Rebamipide Inhibits Ceramide-Induced Interleukin-8 Production in Kato III Human Gastric Cancer Cells

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ABSTRACT
Helicobacter pylori adheres to gastric epithelial cells and stimulates interleukin-8 production. Ceramide, a lipid second messenger, has become known as an important mediator of some actions of several cytokines. We have recently reported that H. pylori-dependent ceramide production may activate nuclear factor-κB and mediate interleukin-8 expression in human gastric cancer cell lines. In this study, we evaluated the effect of rebamipide, an antigastritis and antiulcer agent, on H. pylori-dependent ceramide production and subsequent interleukin-8 expression in Kato III cells. Rebamipide inhibited ceramide-induced interleukin-8 expression in a dose-dependent manner. Rebamipide decreased the ceramide-induced increase of the interleukin-8 mRNA level as assessed by Northern blotting. Rebamipide suppressed interleukin-8 gene transcription and nuclear factor-κB-dependent transcriptional activity as assessed by luciferase assay. Rebamipide inhibited the ceramide-induced degradation of IκB-α (a major cytoplasmic inhibitor of nuclear factor-κB), further supporting that rebamipide inhibits the activation of nuclear factor-κB. Rebamipide also inhibited the ceramide-dependent activation of mitogen-activated protein kinases. Furthermore, rebamipide significantly attenuated the H. pylori-dependent increase in the intracellular ceramide level. These results suggest a novel mechanism by which rebamipide may protect against the mucosal inflammation associated with H. pylori infection.

Helicobacter pylori is regarded as an important pathogen in gastric and duodenal inflammation (Graham, 1989; Marshall, 1994). Although the pathological mechanisms involved in H. pylori-induced mucosal inflammation are not completely known, there is accumulating evidence that activated neutrophils play an important role (Blaser, 1992; Yoshida et al., 1993). Gastric epithelial cells produce and secrete interleukin (IL)-8, a potent chemotactic and activating factor for neutrophils, in response to this bacterium (Ben-Baruch et al., 1995). The expression of the IL-8 gene is primarily controlled at the transcriptional level. Nucleotide sequence analysis of the 5′ regulatory region of the IL-8 gene has revealed potential binding sites for several transcription factors, including nuclear factor-κB (NF-κB), NF-IL6, and activator protein-1 (AP-1) (Mukaida et al., 1994). We and others have shown that the H. pylori-induced transcription of IL-8 gene requires the activation of NF-κB and, to a lesser extent, AP-1 in human gastric epithelial cells (Aihara et al., 1998; Masamune et al., 1999). NF-κB is known to be involved in the control of the cytokine-induced expression of many immune and inflammatory-response genes (Grilli et al., 1993). NF-κB is localized in the cytoplasm in an inactive form where it is associated with the inhibitory protein IκB-α. IκB-α retains the NF-κB complex in the cytoplasm and inhibits DNA binding. Various NF-κB activators cause phosphorylation and degradation of the inhibitory protein IκB-α, allowing NF-κB to be released from the complex. NF-κB then moves to the nucleus where it binds to the DNA recognition site and mediates gene transcription (Grilli et al., 1993).

Sphingolipid metabolites such as ceramide (Hannun, 1994; Kolesnick and Golde, 1994) have been implicated as important signaling molecules in the regulation of diverse cellular functions. Several extracellular stimuli, including tumor necrosis factor-α (TNF-α), IL-1β, and interferon-γ have been shown to activate sphingomyelinases, resulting in sphingomyelin hydrolysis and the generation of ceramide (termed the “sphingomyelin-ceramide pathway”). In turn, ceramide mediates the effects of these agonists on cell growth, differentiation, apoptosis, and inflammatory responses (Hannun,
Experimental Procedures

Materials. C₂₄-ceramide (N-acetylphosphosine, a cell-permeable ceramide analog) and sn-1,2-diacylglycerol kinase were from Calbiochem (San Diego, CA). [α-32P]dCTP and [γ-32P]ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Antibodies used for Western blotting were from New England Biolabs (Beverly, MA). PD98059 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Rebamipide (2-[4-chlorobenzoylamino]-3-[2(1H)-quinolinon-4-yl]propionic acid) (Uchida et al., 1985), provided by Otsuka Pharmaceutical Co. (Tokyo, Japan), was dissolved and stored at 200 mM in dimethyl sulfoxide. All other reagents were from Sigma Chemical Co. (St. Louis, MO) unless specifically described.

H. pylori and Epithelial Cell Culture. H. pylori (caga¹ strain 43504; American Type Culture Collection, Rockville, MD) was grown on brucella agar (Difco, Detroit, MI) plates supplemented with 7% fetal bovine serum and incubated at 37°C in a microaerophilic environment. After an overnight incubation, bacteria were harvested and transferred to brucella broth supplemented with 7% fetal bovine serum. Following another overnight incubation, the bacterial suspension was pelleted by centrifugation and resuspended in F-12 HAM medium (Life Technologies, Rockville, MD), and bacterial numbers were standardized by optical density measurement at 600 nm. The motility of H. pylori in cultures was confirmed by a phase-contrast microscopy before experimental use.

Kato III human gastric cancer cells (American Type Culture Collection) were grown in F-12 Ham medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and streptomycin. Cells were grown as a monolayer on 96-well flat-bottom plates (Costar, Acton, MA) for enzyme-linked immunosorbent assay, or on tissue culture dishes for other experiments. On the day of the experiments, the cells were refed with fresh serum- and antibiotic-free medium. In experiments involving rebamipide or inhibitors of mitogen activated protein (MAP) kinases, it was usually added to the cultures 4 h prior to the addition of C₂₄-ceramide (final concentration at 10 μM) or H. pylori (at 5 × 10⁵ colony forming units/ml) unless specifically indicated.

Enzyme-Linked Immunosorbent Assay. After a 24-h incubation, culture supernatants were harvested and stored at −80°C until the measurement. IL-8 levels in the supernatants were measured by enzyme-linked immunosorbent assay (Endogen, Woburn, MA) according to the manufacturer’s instructions.

Northern Blot Analysis. Following a 4 h-incubation, total RNA was isolated using an RNeasy total RNA preparation kit (Qiagen, Chatsworth, CA). Ten micrograms of total RNA was separated on a 1% agarose-2.2 M formaldehyde gel, and transferred to a nylon membrane filter (Amersham Pharmacia Biotech). Blots were hybridized for 16 h at 42°C to the 32P-labeled DNA probes of IL-8 and β-actin generated by polymerase chain reaction as previously described (Masamune et al., 1999). After the hybridization, the filter was washed twice with 2× standard saline citrate (3 M NaCl and 0.3 M sodium citrate) and 0.1% SDS at room temperature for 10 min followed by two washes with 0.2× standard saline citrate and 0.1% SDS at 42°C for 30 min. The washed filter was subjected to the BAS 1500 system (Fuji Film, Tokyo, Japan).

Luciferase Assay. Luciferase expression vectors containing the minimally essential promoter region of the IL-8 gene (bp −133 to +44), and those containing two NF-κB consensus binding sites (Masamune et al., 1999) were kindly provided by Dr. Naofumi Muikaid (Kanazawa University, Kanazawa, Japan). For the luciferase assay, 1 × 10⁶ Kato III cells were transfected with 2 μg of each luciferase vector along with 40 ng of pRL-TK vector (Promega, Madison, WI) as an internal control, using lipofectAMINE reagent (Life Technologies). After 20 h, the cells were treated with rebamipide at the indicated concentrations for 4 h, and then were stimulated with C₂₄-ceramide in the presence of rebamipide. After another 24-h incubation, cell lysates were prepared using a Pico Gene kit (Toyo Ink Co., Tokyo, Japan), and the light intensities were measured using a model Lumat LB9507 luminescence reader (EG&G Berthold, Bad Wildbad, Germany).

Western Blot Analysis. Kato III cells were treated with C₂₄-ceramide or H. pylori for 30 min and lysed in SDS buffer (62.5 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) for 15 min on ice. The samples were then sonicated, heated to 100°C for 5 min, and centrifuged at 15,000 rpm for 5 min to remove insoluble cell debris. They were fractionated on a 10 to 20% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with a 5% (w/v) solution of dry milk in Tris-buffered saline (pH 7.4). The levels of activated, phosphorylated MAP kinases in the samples were determined using phosphospecific MAP kinase antibodies (extracellular signal-regulated kinase (ERK)1/2, c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 kinases) in a 1:1000 dilution. After incubation with a secondary antibody (goat anti-rabbit antibody, horseradish peroxidase conjugated), proteins were visualized using an ECL kit (Amersham Pharmacia Biotech). The levels of pan-MAP kinases and IκB-α were determined in a similar manner.

Ceramide Measurement. Following treatment with H. Pylori, lipids were extracted as previously described (Bligh and Dyer, 1959) and the ceramide content was quantified using diacylglycerol kinase as previously described (Masamune et al., 1996). Briefly, dried lipids were solubilized in 20 μl of octyl-β-D-glucoside/ cardiolipin solution (7.5% octyl-β-D-glucoside and 5 mM cardiolipin in 1 mM dihydroxyacetone/2-acetic acid) by ultrasonication. The reaction was carried out in a final volume of 100 μl containing the 20-μl sample solution, 50 mM imidazole HCl, 50 mM NaCl, 12.5 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, 6.6 μg of diacylglycerol kinase, and 1 mM [γ-32P]ATP for 30 min at 22°C. The major lipid products of their phosphorylation reaction were phosphatidic acid (from diacylglycerol) and ceramide-1-phosphate (from ceramide). They were completely resolved by thin-layer chromatography using chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v) as a solvent, and visualized by autoradiography. Ceramide-1-phosphate spots were identified by the comparison with the standard sample of ceramide phosphorylated under identical conditions and developed in parallel. Quantification of ceramide-1-phosphate was carried out using the BAS 1500 system and software provided with the instrument by the manufacturer.

Statistical Analysis. Differences between experimental groups were evaluated by the two-tailed unequal paired Student’s t test. A p value less than 0.05 was considered statistically significant.

Results

Rebamipide Inhibited C₂₄-Ceramide-Induced IL-8 Expression in Kato III Cells. We first examined the effects of rebamipide on IL-8 production in Kato III cells by enzyme-
linked immunosorbent assay. As previously reported (Masamune et al., 1999), C2-ceramide increased IL-8 production in a dose-dependent manner (Fig. 1A). C2-ceramide at 10 \( \mu \text{M} \) induced IL-8 production, representing a 9.4-fold increase compared with the unstimulated control. Rebamipide inhibited C2-ceramide-induced IL-8 expression in a dose-dependent manner (Fig. 1B). A statistically significant inhibition in IL-8 protein release was evident at as low as 10 \( \mu \text{M} \) rebamipide \((p < 0.05)\). The maximal effect was observed at 1 mM, where IL-8 expression was approximately 27% of the control value. In these studies, rebamipide was added to the culture medium using dimethyl sulfoxide as a vehicle, and the amounts of dimethyl sulfoxide used (0.5% at 1 mM rebamipide) did not affect cell viability, morphology, or IL-8 production (data not shown). We examined the effect of increasing the time of exposure on IL-8 production. Rebamipide treatment was started 24, 4, 2, and 1 h before, or at 0 and 1 h after C2-ceramide stimulation. The inhibitory effect of rebamipide was more evident in a time-dependent manner up to 4 h, but further enhancement of the effect was not observed even if the exposure time was extended to up to 24 h (Fig. 2). Rebamipide did not inhibit the C2-ceramide-induced IL-8 expression if it was added after the C2-ceramide treatment. Furthermore, washing out the rebamipide abolished the inhibition of C2-ceramide-induced IL-8 production, suggesting that rebamipide must be present during the incubation period of C2-ceramide stimulation to elicit the inhibitory effect. Rebamipide also inhibited the \( H. \text{pylori} \)-induced IL-8 expression. Rebamipide at 1 mM decreased IL-8 production to approximately 55% of the control (Fig. 1B).

Rebamipide Decreased the Level of IL-8 mRNA. We studied the effects of rebamipide on the IL-8 gene expression. We determined the IL-8 mRNA levels by Northern blot analysis. IL-8 mRNA expression was induced by treatment with 10 \( \mu \text{M} \) C2-ceramide, and it was inhibited in the presence of rebamipide in a dose-dependent manner (Fig. 3). These results were roughly consistent with the results of the enzyme-linked immunosorbent assay, and suggested that rebamipide decreased the steady-state level of IL-8 mRNA.

Rebamipide Inhibited Ceramide-Induced IL-8 Gene Transcription. The expression of IL-8 gene is primarily controlled at the transcriptional level (Mukaida et al., 1994). We next examined the effects of rebamipide on the C2-ceramide-induced IL-8 gene transcription. C2-ceramide markedly enhanced the luciferase activity in Kato III cells trans-
fected with luciferase expression vectors containing the minimally essential promoter region of the IL-8 gene (bp −133 to +44). Pretreatment of the cells with rebamipide decreased the induced luciferase activity (Fig. 4), suggesting that rebamipide inhibited the IL-8 gene transcription induced by C2-ceramide.

**Activation of NF-κB Was Inhibited by Rebamipide.**

We and others have shown that both ceramide- and *H. pylori*-induced transcription of IL-8 gene requires the activation of NF-κB and, to a lesser extent, AP-1 in human gastric epithelial cells (Aihara et al., 1997; Masamune et al., 1999). Because activation of NF-κB plays a central role in IL-8 gene transcription, we examined the effect of rebamipide on the activation of NF-κB. As shown in Fig. 5A, rebamipide suppressed the C2-ceramide-induced NF-κB-dependent luciferase activity, suggesting that rebamipide inhibited IL-8 gene transcription at least in part through the inhibition of NF-κB.

Rebamipide Inhibited the Ceramide-Induced Activation of MAP Kinases.

MAP kinases are a family of ubiquitous, highly conserved, cell signaling molecules (Robinson and Cobb, 1997). Upon activation by upstream kinases, MAP kinases phosphorylate downstream kinases and/or mediators, including transcription factors. MAP kinases can be activated by a wide variety of extracellular stimuli such as NF-κB activation. Phosphorylation and degradation of the inhibitory protein IκB-α, and subsequent dissociation of this protein from NF-κB are thought to be necessary for the activation (Grilli et al., 1993). We examined the effect of rebamipide on the degradation of IκB-α by Western blotting. Rebamipide inhibited the C2-ceramide-induced degradation of IκB-α, further supporting that rebamipide inhibited the activation of NF-κB (Fig. 5B).
growth factors, proinflammatory cytokines, and stresses. Three main classes of MAP kinases have been characterized: ERK1/2 (also known as p42/p44 kinases), JNK/SAPK, and p38 MAP kinases (Robinson and Cobb, 1997). H. pylori has been shown to activate these MAP kinases and the activation may be involved in the IL-8 expression (Keates et al., 1999; Naumann et al., 1999). We first examined the effects of inhibitors of MAP kinase pathways on H. pylori-induced IL-8 expression. We used PD98059, a specific inhibitor of MAP kinase activation, which prevents the activation of ERK1/2, and a selective p38 inhibitor, SB203580. In agreement with the previous reports (Keates et al., 1999; Naumann et al., 1999), H. pylori-induced IL-8 expression was partially blocked by PD98059 and SB203580 (Fig. 6A). Both inhibitors did not exhibit cytotoxicity up to 25 \( \mu \)M. PD98059 at 25 \( \mu \)M did not alter the activation of NF-\( \kappa \)B, JNK/SAPK, nor p38 MAP kinases (data not shown). SB203580 at 25 \( \mu \)M did not alter the activation of NF-\( \kappa \)B, JNK/SAPK, or ERK1/2 (data not shown). In addition, \( \text{C}_{2} \)-ceramide-induced IL-8 expression was partially blocked by these inhibitors (Fig. 6B). Rebamipide enhanced inhibitory effects of these inhibitors on IL-8 expression (Fig. 6).

We next examined the effect of rebamipide on the activation of MAP kinases by Western blotting using phospospecific antibodies. Activation of all three classes reportedly reached the maximal level after 30 min (Keates et al., 1999; Naumann et al., 1999). Both H. pylori and \( \text{C}_{2} \)-ceramide induced marked phosphorylation of MAP kinases after treatment for 30 min (Fig. 7). For ERK, two immunoreactive bands were evident, representing phosphorylated p44 (ERK1, upper band) and p42 (ERK2, lower band) MAP kinases. For JNK, two immunoreactive bands were again evident representing p54 (upper band) and p46 (lower band) MAP kinases. Rebamipide inhibited the activation of all three classes of MAP kinases induced by both H. pylori and \( \text{C}_{2} \)-ceramide (Fig. 7).

**Rebamipide Attenuated the H. pylori-Dependent Increase in the Intracellular Ceramide Level.** We previously reported that H. pylori increased the intracellular level of ceramide (Masamune et al., 1999). In Kato III cells, H. pylori infection induced statistically significant increases (approximately 2.7-fold) in the ceramide content that peaked at 60 min. Rebamipide inhibited the H. pylori-dependent increase in the intracellular level of ceramide in a dose-dependent manner (Fig. 8). Rebamipide at 1 mM decreased the H. pylori-dependent increase in the intracellular ceramide level by 59\% compared with the control in the absence of rebamipide.

**Discussion**

H. pylori infection induces the mucosal production of various cytokines in the host, especially IL-8, and the host-parasite interaction has an important role in the pathogenesis of gastric mucosal inflammation (Blaser, 1992; Yoshida et al., 1993). Indeed, gastric mucosal levels of IL-8 are increased and its activity correlates with the histological severity in patients with H. pylori gastritis (Ando et al., 1996). Inhibition of IL-8 production in gastric epithelial cells may reduce the inflammation of the gastric mucosa and may have a potential therapeutic application. Rebamipide, an antulcer and antiinflammatory agent, has several anti-inflammatory actions: it scavenges hydroxyl radicals (Naito et al., 1996) and inhibits the production of oxygen radicals by activated neutrophils (Ogino et al., 1992), neutrophil adhesion to endothelial cells, the expression of adhesion molecules (Suzuki et al., 1994), and IL-8 production by H. pylori in human gastric
Rebamipide appears to exert its inhibitory effect on IL-8 expression at the transcriptional level. Northern blot analysis revealed that rebamipide suppressed the C2-ceramide-induced increase of the steady-state level of IL-8 mRNA. Furthermore, we demonstrated the inhibitory effect of rebamipide on the IL-8 gene transcription using an IL-8 promoter-reporter gene construct. Because activation of NF-κB plays a central role in the expression of IL-8 and rebamipide inhibited the ceramide-induced NF-κB-dependent transcriptional activity, it seems likely that rebamipide inhibited IL-8 expression at least in part through the attenuated activation of NF-κB. Phosphorylation and degradation of the inhibitory protein IκB-α, and subsequent dissociation of this protein from NF-κB are thought to be necessary for the activation (Grilli et al., 1993). Rebamipide inhibited the degradation of IκB-α induced by H. pylori and C2-ceramide, further supporting the inhibitory effects of rebamipide on NF-κB activation. It has been shown that rebamipide suppressed NF-κB activation and subsequent IL-8 expression induced by H. pylori, IL-1β, and TNF-α (Aihara et al., 1998). In addition, rebamipide inhibited C2-ceramide-induced NF-κB activation in the present study, suggesting that rebamipide may interplay with the common pathways leading to NF-κB activation. The importance of the oxidation state for NF-κB activation has been demonstrated (Menon et al., 1993). Indeed, hydrogen peroxide, a reactive oxidant, activates NF-κB and the activation is repressed by antioxidants such as N-acetylcysteine in human gastric epithelial cells (Shimada et al., 1999). The chemical basis for the inhibitory effect of N-acetylcysteine seems to lie in the oxygen radical-scavenging effect of the thiol groups. Pyrrolidine dithiocarbamate, a proven free radical scavenger, also potently inhibits the activation of NF-κB and/ or NF-κB interaction with its upstream regulatory binding site, thereby preventing NF-κB-mediated gene transcription (Muñoz et al., 1996). Yoshida et al. (1996) reported that rebamipide could scavenge active oxygen species and inhibit superoxide production in human neutrophils stimulated by H. pylori extract. It has been also demonstrated that rebamipide inhibits lipid peroxidation and oxidant-mediated activation of NF-κB, thereby decreasing IL-8 production by H. pylori (Kim et al., 2000). The alteration of the oxidation state by rebamipide through its oxygen radical-scavenging effect in gastric epithelial cells may, therefore, account for the inhibition of NF-κB activation and the subsequent IL-8 expression. Since NF-κB binding sites are found in the promoters of a variety of proinflammatory or immune-response genes, including cytokines, acute-phase reactant proteins, and adhesion molecules (Grilli et al., 1993), it would be of interest to examine whether the inhibition of NF-κB activation in gastric epithelial cells by rebamipide could inhibit the expression of multiple pathophysiologically relevant agents.

In this study, we have shown that rebamipide inhibited the ceramide generation induced by H. pylori. As previously reported (Aihara et al., 1998), rebamipide at the concentrations used in this study did not affect the growth of H. pylori in vitro; thus, direct cytotoxic effects could be excluded. It has been shown that rebamipide inhibits the adhesion of H. pylori to human gastric cancer cell lines (Hayashi et al., 1998). This may at least in part account for the inhibition of the H. pylori-dependent ceramide generation by rebamipide. Another possibility is that rebamipide may directly inhibit the enzymatic activity of sphingomyelinase. Cytokine-induced sphingomyelin hydrolysis and the generation of ceramide have been shown to be redox-sensitive; antioxidants such as N-acetylcysteine and pyrrolidine dithiocarbamate potently inhibited the cytokine-induced degradation of sphingomyelin to ceramide (Singh et al., 1998). Furthermore, Liu et al. (1998) reported that glutathione inhibited the TNF-α-induced activation of neutral sphingomyelinase in human acute lymphoblastic leukemic MOLT-4 and human mammary carcinoma MCF-7 cells in vitro. Rebamipide has been shown to increase the glutathione level in the colon in an acetic acid-induced colitis model (Sakurai et al., 1998). Although we did not directly measure the effect of rebamipide...
on H. pylori-induced sphingomyelinase activity, it is possible that rebamipide directly suppressed sphingomyelinase activity through the alterations in the level of glutathione. Experiments to test this hypothesis are underway in our laboratory.

Recent studies have shown that H. pylori activates MAP kinases in human gastric cancer cell lines (Keates et al., 1999; Naumann et al., 1999). In this study, we have shown that rebamipide inhibited the activation of three classes of MAP kinases (JNK/SAPK, ERK1/2, and p38 MAP kinases). MAP kinases are key elements in the regulation of cellular responses to external inflammatory and proliferative signals (Robinson and Cobb, 1997). Several studies have implicated MAP kinases as upstream mediators of NF-κB and AP-1 activation and cytokine gene expression, including IL-8 (Robinson and Cobb 1997). Different strains (cag + and cag− strains) of H. pylori vary in their ability to activate MAP kinase pathways in gastric epithelial cells; H. pylori cag− strains are more potent than cag + strains in inducing MAP kinase activation. Activation of MAP kinases has been suggested to play a role in the H. pylori-induced expression of IL-8 in gastric epithelial cells, and the differential activation of MAP kinases is a possible mechanism for the strain-specific difference in IL-8 in gastric epithelial cells.

References


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