Nitric oxide production during Vibrio cholerae infection

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Nitrergic activity was Ca\textsuperscript{2+} in the form of NO production in association with increases in local smooth muscle (14, 22, 33). These effects are mediated in large part by the multifunctional molecule (35) that also affects mucosal epithelial cell secretion (7, 11, 31). The massive fluid loss often associated with both natural and experimental infection in humans strongly suggested that V. cholerae-induced diarrhea was accompanied by increased NO generation, which characterized the mechanisms and localization of NO production in the intestine using a rabbit ileal loop model in vivo and the bactericidal activity of NO products against V. cholerae.

METHODS

Human Subjects

Natural infection. In March 1995, during the cholera season in Lima, Peru, we collected sera from 10 asymptomatic adults and 26 age- and sex-matched patients (mean age 40 ± 17.3 yr, range 18–80; 14 female, 12 male) with acute watery diarrhea on presentation to the hospital. These asymptomatic patients had been ill (6–7 liquid stools/day) for a mean of 2.1 ± 0.8 days; 11 had moderate and 15 had severe dehydration by World Health Organization criteria. Food intake was limited in the 24–48 h before presentation in the cholera group due to nausea and vomiting. Levels of serum creatinine were similar in both symptomatic and healthy groups (1.0 vs. 0.8 mg/dl).

Experimental infection. At the United States Army Medical Research Institute of Infectious Diseases, 10 healthy volunteers aged 18–40 yr with no underlying medical illnesses, recent travel, intestinal symptoms, or history of cholera were challenged orally with 5 × 10\textsuperscript{8} colony forming units (cfu) of live wild-type V. cholerae 01 El Tor Inaba 9N16961 (Center for Vaccine Development, Baltimore, MD) in bicarbonate-ascorbic acid buffer. Of these 10 volunteers, five had been immunized 1 mo earlier with a live attenuated oral vaccine, the Peru-15 strain, a genetically attenuated strain of V. cholerae01 El Tor Inaba in which the CT genetic element (ctx, zot, and ace) had been replaced with a CT-B subunit gene, as previously described (16). Volunteers were challenged with wild-type V. cholerae01 and closely monitored for vital signs, diarrhea [≥2 loose or watery stools in 48 h (or ≥300 ml)], and fever (>100°F) (16). Whole blood, plasma, and 12-h urine samples were collected each morning on days 0, 2, 4, and 7 postchallenge and stored at −20°C until tested. All volunteers were offered the same diet throughout the course of the study. Written informed consent was obtained from all patients as approved by the Human Subjects Review Board of the Office of the U.S. Army Surgeon General.

Rabbit Ileal Loop Assay

We measured intestinal fluid secretion (fluid-to-length ratio) in isolated ileal loop segments from female New Zealand rabbits (1.5–2 kg; Weaver’s Birchwood Farm, Red Wing, MN) as previously described (5, 15). A blank loop separated each experimental loop injected with 1 ml of phosphate-buffered saline (PBS) with or without CT (0.01–1.0 µg/loop; List Biological Laboratories, Campbell, CA). In selected experiments, V. cholerae lipopolysaccharide (LPS) (1–100 µg/ml; Sigma, St. Louis, MO) was added in the presence or absence of 1 µg/ml dexamethasone to prevent inhibition of NO production by dexamethasone.

V. cholerae is a common and serious cause of diarrhea and dehydration in developing countries. These effects are mediated in large part by the actions of cholera toxin (CT), which irreversibly stimulates local inulinase and intestinal cell secretion (7, 11, 31). The massive fluid loss often associated with V. cholerae diarrhea requires increased regional blood flow. CT may also elicit the production of intestinal neurotransmitters, particularly vasoactive intestinal peptide (VIP), which promote peristalsis (13). The mutually enhanced release of VIP and nitric oxide (NO) provides powerful mediators of relaxation in both intestinal and vascular smooth muscle (14, 22, 33). Thus NO may participate in the regulation of regional blood flow and intestinal motility (8, 18, 19). This multifunctional molecule (35) may also affect the integrity of mucosal epithelial cells in the intestine (3, 10, 36, 37, 40) and exert antibacterial activity (6). Therefore, we determined whether acute cholera infection elicited NO production in humans. Because these studies of...
of CT (0.01–1.0 µg/loop). After 8–18 h (see below), we measured the volume of intestinal fluid and concentrations of NO2/NO3 in each loop. Samples of intestinal fluid were centrifuged at 3,000 g for 5 min and stored at −20°C until tested. Loop fluid was also cultured for both aerobic and anaerobic bacteria. All protocols were approved by the Animal Subjects Committee at the Veterans Affairs Medical Center.

Measurement of NO2/NO3

The stable end products of NO (NO2 and NO3) were assayed as previously described (34) using the Griess reagent after conversion of NO3 to NO2 with Escherichia coli nitrate reductase. In the ileal loops, the amount of NO2 was expressed as nanomoles per centimeter ([nmol NO2/NO3/ml fluid] × (ml fluid/cm loop length)).

Assay for NO Synthase Activity in the Intestinal Wall

After 8 h, washed ileal loop tissues were frozen with liquid nitrogen and stored at −75°C until tested. Frozen tissues were homogenized with a tissue homogenizer (Omi TH; Omni International, Gainesville, VA) in 1 ml of ice-cold buffer (pH 7.4) containing 50 mM tris(hydroxymethyl)aminomethane, 0.1% β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 mM leupeptin, 1 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 3,000 g for 30 min, and the supernatants were stored on ice for up to 1 h before assay.

NO synthase (NOS) activity was determined by the conversion of L-[14C]arginine (Amersham, Arlington Heights, IL) to L-[14C]citrulline, as previously described (17, 30, 32). Ca2+-dependent activity was determined as the difference between the quantity of L-[14C]citrulline produced from control samples and samples containing 3 mM EGTA to bind Ca2+. Ca2+-independent activity was determined as the difference between the activity in samples containing 3 mM EGTA and samples containing both 3 mM EGTA and 1 mM Nω-nitro-l-arginine (l-NNA), an inhibitor of NOS. NO activity was expressed as picomoles of L-[14C]citrulline formed per gram protein per minute (2).

NO2/NO3 Production by Rabbit Intestinal Tissue In Vitro

Sections of small intestine from three healthy control rabbits were washed with ice-cold PBS, cut into small pieces, pooled, and incubated with PBS, CT (0.5 µg/ml), heat-inactivated CT (0.5 µg/ml), V. cholerae PS (10 µg/ml), and CT or LPS with dexamethasone [1 µg/ml (2.6 µM)] in 2 ml of Eagle’s minimum essential medium (Sigma) containing 10% fetal calf serum (GIBCO, Grand Island, NY), 2 mM L-arginine, and 100 U/ml penicillin at 37°C. Released NO, expressed as nanomoles of NO2/NO3 per gram tissue (wet wt), was measured in centrifuged supernatants collected at 48 h.

Tissue Staining for NADPH Diaphorase

To localize NOS activity in the intestine, we stained Formalin-fixed tissues for NADPH diaphorase, a sensitive marker of NOS activity (4, 12), as previously described (38, 39).

Antibacterial Activity of NO

To determine whether the NO produced locally in the intestine may have had direct effects on the organism, the susceptibility of five V. cholerae (El Tor; 2 Inaba and 3 Ogawa) and five E. coli H7:0157 strains was tested using a microbroth dilution technique in M9 medium (thiol-free) as described by De Groot et al. (6) and Reimer et al. (28). Briefly, log phase growth of each isolate was washed in M9 medium and inoculated to a final concentration of 3.8 × 104 cfu/well. 2,2′-(Hydroxynitrosohydrozono)bis-ethanamine (DETA-NO; Research Biochemicals International, Natick, MA), diethylamine diazeniumdiolate ion (DEA-NO; Cayman Chemical, Ann Arbor, MI), 3-morpholinosydnonimine hydrochloride (SIN-1; Cassela, Frankfurt, Germany), hydrogen peroxide (Sigma), hypoxanthine (HX; Sigma), and xanthine oxidase (XO; EC 1.1.3.22; Sigma) were diluted to stock concentrations, filter sterilized, and added to the following final concentrations: 0.0625–4 mM DETA-NO, 0.0625–4 mM DENO, 0.125 mM SIN-1, 6.2–100 µM hydrogen peroxide, 6.2–100 µM HX, and 7.5 × 10−3 to 5 × 10−3 U/ml XO. Tests were incubated at 37°C for 24 h, and minimum inhibitory concentrations (MIC) was read as the lowest concentration giving no visible growth. MICs for DETA-NO above 4 mM were recorded at 8 mM, because higher concentrations were insoluble in the medium. The minimal bactericidal concentration was that concentration at which >99.9% of initial inoculum was killed.

Statistics

Values for NO2/NO3 in human plasma and urine, and rabbit ileal loop measurements were compared between groups by unpaired Student’s t-test, and serial values within human groups were compared with baseline values by paired Student’s t-test, using the Statview 4.0 statistical program (Abacus Concepts, Berkeley, CA).

RESULTS

Clinical Response to Live V. Cholerae Infection in Humans

Patients with natural V. cholerae infection in Peru were more ill and more dehydrated than those with experimental cholera infection in the U.S. After experimental challenge of volunteers in the U.S. with live V. cholerae, 6 of 10 volunteers developed abdominal pain...
and diarrhea (2 of 5 vaccinees and 4 of 5 control subjects) (16). Total intestinal fluid loss in symptomatic patients ranged from 300–3,830 ml, and symptoms were most prominent on days 2–4. Mean blood pressures remained stable throughout the course of infection, independent of the presence or absence of diarrhea.

Levels of NO$_2^-$/NO$_3^-$

Among Peruvians, levels of NO$_2^-$/NO$_3^-$ in serum of patients with acute V. cholerae infection were almost double those of healthy local control subjects (Fig. 1; P < 0.0001). These elevations were independent of the duration of symptoms, level of dehydration, or serum creatinine levels.

In U.S. volunteers, mean levels of NO$_2^-$/NO$_3^-$ in plasma were also increased significantly in patients with diarrhea compared with preinfection values but remained unchanged in subjects without diarrhea (Fig. 2). Among symptomatic patients, peak levels of NO$_2^-$/NO$_3^-$ on day 4 were significantly higher than their baseline values (182 vs. 125 nmol/ml; P < 0.001) and compared with those in asymptomatic subjects (P < 0.04; Fig. 2). Previous immunization had no independent effect on plasma levels of NO$_2^-$/NO$_3^-$. As in plasma, NO$_2^-$/NO$_3^-$ levels in urine increased significantly from preinfection values only in U.S. patients with diarrhea (1.1 nmol NO$_2^-$/NO$_3^-$/µg creatinine preinfection vs. 2.8 and 2.5 nmol NO$_2^-$/NO$_3^-$/µg creatinine on days 2 and 4, respectively; P < 0.03) (Fig. 3). These values in urine were higher on day 2 than those in asymptomatic subjects (P < 0.001), among whom, as in serum, levels did not change. In patients with diarrhea, despite increased levels of NO$_2^-$/NO$_3^-$ in plasma and urine, mean arterial pressure remained stable, and only two developed mild fever. These data suggested a limited systemic inflammatory response to

![Fig. 2. Effect of diarrhea on levels of stable NO metabolites (NO$_2^-$/NO$_3^-$) in human plasma during V. cholerae infection in humans. After challenge with live wild-type V. cholerae El Tor Inaba, levels of NO$_2^-$/NO$_3^-$ increased significantly from preinfection levels in patients with diarrhea (n = 6), whereas levels remained stable in asymptotically infected subjects (n = 4). Data are means ± SD. *P < 0.04.](http://ajpgi.physiology.org/...)

![Fig. 3. Effect of diarrhea on NO$_2^-$/NO$_3^-$ excretion in urine during V. cholerae infection in humans. Excretion of NO$_2^-$/NO$_3^-$ increased significantly above baseline values (*P < 0.03) in patients with diarrhea (n = 6) but not in asymptomatic subjects (n = 4).](http://ajpgi.physiology.org/...)

Table 1. NOS activity in intestinal wall segments from control and CT-stimulated isolated rabbit ileal loops

<table>
<thead>
<tr>
<th>No. of Loops</th>
<th>NOS Activity, nmol·min$^{-1}$·g protein$^{-1}$</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
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<tr>
<td>Cholera toxin (1 µg/ml)</td>
<td>8</td>
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<tr>
<td>Difference</td>
<td>P &lt; 0.05</td>
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Values are means ± SE. NOS, nitric oxide synthase; CT, cholera toxin.
this mucosal infection and a local intestinal source of NO in humans.

Secretory Response to CT and LPS in Rabbits

We further characterized and localized the effects of *V. cholerae* infection on the l-arginine-NO pathway in an in vivo rabbit ileal loop model (5, 15). This model permitted us to examine the independent effects of purified CT and LPS from *V. cholerae* on intestinal fluid secretion and NO production.

The fluid response to CT in isolated rabbit ileal loops (expressed as volume-to-length ratio) was dose dependent, with doses as low as 10 ng/ml producing detectable fluid accumulation (Fig. 4). In pilot experiments, fluid was present as early as 6–8 h after inoculation. Levels of NO$_2$/NO$_3$ in the fluid also showed a dose-dependent response to CT (Fig. 4).

In contrast to the effects of CT alone, purified *V. cholerae* LPS (1–100 µg/ml) elicited no appreciable fluid accumulation over 18 h when injected alone into the lumen of the ileal loops of live animals. Moreover, LPS induced no additive or synergistic accumulation of fluid or NO$_2$/NO$_3$ when added to CT (0.01, 0.1, and 1 µg/ml) in the loops (data not shown).

The NO$_2$/NO$_3$ measured in the lumen was not due to a food source, as the animals were not fed during the trial and loops were sealed. In addition, bacterial contaminants in the loops at the time of harvest were few in number and present in low concentrations (data not shown).

NOS Activity in Tissue

Intestinal tissue specimens obtained from loops stimulated with CT in vivo showed a twofold increase in NOS activity compared with levels in control tissues, as detected by the oxidation of l-arginine to l-citrulline (Table 1; P < 0.05). The activity measured was consistently inhibitable by a specific NOS inhibitor (l-NNA). Moreover, the unstimulated NOS activity, as well as that induced by CT, was predominantly Ca$^{2+}$ dependent (79–97% inhibitable by 3 mM EGTA).

We localized the production of CT-induced NOS in the intestine by staining for NADPH diaphorase, a

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Fig. 5. NADPH diaphorase staining in intestinal tissue. Formalin-fixed tissue from isolated rabbit ileal loops exposed for 8 h to PBS alone (A) or purified CT (B) in vivo were stained for NADPH diaphorase activity, a sensitive marker of NO synthase activity (39).
sensitive indicator of NOS activity, which is normally present in intestinal neuronal and endothelial cells (23, 24, 33). NADPH diaphorase staining was dramatically enhanced in rabbit ileal loops exposed to CT compared with that in control loops. However, the dense staining was restricted primarily to the epithelial cells (Fig. 5). Expression of NADPH diaphorase staining observed in intestinal neurons and vessels did not change discernibly in rabbits with or without CT exposure. Very few acute inflammatory cells were seen by light microscopy either within the epithelium or lamina propria in tissue stained with hematoxylin-eosin in the absence or presence of CT. Moreover, no mononuclear cells stained with NADPH diaphorase in the lamina propria. Thus CT-induced increases in luminal NO$_2$/NO$_3$ levels were associated with a concomitant rise in mucosal NOS activity in the epithelial cells, rather than in neurons, endothelial or smooth muscle cells, or lamina propria macrophages.

We further characterized this local NO synthesis by incubating finely minced intestinal tissue from healthy control rabbits in vitro with CT and LPS. We found that CT elicited high levels of NO$_2$/NO$_3$ in the culture supernatants (Fig. 6), whereas denatured CT did not. The CT-induced NO$_2$/NO$_3$ was not inhibited by pretreatment of the tissue with dexamethasone. In contrast to the lack of response with intact tissue, LPS induced NO$_2$/NO$_3$ production from minced intestinal tissue. However, unlike the activity stimulated by CT, LPS-induced activity was inhibited by dexamethasone. Such inhibition of the LPS effects is typical of a Ca$^{2+}$-independent "macrophage type" NOS (27). No signs of inflammation (e.g., polymorphonuclear, macrophage, or lymphocytic infiltrates) disruption, or morphological changes in epithelial cells were noted 8–12 h after CT exposure (Fig. 7), despite significant fluid and NO$_2$/NO$_3$ accumulation.

**Antibacterial Activity of NO**

In association with superoxide radicals normally found in the intestine (29), NO may combine to form peroxynitrite, a product with potent bactericidal activity (6). SIN-1, a donor of both compounds (NO$^\cdot$ + O$_2$ $\rightarrow$ OONO$^-$), showed dose-dependent inhibition of V. cholerae growth in vitro (Fig. 8A), with identical inhibitory and bactericidal concentrations.

The bactericidal activity of the pure long- and short-acting NO$^\cdot$ donors (DETA-NO and DEA-NO, respectively) was increased in a synergistic fashion by the addition of HX/XO, which releases copious amounts of O$_2$. Moreover, the enhanced inhibitory activity of the NO$^\cdot$ and oxygen radical donors combined was consistent in five strains each of V. cholerae and E. coli O157:H7, a common intestinal pathogen ($P < 0.03$ for each organism with vs. without an O$_2$ donor; data not shown).
DISCUSSION

We have shown that both severe natural cholera infection and symptomatic experimental infection with V. cholerae in humans is associated with increased levels of stable metabolites of NO in both plasma and urine. These elevations were restricted to patients with diarrhea. Our data also demonstrate that the increases in NO2 were unrelated to the presence of systemic symptoms such as hypotension or fever. The known restriction of this noninflammatory noninvasive infection to the superficial mucosa suggested an intestinal source of NO in subjects with V. cholerae-associated diarrhea.

Indeed, we discovered a mucosal source for NO synthesis in a classic animal model. Purified CT given intraluminally induced dose-dependent production of both fluid and NO2 in the intestinal lumen, in association with increased NOS activity in the ileal tissue. Staining of intestinal tissue for NADPH diaphorase, a marker for NOS activity, confirmed the local upregulation of NOS, a change confined exclusively to the epithelial cells. Induction of NOS in intestinal epithelial cells has been described in rats given LPS systemically (37). In contrast, our results demonstrated that in vivo exposure of intact bowel mucosa to LPS on the luminal surface does not induce local production of either fluid secretion or NO, highlighting the importance of unimpaired epithelial cell integrity. LPS was shown to elicit NO2 production from minced intestinal tissue in vitro but not from intact mucosa in vivo.

Studies in animals have demonstrated increased intestinal NO production after local injury and inflammation (10, 21), as have studies in humans with inflammatory bowel disease (1, 20, 26). In tissues from patients with active colitis, corticosteroids decreased intestinal NO production, characteristic of a macrophage type of inducible Ca2+-independent NOS isoenzyme (26). Similarly, corticosteroids inhibited the LPS-induced NO2 production in our disrupted intestinal tissue. In contrast, our data suggest that CT-induced NO production originates from a Ca2+-dependent and dexamethasone-resistant NOS in intact intestinal epithelium in the absence of inflammation. These characteristics are similar to those of a constitutive NOS, such as those identified in endothelial cells, gastrointestinal smooth muscle, and neuronal cells (22, 35), but the specific designation of the intestinal epithelial enzyme described here has not yet been established. Although each of these cells may have contributed to the increases in NO2 we observed, none showed the dramatic increase in NADPH diaphorase staining observed in the epithelial cells.

Our studies are the first to characterize the induction of NOS activity by CT in vivo in epithelial cells, in the absence of obvious local or systemic inflammation. Increased NO production during V. cholerae infection may serve both adaptive and protective functions. NO promotes peristalsis directly through effects on intestinal smooth muscle and indirectly via activation of VIP-secreting neurons (8, 9, 14). This relaxing effect on the bowel may accommodate the dramatic increases in intestinal volume and facilitate clearance of the fluid and bacterial load. Moreover, NO is also an endogenous mediator of relaxation of the vasculature. This powerful vasodilator effect may facilitate the increase in local blood flow required to support the necessary secretion of fluid and electrolytes stimulated by CT (25).

Protective effects of CT-induced NO may include both its bactericidal and cytotoxic effects. Similar to its ability to kill Salmonella (6), NO acts synergistically with oxygen radicals to kill V. cholerae in vitro. Such local bactericidal activity in the intestine may contribute to the host’s ability to limit the magnitude of the infection and subsequent CT release to facilitate spontaneous resolution of infection, which occurs in most cases. However, whether the levels and the precise stoichiometric balance of NO and oxygen radicals...
achieved locally actually result in vibriocidal activity in vivo is unknown. NO also may affect mucosal cell viability. Whereas low levels of NO likely contribute to maintaining cell viability, large amounts of NO can be cytotoxic to the intestinal epithelium (10, 37). Such cytotoxicity may ultimately shorten the duration of diarrhea during acute V. cholerae infection because epithelial cell activation and secretory activity continue relentlessly until the cell dies (7, 11). The effects on both bacterial and epithelial cells in vivo remain speculative at this point.

In summary, our studies show that V. cholerae-associated diarrheal disease activates the NO pathway. This NO is primarily derived from enhancement of a Ca2+-dependent form of NOS produced in intestinal epithelial cells. This local activity is stimulated by CT but not LPS and is not suppressed by dexamethasone. Mucosal production of NO in the intestine during acute diarrheal disease may have adaptive effects by increasing local blood flow and peristalsis to accommodate fluid secretion. Protective effects of NO may include the bactericidal activity of reactive NO metabolites to limit the infectious burden and decreasing epithelial cell viability to promote removal of secreting cells. These data reveal a previously unrecognized aspect of the biology of the host response to this serious diarrheal disease.

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