NOX, a novel nitric oxide scavenger, reduces bacterial translocation in rats after endotoxin challenge

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Dickinson, Eva, Recep Tuncer, Evan Nadler, Patricia Boyle, Sean Alber, Simon Watkins, and Henri Ford. NOX, a novel nitric oxide scavenger, reduces bacterial translocation in rats after endotoxin challenge. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1281–G1287, 1999.—Endotoxemia promotes gut barrier failure and bacterial translocation (BT) by upregulating inducible nitric oxide synthase (iNOS) in the gut. We hypothesized that administration of a dithiocarbamate derivative, NOX, which scavenges nitric oxide (NO), may reduce intestinal injury and BT after lipopolysaccharide (LPS) challenge. Sprague-Dawley rats were randomized to receive NOX or normal saline via subcutaneously placed osmotic pumps before or after LPS challenge. Mesenteric lymph nodes, liver, spleen, and blood were cultured 24 h later. Transmucosal passage of Escherichia coli C-25 or fluorescent beads was measured in an Ussing chamber. Intestinal membranes were examined morphologically for apoptosis, iNOS expression, and nitrotyrosine immunoreactivity. NOX significantly reduced the incidence of bacteremia, BT, and transmucosal passage of bacteria and beads when administered before or up to 12 h after LPS challenge. LPS induced enteroctye apoptosis at the villus tips where bacterial entry was demonstrated by confocal microscopy. NOX significantly decreased the number of apoptotic nuclei and nitrotyrosine residues. NOX prevents LPS-induced gut barrier failure by scavenging NO and its toxic derivative, peroxynitrite.

BACTERIAL TRANSLLOCATION (BT), which is defined as the passage of viable microbes across an intact intestinal barrier (4), plays an integral role in the pathogenesis of gut origin sepsis (1). Spontaneous BT is a normal event that may be important for mucosal antigen sampling in the gut (41). However, a number of factors, including endotoxemia (8), trauma (35), or intestinal obstruction (7), may promote pathological BT. Endotoxemia elicits a severe inflammatory response characterized by the release of various inflammatory mediators, including nitric oxide (NO) (31, 33). An increasing body of evidence suggests that excessive production of NO may promote BT by altering intestinal permeability to microbes. We have shown previously that aminoguanidine, a relatively selective competitive inhibitor of inducible NO synthase (iNOS), significantly decreased BT after lipopolysaccharide (LPS) administration (42). Unno et al. (50) showed that LPS depressed intestinal mucosal mitochondrial function; inhibition of NO production reversed this effect and decreased BT. These results are consistent with those of Chen et al. (5), who showed that treatment with dexamethasone, an inhibitor of iNOS, reduced LPS-induced intestinal hyperpermeability in rats. Intrarectal administration of peroxynitrite, a toxic metabolite generated by the reaction of NO with superoxide, leads to severe colonic inflammation (37). Numerous studies, however, suggest that NO may play a protective role in sepsis. Arginine supplementation has been shown to improve survival in gut-derived sepsis by enhancing bacterial killing (13).

This effect can be abrogated by the administration of Nω-nitro-arginine methyl ester (L-NAME), a competitive inhibitor of NO. This finding suggests that the effect of arginine is mediated through NO, which is known to be cytotoxic and cytostatic for various microorganisms. In addition, iNOS activity is upregulated throughout the gastrointestinal tract of rats after LPS administration (6), a phenomenon that may be important for bacterial killing. Thus NO appears to play a paradoxical role in sepsis, modulating bacterial infections on one hand and increasing BT on the other. In this study, we examined the role of a water-soluble dithiocarbamate derivative, NOX, which scavenges NO in vitro and in vivo (25, 27), on LPS-induced BT.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 250–300 g were acclimatized for a minimum of 1 wk before experimentation. The experimental protocol was approved by the Animal Research and Care Committee of the Children's Hospital of Pittsburgh. Pentobarbital sodium (50 mg/kg) was administered intraperitoneally to the animals. Osmotic pumps (Alzet model 2ML1, 10 µl/h, 7-day pump; Alza, Palo Alto, CA) were then placed subcutaneously in the back of each rat, and the delivery catheter was tunneled subcutaneously in the neck. The animals were randomized to receive either 2 ml of normal saline (NS) solution or 450 mg of NOX (a kind gift of Dr. Ching-San Lai, Medinox, San Diego, CA) in a final volume of 2 ml via the osmotic pumps. Preliminary experiments in our lab using different doses of the compound revealed that this dose of NOX most consistently reduced the incidence of BT after LPS challenge without any adverse side effects. Eighteen hours after pump placement, the animals were challenged with 10 mg/kg of LPS (Escherichia coli 0111:B4; Difco, Detroit, MI) intraperitoneally. In our posttreatment groups, the pump was placed subcutaneously in the back of the animals; however, the catheter was not tunneled subcutaneously until 4, 8, 12, or 18 h after LPS (10 mg/kg) administration. All animals were
killed 24 h after LPS challenge. Quantitative cultures of the peritoneal fluid, blood, mesenteric lymph nodes (MLN), spleen, liver, and cecum were obtained, and these cultures were processed as previously described (12).

Nitrite and nitrate levels. Plasma was obtained by cardiac puncture. An automated procedure based on the Greiss reaction was used to measure the stable end products of NO metabolism, nitrite, and nitrate (42).

In vitro studies. The passage of FITC-labeled beads, measuring 1 µm in diameter, from the mucosal to the submucosal side of the ileal mucosal membrane was determined in the Ussing chamber. Our current adaptation of the Ussing chamber for the study of transmucosal passage of bacteria has been previously described (12, 26, 42). A volume of 1 ml of fluorescent beads (Fluoresbeire; Polyscience, Warrington, PA) was loaded on the mucosal side. Samples were collected every hour for 3 h from the submucosal and mucosal chambers and examined by fluorescence-activated cell sorting at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Similar studies were conducted with FITC-labeled antibodies used. Sections were then incubated for 60 min with Gelvatol (Monsata, St. Louis, MO) and then cover-slipped for fluorescent microscopy.

Confocal laser microscopy. FITC-labeled E. coli C-25 (final concentration of 10^9 colony-forming units per ml) was placed on the mucosal side in the Ussing chamber. Ileal mucosal membranes were removed after 180 min and fixed in 2.5% paraformaldehyde for 1 h and 30% sucrose overnight. The tissue was frozen in liquid nitrogen, stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) to reveal cellular actin, and imaged using a confocal laser microscope (Multiconfocal laser microscope. The protocol was conducted as per standard published procedure (11). Briefly after the ileal specimens were sectioned, three washes in PBS were performed, followed by fixation in cold methanol for 30 min. The specimens were washed twice more in PBS. Next, 10 ml of the TDt reaction mixture (distilled water, 48 ml) were added to the slides and incubated at 37°C for 90 min. Slides were then washed with PBS three more times. The slides were mounted with Gelvatol (Monsata, St. Louis, MO) and then cover-slipped.

Immunocytochemical labeling for iNOS and nitrotyrosine. Intestinal segments were removed and immediately snap-frozen in liquid nitrogen. Sections (3 µm) were cut on a Micron cryostat, mounted on Superfrost slides (Fischer Scientific, Pittsburgh, PA), and labeled as described. Frozen sections were washed three times in PBS containing 0.5% BSA and 0.15% glycerine, pH 7.5 (buffer A), followed by a 30-min incubation with purified goat IgG (50 µg/ml) at 25°C and three additional washes with buffer A. The preceding steps were designed to ensure minimal nonspecific reaction to the antibodies used. Sections were then incubated for 60 min with a primary polyclonal antimurine iNOS antibody (Transduction Laboratories, Lexington, KY) or polyclonal antimurine nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). These antibodies cross-react with the rat protein. Antibody incubations were then followed by three washes in buffer A and a 60-min incubation with antiimmuneroxidase (Vector Laboratories, Burlingame, CA) to label the tissue. Specimens underwent three washes in buffer A and then were mounted in gelvatol and cover-slipped for fluorescent microscopy.

Statistical analysis. Data are presented as means ± SE. Bacterial translocation was compared between groups by using χ2 analysis. Transmucosal passage of bacteria in the

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On day 1, rats were randomized to receive Alzet osmotic pumps filled with either novel nitric oxide scavenger NOX or normal saline. On day 2, all rats received 10 mg/kg of lipopolysaccharide (LPS) intraperitoneally. Blood cultures and samples from mesenteric lymph nodes (MLN), liver, or spleen were taken 24 h after LPS challenge. Results represent the number (and percentage) of animals that translocated or had positive blood cultures in each group. All animals with positive blood cultures translocated to the MLN. *P = 0.004 (χ2); ††P = 0.008 (Fisher’s exact mean test).

Ussing chamber and positive blood cultures were compared between groups by using Fisher’s exact mean test. All other analyses were discrete comparisons between groups, and unpaired Student’s t-test was used.

**RESULTS**

Effect of NOX on BT and bacteremia after LPS challenge. Rats were randomized to receive either NOX or NS via Alzet osmotic pumps. The results are summarized in Table 1. Pretreatment with NOX resulted in a substantial decrease in the incidence of BT and bacteremia after LPS challenge. Similarly, at 4, 8, and 12 h after LPS challenge, NOX significantly reduced BT to the MLN compared with the NS group; administration of NOX at 18 h had no effect on BT (Table 2). There was no difference between the cecal flora of animals treated with NS or NOX before or after LPS challenge (data not shown). Ileal mucosal membranes from rats challenged with LPS showed submucosal edema and sloughing of enterocytes at the apices of the intestinal villi (Fig. 1A). These changes were more pronounced in the control specimens than in specimens from NOX-treated animals (Fig. 1B). There was no difference in plasma nitrite and nitrate levels between the NS and the NOX groups 24 h after LPS challenge (783 ± 121 µM vs. 687 ± 101 µM). However, nitrite/nitrate levels were significantly higher in animals with positive blood cultures (1,147 ± 88 µM) than in animals with negative blood cultures (323 ± 23 µM) (P < 0.05). There was no

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On day 1, rats were randomized to receive Alzet osmotic pumps filled with either NOX or normal saline; however, catheters were not tunneled. On day 2, all rats received 10 mg/kg of LPS intraperitoneally. Catheters were tunneled subcutaneously at 4, 8, 12, or 18 h after LPS challenge. Blood cultures and samples from MLN, liver, and spleen were taken 24 h after LPS challenge. Results represent number (and percentage) of animals that translocated in each group. *P < 0.03 (Fisher’s exact mean test).
difference in mortality between animals receiving NS (10.1%) or NOX (9.4%) either before or after LPS challenge.

Effect of NOX on intestinal permeability. We measured intestinal permeability to FITC-labeled E. coli C-25 and FITC-labeled beads using the Ussing chamber. There was no difference in the potential difference across ileal mucosal membranes from NS- or NOX-treated rats after LPS challenge (data not shown). Transmucosal passage of E. coli C-25 was detected in five of eight (62.5%) ileal mucosal membranes in the NS group, compared with one of seven (14.3%) in the NOX-treated animals (P < 0.02, Fisher’s exact mean test). Similarly, significantly fewer beads passed across ileal mucosal membranes from NOX-treated rats (25 ± 19 beads) compared with membranes from NS-treated animals (3,432 ± 24 beads) (P < 0.05, Student’s t-test). Confocal microscopy confirmed decreased intestinal permeability to FITC-labeled E. coli C-25 in ileal mucosal membranes from NOX-treated animals (Fig. 1, A and B).

Immunohistochemistry for iNOS. Immunohistochemical analysis of freshly harvested sections of ileum from animals in both groups revealed that iNOS protein was localized predominantly at the apices of the villi in all LPS-challenged animals. There was no significant difference in iNOS expression between the two groups (Fig. 2).
NO and enterocyte viability. The previous sections documented iNOS protein in the intestinal segments. NO has been shown to induce either apoptosis or necrosis in a number of different cell types (21, 48). Therefore, we sought to determine whether enterocyte apoptosis contributed to the increase in BT after LPS challenge. Figure 1C shows increased enterocyte apoptosis in the apical villi of NS-treated animals. In contrast, animals treated with NOX had fewer apoptotic nuclei in their villi (Fig. 1D). Quantitative analysis of 372 villi from NS-treated rats revealed 4.9 ± 2.3 apoptotic nuclei per villus compared with 2.8 ± 0.8 apoptotic nuclei per villus in 421 villi from NOX-treated rats (P < 0.001, Student’s t-test).

Determination of immunoreactivity to nitrotyrosine. One of the more biologically significant reactions of NO is with superoxide to produce the potent oxidizing agent peroxynitrite, which in turn can cause direct tissue injury. Peroxynitrite has a very short half-life (<1 s) but forms nitrotyrosine residues in proteins that can be detected immunohistochemically using specific antibodies. If enterocyte apoptosis or gut barrier failure in sepsis is caused by NO-induced peroxynitrite formation, then increased nitrotyrosine residues should be detected when iNOS protein is upregulated. Figure 1E shows that nitrotyrosine residues are more prevalent in the NS-treated animals compared with the animals treated with NOX (Fig. 1F). Quantitative analysis revealed 14.5 ± 7.3 cells positive for 3-nitrotyrosine per villus in the NS-treated rats compared with 11.3 ± 6.8 for the NOX-treated group (P < 0.001, Student’s t-test).

DISCUSSION

NO is a molecule that exerts diverse biological effects. Its complexity is underscored by the abundance of reports corroborating both its protective and deleterious roles in the pathophysiology of sepsis or shock (5, 6, 13, 16–18, 42, 50, 52). These studies are not necessarily contradictory. Khan et al. (24) illustrated this dichotomy in a recent report in which iNOS-null mice challenged with Toxoplasma gondii exhibited impaired microbial killing. However, these animals showed a decrease in tissue damage and an increase in survival rate. Various inflammatory stimuli, including LPS, induce large (micromolar) quantities of iNOS. Complete or nonselective inhibition of NO after LPS challenge may result in increased tissue damage, delayed repair, and increased mortality (30, 45). This observation is due to the fact that NO is beneficial especially when produced in low quantities, where it acts to prevent platelet aggregation and microvascular thrombosis. It is the sustained upregulation of iNOS that is likely to be deleterious. NO directly inhibits heme proteins and nonheme iron proteins, as well as ribonucleotide reductase, the rate-limiting enzyme in DNA synthesis (51). Furthermore, the oxidation products of NO can, through nitrosation reactions, cause direct DNA damage through deamination reaction and strand breaks that result in activation of polyadenosine diphosphate-ribosyl synthetase and energy depletion (51). Although these mechanisms may be beneficial for the elimination of microbes, they may also severely damage the host tissue. At high concentrations, NO disrupts the actin cytoskeleton, inhibits ATP formation, and dilates intercellular tight junctions (39, 49). Kennedy et al. (23) reported that NO dilates tight junctions and depletes ATP in cultured Caco-2BBe intestinal epithelial monolayers. Tepperman et al. (47, 48) showed that LPS induces iNOS enzymatic activity in rat small intestinal and colonic epithelial cells and decreases viability of these cells. Unno et al. (50) have shown that NO gas and various NO donors decrease intracellular ATP levels and increase intestinal permeability. We previously showed that competitive inhibition of iNOS with aminoguanidine reduces intestinal permeability and decreases BT after endotoxemia (42). Other investigators have reported that selective iNOS inhibitors protected against LPS-induced mortality (52). Our data suggest that scavenging excess NO may preserve or enhance enterocyte viability in vitro and in vivo. The NO scavenger NOX markedly reduced transmucosal bacterial passage in the Ussing chamber. LPS increased the permeability of ileal mucosal membranes, as evidenced by the increase in BT in vivo and in the Ussing chamber in vitro. Pretreatment with NOX or aminoguanidine decreases BT and transmucosal passage of E. coli C-25 and fluorescent beads in vitro. However, of more clinical relevance is the fact that NOX significantly decreased BT even when administered up to 12 h after LPS challenge. These observations are in contrast to other reports that showed that inhibition of NO production increases epithelial permeability (22). There was a trend toward a linear increase in the incidence of BT with incremental delay in the...
administration of NOX. The lack of effect of NOX at 18 h after LPS challenge probably reflects the fact that BT had already occurred by that time in most of the animals.

The decrease in BT and bacteremia in the NOX-treated animals was not due to alterations in their intestinal microbial flora because the latter was similar to that of NS-treated animals. Rather, this reduction in BT and bacteremia was associated with decreased peroxynitrite formation, thus suggesting that LPS causes gut barrier failure through NO-induced peroxynitrite generation. There was no change in the expression of iNOS protein in the epithelium, and the stable end products of NO metabolism, nitrite and nitrate, were readily detected in the plasma. Peroxynitrite has been implicated in the pathophysiology of a wide spectrum of diseases, including atherosclerosis, arthritis, endotoxemia, ischemia-reperfusion injury, and acute respiratory distress syndrome (9). Peroxynitrite is formed by the reaction between NO and superoxide (2). Peroxynitrite is particularly efficient at oxidizing iron sulfur centers, zinc fingers, and protein thiol groups, causing direct DNA damage and affecting the viability of the cell (2, 10, 38, 46). Kennedy and colleagues (23) reported that peroxynitrite, but not NO, increases transepithelial permeability by inducing DNA strand breaks, activating the poly(ADP-ribose) synthetase system, and causing depletion of ATP in Caco-2BBe cells. In addition, peroxynitrite can induce lipid peroxidation, inactivate sodium channels, or react with transmembrane proteins. Peroxynitrite also reacts with aromatic amino acids, resulting in the modification of free or protein-associated tyrosine residues and subsequent formation of 3-nitrotyrosine, the footprint of peroxynitrite (2). NOX presumably decreased peroxynitrite formation because immunoreactivity to 3-nitrotyrosine was diminished in the NOX-treated groups.

Previous reports have shown that endotoxic shock leads to apoptosis of a variety of cells (15, 21). Infection with Mycobacterium tuberculosis induces apoptosis in murine macrophages, and this process is dependent on NO. Compared with animals treated with NS, we found a lower rate of apoptosis in animals treated with NOX after LPS challenge. Numerous reports have shown that peroxynitrite is capable of inducing apoptosis and necrosis in various cells (20, 40, 44). Although immunohistochemical analysis localized nitrated proteins predominantly in the lamina propria, we cannot exclude the possibility that decreased peroxynitrite generation as a result of NOX treatment may have contributed to preservation of gut epithelial barrier function after LPS challenge. It is less likely that other compounds, such as nitrogen dioxide or nitrous acid, may have been responsible for nitrination of tyrosine residue in the gut because the former, for instance, would have to lose an electron to become nitrosonium ion (NO$_2^+$) first, and furthermore, both species are effectively scavenged in vivo (19).

Our data show a 28% incidence of positive blood cultures in NS-treated rats after LPS challenge compared with 0% in the NOX-treated animals. Animals with positive blood cultures had the highest nitrite and nitrate levels, consistent with the observations of Groeneveld et al. (14), who reported that serum nitrate levels were higher in patients with positive blood cultures compared with patients with unproven bacteremia.

NOX is a water-soluble dithiocarbamate derivative that chelates reduced iron and binds NO in solution in vivo and in vitro (25, 27). There are several classes of dithiocarbamate derivatives that differ in their NO binding properties; these include diethylthiocarbamate, which is hydrophobic, and N-methyl-$\alpha$-glucamine dithiocarbamate (MGD) and proline dithiocarbamate, which are hydrophilic (28, 32, 34). Complexes of Fe$^{2+}$ with these compounds effectively bind NO, resulting in the formation of a paramagnetic mononitrosyl iron complex that can be detected by electron paramagnetic resonance spectroscopy. NOX, which has a molecular mass of 300 Da, most closely resembles MGD in its physical and biochemical properties. This water-soluble compound has been shown to scavenge free iron and to bind NO in vitro and in vivo after LPS challenge, with maximal trapping occurring 6–8 h after LPS administration (25, 27, 28). In vitro evidence suggests that NOX is less likely to bind superoxide or peroxynitrite (36). The distribution of the mononitrosyl iron complex in tissues correlates with the sites of NO production, which include liver, small intestine, lung, and kidney (27, 32). Significant levels can also be detected in the blood and urine. The complexes appear to decay or decompose rapidly in tissues (within 60 min) or may be transferred to blood or urine (32). Although we did not look for evidence of mononitrosyl iron complexes in our system, previous investigators have clearly documented their presence in blood and tissues after LPS administration (28, 32). The fact that plasma nitrite and nitrate levels were not affected in the NOX-treated rats may be partly due to our assay. When dissolved in water, NOX produces a yellow solution that may interfere with the Griess reagent, which detects nitrite and nitrate by means of a colorimetric reaction. A recent report by Pieper et al. (36) showed that long-term administration of another derivative of the NOX compound, NOX-101, prevented diabetes-induced endothelial dysfunction. In contrast to our findings, NOX-101 abolished total urinary nitrate and nitrite in rats made diabetic by intravenous administration of streptozotocin. These differences may reflect the distinct properties of these two dithiocarbamate derivatives or differences in our model utilizing LPS challenge. Alternatively, it is possible, although unlikely, that the mononitrosyl iron complex yields nitrate or nitrite upon decomposition. Our findings suggest that urinary nitrate and nitrite, as determined by the Griess reaction, may not be reliable indicators for monitoring NO metabolism after NOX treatment.

NOX presumably inactivates or neutralizes the toxic effect of NO and reduces peroxynitrite formation, as evidenced by the decrease in nitrotyrosine immunoreactivity. The apparent lack of toxicity of NOX may represent a relative advantage of this compound over...
competitive inhibitors of NOS such as aminoguanidine, which has to be administered in relatively high doses in order to achieve efficacy in vivo (29, 42). Aminoguanidine loses its selectivity at high doses and inhibits all three isoforms of NOS: NOS1 or neuronal NOS; NOS2 (iNOS); and NOS3 or endothelial NOS (43). Despite the fact that NOX can scavenge NO derived from all three NOS isoforms, no adverse effect was noted even in the pretreatment group, in which one would expect some potentially deleterious effect from scavenging NO produced by NOS3. Furthermore, the fact that NOX decreases the incidence of BT up to 12 h after LPS challenge suggests that it may be effective clinically, even after the onset of sepsis.

In conclusion, our data corroborate our previous report and others that showed an increased incidence of BT after LPS challenge. This finding was associated with sustained upregulation of iNOS and excessive production of NO. NO increases the formation of peroxynitrite in the intestinal villi, which results in enterocyte apoptosis or necrosis and, consequently, gut barrier failure. Pretreatment with NOX selectively scavenges NO, thus maintaining enterocyte viability and reducing BT. NOX may have important implications for the treatment of septic shock and other diseases characterized by the sustained production of NO. Posttreatment of sepsis with NOX at 4 and 8 h offers the same benefit as pretreatment; however, administration of NOX 12 h after LPS challenge was somewhat less efficacious, but significantly better than no treatment or treatment at 18 h.

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