.79±0.2†
Proteinuria (mg/dL)	6±1	528±97†

Data are presented as mean±SEM. N=12 each
CRF, chronic renal failure; MAP, mean arterial pressure
*$p<0.05$, †$p<0.01$, †$p<0.001$, compared with control

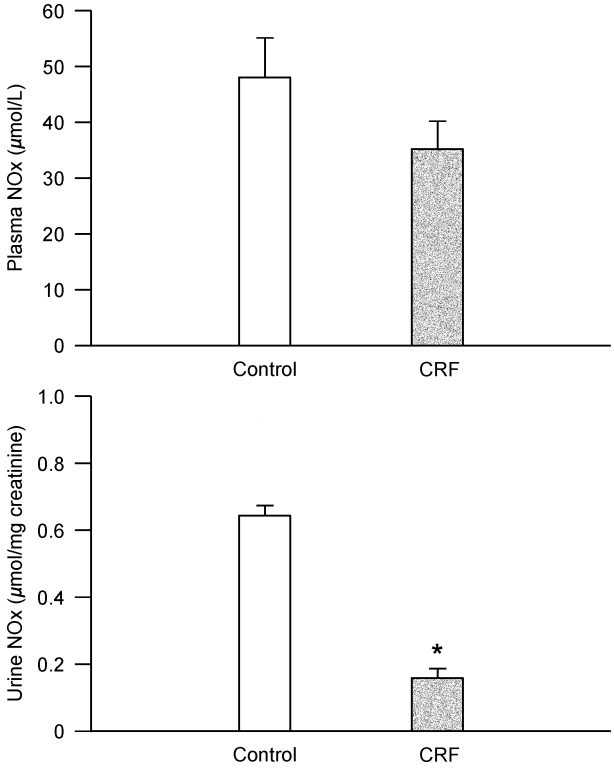

Fig. 1. NO metabolites in the plasma and urine. Each column represents mean±SEM of 8 experiments. *$p<0.05$ compared with the control.

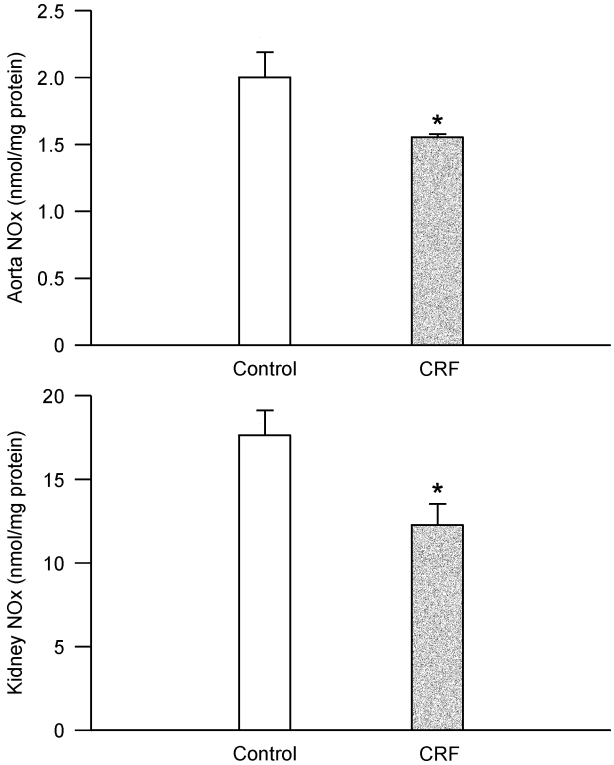

Fig. 2. NO metabolites in the aorta and kidney. Each column represents mean±SEM of 8 experiments. *$p<0.05$ compared with the control.

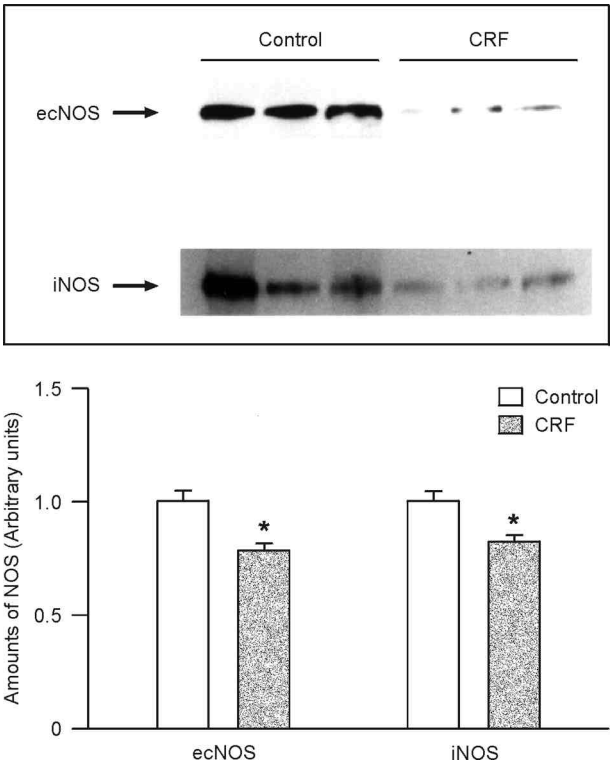

Fig. 3. NOS protein expression in the thoracic aorta. Representative autoradiograms of ecNOS and iNOS, and their densitometric analysis of 10 rats are shown. *$p<0.05$ compared with control.

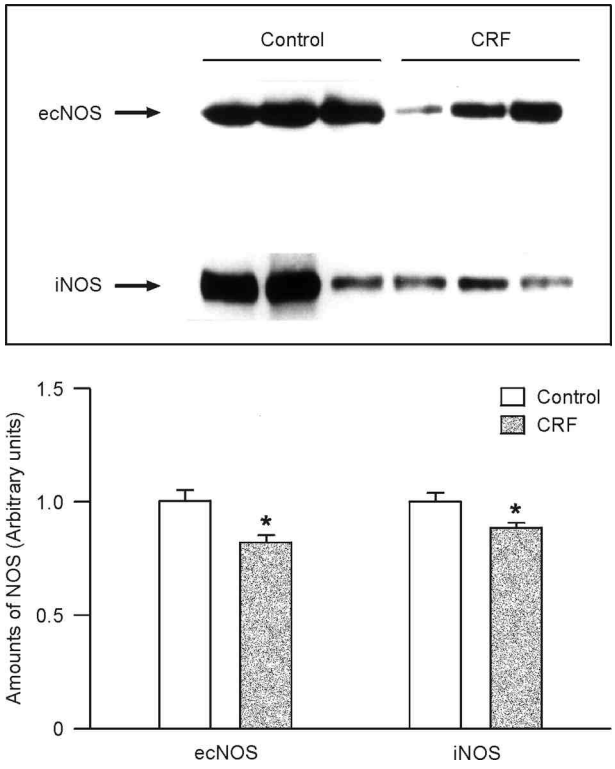

Fig. 4. NOS protein expression in the kidney. Representative autoradiograms of ecNOS and iNOS, and their densitometric analysis of 10 rats are shown. *$p<0.05$ compared with control.

analysis. Anti-ecNOS and anti-iNOS antibodies recognized protein bands with molecular sizes of 140 and 130 kDa, respectively. Fig. 3 shows densitometric analysis of NOS isoforms in the thoracic aorta. The ecNOS expression was decreased to 75.1±5.5% of the control in CRF. There were also significant decreases in iNOS protein expression in CRF, being decreased to 70.2±4.7% of the control.

Fig. 4 shows densitometric analysis of NOS isoforms in the kidney. The ecNOS expression was decreased in CRF to 80.1±4.3% of the control. There were also significant decreases in iNOS protein expression in CRF, being decreased to 85.0±3.5% of the control.

The expression of NOS mRNA was determined in the glomeruli by RT-PCR. The ecNOS expression was decreased in CRF to 82.3±2.6% of the control.. The iNOS protein expression was also decreased in CRF to 83.4± 2.5% of the control (Fig. 5).

DISCUSSION

CRF animals showed a significant rise in arterial pressure, coupled with development of anemia. The hypertension was more marked in the present study, in which

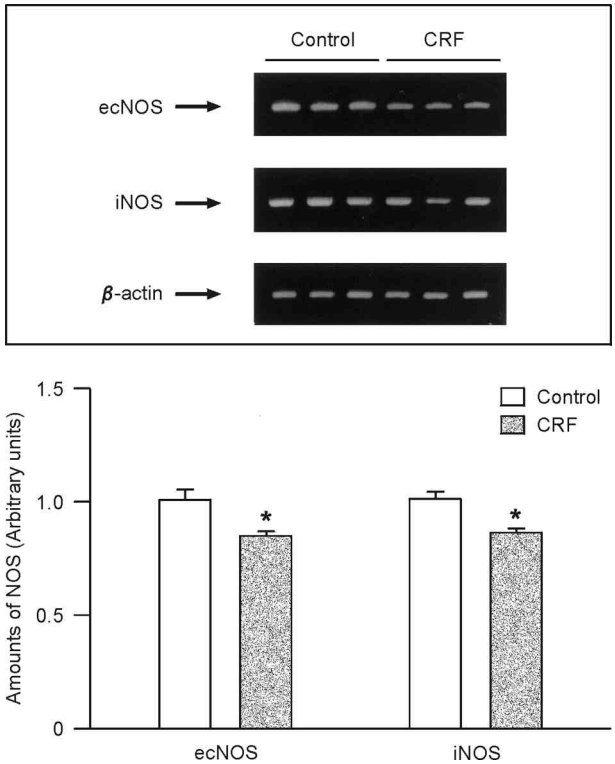

Fig. 5. NOS mRNA expression in the glomeruli. Representative autoradiograms of ecNOS and iNOS, and their densitometric analysis of 10 rats are shown. *$p<0.05$ compared with control.

CRF was associated with infarction of the remaining kidney, than in the CRF model of surgical ablation of the kidney (16). This finding supports the previous notion that renin and aldosterone are important factors contributing to the development of hypertension in infarction model of CRF (17). Glomerular filtration rate as assessed by creatinine clearance was also decreased in CRF, the degree of which was comparable with that observed in the previous studies (9, 16).

Urinary NOx concentrations were significantly decreased along with parallel changes of tissue NOx contents in the remnant kidney, being in agreement with previous observations made by Vaziri et al. (11). Urinary excretion of NOx may depend on various factors such as glomerular filtration, tubular handling and de novo synthesis of NO. If systemic NO generation were normally maintained in the presence of decreased GFR, the plasma NOx would have piled up to show an increase as urea nitrogen. However, plasma NOx levels were not significantly increased despite the impaired glomerular fitration rate. Therefore, the normal plasma NOx concentration may be a reflection of a depressed NO generation in CRF.

It has been previously noted that progressive nephropathies are associated with a reduced capacity of the kidney to generate NO (18, 19). Conversely, it has been shown that chronic administration of NO donors retarded progressive deterioration of renal function and structure in CRF (20). Reyes et al. (18) also observed that NO precursor L-arginine increased glomerular filtration rate and effective renal plasma flow, reduced proteinuria, and preserved renal morphology in rats with CRF.

It is suggested that a decreased NO formation is responsible for renal derangements in CRF. A prolonged inhibition of NO synthesis has been noted to stimulate the renal activity of Na^+, K^+-ATPase (21). Therefore, a decrease in renal NO synthesis in CRF may be causally related with salt retention, and development of systemic hypertension. The vascular NOx contents were also decreased along with decreased NOS expression in CRF. Decreased NO synthesis in the vasculature and kidney may in concert contribute to the development of hypertension in CRF.

One may argue that the plasma and urine NOx concentrations cannot give a precise measure of endogenous NOx production when dietary nitrate intake is uncontrolled (22). Moreover, the rats with CRF may have reduced food intake possibly due to anorexia or uremia. A reduced food intake may result in decreases in plasma levels and urinary excretion of NOx. However, the expression of ecNOS and iNOS proteins was significantly decreased along with parallel changes of renal tissue NOx contents in CRF. It is unlikely that a changed dietary intake is responsible for the altered NO activity in the present study.

The depressed NO system has been ascribed to an accumulation of endogenous NOS inhibitors in uremia. An increased concentration of methylated arginine and guanidino compounds with NOS inhibitory properties has been shown in patients with end-stage renal disease (23, 24). However, ecNOS and iNOS mRNA were reduced in the glomeruli in CRF in the present study. The reduction of ecNOS and iNOS expression is consistent with that observed by Vaziri et al. in the kidney and vascular tissue in CRF (11). It is likely that loss of functional endothelium by glomerular sclerosis reduces ecNOS expression. In addition, in the glomeruli soon after the surgical ablation of renal mass, inflammatory mediators such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) may be formed in excessive amounts, inhibiting iNOS mRNA expression and thus NO synthesis (25, 26). Taken together, it is suggested that NOS availability is decreased in CRF as opposed to a mere competitive inhibition of the pre-existing enzyme.

In summary, the expression of constitutive and inducible isoforms of NOS and NO formation in the kidney and vasculature were decreased in rats with CRF.

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