Urinary tract infection in iNOS-deficient mice with focus on bacterial sensitivity to nitric oxide

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Poljakovic, Mirjana, and Katarina Persson. Urinary tract infection in iNOS-deficient mice with focus on bacterial sensitivity to nitric oxide. Am J Physiol Renal Physiol 284:F22–F31, 2003. First published September 3, 2002; 10.1152/ajprenal.00101.2002.—Inducible nitric oxide synthase (iNOS)-deficient mice were used to examine the role of iNOS in Escherichia coli-induced urinary tract infection (UTI). The toxicity of nitric oxide (NO)/peroxynitrite to bacteria and host was also investigated. The nitrite levels in urine of iNOS+/+ but not iNOS−/− mice increased after infection. No differences in bacterial clearance or persistence were noted between the genotypes. In vitro, the uropathogenic E. coli 1177 was sensitive to 3-morpholinosydnonimine, whereas the avirulent E. coli HB101 was sensitive to both NO and 3-morpholinosydnonimine. E. coli HB101 was statistically (P < 0.05) more sensitive to peroxynitrite than E. coli 1177. Nitrotyrosine immunoreactivity was observed in infected bladders of both genotypes and in infected kidneys of iNOS+/+ mice. Myeloperoxidase, neuronal (n)NOS, and endothelial (e)NOS immunoreactivity was observed in inflammatory cells of both genotypes. Our results indicate that iNOS−/− and iNOS+/+ mice are equally susceptible to E. coli-induced UTI and that the toxicity of NO to E. coli depends on bacterial virulence. Furthermore, myeloperoxidase and nNOS/eNOS may contribute to nitrotyrosine formation in the absence of iNOS.

inducible nitric oxide synthase; myeloperoxidase; Escherichia coli; transgenic; nitrotyrosine

URINARY TRACT INFECTIONS (UTIs), including cystitis and pyelonephritis, are among the most common bacterial infections in man and Escherichia coli is the most causative agent of the infection. The bacteria are cleared from the urinary tract through the action of inflammatory cells, particularly by polymorphonuclear (PMN) cells (23). The production of antimicrobial factors such as nitric oxide (NO) may contribute to control UTIs. It has been demonstrated that patients with UTI have an elevated nitrite concentration in the urine compared with healthy controls (42). Increased gaseous NO concentrations in the urinary bladder in patients with lower UTI have also been reported (30). Previously, we have demonstrated increased urinary nitrite production in E. coli-infected mice (41). However, the actual role of NO in UTI has not been eluci-

dated, and it is not clear whether NO has a bactericidal effect on UTI.

In inflammatory responses, NO is produced by an enzyme known as inducible nitric oxide synthase (iNOS). Genetically altered mice with a mutation of the iNOS gene have been used to define the role of NO production and iNOS expression in infection. Mice lacking iNOS were more susceptible to herpes simplex virus infection than their corresponding wild-type controls (31), and NO production seemed to be important in the host response to extracellular gram-positive bacteria (33). However, disruption of iNOS improved the clearance of Mycobacterium avium, and increased NO production seemed to exacerbate this infection rather than clear it up (19).

NO itself is neither highly reactive nor particularly toxic but forms oxidants that are responsible for its toxicity. The chemical reactivity and toxicity of NO can be increased by its diffusion-limited reaction with superoxide (O2-) to form peroxynitrite (4). Peroxynitrite is a major damaging oxidant that is likely to account for most of the cytotoxicity commonly attributed to NO. Once formed, peroxynitrite reacts with most biological molecules, and modification of structural proteins may be an important pathological target (2). The nitration of protein tyrosine residues by peroxynitrite produces 3-nitrotyrosine, an index of peroxynitrite formation in vivo (4). In inflammatory processes, tissue nitration is preferentially located around areas in which peroxynitrite-producing cells are most abundant, and strong nitrotyrosine immunoreactivity is usually observed in macrophage- or neutrophil-rich areas (7, 14, 28). Macrophages, neutrophils, and other phagocytic cells are known to generate large amounts of toxic molecules, including peroxynitrite, which has been found to be bactericidal (49). Neutrophils in the urinary tract have been shown to express iNOS after a bacterial infection (41, 46), but it remains to be established whether urinary neutrophils also produce peroxynitrite. Extensive nitrotyrosine expression has been observed in proximal tubular epithelial cells in kidneys of LPS-treated rats (6), but nitrotyrosine expression has not been investigated in bladder tissue.

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This study examined the significance of iNOS induction and NO production for the clearance of E. coli from the urinary tract of wild-type and iNOS-deficient mice. Furthermore, the toxicity of NO and peroxynitrite to different E. coli strains was examined in vitro and the toxicity of NO to the host tissue was assessed by investigation of nitrotyrosine formation.

**METHODS**

**Mice**

C57BL/6J and C57BL/6-NOS2−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were constructed as previously described (29). Female mice were used at 9 wk of age. The mice were kept on a nitrate/nitrite-free diet (Altromin 1324N, Petersen, Ringsted, Denmark), starting 1 wk before the experiment. A few days before infection, groups of five mice were separated into individual cages, and urine samples were collected and examined for bacterial growth and the leukocyte cell content was determined microscopically using a hemocytometer chamber. Mice with more than 5 × 10⁶ leukocytes/ml in preinoculation urine samples or with a positive bacterial culture were excluded from the experiment. The experimental protocol was approved by the Animal Ethics Committee, Lund University, Lund, Sweden.

**Bacteria**

**In vivo experiments.** E. coli 1177, of serotype O1:K1:H7, was isolated from a child with acute pyelonephritis (32). The strain is virulent in the mouse UTI model and evokes a strong inflammatory host response (10). It expresses type 1 fimbrial adhesins but is hemolysin negative. E. coli 1177 was maintained in deep agar stabs, passaged on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI), grown overnight at 37°C in static Luria broth, and harvested by centrifugation at 3,200 rpm for 10 min. The pellet was resuspended in sterile PBS, pH 7.2, to a concentration of 10⁹ colony-forming units (CFU)/ml.

**In vitro experiments.** When used for the in vitro experiments, E. coli 1177 was grown overnight at 37°C on TSA and harvested in sterile PBS by centrifugation at 3,200 rpm for 10 min. As an avirulent strain, a nonfimbriated K12 derivative, E. coli HB101, was used. HB101 was grown overnight at 37°C on TSA and harvested in sterile PBS by centrifugation at 3,200 rpm for 10 min. The pellets of both bacterial strains were resuspended and then diluted in sterile PBS to a final concentration of 10⁶ CFU/ml.

**Infection Procedure**

The mouse bladder was emptied by gentle compression on the lower abdomen, and urine was saved for preinfection measurements of nitrite (see below). Experimental UTI was established in the mice by intravesical injection of E. coli 1177 as previously described (22). After anesthesia, 0.1 ml of bacterial suspension was slowly instilled into the bladder transurethrally, using a soft polyethylene catheter (outer diameter 0.61 mm; Kebolab, Malmö, Sweden). The catheter was immediately withdrawn after inoculation, and no further manipulations were carried out. The animals were placed in the cages after instillation and allowed food and water ad libitum. Infection was monitored at 6, 24, and 72 h and 7 days. Urine samples were stored on ice or at 4°C until further analysis.

Infection was quantified by viable bacterial counts on tissue homogenates from mice killed by CO₂ asphyxia at different times after inoculation. One-half of the bladder and one of the kidneys were aseptically harvested and homogenized in 5 ml of sterile PBS in sterile disposable plastic bags using a LAB Stomacher 80 Homogenizer (Seward Medical UAC House, London, UK). Serial dilutions of the tissue homogenates were plated on TSA. After overnight culture, plates with bacterial colonies were scored and bacterial numbers (CFU/ml of tissue homogenate) were determined after adjustment for the dilution factor. The other half of the bladder and the second kidney were processed for immunohistochemistry as described below.

**Immunohistochemistry**

Bladders and kidneys were immersion-fixed for 4 h in cold 4% formaldehyde in PBS (pH 7.4) and then rinsed for 3 days in PBS containing 15% sucrose. Both fixation and rinsing were performed at 4°C, after which the specimens were frozen in isopentane at −40°C and stored at −70°C until sectioning. Sections were cut (10 μm) on a Leica CM3050; Leica Microsystems, Askö, Sweden) and preincubated with PBS containing 0.2% Triton X-100 and 0.1% BSA for 2 h at room temperature. Sections were incubated with the following primary antibodies (diluted with PBS containing 0.2% Triton X-100 and 0.1% BSA): a rabbit polyclonal antibody raised to murine iNOS (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA); a rabbit polyclonal antibody raised to nitrotyrosine (1:470; Upstate Biotechnology, Lake Placid, NY); a sheep polyclonal antibody raised to neuronal (n)NOS (1:1,000; a gift from Drs. I. Charles and P. C. Emson, Cambridge Univ., Cambridge, UK); or a rabbit polyclonal antibody raised to endothelial (e)NOS (1:250; Santa Cruz Biotechnology) overnight in a moisture chamber at room temperature. Sections were incubated with a rabbit polyclonal antibody raised to MPO (1:200; NeoMarkers, Fremont, CA) for 2 h at room temperature. The sections were rinsed in PBS and incubated for 90 min with FITC-conjugated donkey anti-rabbit IgG (1:80), Texas red (TR)-conjugated F(ab′)₂ fragment donkey anti-rabbit IgG (1:160; both from Jackson Immunoresearch Laboratories, West Grove, PA), or FITC-conjugated donkey anti-sheep IgG (1:80; Sigma) diluted in PBS. The sections were rinsed in PBS and mounted in glycercol with p-phenylendiamine to prevent fading.

To identify the inflammatory cells, a double-label immunofluorescence method was used. Sections were first incubated overnight with RB6–8C5, a rat IgG2b monoclonal antibody specific for murine neutrophils (a gift from Dr. A. Jöstedt, Umeå University, Umeå, Sweden). After being rinsed in PBS, the iNOS antibody was added and the sections were incubated again overnight. RB6–8C5 staining was visualized by incubating the sections for 90 min with TR-conjugated F(ab′)₂ fragment donkey anti-rat IgG (1:160; both from Jackson Immunoresearch Laboratories, West Grove, PA), or FITC-conjugated donkey anti-sheep IgG (1:80; Sigma) diluted in PBS. The sections were rinsed in PBS and mounted in glycercol with p-phenylendiamine to prevent fading.

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was used for image handling, and the three-color channels were handled separately. Only the background level, contrast, and brightness of the entire image were changed in the final picture.

**Hematoxylin and Eosin Staining**

The morphology of the inflammatory cells was examined after staining of the tissue sections with hematoxylin and eosin (Apoteksbolaget, Malmö, Sweden).

**DNA Isolation and Genotyping with PCR**

PCR analyses for the intact and disrupted iNOS gene were performed to genotype the mice. Briefly, DNA was extracted from the tip of the tail by incubating the tail in lysis buffer (50 mM Tris·Cl, 5 mM EDTA, 100 mM NaCl, and 0.5% SDS; all from Sigma, St. Louis, MO) containing proteinase K (10 mg/ml; Sigma) overnight at 56°C. PCR was performed according to the Sigma PCR-Core kit with Taq DNA polymerase (Sigma), using 2 µg genomic DNA. The primers for mouse iNOS were obtained from MWG-Biotech (Ebersberg, Germany) and were as follows: sense, 5’-CAG ATC GAG CCC AGG CGT AGC-3’, and antisense, 5’-CCT TGG TGT TGA TGG AAG ACC-3’, amplifying a 533-bp product. PCR was performed in an automated thermal cycler (GeneAmp PCR System 2400, PerkinElmer, Foster City, CA), with one cycle at 94°C for 60 s, at 72°C for 60 s, and a final cycle at 94°C for 5 min. The primers for mouse genomic DNA. The primers for mouse iNOS were designated as nitrite, a stable end product of NO (20). Most uropathogens are members of the family Enterobacteriaceae, known to reduce nitrate to nitrite. Urine contains nitrates from dietary sources, but the mice in our study were fed a nitrate/nitrite-free diet to ensure that we measured nitrite formed from endogenous NO and not from bacterially converted nitrate. The urinary nitrite levels increased in wild-type mice after infection with E. coli 1177 at 6 h postinfection (42 ± 12 µM, n = 12, P < 0.05) compared with 0 h (12 ± 1 µM, n = 12) (Fig. 2A). The urinary nitrite levels were low at 24 and 72 h and 7 days after bacterial instillation. iNOS−/− mice showed no urinary nitrite response to infection (Fig. 2B).

**Bacterial Counts of Infected Tissue**

Bladders and kidneys were harvested from the E. coli 1177-infected iNOS+/+ and iNOS−/− mice, and bacterial persistence was determined by viable counts on tissue homogenates. There was no significant difference in bacterial persistence or clearance in bladders obtained from iNOS wild-type or iNOS-deficient mice (Fig. 3A). The highest bacterial numbers were found 24 h after infection in wild-type mice and 6 h after infection in iNOS-deficient mice. Between 72 h and 7 days after infection, the bacterial numbers were fairly constant and none of the mice strains managed to clear the infection.

**Results**

The genotype of the mice was confirmed by PCR using primers specific for the calmodulin-binding domain of the iNOS gene. A 533-bp product, corresponding to the calmodulin-binding domain of iNOS, was detected in all the investigated C57BL/6J mice (iNOS+/+) but not in the investigated transgenic iNOS−/− mice (Fig. 1).

**Nitrite Levels in Urine**

The production of NO into the urine in response to infection was quantified as nitrite, a stable end product of NO (20). Most uropathogens are members of the family Enterobacteriaceae, known to reduce nitrate to nitrite. Urine contains nitrates from dietary sources, but the mice in our study were fed a nitrate/nitrite-free diet to ensure that we measured nitrite formed from endogenous NO and not from bacterially converted nitrate. The urinary nitrite levels increased in wild-type mice after infection with E. coli 1177 at 6 h postinfection (42 ± 12 µM, n = 12, P < 0.05) compared with 0 h (12 ± 1 µM, n = 12) (Fig. 2A). The urinary nitrite levels were low at 24 and 72 h and 7 days after bacterial instillation. iNOS−/− mice showed no urinary nitrite response to infection (Fig. 2B).

**Bacterial Viability Experiments**

Bacterial viability in response to exogenously applied NO and 3-morpholinosydnonimine (SIN-1), a generator of peroxynitrite in solution, was examined. One milliliter (10⁹ CFU/ml) of E. coli 1177 or E. coli HB101 in PBS was transferred to a sterile test tube. The bacteria were exposed to either NO [2,2-(hydroxynitrosohydrazino)bis-ethanamine (DETA/NO); 500 µM, Alexis Biochemicals, Lausen, Switzerland] or SIN-1 (500 µM, Casella, Frankfurt am Main, Germany) for 24 h. Untreated bacteria were used as control. Serial dilutions were plated on TSA and, after overnight culture, plates with bacterial colonies were scored and bacterial numbers (CFU/ml) were determined after adjustment for the dilution factor.

**Analysis of Data**

Data are expressed as means ± SE. Student’s paired or unpaired t-test was used to compare two means, and ANOVA followed by the Bonferroni-Dunn post hoc test was used for multiple comparisons (GraphPad Prism 3.0). Differences were considered significant at P < 0.05.
Morphological Examination

Bladders and kidneys were examined for iNOS, nNOS, eNOS, nitrotyrosine, and MPO expression by immunohistochemistry at 6, 24, and 72 h and 7 days postinstillation.

Identification of inflammatory cells. Hematoxylin and eosin staining showed that the majority of the inflammatory cells in bladders and kidneys were PMN cells. By double-label immunofluorescence using the neutrophil marker RB6–8C5, the majority of the iNOS-positive cells were identified as neutrophils (data not shown).

iNOS immunoreactivity. Low levels of two abnormal iNOS transcripts have been detected in macrophages isolated from iNOS−/− mice (29). These transcripts may produce low levels of immunoreactive peptides that are enzymatically inactive but recognized by some iNOS antibodies (The Jackson Laboratory, personal communication). The iNOS antibody used in our study detected iNOS in inflammatory cells in bladders of both wild-type and iNOS-deficient mice 6 h after infection (data not shown). iNOS-positive uroepithelial cells were found in the majority of bladders from iNOS+/− mice from 6 h and up to 7 days after infection (Fig. 4A). iNOS immunoreactivity was, however, not observed in the bladder urothelium of iNOS−/− mice (Fig. 4B).

Kidneys of infected iNOS+/+ and iNOS−/− mice were devoid of iNOS-expressing inflammatory cells at all times. iNOS immunoreactivity in transitional and columnar epithelial cells lining the renal pelvis was found in the majority of wild-type mice at 6 and 24 h postinstillation, and this immunoreactivity was further increased at 72 h and 7 days after infection (Fig. 4C). The iNOS-deficient mice did not express iNOS immunoreactivity in epithelial cells lining the renal pelvis (Fig. 4D).

nNOS and eNOS immunoreactivity. We next examined whether a compensatory increase in nNOS and eNOS expression had occurred in iNOS-deficient mice. In the bladder, nNOS-immunoreactive neuronal structures were observed within the smooth muscle and submucosa of E. coli-infected bladders of both wild-type and iNOS-deficient mice. In the kidney, nNOS immunoreactivity was sparse and only observed in the

Fig. 2. Nitrite levels in urine samples obtained after instillation of Escherichia coli strain 1177 in wild-type (A) and iNOS-deficient mice (B). The preinoculation nitrite values are given at time 0. Values are means ± SE (n = 3–24). *P < 0.05.

Fig. 3. Wild-type (○) and iNOS-deficient (●) mice were inoculated with E. coli 1177, and the infection was monitored by viable bacterial counts of bladder (A) and kidney homogenates (B) after 6, 24, and 72 h or 7 days. There was no significant difference in bacterial clearance or persistence between the genotypes. Values are means ± SE expressed as colony-forming units (CFU)/ml (n = 3–6).

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Fig. 4. (A) iNOS immunoreactivity in transitional and columnar epithelial cells lining the renal pelvis. (B) iNOS immunoreactivity in transitional and columnar epithelial cells lining the renal pelvis.
macula densa and in a few nerve fibers associated with blood vessels. eNOS-positive immunoreactivity was observed in vascular endothelium. The inflammatory cells of both genotypes were found to express nNOS and eNOS (Fig. 5, A and B). As judged by visual observation, no difference in nNOS or eNOS expression was observed when infected iNOS-deficient mice were compared with infected wild-type mice.

Nitrotyrosine immunoreactivity. Immunoreactivity to nitrotyrosine was not found in uninfected bladders and kidneys. Nitrotyrosine immunoreactivity was, however, found in bladders of infected iNOS−/+ and iNOS−/− mice at all times (Fig. 6A). Three types of cells/structures were found to express nitrotyrosine. PMN cells and large round cells, resembling inflammatory cells, showed nitrotyrosine staining (Fig. 6B). In addition, small nitrotyrosine-positive structures, with no visible nucleus, were found close to the inflammatory cells in the submucosa (Fig. 6B). In kidneys of wild-type mice, nitrotyrosine was observed in inflammatory cells and in small structures in the renal pelvis primarily at 72 h and 7 days postinfection (Fig. 6C). Nitrotyrosine immunoreactivity was found in the glomeruli of one iNOS−/+ mouse 72 h after bacterial infection (Fig. 6D). Nitrotyrosine was not detected in kidneys of iNOS−/− mice (Fig. 6E).

MPO immunoreactivity. Because nitrotyrosine immunoreactivity was observed in iNOS-deficient mice, possible sources for protein nitration other than peroxynitrite were investigated. MPO is an enzyme used by granulocytes during phagocytosis. In the presence of nitrite, MPO can nitrate protein tyrosines (13). No immunoreactivity to MPO was observed in uninfected bladders and kidneys. Numerous MPO-immunoreactive inflammatory cells were observed in infected bladders of both iNOS−/+ (Fig. 7A) and iNOS−/− (Fig. 7B) mice at all times. The MPO-immunoreactive cells were located within the bladder smooth muscle layer, lamina propria, and close to or within the uroepithelium. In the kidney, inflammatory cells expressing MPO were observed close to the epithelium lining the renal pelvis mainly at 6 h after infection. Some MPO-immu...
noreactive inflammatory cells were also observed in the glomeruli (Fig. 7C) at all times. There was no difference in the number or distribution of MPO-expressing cells between the genotypes as judged by visual observation. However, MPO immunoreactivity was by far more pronounced in the bladder than in the kidney. The uroepithelial cells were not stained for MPO.

**Bacterial Viability**

The effect of exogenously added NO and SIN-1, a peroxynitrite donor in solution, on bacterial viability was investigated. When *E. coli* 1177 was exposed to DETA/NO (500 μM), which spontaneously decomposes and provides a constant NO supply over hours (3), the number of colonies formed was only slightly (14 ± 8.4%, n = 3) decreased compared with untreated controls. However, when exposed to SIN-1 (500 μM), the number of viable colonies of *E. coli* 1177 was significantly decreased by 53 ± 12% (n = 3, P < 0.05) compared with untreated bacteria (Fig. 8A). HB101, the nonfimbriated *E. coli* strain, was sensitive to both NO and SIN-1. DETA/NO (500 μM) and SIN-1 (500 μM) caused a significant decrease in *E. coli* HB101 viability by 25 ± 7.8 (n = 3, P < 0.05) and 85 ± 3.5% (n = 3, P < 0.001), respectively (Fig. 8B). *E. coli* HB101 was statistically (P < 0.05) more sensitive to SIN-1 than *E. coli* 1177.

**DISCUSSION**

In this study, iNOS-deficient mice were used to examine the role of iNOS expression and NO production in bacterial clearance after an *E. coli*-induced UTI. Uropathogenic *E. coli* was found to persist in bladders and kidneys of iNOS+/+ and iNOS−/− mice for up to 7 days after infection, and none of the mice strains managed to clear the infection. Bacteria have previously been reported to persist within the bladder of C57BL/6 mice for days and weeks (26, 37), with the persistence being attributed to the type 1 pili (10, 36). There was no significant difference in bacterial clearance or persistence between wild-type and iNOS−/− mice, suggesting that the bacteria persisted in bladders and kidneys irrespective of iNOS induction. These findings are con-
sistent with an earlier study, showing that pharmacological inhibition of iNOS did not alter the sensitivity of C3H/HeN and C3H/HeJ mice to renal infection caused by two different *E. coli* uropathogens (39). Furthermore, in experimental glomerulonephritis, no difference in the disease was observed when iNOS-deficient mice were compared with wild-type mice (9).

Immunoreactivity to iNOS was observed in bladder inflammatory cells of iNOS−/− mice and, surprisingly, also in the iNOS−/− mice. Low expression of two abnormal iNOS transcripts, which may produce immunoreactive peptides, has been described in macrophages isolated from the iNOS−/− mice used in this study (29). Some iNOS antibodies may recognize the produced peptides and, indeed, several investigators have noticed iNOS-positive labeling in the iNOS−/− mice (The Jackson Laboratory, personal communication). Nevertheless, these transcripts are enzymatically defective and do not produce NO. In our study, no increase in urinary nitrite levels of infected iNOS−/− mice was detected, confirming the absence of iNOS activity in these animals. Genotypic studies further confirmed the disruption of the iNOS gene in transgenic mice. The nitrite levels in urine from *E. coli*-infected iNOS+/+ mice reached a peak 6 h after instillation, which coincided in time with the presence of numerous iNOS-positive inflammatory cells in the bladder. iNOS immunoreactivity in uroepithelial cells was found up to 7 days after infection, but urinary nitrite levels were not increased in samples analyzed 1, 3, or 7 days postinfection. This suggests that inflammatory cells, and not uroepithelial cells, are the main contributors of NO/nitrite in urine samples of infected mice.

We have previously demonstrated iNOS immunoreactivity in uroepithelial cells at 24 and 72 h after infection in C3H/HeN mice infected with *E. coli* (41). In the present study, using C57BL/6 wild-type mice and a different *E. coli* strain, iNOS immunoreactivity in uroepithelial cells was seen as early as 6 h postinfection, and the expression increased with time. Thus the genetic background of the mice as well as the bacterial strain seem to affect the rate and degree of the iNOS response in host uroepithelial cells. iNOS immunoreactivity was not found in uroepithelial cells in bladders and kidneys of iNOS-deficient mice. This may suggest that uroepithelial cells in iNOS−/− mice, unlike inflammatory cells, do not produce the abnormal iNOS transcripts.

It is generally believed that NO in inflammatory cells is antimicrobial and that it participates in the host defense against invading pathogens (15). However, the role of iNOS induction in uroepithelial cells is not clear. Induction of iNOS in uroepithelial cells may be involved in uroepithelial cell shedding by promoting deletion of infected and damaged cells (16, 34). Inhibition of iNOS was found to reduce intestinal permeability and to decrease bacterial translocation by limiting the damage to the gut mucosa (12, 43). This suggests that NO may favor bacterial translocation through the epithelium. Massive bladder uroepithelial cell shedding has been reported within 6 h after infection with type 1 piliated *E. coli* in the same mice strain, C57BL/6, used in our study (38). The degree of uroepithelial cell shedding was not specifically investigated in the present study, and it is unclear whether iNOS-deficient bladders and kidneys showed less shedding than tissue from wild-type mice.
Recent evidence shows that most organisms, including E. coli, are able to metabolize and detoxify NO. Studies have shown that aerobic E. coli strains are protected against NO toxicity by expressing an NO-inducible NO deoxygenase (NOD) (17). NOD is a flavohemoglobin, which oxidizes NO to NO$_3$ (18), thereby protecting E. coli against the toxic effects of NO. Additional protective mechanisms against NO include expression of stress regulons at the DNA level. Studies on E. coli have demonstrated the presence of specific antioxidant regulons, such as the soxRS regulon, which after induction may protect bacteria against NO (44). E. coli carrying a deletion of the soxRS locus is hypersusceptible to NO-dependent killing (40). It is likely that pathogenic bacteria benefit most by developing NO/peroxynitrite resistance. In the present study, the pathogenic E. coli strain 1177 was significantly more resistant to peroxynitrite than the nonpathogenic E. coli strain HB101. This suggests that the outcome of NO toxicity to E. coli in UTI may depend on bacterial virulence. Moreover, the relative resistance of E. coli strain 1177 to NO/peroxynitrite, as found in vitro, may explain that no differences in bacterial clearance were noted between infected iNOS-deficient and wild-type mice.

The cytotoxicity of NO/peroxynitrite is not only directed to invading pathogens but may also affect NO-producing cells and surrounding tissue (35). Increased iNOS expression and NO production have been shown to coincide in time with maximal kidney damage in acute pyelonephritis (27). Nitrotyrosine has become a useful marker of peroxynitrite formation in vivo (5). Immunoreactivity to nitrotyrosine was found in bladder inflammatory cells at all time points, demonstrating that urinary neutrophils produce peroxynitrite. No difference in nitrotyrosine staining in the bladder was detected between the genotypes, suggesting that peroxynitrite formed in iNOS$^{-/-}$ mice must depend on NO sources other than iNOS. It has previously been demonstrated that the lack of iNOS does not fully abolish tyrosine nitration (50). Production of NO by other isoforms of NO synthase is a possible source of NO in iNOS-deficient mice. In our study, nNOS and eNOS immunoreactivity was observed in inflammatory cells in bladders of E. coli-infected iNOS-deficient mice. It was recently demonstrated that human neutrophils express eNOS (11) and that human and rat neutrophils express nNOS mRNA (21). Also, rat neutrophils have been shown to express nNOS protein and spontaneously release nitrite and nitrate anions (21). Purified nNOS was found to produce both O$_2$ and NO and to form peroxynitrite (48). Thus evidence exists to support the notion that nitrotyrosine can be formed by NO derived from nNOS and/or eNOS in mice lacking iNOS. A compensatory increase in NO production from nNOS and/or eNOS may have evolved in iNOS-deficient mice. However, the expression of nNOS and eNOS protein did not appear to differ in wild-type and iNOS$^{-/-}$ mice, at least not after immunohistochemical evaluation.

MPO, an enzyme implicated in various inflammatory diseases, has been suggested as a potential pathway...
for nitrotyrosine formation. MPO is a major neutrophil protein and is stored in granules and released during phagocytosis (47). MPO may use either hydrogen peroxide (H₂O₂) or hypochlorous acid (HOCl) to oxidize nitrite and form reactive nitrogen intermediates that may result in tissue nitration (24, 45). In our study, numerous MPO-stained inflammatory cells were observed in infected bladders of both wild-type and iNOS-deficient mice. This suggests that MPO may contribute to the detected nitrotyrosine formation in bladders of iNOS−/− mice. Nitrotyrosine expression, but no iNOS expression, was observed in inflammatory cells in kidneys of iNOS+/+ mice. We have previously detected iNOS-positive inflammatory cells in the kidney of infected C3H/HeN mice (41). It is likely that iNOS expression in kidney inflammatory cells in the present study, which used a different mouse strain and a more virulent bacterial strain, peaked earlier than 6 h. Furthermore, MPO-positive cells were observed in the kidney and, as discussed above, MPO may contribute to nitrotyrosine formation. Unlike in the bladder, nitrotyrosine was not observed in kidneys of iNOS−/− mice, suggesting that no peroxynitrite- or MPO-derived nitrotyrosine formation occurred in the kidney of iNOS-deficient mice. Indeed, MPO and nNOS/eNOS expression, the possible alternative sources for nitrotyrosine formation in the absence of iNOS, was not as pronounced in the kidney as in the bladder.

Nitrotyrosine was also observed in the vicinity of inflammatory cells in small submucosal structures. The inflammatory cells may secret peroxynitrite and cause nitration in membrane or intracellular compartments of target cells (1). In our study, the small structures observed could be nitrated structural tissue proteins or nitrotyrosine formed on the bacteria (14). The modification of structural proteins may be a particularly important pathological target of nitration by causing disruption of the normal function of cellular structures (2).

In conclusion, our results indicate that wild-type and iNOS-deficient mice are equally susceptible to E. coli-induced UTI and that the outcome of NO toxicity to E. coli may depend on bacterial virulence. Furthermore, the lack of iNOS did not abolish nitrotyrosine formation. Myeloperoxidase and nNOS/eNOS may contribute to nitrotyrosine formation in the absence of iNOS.

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