

Differential Role of ERK and p38 on NF- κ B Activation in *Helicobacter pylori*-Infected Gastric Epithelial Cells

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Gastric cancer, as well as inflammation, caused by *Helicobacter pylori*, activates the production of chemokines by activation of redox-sensitive transcription factor NF- κ B in gastric epithelial cells. Mitogen-activated protein kinases including extracellular signal-regulated kinase (ERK) and p38 kinase (p38) are activated by *Helicobacter pylori*, which may regulate NF- κ B activation in the infected cells. However the mechanisms how ERK and p38 induce NF- κ B activation have not been investigated. Present study aims to investigate the role of ERK and p38 on the activation of NF- κ B in *Helicobacter pylori*-infected AGS cells. Western blot analysis was performed for determining the levels of I κ B, p105, p50 and p65 in gastric epithelial cells infected with *Helicobacter pylori* and treated with ERK inhibitor U0126 and p38 inhibitor SB203580. *Helicobacter pylori* induced the degradation of I κ B α and upregulation of p105, p50 and p65 in the infected cells. U0126 inhibited the degradation of I κ B α while SB203580 suppressed expression of p105, p50 and p65 in *Helicobacter pylori*-infected cells. ERK and p38 differentially activate NF- κ B; ERK induces degradation of I κ B α while p38 upregulates the expression of p50 and p65, subunits of NF- κ B in *Helicobacter pylori*-infected gastric epithelial AGS cells. (*J Cancer Prev* 2013;18:346-350)

Key Words: ERK, p38, NF- κ B, *Helicobacter pylori*

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been considered as a major etiologic agent causing chronic gastritis, along with other features, including lymphoid follicles or lymphoid aggregates, surface epithelial degradation with mucous depletion, and intestinal metaplasia.¹ One of the potential toxic factors involving *H. pylori*-induced gastric injury is oxygen radicals, which are released from activated neutrophils since *H. pylori* exhibits chemotactic activity for neutrophils.² Thus, neutrophil infiltration of the gastric epithelium was the initial pathological abnormality described in *H. pylori* gastritis and remains a hallmark of active infection. Chemoattractant cytokine (chemokine) response

is particularly important in the early stages of *H. pylori*-induced inflammation. Chemokines modulate leukocyte adhesion and activate signal transduction cascades, leading to novel gene expression programs. These also mediate other leukocyte function necessary for leukocytes to leave the circulation and infiltrate tissues. Thus, increase of chemokine production and release is an important mechanism for leukocyte recruitment in response to injury or infection.

NF- κ B is a member of the Rel family including p50 (NF- κ B1), p52 (NF- κ B2), Rel A (p65), c-Rel, Rel B, and *Drosophila* morphogen dorsal gene product.³ In resting cells, NF- κ B is localized in the cytoplasm as a hetero- or homodimer, which are non-covalently associated with

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cytoplasmic inhibitory proteins, including I κ B α . Upon stimulation by a variety of pathogenic inducers such as viruses, mitogens, bacteria, agents providing oxygen radicals and inflammatory cytokines, the NF- κ B complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes.⁴ Previously we found that *H. pylori* increased lipid peroxidation, an indicative of oxidative damage, and induced the activation of two species of NF- κ B dimers (a p50/p65 heterodimer and a p50 homodimer) in gastric epithelial cells.^{5,6} Pyrrolidone dithiocarbamate (PDTC), a proven free radical scavenger and NF- κ B inhibitor, potentially inhibits NF- κ B interaction with its upstream regulatory binding site thereby preventing NF- κ B-mediated transcriptional activation in *H. pylori*-infected gastric epithelial cells.⁷ In addition, p105, the precursor of p50 subunit of NF- κ B is processed by an ATP-dependent process that requires proteasomes and ubiquitin conjugation. The C-terminal region of p105 is rapidly degraded, leaving the N-terminal p50 domain.⁸

Mitogen-activated protein kinases (MAPKs) comprise an important group of serine and threonine signaling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations, leading to activation of transcription factors. Among MAPK, extracellular signal-regulated kinase (ERK) pathway is linked to cellular proliferation and differentiation as well as proinflammatory cellular response. Previously we showed the activation of ERK and p38 in *H. pylori*-infected gastric epithelial cells, which is upstream signaling for NF- κ B activation in gastric epithelial AGS cells.⁹ However, ERK and p38 may differentially activate NF- κ B in gastric epithelial AGS cells infected with *H. pylori*. In this study, we examined the role of ERK and p38 on the activation of NF- κ B in by *H. pylori*-infected AGS cells, by determining the levels of I κ B, p105, p50 and p65 in gastric epithelial cells infected by *H. pylori* and treated with ERK inhibitor U0126 and p38 inhibitor SB203580.

MATERIALS AND METHODS

1. *H. pylori* strain

H. pylori strains NCTC 11637 was obtained from the

National Collection of Type Cultures (NCTC; Colindale, United Kingdom). *H. pylori* was grown on chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Maryland) for 24 h in a microaerobic and humidified atmosphere at 37°C.

2. Cell culture and *H. pylori* infection

Human gastric cancer AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (Rockville, Maryland) and grown in RPMI-1640 medium (pH 7.4; Sigma, St. Louis, Missouri) media with 10% fetal bovine serum, 4 mM glutamine (GIBCO-BRL, Grand Island, New York) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cells were seeded in 12-well cell culture plates at 10⁵ cells per well in a volume of 1 ml and cultured to reach 80% confluency. Prior to stimulation, each well was washed twice with 1 ml of fresh cell culture medium containing no antibiotics. Bacterial cells were harvested, washed with phosphate buffered saline (PBS), and then resuspended in antibiotic-free cell culture medium. The bacterial cells were added to the cultured cells at a bacterium/cell ratio of 500:1 in a 1 ml volume. A ratio of bacterium/cell was adapted from previous studies.^{5,6} An ERK inhibitor U0126 (Catalog # 9903, Cell Signaling Technology, Inc., Beverly, MA, USA) and a p38 inhibitor SB203580 (Catalog # 559389, Calbiochem Biochemicals, San Diego, CA, USA) were dissolved in dimethylsulfoxide at 50 mM stock solution. The inhibitors, at 20 μ M final concentration, were pre-treated to the culture medium before the treatment of *H. pylori*.

3. Preparation of extracts

Whole cell and nuclear extracts were prepared for respective Western blot analysis. Briefly, the harvested cells were extracted with lysis buffer (10 mM Tris-HCl, pH7.4, 10% Nonidet P-40 and protease inhibitor cocktail) and centrifuged. The supernatants were used for whole cell extracts. To prepare nuclear extracts, the cells were rinsed with ice-cold PBS, harvested by scraping into PBS, and pelleted by centrifugation at 1,500 g for 5 min. The cells were lysed in buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.5

mM MgCl₂, 0.2% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonylfluoride (PMSF). The nuclear pellet was resuspended on ice in nuclear extraction buffer containing 20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl₂, 25% glycerol, 1 mM DTT, and 0.5 mM PMSF. Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

4. Western blot analysis

100 μ g of cellular protein was loaded per lane, separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions, and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, Illinois) by electroblotting. The transfer of protein and equality of loading in all lanes was verified using reversible staining with Ponceau S. The membranes were blocked using 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.15% Tween 20) for 3 h at room temperature. The proteins were detected with polyclonal antibodies for I κ B α , p105, p50 and p65 at 1:2000 dilution (all from Cell Signaling Technology, Inc., Beverly, Massachusetts) diluted in TBS-T containing 5% dry milk, and incubated at 4°C overnight.

After washing in TBS-T, the immunoreactive proteins were visualized using goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase, which was followed by enhanced chemiluminescence (Amersham). Exactly equal amount of protein, determined by Bradford method,¹⁰ was loaded in each lane. The Western result presented in each figure is the representative of five separate experiments.

RESULTS

The cells with *H. pylori* were cultured for 4 h. Fig. 1A shows that *H. pylori* induced the degradation of I κ B α at 1 h (Fig. 1A). To confirm that observed increase in NF- κ B gene transactivation parallels increased p50 and p65 nuclear translocation, we determined cytosolic and nuclear levels of p50 and p65 (Fig. 1B). Western blot analyses showed significant decrease in p50 and p65 levels in cytosol 1 h and 2 h after *H. pylori* respectively in the gastric epithelial AGS cells. At the same time, nuclear p50 and p65 levels increased.

H. pylori-induced upregulation of p105, p50 and p65 was shown at 1 h, which was increased until 4 h (Fig. 1C). Since

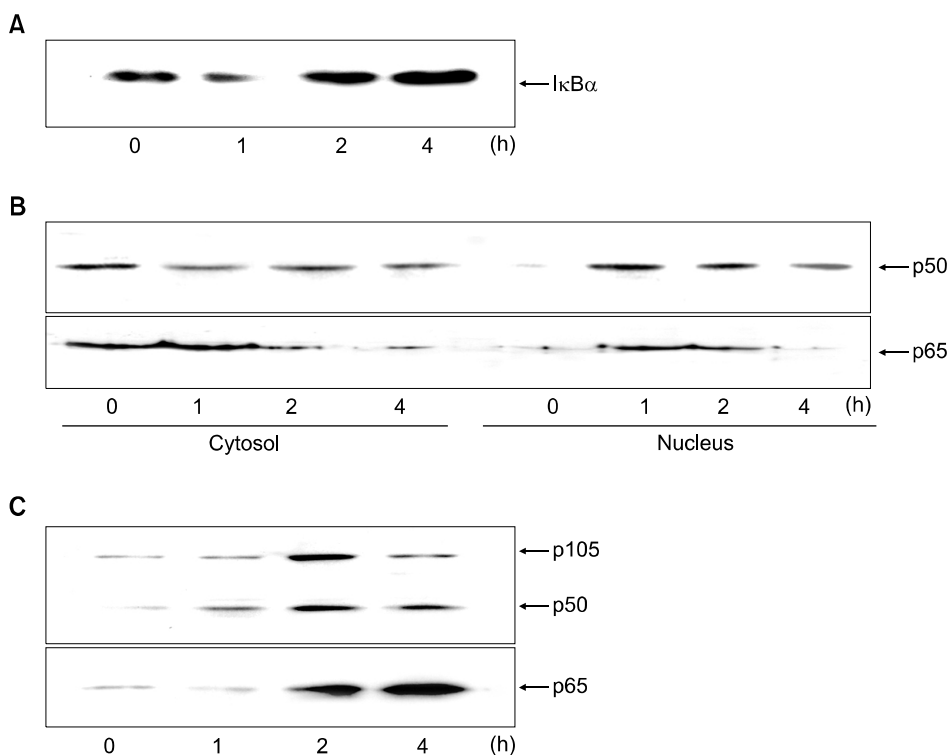


Fig. 1. Protein levels of I κ B α , p105, p50 and p65 in *H. pylori*-infected AGS cells. The cells were seeded in 12-well culture plates at 10⁵ cells per well and cultured to reach 80% confluency. The bacterial cells were added to the cultured cells at a bacterium/cell ratio of 500:1. The cells were cultured for 4 h. The protein levels in whole cell extracts (A, C) and the protein levels in cytosolic extracts and nuclear extracts (B) were shown. Western blot result in each lane is the representative of five separate experiments.

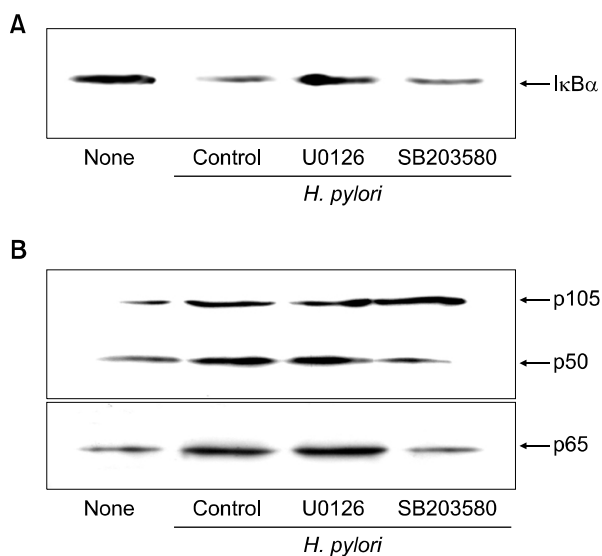


Fig. 2. Protein levels of I κ B α , p105, p50 and p65 in *H. pylori*-infected AGS cells treated with MAPK inhibitors. AGS cells were seeded in 12-well culture plates at 10^5 cells per well and cultured to reach 80% confluency. The MAP kinase inhibitors, U0126 (an ERK inhibitor) and SB203580 (a p38 inhibitor) (at 20 μ M final concentration), were pretreated to the culture medium 1 h before the treatment of *H. pylori*. The bacterial cells were added to the cultured cells at a bacterium/cell ratio of 500:1 for 1 h (I κ B α , A) and 2 h (p105, p50 and p65, B), respectively. None, AGS cells without treatment and cultured in the absence of *H. pylori*; Control, AGS cells without treatment and cultured in the presence of *H. pylori*; U0126, AGS cells treated with U0126 and cultured in the presence of *H. pylori*; SB203580, AGS cells treated with SB203580 and cultured in the presence of *H. pylori*.

p105 is a precursor of p50, increased expression of p105 may induce upregulation of p50. These results suggest that NF- κ B activation may be induced by upregulation of NF- κ B subunits (p50, p65) as well as degradation of I κ B α in the infected cells.

The cells were pretreated with the MAP kinase inhibitors, U0126 (an ERK inhibitor) and SB203580 (a p38 inhibitor) at 20 μ M final concentration for 1 h, and then infected with *H. pylori* for 1 h (of I κ B α degradation) and 2 h (expression levels of p105, p50, and p65). U0126 inhibited the degradation of I κ B α (Fig. 2A) while SB203580 suppressed the expression of p105, p50 and p65 in *H. pylori*-infected cells (Fig. 2B). These results demonstrate involvement of ERK and p38 in the activation of NF- κ B differentially in *H. pylori*-infected AGS cells.

DISCUSSION

MAPKs are known to be involved in signaling, which leads to the synthesis of pro-inflammatory cytokines and chemokines. The NF- κ B element is believed to be the main regulator of inducible expression of inflammatory genes. Expression of IL-8 gene was markedly up-regulated at the levels of mRNA and protein, which was in parallel with the activation of NF- κ B and increase in phospho-specific ERK1/2, JNK2/1 and p38 by *H. pylori*, in AGS cells. We observed that activation of NF- κ B was inhibited by treatment with MAPK inhibitors (U0126 as an ERK inhibitor or SB203580 as a p38 inhibitor) in *H. pylori*-infected AGS cells. The results suggest that induction of cytokines by *H. pylori* may depend on the activation of MAPK cascade and NF- κ B in gastric epithelial cells.

Keates et al.¹¹ reported that direct contact of *H. pylori* with gastric epithelial cells activates NF- κ B *in vitro*. Both the phosphorylation and proteolytic degradation of I κ B α allows the release and nuclear transmigration of NF- κ B, which may be induced by oxygen radicals.^{3,4} We previously demonstrated that lipid peroxidation, an index of oxidative membrane damage, increased by *H. pylori* in gastric epithelial AGS and Kato III cells, which was in parallel with a time course stimulation of IL-8 production.^{5,6} More recently Shimoyama et al.¹² reported that oxygen radicals are important mediators for chemokine expression in human neutrophils stimulated by *H. pylori*. Since oxidative stress is an important regulator of chemokine gene expression¹³ and an inducer of the NF- κ B,^{3,4,14} antioxidants might be beneficial for the treatment of *H. pylori*-induced gastric mucosal injury and inflammation caused by oxidant-mediated chemokine production.

MAPK family are composed with JNK1/2, ERK and p38 kinase. Several lines of evidence support the role of JNK pathway, which plays a major role in cellular functions, such as cell proliferation and transformation, whereas the ERK pathway was shown to suppress apoptosis and enhance cell survival or tumorigenesis.¹⁵

It has been suggested that NF- κ B activation may be mediated by two distinct signaling pathways. First, the NF- κ B translocation dependent I κ B α phosphorylation and degradation.¹⁶ Second, phosphorylation of ERK and p38

MAPK leads to NF- κ B translocation.¹⁷ Although the effect of *H. pylori* on NF- κ B activation was well studied, the modulation of NF- κ B activation through the IKK/NIK pathway between the p38 sub-group of MAP kinase has not been reported.

Furthermore, our data suggest that a reduction in ERK activation by U0126 may inhibit I κ B α degradation. Although, previous reports indicate that MAP kinase activation may be involved in the activation of NF- κ B,¹⁸ very little is known about interaction of MEK/ERK pathway with I κ B α /NF- κ B. Previous study along with the present data support the idea of the potential crosstalk between the ERK and the I κ B α /NF- κ B signal transduction pathways following *H. pylori* infection.

In conclusion, MAP kinases, such as ERK and p38, may control the activation of NF- κ B in *H. pylori*-infected AGS cells in a different way. Activated ERK induces the dissociation of I κ B α from NF- κ B, therefore allowing nuclear translocation and DNA-binding of NF- κ B. p38 induces the expression of p65 and p50. Therefore, *H. pylori* activates transcription factor NF- κ B, which is mediated by ERK and p38 in gastric epithelial cells.

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