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

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Submitted 25 June 2004; accepted in final form 20 September 2004

Abstract

Ecabet sodium inhibits *Helicobacter pylori* lipopolysaccharide-induced activation of NADPH oxidase 1 and apoptosis of 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. RT-PCR 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 (O$_2^-$) generation. Pretreatment with 10 mg/ml of a nonabsorbable antigenic ulcer drug, ecabet sodium (ecabet), completely blocked these two mRNA expressions and the upregulation of O$_2^-$ 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 8, loss of mitochondrial membrane potential, release of cytochrome c, 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 nonadenated 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

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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 dehydroabietic acid derivative 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 pepsin (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 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 amoeboocyte lysate assay (19). After being cultured for 2 days, growing cells consisted of pit cells (≥90%), pre-pit cells (∼5%), parietal cells (4–5%), mucus neck cells (<1%), and fibroblasts (<1%) (19, 40). Pit cells are responsible for O$_2^-$ generation (19, 40). The rate of O$_2^-$ 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 100 endotoxin unit (EU)/μg by the Limulus amoeboocyte lysate assay as previously described (19). LPS from Escherichia coli K-235 was purchased from Sigma (St. Louis, MO), and its endotoxin activity was also determined to be 344 EU/μg by the same assay.

Assessment of apoptosis. Apoptosis was determined by DNA fragmentation and chromatin condensation. For detecting DNA ladder formation, both floating and attached cells were collected in microcentrifuge tubes and centrifuged at 900 g for 5 min at 4°C. The pellets were lysed for 30 min at 4°C in 200 μl of 10 mM Tris-HCl buffer (pH 8.0), containing 10 mM EDTA and 0.5% (vol/vol) Triton X-100. Fragmented DNAs were extracted and analyzed as described previously (13). For morphological detection of apoptosis, cells were fixed with 4% formalin in PBS for 30 min, washed twice with PBS, and stained for 20 min with 5 μg/ml of a fluorescent dye Hoechst 33342 (Sigma). Chromosomal condensation was observed using a fluorescence microscopy (TMD 3000-EF; Nikon, Tokyo, Japan). Mitochondrial membrane potential was cytochemically determined using a MitoLight apoptosis detection kit (Chemicon, Temecula, CA) according to the manufacturer’s protocol. Activities of caspase 3- and 8-like enzymes were measured using the synthetic substrates N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) and N-acetyl-Ile-Glu-Thr-Asp-p-nitroanilide (Ac-IETD-pNA), respectively (13).

Immunoblot analysis. Whole cell proteins were extracted from cultured cells as previously described (19). Sample of 35-μg protein per lane was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After nonspecific binding sites were blocked with 4% purified casein, the membrane was incubated for 1 h at room temperature with a 1,000× diluted antibody against transforming growth factor-β-activated kinase 1 (TAK1; Santa Cruz Biotechnology, Santa Cruz, CA) or TAK1-binding protein 1 (TAB1; Santa Cruz Biotechnology). Bound antibodies were detected with an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia, Piscataway, NJ). Cytosol and mitochondria fractions were prepared, and the release of cytochrome c from mitochondria was determined by immunoblot analysis with an antibody against cytochrome c (Pharmingen, San Diego, CA) (18). Bound antibodies were then visualized 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 subjected to immunoblotting with an antibody against β-actin (OncoGene, Cambridge, MA).

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′-TTTGCTTCCTCTGTGCCGG-3′ (sense) and 5′-TCTGGGTGTTGGCAGATACC-3′ (antisense); Nox1, 5′-ATGGAACACTGGGTGGTTAA-3′ (sense) and 5′-TAGCTGAGTTACTCATGAGA-3′a gap GDPH, 5′-TCATGACCACAGTCATG-3′ (sense) and 5′-GCGTCTCTACACCCCTTCTTTGATGT-3′ (antisense). PCR products were sequenced with a DNA sequencer and confirmed to be the corresponding cDNA fragments.

For Northern blot analysis, samples of 30 μg of total RNA were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde and transferred to nylon membrane filters (Hybond-N+; Amersham Pharmacia). After prehybridization, the membrane was hybridized for 4 h at 65°C with a CDNA probe for Nox1, Nox1, or GAPDH, which had been preblabeled with [α-32P]deoxycytidine triphosphate using a random primer kit (TaKaRa). The membrane was washed twice with 2× standard saline citrate (SSC; 0.3 M NaCl + 0.03 M sodium citrate) containing 0.5% SDS for 10 min at 65°C and then three times with 0.2× SSC containing 1% SDS. Bound probes were autoradiographed by exposure to a Kodak X-Omat film for an appropriate time at −80°C.

Statistical analysis. ANOVA and Scheffe’s test were used to determine statistically significant differences. Differences were considered significant if P <0.01.

RESULTS

Effects of ecabet on H. pylori LPS-induced upregulation of O$_2^-$ release from gastric mucosal cells. Guine pig gastric mucosal cells (pit cells) express Nox1 and spontaneously release a small amount of O$_2^-$ (∼10 nmol·mg protein$^{-1}$·h$^{-1}$) under LPS-free conditions (19). These cells respond to H. pylori LPS (EC$_{50}$, 8 EU/ml) or E. coli LPS (EC$_{50}$, 0.3 EU/ml) and increase O$_2^-$ 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 O$_2^-$ 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
Guinea pig gastric mucosal cells constitutively express mRNAs for p67phox, its new homolog Noxa1, p22phox, and Rac1, whereas the levels of these mRNA were not changed after exposure to 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 p67phox increased in parallel with elevation of O2 generation after treatment with H. pylori LPS (19). To explore this inconsistency, we developed a novel polyclonal antibody against human recombinant p67phox which recognized the guinea pig p67phox with a molecular mass of 63 kDa, and the amount was not affected by H. pylori LPS (data not shown).

Effects of ecabet on H. pylori LPS-induced apoptosis. Higher concentrations of H. pylori LPS (>200 EU/ml) caused apoptosis of primary cultured guinea pig gastric mucosal cells in 10% FBS-containing media. 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 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 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). Ecbat 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, ecbat suppressed both LPS-induced release of cytochrome c

mg/ml ecabet, respectively, and 10 mg/ml of this compound completely blocked the upregulation of O2 production stimulated by H. pylori or E. coli LPS (Fig. 1).

Although extremely high concentrations of ecabet were required for the inhibition, the effective concentrations were similar to those reported in experiments in vitro (12, 21). We also confirmed that treatment with ecabet at 20 mg/ml did not change the cell viability throughout the experiments. This was based on continued trypan blue exclusion, adherence to the culture plates, and unchanged morphological features (data not shown). Untreated and H. pylori LPS (20 EU/ml)-treated cells released 3.03 ± 0.68 (means ± SD, n = 4) and 5.93 ± 0.74% (n = 4) of the total cellular lactate dehydrogenase activity during 12-h incubation, respectively. Cells treated with 20 mg/ml ecabet or with 20 mg/ml ecabet plus 20 EU/ml H. pylori LPS, respectively, released 2.66 ± 0.71 (n = 4) or 2.42 ± 0.36% (n = 4) of the activity during the same period. Thus the concentrations used in this study were not toxic and may be clinically relevant, because patients are usually taking ecbat 1 (1) or 2 g twice a day (16).

Effects of ecabet on H. pylori LPS-induced expression of Nox1 and Noxo1 mRNAs. Primary cultures of guinea pig gastric mucosal cells are more sensitive to E. coli LPS than H. pylori LPS. However, H. pylori LPS is important for the pathogenesis of gastric disorders. We next examined the mechanism by which ecabet blocked the priming effect of H. pylori LPS. RT-PCT and Northern hybridization showed that on stimulation by 20 EU/ml of H. pylori LPS, guinea pig gastric mucosal cells expressed Nox1 and Noxo1 mRNAs (Fig. 2), followed by upregulation of O2 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 Nox1 and the Noxo1 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 phyllotoxin 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 \textit{H. pylori} LPS partially inhibited the LPS effects (Fig. 8, A–C, e), whereas \textit{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 \textit{H. pylori} LPS, because ecabet-pretreated cells similarly upregulated \( \text{O}_2^- \) generation or underwent apoptosis in response to \textit{H. pylori} LPS, when ecabet was removed by washing before stimulation by \textit{H. pylori} LPS (Fig. 8, A–C; g). These data suggest that this compound may preferentially interact with \textit{H. pylori} LPS and inhibit \textit{H. pylori} LPS-stimulated TLR4 signaling.

On the basis of the above findings, we tested whether ecabet directly bound to \textit{H. pylori} LPS. Ecabet precipitates under acidic conditions. After preincubation of 40 EU (366 ng/ml) of \textit{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 \( \text{g} \) for 20 min at 4°C. Endotoxin activities of these supernatants neutralized with sodium hydroxide were determined by the \textit{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 \textit{H. pylori} LPS, and the resultant supernatant contained only <0.4 EU/ml of the LPS.

To further confirm the interaction between \textit{H. pylori} LPS and ecabet, 366 ng/ml \textit{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 non-denatured gel electrophoresis using a 5% polyacrylamide gel. Changes in molecular mass of \textit{H. pylori} LPS was indirectly assessed by the silver staining. As shown in Fig. 9A, incubation of \textit{H. pylori} LPS with 2 mg/ml or higher concentrations of ecabet produced slower migration of \textit{H. pylori} LPS. We also examined the interaction between \textit{E. coli} LPS and ecabet. In this case, a higher concentration of \textit{E. coli} LPS (71 \( \mu \text{g/ml} \)) was incubated with 10 mg/ml ecabet to visualize \textit{E. coli} LPS by the silver staining. The non-denatured gel electrophoresis showed more slowly migrated bands of \textit{E. coli} LPS after incubation with ecabet (Fig. 9B).

**DISCUSSION**

Because endotoxic activity of \textit{H. pylori} LPS is much lower than LPS from the other enterobacteria (4, 26, 30), \textit{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 \textit{H. pylori} LPS can trigger distinct inflammatory responses in rat gastric mucosa (32). LPS is a complex molecule composed of carbohydrates and lipid fragments. It has recently been shown that a chemically synthesized lipid A of \textit{H. pylori} as well as its natural lipid A are able to activate TLR4 signaling and trigger a weaker but significant pro-inflammatory response in human gastric epithelium (MKN-1 cells), although they exert no or

**Fig. 6.** Effects of ecabet on ethanol- or etoposide-induced apoptosis of gastric mucosal cells. Gastric mucosal cells pretreated with 10 mg/ml ecabet (ecabet +) or saline alone (ecabet −) for 30 min in 0.1% FBS-containing RPMI 1640 medium were exposed to \textit{H. pylori} LPS (40 EU/ml), 5% ethanol, or 1 mM etoposide for 12 h. Caspase 8 (A)- or 3-like (B) enzyme activity and apoptotic cells (C) were measured as described in the Figs. 3 and 4. *Significantly increased, compared with untreated control cell (P < 0.01 by ANOVA and Scheffe’s test). #Significantly different, compared with ecabet-treated cells (P < 0.01 by ANOVA and Scheffe’s test).

**Fig. 7.** Effects of ecabet on \textit{H. pylori} LPS-triggered phosphorylation of TAK1 and TAB1. Gastric mucosal cells pretreated with saline or 10 mg/ml ecabet were exposed to 40 EU/ml of \textit{H. pylori} LPS for the indicated times in 0.1% FBS-containing RPMI 1640 medium. Samples of whole cell proteins were separated by SDS-PAGE in a 6% acrylamide gel and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis with an antibody against TAK1 (A) or TAB1 (B) was performed as described in MATERIALS AND METHODS. Bacterial alkaline phosphatase (BAP)-treated samples were prepared from cells exposed to 40 EU/ml \textit{H. pylori} LPS for 60 min (18).
Anti-\textit{H. pylori} Actions of Ecabet Sodium

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The anti-\textit{H. pylori} actions of ecabet sodium were studied in a gastric mucosal model. Gastric mucosal cells maintained in 10% FBS-containing RPMI 1640 medium were pretreated with 10 mg/ml of ecabet for 30 min, and then exposed to 20 or 40 EU/ml of \textit{H. pylori} LPS for 30 min at 37°C, and these mixtures were added to the cells maintained in 10% or 0.1% FBS-containing RPMI 1640 medium. After the cells in 10% or 0.1% FBS-containing RPMI 1640 medium were pretreated with 10 mg/ml of ecabet, \textit{H. pylori} LPS (20 or 40 EU/ml) were incubated with 10 mg/ml of ecabet for 30 min at 37°C, and these mixtures were added to the cells maintained in 10% or 0.1% FBS-containing RPMI 1640 medium. After the cells in 10% or 0.1% FBS-containing RPMI 1640 medium were pretreated with 10 mg/ml of ecabet for 30 min, ecabet was removed by vigorously washing with saline, and they were exposed to 20 or 40 EU/ml of \textit{H. pylori} LPS (g). Twelve hours after these treatments, \textit{O}_2^- production (A), number of apoptotic cells (B), and DNA ladder generation (C) were measured as described in MATERIALS AND METHODS. Values are means ± SD, n = 6. *Significantly increased, compared with untreated control cells (P < 0.01 by ANOVA and Scheffé’s test).

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 \textit{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 \textit{H. pylori}-induced NF-\textit{kB} activation and chemokine expression. On the other hand, Ishihara et al. (14) have shown that \textit{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 \textit{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 \textit{H. pylori} as well as \textit{E. coli}. However, it is still unknown whether \textit{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 \textit{O}_2^- without any cytosolic components of phagocyte NADPH oxidase, such as \textit{p47}\textsuperscript{phox} or \textit{p67}\textsuperscript{phox} (38). But recently, it has been shown that Nox1 might also be activated by its homolog Noxa1 (3, 10, 39). Human and mouse Nox1 proteins may directly bind via their SH3 domains to the COOH terminus of \textit{p22}\textsuperscript{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 \textit{p67}\textsuperscript{phox}, \textit{p51}\textsuperscript{phox}, or \textit{p22}\textsuperscript{phox}, similarly as human Nox1 does. Guinea pig gastric pit cells constitutively express \textit{p22}\textsuperscript{phox}, \textit{p67}\textsuperscript{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 \textit{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...
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 Nox1 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 O$_2^-$ 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 inhibition of upregulation of O$_2^-$ 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.

**GRANTS**

This study was supported by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (14370184; to K. Rokutan) and a grant-in-aid for scientific research from the 21st Century Center of Excellence Program, Human Nutritional Science on Stress Control, Tokushima, Japan.

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