Bladder Instillation and Intraperitoneal Injection of Escherichia coli Lipopolysaccharide Up-regulate Cytokines and iNOS in Rat Urinary Bladder

LIEF ERIC OLSSON, MARCIA A. WHEELER, WILLIAM C. SESSA and ROBERT M. WEISS

Section of Urology and Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

Systemic bacterial lipopolysaccharides (LPS) induce inflammatory responses characteristic of sepsis. Instillation of LPS into rat bladder produces a localized inflammatory response similar to that seen in urinary tract infections (UTIs). Four hours after intravesical instillation of LPS, neutrophils infiltrate into the bladder, and mRNA for inducible nitric oxide synthase (iNOS) and the cytokines, interleukin (IL)-6 and IL-10, is detected in rat bladder but not in the kidney. Induction of iNOS protein is inferred because urinary nitrate and cGMP levels are increased 4 hr after LPS intravesical instillation and remain elevated for at least 24 hr. When LPS is injected intraperitoneally, iNOS and IL-6 mRNA are induced both in the bladder and in the kidney. These data are consistent with the effects of intravesical instillation of LPS remaining localized. iNOS activity increases in both particulate and soluble bladder fractions when measured 4 hr after intravesical instillation of LPS. The magnitude of these increases in iNOS activity in the bladder is not as great as when LPS is injected intraperitoneally. Intravesical instillation of LPS induces no increase in lung or kidney NOS activity. The localized inflammatory response produced by intravesical instillation of LPS demonstrates the importance of LPS as a mediator of the host response in UTIs and supports the use of urinary measurements of nitrate and cGMP in humans as indicative of the localized induction of iNOS in UTIs.

During the course of a human UTI, bacteria ascend into the bladder and attach to the urothelium, which causes urothelial cells to secrete cytokines including IL-1α, IL-6 and IL-8 (Svanborg et al., 1994). Urinary levels of iNOS (Wheeler et al., 1997), cyclic GMP (Smith et al., 1996a) and several cytokines, including TNFa, IL-1α, IL-1β, IL-6 and IL-8 (Hedges et al., 1992; Ko et al., 1993; Davidoff et al., 1997) also are up-regulated during human UTIs, although determining the time course of induction is difficult. LPS, the cell wall component of many bacteria including Escherichia coli, can induce many of the host defenses required for bacterial killing (Cross et al., 1995) and up-regulates nitric oxide and cytokine production (Szabo and Thiemermann, 1995) in single cells (Stuehr and Marletta, 1990; Radomski et al., 1990), in isolated tissues (Fleming et al., 1990) and in whole animal models (Knowles et al., 1990). Intraperitoneal injection of LPS induces the expression of iNOS protein in many cells and tissues including mononuclear cells, liver, spleen, lung, kidney and bladder epithelium (Cook et al., 1994). To determine factors that are necessary to induce iNOS with conditions that mimic a UTI, we adapted the procedure of Stein et al. (1996) in which protamine sulfate followed by E. coli LPS is instilled into rat bladders to produce inflammation.

We inject LPS intraperitoneally or instill LPS into the female rat bladder and measure changes in iNOS mRNA and activity in the bladder and the levels of its enzymatic products including NOx and cyclic GMP in the urine. Because iNOS is the Ca++-calmodulin-independent isoform of NOS, whereas the constitutive isoforms nNOS and eNOS are Ca++-calmodulin dependent, we monitor iNOS activity as activity in the presence and absence of EGTA. Nitrate and nitrite are oxidation products of nitric oxide and also are used to monitor changes in NOS (Evans et al., 1994), whereas cyclic GMP is increased when NO reacts with the heme moiety of the soluble guanylyl cyclase enzyme. Cyclic GMP also can be increased via other pathways including by activation of particulate guanylyl cyclase enzyme by atrial natriuretic factor and related peptides (Chinkers and Garbers, 1991).

The pro-inflammatory cytokine, IL-6, produces fever and C-reactive protein response (Nijsten et al., 1987) and up-

Received for publication August 12, 1997.

1 Supported in part by Public Health Service DK38311 and DK47548 from the National Institute of Diabetes and Digestive and Kidney Diseases.

ABBREVIATIONS: b.p., base pair; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FAD, flavin adenine dinucleotide; iNOS, inducible nitric oxide synthase; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IL, interleukin; L-NMMA, NG-monomethyl-L-arginine; LPS, lipopolysaccharide; NOS, nitric oxide synthase; NOx, nitrate plus nitrite; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TNF, tumor necrosis factor; UTI, urinary tract infection.
regulates iNOS in human hepatocytes (Nussler et al., 1995), whereas IL-10 is an anti-inflammatory cytokine that downregulates iNOS in rat monocytes (Warner et al., 1995) and macrophages (Cenci et al., 1993). Thus, we correlated changes in NOS activity and mRNA with changes in mRNA for cytokines, IL-6 and IL-10, not only in the bladder and urine, but also in lung and kidney. When LPS is injected intraperitoneally, systemic changes in NOS and cytokines would be expected not only in the bladder but also in the lung and kidney (Cook et al., 1994), but when LPS is instilled intravesically, changes in cytokines and iNOS would be expected only in the bladder.

**Methods**

**Induction of inflammation.** Adult female Sprague Dawley rats (200–250 g) were sedated with xylazine (4 mg/kg) and ketamine (90 mg/kg) and then either injected intraperitoneal with LPS (10 mg/kg) or instilled intravesically with protamine followed by LPS according to our institutionally approved Animal Use protocol. LPS prepared by trichloroacetic acid extraction from *E. coli*, serotype 026:B6 or serotype 0127:B8, and protamine sulfate, Grade X, was purchased from Sigma, St. Louis, MO.

The procedure of Stein et al. (1996) was followed to instill LPS into the bladder. After sedation, rats were catheterized with a lubricated soft catheter (PE20) attached to a syringe. The bladder was emptied and the urine saved for zero time point measurements. Protamine sulfate (10 mg) in 500 μl sterile PBS was instilled into the bladder through the catheter. After 30 min, the bladder was emptied again, and LPS (1 mg) in 500 μl sterile PBS was instilled through the catheter. The catheter was removed and the rats allowed to recover. Sterile PBS (500 μl) or protamine (10 mg in 500 μl sterile PBS) alone was instilled in control animals. For instillation, rats were fixed in paraformaldehyde and paraffin-embedded sections were stained with hematoxylin and eosin stain. Leukocytes and epithelial cells were identified.

**Detection of cytokines and iNOS cDNAs after RT-PCR.** Tissues were homogenized in TRIzol (Life Technologies, Gaithersburg, MD) (100 mg tissue/ml TRIzol) and total RNA was extracted. Single-stranded cDNAs were synthesized with oligo dT priming and Superscript II Reverse Transcriptase (Gibco BRL, Life Technologies, Burg, MD) (100 mg tissue/ml TRIzol) and total RNA was extracted.

**Measurement of urinary NOx levels.** NOx levels were measured after reduction of nitrate to nitrite with bacterial nitrate reductase (Schmidt et al., 1992). Nitrate standards (1–100 μM) and rat urines which had been diluted 10-fold in PBS were incubated (30 min, 37°C) in a reaction mixture (final volume, 100 μl) containing 0.25 U/ml nitrate reductase (Sigma N7265, from *Aspergillus*), 250 μM NADPH and 20 μM FAD in PBS. To oxidize NADPH, 10 mM pyruvate and 10 U lactate dehydrogenase (Sigma L2500 from rabbit muscle) were added, the mixture was incubated for an additional 10 min (37°C) and the nitrite concentration was determined after addition of 100 μl Greiss reagent. Nitrate was linear over this concentration range, and there was a 100% conversion of nitrate to nitrite.

**Measurement of urinary cyclic GMP levels.** Cyclic GMP levels were determined in 1:30 dilutions of urine supernatants by an 125I-radioimmunoassay (Biomedical Technologies, Stoughton, MA) (Smith et al., 1996a).

**Assay of NOS activity.** Tissues were minced and suspended in 10 volumes ice-cold 20 mM HEPES buffer (pH 7.4), containing 1.0 mM diethiothreitol and protease inhibitors (Dokita et al., 1994). Bladders were homogenized (10 sec, at 70% power, three times, and 20 sec at 50% power) by a Polytron (Brinkmann Instruments, Westbury, NY). Lungs and kidneys were homogenized (10 sec, 70% power) by an UltraTurrax, T25 (IKA Labortechnik, Staufen, KG). All tissue homogenates were centrifuged (20,000 × g, 20 min, 4°C). The particulate and soluble fractions were stored at −80°C until analysis. After thawing on ice, the particulate fractions were resuspended and washed two times in 10 volumes HEPES buffer plus DTT and protease inhibitors to remove endogenous l-arginine (20,000 × g, 20 min, 4°C). The soluble fractions were centrifuged at 50,000 × g for 20 min, and applied to a 0.5-ml column of AG 50W-X8, Na+ form (Bio-Rad, Hercules, CA) to remove endogenous l-arginine.

**NOS activity was measured in soluble and particulate fractions as the formation of L-[14C]citrulline from L-[14C]arginine (DuPont/NEN, Wilmington, DE). The lung and bladder enzymes and buffer controls were incubated (37°C, 45 min) with homogenizing buffer containing l-[14C]arginine (0.2 μCi, 3 μM), 10 mM valine, 2.0 mM NADPH, 15 μM tetrahydrobiopterin and 10 μM FAD, in the presence of 1 mM CaCl2 or 1 mM EGTA. The conditions for measuring NOS activity in the kidney were identical with those used for bladder and lung, except the l-arginine concentration was 10 μM. The assay was terminated by the addition of 1.0 ml of 20 mM HEPES (pH 5.5) containing 2.0 mM ethylenediaminetetraacetic acid. The sample was then applied to a 1.0-ml column of AG 50W-X8, Na+ form and eluted with 1.0 ml of HEPES (pH 5.5). The radioactivity of the column effluent was measured in a liquid scintillation counter, after addition of 15 ml of Optifluor (Packard, Meriden, CT). iNOS activity was calculated as EGTA (1.0 mM)-dependent activity, after subtraction of counts per minute measured in the presence of the NOS inhibitor L-NMMA (1 mM). Before measurement of protein levels, particulate samples were hydrolyzed with 1 N NaOH. Protein concentrations were measured (Bradford, 1976) with bovine γ-globulin as a standard (Bio-Rad, Hercules, CA).

**Statistics.** Data are presented as mean ± S.E.M. Assessment of LPS effects was obtained by analysis of variance. Significant differences from controls or from one another were obtained with the Scheffe procedure.

**Results**

**Neutrophil influx.** Hematoxylin and eosin-stained sections from bladder from control rats had zero to one leukocyte/high power field. Four hours after intravesical instillation of protamine followed by LPS, hematoxylin and eosin-stained sections of bladder contained approximately 10.7 ± 2.0 leukocytes per high power field (×400, n = 4), and these leukocytes were frequently in or near blood vessels (fig. 1). Some urothelial cell shedding was also visualized at 4 hr. Twenty-four hours after protamine-LPS instillation, urothelial shedding was more prominent, and many leukocytes could be visualized in the subepithelial layer.

**Changes in iNOS and cytokine mRNA.** Four hours after intraperitoneal injection of LPS, iNOS mRNA was de-
tected in the bladder (fig. 2, lane 1) and kidney (fig. 2, lane 4) of female rats. iNOS mRNA was not detected in the bladder (fig. 2, lane 3) and kidney (fig 2, lane 6) of control animals. Intravesical instillation of protamine followed by LPS induced iNOS mRNA in the bladder (fig. 2, lane 2), however, no iNOS RT-PCR product was detected in the kidney (fig 2, lane 5). Intravesical instillation of either LPS or protamine alone did not increase bladder iNOS mRNA.

IL-6 mRNA was induced in the bladder and kidney of rats in which LPS was systemically (intraperitoneally) introduced, but only in the bladder when LPS was intravesically instilled (fig. 3A). IL-6 was not detected in the bladder or kidney of control animals. IL-10 RT-PCR product was detected in the bladder, but not in the kidney of LPS intravesically instilled or intraperitoneally injected rats (fig. 3B). IL-10 mRNA was not detected in the bladder or kidney of control rats.

**Changes in urinary NOx and cyclic GMP levels.** Urinary NOx in rats that had been instilled intravesically with LPS after protamine instillation or injected intraperitoneally with LPS increased significantly between 3 and 5 hr, but not at 1 or 2 hr. The level of NOx in the urine of rats before LPS intravesical instillation or intraperitoneal injection was 120 ± 16 nmol/ml. Urinary NOx increased 440% and 580%, respectively, when measured 4 and 24 hr after LPS was instilled intravesically (fig. 4A). Urinary NOx was 84 ± 31 nmol/ml, 24 hr after intravesical instillation with protamine sulfate alone. Four hours after LPS was injected intraperitoneally, urinary NOx increased to 1449 ± 575 nmol/ml.

Four hours after intravesical instillation of LPS, urinary cyclic GMP levels increased 168% over control levels and remained elevated (142%) when measured 24 hr after intravesical instillation (fig. 4B).

**Changes in iNOS activity.** In control rat bladder, there was no detectable Ca\(^{++}\)-independent particulate or soluble NOS activity. When LPS was instilled into the bladder after instillation of protamine sulfate, Ca\(^{++}\)-independent NOS activity was induced in both particulate and soluble bladder preparations. Seventy-two percent of Ca\(^{++}\)-independent activity was particulate. When LPS was introduced systemically (injected intraperitoneally), there also was a significant induction in both particulate and soluble Ca\(^{++}\)-independent (iNOS) activity in the bladder (fig. 5), 35% of the total activity being particulate.

Kidney soluble and particulate fractions contained large amounts of l-NMMA-independent activity, which probably can be attributed to the presence of arginase activity (Zamecka and Porembka, 1988). Small amounts of Ca\(^{++}\)-independent NOS activity (l-NMMA-inhibitable activity) were detected in control kidney soluble and particulate fractions. Intravesical instillation of LPS after protamine instil-
lation did not significantly change Ca\(^{2+}\)-independent NOS activity in the kidney, whereas intraperitoneal injection of LPS increased kidney Ca\(^{2+}\)-independent NOS activity 7.3 times in the soluble fraction (fig. 6A).

Control rat lung preparations contained small amounts of Ca\(^{2+}\)-independent NOS activity (fig. 6B). Neither soluble nor particulate Ca\(^{2+}\)-independent NOS activity in the lung was altered by intravesical LPS-protamine treatment. In contrast, there were large increases of particulate and soluble Ca\(^{2+}\)-independent NOS activity (shown on log scale) when LPS was injected intraperitoneally. Forty-seven percent of the total EGTA-independent NOS activity was particulate.

**Discussion**

Systemic administration of LPS produces pain, inflammation and changes in immune function that mimic septic shock. Many of these effects are mediated by production of cytokines and nitric oxide. During a UTI, attachment of bacteria to the urothelium causes epithelial hyperplasia (Uchida et al., 1989), increase in cytokines (Hedges et al., 1992; Ko et al., 1993; Svanborg et al., 1994; Davidoff et al., 1997), influx of neutrophils (Fukushi and Orikasa, 1981) and induction of iNOS (Wheeler et al., 1997). Intravesical instillation of LPS in rats after pretreatment with protamine sulfate also causes urothelial shedding and an influx of neutrophils (Stein et al., 1996). In our present experiments, we show that LPS induces significant increases in mRNA message for the cytokines, IL-6 and IL-10, and for iNOS in the bladder of rats. Bladder iNOS activity, defined as Ca\(^{2+}\)-independent NOS activity, also is induced within 4 hr after intravesical instillation of LPS, with resultant increases in urinary NO oxidation products and cGMP. Although systemic (intraperitoneal) LPS treatment up-regulates iNOS and cytokine production in the bladder and kidney, intravesical LPS treatment does not induce iNOS or cytokine production in the kidney, which indicates that intravesical application of LPS remains localized. Neither iNOS mRNA nor iNOS activity is detected in bladders of control rats. Systemic injection of LPS produces higher iNOS activity in the bladder and higher levels of urinary NOx than does intravesical LPS instillation.

**Fig. 3.** Induction of IL-6 (A) and IL-10 (B) in the bladder but not the kidney after intravesical instillation of LPS. mRNA was isolated from bladder (lanes 1–3) and kidney (lanes 4–6) 4 hr after control (protamine) (lanes 3 and 6), intravesical protamine/LPS (lanes 2 and 5) or intraperitoneal LPS (lanes 1 and 4) treatment. Rodent-specific IL-6 (A) or IL-10 (B) primers allowed amplifications of desired cDNA. Results are shown for a representative gel (n = 2).

**Fig. 4.** Intravesical instillation of LPS increases urinary NOx levels (A) and cGMP levels (B). (A) Urinary NOx levels were measured in the urine at zero time (n = 43), 4 hr (n = 11) and 24 hr (n = 10) after LPS instillation. Urinary cGMP levels were measured in the urine at zero time (n = 18), 4 hr (n = 7) and 24 hr (n = 6) after LPS instillation. Data are expressed as mean ± S.E.M. *P < .05 compared with values at zero time.
ever, recent reports (Schmidt et al., 1992; Wheeler et al., 1997; Moy et al., 1997) indicate that iNOS is at least partially membrane associated. iNOS activity in neutrophils isolated from patients with UTIs is primarily (>90%) particulate (Wheeler et al., 1997). Transport of eNOS to the caveolae has been found to be important for function and iNOS has been found to be active only when it is a dimer (Albakri and Stuehr, 1996). Both post-translational modifications and localization may affect iNOS activity.

Cytokine production regulates the host response to inflammation and infection. In bladders instilled with LPS, we have identified mRNA for both pro-inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines. Although TNFα and IL-1 are regarded as major mediators of septic shock, IL-1 and to a greater extent TNFα production may be transitory (Ohira et al., 1995). TNFα and IL-1, in turn, induce production of other cytokines such as IL-6. IL-6 levels predict mortality in septic shock and may represent the net effect of biologically active TNFα and IL-1 (Dinarello, 1996). Davidoiff et al. (1997) found that IL-6 is elevated in the urine of most UTI patients with elevated urinary levels of IL-1β. Urinary cytokine up-regulation has been observed not only in UTI, but also after intravesical instillation of bacillus Calmette-Guerin for treatment of superficial bladder cancer, where elevations in urinary levels of IL-2, IL-6, IL-8 and TNFα have been measured (Stassar et al., 1994). Both epidemiological studies (Kantor et al., 1984) and animal studies (Davis et al., 1984) indicate that UTI is a significant risk factor for the development of bladder cancer and IL-6, which is elevated in UTIs, has been found to markedly enhance growth of transformed rat bladder cells (Okamoto et al., 1995).

Anti-inflammatory cytokines like IL-10 are important in counteracting the effects of LPS and pro-inflammatory cytokines. IL-10 also inhibits the induction of NO and improves survival rates in a murine model of endotoxic shock (Cenci et al., 1993; Thiemermann, 1994; Warner et al., 1995). IL-10 is up-regulated in the bladder during both intravesical instillation and intraperitoneal injection of LPS.

Both LPS injection and intravesical instillation induce production of cytokines and iNOS; however, with intravesical instillation, induction of both cytokines and iNOS is relegated to the bladder. Previously, we have measured increases in the urinary levels of iNOS, cGMP, NOx and IL-8 in patients with UTIs (Smith et al., 1996a; Wheeler et al., 1997; Saito et al., 1997), kidney transplant rejection (Smith et al., 1996b) and interstitial cystitis (Smith et al., 1996a; Wheeler et al., 1997). From our findings in the rat model, we can infer that changes in cytokines and NOx-related products during disease processes that affect the human urinary tract can be measured in the urine of these patients. In human UTIs, cytokines and iNOS are also induced; however, the time course of this induction is more difficult to ascertain than with an animal model, where we can observe induction within 4 hr.

Induction of iNOS protein after systemic LPS treatment has been observed in the bladder urothelium (Cook et al., 1994) where NO production may be involved in bacterial killing (Cross et al., 1995) and in the regulation of inflammation. Increases in urinary frequency during UTIs also may be regulated through iNOS, because in the ileum (Weisbrodt et al., 1996) and in blood vessels (Schneider et al., 1994), LPS treatment reduces contractility, and this
reduction in contractility is reversed by treatment with NOS inhibitors. LPS bladder instillation provides a model to both study and intervene in infectious/inflammatory processes.

Acknowledgments

The authors thank Shannon Smith for helpful discussions.

References


Send reprint requests to: Robert M. Weiss, Section of Urology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 208041, New Haven CT 06520-8041.