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Alteration of Nitric Oxide Synthase and Guanylyl Cyclase Activity in Rats with Ischemia/Reperfusion Renal Injury

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The present study was designed to investigate the protein expression of nitric oxide synthase (NOS) and guanylyl cyclase (GC) activity in ischemia/perfusion (I/R) renal injury in rats. Renal I/R injury was experimentally induced by clamping the both renal pedicle for 40 min in Sprague-Dawley male rats. The renal expression of NOS isoforms was determined by Western blot analysis, and the activity of guanylyl cyclase was determined by the amount of guanosine 3', 5'-cyclic monophosphate (cGMP) formed in response to sodium nitroprusside (SNP), NO donor. I/R injury resulted in renal failure associated with decreased urine osmolality. The expression of inducible NOS (iNOS) was increased in I/R injury rats compared with controls, while endothelial NOS (eNOS) and neuronal NOS (nNOS) expression was decreased. The urinary excretion of NO metabolites was decreased in I/R injury rats. The cGMP production provoked by SNP was decreased in the papilla, but not in glomerulus. These results indicate an altered regulation of NOS expression and guanylyl cyclase activity in I/R-induced nephropathy.

Key Words: Nitric oxide synthase, Ischemic/perfusion injury, cGMP

INTRODUCTION

Ischemia/reperfusion (I/R) renal injury is a major cause of acute renal failure (ARF) after major surgery or renal transplantation (Dragun et al, 2000). The mechanisms underlying renal I/R injury are complex, including ATP depletion, accumulation of intracellular Ca^{2+}, mitochondrial dysfunction, multiple enzyme production and reactive oxygen species (Spandou et al, 2006). Nirtic oxide (NO) has also been suggested, but the role of NO in I/R-induced ARF is controversial. NO synthase (NOS) inhibitor was reported to prevent I/R injury in rat proximal tubules, thus suggesting that NO is synthesized in proximal tubules and is involved in tubular I/R injury (Yu et al, 1994). In contrast, it has also been noted that the inhibition of NO production with an NOS inhibitor significantly deteriorated renal function of the postischemic kidney in anesthetized rats, whereas pretreatment with L-arginine, NO precursor, abolished the NOS inhibitor-induced deterioration of renal function (Chintala et al, 1993). These seemingly opposite effects of NO may depend upon the site of NO production as well as the existence of different isoforms of NOS in mammalian kidney.

NO induces the generation of cGMP, which is activated by different isoforms of guanylyl cyclase (GC) (Forstermann et al, 1986; Chinkers et al, 1989), and also activates the cytoplasmic heterodimeric haemoprotein, soluble GC (Hobbs, 1997). Stimulation of either GC results in the conversion of GTP to the intracellular second messenger cGMP, which exerts potent effects on kidney, especially on renal blood flow (RBF), glomerular hemodynamics, and urinary sodium excretion ($U_{Na}V$) (Alberola et al, 1992; Majid et al, 1993). The overall activity of the NO system may be determined by the tissue expression of different isoforms of NOS as well as the soluble GC acticity.

Therefore, altered protein expression of different NOS isoforms in kidney and soluble GC activity might provide some important clues regarding the pathophysiology in ARF.

METHODS

Animals

Sprague-Dawley male rats, weighing 180 to 200 g, were used. The experimental procedure conformed to the institutional guidelines for experimental animal care and use. To induce I/R injury, both renal pedicles were clamped for 40 min under ketamine anesthesia (50 mg/kg, IP). Control rats underwent sham operation without clamping the renal pedicle. They were returned to the metabolic cages and were kept for 48 hr to collect urine. On the experimental day, the rats were decapitated under a conscious state, and trunk blood was taken. The right kidney was rapidly removed, dissected into three zones [cortex and outer stripe of outer medulla (cortex/OSOM), inner stripe of outer medulla (ISOM) and inner medulla], and processed for semi-

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ABBREVIATIONS: I/R, ischemia/reperfusion; ARF, acute renal failure; NO, nitric oxide; GC, guanylyl cyclase; RBF, renal blood flow; $U_{Na}V$, urine sodium excretion; ANP, atrial nitriuretic peptide; SNP, sodium nitroprusside.

quantitative immunoblotting as described below. The left kidney was rapidly taken and kept at −70°C until assayed.

Protein preparation and Western blot analyses

The dissected renal cortex/OSOM, ISOM, and inner medulla were homogenized (Ultra-Turrax T8 homogenizer, IKA Labortechnik, Staufen, Germany) in ice-cold isolation solution (pH 7.2) containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 4,000 g for 15 min at 4°C to remove whole cells, nuclei and mitochondria, and the supernatants were pipetted off and kept on ice. The total protein concentration was measured (Pierce BCA protein assay reagent kit, Pierce, Rockford, IL). All samples were adjusted with isolation solution to have same final protein concentrations, solubilized at 65°C for 15 min in SDS-containing sample buffer, and then stored at −20°C. To ascertain equal protein loading, an initial gel was stained with Coomassie Blue. SDS-PAGE was performed on 8% polyacrylamide gels. The proteins were transferred by gel electrophoresis (BioRad Mini Protean II) onto nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hr. The membranes were then incubated overnight at 4°C with anti-mouse polyclonal antibodies against endothelial NOS (eNOS), inducible NOS (iNOS), and nNOS (1 : 2,500; Transduction Laboratories, Lexington, KY). The membranes were then incubated for 2 hr with horseradish peroxidase-labeled goat anti-mouse IgG (1 : 1,000). The labeling was visualized by enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) and analyzed by Image Reader (LAS-3000 Imaging System, Fuji Photo Film).

Colorimetric assay of nitrite/nitrate

As an index of NO synthesis in tissues, its stable metabolites (nitrite/nitrate, NOx) in urine were measured by a colorimetric NO assay kit (Oxford Biochemical Research Inc., Oxford, MI). A microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrite with Griess reagent, 80 μl of MOPS (50 mmol/L)/EDTA (1 mmol/L) buffer and 5 μl of urine samples were added to wells. Nitrate reductase (0.01 U) and 10 μl of 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 then absorbance at 540 nm was 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.

Soluble guanylyl cyclase activity

The glomerulus was isolated from cortex by graded sieve methods (Torres et al, 1978). In brief, the kidney was decapsulated, and the cortex was consecutively filtered through standard sieves (250, 150, 125, and 75 μm). The glomeruli on the 75 μm sieve were collected by centrifugation (1,000 g for 15 min at 4°C). The glomerulus or papilla was homogenized in an ice-cold homogenization buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM ethylenediaminetetraacetate, 0.2 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose). The homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was centrifused again at 100,000 g for 60 min at 4°C. The resulting supernatant was used for determination of soluble guanylyl cyclase activity. Protein concentrations were determined using bicinchoninic acid assay kit (Bio-Rad, Hercules, CA, USA).

Soluble GC activity was measured in the glomerulus and papillary supernatant aliquots by the method of Winquist et al. (Winquist et al, 1984) with a slight modification. The aliquots were incubated for 15 min at 37°C in 50 mmol/L Tris HCl (pH 7.6) containing 1 mmol/L 3-isobutyl-1-methylxanthine, 1 mmol/L GTP, 1 mmol/L ATP, and 15 mmol/L $MgCl_2$ in the presence of sodium nitroprusside (SNP; 10^{-7} to 10^{-3} mol/L). Incubation was stopped by adding ice-cold 50 mmol/L sodium acetate (pH 5.0) and boiling for 5 min. Samples were then centrifuged (10,000 g for 10 min at 4°C).

cGMP in the supernatant was measured by equilibrated radioimmunoassay. In brief, standards and samples were introduced in a final volume of 100 μl of 50 mmol/L sodium acetate buffer (pH 4.8), and then 100 μl of dilute cGMP antiserum (Calbiochem-Novabiochem, San Diego, CA, USA) and iodinated cGMP (10,000 cpm/100 ul, specific activity) were further added. Results are expressed as pmol of cGMP generated/mg protein/min.

Statistical analysis

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

Table 1. Changes in renal function

	Control (n=5)	Exp (n=5)
Body weight (g)	250±4.08	251±11.87
UO (ml/day)	11.25±0.85	48.5±2.81*
P-Cr (umol/L)	0.26±0.02	2.28±0.47*
Ccr (ml/min)	5.68±0.59	0.20±0.04*
$U_{Na}\times UO$ (mmol)	3.99±0.23	3.94±0.60
FE_{Na} (%)	0.90±0.08	6.12±1.5*
FE_K (%)	14.00±1.35	79.79±17.70*
U-Osm (mosm/KgH_2O)	2276.2±15.43	523.25±22.91*
P-Osm (mosm/KgH_2O)	298.2±1.88	341.25±7.91*
U/P osm	7.64±0.09	1.54±0.07*
T^cH_2O (μl/min/kg)	209.76±11.19	36.24±6.23*

Values are expressed as mean±SEM. These values were obtained on the last day of experiments. UO, urine output; P-Cr, plasma creatinine; Ccr, creatinine clearance; $U_{Na}\times UO$, rate of urinary sodium excretion; FE_{Na}, fractional excretion of sodium into urine; FE_K, fractional excretion of potassium into urine; U-osm, urine osmolality; P-osm, plasma osmolality; U/P osm, urine-to-plasma ratio of osmolality, T^cH_2O, solute-free water absorption. *$p<0.05$, when experimental group was compared with control group.

Table 2. The expression of iNOS, eNOS and nNOS proteins in the kidney

		Control (n=5)	Exp (n=5)
iNOS	Cortex/OSOM	1.00±0.11	1.19±0.22
	ISOM	1.00±0.12	1.89±0.93*
	Inner medulla	1.00±0.24	1.46±0.52*
eNOS	Cortex/OSOM	1.00±0.39	0.71±0.12
	ISOM	1.00±0.05	0.41±0.09*
	Inner medulla	1.00±0.01	0.74±0.12
nNOS	Cortex/OSOM	1.00±0.21	0.54±0.07*
	ISOM	1.00±0.11	0.59±0.12*
	Inner medulla	1.00±0.10	0.38±0.09*

Values are expressed as mean±SEM. iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; OSOM, outer stripe of outer medulla; ISOM, inner stripe of outer medulla. *p<0.05, when experimental group was compared with control group.

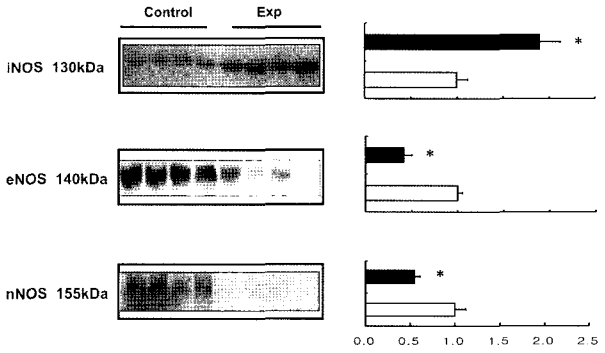

Fig. 1. Expression of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS) protein in the inner stripe of outer medulla. Data are mean±SEM of five rats for each group (open column, control; closed column, experimental). *p<0.05 vs. control.

RESULTS

Renal functions

Table 1 shows renal functional data. I/R injury resulted in a renal failure evidenced by the increase of serum creatinine concentration along with the decrease of its renal clearance. The urine volume was increased, whereas urine osmolality, urine to plasma osmolality ratio (U/Posm) and free water reabsorption were decreased, indicating impaired urinary concentration. The urinary excretion of sodium and fractional excretion of sodium were increased.

Expression of nitric oxide synthase (NOS)

The protein expression of iNOS was increased in ISOM and inner medulla in I/R injury rats compared with controls, however not in cortex/OSOM. The expression of eNOS was decreased in ISOM, but unchanged in cortex/OSOM and inner medulla. The protein expression of nNOS was decreased in cortex/OSOM, ISOM, and inner medulla (Table 2, Fig. 1).

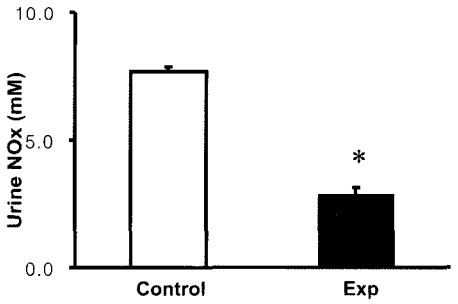

Fig. 2. Amount of urinary nitric oxide metabolite (NOx). Data are mean±SEM of five rats for each group. *p<0.05 vs. control.

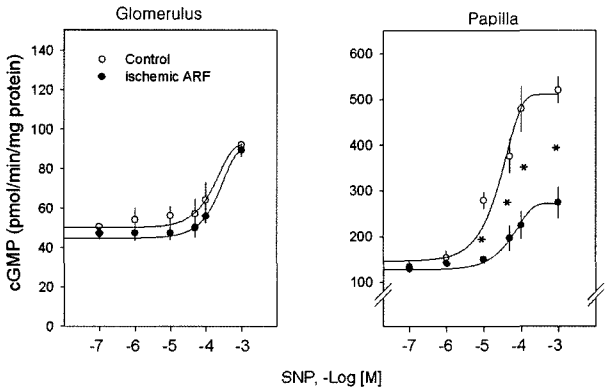

Fig. 3. cGMP production provoked by SNP in the glomerulus and papilla. Each point represents mean±SEM of three rats. *p<0.05 vs. control.

Urinary excretion of nitric oxide metabolite (NOx)

The amount of urinary NOx was decreased in I/R injury rats compared with controls (Fig. 2).

Guanylyl cyclase activity

Soluble GC activity evoked by sodium nitroprusside (SNP) was not changed in the glomerulus, whereas it was decreased in the papilla of I/R injury rats compared with controls (Fig. 3).

DISCUSSION

NO plays important physiological roles in the regulation of blood flow and Na balance via the Na-K ATPase in the kidney (Guzman et al, 1995). On the other hand, NO has a cytotoxic effect on renal tubular cells (Yaqoob et al, 1996). This paradoxical effect has been attributed to the existence of different isoforms of NOS in mammalian kidney. NO is synthesized by endothelial and neuronal NO synthase isoforms localized in vascular endothelium and macular densa

(Bachmann et al, 1995), and renal vascular regulation is mainly governed by NO (Baylis et al, 1990). Inhibition of constitutive isoforms of NOS may contribute to the development of endothelial cell dysfunction in I/R injury. The constitutive NOS activation is critical for turning on heme-containing enzymes such as soluble guanylyl cyclase, mediation of vasorelaxation, and anti-apoptosis and anti-oxidant phenotype (Goligorsky et al, 2004). It has been known that ischemic kidneys do not respond to acetylcholine, but rather show an increase in vascular resistance (Conger et al, 1981). The lack of responsiveness to acetylcholine in rats with acute renal failure is one of many examples of poor responses to endothelium-dependent vasodilators in this condition. The question is whether this phenomenon is a reflection of inhibition of the enzyme or rather its maximal stimulation and possible reduction in NO availability. In the present study, protein expressions of constitutive isoforms of NOS as well as soluble GC activity were found to decrease in I/R rats, suggesting that decreased production of NO and blunted responsiveness to NO may play a role in vascular endothelial dysfunction and ischemic damage to the renal parenchyma in I/R injury induced acute renal failure.

On the other hand, iNOS is present in the rat proximal tubule and inner medullary collecting duct (Markewitz et al, 1993) and has been suggested as a key regulator of inflammatory processes (Maeda & Akaike, 1998) such as septic shock (Titheradge, 1999) or ischemia/reperfusion injury (del Zoppo et al, 2000). Sustained, high-output generation of NO by iNOS, depending on the cellular context, may turn on sequelae as broad as lipid peroxidation and DNA damage (Davis et al, 2001). Recent data provided evidence that activation of iNOS, thus leading to excessive NO production, causes tubular cytotoxicity and aggravates renal failure (Goligorsky et al, 2004). Consistent with this, iNOS expression was found to increase in I/R rats in the present study. These findings suggest that NO generated by iNOS contributes to renal tubular cytotoxicity.

In the present study, the urinary NOx excretion which represents the total NO production in the kidney was decreased along with the imbalanced expression of NOS isoforms. Regardless of enhanced iNOS expression, the decreased NO production can be interpreted to mean that decreased activity of constitutive NOS dominated overall iNOS activity. The imbalance between the expression and activity of inducible and constitutive isoforms of NOS is an important contributor to the pathophysiology in acute renal failure, because effects of NO effects are heterogenous, depending on the site of its production, duration of its effects, and the level of reactive oxygen intermediates concomitantly present.

In I/R injury, inflammatory reactions are most pronounced in the outer medulla, a part of the kidney that is extremely susceptible to hypoperfusion and hypoxic damage (Ysebaert et al, 2000). Other compartments such as the corticomedullary junction are also injured, however, iNOS and eNOS in this study did not show any significant difference in cortex/OSOM. This anatomical heterogeneity in the expression of NOS isoforms may be associated with severity of segmental renal injury.

NO induces vasodilation by stimulation of soluble guanylyl cyclase, which catalyzes the synthesis of cyclic guanosine monophosphate (cGMP) (Moro et al, 1996). sGC is present in the cytosol as a heterodimer, consisting of α and β subunits (Hobbs, 1997). In the present study, cGMP production in response to SNP was decreased in the papilla, but not in glomerulus. Segmental differences of impaired GC activity may attributed to segmental differences in the ischemic renal injury. If this blunted generation of cGMP occurs in response to NO in the renal vasculature, an opposed vasoconstriction during I/R renal injury would contribute to the pathogenesis of acute renal failure.

In summary, the activity of constitutive NOS was suppressed, while the expression of iNOS was enhanced. The functional consequences of such an imbalance could explain peculiar vascular and tubular phenomena in I/R-induced nephropathy.

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