

Altered Regulation of Renal Nitric Oxide and Atrial Natriuretic Peptide Systems in Lipopolysaccharide-induced Kidney Injury

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Nitric oxide (NO) and atrial natriuretic peptide (ANP) may induce vascular relaxation by increasing the production of cyclic guanosine monophosphate (cGMP), an important mediator of vascular tone during sepsis. This study aimed to determine whether regulation of NO and the ANP system is altered in lipopolysaccharide (LPS)-induced kidney injury. LPS (10 mg · kg⁻¹) was injected in the tail veins of male Sprague-Dawley rats; 12 hours later, the kidneys were removed. Protein expression of NO synthase (NOS) and neutral endopeptidase (NEP) was determined by semiquantitative immunoblotting. As an index of synthesis of NO, its stable metabolites (nitrite/nitrate, NO_x) were measured using colorimetric assays. mRNA expression of the ANP system was determined by real-time polymerase chain reaction. To determine the activity of guanylyl cyclase (GC), the amount of cGMP generated in response to sodium nitroprusside (SNP) and ANP was calculated. Creatinine clearance decreased and fractional excretion of sodium increased in LPS-treated rats compared with the controls. Inducible NOS protein expression increased in LPS-treated rats, while that of endothelial NOS, neuronal NOS, and NEP remained unchanged. Additionally, urinary and plasma NO_x levels increased in LPS-treated rats. SNP-stimulated GC activity remained unchanged in the glomerulus and papilla in the LPS-treated rats. mRNA expression of natriuretic peptide receptor (NPR)-C decreased in LPS-treated rats, while that of ANP and NPR-A did not change. ANP-stimulated GC activity reduced in the glomerulus and papilla. In conclusion, enhancement of the NO/cGMP pathway and decrease in ANP clearance were found play a role in the pathogenesis of LPS-induced kidney injury.

Key Words: Lipopolysaccharide, Atrial natriuretic peptide, Nitric oxide, Guanylyl cyclase

INTRODUCTION

Sepsis and its common complication, septic shock, are generally induced by the action of endotoxins or lipopolysaccharide (LPS), originating from gram-negative bacteria [1]. LPS is commonly used to induce endotoxemia or sepsis in rodent models both in vivo and in vitro, and an LPS-induced decrease in blood volume may lead to dysfunction or failure of major organs [2].

Sepsis alters the concentrations of nitric oxide (NO) in plasma and endothelial cells [3,4]. NO is a free radical released from various types of cells such as macrophages, cardiac myocytes, and vascular smooth muscle and glial cells. Several studies have suggested that overproduction of NO plays a major role in the pathophysiology of septic shock, and induction of nitric oxide synthase (NOS) with consequent excessive NO formation has been proposed as a major factor in pathologic vasodilatation and tissue damage [4-6].

The role of NO in an LPS-induced shock model has been

suggested to be associated with atrial natriuretic peptide (ANP) [7]. ANP, a 28-amino acid polypeptide, is secreted from atrial myocytes in response to volume or pressure loading. Physiologically, it has an important role in salt and water homeostasis and renal and cardiac function [8]. One of the main target organs for ANP is the kidney, which has natriuretic peptide receptors (NPR) mainly in the glomeruli and inner medulla. Studies have also shown that the kidney itself produces ANP [9]. However, changes in renal ANP and its receptor system have not yet been demonstrated in LPS-induced sepsis. Thus, this study aimed to determine whether there is altered regulation of local renal NO and ANP system-associated guanylyl cyclase (GC) activity in an LPS-induced septic shock rat model.

METHODS

Animals

Male Sprague-Dawley rats weighing 180~200 g were used. Rats were kept in a temperature-controlled room

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ABBREVIATIONS: LPS, lipopolysaccharide; NO, nitric oxide; ANP, atrial natriuretic peptide; NPR, natriuretic peptide receptor; NEP, neutral endopeptidase; GC, Guanylyl cyclases; SNP, sodium nitroprusside.

regulated on a 12 : 12-h light-dark cycle. The study was approved by the Ethics Committee of Chonnam National University Medical School.

The rats were fed once daily in the morning and ate all of the offered food during the course of the day. LPS from *Escherichia coli* serotype 026 : B6 was obtained from Sigma Chemical Co. (Sigma; St. Louis, MO, USA) dissolved in pyrogen-free sterile phosphate based saline. LPS ($10 \text{ mg} \cdot \text{kg}^{-1}$) was injected via tail vein of rat. Twelve hours later the kidneys were taken. The rats were maintained individually in the metabolic cages to allow urine collections for the measurement of Na^+ and creatinine. Under anesthesia with isoflurane, blood samples were collected from the inferior vena cava and analyzed for plasma levels of Na^+ and creatinine. The right kidney was removed and processed for immunoblotting. The left kidney was removed and assayed for mRNA expression by real time-polymerase chain (PCR) reaction. Another set of experiment was performed for GC enzyme activity.

Expression of natriuretic peptide system

Total RNA was extracted from kidney with Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in distilled water. The RNA concentration was determined by the absorbance read at 260 nm (Ultraspec 2000; Pharmacia Biotech, Cambridge, UK). The mRNA expression of ANP, natriuretic peptide receptor (NPR)-A, and NPR-C was determined by real-time PCR. cDNA was made by reverse transcribing $5 \mu\text{g}$ of total RNA using oligo (dT) priming and superscript reverse transcriptase II (Invitrogen; Carlsbad, CA, USA). cDNA was quantified using Smart Cycler II System (Cepheid; Sunnyvale, CA, USA) and SYBR Green was used for detection. PCR was done using Rotor-Gene™ 3,000 Detector System (Corbett Research; Mortlake, New South Wales, Australia). Primers were prepared as described previously [10]. Data from the reaction were collected and analyzed with the Corbett Research Software [11].

Membrane preparation and guanylyl cyclase activity

The biological effect of NO is mediated by activation of sGC and subsequent generation of cGMP, whereas most of the biological effects of ANP are mediated by secondary formation of cGMP catalyzed by pGC coupled with NPR-A [12]. Therefore, the tissue was fractionated into cytosolic and membrane portions.

The glomerulus was isolated by graded sieve methods. In brief, the kidney was decapsulated, and the cortex was consecutively filtered through standard sieves (250, 150, 125, and $75 \mu\text{m}$). The glomeruli on the $75 \mu\text{m}$ 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 15 min at 4°C and the supernatant was recentrifuged at 100,000 g for 60 min at 4°C . The resulting pellet was used as membrane preparation for the measurement of pGC activity. The supernatant was used as soluble fraction for the measurement of sGC activity. Protein concentrations were determined using bicinchoninic acid assay kit (Pierce; Rockford, IL, USA).

pGC activity was measured in the glomerular and papil-

lary membrane aliquots by the method of Winquist et al. [10] with a slight modification. The aliquots were incubated for 15 min at 37°C in 50 mM Tris HCl (pH 7.6) containing 1 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 1 mM ATP, 15 mM MgCl_2 , in the presence of ANP (10^{-10} to 10^{-6} M). The reaction was stopped by adding ice-cold 50 mM sodium acetate (pH 5.0) and boiling for five min. Samples were then centrifuged (10,000 g for 5 min at 4°C).

For the measurement of sGC activity, the protein samples were incubated for 15 min at 37°C in 50 mM Tris HCl (pH 7.6), containing 1 mM 3-isobutyl-1-methylxanthine, 1 mM GTP, 1 mM ATP, and 15 mM MgCl_2 , in the presence of sodium nitroprusside (SNP, 10^{-7} to 10^{-3} M). Incubation was stopped by adding ice-cold 50 mM sodium acetate (pH 5.0) and boiling for 5 min. Samples were then centrifuged (10,000 g, 4°C , 10 min), of which supernatant was used to measure cGMP by equilibrated radioimmunoassay. In brief, standards and samples were introduced in a final volume of $100 \mu\text{l}$ of 50 mM sodium acetate buffer (pH 4.8), and $100 \mu\text{l}$ of dilute cGMP antiserum (Calbiochem-Novabiochem, San Diego, CA, USA) and iodinated cGMP (10,000 cpm/ $100 \mu\text{l}$). Results are expressed as pmol cGMP generated/mg protein per min.

Semiquantitative immunoblotting

The dissected kidney was homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM ethylenediaminetetraacetic acid (EDTA), $8.5 \mu\text{M}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), with pH 7.2. The homogenates were centrifuged at 1,000 g for 15 min at 4°C to remove whole cells, nuclei and mitochondria. The total protein concentration was measured by BCA assay kit (Pierce; Rockford, IL, USA). All samples were adjusted with isolation solution to reach the same final protein concentrations. They were then dissolved at 65°C for 15 min in SDS-containing sample buffer and stored at -20°C . To confirm equal loading of protein, an initial gel was stained with Coomassie blue. SDS-PAGE was performed on 9 or 12% polyacrylamide gels.

The proteins were transferred by gel electrophoresis (Bio-Rad, Mini Protean II; Hercules, CA, USA) onto nitrocellulose membranes (Amersham Pharmacia Biotech, Hybond ECL RPN3032D; Little Chalfont, UK). The blots were subsequently blocked with 5% milk in PBS with tween 20 (80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 hr and incubated overnight at 4°C with primary antibodies, followed by incubation with secondary anti-rabbit (Dako; Glostrup, Denmark) or anti-mouse (Dako) horseradish peroxidase-conjugated antibodies. The labeling was visualized by an enhanced chemiluminescence system. The monoclonal antibodies to endothelial NOS (eNOS), neuronal NOS (nNOS), inducible NOS (iNOS) (Transduction Laboratories; Lexington, KY, USA), sGC, neutral endopeptidase (NEP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti- β -actin antibody (Sigma; St. Louis, MO, USA) were commercially obtained.

Colorimetric assay of nitrite/nitrate

As an index of synthesis of NO, its stable metabolites (nitrite/nitrate, NOx) were measured by a colorimetric NO assay kit (Oxford Biochemical; Oxford, MI, USA). Microplate was used to perform enzyme reactions in vitro. For spectrophotometric assay of nitrite with griess reagent, $80 \mu\text{l}$

MOPS (50 mM) / EDTA (1 mM) buffer and 5 μ l samples were added to wells. Nitrate reductase (0.01 U) and 10 μ l NADH (2 mM) 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 absorbance values were read at 540 nm in a microtitre plate reader (Bio-Rad, Model 3,550).

Statistical analyses

Results are expressed as mean \pm SEM. The statistical significance of differences between the groups was determined using an unpaired *t*-test. Differences with values of $p < 0.05$ were considered significant.

RESULTS

Renal function

Table 1 shows body weight, creatinine clearance, and fractional excretion of sodium. Creatinine clearance was de-

Table 1. Changes in blood pressure and renal functional data

	Control (n=8)	LPS (n=9)
Body weight (g)	200 \pm 5.0	200 \pm 6.3
UO (ml/12 hr)	9.9 \pm 2.6	8.4 \pm 2.4
P-Cr (mg/dl)	0.25 \pm 0.07	0.73 \pm 0.15*
Ccr (ml/min)	1.16 \pm 0.34	0.26 \pm 0.14*
FE _{Na} (%)	0.47 \pm 0.18	1.36 \pm 0.19*

Values are expressed as mean \pm SEM. These values are measured on the last day of experiments. LPS, lipopolysaccharide induced endotoxemia rat model; UO, urine output; P-Cr, plasma creatinine; Ccr, creatinine clearance; FE_{Na}, fractional excretion of sodium into urine. * $p < 0.05$ compared with control.

creased, while fractional excretion of sodium increased in LPS rats compared with controls. However, body weight and urinary output were not different between two groups.

Alteration of nitric oxide system

Semiquantitative immunoblotting revealed increased abundance of iNOS proteins in the LPS injection rats, while protein expression of eNOS and nNOS showed no change between two groups (Fig. 1). The amount of plasma and urinary NOx excretion was increased in LPS injection rats (Fig. 2). Fig. 3 shows semiquantitative immunoblotting of sGC and cGMP generation in response to graded doses of SNP. The expression of sGC protein was not altered in the cortex. The cGMP generation in response to SNP was not different in the glomerulus and papilla in LPS rats.

Alteration of natriuretic peptide system

Fig. 4 shows mRNA expression of ANP, NPR-A and NPR-C in the kidney. The expression of NPR-C was decreased in LPS injection rats. In contrast, the expression ANP and NPR-A was not different between two groups. Fig. 5 shows semiquantitative immunoblotting of NEP and cGMP generation in response to graded doses of ANP. The protein expression of NEP was not changed in the cortex in LPS injection rats. The cGMP generation in response to ANP was blunted in glomerulus and papilla on LPS injection rats.

DISCUSSION

Endotoxins, LPS is product of the cell walls of gram-negative bacteria that cause septic shock. Acute kidney injury due to septicemia may be caused by decreased glomerular blood pressure and renal blood flow [13]. In the present study, LPS caused marked renal damage, as demonstrated by a significant decrease in the glomerular filtration rate

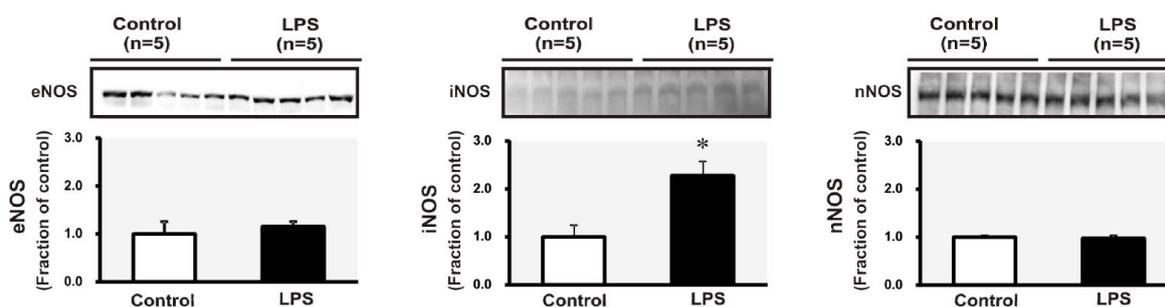


Fig. 1. Semiquantitative immunoblotting of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) in the kidney. * $p < 0.05$ compared with control.

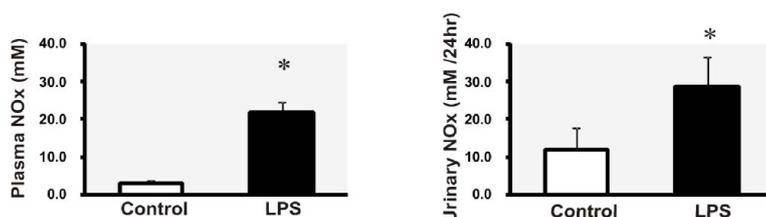


Fig. 2. Nitric oxide metabolites (nitrite/nitrate, NOx) in plasma and urine. * $p < 0.05$ compared with control.

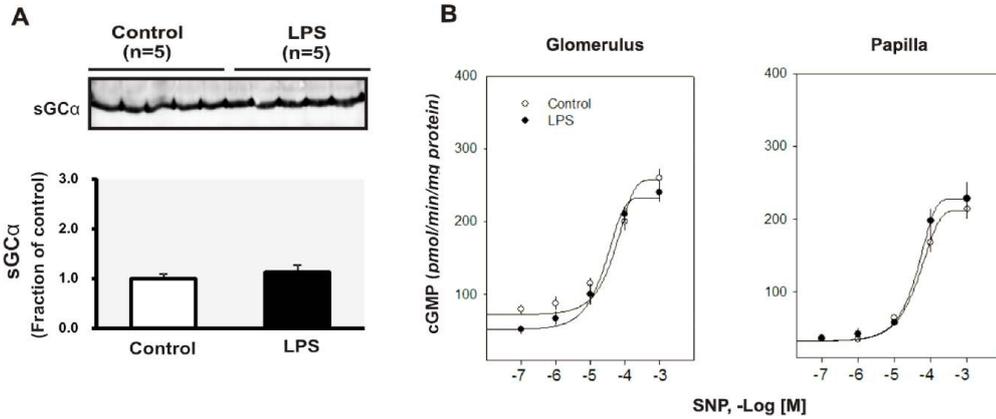


Fig. 3. (A) Semiquantitative immunoblotting of soluble guanylyl cyclase (sGC) in the kidney. (B) cGMP production in response to sodium nitroprusside (SNP) in the glomerulus and papilla. Each point represents mean \pm SEM of experimental rats. * $p < 0.05$ compared with control.

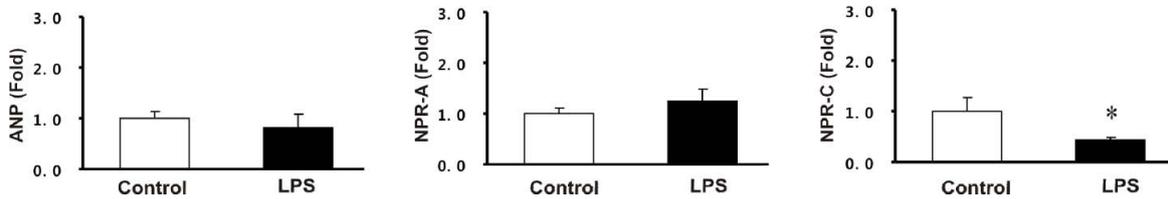


Fig. 4. The mRNA expression of atrial natriuretic peptide (ANP), natriuretic peptide receptor type A and C (NPR-A, NPR-C) in whole kidney. Columns show real-time PCR data representing control and LPS groups. * $p < 0.05$ compared with control.

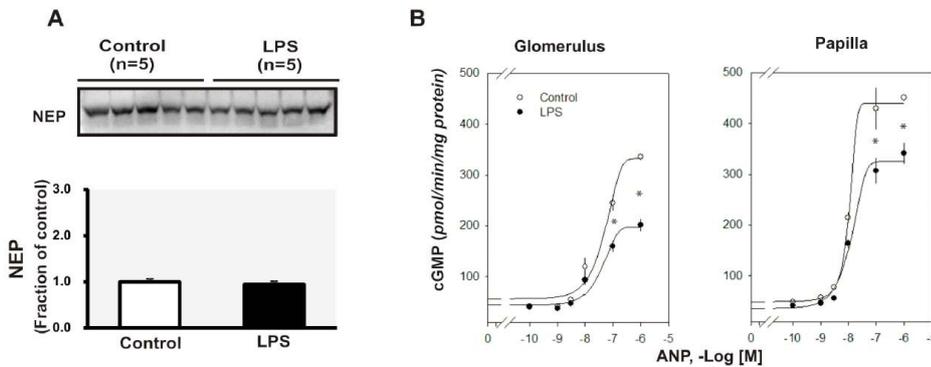


Fig. 5. (A) Semiquantitative immunoblotting of neutral endopeptidase (NEP) in the kidney. (B) cGMP production in response to atrial natriuretic peptide (ANP) in the glomerulus and papilla. Each point represents mean \pm SEM of experimental rats. * $p < 0.05$ compared with control.

(GFR). Acute kidney injury during sepsis is associated with increased NO production [14]. Consistent with this, the present study demonstrated that urinary and plasma NOx levels markedly increased in LPS rats.

NO is synthesized by eNOS and nNOS localized in the vascular endothelium and macula densa [15], whereas iNOS is present in the proximal tubule and inner medullary collecting duct [16]. iNOS may be a key mediator of inflammatory processes such as septic shock [17] and ischemic/reperfusion injury [18]. Recent data suggest that eNOS restores renal function after injury, whereas activation of iNOS leads to excessive NO production and aggravation of kidney injury [19]. In the present study, iNOS expression was found to increase in the kidney in association with decreased GFR in LPS-treated rats, whereas eNOS and nNOS expression did not change. iNOS increases the production of NO up to 1,000 times more than the normal production

by constitutive NOS. Therefore, although NO generation in the kidney is essential for preservation of renal perfusion and function, high levels of NO secondary to an increase in iNOS activity may inhibit eNOS activity, resulting in renal vasoconstriction, decreased GFR [14], and cytotoxic injury, leading to LPS-induced kidney injury.

The present study demonstrated that SNP-induced cyclic guanosine monophosphate (cGMP) generation did not change in the glomerulus and papilla, which suggests that increased production of cytotoxic NO by iNOS combined with unaltered soluble-GC activity plays a role in the pathogenesis of LPS-induced kidney injury.

Increased plasma ANP concentrations have been previously reported during early sepsis in vivo [20]. In the present study, mRNA expression of ANP did not change, but that of NPR-C decreased in LPS-treated rats. Binding of ANP to NPR-C results in internalization of the re-

ceptor-ligand complex, followed by hydrolytic degradation of ANP. Moreover, the widely distributed ectoenzyme NEP cleaves the ANP Cys7-Phe8 bond close to the disulfide bond, breaking the ring structure and rendering ANP biologically inactive [21]. Consequently, a reduction in ANP clearance may result in augmentation of its plasma levels. ANP is linked to particulate GC, and its activation results in secondary cGMP formation [22]. In the present study, pGC activity in response to ANP decreased in the glomerulus and papilla in the kidneys of LPS-treated rats. These findings suggest dissociation of ANP synthesis and reduction in its responsiveness in the kidney. We believe that decreased GC activity in this model may be a compensatory mechanism in response to enhanced ANP activity in the kidney [23], and may play a compensatory role against hypotension and decreased tissue perfusion.

In conclusion, enhancement of the NO/cGMP pathway, as well as decreased ANP clearance, plays a role in the pathogenesis of LPS-induced kidney injury.

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