B.L. SLOMIANY, J. PIOTROWSKI, A. SLOMIANY

EFFECT OF EBROTIDINE ON GASTRIC MUCOSAL INFLAMMATORY RESPONSES TO HELICOBACTER PYLORI LIPOPOLYSACCHARIDE

Research Center, University of Medicine and Dentistry of New Jersey, Newark, USA

*Helicobacter pylori* lipopolysaccharide is a primary virulence factor responsible for eliciting acute mucosal inflammatory responses associated with *H. pylori* infection. In this study, we applied the animal model of *H. pylori* lipopolysaccharide-induced acute gastritis to assess the effect of antiulcer agent, ebrotidine, on the gastric mucosal inflammatory responses by analyzing the interplay between the activity of a key apoptotic caspase, caspase-3, epithelial cell apoptosis, and the expression of inducible nitric oxide synthase (NOS-2).

**Methods:** Rats, pretreated twice daily with ebrotidine at 100 mg/kg, or the vehicle, were subjected to intragastric application of *H. pylori* lipopolysaccharide at 50 µg/animal, and after 4 additional days on the antiulcer drug or vehicle regimen their mucosal tissue used for histologic assessment, assays of epithelial cells apoptosis, and the measurements of caspase-3 and NOS-2 activities.

**Results:** In the absence of antiulcer agent, *H. pylori* lipopolysaccharide induced acute reaction characterized by the inflammatory infiltration of the lamina propria, hyperemia, and epithelial hemorrhage. This was accompanied by an 11.2-fold increase in epithelial cell apoptosis, a 6.5-fold increase in mucosal expression of NOS-2, and a 5.4-fold increase in caspase-3 activity. Treatment with H2-receptor antagonist ebrotidine, also known for its gastroprotective effects, produced a 50.9% reduction in the extent of mucosal inflammatory changes elicited by *H. pylori* lipopolysaccharide and an 82.5% decrease in the epithelial cells apoptosis, while the activity of caspase-3 decreased by 33.7% and that of NOS-2 showed a 72.8% decline.

**Conclusions:** The findings implicate caspase-3 involvement in gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide, and point towards participation of NOS-2 in the amplification of the cell death-signaling cascade. Our study also demonstrate that ebrotidine exerts modulatory effect on the *H. pylori*-induced mucosal inflammatory responses by interfering with the events propagated by NOS-2 and caspase-3.

**Key words:** *Helicobacter pylori*, lipopolysaccharide, acute gastritis, NOS-2, caspase-3, ebrotidine.
INTRODUCTION

Infection with Helicobacter pylori is a primary factor in the etiology of gastric disease, and the relationship between the bacterium and gastric mucosal inflammatory changes that characterize gastritis and duodenal ulcers is well established (1—4). The product of particular significance to the virulent action of H. pylori is its cell wall lipopolysaccharide (5—8). The pathogenic effects of H. pylori lipopolysaccharide are manifested by a marked up-regulation in gastric mucosal proinflammatory cytokine expression, excessive nitric oxide generation, repression of regulatory cytokine production, and abrogation of the processes associated with cell cycle progression and cellular proliferation (5, 8—10). Other mucosal responses elicited by the lipopolysaccharide involve stimulation transcriptional factor NFκB nuclear translocation, and the disturbances in nitric oxide synthase (NOS) activity responsible for the production of NO (11—14).

Of the three characterized NOS isozymes, the two constitutively expressed forms are Ca$^{2+}$-dependent and provide NO pulses for a fine modulation of the cellular processes, including those involved in the inhibition of apoptogenic signals (14—18). The third isoform, known as inducible NOS or NOS-2, is Ca$^{2+}$-independent and its expression undergoes induction in response to proinflammatory cytokines and bacterial lipopolysaccharide (12, 13). The activation of NOS-2 has been identified as a culprit of transcriptional disturbances leading to apoptosis (14, 16, 17, 19), and the sustained NO generation following NOS-2 induction by lipopolysaccharide is recognized as a major cause of neuronal death during trauma, hypotension during endotoxic shock, and degenerative changes observed in the pathogenesis of liver disease (12, 13, 16).

The data obtained with other Gram-negative bacteria indicate that the induction of apoptosis by lipopolysaccharide is mediated by cytokines of the TNF family and involves the transmembrane receptor, TNFR1 (12, 13). The ligand binding induces trimerization of the receptor molecules, eliciting recruitment to its cytosolic face of several death domain-containing adaptor proteins that interact with caspase zymogens causing their autocatalytic activation (17, 18). The activation of caspases is recognized as an irreversible commitment to the execution phase of apoptosis characterized by cytoplasmic shrinkage, cleavage of cytoskeletal and nuclear proteins, and DNA fragmentation (17, 18). An extensive apoptotic DNA fragmentation and up-regulation of proinflammatory cytokine expression are also characteristic feature of gastric mucosa exposed to H. pylori lipopolysaccharide and in patients with H. pylori-associated gastritis (6, 8, 20).
Using animal model of *H. pylori* lipopolysaccharide-induced acute gastritis (6, 8), in this study, we assessed the effect of antiulcer agent, ebrotidine, on gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide by analyzing the interplay between activity of a key executioner caspase, caspase-3, apoptotic DNA fragmentation, and the expression of NOS-2.

**MATERIALS AND METHODS**

**Animal preparation**

The study was conducted with Sprague-Dawley rats weighing 180 to 200 g, and cared for by the professional personnel of the Research Animal Facility. The animals were maintained on a regular chow diet, and deprived of food 24 h before the experimentation. Water was withheld for 2 h before the procedure. All experiments were conducted with groups of eight animals per treatment. The animals received twice daily for 3 consecutive days the intragastric pretreatment with either ebrotidine at 100 mg/kg or the vehicle, and were then subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide at 50 μg per animal, and maintained on the drug or vehicle regimen for an additional 4 days (10). The rats in each group were killed 16 h after the last treatment, their stomachs dissected, and the mucosal tissue used for histologic assessment, quantization of gastric epithelial cell apoptosis, and the assays of the mucosal expression of caspase-3 and NOS-2 activity.

**Mucosal histology**

The sections of gastric mucosa were cut into 4-μm strips, fixed in 10% buffered formalin, and stained with hematoxylin and eosin (6). The morphological pattern of gastritis was graded in accordance with the Sydney system (22), and the changes in mucosal histology were quantified on the basis of the scoring system of Rauws et al. (22), as described earlier (6). The intact mucosa with no infiltration was graded as 0; the density of the inflammatory infiltrate (neutrophils, granulocytes, and plasma cells) in the lamina propria, 0—2; the density of polymorphonuclear leukocytes in the lamina propria, 0—3; the presence of intraepithelial polymorphonuclear leukocytes, 0—3; and superficial erosions, 0—2.

**Apoptosis assay**

Quantitative measurements of apoptosis was carried out with epithelial cells prepared from gastric mucosal scrapings (15). The cells were incubated in the lysis buffer in accordance with the manufacturer's (Boehringer Mannheim) instruction, centrifuged, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments reacted in the microtiter wells with immobilized anti-histone antibody. Following washing, the retained complex was reacted with anti-DNA peroxidase, and probed with ABTS reagent for spectrophotometric quantitization (15).
**NOS-2 activity assay**

Inducible nitric oxide synthase activity of gastric mucosa was measured using a NOS-detect kit (Stratagene). The individual specimens of gastric mucosa were homogenized in a sample buffer containing 10 mM EDTA, and centrifuged at 800×g for 10 min (8). The aliquots of the resulting supernatants were incubated for 30 min at 25°C in the presence of 50 μCi/μl L-[2,3,4,5-3H]arginine, 10 mM NADPH, 5 μM tetrahydrobiopterin, and 50 μM Tris-HCl buffer, pH 7.4, in a final volume of 250 μl. The reaction was terminated by adding to each sample a 0.4 ml of stop buffer followed by 0.1 ml of Dowex-50W (Na+) resin. The mixtures were transferred to spin cups, centrifuged, and the formed L-[3H]citrulline contained in the flow through was quantitated by scintillation counting (15).

**Caspase3 activity assay**

Caspase—3 activity measurements were carried out with gastric epithelial cells using a QuantiZyme assay system (Biomol Res. Lab., Inc.). The epithelial cells, prepared from gastric mucosal scrapings (15), were incubated at 4°C with the lysis buffer according to the manufacturer’s instruction, and the lysates were centrifuged at 10,000×g for 10 min. The aliquots of the resulting cytosolic fraction, diluted with the reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM EDTA, and 10% glycerol) to contain 30 μg protein, were incubated in the microtitrator wells with 50 μM of DEVD-pNA (Asp-Glu-Val-Asp-p-nitroanilide) substrate for 1 h at 37°C, and the caspase—3 activity measured spectrophotometrically (8).

**H. pylori lipopolysaccharide**

*H. pylori* ATCC No. 4350 clinical isolate was used for lipopolysaccharide preparation (6). The bacterium was cultured on Brucella broth supplemented with 10% horse serum and 5% tryptone soya in a microaerophilic atmosphere. The organisms were maintained at 37°C yielding, after 72 h, a viable count of 5×10^7 CFU/ml (23). The bacterium was harvested, washed with water, treated with ethanol and acetone, dried and homogenized with liquid phenol-chloroform-petroleum ether (6). The resulting suspension was centrifuged, and the lipopolysaccharide contained in the supernatant was precipitated with water, washed with 80% phenol solution, and dried with ether. The dry residue was dissolved in a small volume of water at 45°C, centrifuged at 100,000×g for 4 h, and the resulting lipopolysaccharide sediment subjected to lyophilization (6).

**Data analysis**

All experiments were carried out in duplicate, and the results are expressed as the means ± SD. The significance level was set at p > 0.05. The tests were performed using Soft Stat, STATISTICA, software. The protein content of samples was measured with BCA protein assay kit (Pierce), and the antiulcer agent, ebrotidine was, kindly donated by Ferrer Internacional, SA., Barcelona, Spain.
RESULTS

The effect of antiulcer agent, ebrotidine, on the course of events associated with gastric mucosal inflammatory reaction to *H. pylori* infection was assessed in the animal model, using rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (6). The results of histologic examination revealed that the lipopolysaccharide at 50 μg per animal produced within 4 days a pattern of inflammatory responses characterized by the infiltration of lamina propria with lymphocytes and plasma cells, edema, hyperemia, and epithelial hemorrhage extending from the lamina propria to the surface of the mucosa (Fig. 1A, B). Treatment with H2-receptor antagonist ebrotidine, also known for its gastroprotective effects, led to a marked reduction in the severity pattern of mucosal inflammatory changes caused by *H. pylori* lipopolysaccharide (Fig. 1C). In the absence of the treatment, the mean grade of the mucosal pathologic condition caused by *H. pylori* lipopolysaccharide and quantitated according to the scoring system developed by Rauws et al. (22) was 5.3, while that of the animals treated with ebrotidine showed a 50.9% reduction in the severity of changes (mean score 2.6).

The results of apoptotic DNA fragmentation assays conducted with the epithelial cells isolated from gastric mucosa during *H. pylori* lipopolysaccharide-induced inflammatory reaction are summarized in Fig. 2. The data obtained revealed that comparing to the controls, the animals subjected to *H. pylori* lipopolysaccharide application produced an 11.2-fold increase in gastric epithelial cells DNA fragmentation. On the other hand, the group treated with ebrotidine showed an 82.5% reduction in the extent of epithelial cell apoptosis.

The data on the expression of gastric mucosal NOS-2 activity during *H. pylori* lipopolysaccharide-induced mucosal inflammatory reaction are shown in Fig. 3. In the absence of antiulcer agents, the lipopolysaccharide evoked a 6.5-fold increase in gastric epithelial expression of NOS-2 activity over that of the controls. Treatment with ebrotidine reduced the lipopolysaccharide-induced increase in NOS-2 activity level by a 72.8%.

The expression of gastric mucosal caspase-3 activity in response to surface epithelial application of *H. pylori* lipopolysaccharide in the absence and the presence of treatment with ebrotidine is presented in Fig. 4. The assays established the mean value for caspase-3 activity in the controls at 3.4 pmoI/mg protein, while that in gastric epithelial cells following lipopolysaccharide application reached the mean value of 18.3 pmoI/mg protein. Treatment with ebrotidine caused a 33.7% decline in gastric epithelial level of caspase-3 activity.
Fig. 1. Effect of ebrotidine treatment on the histology of rat gastric mucosa elicited by the intragastric surface epithelial application of *H. pylori* lipopolysaccharide. A — Normal gastric mucosa. B — Gastric mucosa 4 days after the intragastric surface epithelial application of *H. pylori* lipopolysaccharide in the absence of ebrotidine. C — In the presence of treatment with ebrotidine. (Original magnification, × 200).
Fig. 2. Effect of treatment with ebrotidine on gastric epithelial cell apoptosis in rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group. *P < 0.05 compared with that of the LPS.

Fig. 3. Effect of treatment with ebrotidine on the expression of nitric oxide synthase-2 (NOS-2) activity in gastric mucosa of the rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group. *P<0.05 compared with that of the LPS.
Fig. 4. Effect of treatment with ebrotidine on the expression of caspase—3 activity in gastric epithelial cells of the rats subjected to intragastric surface epithelial application of *H. pylori* lipopolysaccharide (LPS). Values represent the means ± SD of duplicate analyses performed on 8 animals in each group. *P* < 0.05 compared with that of the LPS.

**DISCUSSION**

Ebrotidine is a new H2-receptor antagonist with antisecretory potency comparable to that of ranitidine (24, 25), combining acid suppressant activity with remarkable gastroprotective and anti-*H. pylori* properties (5, 24, 25). Moreover, the successful ulcer therapy with ebrotidine evokes a significant increase in the *H. pylori* aggregating capacity of gastric mucin, the agent exhibits a strong inhibitory effect on the *H. pylori* urease activity, and shows the ability to reverse the impairment caused by *H. pylori* lipopolysaccharide in feedback inhibition of gastrin release by somatostatin (5, 7, 23, 26). As the primary virulence factor of *H. pylori* that elicits mucosal inflammatory responses that characterize gastritis is its cell wall lipopolysaccharide (6, 10), in this study, we investigated the effect of ebrotidine on the interplay between the extent of mucosal pathology, epithelial cell apoptosis, activity of a key apoptotic caspase, caspase-3, and the mucosal expression of NOS-2.

The results revealed that gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide, in the absence of ebrotidine treatment, show a pattern resembling that of acute gastritis (6), and are accompanied by an 11.2-fold enhancement in epithelial cell apoptosis, 5.4-fold increase in caspase-3
activity, and a 6.5-fold induction in mucosal expression of NOS-2. Treatment with ebrotidine elicited a 50.9% reduction in the extent of mucosal inflammatory changes caused by H. pylori lipopolysaccharide, and this effect of ebrotidine was reflected in an 82.5% decrease in epithelial cell apoptosis, a 72.8 decline in NOS-2, and a 33.7% reduction in caspase-3 activity. These findings, together with the earlier results indicating that H. pylori as well as its cell wall lipopolysaccharide cause up-regulation of TNF-α and other proinflammatory cytokine expression (10, 27, 28), point toward the H. pylori lipopolysaccharide as a trigger initiating gastric mucosal inflammatory events that lead to caspase cascade activation and apoptotic death.

The caspase family of cysteine proteases consists of 12 members, of which caspases 8, 9 and 3 are situated at pivotal junctions in apoptosis pathways (17, 29). All caspases exist in the cytoplasm as inert precursors or procaspases, which in response to apoptotic signals undergo proteolytic cleavage adjacent to aspartate residues (17). The implementation of the apoptotic program requires the participation of two classes of caspases, the initiator caspases with long N-terminal prodomains (caspases-8, -9, and -10) which activate the executioner caspases with short N-terminal prodomains (caspases-3, -6, and -7), that in turn cleave the targeted intracellular substrates. Different initiator caspases mediate distinct sets of signals. The activation of caspase-8, positioned to respond to extracellular apoptosis-inducing ligands, requires association with FADD (Fas-associated death domain) through the death effector domain, and this adaptor-mediated oligomerization brings procaspases molecules into close proximity to allow autocatalytic activation (17, 18, 30). On the other hand, caspase-9 is positioned at the apex of in the apoptotic signaling cascade activated by the release of cytochrome c. The activation of procaspase-9 occurs in response to the alteration in mitochondrial membrane permeability and the escape of cytochrome c from mitochondria, which, along with dATP, binds to APAF-1(apoptotic protease activating factor-1), inducing its oligomerization and association through the CARD (caspase recruitment domain) with procaspase-9 (29—31).

Thus activated initiator caspases 8 and 9 then activate the pivotal executioner caspase, caspase-3, which lacks the long N-terminal prodomain required for the recruitment of APAF-1 complex (18, 29—31). The importance of caspase-3 to the apoptotic process is underscored by a recent finding that this caspase liberates a DNase termed CAD (caspase- activating DNase) from an inhibitor of CAD (ICAD) by cleaving the ICAD protein, thus leading to DNA degradation (29). The fact that gastric mucosal responses to H. pylori lipopolysaccharide were reflected by a marked increase (5.4-fold) in caspase-3 activity supports the
notion that the apoptotic events elicited in mucosa by the lipopolysaccharide display a pattern of apoptogenic signal propagation consistent with that of type 1 TNF receptor (TNFR1) engagement.

Since the executioner caspases, including caspase-3, remain under the regulatory control of nitric oxide and since lipopolysaccharide of other Gram-negative bacteria cause the induction of NOS-2 (11—13, 32), it was of interest to examine the effect of H. pylori lipopolysaccharide on the interplay between the gastric mucosal caspase-3 activity and the expression of NOS-2. The results revealed that the mucosal inflammatory responses to H. pylori lipopolysaccharide associated with up-regulation of caspase-3 activity were manifested in a massive induction in NOS-2, while the reduction in the extent of inflammatory changes achieved with ebrotidine treatment was also reflected in a decline in the mucosal expression of NOS-2. Thus the observed induction of NOS-2 by H. pylori lipopolysaccharide may be an important factor in H. pylori cytotoxicity associated with mucosal inflammatory conditions that characterize gastritis in patients infected with this bacterium (2, 4). Indeed, the up-regulation in NOS-2 expression associated with infiltrating macrophages and the production of proinflammatory cytokines has been implicated in the pathogenesis of degenerative diseases, such as stroke, ischemic and traumatic injury, and stress ulcers (11—13, 33).

Although the exact molecular mechanism of the lipopolysaccharide-mediated events is not clear, the available data point toward essential role of proteins of the NFκB/Rel transcription factor family (12, 13, 34). These proteins have been shown to be essential for the induction of NOS-2 gene expression in macrophages exposed to E. coli lipopolysaccharide as well as in astrocytes responding to challenge by lipopolysaccharide from S. typhimurium (12, 35). In the absence of challenge, NFκB exists in the cytoplasm in inactivated form through association with the inhibitory proteins, IκB. Following activation, the IκB proteins undergo phosphorylation and are selectively degraded, leading to the release of NFκB, its nuclear translocation, and binding to the affinity sites in the promoters of inducible genes to activate transcription (36).

The lipopolysaccharide-induced NFκB activation apparently occurs through the glycophosphatidylinositol membrane-anchored protein, known as mCD14 (12, 13). A second form of CD, present in serum and referred to as soluble, is involved in conferring responsiveness to lipopolysaccharide upon cells that lack mCD, as in the case of epithelial cells (13). The complex formed between CD and lipopolysaccharide is then recognized by a specific transmembrane receptor that triggers signal transduction for NFκB activation, and nuclear translocation to initiate the expression of NOS-2 and nitric oxide release (12, 35). The termination of NFκB activity occurs upon IκB resynthesis,
which leads to dissociation of DNA-bound NFκB and its sequestration from the nuclear compartment and redistribution back into the cytoplasm (36).

Understanding the mechanism involved in termination of NFκB activity may identify an important target for a new generation of agents designed to treat H. pylori-associated gastric disease. Our study imply that ebrotidine, by exerting inhibitory effect on the H. pylori lipopolysaccharide-mediated NFκB signaling pathway, is capable to modulate the extent of mucosal inflammatory involvement in response to H. pylori infection.

REFERENCES


Received February 22, 1999
Accepted: June 30, 1999

Author’s address: Dr. B.L. Slomiany, Research Center UMDNJ-NJ Dental School 110 Bergen Street Newark, NJ 07103-2400
Phone 973-972-7052 Fax 973-972-7020 E-mail slomiabr@umdnj.edu