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 CO2 and H2 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 NaHCO3 but not by the addition of 20 mM NH3. In addition, the nitration of L-tyrosine of H. pylori after treatment with ONOO− was significantly reduced by the addition of urea or NaHCO3, 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, CO2/HCO3−, 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 (O2−) 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 O2− 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 (CO2) and ammonia (NH3). It is reported that urease functions in H. pylori infection to neutralize gastric acid by producing NH3 (31). Enhanced production of NH3 also may facilitate the formation of NH3-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 NH3 produced by the enzymatic reaction. In contrast, little attention has been paid to the roles of CO2/HCO3− produced in the same process. It is noteworthy that the chemical reactivity of ONOO− is reported to be modulated by CO2/HCO3− (26, 28, 54). Specifically, ONOO− reacts rapidly with CO2, and through the formation of NO−/O2− , not only is isomerization of ONOO− to NO− accelerated (27, 50), but also the nitration potency of ONOO− is significantly enhanced and the oxidation potential is markedly attenuated (54, 56). For example, CO2/HCO3− facilitates ONOO−-induced nitration of aromatic compounds, such as tyrosine and guanine (guanosine); however, it suppresses their oxidation (26, 54, 56). In addition, the in vitro bactericidal activity of ONOO− on Escherichia coli was reduced by the addition of NaHCO3 (22, 57).

Therefore, the purpose of this study was to clarify the role of urease in persistent colonization of H. pylori, especially to

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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 isolated from a clinical source was also used in this study. These bacteria were grown in brain heart infusion broth (Becton Dickinson & Co., Cockeysville, Md.) supplemented with 10% fetal calf serum (Intergen Co., Purchase, N.Y.) in microaerophilic conditions (5% O2, 10% CO2-generating agent, CampyPak [Becton Dickinson & Co.,] under microaerobic conditions, the number of colo-

**Reagents.** ONOO− was prepared from nitrite and hydrogen peroxide in a quenched-flow reactor as previously described (5). The NO-liberating agent propylene NONOate (CH3N[(NO)NO]) (CH3)2NH, CH2-1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene) (P-NONOate) which has a half-life of 7.6 min in aqueous solutions at a neutral pH under our experimental conditions, was obtained from Dojindo Laboratories, Kumamoto, Japan. Urea, NaHCO3, or NH4OH as described above. Aliquots (120 µl) were removed from the bacterial suspension in 10 mM NaOH into 0.5 M PBS (1.2 ml) at a flow rate of 240 ml/min, the reaction mixture was then treated with 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, and 750 mV. Peaks of nitrotyrosine and L-tyrosine were identified and quantified based on comigration with known concentrations of authentic standards and their electrochemical activation profiles. Identification of nitrotyrosine was confirmed by the disappearance of the peak after reduction of nitrotyrosine to aminotyrosine by 20 mM sodium dithionite. The amounts of nitrotyrosine and tyrosine were quantified from the peak areas obtained at 750 and 600 mV, respectively.

**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 nitrosy-

**Concentration of ONOO−.** To examine the bactericidal effect of ONOO− on H. pylori-infected stomach, we 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-NONOate 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-NONOate at 1, 10, or 100 µM did not affect the viability of the bacteria (Fig. 1B). It is known that one molecule of P-NONOate 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 a sufficient amount of bactericidal metabolites of NO, such as ONOO−, formed during the reaction period. In any event, these results indicate that NO per se exhibits very little bactericidal action, which is a great contrast to ONOO−.

**Effect of urea on bactericidal action of ONOO− on H. pylori and C. jejuni.** As shown in Fig. 1C, in the presence of a physiological concentration (10 mM) of urea, survival of H. pylori was improved by NO-mediated bactericidal activity. Because NO 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 mM 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 fluoroamide is a specific inhibitor of extracellular urease and that AHX is effective on both intracellular and extracellular urease (35). In the pres-
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 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 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 (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 urease 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 ureIEFEGH 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

FIG. 2. (A) Effects of urease inhibitors and additional urease on the bactericidal action of ONOO− on H. pylori. H. pylori ATCC 43504 organisms (10⁶ CFU/ml) were exposed to a constant concentration of ONOO− (1 μM) for 3 min in the absence (a) or presence (b to e) of 10 mM urea. Reactions were performed in the presence of 10 μM fluoroamide (c), 70 mM AHX (d), or 48 U of B. pasteurii urease per ml (e). (B) Effects of NaHCO3 and NH3 on bacterial action of ONOO− on H. pylori. H. pylori ATCC 43504 organisms (10⁶ CFU/ml) were exposed to a constant concentration of ONOO− (1 μM) for 3 min in the presence of 10 mM urea (b), 20 mM NH3 (c), 20 mM NaHCO3 (d), or 10 mM urea plus 20 mM NaHCO3 (e) or in the absence of all compounds (a), and the colony-forming assay was performed. Data from three independent experiments are expressed as means ± SD. *, P < 0.01 versus a; †, P < 0.05 versus b; **, P < 0.005 versus b; and ‡, P < 0.005 versus d.

FIG. 3. Nitrotyrosine formation in H. pylori cells after treatment with ONOO−. 3-Nitro-L-tyrosine (3-Nitro-Tyr) was quantified by HPLC coupled to electrode detectors, with the pronase digests of non-ONOO−-treated H. pylori ATCC 43504 cells (a) or those exposed to 1 μM ONOO− (b to d) for 3 min in the absence (b) or presence of 10 mM urea (c) and 20 mM NaHCO3 (d). (A) Elution profile of the pronase digest of H. pylori exposed to 1 μM ONOO− in the absence of urea or NaHCO3. (B) The amounts of 3-Nitro-Tyr formed in H. pylori were expressed as the ratio of 3-Nitro-Tyr to total L-tyrosine (Tyr) recovered from bacterial cells. ND, 3-Nitro-Tyr was not detected (<0.1 pmol/10⁸ CFU). *, P < 0.05 versus b. Data are means ± SD of three independent experiments.
were directly exposed to a 1 μM effective concentration of ONOO\textsuperscript{−} 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\textsuperscript{−} (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\textsuperscript{−} 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\textsuperscript{−} produced in vivo.

Although it is reported that ONOO\textsuperscript{−}-dependent nitration of aromatic compounds, including tyrosine, is enhanced in the presence of CO\textsubscript{2}, it is formed of nitroproline in H. pylori was suppressed by the addition of urea or NaHCO\textsubscript{3} (Fig. 3B). Recently, Romero et al. reported that CO\textsubscript{2} shortened the half-life and the diffusion distance of ONOO\textsuperscript{−} and hence inhibited the oxidation of oxyhemoglobin in red blood cells by ONOO\textsuperscript{−} (43). Therefore, the results obtained in this study suggest that CO\textsubscript{2} formed by bacterial urease inhibits the reactivity of ONOO\textsuperscript{−} 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\textsuperscript{−} (Fig. 2A).

In conclusion, urease of H. pylori plays a role in the defense against the toxicity of ONOO\textsuperscript{−} via production of CO\textsubscript{2}, 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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