CONCISE COMMUNICATIONS

Effect of Nitric Oxide on Helicobacter pylori Morphology

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Helicobacter pylori causes chronic gastritis punctuated with fluctuating episodes of acute distress that can lead to peptic ulcer disease. Several factors produced by the bacterium have been shown to initiate the inflammatory response, but mechanisms potentially involved in the down-regulation of inflammation have not been described. We show that nitric oxide (NO) released from synthetic NO generators causes a rapid and dose-dependent morphologic conversion of H. pylori from the replicating spiral form to the nonreplicating, but viable, coccoid form. Because only spiral organisms—and not coccoid forms—are capable of inducing interleukin-8 secretion by epithelial cells, this conversion could result in down-regulation of the inflammatory response. These data suggest that the increase in NO synthase activity observed during gastritis results in morphologic conversion to a potentially dormant but viable H. pylori.

In the absence of effective treatment, chronic gastritis induced by Helicobacter pylori persists for the life of an individual. Maintenance of such a long-term infection must involve a balance between inflammatory up-regulation and suppression (suggested in [1]). Although it has been well-documented that H. pylori induces proinflammatory cytokine production in the gastric mucosa, little is known about mechanisms leading to the down-regulation of the inflammatory response to H. pylori infection.

Several investigators have noted an up-regulation of nitric oxide (NO) synthesis during gastritis. Inducible NO synthase (iNOS) activity has been shown to be elevated in the antrum and fundus of duodenal ulcer patients infected with H. pylori [2, 3]. These levels were reduced 10-fold after eradication of H. pylori [4]. iNOS immunopositive cells were detected in the vascular smooth muscle, gastric epithelium, gastric smooth muscle, and inflammatory cells of patients with ulcer and H. pylori-associated gastritis, and reactive nitrogen oxides were greatly elevated in biopsy samples from the centers of peptic ulcerations [5]. Elevated levels of iNOS and nitrotyrosine (a product of peroxynitrite) in gastric biopsy samples, as well as increased apoptosis of gastric epithelial cells, were hallmarks of H. pylori infection in a Columbian population at high risk for gastric cancer [6]. iNOS was detected in polymorphonuclear leukocytes and mononuclear cells, both of which are found near the epithelial surface in gastritis [6]. In addition, indices for iNOS activity, apoptosis and inflammation, were significantly correlated in patients before and after H. pylori eradication [4, 6]. NADPH-diaphorase activity, a measure of NO synthase activity, was elevated in both the lamina propria and the surface epithelium of biopsy samples from children with antral gastritis [7]. At the epithelial surface, focally intense areas of staining occurred in epithelial cells, particularly in mucosal extruded areas [7, 8] most likely to be in contact with H. pylori. Again, this activity disappeared when H. pylori was eradicated [7]. However, although NO levels are elevated during gastritis, this gas or its products are not sufficient to clear Helicobacter infections. Patients are not spontaneously cured, and inflammatory episodes continue for many years in the absence of successful antibiotic therapy. In addition to cellular sources of NO, dietary nitrate provides a considerable amount of gastric NO, catalyzed in the acidic environment of the stomach [9]. Thus, H. pylori has ample access to dietary and cellular sources of NO in the gastric mucosa.

The role of the coccoid form in the life cycle of Helicobacter is currently a subject of debate. The coccoid form has a low level of metabolism and of protein and DNA synthesis [10, 11], yet it can withstand many environmental changes, such as increased pH [12], antibiotics [10, 13], and starvation [12]. However, spiral H. pylori are the only forms capable of adhering to gastric epithelial cells or inducing interleukin (IL)–8 from these cells [14]. Recently, a regeneration medium has been described that causes conversion from coccoid to spiral forms at a rate faster than replication [15]. This suggests that H. pylori coccoid forms have the potential to revert to spiral, more virulent bacteria under some conditions.


Figure 1. Effect of nitric oxide (NO) donors on Helicobacter pylori SD14 growth and morphology ($n = 2$). Black bars represent results from 24 h, striped bars from 48 h. GSH, glutathione; GSSG, oxidized glutathione; GSNO, nitrosoglutathione; PAPA, PAPA NONOate (see text).

A, Direct bacterial counts determined by using Petroff-Hauser counter immediately after incubation with NO donors. B, Plate counts of dilutions of bacteria 5 days after the experiment. $P$ values were determined relative to the means of the controls and in all cases were $<.005$. C, Determination of percentage of coccoid forms in the culture by Wright-Giemsa stain of bacteria that were fixed immediately after the experiment. In all cases, $P$ values were $<.025$, relative to controls.

*H. pylori* exposed to NO at low oxygen tension form peroxynitrite, and respiration is irreversibly inhibited [16]. However, because coccoid *H. pylori* also have minimal respiratory activity, these forms could possibly survive exposure to NOs. The inability of infected individuals to spontaneously eradicate *H. pylori*, despite the generation of copious quantities of NO in the inflamed stomach, suggests that the NO produced does not effectively kill the bacterium. Therefore, we decided to investigate the effect of NO on *H. pylori* morphologic conversion.

**Methods**

Bacterial strains were isolated from biopsy samples from patients with duodenal ulcer (with approval of the UCSD Human Subjects Committee), as described elsewhere [14]. Bacteria were grown on Columbia agar with 7% laked horse blood, IsoVitalex (Becton Dickinson Microbiology Systems, Cockeysville, MD), and Dent supplement (Oxoid Ltd., Basingstoke, UK) [14], and the plates were incubated with Campy Paks (Becton Dickinson).

For incubation with NO donors, bacteria from 1- or 2-day-old plates were harvested and counted (by use of a Petroff-Hauser chamber), and $10^8$ bacteria were inoculated in 1 mL of Brucella broth containing 5% fetal calf serum per well in a 24-well microtiter tray. NO donors (Alexis Corp., San Diego) were suspended either in 40 mM NaOH for the diazenium diolates (NONOates) or in PBS for S-nitroso-L-glutathione (GSNO) and controls and sterile filtered just prior to use at 1 mM. The NO generators were added to wells in duplicates, and the trays were incubated in Campy pouches (Becton Dickinson) at 37°C. Half-lives of NO donors in neutral aqueous solution at 37°C are as follows (from Alexis Corp.): MAHMA NONOate, or 1-[(N-methyl-N-[6-(N-methylammoniohexyl)amino]diazen-1-ium-1,2-diolate] (MAHMA), 1 min; PAPA NONOate, or 1-[N-(3-ammoniopropyl)-N-[(n-propyl)amino]diazen-1-ium-1,2-diolate (PAPA), 15 min; spermine NO, 39 min; DETA NONOate, or 1-[2-(2-aminoethyl)-N-(2-ammonioethy]amino]diazen-1-ium-1,2-diolate] (DETA), $>500$ min. We determined the half-life of GSNO in Brucella broth with 5% fetal calf serum at 37°C to be 3 days by using the Saville method for measurement of nitrosothiols [17].

Direct bacterial counts were determined on live cultures with a Petroff-Hauser counter under light microscopy, separating clumps of bacteria by multiple passages through a 25-gauge needle. Bacterial plate counts were done in duplicate and averaged at 5 days. To determine the percentage of bacteria that were coccoid, bacteria were fixed on slides and stained with Wright-Giemsa stain (Fisher
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**Results**

Figure 1 shows typical results of incubation of spiral cultures of *H. pylori* SD14 with 1 mM concentrations of NO donors. *Panel A* shows that the direct bacterial counts decreased by ~10-fold in the presence of GSNO and PAPA, whereas *panel B* shows that the bacterial colony forming units were reduced 10^4-fold after incubation with these NO donors. This difference was particularly dramatic at 48 h, but it was readily apparent at 24 h. In contrast, the numbers of cultivable bacteria after the experiment in the reduced and oxidized glutathione controls were similar to those in the untreated controls at both 24 and 48 h. The explanation for this phenomenon, shown in *panel C*, is that the NO donors significantly increased the percentage of the culture that converted to the nonculturable, coccoid form. Nevertheless, bacteria in these cultures were viable, because coccoid forms induced by GSNO and MAHMA stained predominantly green with the BacLight kit, indicating their viability.

Figure 2 shows the effect of a variety of NO donors on a different strain of *Helicobacter*. *H. pylori* strain SD3 was incubated with 1 mM of NO donors for 24 h, and the proportion of coccoid versus spiral bacteria was determined. All of the NO donors tested caused considerable conversion of *H. pylori* to the coccoid form. Interestingly, DETA NONOate, which has a half-life of >500 min (according to product literature), caused as much conversion to the coccoid form as MAHMA and PAPA, with half-lives of 1 min and 15 min, respectively, at pH 7.4, 37°C. For strain SD3, GSNO caused considerable cell death; many spiral bacteria at the end of the experiment were nonmotile and stained red with the BacLight kit. However, many coccoid, green fluorescent bacteria were also present. Thus, a rapid or slow release of NO is sufficient to induce conversion of *H. pylori* to viable, coccoid forms, but NO can be bactericidal to the spiral forms at sufficient doses.

To further show the effect of NO on *H. pylori*, we incubated spiral bacteria with various concentrations of GSNO at several time points (figure 3). The graph shows the results of 3 separate counts at each dose and time point when *H. pylori* SD3 was used. The percentage of coccoid bacteria increased in a time- and dose-dependent manner in the presence of GSNO. Significant GSNO effects were observed at a concentration as low as 0.1 mM at 24 h and as early as 4 h after addition at the highest GSNO concentration tested.

**Discussion**

Because of the facile permeability of NO, a sufficient NO concentration may be present at the surface of gastric epithelial cells to induce morphologic conversion of *H. pylori* in the vicinity. We suggest that a major effect of NO produced by the host as a response to *Helicobacter* infections is to down-regulate the inflammatory response by increasing the conversion to coccoid organisms. Because coccoid forms neither replicate nor induce IL-8 secretion from epithelial cells, NO may have the overall effect of decreasing the burden of spiral forms able to...
induce a gastric inflammatory response. The NO generated during gastritis may enhance the spiral-to-coccoid form conversion in focal areas.

In a study using anti-\textit{H. pylori} antibody, coccoid forms were detectable in 83% of gastrectomy tissues from infected individuals [18]. A particularly relevant finding is that the mucosa at the margins of adenocarcinoma tumors contained predominantly coccoid forms [18]. The intensity of iNOS immunoactivity at the epithelium was greatest in dysplasia, compared with atrophic or superficial gastritis [3]. These findings are consistent with our hypothesis that NO in the gastric mucosa may influence conversion of \textit{H. pylori} from spiral to coccoid forms.

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References


