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Short Communication

EFFECT OF LIPOPOLYSACCHARIDE ON NITRIC OXIDE SYNTHASE ACTIVITY IN RAT PROXIMAL TUBULES

PHILIP R. MAYEUX,* HOLLYE R. GARNER, JENNIFER D. GIBSON and VICTOR C. BEANUM

Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, U.S.A.

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Abstract—Renal proximal tubules isolated from the rat possess nitric oxide synthase (NOS) activity that is calcium/calmodulin dependent and stereoselectively inhibited by N^G-monomethyl-arginine (NMMA). In the absence of added Ca^{2+} and calmodulin, activity was reduced 84 ± 13% compared with the activity in the presence of 2 mM Ca^{2+} and 25 μg/mL calmodulin. Inhibition by EGTA (10 mM) was 95 ± 5% and by calmidazolium (R24571, 250 μM) was 99 ± 1%. Inhibition by L-NMMA (100 μM) was 78 ± 13% and by D-NMMA (100 μM) was 7 ± 7%. The majority of NOS activity was found in the soluble fraction. NOS activity in isolated proximal tubules was also examined 6 hr after a single i.v. injection of lipopolysaccharide. Activity was increased significantly ($P < 0.05$) in the soluble fraction by 2-fold [from 0.320 ± 0.052 to 0.648 ± 0.046 (nmol/mg protein/30 min)] and in the particulate fraction by 3-fold [from 0.081 ± 0.030 to 0.256 ± 0.034 (nmol/mg protein/30 min)]. All activities were inhibited by EGTA. These data demonstrate that proximal tubules express a calcium/calmodulin-dependent NOS activity that is increased *in vivo* by lipopolysaccharide.

Key words: proximal tubule; nitric oxide synthase; lipopolysaccharide; calmidazolium; N^G-monomethyl-L-arginine; L-arginine; citrulline

NOS† converts L-arginine to citrulline with the concomitant release of NO [1, 2]. The synthesis of NO and its role in signal transduction have been studied extensively in vascular and nervous tissues [3], but only recently have studies been directed toward establishing a role for NO in renal physiology and pathophysiology. In the rat proximal tubule, physiological evidence indicates a basal release of NO that participates in the regulation of proximal tubule function [4–6]. There is also biochemical evidence demonstrating the presence of constitutive NOS activity in the proximal tubule and that NO generation participates in the development of hypoxia/reoxygenation injury [7].

LPS has been shown to induce NOS *in vivo* in a number of tissues [8–10], and NO has been shown to play a role in the development of hypotension associated with LPS administration [11]. Acute renal failure is a frequent and serious complication of endotoxemia in humans [12]. Changes in NOS activity within the kidney in response to LPS could conceivably alter renal function given the evidence that glomerular and proximal tubule function may be regulated by NO [4–6, 13]. The purpose of the present study, therefore, was to determine if NOS activity in the proximal tubule is altered after LPS administration.

Materials and Methods

Materials. L-[2,3,4,5-^{3}H]Arginine (57 Ci/mmol) was purchased from the Amersham Corp. (Arlington Heights, IL). Dowex AG 50W-X8 (Na^+ form) and Dowex AG 1-X8 (Cl^- form) were purchased from Bio-Rad Laboratories (Hercules, CA). Tetrahydrobiopterin was purchased from Research Biochemicals Inc. (Natick, MA). L-Arginine, L-citrulline, L-NMMA, D-NMMA, calmodulin, R24571 (calmidazolium), LPS (*Escherichia coli*, serotype 055:B5) and all other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO).

Preparation of tubule fractions. Animals were housed and killed in accord with the NIH Guide for the Care and Use of Laboratory Animals. Rat proximal tubules were isolated from the kidneys of male Sprague–Dawley rats (250–300 g) by collagenase digestion and Percoll density gradient centrifugation using published methods [14, 15]. The purity of each preparation was assessed by light microscopy and used only if it was greater than 95% tubules. Proximal tubules were washed once and resuspended in 4 vol. assay buffer (wet weight:vol) containing 40 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1.5 μM pepstatin A, 2 μM leupeptin, and 25 μM tetrahydrobiopterin (pH 7.4). Homogenization was performed at 4° using a teflon tissue grinder. The homogenate was centrifuged for 20 min at 10,000 *g*, and the resulting supernatant was centrifuged for 1 hr at 100,000 *g*. The resulting pellet was resuspended in assay buffer. Both the 100,000 *g* supernatant and pellet were stored at −70° in assay buffer containing 10% glycerol. Protein concentration was determined using the Bradford method as described in the Bio-Rad protein assay kit with bovine serum albumin standards.

Citrulline determination. The conversion of arginine to citrulline was monitored using [^{3}H]L-arginine as described [1]. [^{3}H]L-Arginine was first purified using Dowex AG 1-

* Corresponding author: Philip R. Mayeux, Ph.D., University of Arkansas for Medical Sciences, Department of Pharmacology and Toxicology, 4301 West Markham Street, Slot 611, Little Rock, AR 72205. Tel. (501) 686-5511; FAX (501) 686-5521.

† Abbreviations: NOS, nitric oxide synthase; LPS, bacterial lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; and D-NMMA, N^G-monomethyl-D-arginine.

Table 1. Effect of inhibitors on proximal tubule NOS activity

	% Inhibition
L-NMMA (100 μM)	78 ± 13*
D-NMMA (100 μM)	7 ± 7
EGTA (10 mM)	95 ± 5*
Calmidazolium (250 μM)	99 ± 1*
No added Ca^{2+} or calmodulin	84 ± 13*

Each assay of the soluble fraction (100,000 *g* supernatant) was incubated for 30 min at 37° in the presence of 100 μM arginine, 2 mM Ca^{2+} and 25 μg/mL calmodulin, unless noted otherwise. Data are expressed as percent inhibition (mean ± SEM of 4–5 preparations) of NADPH-dependent citrulline formation in the presence of inhibitor. Control activity was in the range of 0.243 to 0.424 nmol/mg/30 min.

* $P < 0.05$ compared with activity in the absence of inhibitor and in the presence of Ca^{2+} and calmodulin.

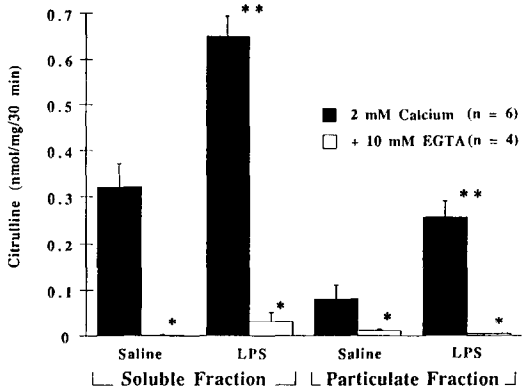

Fig. 1. Effect of LPS administration on NOS activity in isolated proximal tubules. Kidney proximal tubules were isolated 6 hr after a single i.v. injection of saline or LPS (7.5 mg/kg). NOS activity was assayed in the soluble fraction (100,000 *g* supernatant) and the particulate fraction (100,000 *g* pellet). Values are means ± SEM. Key: (*) $P < 0.05$ compared with activity in the absence of EGTA, and (**) $P < 0.05$ compared with saline-treated values.

X8. Unless stated otherwise, the assay mixture contained 100 μM L-arginine (300,000–400,000 dpm [^{3}H]L-arginine), 2 mM $CaCl_2$, 1 mM NADPH, 25 μg/mL calmodulin, 25 μM FAD and 100 μg protein in a final volume of 200 μL. At the end of a 30-min incubation at 37°, 800 μL of stop solution containing 20 mM HEPES and 2 mM EDTA (pH 4.0) was added, and the samples were placed on ice. [^{3}H]Citrulline was extracted by passing the mixture through Dowex AG 50W-X8 and extracting with 1 mL water twice. Citrulline formation was quantified using liquid scintillation spectrometry and the specific activity of [^{3}H]L-arginine. Each preparation was assayed in duplicate in the presence and absence of NADPH to establish the NADPH-dependent citrulline formation.

NO generation. The generation of NO was quantified by assaying the conversion of oxyhemoglobin to methemoglobin with a dual beam spectrophotometer (model U2000, Hitachi Instruments, Danbury, CT). The reaction mixture contained the same components as the citrulline assay except: [^{3}H]L-arginine was omitted, 200 μg protein was used, and the mixture contained 1.4 to 1.6 μM oxyhemoglobin in a final volume of 1 mL. The reference cuvette contained the reaction mixture without L-arginine. After a 30-min incubation at 37° the difference spectrum was obtained against the reference. The extinction coefficient ($E = 77{,}200\ M^{-1}\ cm^{-1}$) of the wavelength shift from 401–421 nm was used to quantitate NO generation [8, 16].

Administration of lipopolysaccharide. Male Sprague–Dawley rats (275–300 g) were anesthetized with halothane and given an i.v. injection of LPS prepared in sterile saline at a dose of 7.5 mg/kg. Control animals received an equal volume of sterile saline. Proximal tubules were isolated 6 hr after LPS administration.

Statistical analysis. Each N represents a proximal tubule preparation derived from the kidneys of two rats. Data are presented as means ± SEM and were analyzed by a paired *t*-test (to evaluate the effect of inhibitors) or an unpaired *t*-test (to compare saline with LPS-treated rats). Statements of significance were based on $P < 0.05$.

Results and Discussion

Initial studies were performed using the citrulline assay to measure NOS activity. NADPH-dependent metabolism of L-arginine was taken as NOS activity because NOS is an NADPH-dependent enzyme and proximal tubules contain other enzymes capable of metabolizing arginine to additional uncharged metabolites (e.g. ornithine) [17]. These metabolites might be interpreted as citrulline. In experiments with the soluble fraction, 24 ± 2% (N = 3) of the total apparent citrulline formation was NADPH dependent (total = 1.786 ± 0.313 nmol/mg/30 min; NADPH dependent = 0.390 ± 0.101 nmol/mg/30 min). This percentage did not change even when the soluble fraction was first passed through a Sephadex G75 column to remove possible contaminating NADPH (28 ± 5% of the total). The validity of the citrulline assay was further confirmed by monitoring the conversion of oxyhemoglobin to methemoglobin by generated NO in the soluble fraction. NOS activity was 0.359 ± 0.113 nmol/mg/30 min (N = 4) and was inhibited 74 ± 20% (N = 4) by the addition of 100 μM L-NMMA. Both the methemoglobin assay and the citrulline assay (Table 1) yielded similar activities, and both were inhibited to a similar extent by L-NMMA. The citrulline assay was used in subsequent experiments because this assay could be performed in a smaller reaction volume and required less protein. Further characterization of NOS activity in the soluble fraction is presented in Table 1. Inhibition of NOS by *N*-monomethyl arginine was stereoselective in that L-NMMA (100 μM) was inhibitory whereas D-NMMA (100 μM) was not. Activity was dependent on added Ca^{2+} and calmodulin in that activity in the presence of 2 mM Ca^{2+} and 25 μg/mL calmodulin was decreased significantly when Ca^{2+} and calmodulin were omitted. Activity in the presence of Ca^{2+} and calmodulin was inhibited significantly by EGTA (10 mM) or the calmodulin antagonist calmidazolium (250 μM), further indicating a Ca^{2+}/calmodulin-dependent activity.

These results are in sharp contrast to those in a recent report by Yu *et al.* [7]. In their study, rat proximal tubule homogenates (20,000 *g* supernatant) were assayed for NOS activity using the citrulline assay. They reported a total apparent NOS activity of 2.6 ± 0.2 nmol/mg/40 min. This value is in close agreement with our total apparent citrulline formation of 1.786 ± 0.313 nmol/mg/30 min. However, we think the authors have overestimated NOS activity because the NADPH-dependence was not determined. This overestimation could explain the reported relatively weak ability of the NOS inhibitor L-nitro-arginine methyl ester to inhibit activity (62% at 5 mM) as well as the reported lack of an inhibitory effect of 2 mM EGTA. We found constitutive NOS activity in the isolated proximal tubule

that was NADPH dependent and stereoselectively inhibited by NMMA. Furthermore, this activity was both Ca^{2+} and calmodulin dependent and present predominately in the soluble fraction (0.320 ± 0.052 nmol/mg/30 min in the soluble fraction and 0.081 ± 0.030 nmol/mg/30 min in the particulate fraction; from Fig. 1, saline control).

In vivo induction of NOS activity by LPS in the rat has been reported for several organs and tissues [8–10]. The effect of LPS on NOS activity in the proximal tubule was examined by assaying NOS activity in tubule fractions isolated 6 hr after a single i.v. injection of LPS or saline. These data are presented in Fig. 1. NOS activity from LPS-treated rats was increased significantly ($P < 0.05$) by 2-fold in the soluble fraction and by 3-fold in the particulate fraction compared with saline controls. Figure 1 also shows that all activities in both groups were Ca^{2+} dependent in that activity in the presence of 2 mM Ca^{2+} was inhibited ($P < 0.05$) by the addition of 10 mM EGTA. These data indicate that LPS administration resulted in a significant increase in NOS activity, and that this activity may be regulated by Ca^{2+} *in vivo*.

Several isoforms of NOS have been described [3]. The three best characterized isoforms are the constitutive Ca^{2+}/calmodulin-dependent neuronal and endothelial forms and the inducible Ca^{2+}-independent macrophage form. Activity found in the proximal tubule is apparently not from neuronal NOS. Immunohistochemical staining with a polyclonal rabbit antibody to rat neuronal NOS was reported positive in the macula densa but negative in the proximal tubule [13], and mRNA coding neuronal NOS has been found in several regions of the kidney but not in the proximal tubule [18]. mRNA of the macrophage NOS has also not been detected in the proximal tubule even after LPS administration [19]. The presence of endothelial NOS has not yet been examined in kidney tubules using either immunohistochemical or molecular biology techniques.

The Ca^{2+}/calmodulin dependence of the proximal tubule NOS activity and its location in both the soluble and particulate fractions, even after LPS, suggest a similarity to endothelial NOS [20, 21]. Recently Tracey *et al.* [22] reported the expression of an endothelial-like NOS by the tubule cell line LLC-PK_1. This isoform is Ca^{2+}/calmodulin dependent but, in contrast to our findings with isolated proximal tubules, is found predominately in the particulate fraction. We did observe a shift in the ratio of activities between the soluble and particulate fractions in the two groups from 4:1 in the saline group to 2.5:1 in the LPS group. The potential physiological significance of the subcellular location of NOS is unknown. Additional studies are required to establish which isoform is expressed by the proximal tubule or whether this activity represents a new isoform.

Several studies suggest that basal NO production in the kidney may participate in local control of glomerular and tubular function [4–6, 13]. Specifically, basal NO production appears to maintain sodium reabsorption [4–6], an important function of the proximal tubule. We have shown that rat proximal tubules express constitutive NOS activity that may be regulated *in vivo* by Ca^{2+}. The ability of the proximal tubule to generate NO and the increase of this activity by LPS indicate a potential for proximal tubule-derived NO to participate in the derangement of kidney function associated with endotoxemia.

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