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β-CAROTENE INHIBITS HELICOBACTER PYLORI-INDUCED EXPRESSION OF INDUCIBLE NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE-2 IN HUMAN GASTRIC EPITHELIAL AGS CELLS

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Reactive oxygen species (ROS) play critical roles in Helicobacter pylori (H. pylori)-associated gastric ulceration and carcinogenesis. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are involved in H. pylori-induced gastric diseases. Previously we demonstrated that H. pylori in Korean isolates induced the activation of mitogen-activated protein kinases (MAPK) and oxidant-sensitive transcription factors NF-κB and AP-1 which mediates the expression of iNOS and COX-2 in gastric epithelial AGS cells. β-Carotene shows antioxidant activity and inhibits NF-κB-dependent gene expression in various cells. Present study aims to investigate whether β-carotene inhibits H. pylori-induced expression of iNOS and COX-2 by suppressing the activation of MAPK, NF-κB, and AP-1 in gastric epithelial AGS cells. HP99 (H. pylori in Korean isolates) was added to AGS cells at the ratio of bacterium/cell, 300/1. β-carotene inhibited H. pylori-induced increase in ROS level, the activation of MAPK (p38, the c-Jun NH2-terminal protein kinases, the extracellular signal-regulated kinases), NF-κB, and AP-1 and the expression of iNOS and COX-2 in AGS cells. Conclusion: β-carotene inhibits oxidant-mediated activation of inflammatory signaling and suppresses the expression of iNOS and COX-2 in gastric epithelial AGS cells infected with H. pylori.

K e y w o r d s: Helicobacter pylori, β-carotene, inducible nitric oxide synthase, cyclooxygenase-2, reactive oxygen species

INTRODUCTION

One of the potential toxic factors in the pathogenesis of Helicobacter pylori (H. pylori)-induced gastric injury is reactive oxygen species (ROS) (1, 2). Previously we showed that H. pylori induced ROS in gastric epithelial cells in the absence of inflammatory cells such as neutrophils (3, 4). The results demonstrate that H. pylori may directly turn on transcription of inflammatory genes prior to the recruitment of inflammatory cells. Since ROS are involved in the expression of chemokines IL-8 and MCP-1 (5, 6), ROS produced in the epithelial cells may mediate the chemotactic action for neutrophils and monocytes in H. pylori-infected tissues. Infiltration of immune/inflammatory cells may propagate the inflammatory events by producing large amounts of ROS in the infected tissues.

Transcription of iNOS is induced by various stimuli including lipopolysaccharide (LPS), cytokines, and bacterial wall products. Inducible nitric oxide synthase (iNOS) is a potent pro-inflammatory mediator. NO produced by iNOS is a critical component of host defenses against bacteria, viruses, and parasites (7, 8). However, large amounts of NO produced by iNOS contribute to gastric cell injury by producing peroxynitrite, which is a reaction product between NO and superoxide. iNOS activity was enhanced in gastric mucosa of the patients with H. pylori-positive duodenal ulcers (9-11).

Cyclooxygenase-2 (COX-2) is induced in gastrointestinal epithelial cells in response to inflammatory cytokines, LPS, mitogens, and ROS (12). COX-2 is responsible for tumor cell proliferation, angiogenesis, and tumor invasion in various tissues. Our previous study showed that COX-2 expression is related to gastric epithelial cell proliferation (13). Expression of COX-2 is strongly correlated with the extent of chronic inflammatory cell infiltration which is related to H. pylori-associated acute and chronic antral inflammation (9, 14-16). Although the role of COX-2 in gastric tissue is uncertain, highly and chronically expressed COX-2 may play an important role in gastric carcinogenesis and propagation of gastric inflammation in H. pylori-infected gastric tissues since prostaglandins produced via COX-2 are believed to be the major contributors to the inflammatory process and can be mutagenic and tumorigenic (17, 18).

Expression of inflammatory genes such as iNOS and COX-2 is primarily controlled at the transcriptional level mediated by nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (10, 13, 19). These transcription factors are reported to be activated by ROS. Therefore, the balance between oxidants and antioxidants ultimately determines the activity profile for these transcription factors. In resting cells, NF-κB is localized in the cytoplasm as a hetero- or homo-dimer, which is noncovalently associated with cytoplasmic inhibitory proteins, including IkBα. Upon stimulation by a variety of pathogenic inducers, the NF-κB...
complex migrates into the nucleus and binds DNA recognition sites in the regulatory regions of the target genes (20). The pro-inflammatory proteins such as COX-2 and iNOS act through distinct signaling pathways that converge on the phosphorylation and degradation of IkBζ (21-23). AP-1 is composed of homodimer or heterodimer of members of the Fos and Jun families, which bind to the TRE (TPA responsive element) motif to mediate gene transcription. AP-1 regulates the gene expression in response to a wide array of extracellular stimuli including growth factors, tumor promoters, and cytokines (24). In relation to H. pylori infection, the expression of iNOS and COX-2 gene was reported to require the activation of NF-κB and AP-1 in human gastric epithelial AGS cells (6, 25, 26).

The activation of mitogen activated protein kinase (MAPK) cascade was observed in H. pylori-infected gastric epithelial cells (6, 27). Three major subfamilies of MAPK have been identified in mammalian cells; the extracellular signal-regulated kinases (ERK), the c-Jun NH2-terminal protein kinases (JNK), and p38. In our previous study, we demonstrated that inhibition of MAPK using MAPK inhibitors suppressed the activation of NF-κB and AP-1 by H. pylori. The results suggest that MAPK cascade may act as the upstream signaling for the activation of NF-kB and AP-1 (6). The scavengers of hydroxyl radicals such as N-acetylcysteine (NAC), mannitol, dimethylthiourea, and rehampide inhibited NF-κB and AP-1 activation in gastric epithelial AGS cells (3, 4, 28). The studies demonstrate that ROS may be involved in the activation of the inflammatory mediators and redox-sensitive transcription factors in H. pylori-infected gastric epithelial cells.

β-Carotene is responsible for the orange color of the many fruits and vegetables such as carrots and sweet potatoes. As an anti-cancer agent, β-carotene induces cell growth inhibition and apoptosis in human breast cancer and colon cancer (29, 30). Moreover, β-carotene prevents the development of inflammatory diseases such as atherosclerosis and inhibits inflammatory gene expression as an anti-inflammatory agent (31, 32). β-Carotene inhibited COX-2 signaling in colon cancer cells (30) and suppressed the inflammatory signaling through the inhibition of NF-κB activation (33). In Korea, β-carotene is more easily available than other carotenoids such as lycopene because a traditional Korean diet is mainly composed of dark green vegetables. β-Carotene is more readily absorbed than other β-carotenoids such as lycopene because a traditional Korean diet is mainly composed of dark green vegetables. β-Carotene was reported to require the activation of NF-κB (34, 35). Although many studies have demonstrated the beneficial effect of β-carotene on inflammation, the detailed mechanism has not been clarified yet.

Present study is purposed to investigate whether β-carotene inhibits the expression of iNOS and COX-2 by suppressing the activation of inflammatory mediators and transcription factors such as MAPK, NF-κB, and AP-1 in gastric epithelial AGS cells infected with H. pylori in Korean isolates.

**MATERIALS AND METHODS**

**Bacterial strain**

H. pylori in Korean isolates (HP99) containing cagA+, vacA s1b, vacA m2, iceA1 (6) was kindly provided by Dr. HC Jung (Seoul National University College of Medicine, Seoul, Korea). These bacteria were inoculated onto chocolate agar plates (Seoul National University College of Medicine, Seoul, Korea). H. pylori strain ATCC CRL 1739 was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 4 mM glutamine, and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin, all from GibCO-BRL, Grand Island, NY, USA). The cells were seeded in 6-well cell culture plate at 3 x 10⁴ cells per well and cultured to reach 80% confluency. Prior to stimulation, each well was washed with 2 ml of fresh culture medium containing no antibiotics. H. pylori was harvested from a chocolate agar plate and suspended in antibiotic-free RPMI-1640 medium supplemented with 10% fetal bovine serum. The bacterial cells were added to the cultured cells at a bacterium/cell ratio of 300:1. A ratio of bacterium/cell was adapted from previous study (25).

**Experimental protocol**

To determine whether β-carotene inhibits H. pylori-induced activation of NF-κB, AP-1 and MAPK and thus suppresses the expression of iNOS and COX-2 in gastric epithelial AGS cells, β-carotene (Sigma, St. Louis, MO, USA) was dissolved in tetrahydrofuran (final concentration of 2, 10, 20 µM) was treated to the cells 2 h prior to H. pylori stimulation. mRNA and protein expression of COX-2 and iNOS were determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis (at 12 h) and Western blot analysis (at 24 h). Activation of transcription factors was assessed by electrophoretic mobility shift assay (EMSA) using nuclear extracts of the cells (at 1 h). Western blotting for total and phospho-specific forms of MAPK was performed at 30 min while protein level of IκBα was determined at 1 h. The level of ROS in the cells was determined at 30 min by confocal microscopy. The concentration of tetrahydrofuran was not exceeded to 0.1%. The control group received tetrahydrofuran instead of β-carotene.

**RT-PCR analysis for iNOS and COX-2**

Gene expressions of COX-2 and iNOS mRNA were assessed using RT-PCR standardized by coamplifying the genes with the housekeeping gene β-actin, which served as an internal control. Total RNA was isolated from the cells by guanidine thiocyanate extraction method (25). Total RNA was reverse transcribed into cDNA and used for PCR with human specific primers for COX-2, iNOS, and β-actin. Sequences of COX-2 primers were 5'-TTCATAATGAGATGTGGAATAATTTGCT-3' (forward primer) and 5'-AGATCATCTCTGCTTGATCTT-3' (reverse primer), giving a 305 bp PCR product (25). Sequences of iNOS primers were 5'-CTGCTATGTAAGTACGGGTG-3' (forward primer) and 5'-AGCCTTGCACAAGGTCTCATGAGCAA-3' (reverse primer), giving rise to a 225 bp PCR product (25). For β-actin, forward primer was 5'-GCCACATTGAGCAGCATGAGG-3' and the reverse primer was 5'-GTGAGATTGTGGGAAAATTGCT-3', giving a 353 bp PCR product. Briefly, the PCR was amplified by 25-30 repeat denaturation cycles at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. During the first cycle, the 95°C step extended to 2 min, and on the final cycle the 72°C step extended to 5 min. PCR products were separated on 1.5% agarose gels containing 0.5 µg/ml ethidium bromide and visualized by UV transillumination.

**Western blot analysis for iNOS, COX-2, and IκBα**

The cells were trypsinized, washed, and then homogenized in Tris-HCl (pH 7.4) buffer containing 1% NP-40 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). The protein concentration of each sample was determined by Bradford assay (Bio-Rad laboratories, Hercules, CA, USA). Total cell extracts (50-100 µg) isolated from the
cells were loaded per lane, separated by 7-12% SDS polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL, USA) by electroblotting. After blocking using 3% nonfat dry milk for 2 h, the membrane was incubated with polyclonal antibodies for COX-2, iNOS, IkBα, actin (all from Santa Cruz Biotechnology, Santa Cruz, CA), and total and phospho-specific forms of p38, JNK, and ERK (Cell Signaling Technology, Inc., Beverly, MA, USA) in TBS-T containing 3% nonfat dry milk at 4°C overnight. After washing with TBS-T, the immunoreactive proteins were visualized by using goat anti-mouse (COX-2), goat anti-rabbit (iNOS, IkBα, p38, JNK, ERK), or donkey anti-goat (actin) secondary antibodies conjugated to horseradish peroxidase, which was followed by enhanced chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA).

**EMSA for the activation of NF-κB and AP-1**

The cells were rinsed with ice-cold phosphate buffered saline (PBS), harvested by scraping into PBS, and pelleted by centrifugation at 1500 rpm for 5 min. The cells were extracted 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 phenylmethyl-sulfonyl fluoride (PMSF). After centrifugation at 13000 rpm for 10 min, the nuclear pellet was resuspended on ice in a 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. The nuclear protein concentration was determined by Bradford assay solution (Bio-Rad laboratories, Hercules, CA, USA). A NF-κB gel shift oligonucleotide (5'-AGTTGAGGAGCTTCCAGGC-3') and a AP-1 gel-shift oligonucleotide (5'-CGCTTGATAGTCAGCCGGAA-3', all from Promega, Madison, WI, USA) were labeled with [³²P]dATP (Amersham) using the T4 polynucleotide kinase (GIBCO, Grand Island, NY, USA). The end-labeled probe was purified from an unincorporated [³²P]dATP using a Bio-Rad purification column (Bio-Rad Laboratories, Hercules, CA, USA) and recovered in Tris-EDTA buffer (TE). Nuclear extracts (3 µg) were incubated with the buffer containing [³²P]-labeled NF-κB or AP-1 consensus oligonucleotide for 30 min, and subjected to electrophoretic separation on a nondenaturing acrylamide gel. The gels were dried at 80°C for 2 h and exposed to a radiography film for 6-18 h at -70°C with intensifying screens.

**Confocal microscopy for determination of ROS**

The cells were seeded in a chamber slide (Nalge Nunc, Naperville, IL, USA) at 5×10⁴ cells per well and cultured...
overnight. The cells were treated with β-carotene for 2 h and stimulated with H. pylori for 30 min. The cells were loaded with 5 µM 2', 7-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Eugene, OR, USA) for 5 min. The fluorescent dichlorofluorescein was detected by a laser scanning confocal microscope (Leica TCS-NT, Heidelberg, Germany) with excitation and emission wavelengths of 488 and 520 nm, respectively (35).

RESULTS

Effect of β-carotene on the expression of iNOS and COX-2

To investigate the effect of β-carotene on mRNA and protein expression of iNOS and COX-2 in H. pylori-infected AGS cells, the cells were infected with H. pylori for 12 h (mRNA) and 24 h (protein). As shown Fig. 1A, H. pylori induced mRNA expression of iNOS and COX-2, which was inhibited by β-carotene dose-dependently. The protein level of iNOS and COX-2 increased by H. pylori was suppressed by β-carotene in a dose-dependent manner. Actin was not changed by both H. pylori infection and β-carotene treatment at the levels of mRNA and protein for iNOS and COX-2 in AGS cells.

Effect of β-carotene on the increase in ROS

Confocal microscopic observation for ROS level in AGS cells using DCF-DA fluorescence dye showed the increase in ROS level in the cells infected with H. pylori (Fig. 1B). Increase in ROS induced by H. pylori was inhibited by treatment of β-carotene dose-dependently. Treatment of β-carotene (10 µM, 20 µM) significantly reduced the level of ROS in H. pylori-infected AGS cells.

Effect of β-carotene on the activation of MAPK

At 30 min stimulation of H. pylori, MAPK was activated by determining phospho-specific forms of p38, JNK and ERK. Total forms of p38, JNK, and ERK were not changed by H. pylori in AGS cells (Fig. 2A). β-Carotene inhibited phosphorylation of three MAPK (p38, JNK, ERK) induced by H. pylori. However, total forms of MAPK were not affected by β-carotene treatment.

Effect of β-carotene on the activation of NF-κB and AP-1

The DNA binding activity of NF-κB and AP-1 in H. pylori-infected cells was determined by EMSA (Fig. 2B). H. pylori induced the increase in DNA binding activities of NF-κB and

Fig. 2. Effect of β-carotene on H. pylori-induced activation of MAPK, NF-κB and AP-1 as well as decrease in IκBα in AGS cells. (A) The levels of phospho-specific and total forms of MAPK (p38, JNK, ERK) were determined by Western blotting at 30 min culture. (B) The activations of NF-κB and AP-1 were determined by DNA binding activities at 1 h culture using EMSA. (C) The level of IκBα in the cells was determined at 1 h culture by Western blotting.
AP-1 at 1 h culture, which was suppressed by β-c[aro]teno[de]-
dependently. The level of IκBα decreased after H. pylori
infection at 1 h. Since the activation of NF-κB is followed by
the degradation of IκBα, the decrease in IκBα reflects the increase
in DNA binding activity of NF-κB in the cells. The degradation of
IκBα by H. pylori was prevented by β-carotene treatment in
a dose-dependent manner (Fig. 2C).

DISCUSSION

Previously we showed that MAPK, NF-κB and AP-1 may be
the critical inflammatory mediators in H. pylori-induced
expression of iNOS and COX-2 in AGS cells (25). Since ROS
production is stimulated by H. pylori in AGS cells (3, 4) and NF-
κB and AP-1 are oxidant-sensitive transcription factor (10), ROS
may have an important role in the expression of iNOS and COX-
2 by activating NF-κB and AP-1 in H. pylori-infected AGS cells.

In the present study, we determined whether an antioxidant β-
caro[teno]-, which is abundant in fruits and vegetables, inhibits H.
pylori -induced activation of inflammatory mediators and the
expression of iNOS and COX-2 in AGS cells. We found that up-
regulation of iNOS and COX-2 was followed by the increase in
the level of ROS and activation of MAPK, NF-κB and AP-1 in H.
pylori-infected gastric epithelial AGS cells. H pylori-induced
production of ROS was inhibited by β-carotene, which resulted in
decrease in the activation of inflammatory signaling (the activation of MAPK, NF-κB and AP-1) and inhibited the expression of iNOS and COX-2 in AGS cells. The results demonstrate that ROS are the upstream activators of H. pylori-
induced inflammatory signaling in gastric epithelial cells.

In our previous study, we found that U0126, an ERK inhibitor,
and SB203580, an inhibitor of p38, suppressed the activation of
both NF-κB and AP-1 in H. pylori-infected gastric epithelial cells
(6). The results suggest that the activation of MAPK may be the
upstream signaling for the activation of NF-κB and AP-1 in H.
pylori-infected gastric epithelial cells. MAPK activation was
involved in H. pylori-induced expression of IL-8 through the
activation of NF-κB and AP-1 as well as the expression of iNOS
and COX-2 induced by various stimuli in diverse cells (6, 36, 37).
One candidate for the sources of ROS in H. pylori-infected gastric
epithelial cells is nicotinamide adenine dinucleotide phosphate
(NADPH) oxidase. Several studies suggest that NADPH oxidase is
involved in ROS production of H. pylori-infected gastric mucosa
of humans and mice (38, 39). NADPH oxidase requires the
assembly of membrane-integrated cytochrome b558 (a heterodimer formed by gp91phox and p22 phox) with cytosolic
components p47 phox, p67 phox, and GTPase Rac (40), thereby
participates in electron transfer from NADPH to molecular O2- and
O2 - is spontaneously converted to H2O2 (40-42). NADPH oxidase
activation activated by lipopolysaccharide (LPS), H2O2, and inflammatory
cytokines is related with MAPK activation (3, 4, 43-45). Therefore,
we suggest that NADPH oxidase activated by H. pylori may
stimulate the production of ROS and turns on the transcription and
translation of iNOS and COX-2 via the activation of MAPK, and
transcription factors NF-κB and AP-1 in AGS cells.

Chu et al. (28) reported that H. pylori induced the decrease in
cellular level of IκBα and increase in nuclear level of NF-κB
in AGS cells. In response to various inflammatory stimuli, IκBα
is degraded and the NF-κB complex migrates into the nucleus
and binds DNA recognition sites in the regulatory regions of the
target genes including iNOS and COX-2 (20, 23). Therefore, the
present decrease in cellular level of IκBα confirms the activation of
NF-κB by H. pylori in AGS cells. The prevention of decrease in
IκBα by β-carotene demonstrates the inhibition of NF-κB activation
by H. pylori in AGS cells.

Transcription of COX-2 and iNOS was mediated by the
activation of NF-κB and AP-1 in H. pylori-infected AGS cells
(10, 25, 46). Higher expression of COX-2 was associated with
functional activity in H. pylori infection. Large amounts of PGE2
produced by COX-2 contribute to inflammatory events. The
activity of iNOS was enhanced in the gastric mucosa of patients
with H. pylori-positive duodenal ulcers (47). iNOS expression
and activity was activated by H. pylori-infection in a macrophage cell line (48). Besides, previously we showed that
cross-coupled activation of NF-κB and AP-1 mediates the
induction of COX-2 and iNOS in H. pylori-infected gastric
epithelial cells (25). Therefore, inhibitory effect of β-carotene
in the activation of MAPK, NF-κB and AP-1 is evidently related to
the suppression on the expression of iNOS and COX-2 in AGS
cells infected with H. pylori. The present results were supported by
the previous studies showing that carotenoids reduced the
expression of inflammatory enzymes and cytokines by inhibiting
ROS production (33, 49, 50) and the activation of oxidant-
sensitive transcription factors (3, 4, 28).

Carotenoids are a class of natural fat-soluble pigments found
principally in plants, algae, and photosynthetic bacteria. In
humans, carotenoids such as β-carotene are the precursors to
vitamin A, and act as antioxidants (33, 49, 50). β-Carotene,
a major lipid soluble antioxidant nutrient, presents in a wide range
of fruits and vegetables. It prevents the development of
inflammatory diseases including atherosclerosis and rheumatoid
arthritis (31-33) as well as cardiovascular diseases (51).

Although anti-inflammatory effects of β-carotene have been
reported in various cells and tissues, the action mechanism of β-
carotene has not been clarified yet. β-Carotene inhibited in vitro
proliferation of oval cells during neoplastic liver injuries in rats
(52). In contrast, β-carotene and arachidonic acid induced DNA
hypomethylation in human umbilical vein endothelial cells and
endothelial progenitor cells. The results show that β-carotene
and arachidonic acid may alter gene expression in endothelial
cells and in certain conditions these compounds may be
connected with pro-malignant effect (53). The effect of β-
carotene on inflammation and carcinogenesis may depend on
cell-type, concentration of β-carotene, and the environmental
factors. Therefore, the precise action mechanism of β-carotene
should be investigated to determine the effect of β-carotene in
certain disease conditions.

In the present study, β-carotene induced decrease in ROS
level, and inhibited the activation of MAPK, NF-κB and AP-1 as
well as the expression of iNOS and COX-2 in H. pylori-infected
AGS cells. Further study should be performed to investigate
whether β-carotene scavenges ROS or directly inhibit the
activation of ROS production machinery including NADPH
oxidase in gastric epithelial cells.

In summary, ROS produced by H. pylori infection may
trigger inflammatory signaling mediated by MAPK and activate
NF-κB and AP-1, resulting in the induction of iNOS and COX-2
in gastric epithelial AGS cells. β-Carotene inhibits the
activation of inflammatory mediators for the expression of iNOS
and COX-2 by reducing the level of ROS induced by H. pylori
in gastric epithelial AGS cells. β-carotene may be beneficial for
preventing and treating H. pylori-induced gastric inflammation
by suppressing ROS-mediated inflammatory signaling and the
expression of iNOS and COX-2 in gastric epithelial cells.

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