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

Kenji Kusumoto,1 Tsukasa Kawahara,1 Yuki Kuwano,1 Shigetada Teshima-Kondo,2 Kyoko Morita,2 Kyoichi Kishi, and Kazuhito Rokutan.

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 (O2−) generation. Pretreatment with 10 mg/ml of a nonabsorbable antioxidant 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 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 nonnated 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.

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

ABSTRACT

*Helicobacter pylori* lipopolysaccharide (LPS) activates NADPH oxidase 1 (Nox1) expression, resulting in the formation of superoxide anion (O2−) and triggers apoptosis in guinea pig gastric mucosal cells. Ecabet sodium eliminates the toxic effects of *H. pylori* LPS on guinea pig gastric mucosal cells, suggesting that ecabet may suppress TLR4-mediated inflammation or apoptosis caused by *H. pylori* infection.
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 to model to examine the TLR4-mediated epithelial cell responses. Ecabet was also suggested to improve efficacy of antibiotic therapy for gastritis: enhanced apoptosis occurs in the superficial compartment of gastric mucosa originally (13). For morphological detection of apoptosis, cells were fixed with 4% formaldehyde and transferred to a polyvinylidene difluoride membrane. After nonspecific binding sites were blocked, 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 (Amer sham Pharmacia, Piscataway, NJ). Cytosol and 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 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 (Onco gene, 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 primer pairs used were as follows: Nox1, 5′-TTTGGCTTCTCGGCTGTT-3′ (sense) and 5′-TCTGGGTTGCGAGATCCC-3′ (antisense); Nox1, 5′-ATGGGAAACTGGGTGTATT-3′ (sense) and 5′-TAGCTGAAGTACCATGAGAA-3′ (antisense); GAPDH, 5′-TCATGACCACTCTGCTAC-3′ (sense) and 5′-GCCGTCTTACCACTTCTT-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 prelabeled with [α-32P] deoxyctydine 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 Scheffé’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 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/μl) or E. coli LPS (EC50, 0.3 EU/μl) 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 g/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 mg/ml or 2
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 O\textsubscript{2} 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 \textit{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 \textit{c}
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- and 3-like enzyme (Fig. 6B) activities and apoptosis (Fig. 6C) of the cells exposed to 5% ethanol for 12 h. A phytoalexin 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.

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-triggered release of cytochrome c (cyto. c) from mitochondria and decline of mitochondrial membrane potential. Gastric mucosal cells pretreated with saline or ecabet were exposed to H. pylori LPS (40 EU/ml) for the indicated times in 0.1% FBS-containing RPMI 1640 medium. Mitochondria-free cytosolic extracts (cytosol) and mitochondrial pellets (mitochondria) were prepared from these cells (18), and the amounts of cytochrome c in these fractions were measured by immunoblot analysis using a 12% polyacrylamide gel, as described in MATERIALS AND METHODS (A). Mitochondrial membrane potential was monitored using a Mitolight kit (B). Mitochondria having intact membrane potentials are colored in red, and green indicates free fluorescence dye in the cytoplasm.

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removed by washing before stimulation by LPS partially inhibited the LPS effects (Fig. 8A) or saline alone (ecabet +) for 30 min in 0.1% FBS-containing RPMI 1640 medium were exposed to 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 cells (P < 0.01 by ANOVA and Scheffé’s test). #Significantly different, compared with ecabet-treated cells (P < 0.01 by ANOVA and Scheffé’s test).

Fig. 7. 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 H. pylori LPS (40 EU/ml), 5% ethanol, or 1 mM etoposide 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...
The TLR4/MD-2 system is a potent receptor complex involved in the response to H. pylori LPS in human gastric mucosa and has been suggested to play an essential role in the mucosal immunity to H. pylori (27). 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 TLR5-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 Noxa 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 (Noxa1) 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...
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 Nox01 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 LPS action of ecabet was also examined in guinea pig gastric mucosal cells was incubated with ecabet, acid-precipitated ecabet almost completely absorbed the endotoxin activity. Furthermore, 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.

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REFERENCES


