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Identification of a signaling cascade for interleukin-8 production by *Helicobacter pylori* in human gastric epithelial cells

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Abstract

Infecting gastric epithelial cells with *Helicobacter pylori* (*H. pylori*) has been shown to induce interleukin-8 (IL-8) production, but the signal transduction mechanism leading to IL-8 production is not defined clearly. In the present study, we investigated the molecular mechanism responsible for *H. pylori*-induced IL-8 release in human gastric epithelial cells. IL-8 levels in culture supernatants were determined by an enzyme linked-immunosorbent assay. Extracellular signal-regulated kinase (ERK) activity was tested using an *in vitro* kinase assay, which measured the incorporation of [γ-^{33}P]ATP into a synthetic peptide that is a specific ERK substrate. ERK phosphorylation and IκBα degradation by *H. pylori* infection were assessed by western blotting. In MKN45 cells, *H. pylori*-induced IL-8 release in a time-dependent manner. This IL-8 release was abolished by treatment with intracellular Ca^{2+} chelators (BAPTA-AM and TMB-8) but not by EGTA or nifedipine. The Ca^{2+} ionophore A23187 also induced IL-8 release to an extent similar to that of *H. pylori* infection. Calmodulin inhibitors (W7 and calmidazolium) and tyrosine kinase inhibitors (genistein and ST638) completely blocked IL-8 release by *H. pylori* and A23187. PD98059, an ERK pathway inhibitor, completely abolished *H. pylori*-induced IL-8 release. Moreover, BAPTA-AM, calmidazolium, and genistein, but not nifedipine, suppressed the ERK activation induced by *H. pylori* infection. PD98059 as well as MG132, an NF-κB pathway inhibitor, blocked both IL-8 production and degradation of IκBα induced by *H. pylori* infection, whereas only PD98059 inhibited ERK activity in response to *H. pylori*. There was no significant difference between IL-8 production induced by the *cagA* positive wild-type strain and the *cagA* negative isogenic mutant strain of *H. pylori*; therefore, CagA is not involved in the IL-8 production pathway. *H. pylori*-induced IL-8 production is dominantly regulated by Ca^{2+}/calmodulin signaling, and ERK plays an important role in signal transmission for the efficient activation of *H. pylori*-induced NF-κB activity, resulting in IL-8 production.

Keywords: *H. pylori*; Interleukin-8; NF-κB; Extracellular signal-regulated kinase; Calcium; MKN45 cells

1. Introduction

Helicobacter pylori (*H. pylori*) plays an important role in the pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma. The *cagA* gene of *H. pylori* that encodes over 40 putative bacterial proteins is considered to be associated with the pathogenicity island of *H. pylori* [1,2]. *CagA*-positive *H. pylori* strains are known to induce IL-8 production from gastric epithelial cells *in vitro* and *in vivo* [3,4]. IL-8 is a potent neutrophil-activating chemotactic cytokine or chemokine. Thus, IL-8 release by infected gastric epithelial cells may be instrumental in regulating neutrophil infiltration of the gastric mucosa in *H. pylori* gastritis.

Several recent studies indicate that NF-κB activation plays a dominant role in *H. pylori*-induced IL-8 production in gastric epithelial cells [5,6]. NF-κB is activated as a consequence of phosphorylation and subsequent proteolytic degradation of the IκB protein [7]. Thus, NF-κB activity directly depends on IκB degradation. Activated NF-κB then translocates to the nucleus where it up-regulates IL-8 gene transcription. However, Keates *et al.* [8] reported that

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Abbreviations: IL, interleukin; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor.

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MAPK, rather than NF-κB, plays a critical role for IL-8 production in AGS gastric epithelial cells. Moreover, Aihara *et al.* [9], using MKN45 cells, showed that a tyrosine kinase-mediated pathway may play an important role in *H. pylori*-induced IL-8 production. Thus, because the signal transduction mechanism leading to IL-8 production after *H. pylori* infection has not been defined clearly, we attempted to examine the roles of various signaling molecules activated by *H. pylori*, using MKN45 gastric epithelial cells. In this study, we show a novel signaling pathway in gastric epithelial cells by which *H. pylori* infection signals NF-κB and subsequently IL-8 production, mainly through the Ca^{2+}/calmodulin system, and we propose that ERK-activated downstream of the Ca^{2+}-sensitive tyrosine kinase plays an important role in the efficient activation of the *H. pylori* infection/NF-κB/IL-8 signaling pathway.

2. Materials and methods

2.1. Cell culture

Cells from the human MKN45 gastric epithelial cancer cell line (RIKEN Gene Bank) were maintained in RPMI 1640 containing 10% FBS, l-glutamine, 100 U/mL of penicillin G, and 100 μg/mL of streptomycin. Cell cultures were maintained at 37° in a humidified atmosphere of 95% air and 5% CO_2. For the experiments, cells grown to ~80% confluence on culture wells were incubated with serum-free RPMI 1640 for 12 hr to reduce serum-induced IL-8 release, unless stated otherwise.

2.2. H. pylori culture

H. pylori (*cagA*$^+$ strain 43504, American Type Culture Collection) was plated in blood agar supplemented with 5% FBS in a microaerobic atmosphere at 37°. For the experiments, *H. pylori* was harvested from plates and suspended in RPMI 1640 without FBS.

The isogenic *H. pylori* mutant lacking the *cagA* gene and the wild-type strain (no. 60190) were also studied. The *H. pylori cagA*$^+$ strain 60190 and the *cagA*$^-$ isogenic mutant were obtained from the culture collection of the Campylobacter and Helicobacter Laboratory at Vanderbilt University and have been described previously [10,11]. The bacteria:cell ratio in stimulation experiments ranged between 75:1 and 100:1.

2.3. Reagents

[γ-^{33}P]ATP (111 TBq/mmol) was purchased from New England Nuclear. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was obtained from the Sigma Chemical Co. Antibodies were obtained from the commercial sources listed in parentheses: rabbit polyclonal anti-*H. pylori* antibody and fluorescent isothiocyanate-conjugated swine anti-rabbit immunoglobulin G antibody (DAKO); polyclonal anti-IκBα antibody (Santa Cruz Biotechnology); polyclonal anti-ERK antibody and polyclonal anti-phospho-specific-ERK antibody (New England Biolabs); monoclonal anti-human IL-1β antibody (R&D Systems); and monoclonal anti-human TNF-α antibody (Genzyme). Human recombinant IL-1β and human recombinant TNF-α were obtained from Upstate Biotechnology. All antibodies were dissolved in saline. A23187, BAPTA-AM [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester], W7 [*N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide, HCl], ST638 [α-cyano-(3-ethoxy-4-hydroxy-5-phenylthiomethyl)cinnamide], and PD98059 (2′-amino-3′-methoxyflavone) were purchased from Calbiochem. TMB-8 (3,3′,5,5′-tetramethylbenzidine), EGTA, nifedipine, calmidazolium, genistein, and MG132 (carbobenzoxy-l-leucyl-l-leucyl-l-norvalinal) were purchased from the Sigma Chemical Co. All inhibitors were dissolved in DMSO and diluted with cell culture medium.

2.4. IL-8 measurement

Cells grown on 24-well plates were stimulated with *H. pylori* at 37° in serum-free RPMI 1640 for specified times. Supernatants were then aspirated, centrifuged at 500 *g* at 4° for 10 min, and stored at −80° until assayed. IL-8 levels in the culture supernatants were determined by means of a specific enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech) against human IL-8, according to the instructions of the manufacturer. The detection limit of this assay was 25.6 pg/mL.

2.5. ERK activity

ERK activity was determined as previously reported [12]. Briefly, cells grown on 24-well plates were stimulated with *H. pylori* at 37° in serum-free RPMI 1640 for 60 min. The reaction was terminated by replacement of the medium with ice-cold lysis buffer. The cell lysate (~1 μg of protein) was centrifuged at 14,000 *g* for 10 min at 4° after brief sonication, and the supernatant was assayed with an ERK assay kit (Amersham Pharmacia Biotech) that measured the incorporation of [γ-^{33}P]ATP into a synthetic peptide (KRELVEPLTPAGEAPNQALLR) that is a specific ERK substrate.

2.6. MTT assay for cellular viability

A modified colorimetric assay based on the selective ability of living cells to reduce the yellow salt MTT to formazan was used to quantitate cell viability. After a culture period of 6 hr in the presence of inhibitors, MTT at 5 mg/mL was added to each well for a 2-hr period. After the formation of formazan crystals, the culture medium supernatant was removed from the wells without disruption

of the formazan precipitate. The formazan crystals were then dissolved in 150 μL DMSO/well. The absorbance was measured at 570 nm using a microplate spectrophotometer (SPECTRAmax 340, Molecular Devices). It has been shown previously that viable cell numbers correlate with optical density, as determined by the MTT assay [13].

2.7. H. pylori adherence assay

The adherence assay was carried out as previously reported [14]. Briefly, cells grown on 24-well plates were stimulated for 6 hr with *H. pylori* at 37° in serum-free RPMI 1640. The medium and unbound *H. pylori* were discarded, and the cells with bound *H. pylori* were washed with assay medium and then fixed with 10% formaldehyde. They were incubated overnight with a 1:10 dilution of rabbit anti-*H. pylori* antibody at 4° and washed with PBS. They were incubated further with a 1:20 dilution of fluorescent isothiocyanate-conjugated pig anti-rabbit immunoglobulin G antibody at 24° for 20 min. Fluorescence intensity was determined using a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems).

2.8. Western blotting

Cells grown on 100-mm dishes were stimulated with *H. pylori* at 37° in serum-free RPMI 1640 for specified times. The reaction was terminated by replacement of the medium with 1 mL of ice-cold buffer, pH 7.2, containing 20 mM HEPES, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 1 mM Na_3VO_4, and 1 mM NaF. The cells were collected with a cell scraper into the ice-cold buffer and then were homogenized at 4°. The homogenates were centrifuged at 14,000 *g* for 10 min at 4°, and the cytosolic fraction was subjected to electrophoresis on 10% SDS gels. Proteins in the gel were transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was treated with an anti-IκBα antibody or an anti-phospho-specific ERK antibody. After incubation with secondary anti-rabbit antibodies, immunoreactive proteins were detected with the ECL system (Amersham Pharmacia Biotech).

3. Results

3.1. IL-8 production by H. pylori

H. pylori significantly enhanced IL-8 production in a time-dependent manner for up to 12 hr in MKN45 cells (Fig. 1A). After 3, 4.5, 6, 12, and 24 hr of coculture with *H. pylori* (2×10^7 CFU/mL), IL-8 production was increased by 46.6 ± 5.8, 177.3 ± 47.2, 502.9 ± 88.9, 804.3 ± 30.8, and 816.8 ± 53.7 pg/mL, respectively. The production of IL-8 was dependent upon the number of bacterial cells in

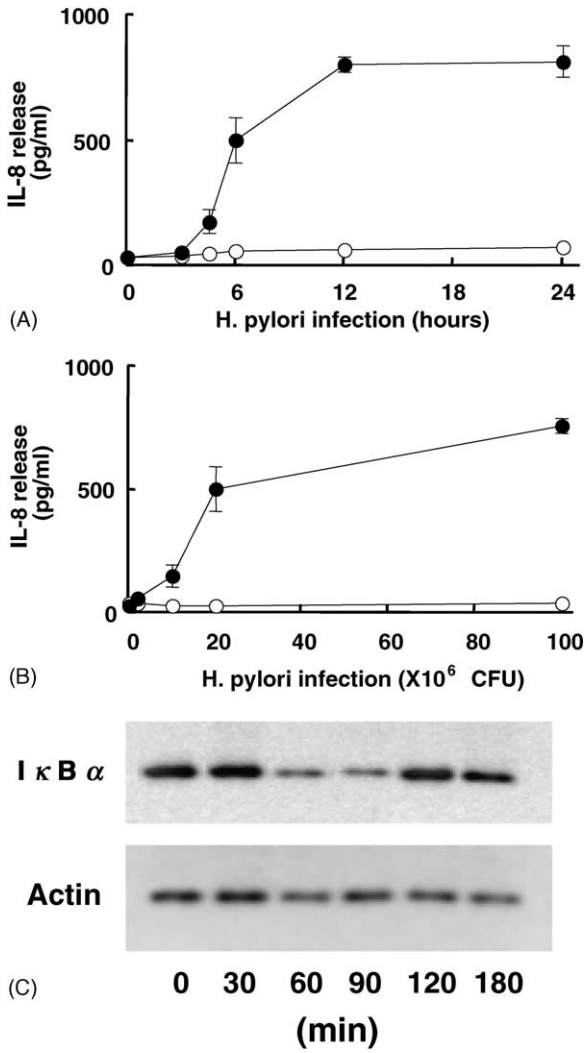

Fig. 1. *H. pylori*-induced IL-8 production and IκBα degradation. (A) MKN45 cells were cocultured with (●) or without (○) *H. pylori* (2×10^7 CFU/mL) for the indicated periods. (B) Cells were cocultured with *H. pylori*, as indicated, for 6 hr. (C) Cells were cocultured with *H. pylori* (2×10^7 CFU/mL) for the indicated periods, and the cell lysate was analyzed by western blotting using an anti-IκBα antibody. The cell lysate was also probed with an anti-actin antibody to confirm equal loading of cell protein. The results in panels A and B are means ± SEM of 4 experiments. The blots are representative of 3 separate experiments.

the range between 1×10^6 and 1×10^8 CFU/mL (Fig. 1B). Treatment with 2×10^7 CFU/mL of *H. pylori* for 6 hr resulted in a submaximal production of IL-8 in the MKN45 cells, whereas no significant production of IL-8 was observed without stimulation. Therefore, subsequent IL-8 experiments were performed with 2×10^7 CFU/mL of *H. pylori* stimulation for 6 hr. NF-κB is sequestered in the cytosol in a complex with IκBα of the inhibitor protein, and is released and activated after phosphorylation of IκBα, which regulates its ubiquitin-dependent degradation [7]. In this study, we assessed the degradation of IκBα protein levels to determine NF-κB activation. *H. pylori* also facilitated the degradation of IκBα in a time-dependent manner. IκBα was degraded from 60 min after *H. pylori* treatment

and recovered to the basal level after 120 min (Fig. 1C). After 180 min, no further degradation was observed until 12 hr (data not shown).

3.2. Calcium and calmodulin-dependent IL-8 production by H. pylori

Since intracellular Ca^{2+} elevation has been reported to be a sufficient stimulus for NF-κB activation [7], we sought to determine whether IL-8 production induced by *H. pylori* is Ca^{2+}-dependent in MKN45 cells. As shown in Fig. 2A, *H. pylori* (2×10^7 CFU/mL)-induced IL-8 production was inhibited completely by pretreatment with BAPTA-AM (10 μM) and TMB-8 (100 μM), commonly used as intracellular Ca^{2+} chelators, but not by pretreatment with EGTA (5 mM) or blockage of L-type Ca^{2+} channels with nifedipine (1 μM). Elevation of cytosolic Ca^{2+} activates a variety of enzymes through interaction with calmodulin [15]. To examine whether calmodulin mediates IL-8 production in response to *H. pylori*, MKN45 cells were preincubated with the calmodulin inhibitors W7 (10 μM) and calmidazolium (10 μM). These drugs completely blocked *H. pylori*- and A23187- (10 μM) induced IL-8 production with no effect on basal IL-8 release (Fig. 2B). These results suggest that *H. pylori* stimulates IL-8 production through a Ca^{2+}/calmodulin-dependent mechanism.

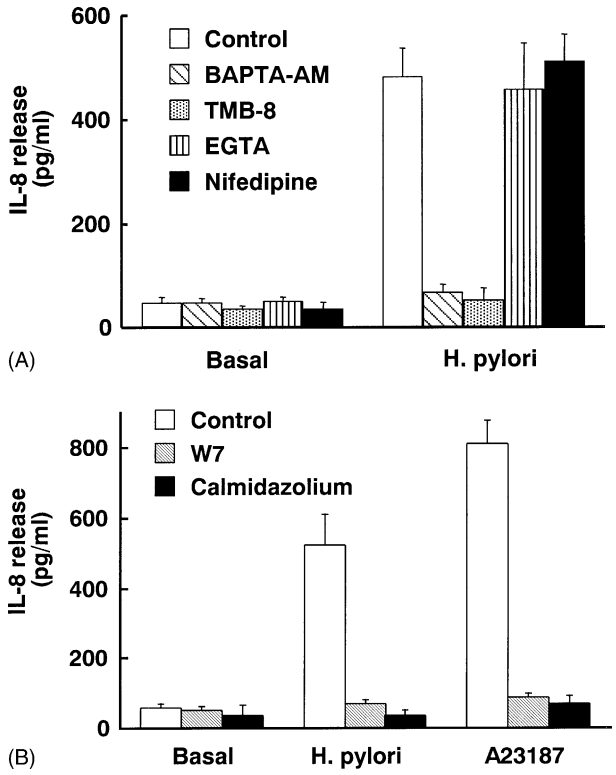

Fig. 2. Effects of Ca^{2+} signal inhibitors on *H. pylori*- and A23187-induced IL-8 production. (A and B) MKN45 cells were pretreated with or without the intracellular Ca^{2+} chelators BAPTA-AM (10 μM) or TMB-8 (100 μM) for 30 min, the extracellular Ca^{2+} chelator EGTA (5 mM) for 3 min, the L-type Ca^{2+} channel blocker nifedipine (1 μM) for 3 min, or the calmodulin inhibitors W7 (10 μM) and calmidazolium (10 μM) for 30 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) or A23187 (10 μM) for 6 hr. Results are the means ± SEM of 3–5 experiments.

3.3. Role of protein tyrosine kinase in calcium-dependent IL-8 production

To determine whether tyrosine kinase activity is required for Ca^{2+}-dependent IL-8 production in response to *H. pylori*, MKN45 cells were preincubated with genistein (30 μM) and ST638 (30 μM), protein kinase inhibitors with a strong preference for tyrosine-specific kinases [16,17], and stimulated by either *H. pylori* (2×10^7 CFU/mL) or A23187 (10 μM). These inhibitors abolished both *H. pylori*- and A23187-induced IL-8 production completely with no effects on basal IL-8 production (Fig. 3). These findings suggest that protein tyrosine kinases activated downstream of the Ca^{2+}/calmodulin pathway are closely involved in *H. pylori*-induced IL-8 production.

3.4. IL-8 production by the cagA negative mutant of H. pylori

H. pylori translocates the bacterial protein CagA into gastric epithelial cells, encoded by the *cag* pathogenicity island, and CagA is tyrosine-phosphorylated and induces changes in the tyrosine phosphorylation state of distinct cellular proteins [18,19]. However, Figs. 2 and 3 demonstrated that treating cells with the Ca^{2+} ionophore A23187-induced IL-8 production, an effect blocked by tyrosine kinase inhibitors and calmodulin inhibitors. To determine whether the CagA protein is the bacterial mediator inducing IL-8 production in the MKN45 cells, we constructed an isogenic *cagA* negative mutant strain of *H. pylori*. There was no significant difference in IL-8 production induced by the *cagA* positive wild-type strain (60190) and the *cagA* negative isogenic mutant, although the production of IL-8

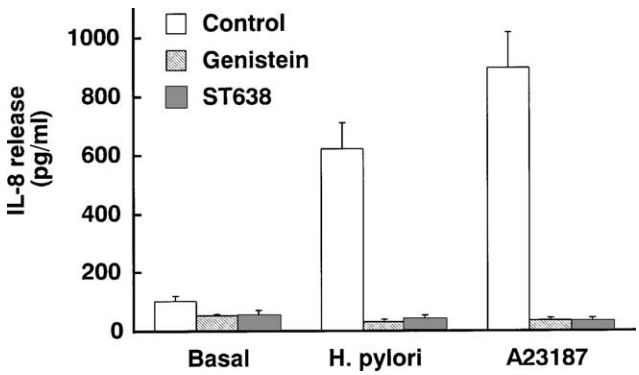

Fig. 3. Effects of tyrosine kinase inhibitors on *H. pylori*- and A23187-induced IL-8 production. MKN45 cells were pretreated with or without the tyrosine kinase inhibitors genistein (30 μM) or ST638 (30 μM) for 30 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) or A23187 (10 μM) for 6 hr. Results are the means ± SEM of 4–5 experiments.

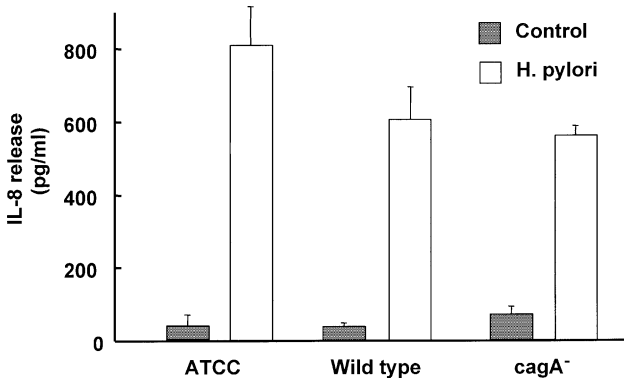

Fig. 4. *H. pylori*-induced IL-8 production. MKN45 cells were cocultured with the *H. pylori cagA*$^+$ strain (ATCC43504; 2×10^7 CFU/mL), the wild type strain (60190; 2×10^7 CFU/mL), or the isogenic mutant lacking the *cagA* gene (*cagA*$^-$; 2×10^7 CFU/mL) for 6 hr. Results are the means ± SEM of 5 experiments.

in the infected cells was decreased (albeit not significantly) compared to that of the uninfected ATCC strain (Fig. 4). Therefore, after *H. pylori* infection, CagA may not be essential for IL-8 production.

3.5. Roles of ERK in IL-8 production by H. pylori

Whereas the synthesis of cytokines has been reported to be mediated via activation of the MAPK family [20,21], we investigated whether IL-8 production induced by *H. pylori* is ERK-dependent in MKN45 cells. As shown in Fig. 5A, *H. pylori* (2×10^7 CFU/mL)-induced IL-8 production was inhibited completely by pretreatment with PD98059 (30 μM), a specific inhibitor of the upstream kinase that activates ERK [22,23] with no effect on basal IL-8 production. PD98059 reduced the *H. pylori*-induced ERK activity

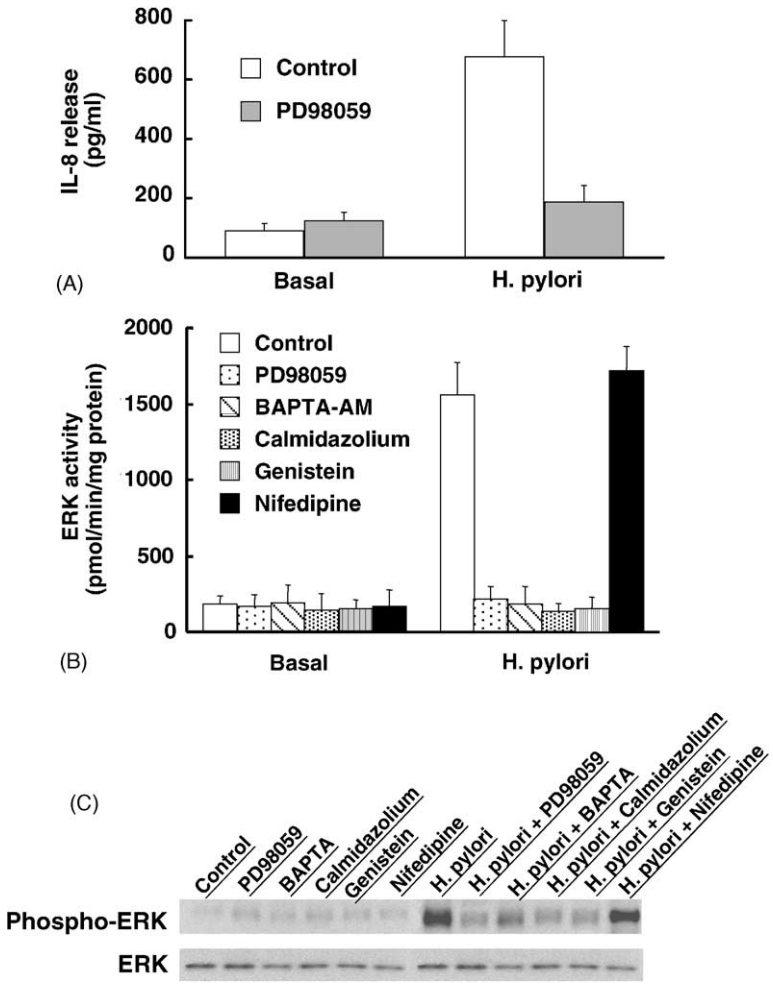

Fig. 5. Effects of an ERK signal inhibitor on *H. pylori*-induced IL-8 production, and of Ca^{2+} signal inhibitors on *H. pylori*-induced ERK activation. (A) MKN45 cells were pretreated with or without the ERK signal inhibitor PD98059 (30 μM) for 30 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) for 6 hr. (B) Cells were pretreated with or without PD98059 (30 μM) for 30 min, the intracellular Ca^{2+} chelator BAPTA-AM (10 μM) for 30 min, the calmodulin inhibitor, calmidazolium (10 μM) for 30 min, the tyrosine kinase inhibitor genistein (30 μM) for 30 min, and the L-type Ca^{2+} channel blocker nifedipine (1 μM) for 3 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) for 60 min. (C) Cells were cocultured with *H. pylori* (2×10^7 CFU/mL) for 60 min, and then the cell lysate was analyzed by western blotting using an anti-phospho-ERK antibody. The cell lysate was also probed with an anti-ERK antibody to confirm equal loading of cell protein. The results in panels A and B are the means ± SEM of 3–5 experiments. The blots are representative of 3 separate experiments.

in MKN45 cells, as did BAPTA-AM (10 μM), calmidazolium (10 μM), and genistein (30 μM), but not nifedipine (1 μM) (Fig. 5B). These findings were also confirmed by determining the phosphorylation level of ERK (Fig. 5C). Thus, it is suggested that *H. pylori*-induced IL-8 production may mediate an ERK pathway through Ca^{2+}/calmodulin-dependent protein tyrosine kinases in MKN45 cells.

3.6. Effects of inhibitors on cytotoxicity and H. pylori adhesion to cells

To confirm that pharmacological inhibitors were not toxic for MKN45 cells, cell viability was assessed using the MTT assay, which measures mitochondrial function. BAPTA-AM (10 μM), TMB-8 (100 μM), W7 (10 μM), calmidazolium (10 μM), genistein (30 μM), ST638 (30 μM), and PD98059 (30 μM) had no effect on cell viability as indicated by the equivalent mitochondrial activities in control and inhibitor-treated cells under the conditions used for all the experiments described in this study (Fig. 6A). In addition, treatment of these inhibitors did not change the adhesion of *H. pylori* (2×10^7 CFU/mL) to MKN45 cells (Fig. 6B).

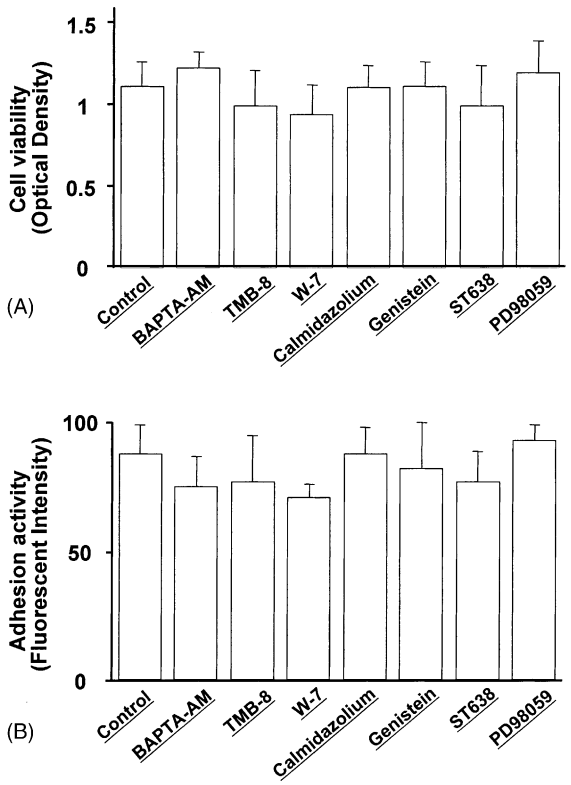

Fig. 6. Effects of pharmacological inhibitors on cytotoxicity and *H. pylori* adhesion. (A) MKN45 cells were pretreated with or without BAPTA-AM (10 μM), TMB-8 (100 μM), W7 (10 μM), calmidazolium (10 μM), genistein (30 μM), ST638 (30 μM), or PD98059 (30 μM) for 8 hr, and then an MTT assay was performed. (B) Cells were pretreated with or without pharmacological inhibitors for 30 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) for 6 hr. *H. pylori* adhesion was determined using an anti-*H. pylori* antibody and a fluorescent isothiocyanate-conjugated immunoglobulin G antibody. Results are the means ± SEM of 3 experiments.

3.7. Effects of IL-1β and TNF-α on H. pylori-induced IL-8 production

It has been reported that *H. pylori* induces the expression of several proinflammatory cytokines, including IL-1β and TNF-α, in gastric mucosa [24], and that these cytokines induce IL-8 production. One possible explanation for the IL-8 production induced by *H. pylori* is that the release of IL-8 is stimulated by autocrine/paracrine secretions of IL-1β and TNF-α produced by epithelial cells. To examine this possibility, we preincubated cells with an anti-IL-1β neutralizing antibody (1 μg/mL) before *H. pylori* coculture or IL-1β stimulation. IL-1β (10 ng/mL)- but not *H. pylori* (2×10^7 CFU/mL)-mediated IL-8 production was inhibited by the anti-IL-1β antibody (Fig. 7A). We also investigated whether TNF-α is associated with *H. pylori* (2×10^7 CFU/mL)-mediated IL-8 production. We preincubated cells with an anti-TNF-α neutralizing antibody (1 μg/mL) before *H. pylori* coculture or TNF-α stimulation. TNF-α (10 ng/mL)- but not *H. pylori* (2×10^7 CFU/mL)-mediated IL-8 production was inhibited by the anti-TNF-α antibody (Fig. 7B). These results suggest that

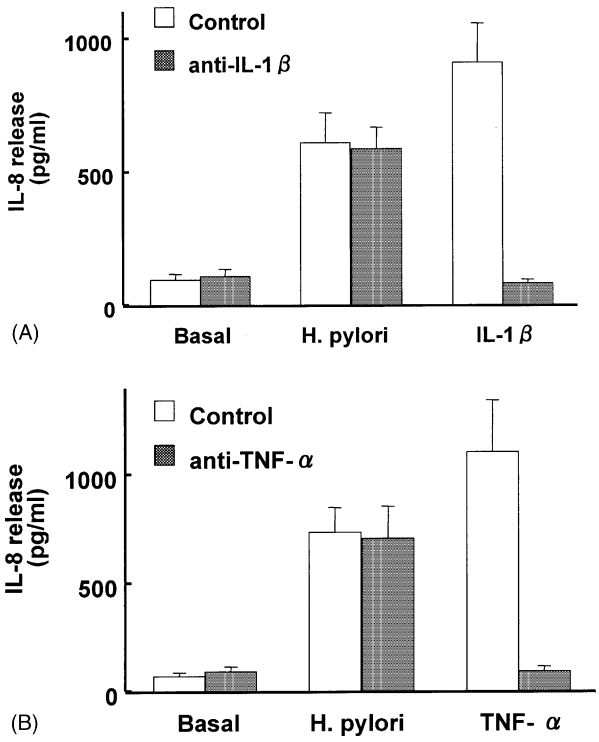

Fig. 7. Effects of neutralizing antibodies on *H. pylori*- and cytokine-induced IL-8 production. (A) MKN45 cells were pretreated with or without an anti-IL-1β antibody (100 ng/mL) for 5 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) or IL-1β (10 ng/mL) for 6 hr. (B) Cells were pretreated with or without an anti-TNF-α antibody (1 μg/mL) for 5 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) or TNF-α (10 ng/mL) for 6 hr. Results are the means ± SEM of 3 experiments.

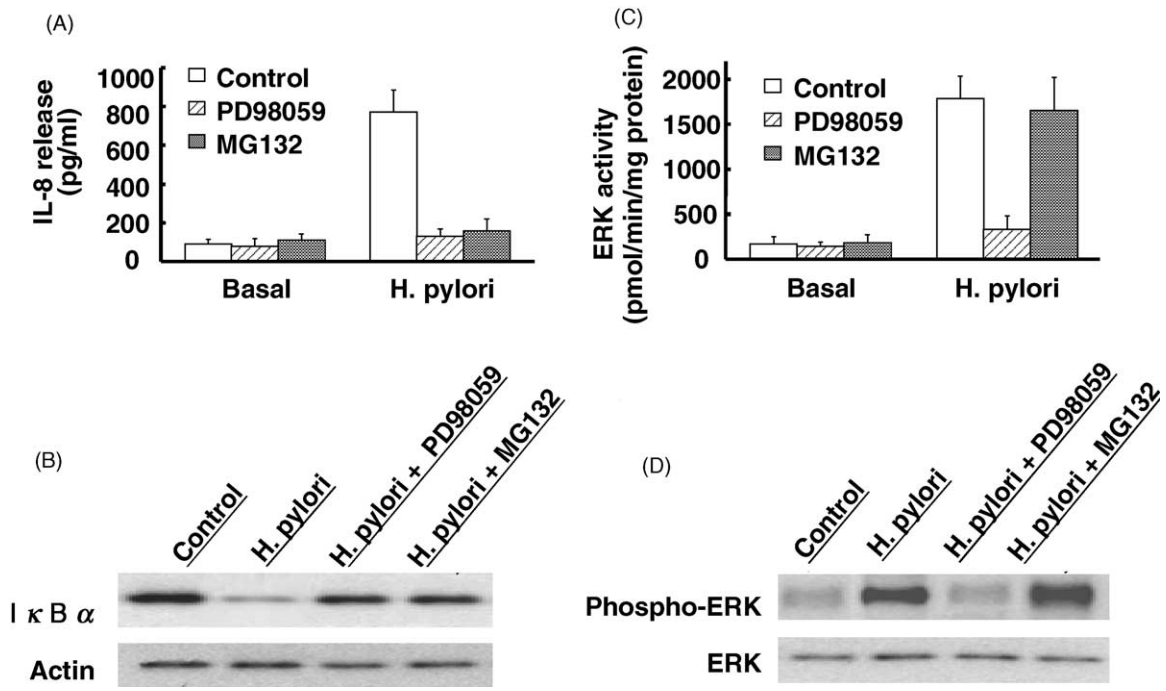

Fig. 8. Effects of PD98059 and MG132 on *H. pylori*-induced IL-8 production, IκBα degradation, and ERK activation. (A) MKN45 cells were pretreated with or without the ERK signal inhibitor PD98059 (30 μM) and the NF-κB pathway inhibitor MG132 (30 μM) for 30 min, and then were cocultured with *H. pylori* (2×10^7 CFU/mL) for 6 hr. (B–D) Cells were cocultured with or without PD98059 (30 μM) and MG132 (30 μM) for 30 min, and then were cocultured with *H pylori* (2×10^7 CFU/mL) for 60 min. Cell lysate was analyzed by western blotting using an anti-IκBα antibody and an anti-phospho-ERK antibody. The results in panels A and C are the means ± SEM of 3–4 experiments. The blots (panels B and D) are representative of 3 separate experiments.

neither IL-1β nor TNF-α affects the release of IL-8 from MKN45 cells after coculture with *H. pylori*.

3.8. NF-κB activation through the ERK pathway

To further characterize the signaling cascade leading to IL-8 production in response to *H. pylori*, we determined whether ERK signaling is involved in NF-κB activation. We tested the effects of PD98059, a specific MEK inhibitor, and MG132, a specific inhibitor of the IκB/NF-κB pathway, through a mechanism that blocks a proteasome that specifically degrades ubiquitinated IκB [25,26], upon *H. pylori*-induced ERK activation and IκBα degradation. Pretreatment of MKN45 cells with PD98059 (30 μM) or MG132 (30 μM) completely blocked both IL-8 production and the degradation of IκBα induced by *H. pylori* (2×10^7 CFU/mL) infection (Fig. 8A and B), whereas PD98059, but not MG132 suppressed the ERK activity and phosphorylation in response to *H. pylori* (Fig. 8C and D). These results suggest that *H. pylori*-mediated downstream signaling of ERK is involved in NF-κB activation.

4. Discussion

The signaling mechanism leading to IL-8 production from *H. pylori* infection has not been clearly defined. In the present report, we have proposed a novel signaling pathway for IL-8 production in gastric epithelial cells infected with *H. pylori* involving NF-κB activation through the ERK cascade and mediated by a Ca^{2+}/calmodulin-dependent tyrosine kinase.

H. pylori infection increased the release of IL-8 from MKN45 cells. IL-8 production was evident within 3 hr of *H. pylori* infection and had peaked at 12 hr. These findings are consistent with previous observations in human epithelial cells, including MKN45 cells [9,27]. *H. pylori* adhesion to gastric epithelial cells *in vitro* and *in vivo* activates NF-κB, and NF-κB is well characterized as a transcriptional regulator of IL-8 production [5,28–32]. Whereas NF-κB is activated as a consequence of phosphorylation and subsequent proteolytic degradation of the IκB protein, NF-κB activation directly depends upon IκB degradation. Therefore, in this study, we assessed the degradation of IκB protein, by western blotting methods, to determine NF-κB activation. *H. pylori* infection also resulted in a marked degradation of IκBα. A previous study suggested that calcium is required to trigger NF-κB activation in T cells [33]. In the present study, the inhibitory effects of intracellular Ca^{2+} chelators suggest a critical role for Ca^{2+} in *H. pylori*-induced IL-8 production in MKN45 cells. We showed further evidence that this Ca^{2+}-dependent IL-8 production by *H. pylori* was mediated predominantly by the release of Ca^{2+} from intracellular stores, rather than by Ca^{2+} influx through the L-type Ca^{2+} channel, by demonstrating that the *H. pylori*-induced IL-8 production was

abolished by intracellular Ca^{2+} chelators and yet was insensitive to either an L-type Ca^{2+} channel blocker or extracellular Ca^{2+} chelation.

Aihara *et al.* [9] reported that in gastric epithelial cells *H. pylori*-induced IL-8 production as well as NF-κB activation through a tyrosine kinase-sensitive pathway, which agrees well with the present observations. We extended their study further by examining the transduction pathways induced by *H. pylori* infection for the production of IL-8 in more detail and found a key role for a Ca^{2+}/calmodulin-dependent tyrosine kinase. The results presented here show that both *H. pylori*- and A23187-induced IL-8 production were blocked completely by two well-characterized calmodulin inhibitors, W-7 and calmidazolium, and two different tyrosine kinase inhibitors, genistein and ST638, suggesting that tyrosine kinases activated by Ca^{2+}/calmodulin-dependent pathways play a central role in IL-8 production after *H. pylori* infection. However, it remains to be determined whether Ca^{2+}/calmodulin-dependent IL-8 production is due to the activation of nonreceptor tyrosine kinases, inhibition of a phosphotyrosine phosphatase, or an unidentified mechanism. Ca^{2+}-dependent activation of a novel focal adhesion kinase family protein tyrosine kinase, PYK2, purified from the bovine uterus [34], has been shown to mediate ERK activation in neuronal cells [35]. PYK2 may be involved in this pathway. However, PYK2, which has been shown to transmit a Ca^{2+} signal, lacks a calmodulin-binding motif and is not directly activated by Ca^{2+} [35]. Moreover, it has been reported that PYK2 was expressed in cells of neuronal origin and in the epithelial cells of the small intestine [36]. Thus, it is unlikely that PYK2 is a direct candidate, and further studies are required to identify a Ca^{2+}/calmodulin-dependent pathway leading to IL-8 production.

Interestingly, Odenbreit *et al.* [18] and Asahi *et al.* [19] recently reported that *H. pylori* translocates the bacterial protein CagA into gastric epithelial cells. CagA is tyrosine-phosphorylated and induces changes in the tyrosine phosphorylation state of distinct cellular proteins; moreover, another report indicated that there was a correlation between the ability to phosphorylate and IL-8 production [37]. However, we found that deletion of the CagA protein did not affect the ability of *H. pylori* to induce the production of IL-8 from gastric epithelial cells by using an isogenic *cagA* negative mutant strain of *H. pylori*. In agreement with our results, Sharma *et al.* [4] and Crabtree *et al.* [38] previously reported that CagA is not an inducer of IL-8 secretion from gastric epithelial cells. Also, activation of the Rho GTPases Rac1 and Cdc42 by *H. pylori* is *cagA*-independent [39]. Therefore, the CagA protein is not functionally involved in the IL-8 production pathway. The function of the CagA protein remains to be determined.

In this study, we confirmed the effects of pharmacological inhibitors, including tyrosine kinases, on *H. pylori* adhesion to MKN45 cells and also cell viability for 6 hr after *H. pylori* infection. These inhibitors did not affect the adhesion of *H. pylori* to MKN45 cells or cell viability under our experimental conditions. Recently, Su *et al.* [40] showed that *H. pylori* adherence to AGS cells, a gastric adenocarcinoma cell line, was reduced by genistein, a tyrosine kinase inhibitor. However, they used the inhibitor at cytotoxic concentrations (100–200 μM). We found that genistein treatment at a concentration of 100 μM decreased the adhesion of *H. pylori* to AGS and MKN28 cells, but it also significantly reduced cell viability (data not shown).

Our present data demonstrate that the MEK-specific inhibitor PD98059 [22] nearly abolished *H. pylori*-induced IL-8 production in MKN45 cells. Furthermore, *H. pylori*-induced ERK activation was blocked by either chelation of intracellular Ca^{2+} or a tyrosine kinase inhibitor, suggesting the involvement of a Ca^{2+}/calmodulin-dependent ERK pathway in the induction of IL-8 production by *H. pylori*. Recently, Meyer-ter-Vehn *et al.* [41] reported that *H. pylori* activates the ERK pathway in AGS cells. In addition, Hobbie *et al.* [20] reported that *Salmonella typhimurium* leads to the activation of ERK, which results in the expression of the IL-8 gene in cultured intestinal epithelial cells. Taken together, these data suggest that IL-8 production induced by *H. pylori* infection is mediated by a Ca^{2+}/calmodulin-dependent tyrosine kinase, resulting in the activation of ERK. ERK regulates cell proliferation and differentiation; hence, the activation of gastric epithelial cell ERK by *H. pylori* may be instrumental in inducing gastroduodenal ulceration and neoplasia.

H. pylori elicits the release of cytokines, such as IL-1β and TNF-α [42], including IL-8. In view of this, we evaluated whether autocrine release of IL-1β and TNF-α plays a role in IL-8 production mediated by *H. pylori*. However, direct involvement of IL-1β and TNF-α in *H. pylori*-mediated IL-8 production was excluded in our study, showing that neither anti-IL-1β nor anti-TNF-α neutralizing antibody inhibited *H. pylori*-mediated IL-8 production.

The involvement of the MAPK (ERK, p38, and JNK) pathways in NF-κB activation has been very well documented [43–45]. However, more recently, Keates *et al.* [8] reported that ERKs are not required for *H. pylori*-mediated IκB degradation or NF-κB activation in AGS cells. Thus, we examined whether ERK activation by *H. pylori* is an upstream signaling pathway of IκB degradation and, hence, IL-8 production. Although both PD98059, an ERK cascade inhibitor, and MG132, an NF-κB pathway inhibitor, were effective in blocking IL-8 production and IκB degradation, PD98059, but not MG132, suppressed ERK activity in response to *H. pylori*. Our results indicate that the stimulation of the ERK pathways by *H. pylori* may be directly responsible for the activation of the NF-κB transcription factors and subsequent synthesis of IL-8.

In conclusion, we have demonstrated that in gastric epithelial cells, *H. pylori*-induced IL-8 production is dominantly regulated by Ca^{2+}/calmodulin signaling and that

ERK plays an important role in signal transmission for the efficient activation of *H. pylori*-induced NF-κB activation, resulting in the production of IL-8. The characterization and identification of the putative effector molecules that transmit *H. pylori*-induced calcium signals and lead to ERK activation are under investigation.

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