Ecabet sodium inhibits *Helicobacter pylori* lipopolysaccharide-induced activation of NADPH oxidase 1 and apoptosis of guinea pig gastric mucosal cells

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Ecabet sodium inhibits *Helicobacter pylori* lipopolysaccharide-induced activation of NADPH oxidase 1 in guinea pig gastric mucosal cells. Am J Physiol Gastrointest Liver Physiol 288: G300–G307, 2005. First published September 30, 2004; doi:10.1152/ajpgi.00274.2004.—Helicobacter pylori LPS activates a homolog of gp91phox, NADPH oxidase 1 (Nox1), in guinea pig gastric mucosal cells cultured in 10% FBS-containing medium. RTPCR and Northern hybridization demonstrated that H. pylori LPS stimulated expression of Nox1 and a novel p47phox homolog (Nox01) mRNAs with a peak at 4 h, followed by upregulation of superoxide anion (O2-) generation. Pretreatment with 10 mg/ml of a nonabsorbable antiagastic ulcer drug, ecabet sodium (ecabet), completely blocked these two mRNA expressions and the upregulation of O2- production. Under low (0.1%)-FBS conditions, H. pylori LPS predominantly caused apoptosis of the cells. Ecabet completely blocked the LPS-triggered phosphorylation of transforming growth factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 1, activation of caspase 3, and appearance of apoptotic cells. In contrast, ecabet had no effect on ethanol- or etoposide-initiated apoptosis. The ecabet-pretreated cells exhibited the responsiveness to H. pylori LPS, similarly as untreated control cells did, when ecabet was removed by washing before the addition of H. pylori LPS. Incubation of H. pylori LPS with ecabet eliminated the toxic effects of the LPS, and nondenatured polyacrylamide gel electrophoresis indicated the formation of higher molecular mass complexes between H. pylori LPS and ecabet, suggesting that ecabet may interact with H. pylori LPS and block the activation of Toll-like receptor 4 (TLR4). Our results suggest that ecabet may suppress TLR4-mediated inflammation or accelerated apoptosis caused H. pylori infection.

Toll-like receptor 4; NADPH oxidase 1; NADPH oxidase organizer 1; superoxide anion

ABOUT ONE-HALF OF THE WORLD’S population carries a gram-negative bacterium, *Helicobacter pylori*, that colonizes the human stomach. *H. pylori* infection causes persistent inflammation and enhanced Th1 immune response in human gastric mucosa. Environmental factors, *H. pylori* virulence factors, and host factors that modify the interaction between this bacterium and host alter the outcome of *H. pylori*-associated diseases (31). The most distinguishing feature of *H. pylori* is the presence of cag pathogenicity island (cagPAI) that carries a number of pathogenic genes including the caga and the cagE genes (2, 42). The vacA (8, 33) and the babA2 (11) genes also affect epithelial cell dynamics and inflammatory responses. As for host genomics, several human gene polymorphisms, including the IL-1β, IL-1β receptor, and TNF-α genes, which result in an enhanced Th1 response, were reported to be associated with an increased risk of gastric cancer (6, 7, 31). The chemical structure of *H. pylori* LPS is different from those of other enterobacterial LPSs (5, 9, 25), and its endotoxic properties are very low compared with other enterobacterial preparations (4, 26, 30). On the other hand, it was reported that *H. felis* caused severe atrophic gastritis in C3H/He mice, whereas C3H/HeJ mice with a mutated Toll-like receptor 4 (TLR4) gene showed heavy colonization but minimal atrophic gastritis with much less macrophage infiltration (34). Subsequently, orally administered *H. pylori* LPS was shown to trigger inflammatory responses in rat gastric mucosa, characterized by enhanced production of inflammatory mediators and accelerated apoptosis (32). Recently, it has been shown that *H. pylori* LPS upregulates TLR4 induction in human gastric epithelial cells (14, 17, 35), and TLR4 is suggested to serve as a receptor for *H. pylori* binding (37). Those observations suggest a crucial role of *H. pylori* LPS and TLR4 in mucosal inflammation caused by *H. pylori* infection.

Enterobacterial LPS is a potent activator of the phagocyte NADPH oxidase. Recently, six new homologs of gp91phox expressed in nonphagocytic cells have been identified and named systematically as the NADPH oxidase (Nox)/dual oxidase (Duox) family (23, 24). Subsequently, novel homologs of p47phox and p67phox, designated Nox oxidizer 1 (Noxo1) and Nox activator 1 (Nox1), respectively, have been identified to be crucial adaptor proteins for Nox1 (3, 10, 39). With the use of cDNA libraries from guinea pig gastric mucosal cells, we had cloned the guinea pig homologs of Nox1 (GenBank Accession no. AF539796), Noxa1 cDNA (GenBank Accession no. AB105907), and Nox1 cDNAs (GenBank Accession no. AB099629). In addition to guinea pig enterochromaffin-like (20) and chief cells (46), primary cultures of guinea pig gastric pit cells are sensitive to *H. pylori* LPS (17–19, 40). Gastric pit cells express Nox1 and TLR4 as well as MD-2, a molecule associated with the extracellular domain of TLR4, and markedly upregulated superoxide anion (O2-) generation in response to *H. pylori* LPS (17, 19, 40). Nox1-derived reactive oxygen species (ROS), particularly hydrogen peroxide, stimulated their proliferation and inflammatory responses through activating NF-κB (41). On the other hand, when these cells were maintained in low-serum conditions and exposed to *H. pylori* LPS,
the LPS dominantly caused apoptosis (18). These diverse events mediated by TLR4 and Nox1 may be relevant to histopathological features characteristic of chronic atrophic gastritis: enhanced apoptosis occurs in the superficial compartment of gastric mucosa where the bulk of bacteria is found, while at the same time, compensatory hyperproliferation is accompanied in the proliferation zone (45). Thus primary cultures of guinea pig gastric mucosal cells may be an excellent model to examine the TLR4-mediated epithelial cell responses to *H. pylori* infection.

Ecabet sodium (ecabet) is a dehydroacetic acid derivative originally purified from pine resin (28) and is now widely used for the treatment of gastric ulcer and gastritis in Japan. This originally purified from pine resin (28) and is now widely used for the treatment of gastric ulcer and gastritis in Japan. This agent has a high affinity for gastric mucus and is known to inhibit pepisin (29) and urease activities (15). Ecabet is also suggested to improve efficacy of antibiotic therapy for *H. pylori* infection in patients with peptic ulcer (1, 16). A recent study showed that ecabet inhibited transcription of the IL-8 gene and secretion of IL-8 by gastric epithelial cells infected with *H. pylori* at a concentration of 5 mg/ml. However, the precise pharmacological actions are still unclear. In this study, we found that ecabet blocked the *H. pylori* LPS-triggered activation of Nox1 and apoptosis of guinea pig gastric mucosal cells.

**MATERIALS AND METHODS**

**Isolation and culture of guinea pig gastric mucosal cells under LPS-free conditions.** The care and healing of the animals were in accordance with the National Institutes of Health guidelines, and the present study was approved by the Animal Care Committee of the University of Tokushima. Gastric mucosal cells were prepared aseptically from guinea pig fundic glands under LPS-free conditions, as previously described (19). Isolated gastric mucosal cells were cultured for 2 days in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% FBS (ICN Biomedicals, Aurora, OH), 100 U/ml penicillin, and 100 µg/ml streptomycin. All reagents and media were free of detectable amounts of LPS, and the final complete culture medium contained <100 pg/ml LPS, which was determined by the *Limulus amoeobocyte* lysate assay (19). After being cultured for 2 days, growing cells consisted of pit cells (>90%), pre-pit cells (~5%), parietal cells (~4%), mucus neck cells (~1%), and fibroblasts (~1%) (19, 40). Pit cells are responsible for O₂⁻ generation (19, 40). The rate of O₂⁻ production was assayed by measuring the superoxide dismutase-inhibitable reduction of cytochrome c and was expressed as nanomoles per milligram of protein per hour (40). *H. pylori* LPS was purified from a clinical isolate, and its endotoxin activity was determined to be 344 EU/ml (183 ng/ml) of Tris-HCl buffer (pH 6.8) containing 0.1 mM 2-mercaptoethanol and 2% SDS. After being washed with PBS, the membrane was again stained for 20 min with 5 µl of 10 mM Tris-HCl buffer (pH 6.8). For detecting DNA ladder generation (19, 40). The rate of O₂⁻ production was assayed by measuring the superoxide dismutase-inhibitable reduction of cytochrome c and was expressed as nanomoles per milligram of protein per hour (40). *H. pylori* LPS was purified from a clinical isolate, and its endotoxin activity was determined to be 344 EU/ml (183 ng/ml) of Tris-HCl buffer (pH 6.8) containing 0.1 mM 2-mercaptoethanol and 2% SDS. After being washed with PBS, the membrane was again stained for 20 min with 5 µl of 10 mM Tris-HCl buffer (pH 6.8).

**RT-PCR and Northern blot analysis.** Total RNA was isolated with an acid guanidium thiocyanate-phenol-chloroform mixture (40). RT reaction and PCR were performed using a TaKaRa RT-PCR kit (TaKaRa, Tokyo, Japan). The sequences of primer sets used were as follows: Nox1, 5'-TTTGCCTTCTTGCCCTGG-3' (sense) and 5'-TCTGGGTTGCGGATACC-3' (antisense); Nox1, 5'-ATGGAAACTCTGGGTGTAAA-3' (sense) and 5'-TAGCTGAAGTTACCATGAGA-3' (antisense). Mitochondria fractions were prepared, and the release of cytochrome c from mitochondria was detected by immunoblot analysis with an antibody against cytochrome c (Pharmingen, San Diego, CA) (18). Bound antibodies were then detected by rinsing the membrane for 15 min at 50°C in 10 mM Tris-HCl buffer (pH 6.8) containing 0.1 mM 2-mercaptoethanol and 2% SDS. After being washed with PBS, the membrane was again stained for 20 min with 5 µl of 10 mM Tris-HCl buffer (pH 6.8). For detecting DNA ladder generation (19, 40). The rate of O₂⁻ production was assayed by measuring the superoxide dismutase-inhibitable reduction of cytochrome c and was expressed as nanomoles per milligram of protein per hour (40). *H. pylori* LPS was purified from a clinical isolate, and its endotoxin activity was determined to be 344 EU/ml (183 ng/ml) of Tris-HCl buffer (pH 6.8) containing 0.1 mM 2-mercaptoethanol and 2% SDS. After being washed with PBS, the membrane was again stained for 20 min with 5 µl of 10 mM Tris-HCl buffer (pH 6.8). For detecting DNA ladder generation (19, 40). The rate of O₂⁻ production was assayed by measuring the superoxide dismutase-inhibitable reduction of cytochrome c and was expressed as nanomoles per milligram of protein per hour (40). *H. pylori* LPS was purified from a clinical isolate, and its endotoxin activity was determined to be 344 EU/ml (183 ng/ml) of Tris-HCl buffer (pH 6.8) containing 0.1 mM 2-mercaptoethanol and 2% SDS. After being washed with PBS, the membrane was again stained for 20 min with 5 µl of 10 mM Tris-HCl buffer (pH 6.8). For detecting DNA ladder generation (19, 40). The rate of O₂⁻ production was assayed by measuring the superoxide dismutase-inhibitable reduction of cytochrome c and was expressed as nanomoles per milligram of protein per hour (40). *H. pylori* LPS was purified from a clinical isolate, and its endotoxin activity was determined to be 344 EU/ml (183 ng/ml) of Tris-HCl buffer (pH 6.8).

**RESULTS**

**Effects of ecabet on *H. pylori* LPS-induced upregulation of O₂⁻ release from gastric mucosal cells.** Guinea pig gastric mucosal cells (pit cells) express Nox1 and spontaneously release a small amount of O₂⁻ (~10 nmol·mg protein⁻¹·h⁻¹) under LPS-free conditions (19). These cells respond to *H. pylori* LPS (EC₅₀, 8 EU/ml) or *E. coli* LPS (EC₅₀, 0.3 EU/ml) and increase O₂⁻ producing capability up to 10-fold in the presence of 10% FBS (Fig. 1; see also Ref. 19). Lipid A is a bioactive component for the priming effect of LPS from *H. pylori* or *E. coli* (19). As shown in Fig. 1, treatment of the cells with ecabet alone at 0.01–20 µg/ml did not change the basal level of O₂⁻ production, whereas the priming effect with 20 EU/ml (183 ng/ml) of *H. pylori* LPS or 3.44 EU/ml (10 ng/ml) of *E. coli* LPS was significantly interfered with 5 µg/ml or 2 µg/ml of *E. coli* LPS.
Guinea pig gastric mucosal cells constitutively express mRNAs for p67\textsuperscript{phox}, its new homolog Nox1, p22\textsuperscript{phox}, and Rac1, whereas the levels of these mRNA were not changed after exposure to \textit{H. pylori} LPS (data not shown). The finding is inconsistent with our previous report that in guinea pig gastric mucosal cells, the amount of a 67-kDa protein that cross-reacted with an antibody against human p67\textsuperscript{phox} increased in parallel with elevation of \( \text{O}_2^\text{-} \) generation after treatment with \textit{H. pylori} LPS (19). To explore this inconsistency, we developed a novel polyclonal antibody against human recombinant p67\textsuperscript{phox} which recognized the guinea pig p67\textsuperscript{phox} with a molecular mass of 63 kDa, and the amount was not affected by \textit{H. pylori} LPS (data not shown).

Effects of ecabet on \textit{H. pylori} LPS-induced apoptosis. Higher concentrations of \textit{H. pylori} LPS (>200 EU/ml) caused apoptosis of primary cultured guinea pig gastric mucosal cells in 10% FBS-containing media. \textit{H. pylori} LPS more effectively induced apoptosis of the cells in a low (0.1%) FBS-containing RPMI 1640 medium at 2 EU/ml (18.3 ng/ml) or higher concentrations (18). Lipid A mediated this apoptosis (18). We also tested whether ecabet could inhibit the apoptosis. Treatment with 10 mg/ml ecabet alone did not initiate apoptosis of the cells (Fig. 3A). Pretreatment with 5 or 10 mg/ml of ecabet for 30 min significantly suppressed the apoptosis induced with 40 EU/ml (366 ng/ml) of \textit{H. pylori} LPS, which was estimated by counting the number of cells having condensed and fragmented chromatin (Fig. 3, A and B). The pretreatment with ecabet also inhibited the apoptotic DNA ladder formation in a dose-dependent manner (Fig. 3C).

LPS initially activates caspase 8, stimulates of mitochondria-dependent apoptotic processes, and finally activates effector caspase 3 in guinea pig gastric mucosal cells (18). As shown in Fig. 4A, after treatment of cells with 40 EU/ml of \textit{H. pylori} LPS, caspase 8-like enzyme activity started to increase within 4 h (Fig. 4A). In association with this activation, the cells began to release cytochrome \( c \) from mitochondria into the cytoplasm at this time point (Fig. 5A), followed by a significant elevation of caspase 3-like enzyme activity at 8 h (Fig. 4B). Ecabet alone at 10 mg/ml did not modify caspase 8- and 3-like enzyme activities during the experimental period, whereas it completely suppressed the LPS-triggered increases in caspase 8- and 3-like enzyme activities (Fig. 4). At the same time, ecabet suppressed both LPS-induced release of cytochrome \( c \) into the cytosol (1) or 2 g twice a day (16).

Effects of ecabet on \textit{H. pylori} LPS-induced expression of Nox1 and Noxo1 mRNAs. Primary cultures of guinea pig gastric mucosal cells are more sensitive to \textit{E. coli} LPS than \textit{H. pylori} LPS. However, \textit{H. pylori} LPS is important for the pathogenesis of gastric disorders. We next examined the mechanism by which ecabet blocked the priming effect of \textit{H. pylori} LPS. RT-PCT and Northern hybridization showed that on stimulation by 20 EU/ml of \textit{H. pylori} LPS, guinea pig gastric mucosal cells expressed Nox1 and Noxo1 mRNAs (Fig. 2), followed by upregulation of \( \text{O}_2^\text{-} \) generation (Fig. 1). Pretreatment with 10 mg/ml ecabet inhibited the LPS-induced expression of these two mRNAs, which was confirmed by RT-PCR (Fig. 2A) and Northern blot analysis (Fig. 2B). These results suggest that ecabet may block the priming effect by inhibiting the LPS-induced transcriptional activation of the \textit{Nox}1 and the \textit{Noxo}1 genes.
Effects of ecabet on ethanol- and etoposide-induced apoptosis.

We also examined whether ecabet similarly inhibited apoptosis caused by other agents. Ethanol activates an initiator caspase, caspase 8, and stimulates mitochondria-mediated apoptosis (43). Ecabet failed to block the activation of caspase 8 (Fig. 6A) - and 3-like enzyme (Fig. 6B) activities and apoptosis (Fig. 6C) of the cells exposed to 5% ethanol for 12 h. A phyllostanin analog, etoposide, induces DNA double-strand breaks and apoptosis. Treatment of the cells with this agent for 12 h activated caspase 3 (Fig. 6B) and caused apoptosis (Fig. 6C) without affecting caspase 8-like enzyme activity (Fig. 6A). Ecabet again failed to suppress this apoptosis stimulated by the genotoxic agent.

Effects of ecabet on stimulation of TLR4 by H. pylori LPS.

TAK1 is one of the common signal-transduction molecules for TLR4 and IL-1 receptor-signaling pathways, and TAB1 functions as an activator for the TAK1. As previously described (18), treatment with 40 EU/ml of H. pylori LPS phosphorylated TAK1 and TAB1 within 30 min (Fig. 7, A and B, lanes 1–6). Pretreatment of ecabet completely blocked the LPS-triggered phosphorylation of these molecules (lanes 7–12 in Fig. 7, A and B). Thus ecabet was likely to interfere the activation of TLR4 signaling by H. pylori LPS.

Interaction between ecabet and H. pylori LPS.

Once guinea pig gastric mucosal cells were stimulated by H. pylori LPS for 30 min, subsequently added ecabet failed to block the LPS-
induced activation of Nox1 (Fig. 8A; d) and apoptosis (d in Fig. 8, B or C). A simultaneous addition of ecabet and H. pylori LPS partially inhibited the LPS effects (Fig. 8, A–C, e), whereas H. pylori LPS lost its stimulatory actions when preincubated with ecabet for 30 min before an addition (Fig. 8, A–C; f). Pretreatment with ecabet appeared not to modify the responsiveness of the cells to H. pylori LPS, because ecabet-pretreated cells similarly upregulated O$_2^-$ generation or underwent apoptosis in response to H. pylori LPS, when ecabet was removed by washing before stimulation by H. pylori LPS (Fig. 8, A–C; g). These data suggest that this compound may preferentially interact with H. pylori LPS and inhibit H. pylori LPS-stimulated TLR4 signaling.

On the basis of the above findings, we tested whether ecabet directly bound to H. pylori LPS. Ecabet precipitates under acidic conditions. After preincubation of 40 EU (366 ng/ml) of H. pylori LPS with 10 mg/ml ecabet or saline for 30 min at 37°C, these mixtures were left untreated or acidified to pH 1.2 with hydrochloric acid and subjected to centrifugation at 15,000 g for 20 min at 4°C. Endotoxin activities of these supernatants neutralized with sodium hydroxide were determined by the Limulus ameobocyte lysate assay. The acidification itself decreased to the activity from 40 to 14 EU/ml, probably due to the removal of phosphates from lipid A (19). Acid-precipitated ecabet absorbed H. pylori LPS, and the resultant supernatant contained only <0.4 EU/ml of the LPS.

To further confirm the interaction between H. pylori LPS and ecabet, 366 ng/ml H. pylori LPS was incubated with different concentrations of ecabet in RPMI 1640 medium for 30 min at 37°C, and the mixture was subjected to nondenatured gel electrophoresis using a 5% polyacrylamide gel. Changes in molecular mass of H. pylori LPS was indirectly assessed by the silver staining. As shown in Fig. 9A, incubation of H. pylori LPS with 2 mg/ml or higher concentrations of ecabet produced slower migration of H. pylori LPS. We also examined the interaction between E. coli LPS and ecabet. In this case, a higher concentration of E. coli LPS (71 µg/ml) was incubated with 10 mg/ml ecabet to visualize E. coli LPS by the silver staining. The nondenatured gel electrophoresis showed more slowly migrated bands of E. coli LPS after incubation with ecabet (Fig. 9B).

**DISCUSSION**

Because endotoxic activity of H. pylori LPS is much lower than LPS from the other enterobacteria (4, 26, 30), H. pylori LPS is not generally considered as a crucial virulence. On the other hand, guinea pig gastric pit cells, chief cells (46), and enterochromaffin-like cells (20) are highly responsive to LPS. Furthermore, an oral administration of H. pylori LPS can trigger distinct inflammatory responses in rat gastric mucosa (32). LPS is a complex molecule composed of carbohydrate and lipid fragments. It has recently been shown that a chemically synthesized lipid A of H. pylori as well as its natural lipid A are able to activate TLR4 signaling and trigger a weaker but significant proinflammatory response in human gastric epithelium (MKN-1 cells), although they exert no or
tor complex involved in the response to H. pylori (14, 35, 36). With the use of HEK293 cells transfected with TLR2, TLR4, and MD-2 or TLR5 expression plasmid, Smith et al. (36) suggested that TLR2 and TLR5 but not TLR4 are required for H. pylori-induced NF-κB activation and chemokine expression. On the other hand, Ishihara et al. (14) have shown that H. pylori infection upregulates the TLR4 and MD-2 expression in human gastric mucosa and have suggested that the TLR4/MD-2 system is a potent receptor complex involved in the response to H. pylori LPS in gastric epithelium. A majority of guinea pig gastric mucosal cells used in this study consisted of pit cells (>95%), and fibroblasts were <1%. These cultures constitutively express TLR4 and MD-2 but not TLR2 or TLR9 (17, 19), and TLR4 mRNA and protein were easily detectable by Northern blot analysis and immunoblotting, respectively (17). Furthermore, immunohistochemistry showed that gastric pit cells possessed significant amounts of TLR4 protein preferentially on the plasma membrane (17), similarly as reported by Ishihara et al. (14) and Schmausser et al. (35). Thus TLR4 expressed in gastric pit cells is likely to play an essential role in the responses to LPS from H. pylori as well as E. coli. However, it is still unknown whether H. pylori LPS actually stimulates human gastric epithelial cells in vivo.

First, we examined the mechanism for the activation of Nox1. Nox1 was thought to generate O$_2^-$ without any cytosolic components of phagocyte NADPH oxidase, such as p47$^{phox}$ or p67$^{phox}$ (38). But recently, it has been shown that Nox1 plus either p67$^{phox}$ or its homolog Noxa1 are necessary for O$_2^-$ producing activity of Nox1 (3, 10, 39). H. pylori LPS did not change the levels of p67$^{phox}$ and its homolog (Nox1) mRNAs (unpublished observations), but it stimulated the expression of Nox1 mRNA in association with upregulation of Nox1 mRNA expression and O$_2^-$ generation. Nox1 conserves most of functional domains for protein-protein interactions (39), whereas it lacks the autoinhibitory region of p47$^{phox}$ (3, 10, 39). Human and mouse Nox1 proteins may directly bind via its SH3 domains to the COOH terminus of p22$^{phox}$ without any conformational changes (39). Predicted amino acid sequence of guinea pig Nox1 (GenBank accession number AB105906) suggests that this component may interact with p67$^{phox}$, p51$^{phox}$, or p22$^{phox}$, similarly as human Nox1 does. Guinea pig gastric pit cells constitutively express p22$^{phox}$, p67$^{phox}$, and Noxa1 (data not shown); therefore, once Nox1 is synthesized together with Nox1 in gastric mucosal cells, Nox1 may result in a “self-activated state” under constant O$_2^-$-generating conditions.

Ecabet was purified from pine resin and has been widely used as a nonabsorbable antigastric ulcer agent in Japan (28). This compound adheres to ulcer regions and topically enhances the mucosal integrity possibly through enhancing mucosal

very low endotoxic activities and mitogenic properties on immune cells (27).

Recently, the TLR family expressed by gastric epithelium has been suggested to play an essential role in the mucosal immunity to H. pylori (14, 35, 36). With the use of HEK293 cells transfected with TLR2, TLR4, and MD-2 or TLR5 expression plasmid, Smith et al. (36) suggested that TLR2 and TLR5 but not TLR4 are required for H. pylori-induced NF-κB activation and chemokine expression. On the other hand, Ishihara et al. (14) have shown that H. pylori infection upregulates the TLR4 and MD-2 expression in human gastric mucosa and have suggested that the TLR4/MD-2 system is a potent receptor complex involved in the response to H. pylori LPS in human gastric epithelial cells.

![Fig. 8. Effects of ecabet on O$_2^-$ generation, apoptosis, and DNA ladder formation in gastric mucosal cells.](image)

![Fig. 9. Interaction between H. pylori LPS and ecabet.](image)
defensive factors (22, 44). Ecabet improves the efficacy of antibiotic therapy for *H. pylori*-infected patients with peptic ulcer (1, 16) and has been suggested to exert unknown anti-*H. pylori* actions besides inhibition of urease activity (1, 16). In this study, we found that ecabet inhibited the *H. pylori* LPS-triggered expression of both Nox1 and Nox0 mRNAs in the 10% FBS-containing medium, resulting in complete inhibition of the priming effect of *H. pylori* LPS (Fig. 1). The anti-*H. pylori* LPS action of ecabet was also examined in guinea pig gastric mucosal cells maintained in the 0.1% FBS-containing medium, in which *H. pylori* LPS predominantly causes their apoptosis (18). Ecabet blocked the *H. pylori* LPS-triggered activation of caspase 8, loss of mitochondrial membrane potential, release of cytochrome c from mitochondria, and activation of caspase 3, leading to significant inhibition of DNA ladder formation and appearance of apoptotic cells. IL-1β and TNF-α share distinct signaling pathways of apoptosis with TLR4. We also examined whether ecabet blocked the IL-1β- or TNF-α-triggered events. Unfortunately, guinea pig gastric mucosal cells do not constitutively express the specific receptor for these cytokines, and neither IL-1β nor TNF-α upregulated O2− production (40) and stimulated apoptosis (data not shown). However, we showed that ecabet did not inhibit apoptosis initiated by a common apoptosis-inducing irritant (ethanol) or a genotoxic agent (etoposide). These results suggest that ecabet may exert anti-*H. pylori* actions at least in part through blocking *H. pylori* LPS-mediated toxic effects.

Next, we examined the mechanism by which ecabet blocked the LPS actions. *H. pylori* LPS stimulates TLR4 signal-transduction molecules downstream to myeloid differentiation factor 88, such as TAK1 and TAB1. We confirmed that ecabet inhibited the *H. pylori* LPS-initiated phosphorylation of TAK1 and TAB1. A series of experiments with ecabet and *H. pylori* LPS (Fig. 8) suggested that ecabet may interact with *H. pylori* LPS and inhibit the activation of TLR4 signaling. A recent study suggests that TLR4 may serve as a receptor for *H. pylori* binding (37), and ecabet was shown to inhibit *H. pylori* adhesion to gastric epithelial cells possibly by directly targeting *H. pylori* (12), also supporting an interaction between ecabet and *H. pylori* LPS.

Ecabet becomes insoluble in acidic conditions. When *H. pylori* LPS was incubated with ecabet, acid-precipitated ecabet almost completely absorbed the endotoxin activity. Furthermore, non-denatured polyacrylamide gel electrophoresis indicated a direct interaction between *H. pylori* LPS and ecabet (Fig. 9). The concentrations of ecabet, which produced the same effect of O2− generation or apoptosis of the cells. Considering these results, ecabet is likely to have a lower, but significant affinity for *H. pylori* LPS. This weak interaction may be enough to block the effects of *H. pylori* LPS in damaging mucosa, because patients are usually taking a high dose of ecabet (1 or 2 g twice a day).

The present study suggests that ecabet is a new type of drug having novel pharmacological actions that may specifically target *H. pylori* LPS and suppress the TLR4-mediated inflammation and accelerated apoptosis in gastric mucosa infected with *H. pylori*. Considering the safety and efficacy demonstrated in Japan, this compound may have a therapeutic benefit for long-term management of *H. pylori*-infected gastric mucosa. At present, however, the molecular interaction between LPS and TLR4-MD-2 complex has not been fully elucidated. Further experiments are still necessary to reveal the specific molecular target(s) for ecabet.

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