Helicobacter pylori Generates Superoxide Radicals and Modulates Nitric Oxide Metabolism*

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During studies of the bacterial action of nitric oxide (NO), we found that it reversibly inhibited the respiration of Escherichia coli and irreversibly inhibited the respiration of Helicobacter pylori. Peroxynitrite, a reaction product of NO and superoxide, irreversibly inhibited the respiration of both H. pylori and E. coli. H. pylori, but not E. coli, generated substantial amounts of superoxide radicals. These results suggest that NO directly inhibits the respiration of E. coli whereas it rapidly reacts with endogenously generated superoxide radicals in H. pylori. The resulting peroxynitrite inactivates the respiration of H. pylori.

Nitric oxide (NO) is a multifunctional gaseous free radical produced by NO synthase in various types of cells, such as endothelial cells, neurons, neutrophils, and macrophages (1). Nitric oxide is also generated from nitrite in saliva and from food by microorganisms in the oral cavity as well as by enzymatic mechanisms under acidic conditions, such as in gastric juice (2). Because NO synthase is also present in gastric mucosa (13, 14), physiological concentrations of NO in gastric juice are fairly high.

We have shown that the biological activity of NO is augmented significantly by physiologically low levels of oxygen tension (2–5). Although NO plays important roles in defense mechanisms against enteric bacteria (6, 7), few studies have explored the mechanism of its bactericidal action at physiological levels of oxygen tension.

Helicobacter pylori is a Gram-negative and microaerophilic bacterium that resides in the mucus layer overlying the gastric epithelium of the human stomach. This organism is thought to play essential roles in the pathogenesis of gastric inflammation, ulceration, and carcinogenesis (8–11). Although H. pylori is exposed to fairly high concentrations of NO in gastric juice, which has low oxygen tension, the effect of NO on the metabolism of H. pylori remains to be defined. We therefore studied the effects of NO on the respiration of H. pylori and Escherichia coli under physiologically low levels of oxygen tension.

MATERIALS AND METHODS

Reagents—Peroxynitrite solution and carboxy-2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide (cPTIO) were obtained from Dojin Co. (Kumamoto, Japan). Mn-type superoxide dismutase (SOD) from E. coli and 2-methyl-6-[[p-methoxyphenyl]-3,7-dihydroimidazol[1,2-α]pyrazin-3-one (MCLA) were obtained from Sigma and Tokyo Kasei Kogyo Co. (Tokyo, Japan), respectively. Nitric oxide solution was prepared as described previously (5).

Bacterial Strains and Their Culture—Two types of enteric bacteria, E. coli K-12 JM109 and H. pylori NCTC-11637, were used in all experiments. E. coli was cultured at 37 °C with shaking in nutrient broth (Difco) containing 0.5% NaCl. H. pylori was cultured in Brucella broth containing 5% horse serum under a microaerophilic atmosphere, produced with the use of a gas pack BB1 CampyPak (Becton, MD), at 37 °C for 20 h as described previously (15). The cultured E. coli and H. pylori were harvested at the logarithmic growth phase, suspended in 10 mM HEPES buffer (pH 7.0) containing 0.9% NaCl, and used in the experiments.

Assay of Bacterial Respiration—Respiration of E. coli and H. pylori was monitored polarographically at 37 °C in 10 mM HEPES buffer (pH 7.0) containing 0.9% NaCl and 5 mM succinate. A Clark-type oxygen electrode was used as described previously (5).

RESULTS AND DISCUSSION

Fig. 1 shows the succinate-dependent respiration of E. coli and H. pylori. Nitric oxide reversibly inhibited the respiration of E. coli. Consistent with our previous observations with mitochondria (2–4) and E. coli (5), the inhibitory effect of NO increased with a decrease in oxygen concentration of the medium. Although NO also inhibited the respiration of H. pylori in an oxygen concentration-dependent manner, it was significantly less active than with E. coli, and inhibition persisted throughout the observation period. Washing the NO-treated cells did not restore respiratory activity of H. pylori (data not shown).

Because NO is rapidly metabolized into various compounds, such as nitrite and nitrate, these metabolite(s) might act to inhibit cellular respiration. To test this hypothesis, we studied the effects of NO metabolites and NO-trapping agents on respiration. Fairly high concentrations of nitrite and nitrate (100 μM) did not inhibit the respiration of E. coli or H. pylori (Fig. 2). In contrast, both cPTIO and oxyhemoglobin, NO-trapping agents (20, 21), instantaneously abolished the inhibitory effect of NO on the respiration of E. coli. Thus, NO, rather than its metabolites, might directly interact with E. coli and thereby inhibit cellular respiration. In the case of H. pylori, neither cPTIO nor oxyhemoglobin abolished the inhibitory effect of NO. Thus, some metabolite(s) of NO other than nitrite and nitrate might irreversibly inhibit the respiration of H. pylori.

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† The abbreviations used are: NO, nitric oxide; cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide; SOD, superoxide dismutase; MCLA, 2-methyl-6-[[p-methoxyphenyl]-3,7-dihydroimidazol[1,2-α]pyrazin-3-one.
Because NO rapidly reacts with peroxynitrite radicals (k = 6.9 × 10^9 M^−1 s^−1) and generates cytotoxic peroxynitrite, effects of this metabolite on the respiration of E. coli and H. pylori were tested. When added to the incubation mixture, peroxynitrite inhibited the respiration of both organisms (Fig. 2). The characteristics of inhibition by peroxynitrite were similar to those of inhibition of H. pylori by NO. Inhibited respiration of both organisms persisted even after washing the cells with incubation medium (data not shown). Thus, peroxynitrite rather than NO and other metabolites might cause the irreversible inhibition of the respiration of H. pylori. These observations suggest that H. pylori might generate superoxide radicals that rapidly react with NO and generate peroxynitrite.

To test this hypothesis, the rate of superoxide generation and cellular levels of SOD were compared between the two organisms. When incubated in a medium containing MCLA, a chemiluminescence probe, H. pylori, elicited marked chemiluminescence that peaked after 2–3 min of incubation (Fig. 3). The MCLA chemiluminescence was strongly inhibited by Mn-SOD, suggesting the involvement of superoxide radicals. Under identical conditions, MCLA chemiluminescence was negligible with E. coli. In contrast to the superoxide generating activity, the cellular level of SOD was significantly lower in H. pylori than in E. coli (Table I). Based on the chemiluminescence intensity of MCLA, the steady-state level of superoxide generated by H. pylori was calculated to be 21 ± 9 μM. Because this superoxide concentration approximates that of NO used in the present experiments, NO might be rapidly trapped by superoxide radicals in and around H. pylori, forming peroxynitrite. Because peroxynitrite is cytotoxic, this metabolite, rather than NO, might irreversibly inhibit the respiration of H. pylori. Consistent with this notion is the finding that peroxynitrite, similar to NO, irreversibly inhibited the respiration of H. pylori.

Because peroxynitrite reacts with metals and Fe-S clusters in various proteins and oxidizes sulfhydryl groups of proteins (22–26), it might affect some component(s) in the respiratory systems of bacteria. The highly toxic nature of peroxynitrite and its metabolites (hydroxyl radical) may also impair the structure and functions of cell constituents, including proteins and DNA. Preliminary experiments using anti-nitrotyrosine antibody and Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed significant amounts of immunoreactive protein bands in NO-treated H. pylori but not in NO-treated E. coli. Helicobacter species show considerable genomic variation (27, 28). This genomic diversity
may be one reason for their success as ubiquitous pathogens. Preliminary polymerase chain reaction-based RFLP analysis of the gene encoding urease showed that NO induced mutation in H. pylori. Under identical conditions, NO had no appreciable effect on DNA samples obtained from NO-treated E. coli. These observations are consistent with the hypothesis that NO reacted with endogenously generated superoxide radicals and the resulting peroxynitrite reacted with proteins and DNA of H. pylori. Reactive oxygen species, including peroxynitrite and hydroxyl radicals, have been well documented to induce gene mutations and cancer. Thus, generation of superoxide and related metabolites might underlie the mechanisms of their genetic diversity and their role in the pathogenesis of gastric inflammation and cancer (29–33). However, these possibilities require further investigation before firm conclusions can be made.

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