**Helicobacter pylori Urease Suppresses Bactericidal Activity of Peroxynitrite via Carbon Dioxide Production**

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**Helicobacter pylori can produce a persistent infection in the human stomach, where chronic and active inflammation, including the infiltration of phagocytes such as neutrophils and monocytes, is induced. H. pylori may have a defense system against the antimicrobial actions of phagocytes. We studied the defense mechanism of H. pylori against host-derived peroxynitrite (ONOO^-), a bactericidal metabolite of nitric oxide, focusing on the role of H. pylori urease, which produces CO_2 and NH_3 from urea and is known to be an essential factor for colonization. The viability of H. pylori decreased in a time-dependent manner with continuous exposure to 1 μM ONOO^-, i.e., 0.2% of the initial bacteria remained after a 5-min treatment without urea. The bactericidal action of ONOO^- against H. pylori was significantly attenuated by the addition of 10 mM urea, the substrate for urease, whereas ONOO^--induced killing of a urease-deficient mutant of H. pylori or Campylobacter jejuni, another microaerophilic bacterium lacking urease, was not affected by the addition of urea. Such a protective effect of urea was potentiated by supplementation with exogenous urease, and it was almost completely nullified by 10 μM flurofamide, a specific inhibitor of urease. The bactericidal action of ONOO^- was also suppressed by the addition of 20 mM NaHCO_3 but not by the addition of 20 mM NH_3. In addition, the nitration of L-tyrosine of H. pylori after treatment with ONOO^- was significantly reduced by the addition of urea or NaHCO_3, as assessed by high-performance liquid chromatography with electrochemical detection. These results suggest that H. pylori-associated urease functions to produce a potent ONOO^- scavenger, CO_2/HCO_3^-, that defends the bacteria from ONOO^- cytotoxicity. The protective effect of urease may thus facilitate sustained bacterial colonization in the infected gastric mucosa.**

Nitric oxide (NO) is known to play an important role in host defense against a variety of microbes (1, 12, 15, 20, 36, 37), although NO itself does not show sufficient antimicrobial activity (24, 55). Some metabolites of NO, such as peroxynitrite (ONOO^-), are considered to be responsible for the antimicrobial as well as the pathogenic effects of NO. NO and superoxide (O_2^-) react in a diffusion-limited manner, forming ONOO^- (5), a strong oxidant and nitrating agent (4, 5, 23) that exhibits potent bactericidal activity (22, 57) as well as cytotoxicity for mammalian cells in vitro and in vivo (4, 5). It has been reported that both NO and O_2^- that are simultaneously produced in local areas of infection are critically involved in antimicrobial defense in murine salmonellosis (Salmonella enterica serovar Typhimurium infection), possibly through formation of ONOO^- (49).

**Helicobacter pylori** can infect human gastric mucosa chronically; such infection is known to be associated with gastritis, peptic gastric ulcer, duodenal ulcer, and an increased risk for gastric cancer (3, 6, 21, 45, 52). A unique feature of *H. pylori* infection is its persistence, which causes prolonged active inflammation, including infiltration of neutrophils and monocytes in gastric mucosa (11, 39). Increased expression of the inducible type of NO synthase (iNOS) (16–18, 30, 42, 47) and elevated formation of nitrotyrosine (17, 30) are also observed in the gastric mucosa of patients with *H. pylori* infection. However, the mechanism of the persistent infection of *H. pylori*, despite the production of highly bactericidal ONOO^- and other reactive nitrogen species, is not clear.

Several investigations have suggested a role for *H. pylori* urease in the survival and pathogenesis of the bacteria (29, 31, 35, 46). Urease catalyzes the hydrolysis of urea to form carbon dioxide (CO_2) and ammonia (NH_3). It is reported that urease functions in *H. pylori* infection to neutralize gastric acid by producing NH_3 (31). Enhanced production of NH_3 also may facilitate the formation of NH_3-derived compounds, such as monochloramine, which shows cytotoxic effects on host cells (46). Enhancement of bacterial motility (35) and inhibition of phagocytic clearance of bacteria (29) were also reported as functions of urease. The pathogenic potential of urease is so far mainly attributed to NH_3 produced by the enzymatic reaction. In contrast, little attention has been paid to the roles of CO_2/HCO_3^- produced in the same process. It is noteworthy that the chemical reactivity of ONOO^- is reported to be modulated by CO_2/HCO_3^- (26, 28, 54). Specifically, ONOO^- reacts rapidly with CO_2 and through the formation of ONOO^-CO_3^-, not only is isomerization of ONOO^- to NO_3^—accelerated (27, 50), but also the nitration potency of ONOO^- is significantly enhanced and the oxidation potential is markedly attenuated (54, 56). For example, CO_2/HCO_3^- facilitates ONOO^-induced nitration of aromatic compounds, such as tyrosine and guanine (guanosine); however, it suppresses their oxidation (26, 54, 56). In addition, the intracellular activity of ONOO^- on Escherichia coli was reduced by the addition of NaHCO_3 (22, 57).

Therefore, the purpose of this study was to clarify the role of urease in persistent colonization of *H. pylori*, especially to...
examine the protective effects of CO₂ produced by urease against the bactericidal activity of ONOO⁻ in vitro.

MATERIALS AND METHODS

Bacteria. H. pylori ATCC 43504 was obtained from the American Type Culture Collection (Manassas, Va.). H. pylori HPK5 and its isogenic ureB mutant HPT209 (lacking urease), which was produced by allelic exchange mutagenesis, were generously provided by T. Nakazawa, Department of Microbiology, Yamaguchi University School of Medicine, Ube, Japan (35). Campylobacter jejuni was isolated from a clinical source and was also used in this study. These bacteria were routinely grown in brucella broth (Becton Dickinson & Co., Cockeysville, Md.) supplemented with 10% fetal calf serum (Intergen Co., Purchase, N.Y.) in MacConkey’s and Enterotube supplement (BBL, Sparks, Md.).

Reagents. ONOO⁻ was prepared from nitrite and hydrogen peroxide in a quenched-flow reactor as previously described (5). The NO-liberating agent propamylamine NONOate (CH₃[N(NO)₂][CH₃]NHCH₃) (3-hydroxy-2-oxo-

lysine (nitrotyrosine), and L-tyrosine were purchased from Sigma Chemical Co., St. Louis, Mo. Dihydorhodamine 123 (DHR) was purchased from Molecular Probes, Inc., Eugene, Ore. Two urease inhibitors, N-(diaminophenyl)-1-fluorobenzamide (flurofamide) and acetohydroxamic acid (AHX), were from a commercial source.

Bacterial assays. We used 0.5 M phosphate buffer (pH 7.6) containing 0.15 M NaCl (250 mM phosphate-buffered saline [PBS]) for the bactericidal assays of ONOO⁻. Bacteria were cultured for 36 to 48 h and of C. jejuni were cultured for 36 to 48 h of C. jejuni cultured for 24 h were washed and resuspended in 0.5 M PBS immediately before the experiment. Bacteria were from commercial sources.

RESULTS

Bactericidal effect of ONOO⁻ on H. pylori. It is now known that ONOO⁻ is a key intermediate in the NO-dependent bactericidal effect. Induction of iNOS and formation of nitrotyrosine, an indicator of ONOO⁻ formation, in H. pylori-infected stomach have also been documented (16–18, 30, 42, 47). Therefore, we examined the bactericidal activity of authentic ONOO⁻ (5) by using the constant-flux infusion method (7, 40). The number of viable bacteria expressed as CFU declined after exposure to ONOO⁻ in a dose- and time-dependent manner (Fig. 1A). Products of ONOO⁻ decomposition, mainly nitrite anion (28), showed no bactericidal activity against H. pylori (Fig. 1B).

The NO-liberating agent P-ONOate was examined by incubation with H. pylori ATCC 43504 (10⁸ CFU/ml) in 0.5 M PBS (pH 7.6) for up to 10 min. P-ONOate at 1, 10, or 100 µM did not affect the viability of the bacteria (Fig. 1B). It is known that one molecule of P-ONOate releases two molecules of NO with a half-life of 7.6 min at a neutral pH (25, 40). Hence, an appreciable concentration of NO (up to 200 µM) did not kill this bacterium, nor was there an amount of NO-dependent bactericidal activity of NO, such as ONOO⁻, formed during the reaction period. In any event, these results indicate that NO per se exhibits only a little bactericidal action, which is a great contrast to ONOO⁻.

Effect of urea on bacterial action of ONOO⁻ on H. pylori and C. jejuni. As shown in Fig. 1C, in the presence of a physiological concentration (10 µM) of urea, survival of H. pylori with NO exposure was not affected. Because urea did not affect the decomposition rate of ONOO⁻ at the pH range 7.0 to 10.0, as assessed by measuring absorbance at 302 nm (data not shown), direct detoxification of ONOO⁻ by urea itself was not plausible. When clinically isolated C. jejuni, another microaerophilic bacteria lacking urease activity, was treated with ONOO⁻ in the same experimental settings in the presence or absence of urea, the susceptibility of C. jejuni to ONOO⁻ was not affected by the addition of urea (Fig. 1D), suggesting that the contribution of urease produced by H. pylori to the suppression of the cytotoxicity of ONOO⁻ was required. To further verify this notion, the bactericidal action of ONOO⁻ against H. pylori HPK5 and its isogenic mutant HPT209, lacking urease, was examined with or without the addition of 10 µM urea. The strains showed similar sensitivities to ONOO⁻ in the absence of urea (Fig. 1E and F). In contrast, urea attenuated the bactericidal effect of ONOO⁻ on the wild-type strain, HPK5 (Fig. 1E), but it did not affect the bacterial killing of ONOO⁻ for the mutant with the urease gene disruption, HPT209 (Fig. 1F). This result indicates again that urease activity is required for urea-dependent attenuation of the ONOO⁻ cytotoxicity.

The role of urease was further investigated with two urease inhibitors. It has been reported that flurofamide is a specific inhibitor of extracellular urease and that AHX is effective on both intracellular and extracellular urease (35). In the presence...
ence of 10 μM flurofamide or 70 mM AHX, the protective effect of urea was almost completely nullified (Fig. 2A), while in the absence of urea, these urease inhibitors did not affect the bactericidal action of ONOO− on H. pylori (data not shown). Flurofamide seemed to be more effective than AHX, so extracellular urease localized on the surface of bacterial cells plays an important role in suppressing the bactericidal action of ONOO−. In contrast to urease inhibitors, the addition of urease derived from B. pasteurii augmented the protective effect of urea (Fig. 2A). These data indicate that the bactericidal effect of ONOO− against H. pylori is diminished by bacterial urease activity.

Effects of NaHCO3 and NH3 on bactericidal action of ONOO− on H. pylori. We examined the effects of the products of the urea-urease reaction, CO2 and NH3, on the bactericidal activity of ONOO−. NaHCO3 (20 mM) suppressed bacterial killing by ONOO− to the same degree as 10 mM urea, whereas NH4OH (20 mM) did not (Fig. 2B). Furthermore, urea (10 mM) plus NaHCO3 (20 mM) showed an additive protective effect for the survival of H. pylori exposed to ONOO− (Fig. 2B), suggesting that urease increases bacterial survival in in vivo situations in which physiological concentrations of HCO3− and urea are close to those used in this experiment, i.e., about 20 and 10 mM, respectively (38).

A change in the pH of the media might affect the chemical reactivity of ONOO− (26, 27, 56). In our experimental settings, however, NH3 released after urea hydrolysis by H. pylori urease did not alter the pH of the reaction mixture. The pH values of the suspension of 106 CFU of H. pylori ATCC ATCC 43504 per ml in 0.5 M PBS after 0, 1, 2, 3, 4, and 5 min of infusion of 100 μM ONOO− at a flow rate of 240 μl/min in the absence of urea were 7.57 ± 0.01, 7.60 ± 0.01, 7.63 ± 0.01, 7.66 ± 0.02, 7.68 ± 0.02, and 7.69 ± 0.01, respectively, and those obtained in the presence of 10 mM urea were 7.57 ± 0.01, 7.60 ± 0.01, 7.63 ± 0.02, 7.65 ± 0.02, 7.67 ± 0.02, and 7.69 ± 0.01 (means ± standard deviations [SD] of three independent experiments). In addition, as shown in Fig. 1A, infusion of an alkaline solution alone (decomposed ONOO− in 10 mM NaOH) did not affect the viability of H. pylori. Also, NH3 per se had no appreciable effect on the bactericidal action of ONOO− (Fig. 2B). We therefore deduced that the protective effect of urease against ONOO− is dependent on its CO2 production but is not dependent on NH3 release or the change in pH.

Nitrotyrosine formation in H. pylori after treatment with ONOO−. ONOO− is known to nitrate aromatic compounds, including tyrosine (4, 23). To assess the effect of urea activity on the chemical reactivity of ONOO− with the bacterial components, we quantified nitrotyrosine in H. pylori cells by using HPLC coupled to electrochemical detection with 12 electrodes (Fig. 3A). The amount of nitrotyrosine in the bacterial cells exposed to 1 μM ONOO− for 3 min was 267 ± 22 pmol/10⁶ CFU, or 7.48% ± 1.2% of the total tyrosine (Fig. 3B). Nitrotyrosine was not detected (less than 0.1 pmol/10⁶ CFU) in the control bacterial cells (no exposure to ONOO−). In contrast, we could not detect any appreciable amount of nitrotyrosine in the bacterial cells treated with P-NONOate (data not shown), indicating that ONOO−, but not NO, exhibits a strong tyrosine-nitrating potential in H. pylori. The addition of 10 mM urea or 20 mM NaHCO3 to the reaction mixture of ONOO− lowered the formation of nitrotyrosine by 50% (Fig. 3B). Since CO2 accelerates decomposition of ONOO− (27, 50), it is plausible that CO2/HCO3− added or formed by bacterial urease might increase the decomposition rate of ONOO− and thus suppress the reactivity of ONOO− with the bacteria.

**DISCUSSION**

*H. pylori* produces a large quantity of urease, which amounts to 5% of the total protein of the bacterium (14). Urease genes in the *H. pylori* genome are composed of two gene clusters: *ureAB* genes and *ureIEFGH* genes (10). Colonization of *H. pylori* mutants whose *ureA*, *ureB*, *ureG*, or *ureF* gene was disrupted in experimental animals was known to be suppressed (13, 44, 48, 53). In addition, proton pump inhibitors used for treatment of *H. pylori* infection inhibit bacterial urease in an irreversible fashion (33). All these studies imply that *H. pylori* urease is essential for *H. pylori* colonization in the stomach.

Several studies were carried out to elucidate the roles of *H. pylori* urease in bacterial colonization in the stomach. Neutralization of gastric acid with NH3 produced by the enzyme might allow the bacterium to survive in the acidic milieu (31). It is reported that the motility of *H. pylori*, which is known to be an important characteristic of the bacterium in the colonization of...
experimental animals, is enhanced by the urea-urease reaction, particularly in a viscous environment (35). Inhibition of neutrophil function by NH₃ was also proposed as a pathogenic mechanism of this enzyme (32).

In addition to these possible roles of urease, the results obtained in this study clearly demonstrate that *H. pylori* urease functions as a part of the defense system of the bacteria themselves against ONOO⁻ (Fig. 1 and 2).

In previous work, elevated generation of ONOO⁻ in vivo and its involvement in antimicrobial host defense were reported for a murine salmonellosis model. Results indicated that suppressing ONOO⁻ generation by inhibiting either NO or O₂⁻ production or by scavenging these radicals accelerated the growth of *S. enterica* serovar Typhimurium in the liver and further augmented its pathogenicity, as evidenced by the increased mortality of infected mice (49). It was thus suggested that ONOO⁻ effectively clears bacteria from sites of infection in vivo (1, 49). In recent years, increased expression of iNOS mRNA and its product has been confirmed in *H. pylori*-infected gastric tissues of patients and experimental animals (16–18, 30, 42, 47). Formation of ONOO⁻ and/or other reactive nitrogen species produced by the NO₂⁻-H₂O₂-myeloperoxidase system at sites of infection by *H. pylori* is also suggested by the immunohistochemical detection of nitrotyrosine (17, 30, 51). Furthermore, it has recently been reported that not only phagocytic inflammatory cells but also *H. pylori* itself produce O₂⁻ (34), which indicates that ONOO⁻ may be formed in and around the bacteria in vivo, where production of NO and O₂⁻ is simultaneously elevated as described above. Consequently, ONOO⁻ may function as a major bactericidal effector for *H. pylori* in the stomach. In a separate experiment, however, no significant difference was found between the number of *H. pylori* organisms colonizing iNOS-knockout mice and that in wild-type mice (unpublished observation). In this context, it is quite reasonable that *H. pylori* has evolved with the system, such as urease, that is capable of detoxifying ONOO⁻, and hence steady and sustained colonization in the infected stomach is facilitated.

A high concentration of ONOO⁻ was used in the present study so that we could obtain reproducible results and clearly demonstrate the bactericidal action of ONOO⁻. The bacteria
were directly exposed to a 1 μM effective concentration of ONOO− in vitro, which is considered to be an extremely severe condition for the bacteria compared with the in vivo setting in infected foci containing various endogenous substances that affect the reactivity of ONOO− (2). Even under such conditions, the physiological concentration of urea increased the survival fractions of two strains of *H. pylori* (ATCC 43504 and HPK5) 3.7- to 8.4-fold after exposure to ONOO− for 5 min (Fig. 1C and E). Therefore, it is conceivable that the urease could function efficiently as a protective factor of *H. pylori* against ONOO− produced in vivo. Although it is reported that ONOO−-dependent nitrination of aromatic compounds, including tyrosine, is enhanced in the presence of CO2 (26, 54), formation of nitrotyrosine in *H. pylori* was suppressed by the addition of urea or NaHCO3 (Fig. 3B). Recently, Romero et al. reported that CO2 shortened the half-life and the diffusion distance of ONOO− and hence inhibited the oxidation of oxyhemoglobin in red blood cells by ONOO− (43). Therefore, the results obtained in this study suggest that CO2 formed by bacterial urease inhibits the reactivity of ONOO− with the bacterial components and accelerates its decomposition outside the bacterial cells. It is of great importance, then, that *H. pylori* urease is localized not only in the cytoplasm but also on the surface of the bacteria (41). In our experimental settings, surface-bound urease seemed to play an important role in the decomposition of ONOO− (Fig. 2A).

In conclusion, urease of *H. pylori* plays a role in the defense against the toxicity of ONOO− via production of CO2, and it may confer the capacity for sustained infection in vivo. Improved understanding of the pathogenic role of urease, in view of a host-pathogen interaction, will help in the exploration of effective therapeutic treatments for *H. pylori* infection and its related gastric diseases, including gastric cancer.

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