Bacterial Infection Induces Nitric Oxide Synthase in Human Neutrophils

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Abstract

The identification of human inflammatory cells that express inducible nitric oxide synthase and the clarification of the role of inducible nitric oxide synthase in human infectious or inflammatory processes have been elusive. In neutrophil-enriched fractions from urine, we demonstrate a 43-fold increase in nitric oxide synthase activity in patients with urinary tract infections compared with that in neutrophil-enriched fractions from noninfected controls. Partially purified inducible nitric oxide synthase is primarily membrane associated, calcium independent, and inhibited by arginine analogues with a rank order consistent with that of purified human inducible nitric oxide synthase. Molecular, biochemical, and immunocytochemical evidence unequivocally identifies inducible nitric oxide synthase as the major nitric oxide synthase isoform found in neutrophils isolated from urine during urinary tract infections. Elevated inducible nitric oxide synthase activity and elevated nitric oxide synthase protein measured in patients with urinary tract infections and treated with antibiotics does not decrease until 6–10 d of antibiotic treatment. The extended elevation of neutrophil inducible nitric oxide synthase during urinary tract infections may have both antimicrobial and proinflammatory functions. (*J. Clin. Invest.* 1997. 99:110–116.) Key words: leukocytes • urinary tract infection • inflammation • immunocytochemistry • nitric oxide

Introduction

In rodent macrophages and neutrophils, inducible nitric oxide synthase (iNOS)[1] produces large quantities of nitric oxide (NO) that can modulate immune, inflammatory and cardiovascular responses (1, 2). Even though much effort has been expended on the development of iNOS-selective inhibitors as therapeutic agents (3, 4), the role of iNOS in human infectious and inflammatory processes is poorly understood. Despite some evidence suggesting the presence of iNOS in human macrophages, nitric oxide synthase (NOS) in human neutrophils is not well characterized. Early reports indicate that human neutrophils can generate small amounts of NO (5) and may contain both a constitutive NOS (6) and an NO-sensitive guanylyl cyclase (7, 8). More recent studies have failed to detect either constitutive or inducible NOS activity (9).

Previously, we found that particulate, NADPH-dependent, calcium-independent NOS activity is elevated in urine of patients with bacterial urinary tract infections (UTIs) (10). During bacterial infection, high levels of several cytokines (11, 12) are secreted by the uroepithelial cells and a large number of inflammatory cells, primarily neutrophils infiltrate the bladder. Since large numbers of neutrophils are found in urine from many patients with UTIs (13) this disease represents an opportunity to evaluate NOS in human neutrophils during infectious and/or inflammatory processes. Therefore, the goals of the present study are to identify the NOS isoform(s) in urinary leukocytes, to characterize the NOS expressing cells, and to examine NOS during the course of infection.

Methods

Sample acquisition. Both clean catch and catheterized urines were collected from control patients (n = 16, 75% female; average age, 60.5±5.2 yr old; age range, 25–75 yr old) and patients with UTIs (n = 40, 82% female; average age, 52.5±3.7 yr old; age range 21–80 yr old). To obtain sufficient leukocytes for assay, control urines that were selected, contained $< 10^4$ CFU/ml, and were negative for nitrite and positive for leukocyte esterase. Nitrite and leukocyte esterase were determined on whole urine using LN 2 Chemstrips (Boehringer Mannheim Biochemicals, Indianapolis, IN). Contributing factors in patients with a positive leukocyte esterase test and a negative urine culture included: kidney transplantation (n = 2), pregnancy (n = 3), Foley catheter drainage (n = 4), candida vaginitis (n = 1), bladder carcinoma (n = 1), appendicial abscess (n = 1), large residual urine volume (n = 1), and interstitial cystitis (n = 3). Of the bacterially infected urines collected, $> 10^5$ CFU/ml of the following organisms were cultured: *Escherichia coli* (55%), *Klebsiella* spp (15%), *Pseudomonas aeruginosa* (10%), *Staphylococcus* spp (5%), and other (15%). *E. coli*, *Klebsiella* spp, and *Enterococcus* isolated from urine cultures grown on blood and MacConkey agar plates also were used as controls.

Neutrophil membrane preparation and solubilization of iNOS activity. Pellets isolated from whole urine by centrifugation (400 *g*, 5 min, 4°C) were resuspended in PBS, underlayered with Ficoll-sodium diatrizoate (LSM, Organon Teknika Corp, Durham, NC) and centrifuged (400 *g*, 20 min, 4°C) to isolate an activated population of neutophils. The cells adherent to the side of the tube contained 92±5% leukocytes, while the cells in the pellet contained 90±6% leukocytes (n = 5). The vast majority of these cells are neutrophils as determined by microscopic analysis of the preparations after Papanicolau staining. No significant difference in NOS activity or immunoreactivity was seen between these two fractions. Neutrophil-enriched fractions were resuspended in PBS, centrifuged (400 *g*, 5 min, 4°C), and

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1. *Abbreviations used in this paper:* eNOS, endothelial nitric oxide synthase; iNOS, inducible NOS; RT-PCR, reverse transcriptase PCR; UTI, urinary tract infection.

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resuspended in an ice-cold homgenization buffer (1 vol pellet/10 vol buffer) which consisted of 20 mM Hepes (pH 7.2), 1.0 mM DTT, 0.05 mM phenylmethylsulfonyl fluoride, leupeptin (0.2 mg/100 ml) and 1.0 mg/100 ml of soybean trypsin inhibitor, pepstatin, antipain, and chymostatin (Buffer H). The resuspended pellet was homogenized with an Ultra-turrax T25 (IKA Labortechnik, Staufen, Germany) and centrifuged (20,000 *g*, 20 min, 4°C). The membrane fraction was stored at −80°C until assay. Membranes were resuspended and washed two times to remove exogenous L-arginine and cofactors.

For partial purification of NOS, membranes prepared from urinary pellets from infected patients were extracted at 4°C with 0.8% deoxycholate in buffer H containing 10% glycerol (buffer A). Lysates were centrifuged (50,000 *g* for 40 min at 4°C) and supernatants were passed over a 1-ml column of 2′, 5′ ADP-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was washed with buffer A containing 200 mM NaCl and then with buffer A containing 2 mM $NADP^+$. NOS was eluted with 10 mM NADPH.

NOS activity assays. NOS activity was measured in crude membranes from neutrophil-enriched fractions and in solubilized, partially purified column eluates. NOS activity was measured (45 min, 37°C) as the formation of [^{14}C]-L-citrulline from [^{14}C]-L-arginine (3 μM) in a reaction mix containing: 2 mM NADPH, 1 mM $CaCl_2$, 10 mM valine, 10 μM FAD and 10 μM tetrahydrobiopterin. L-citrulline was eluted from AG 50W-X8 (Na^+-form) (Bio-Rad, Hercules, CA) and [^{14}C]-L-citrulline was quantified by liquid scintillation counting. The enzyme was diluted so that < 25% of [^{14}C]-L-arginine was hydrolyzed. Thin layer chromatography of the radioactive products of the NOS activity assay showed that 82–97% of the radioactivity migrated as citrulline (10). Protein concentrations were measured using gamma globulin as standard with the Bradford Assay (14).

Reverse transcriptase (RT)-PCR of iNOS and endothelial NOS (eNOS) from neutrophil-enriched leukocytes. Total RNA was isolated from neutrophil enriched pellets using Trisolve reagent (Life Technologies, Inc., Gaithersburg, MD). Single stranded cDNA was synthesized using oligo dT priming and Superscript II Reverse-Transcriptase (Gibco-BRL, Life Technologies, Gaithersburg, MD). In preliminary studies, using generalized human NOS primers (sense 5′-AC$^T/_C$CC$^T/_C$GT$^G/_T$TT$^C/_T$CA$^C/_T$CAGGAG-3′ and antisense 5′-CT-G$^G/_C$CC$^A/_G$C$^A/_T$C$^C/_G$AGCTC$^A/_C$TC$^C/_G$CC-3′), PCR amplified products from human iNOS, eNOS, and neuronal NOS cDNAs. However, Southern blotting of PCR products from neutrophil enriched pellets detected only human iNOS and eNOS products, but not neuronal NOS products (data not shown). Human iNOS specific primers for subsequent PCR analysis were: sense 5′-CACCTTTGATGAGGG-GAC-3′ and antisense 5′-GCATCCAGCTTGACCAG-3′. Human eNOS specific primers were: sense 5′-GTGATGGCGAAGCGAGT-GAAG-3′ and antisense 5′-CCGAGCCCGAACACACAGAAC-3′ (15). As a control for cDNA synthesis, β-actin specific primers (sense 5′-AGCGGGAAATCGTGCGTG-3′ and antisense 5′-CAGGGTA-CATGGTGGTGCC-3′) were used (15). PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. The resultant PCR products were 413 bp for iNOS and 422 bp for eNOS. NOS isoform-specific primers did not amplify the other forms of NOS (i.e., iNOS-specific primers did not amplify human eNOS nor neuronal NOS cDNAs).

Western analysis. Laemmli sample buffer was added to total cell lysates, membranes or 2′,5′ ADP-Sepharose purified iNOS and samples were heated to 95°C for 10 min. Proteins were electrophoresed on 7.5% gels by SDS-PAGE, (16) transferred to nitrocellulose and blotted with human iNOS specific polyclonal antisera (1:30,000) (17), eNOS-specific monoclonal antisera (1:1,000) (18) or neuronal NOS antisera (1:1,500; Transduction Labs, Lexington, KY). A goat, anti–rabbit Ig conjugated to horseradish peroxidase (Amersham International, Buckinghamshire, United Kingdom) was used as a secondary antibody for iNOS and a goat, anti–mouse conjugated to horseradish peroxidase (Amersham International) was used as a secondary antibody for eNOS. Immunoreactive proteins were detected with the enhanced chemiluminescence method (Amersham International).

Leukocyte isolation and immunocytochemistry. Cell pellets from fresh nitrite positive, leukocyte esterase positive urines were incubated with CD45 (Anti-HLe-1) monoclonal antibody or with CD14 (LEU-M3) monoclonal antibody (Becton Dickinson, San Jose, CA) (100 μl antibody/75 μl packed pellet) in PBS containing protease inhibitor cocktail (Boehringer Mannheim Biochemicals) and 0.1% BSA, (PBS A) for 30 min (4°C). The CD45 antigen is present on all human leukocytes, while the CD14 antigen is present on the majority of normal peripheral blood monocytes. The antibodies were removed by centrifugation (three times with PBS A, 700 *g*, 3 min) and cells were incubated with fluorescein isothiocyanate labeled anti–mouse IgG (FITC, 1:150) in PBS A for 30 min (4°C). In control cells, the CD45 antibody was omitted. Cells were sorted by flow cytometry, centrifuged onto slides (1,200 rpm, 5 min), and fixed with acetone (−20°C) for 10 min.

For iNOS colocalization studies, the CD45 labeled, flow cytometry–sorted cells were permeabilized with 0.1% Triton X-100 for 3 min, extensively washed in PBS A and incubated sequentially with iNOS polyclonal antibody (1:1,000) for 2 h at room temperature and Texas Red anti–rabbit IgG for 1 h at room temperature. Cells were washed three times after each incubation. Slides were mounted with Slowfade (Molecular Probes, Eugene, OR) and photographed using a Nikon Microphot -FXA microscope (Lake Success, NY). The specificity of the iNOS antibody was determined by preabsorption of the antisera with the immunogen peptide (YRASLEMSAL-COOH) and by the lack of staining with the secondary anti sera alone.

Data analysis. IC_{50}s were determined using a computer assisted log-logit plot (n = two to six experiments performed in duplicate for each inhibitor) and curves were constructed using a curve fitting program. Results were expressed as mean±SEM and means were compared usings analysis of variance followed by the Fisher F-test. Multicomparison significance level was 95%.

Results

To identify the NOS isoform(s) present and to characterize the cell types expressing NOS activity, we performed Ficoll density gradient fractionation of cell pellets isolated from patients with UTIs. The majority of NOS activity is found in neutrophil-enriched fractions, i.e., the adherent fraction and the cell pellet. Differential staining of leukocyte subsets confirmed the enrichment with neutrophils in the NOS-positive fractions. NADPH-dependent NOS activity is 43-fold higher in neutrophil-enriched fractions isolated from urines obtained from patients with UTIs compared with that in neutrophil-positive urines obtained from patients without UTIs (23.5±13.8 [n = 11] and 0.55±0.16 [n = 8] pmol citrulline/mg protein per min, respectively). The leukocyte NOS activity is inhibited competitively by the following inhibitors with a rank order of: L-N^5-(1-iminoethyl)ornithine (L-NIO) > L-thiocitrulline > N^G-monomethyl-L-arginine (L-NMMA) > L-canavanine > aminoguanidine > N^G-nitroarginine methyl ester (L-NAME) = N^G-nitro-L-arginine (L-NNA) (Fig. 1 *A*, Table I). Leukocyte iNOS activity is inhibited by the calmodulin antagonists, trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) (Table I).

Deoxycholate (0.8%) solubilizes 60% of NOS activity from membranes and is more effective than other detergents (CHAPS [10–20 mM], NP-40 [1%], or Triton X-100 [0.1–1%]). Solubilized NOS activity binds to the affinity resin, 2′5′ADP-Sepharose, and is eluted with NADPH (10 mM). This detergent solubilized, semipurified NOS is calcium independent and shares the same inhibitor profile as the crude leukocyte-enriched fraction (Fig. 1 *B*).

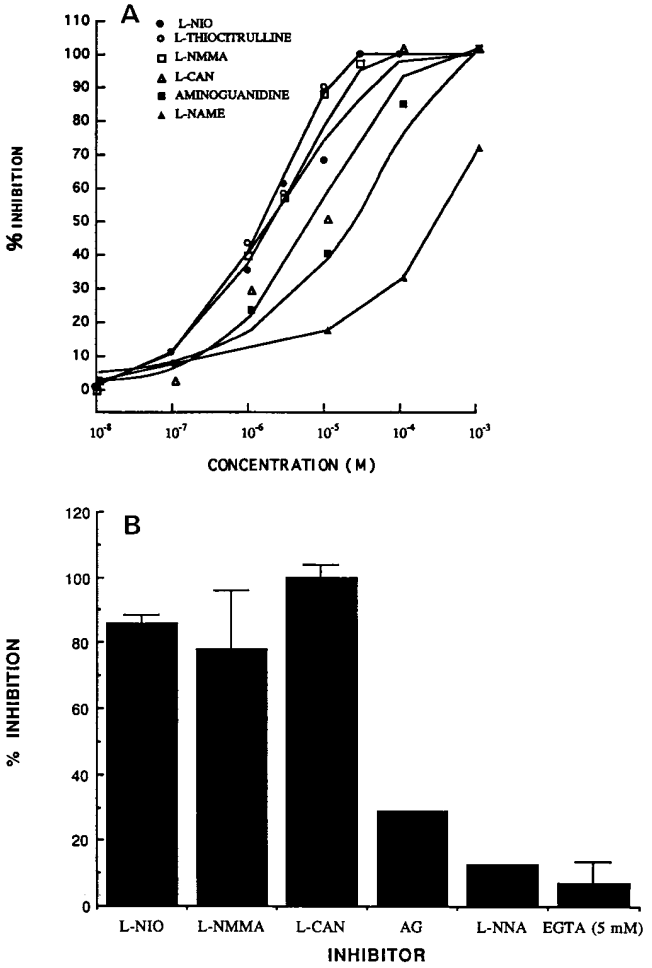


Figure 1. Inhibition of neutrophil-enriched (*A*) or semi-purified (*B*) NOS activity by arginine analogues decreases leukocyte NOS activity. (*A*) Average iNOS values are given in the text. Data are expressed as percentage inhibition of control±SEM for two to three experiments for each inhibitor. Protein concentration of neutrophil-enriched fractions was 0.45±0.08 mg/ml protein. (*B*) Column eluates were assayed for NOS activity in the presence of $CaCl_2$ (*control*), inhibitors (*100 μM*) plus $CaCl_2$ or EGTA (*5 mM, no exogenous $CaCl_2$*).

To molecularly identify the NOS isoform(s) in urine from patients with UTIs, using oligonucleotide primers based on human iNOS and human endothelial NOS (eNOS), we performed RT-PCR on cDNA prepared from leukocyte-enriched total RNA. Fig. 2 reveals a specific 413 bp fragment consistently amplified with human iNOS primers. iNOS primer specific RT-PCR products were found in the eight urines with $> 10^5$ CFU/ml of *E. coli* ($n = 4$), *Enterococcus* ($n = 1$), *Pseudomonas* ($n = 1$), *Klebsiella* ($n = 1$), or *Enterobacter* ($n = 1$). In the same samples, a faint RT-PCR product is seen using eNOS primers. DNA sequencing of the PCR products reveals 99.9% nucleotide sequence identity with their respective cloned human cDNAs. No primer specific RT-PCR product for iNOS was seen in samples prepared from three strains of bacteria.

Next, we used immunological criteria to characterize the iNOS activity in leukocyte enriched pellets using a specific COOH terminal human iNOS polyclonal antibody (17). Western blotting of total lysates or 2′5′ADP-Sepharose purified samples isolated from leukocyte enriched fractions obtained from patients with UTIs identified immunoreactive iNOS protein at ~ 130 kD, identical in M_r to the protein product seen in HEK 293 cells stably transfected with the human hepatocyte iNOS cDNA (Fig. 3, *A* and *B*). No iNOS immunoreactivity is found in leukocyte enriched lysates, that are leukocyte positive, but nitrite and culture negative ($n = 4$, Fig. 3 *A*) or in 2′5′ADP-Sepharose purified samples ($n = 4$) prepared from leukocytes from control patients. Fig. 3 *B* shows that detergent solubilized and ADP-Sepharose purified iNOS from a patient infected with *Pseudomonas aeruginosa* was not detected after 6 d of antibiotic treatment. Preabsorption of the antisera with the immunogen peptide (YRASLEMSAL-COOH) eliminates the iNOS immunoreactivity in the total cell lysate and in ADP-Sepharose fractions as well as in transfected HEK 293 cells (data not shown). Using a specific eNOS monclonal antibody and using bovine eNOS expressed in HEK 293 cells as a positive control, eNOS protein is undetectable by Western blot analysis (18) in six samples that are positive for human iNOS either by Western blotting or by RT-PCR. These samples included two total cell lysates, two leukocyte-enriched lysates and two detergent-solubilized and 2′5′ADP-Sepharose purified samples. Neither iNOS, neuronal NOS, nor eNOS protein was detected in 0.8% deoxycholate extracts from three strains of bacteria which were 2′5′ADP-Sepharose purified.

Table I. NOS Inhibitors and Calmodulin Antagonists Inhibit Leukocyte NOS Activity

	IC_{50}
NOS inhibitor	*μM*
L-NIO	3.0
L-Thiocitrulline	4.1
L-NMMA	7.1
L-Canavanine	12.1
Aminoguanidine	22.0
L-NAME	116
L-NNA	> 200
Calmodulin antagonist	
Trifluoperizine	10
W-7	39

IC_{50} values are determined using a computer-assisted log-logit plot for two to six experiments for each inhibitor, each done in duplicate.

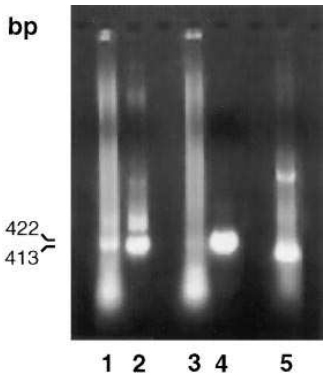


Figure 2. RT-PCR detects iNOS and eNOS mRNA in neutrophils from patients with UTIs. Single stranded cDNA prepared from NOS positive neutrophils was used as a template for RT-PCR with iNOS (lane *1*) and eNOS (lane *3*) specific primers. Lanes *2* and *4* show positive controls using human iNOS and eNOS cDNAs as PCR templates. iNOS and eNOS PCR products were 413 and 422 bp, respectively. β-actin is shown in lane *5*.

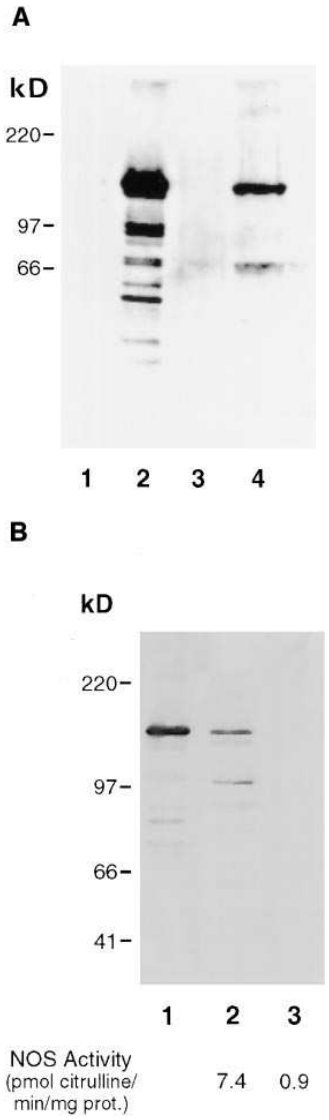


Figure 3. Immunological detection of iNOS in human neutrophils isolated from patients with UTIs. Western blot anaysis was performed on the following samples: (*A*) Equivalent amounts of total cell lysates from nontransfected HEK 293 cells (lane *1*) and HEK 293 cells stably transfected with the human iNOS cDNA (lane *2*), a leukocyte-enriched fraction (100 μg protein) from a leukocyte-positive urine which showed no growth by culture (lane *3*), a leukocyte-enriched fraction (100 μg protein) from a nitrite and leukocyte-positive urine with $> 10^5$ CFU *Pseudomonas* (lane *4*); (*B*) Detergent solubilized and ADP-Sepharose purified NOS from HEK 293 cells stably transfected with the human iNOS cDNA (lane *1*), a leukocyte-enriched fraction (260 μg protein) prepared from a patient infected with $> 10^5$ CFU *Pseudomonas aeruginosa* (leukocyte and nitrite positive) (lane *2*), and a leukocyte-enriched fraction (280 μg protein) prepared from the same patient after 6 d of antibiotic treatment (lane *3*). NOS activity was measured in membrane fractions prepared from each sample. After antibiotic treatment, the urine was leukocyte positive but bacterial growth negative by culture. NOS activity was measured in membrane fractions prepared from each sample.

To determine which cell(s) contained iNOS, cells from patients with UTIs were fluorescently labeled with CD45, sorted by flow cytometry and the CD45 labeled cells were then incubated with iNOS antibody and a fluorescent probe. CD45 antigen is present on all human leukocytes. FACS sorting demonstrates that 86–93% ($n = 3$) of the total fluorescent labeling is found in the CD45 positive peak (Fig. 4 *A*), indicating that the vast majority of cells in patients with UTIs are leukocytes. CD14, a specific marker for human monocytes/macrophages, labels 6±3% of cells, indicating that the predominant population of CD45-labeled cells are neutrophils. Phase contrast microscopy confirmed that > 90% of these cells were neutrophils. After FACS cell sorting of CD45 labeled cells (Fig. 4 *B*), immunocytochemistry with iNOS antibody (Fig. 4 *C*) demonstrates iNOS protein in 82.1±2.9% of the CD45-labeled cells ($n = 10$ fields, from three separate experiments) the vast majority of which are neutrophils. iNOS appears to be localized primarily in the perinuclear region of the CD45-positive neutophils (Fig. 4 *C*).

Neutrophil iNOS activity and protein were analyzed in urine pellets from patients at the time of UTI diagnosis and during the course of antibiotic treatment. In patients with UTIs, average NOS activity in total cell pellet was 10.7±2.9 pmol citrulline/mg protein per min ($n = 5$) at the time of diagnosis, and 23.5±8.6 pmol citrulline/mg protein per min ($n = 5$) after 2–4 d of antibiotic treatment. In these same patients, iNOS activity decreased significantly to 1.5±0.5 pmol citrulline/mg protein per min ($n = 5$) 6–17 d after the diagnosis and initiation of treatment of the UTI. These results are confirmed by Western blot analysis in $^5/_5$ patients; iNOS protein remains elevated after 2–4 d of antibiotic therapy, but is significantly reduced thereafter (Fig. 5).

Discussion

The present study identifies neutrophils as the primary source of iNOS in leukocyte-enriched pellets isolated from the urines of patients with UTIs. It is the first demonstration of biologically active iNOS in human neutrophils isolated during an infectious process. No NOS activity nor immunoreactivity was seen in 16/16 control samples of leukocyte-positive, culture-negative urine (Table II). This agrees with our previous report that NOS activity was significantly lower in 10/10 cell pellets from healthy controls that were leukocyte and urine culture negative compared to cell pellets from patients with UTIs (10). iNOS is markedly elevated in neutrophils (45/48 assays) isolated from the urine of patients (39/40) with UTIs compared with control patients (Table II). NOS activity was not elevated in a single patient with sickle cell anemia, sarcoidosis, and chronic renal failure receiving immunosuppressant therapy who had a history of persistant bacterial (*Enterococcus* and *Klebsiella*) and fungal infections.

The rank order of potency of NOS inhibitors on crude and semipurified neutrophil iNOS is consistent with that seen with human recombinant iNOS activity (19) where L-NMMA and L-NIO are equipotent and both are much more potent than aminoguanidine. L-NNA is a less potent inhibitor of human recombinant iNOS than of human recombinant eNOS or neuronal NOS. In activated rat peritoneal neutrophils, L-NIO and L-NMMA have a higher potency in inhibiting iNOS than does L-NNA (20). The IC_{50}s for L-NMMA and L-NIO are similar for human and rat neutrophil NOS and for human recombinant iNOS, while the ED_{50} for L-NNA is similar for human and rat neutrophil NOS but is higher than that for human recombinant iNOS. Unlike iNOS isolated from rodent sources, human neutrophil iNOS is inhibited by the calmodulin antagonists, TFP and W-7, suggesting that calmodulin is bound less tightly to the human enzyme relative to the rodent form. Indeed, calmodulin is associated tightly, but noncovalently, with purified murine iNOS (21). The affinity of calmodulin binding for human iNOS is presently not known. Irrespective of the potential differences in calmodulin binding affinity to human and rodent iNOS, both enzymes are clearly calcium independent (22).

NOS isolated from rat polymorphonuclear neutrophils (23) and iNOS isolated from transformed macrophages are pri–marily soluble proteins (24). Approximately 40% of iNOS isolated from primary mouse peritoneal macrophages (25) and 25% of the iNOS in the cloned murine macrophage cell line RAW264.7 are membrane associated (26). In contrast, > 90% of human neutrophil iNOS is tightly membrane associated,

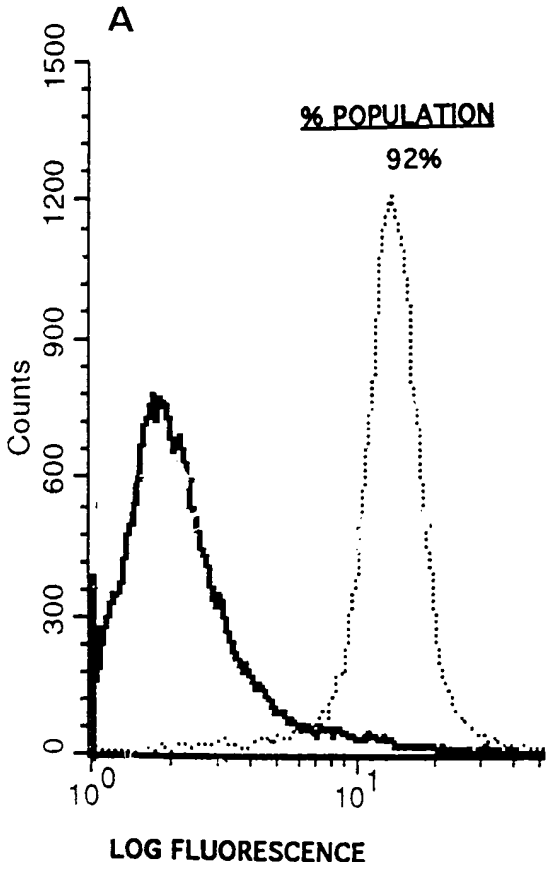
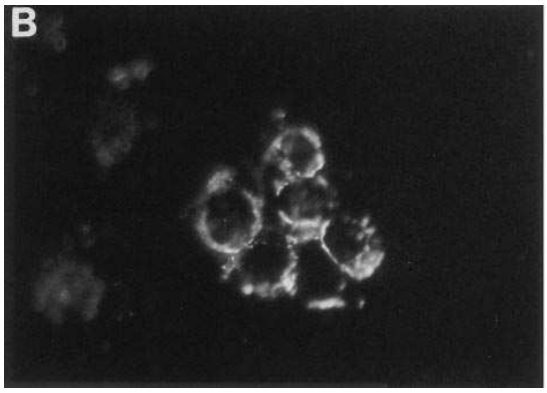
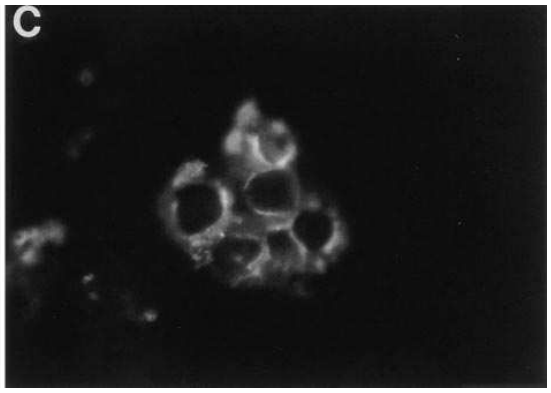


Figure 4. Immunocytochemical demonstration of iNOS in CD45-positive leukocytes. (*A*) FACS-sorted CD45 positive cells were used for immunofluorescence microscopy. Cell pellets from a urine of a patient with a UTI was incubated with CD45. In control cells from the same patient, the CD45 antibody was omitted. Both CD45-labeled and unlabeled cells were then labeled with fluorescein isothiocyanate labeled anti–mouse IgG and labeled and unlabeled cells sorted by flow cytometry, centrifuged onto slides (1,200 rpm, 5 min) and fixed with acetone (−20°C) for 10 min. These CD45 cells (*B*) were visualized for iNOS (*C*) as outlined in the Methods section.





which is similar to the membrane partitioning of endothelial nitric oxide synthase (27). iNOS, eNOS, and neuronal NOS are not integral membrane proteins and do not contain stretches of hydrophobic amino acids consistent with a transmembrane domain. Cotranslational *N*-myristoylation is necessary, but not sufficient for eNOS membrane targeting (28). eNOS also is posttranslationally palmitoylated, but this modification does not contribute to the ability of eNOS to associate with biological membranes but most likely influences specific targeting to endothelial caveolae (29–31). Since iNOS is not myristoylated nor palmitoylated (25), the mechanism for its membrane association remains unclear. Human iNOS is localized primarily in the perinuclear region of the CD45-positive neutrophils. These data are in accord with the perinuclear localization of murine macrophage iNOS in perinuclear, post-Golgi vesicles (25). eNOS also has been localized predominantly in the perinuclear Golgi region of endothelial cells (30). The targeting of iNOS and eNOS to the Golgi regions of cells may allow for NOS to rapidly move to post-Golgi compartments to yield higher local concentrations of NO. Alternatively, the perinuclear localization of NOS isoforms may compartmentalize the two enzymes near critical substrates or cofactors.

RT-PCR amplified both iNOS and eNOS but not neuronal NOS-specific products, and DNA sequencing confirmed the PCR results. These results are consistent with a previous report demonstrating the presence of both iNOS and eNOS RNAs in human blood borne monocytes/macrophages (15). Although eNOS mRNA is detected by RT-PCR, no eNOS protein is demonstrated in samples from patients with UTIs that are positive for human iNOS protein. This evidence, in conjunction with the calcium independence of this NOS activity demonstrates that leukocyte-derived iNOS, not eNOS, is the major source of NOS activity in the urine during infection.

This paper also demonstrates for the first time, isolation of

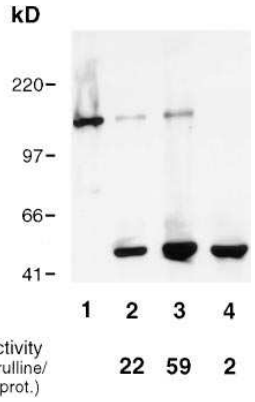


Figure 5. Immunological detection and enzyme activity of iNOS after antibiotic treatment of UTIs. Total cell lysate (25 μg) from urine of a patient with $> 10^5$ CFU/ml *Gardnerella vaginalis* and with symptoms of urinary frequency and dysuria at time of diagnosis (lane *2*), 3 d (lane *3*), and 17 d (lane *4*) after the initiation of antibiotic treatment. NOS activity was measured in membrane fractions prepared from each sample. Total lysate of HEK 293 cells stably transfected with the human iNOS cDNA is shown in lane *1*.

neutrophils from urine using flow cytometry of CD45-positive cells. Neutrophils are the major cell type found in the urine of most patients with UTIs, as CD14, a marker of monocytes/macrophages, labels < 7% of the total cells. This is in agreement with other authors who find that > 90% of the cells present in urine from patients with UTIs are neutrophils (32). Therefore, we have identified human iNOS as the principal isoform of NOS found in human inflammatory neutrophils.

Previously, the expression of iNOS induced by combinations of cytokines and bacterial products was demonstrated in cultured human hepatocytes (33), chondrocytes (34), vascular smooth muscle cells (35), and megakaryocytes (36) and in rodent macrophages (37). Studies in human blood borne neutrophils (9, 38), in extravasated peritoneal polymorphonuclear leukocytes from humans with peritoneal infections (39), and in freshly isolated and cultured human monocytes/macrophages (40) failed to detect significant amounts of iNOS or NO. Recently, neutrophils within human buffy coat preparations stimulated with a mixture of interleukin-1, tumor necrosis factor α, and interferon-γ were shown to contain iNOS mRNA and protein, however iNOS was detected in only 20% of the stimulated neutrophils (41). Studies with monocytes/macrophages indicate that these cells can produce NO after infection with mycobacteria (17) or human immunodeficiency virus type I (42) and also after cross-linking of the surface receptor CD69 (43) or ligation of FcεII/CD23 (44, 45). Because NO is increased under conditions where the inducing agents are undefined, the combination of microbial products and cytokines, that induces iNOS in human disease states, warrants further investigation.

During the course of a UTI, bacterial colonization causes uroepithelial cells in the bladder to rapidly secrete IL-6 (11) and IL-8 (12) and initiates a large influx of neutrophils. Neutrophil numbers can rise to 10^3–10^8 cells/ml urine in UTIs (13). We find a large increase in the NOS activity and NOS protein in these leukocytes at the time of UTI diagnosis. Since leukocyte cytoplasts can generate reactive nitrogen intermediates that kill bacteria (46) and since many UTIs resolve spontaneously, the increased NOS activity seen in urinary leukocytes from patients with UTIs may be involved in bacterial killing. Phagocytosed bacteria can be killed, perhaps by the formation of peroxynitrite, a reaction product of NO and superoxide anions. In studies with cytokine-activated blood borne neutrophils, only neutrophils that had ingested bacteria showed evidence of NOS activity as indicated by the formation of nitrotyrosine (41). While iNOS may be bactericidal, iNOS in UTIs may contribute to the inflammatory response in infection (47). Many UTI symptoms, such as dysuria and frequency, are due to the acute inflammatory response and not to the presence of the bacteria. Bacterial numbers frequently are greatly reduced within hours after initiation of antibiotic treatment (48) and yet the symptoms related to a UTI may persist for days. NOS activity and immunoreactivity persist for a period of time after initiation of antibiotic treatment, suggesting that iNOS is involved in the inflammatory response seen in UTIs. Because UTIs represent an ongoing infectious process with interplay of bacteria and host defense mechanisms, the characterization of iNOS in neutrophils from infected urine suggests an important role for NO as a mediator of inflammation. Measurement of iNOS in neutrophils isolated from urine may aid in diagnosis and evaluation of other inflammatory diseases such as sepsis and renal transplant rejection states.

Table II. Detection of iNOS in Neutrophils Isolated from Urine

	Control samples	UTI samples
	Leukocyte+/Culture−	Leukocyte+/Culture+
	NOS-positive samples/total samples analyzed	
NOS activity	0/8	21/23
iNOS PCR product	−	8/8
Western blot analysis for iNOS	0/8	13/14
Immunohistochemistry of neutrophils for iNOS	−	3/3 (82.1% of cells)

16 control and 40 patients with UTIs were evaluated in this study. More than one test was performed on some patients. NADPH-dependent NOS activity values of > 5 pmol citrulline/mg protein per min were considered to represent iNOS; NADPH-dependent NOS activity values < 2 pmol citrulline/mg protein per min were considered control levels. Two patients were negative for iNOS at the time of diagnosis. A 55-yr-old female with sickle cell anemia and renal failure had little NOS activity in her urine at a time in which *Enterococcus* was cultured from a catherized specimen. This patient who was on immunosuppressives had repeated positive urine cultures with *Klebsiella*, *Torulopsis*, and *Enterococcus* and her NOS activity remained low. A 25-yr-old female was negative for both NOS activity and iNOS Western blot analysis when *E. coli* was cultured in her urine. 3 d after treatment, however, immunoreactivity was visualized by Western blot analysis for iNOS.

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