Digestive Diseases and Sciences, Vol. 41, No. 6 (June 1996), pp. 1139–1144

Rebamipide Protects Against Activation of Neutrophils by *Helicobacter pylori*

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Our objectives were to determine whether rebamipide, a unique antiulcer agent, would inhibit adhesive reactions between neutrophils and endothelial cells as well as the production of active oxygen species from neutrophils elicited by an extract of *H. pylori*. A water extract of *H. pylori* that was prepared from biopsy materials obtained from a patient with gastric ulcer increased the surface expression of CD18 on human neutrophils isolated from peripheral blood, the adhesion of neutrophil-endothelial cells, and the production of active oxygen species by neutrophils. Rebamipide, at concentrations of 10^{-5} and 10^{-6} M, reduced the adherence of neutrophils to endothelial cells as well as the CD18 expression on neutrophils induced by this bacterial extract. Rebamipide also inhibited the production of active oxygen species from neutrophils stimulated by *H. pylori* extract. These results suggest that rebamipide protects against the gastric mucosal inflammation associated with *H. pylori* by inhibiting neutrophil function.

KEY WORDS: *H. pylori;* neutrophil; endothelial cell; adhesion molecule; active oxygen species; rebamipide.

While *Helicobacter pylori* infection is thought to be involved in the pathogenesis of gastric and duodenal inflammation, the mechanisms are not well defined. It has been suggested that *H. pylori* produces and releases chemoattractants that activate neutrophils and other inflammatory cells, with the activated leukocytes causing tissue injury (1, 2). Our studies indicated that *H. pylori* extract, which is acid- and pepsin-resistant, induced the surface expression of neutrophil adhesion molecules CD11b/CD18 and promoted neutrophil adherence to the endothelium followed by transendothelial migration (3). We also found that this *H. pylori* extract induced the production of active oxygen species (AOS) by neutrophils (4). These results are consistent with the clinical observations showing that the degree of *H. pylori* infection and the severity of the mucosal injury are directly correlated with the extent of neutrophil infiltration (5). These findings suggest that *H. pylori* infection can induce gastrointestinal mucosal injury by initiating an acute inflammatory response causing neutrophil–endothelial cell interactions and the activation of neutrophils.

Rebamipide is a new antiulcer agent that scavenges AOS and inhibits AOS production by activated neutrophils, thereby protecting against oxygen-derived free radical-induced gastric mucosal injury (6–8). We conducted this study to evaluate the effects of rebamipide on *H. pylori* extract-induced neutrophil adherence to endothelial cells and neutrophil AOS production.

Manuscript received October 11, 1995; revised manuscript received February 20, 1996; accepted March 8, 1996.

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0163-2116/96/0600-1139$09.50/0

MATERIALS AND METHODS

***H. pylori* Water Extracts.** *H. pylori* extracts were prepared, as previously described (3), from strain H13, which was isolated from the gastric antral biopsy taken from a patient with a gastric ulcer. Briefly, the organism was grown on blood agar plates and harvested with sterile cotton swabs into distilled water, using 1.0 ml/plate (10^9–10^{10} bacteria). The cell suspension was kept at room temperature for 20 min before centrifugation at 12,000 rpm for 15 min. The resultant supernatant is the initial water extract; no preservatives were added, and the extract was stored at −70°C until needed. Before use, the water extract was thawed at room temperature and centrifuged at 18,000 rpm for 20 min. To remove much of the high-molecular-weight material, consisting mainly of membrane vesicles and whole flagellae, the supernatant was clarified by passage through a 0.2-μm syringe-adapted filter.

Endothelial Cells. Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords by collagenase treatment as previously described (9). The cells were plated in Medium 199 (Gibco, Grand Island, New York) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories Inc., Logan, Utah), thymidine (2.4 mg/liter; Sigma Chemical, St. Louis, Missouri), glutamine (230 mg/liter; Gibco), heparin sodium (10 IU/ml; Sigma), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Gibco), and endothelial cell growth factor (80 μg/ml; Biomedical Technologies Inc., Stoughton, Massachusetts). The cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2 and expanded by brief trypsinization (0.25% trypsin in phosphate-buffered saline containing 0.02% ethylenediamine tetraacetic acid). HUVEC of the primary through the third passage were seeded into 0.1% gelatin and 25 μg/ml fibronectin-coated 11-mm, 48-well tissue culture plates (Gibco) and used in adhesion assays when confluent. Endothelial cells were identified by their cobblestone appearance at confluence and positive labeling with acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-1-3,3,3′,3′-tetrametylindocarbocyanine perchlorate (Dil-Ac-LDL; Biomedical Technologies Inc.), and mouse antihuman factor VIII (Calbiochem, San Diego, California).

Neutrophils. Human neutrophilic polymorphonuclear leukocytes (neutrophils) were isolated from venous blood of healthy adults by using standard dextran sedimentation and gradient separation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). This procedure yields a polymorphonuclear leukocyte population that is 95–98% viable (trypan blue exclusion) and 98% pure (acetic acid–crystal violet staining). Isolated neutrophils were resuspended in plasma-free Hanks' balanced salt solution (HBSS).

Effect of Rebamipide on Neutrophil Viability. To investigate the effect of rebamipide on the viability of neutrophils, neutrophils were incubated with rebamipide (generously provided by Otsuka Pharmaceuticals Co., Ltd., Tokushima, Japan) at final concentrations of 10^{-5} or 10^{-6} M for 30 min. Cell viability was assessed by the trypan blue exclusion technique.

Adhesion Assays. Isolated neutrophils were added to HUVEC monolayers at a neutrophil-to-endothelial cell ratio of 10:1 with or without a 50-fold dilution of *H. pylori* extract. After a 30-min coincubation at 37°C, the supernatant was removed and the monolayers were washed with 500 μl of HBSS. Myeloperoxidase (MPO) activity from neutrophils in the supernatant and wash fluid was assessed by the method of Krawisz et al (10).

The percent of added neutrophils that adhered to the HUVEC monolayers was quantitated as follows:

$$\text{Neutrophil adherence (\%)} = \frac{A - (B + C)}{A} \times 100$$

where A is MPO activity from the total neutrophils added to HUVEC monolayers, B is MPO activity from the neutrophils in the supernatant, and C is MPO activity from the neutrophils in the wash.

To investigate the effect of rebamipide on the extract-induced adherence of neutrophils to endothelial cells, rebamipide and neutrophils were simultaneously added to endothelial monolayers. Final drug concentrations were 10^{-5} or 10^{-6} M. Adhesion assays were performed as described above.

Immunofluorescence Flow Cytometry. Surface expression of CD18, the β_2 subunit of CD11/CD18 glycoprotein, on neutrophils was determined by immunofluorescence flow cytometry as previously described (11). Briefly, the reaction mixture consisted of 10^6 neutrophils, *H. pylori* extract (final concentration: 2%), monoclonal antibody to CD18 (L130; Becton Dickinson, San Jose, California; final concentration: 2 μg/ml) in a total volume of 500 μl HBSS. After a 30-min incubation at 37°C, neutrophils were washed with PBS, and a fluorescein isothiocyanate (FITC) -conjugated goat $F(ab')_2$ fragment specific for mouse immunoglobulin G (Cappel, Durham, North Carolina) was added at a final concentration of 10 μg/ml. After a 15-min incubation at 4°C, neutrophils were washed with PBS and the monoclonal antibodies binding to neutrophils were analyzed in an EP-ICS Profile flow cytometer (Coulter Corp., Hialeah, Florida). To determine the effect of rebamipide on neutrophil CD18 expression, rebamipide was added to achieve final concentrations of 10^{-5} or 10^{-6} M in similar flow cytometry assays.

Active Oxygen Species (AOS) Production from Neutrophils. AOS production from neutrophils was determined by using the electron paramagnetic resonance (EPR) spin trapping method as previously described (6, 8, 12). Briefly, neutrophils (2×10^6 cells/ml) in HBSS were incubated with 100 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO; Sigma) and 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC; Wako Pure Chemicals, Osaka, Japan) at 37°C for 2 min. Subsequently, a 50-fold dilution of *H. pylori* extract and rebamipide, at a final concentration of 10^{-5} or 10^{-6} M, were added, and the entire mixture was immediately transferred to a flat quartz EPR cell (Labotec Co., Tokyo, Japan). EPR measurements were performed 1, 6, and 11 min later by using Jeol-JES-FR80 X-band EPR spectrometer (Jeol Co., Tokyo, Japan). The specifications of this spectrometer were as follows: frequency, 9.42 GHz; temperature, 37°C; magnetic field 335.8 mT; microwave power, 8.0 mW; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; sweep width, 10 mT; sweep time, 2.5 mT/min; response time, 0.1 second; and received gain, × 250.

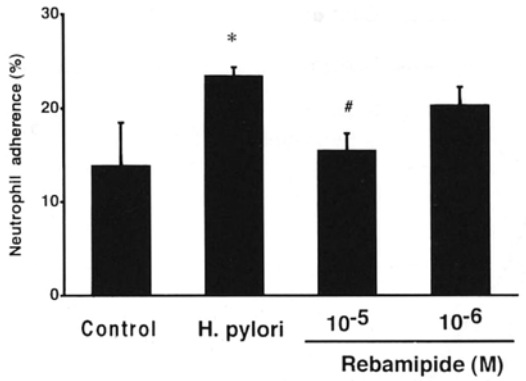

Fig 1. Effect of rebamipide on neutrophil adherence to human umbilical vein endothelial cells (HUVEC) monolayers induced by *H. pylori* extract. Each value represents the mean ± SE of three to five experiments performed in triplicate. $^{*}P < 0.05$ compared with control group without *H. pylori* extract and $^{\#}P < 0.05$ compared with *H. pylori* group without rebamipide.

In parallel experiments, neutrophils (2×10^6 cells/ml) were pretreated with rebamipide at final concentrations of 10^{-5} or 10^{-6} M. After a 30-min incubation, neutrophils were washed to remove rebamipide, and used in the EPR spin trapping assay as described above.

Statistical Analysis. Values are expressed as means ± SE. Data were analyzed using an analysis of variance (ANOVA) followed by Scheffe's test. A probability (*P* value) of <5% was considered significant.

RESULTS

The incubation of neutrophils with rebamipide resulted in more than 97% viable cells, which indicates that rebamipide itself does not decrease cell viability, as previously reported (13). Figure 1 shows the effect of rebamipide on neutrophil adherence to endothelial cells caused by water extract of *H. pylori*. *H. pylori* extract increased neutrophil–endothelial cell adhesive interactions. Importantly, at 10^{-5} M, rebamipide significantly inhibited this increased neutrophil adherence without influencing the adherence of unstimulated neutrophils (control). The ability of rebamipide to prevent the surface expression of CD18 on neutrophils was also investigated. As shown in Figure 2, *H. pylori* extract induced the surface expression of CD18 on neutrophils. The induction of CD18 expression was significantly reduced by rebamipide at either the 10^{-5} or 10^{-6} M concentration. Rebamipide had no significant effect on CD18 expression on unstimulated neutrophils (control).

We further investigated the effect of rebamipide on AOS produced by *H. pylori* extract-stimulated neutrophils. Our results showed that *H. pylori* extract increased the intensity of the DMPO-OH signals that originated from neutrophils in a time-dependent manner from 1 to 11 min (Figure 3). Three experiments performed with neutrophils isolated from three healthy volunteers showed the same EPR signal patterns. The DMPO-OH signal, which is indicative of hydroxyl radical spin adducts (6, 8, 12), was completely suppressed by the addition of superoxide dismutase, suggesting that the DMPO-OH signals (hydroxyl radicals) arise from superoxide radicals produced by neutrophils (data not shown). Figure 4 demonstrates the effect of rebamipide on the intensity of DMPO-OH signal generated from the neutrophils stimulated by *H. pylori* extract. The intensity was compared by measuring the height of DMPO-OH signals at 6 min after stimulation by *H. pylori* extract. Each bar was expressed as a ratio to the value in the absence of rebamipide (percentage of control). The increased intensity of the DMPO-OH signal obtained from *H. pylori* extract-stimulated neutrophils was reduced by coincubation with rebamipide at final concentrations of 10^{-5} or 10^{-6} M as indicated by the closed bar. In addition, to focus on the inhibitory effect of rebamipide on AOS production from *H. pylori* extract-stimulated neutrophils, without evaluating their scavenging characteristics, neutrophils were pretreated with rebamipide, washed, and used in the EPR spin-trapping method. As shown by the open bar, the intensity of DMPO-OH signal from neutrophils exposed to 10^{-5} or 10^{-6} M rebamipide prior to adding the *H. pylori* extract was significantly reduced compared with that from neutrophils not preincubated with rebamipide. DMPO-OH signals could not be detected from unstimulated neutrophils, regardless of rebamipide incubation.

DISCUSSION

We observed that rebamipide, an antiulcer agent, significantly prevented the surface expression of CD18 on neutrophils and neutrophil adherence to endothelial cells, both of which were induced by *H. pylori* extract. The CD11/CD18 glycoprotein complex expressed by activated neutrophils has been shown to participate in neutrophil adherence to endothelial cells in a variety of *in vivo* and *in vitro* models of inflammation (14, 15). In previous studies, we found that a water extract of *H. pylori*, obtained from a patient with asymptomatic gastritis, induced the surface expression of CD11b/CD18 glycoproteins on neutrophils (3). This resulted in the adherence and transendothelial migration of neutrophils both *in vitro* and *in vivo* (3). CD11b/CD18 glycoprotein is stored within specialized granules of resting neutrophils and,

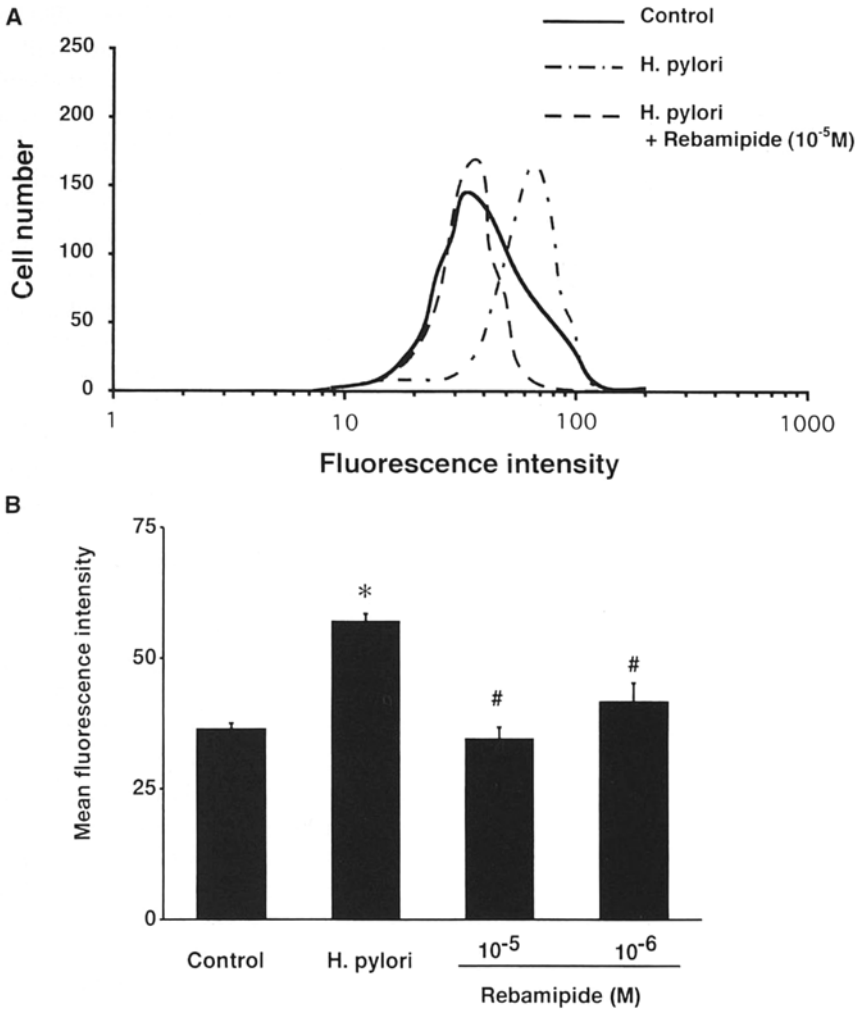

Fig 2. Effect of rebamipide on the CD18 surface expression of neutrophils treated with *H. pylori* extract: (A) representative fluorescence pattern; (B) mean fluorescence intensity. Each value represents the mean ± SE of three experiments. $^{*}P < 0.05$ compared with control group without *H. pylori* extract and $^{\#}P < 0.05$ compared with *H. pylori* group without rebamipide.

when activated, is translocated to the cell surface by granule fusion (16). Neutrophil activation also triggers functional activation of preexisting cell surface CD11b/CD18, presumably through conformational and/or topological alterations (17). We recently found that the mean concentration of rebamipide in the gastric mucosa reached 2×10^{-4} M within 2 hr of its oral administration at a dose of 100 mg, which is used in treating patients with gastric mucosal injury (18). This finding supports the concept that neutrophils and endothelial cells in the human gastric mucosa can be exposed to 10^{-5} or 10^{-6} M rebamipide, the concentrations used in the present study. While the exact molecular mechanisms through which rebamipide inhibits CD18 surface expression on neutrophils are unclear, the present results indicate that rebamipide may protect against gastroduodenal inflammation by inhibiting the infiltration of neutrophils into the extravascular space elicited by infection of *H. pylori.* Recent studies suggest that L-selectin and sialyl Lewis X contribute to the rolling and weak adhesion of neutrophils (19). The effects of *H. pylori* extract and rebamipide on the expression of L-selectin and sialyl Lewis X need to be further investigated to resolve the mechanisms via which rebamipide inhibits CD18 surface expression on the neutrophils.

Our present finding that the coincubation of rebamipide with neutrophils and *H. pylori* extract decreased the DMPO-OH signal intensities obtained from neutrophils indicates that this agent can scavenge AOS and/or inhibit the production of AOS from neutrophils stimulated by *H. pylori* extract. Our pre-

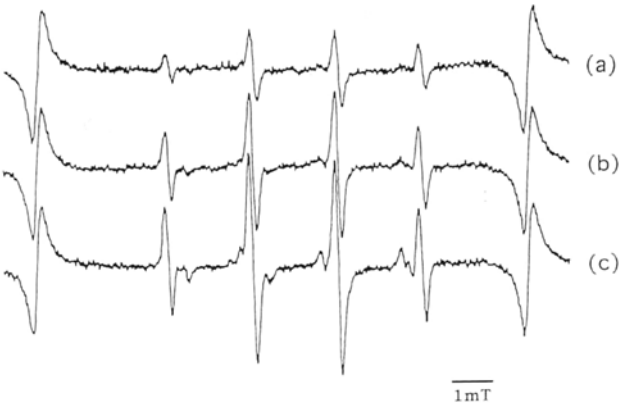

Fig 3. Electron paramagnetic resonance (EPR) signals of the DMPO-OH spin adduct obtained from neutrophils stimulated by *H. pylori* extract. Tracing a: 1 min after the addition of *H. pylori* extract; tracing b, 6 min after the addition of *H. pylori* extract; and tracing c, 11 min after the addition of *H. pylori* extract. These EPR tracings show one of the three experiments.

vious studies showed that rebamipide could scavenge hydroxyl radicals generated from the hydrogen peroxide–ferrous sulfate system (6). The present study also revealed a significant reduction in the intensity of the DMPO-OH signal from neutrophils that were washed after being pretreated with rebamipide. This finding suggests that rebamipide may inhibit the production of AOS by attaching to the cell membrane or passing into the cell. This evidence is consistent with that in previous reports showing that rebamipide reduces the oxidant production from neutrophils elicited by the suspension of *H. pylori* (20) or by formyl-methionyl-leucyl-phenylalanine (13). Our data suggest that rebamipide can both scavenge AOS and inhibit the production of AOS from *H. pylori*-stimulated neutrophils. AOS produced by neutrophils have been implicated in the pathogenesis of *H. pylori*-associated gastric mucosal injury (21). In addition, AOS and lipid peroxidation cause microvascular and parenchymal disorders in other forms of gastric mucosal injury induced by anoxia/reoxygenation or nonsteroidal antiinflammatory drugs (22, 23). Therefore, rebamipide offers a potential for protection against the neutrophil-associated gastric mucosal injury by scavenging AOS and inhibiting their production from neutrophils.

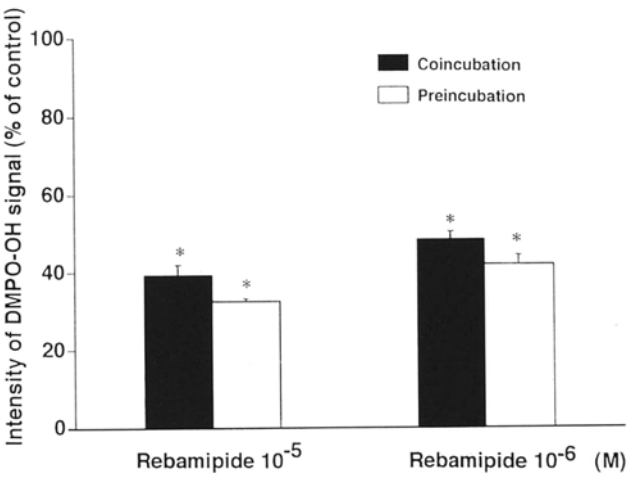

Fig 4. Effect of rebamipide on the intensity of DMPO-OH signals obtained from neutrophils stimulated by *H. pylori* extract. Neutrophils were coincubated or preincubated with rebamipide at final concentrations of 10^{-5} or 10^{-6} M. Each value represents the mean ± SE of three experiments. $^{*}P < 0.05$ compared with control group without rebamipide.

In summary, the present results demonstrate that rebamipide, a unique antiulcer compound, inhibits both the surface expression of CD18 on neutrophils and the production of AOS from neutrophils that were elicited by *H. pylori* extract. While rebamipide has no direct effect on the clearance and eradication of *H. pylori*, it may be useful in preventing the gastric mucosal inflammation associated with this organism.

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