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

HIDEO KUWAHARA,[1,2] YOICHI MIYAMOTO,[1] TAKAAKI AKAIKE,[1]* TATSUO KUBOTA,[1] TOMOHIRO SAWA,[1] SHINICHIRO OKAMOTO,[1] AND HIROSHI MAEDA[1]*

Department of Microbiology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811,[1] and Department of Medical Technology, Ginkyo College of Medical Science, 1-6-2 Okubo, Kumamoto 860-0083,[2] Japan

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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 mucosae 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 $ONOOCO_2^-$, 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 in vitro bactericidal 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

* Corresponding author. Mailing address: Department of Microbiology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan. Phone: (81) 96-373-5098. Fax: (81) 96-362-8362. E-mail: msmaedah@gpo.kumamoto-u.ac.jp.

examine the protective effects of CO_2 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 routinely grown in brucella broth (Becton Dickinson & Co., Cockeysville, Md.) supplemented with 10% fetal calf serum (Intergen Co., Purchase, N.Y.) in the presence (*H. pylori* HPT209) or absence (*H. pylori* ATCC 43504, *H. pylori* HPK5, and *C. jejuni*) of 7 μg of kanamycin sulfate/ml under microaerobic conditions maintained in a GasPak jar (Becton Dickinson & Co.) with an H_2- and CO_2-generating agent, CampyPak (Becton Dickinson & Co.).

Reagents. $ONOO^-$ was prepared from nitrite and hydrogen peroxide in a quenched-flow reactor as previously described (5). The NO-liberating agent propylamine NONOate ($CH_3N[N(O)NO]^-(CH_2)_3NH_2^+CH_3$, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazine) (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, $NaHCO_3$, and an aqueous solution of ammonia (NH_4OH) were from Wako Pure Chemicals Co. Ltd., Osaka, Japan. Urease from *Bacillus pasteurii*, 3-nitro-L-tyrosine (nitrotyrosine), and L-tyrosine were purchased from Sigma Chemical Co., St. Louis, Mo. Dihydrorhodamine 123 (DHR) was purchased from Molecular Probes, Inc., Eugene, Oreg. Two urease inhibitors, *N*-(diaminophosphinyl)-4-fluorobenzamide (flurofamide) and acetohydroxamic acid (AHX), were from ICN Biomedicals Inc., Aurora, Ohio, and Nacalai Tesque Inc., Kyoto, Japan, respectively. Pronase was obtained from Calbiochem-Novobiochem Co., La Jolla, Calif. All other reagents were from commercial sources.

Bactericidal assays. We used 0.5 M phosphate buffer (pH 7.6) containing 0.15 M NaCl (0.5 M phosphate-buffered saline [PBS]) for the bactericidal assays of $ONOO^-$ and other effector substances to minimize changes in pH during the reaction because of infusion of the alkaline solution of $ONOO^-$ (in 10 mM NaOH) and production of NH_3 and HCO_3^-, which might have affected the pH of the reaction mixture. Suspensions of *H. pylori* cultured for 36 to 48 h and of *C. jejuni* cultured for 24 h were washed with and resuspended in 0.5 M PBS immediately before use. Bacterial suspensions were kept on ice until use. Urease activity and bacterial motility and morphology were checked before each use. The constant-flux infusion method (7, 40) was used to treat the bacteria with steady concentrations of $ONOO^-$. In the constant-flux infusion process, the effective and constant concentration of $ONOO^-$ is maintained by balancing infusion and decomposition of $ONOO^-$ in the system. The concentrations of $ONOO^-$ maintained constant were estimated by the DHR oxidation assay, as described earlier (8). Specifically, DHR (28 μM) was added to the reaction mixture of $ONOO^-$ without bacteria; simultaneously, the $ONOO^-$ infusion was stopped, and the amount of the oxidized product rhodamine was measured fluorometrically. The concentration of $ONOO^-$ was then estimated by using a standard curve of the amount of rhodamine generated as a function of $ONOO^-$, which was prepared separately by reaction of DHR with a bolus of $ONOO^-$ injected into 0.5 M PBS. As a result, by infusion 10, 100, and 1,000 μM $ONOO^-$ in 10 mM NaOH into 0.5 M PBS (1.2 ml) at a flow rate of 240 μl/min, the concentrations of $ONOO^-$ remained constant at 0.3, 1, and 3 μM, respectively. *H. pylori* or *C. jejuni* (10^8 CFU/ml each) samples were treated constantly with 1 μM $ONOO^-$ by infusing 100 μM $ONOO^-$ in the absence or presence of urea, $NaHCO_3$, or NH_4OH as described above. Aliquots (120 μl) were removed from the reaction mixture at 30-s intervals and were immediately diluted with nutrient broth (Eiken Chemical Co. Ltd., Tokyo, Japan) and seeded on brucella agar plates containing 5% lysed horse blood (Nippon Bio-Test Laboratories Inc., Tokyo, Japan) for the colony-forming assay. After cultivation for 5 days (*H. pylori*) or 2 days (*C. jejuni*) under microaerobic conditions, the number of colonies formed was determined.

To examine the bactericidal effect of NO on *H. pylori*, P-NONOate at 1, 10, and 100 μM was added to the suspension of *H. pylori* ATCC 43504 (10^8 CFU/ml) in 0.5 M PBS. After incubation for 3, 5, and 10 min, aliquots of the bacterial suspension were diluted with nutrient broth and seeded on brucella agar plates containing 5% lysed horse blood. Viable *H. pylori* organisms were quantified by the colony-forming assay, as just described.

Measurement of nitrotyrosine. Suspensions of *H. pylori* (5×10^8 CFU/ml) treated continuously with 1 μM $ONOO^-$ for 3 min in the presence or absence of 20 mM $NaHCO_3$ or 10 mM urea, as described above, were centrifuged at 1,600 × *g* and then resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl (250 μl). Aliquots (100 μl) were treated with 60 μg of pronase/ml for 18 h at 50°C. The bacterial suspension became clear and no precipitate was seen in the resultant reaction mixture even after centrifugation at 10,000 × *g*, indicating that the bacterial cells were completely digested by the pronase treatment. After filtration through a centrifugal filter unit (Ultrafree-MC with a 10,000 nominal molecular weight limit; Millipore Corp., Bedford, Mass.), the filtrate was processed by high-performance liquid chromatography (HPLC) coupled to electrochemical detection with 12 CoulArray electrode cells (ESA, Inc., Chelmsford, Mass.) (9, 19). Nitrotyrosine recovered from the bacterial cells was separated on a reverse-phase column (4.6 by 250 mm) (TSKgel ODS-80Ts; Tosoh Co., Tokyo, Japan) and eluted with 50 mM sodium acetate buffer (pH 4.7) containing 5% methanol at a flow rate of 0.8 ml/min. The CoulArray electrode array detector was operated with applied potentials at 200, 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.

Statistical analysis. Statistical analyses were done with the two-tailed *t* test for unpaired data.

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 nitrate anion (28), showed no bactericidal activity against *H. pylori* (Fig. 1A).

The NO-liberating agent P-NONOate was examined by incubation with *H. pylori* ATCC 43504 (10^8 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 significantly increased. 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 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 flurofamide is a specific inhibitor of extracellular urease and that AHX is effective on both intracellular and extracellular urease (35). In the pres-

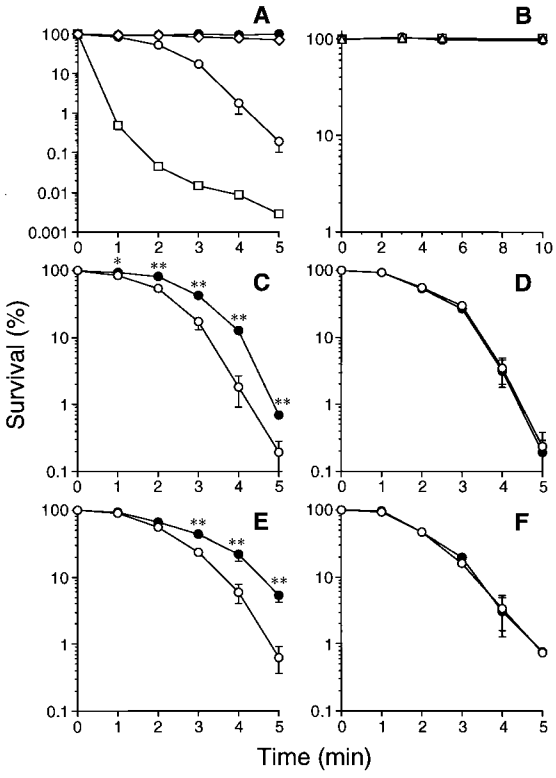

FIG. 1. (A) Bactericidal effect of $ONOO^-$ on *H. pylori* ATCC 43504. Decomposed $ONOO^-$ (equivalent to 1 mM $ONOO^-$) (●) or $ONOO^-$ at 10 μM (◇), 100 μM (○), or 1 mM (□) in 10 mM NaOH was infused into 1.2 ml of bacterial suspension (10^8 CFU/ml) at a flow rate of 240 μl/min. Every 30 s, 120-μl aliquots were removed from the reaction mixture, and the number of viable bacteria was determined by the colony-forming assay. Concentrations of $ONOO^-$ in the reaction mixture during infusion of 10 μM, 100 μM, and 1 mM $ONOO^-$ were assumed to be maintained at constant 0.3, 1, and 3 μM concentrations, respectively. Data are means ± SD from three independent experiments. (B) Survival of *H. pylori* after exposure to the NO-donor P-NONOate. *H. pylori* ATCC 43504 organisms (10^8 CFU/ml) were incubated for the indicated periods with 1 (○), 10 (□), or 100 (△) μM P-NONOate, followed by colony-forming assay. (C and D) Effects of urea on bactericidal action of $ONOO^-$ on *H. pylori* ATCC 43504 (C) and *C. jejuni* (D). (E and F) Effects of urea on the bactericidal action of $ONOO^-$ on *H. pylori* HPK5 (E) and its isogenic *ureB* mutant, HPT209 (F). Bacteria (10^8 CFU/ml) were exposed to a constant concentration of $ONOO^-$ (1 μM) in the presence (●) or absence (○) of 10 mM urea for the indicated periods, and the number of viable bacteria at each time point was determined. Data from three independent experiments are expressed as means ± SD. * and **, $P < 0.05$ and $P < 0.01$ versus control without urea, respectively.

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 $NaHCO_3$ and NH_3 on bactericidal action of $ONOO^-$ on *H. pylori*. We examined the effects of the products of the urea-urease reaction, CO_2 and NH_3, on the bactericidal activity of $ONOO^-$. $NaHCO_3$ (20 mM) suppressed bacterial killing by $ONOO^-$ to the same degree as 10 mM urea, whereas NH_4OH (20 mM) did not (Fig. 2B). Furthermore, urea (10 mM) plus $NaHCO_3$ (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 HCO_3^- 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, NH_3 released after urea hydrolysis by *H. pylori* urease did not alter the pH of the reaction mixture. The pH values of the suspension of 10^8 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 alone (decomposed $ONOO^-$ in 10 mM NaOH) did not affect the viability of *H. pylori*. Also, NH_3 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 CO_2 production but is not dependent on NH_3 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/10^8 CFU, or 7.48% ± 1.2% of the total tyrosine (Fig. 3B). Nitrotyrosine was not detected (less than 0.1 pmol/10^8 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 $NaHCO_3$ to the reaction mixture of $ONOO^-$ lowered the formation of nitrotyrosine by 50% (Fig. 3B). Since CO_2 accelerates decomposition of $ONOO^-$ (27, 50), it is plausible that CO_2/HCO_3^- 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 *ureI* 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 NH_3 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

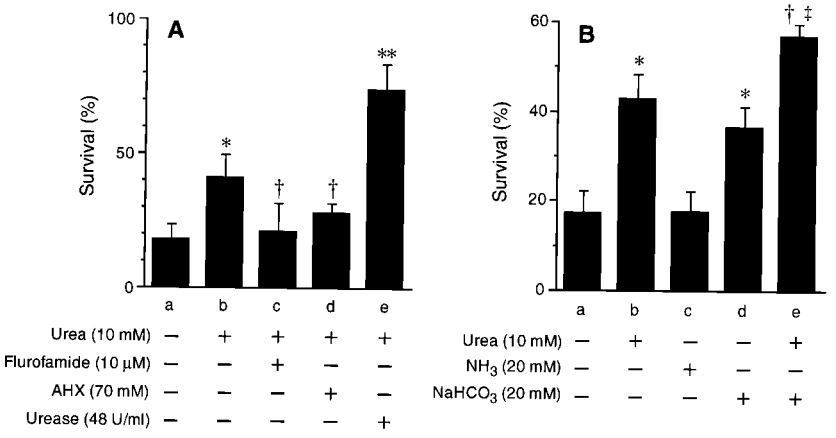

FIG. 2. (A) Effects of urease inhibitors and additional urease on the bactericidal action of $ONOO^-$ on *H. pylori*. *H. pylori* ATCC 43504 organisms (10^8 CFU/ml) were exposed to a constant concentration of $ONOO^-$ (1 μM) for 3 min in the absence (a) or presence (b to e) of 10 mM urea. Reactions were performed in the presence of 10 μM flurofamide (c), 70 mM AHX (d), or 48 U of *B. pasteurii* urease per ml (e). (B) Effects of $NaHCO_3$ and NH_3 on bactericidal action of $ONOO^-$ on *H. pylori*. *H. pylori* ATCC 43504 organisms (10^8 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 NH_3 (c), 20 mM $NaHCO_3$ (d), or 10 mM urea plus 20 mM $NaHCO_3$ (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.

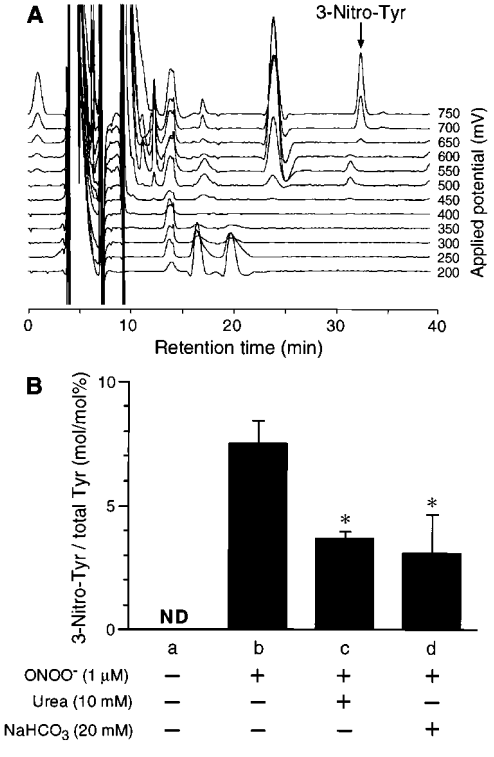

FIG. 3. Nitrotyrosine formation in *H. pylori* cells after treatment with $ONOO^-$. 3-Nitro-L-tyrosine (3-Nitro-Tyr) was quantified by HPLC coupled to electrode detectors, with the pronase digests of non-$ONOO^-$-treated *H. pylori* ATCC 43504 cells (a) or those exposed to 1 μM $ONOO^-$ (b to d) for 3 min in the absence (b) or presence of 10 mM urea (c) and 20 mM $NaHCO_3$ (d). (A) Elution profile of the pronase digest of *H. pylori* exposed to 1 μM $ONOO^-$ in the absence of urea or $NaHCO_3$. (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/10^8 CFU). *, $P < 0.05$ versus b. Data are means ± SD of three independent experiments.

experimental animals, is enhanced by the urea-urease reaction, particularly in a viscous environment (35). Inhibition of neutrophil function by NH_3 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 O_2^- 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 NO_2^--H_2O_2-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 O_2^- (34), which indicates that $ONOO^-$ may be formed in and around the bacteria in vivo, where production of NO and O_2^- 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 nitration of aromatic compounds, including tyrosine, is enhanced in the presence of CO_2 (26, 54), formation of nitrotyrosine in *H. pylori* was suppressed by the addition of urea or $NaHCO_3$ (Fig. 3B). Recently, Romero et al. reported that CO_2 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 CO_2 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 CO_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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