Astaxanthin Inhibits Helicobacter pylori-induced Inflammatory and Oncogenic Responses in Gastric Mucosal Tissues of Mice

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INTRODUCTION

Helicobacter pylori (H. pylori) is widely recognized as a major risk factor for gastric inflammation. Moreover, the continued gastric mucosal exposure to H. pylori results in a chronic inflammatory state that may progress to gastric cancer. Astaxanthin, a pinkish antioxidant carotenoid, abundant in marine organisms, is known for its protective effect against inflammation and multiple types of cancer. The purpose of this study was to examine the effect of astaxanthin on H. pylori-induced oxidative injury, inflammation, and oncogene expression in gastric mucosal tissues of the infected mice. Mice were inoculated using oral gavage with H. pylori suspension (10^8 colony forming unit of H. pylori/0.1 mL) for three days, after which they were fed astaxanthin-supplemented diet (5 mg/kg body weight/day for seven weeks). The effects of astaxanthin on H. pylori-induced increase in lipid peroxide (LPO) production, myeloperoxidase (MPO) activity, expression of the inflammatory cytokine IFN-γ and oncogenes (c-myc and cyclin D1), and the accompanying histologic changes in gastric mucosal tissues were evaluated. H. pylori infection increased the level of LPO, MPO activity, and the expression of IFN-γ, c-myc, and cyclin D1 in gastric mucosal tissues of mice. H. pylori infection induced neutrophil infiltration and hyperplasia of gastric mucosa. Astaxanthin supplementation attenuated these effects. In conclusion, consumption of astaxanthin-rich foods may prevent H. pylori-associated oxidative damage and inflammatory and oncogenic responses in gastric mucosal tissues.
Astaxanthin is the ketocarotenoid responsible for the red-orange pigmentation observed in aquatic organisms such as salmonids and lobsters [16]. Astaxanthin has attracted substantial interest due to its anti-inflammatory and anti-cancer effects [17]. Our previous study has revealed that astaxanthin protects gastric epithelial cells from the harmful effects of H. pylori infection by attenuating the induction of ROS production and increasing the activity of antioxidant enzymes [8]. These results led us to propose the hypothesis that astaxanthin may avert H. pylori-associated gastric alterations including oxidative damage (increase in lipid peroxide [LPO] levels and myeloperoxidase [MPO] activity) and the expression of the inflammatory cytokine IFN-γ as well as oncogenes (c-myc and cyclin D1) in gastric mucosal tissues of the infected hosts.

MATERIALS AND METHODS

Animals
Male C57BL/6 mice (6 weeks of age), purchased from Orient Bio Inc. (Seongnam, Korea), were maintained in a controlled room in the animal facility of Yonsei University College of Medicine under the following conditions: temperature, 23.0°C ± 3.0°C; humidity, 50% ± 10%; 12-hour light/dark cycle. Animals were housed in polypropylene cages furnished with hardwood chip bedding (5 animals per cage) and had free access to food and water. All experimental procedures were approved by the Institutional Review Board (IRB) at Animal Ethical and Experimental Committee of Yonsei University College of Medicine, Seoul, Korea (IRB No. IACUC No. 2018-0127).

Bacterial inoculation
The H. pylori strain NCTC 11637 (CagA+ and VacA+) was obtained from the American Type Culture Collection (Rockville, MD, USA) and stored at −80°C in BBL Brucella Broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 10% FBS (Gibco; Grans Island, NY, USA). Bacterial cells were grown and prepared under previously described conditions [8]. After reaching the exponential phase, bacteria were suspended in PBS at a density of 10^8 colony forming unit (CFU)/mL. No specific pretreatments (such as acid inhibition or antibiotics) were used before orogastric H. pylori inoculation or before the animals were sacrificed. Mice were orogastrically inoculated 3 times (days 0, 1, and 2) with 0.1 mL of the inoculum preparation of H. pylori (10^8 CFU of H. pylori/0.1 mL) through a feeding needle. Prior to each inoculation, mice were fasted for 12 hours. Non-inoculated control mice were administered 0.1 mL of sterile PBS under identical conditions.

Experimental protocol
The mice were randomly assigned to the following 3 groups (n = 15 per group): (1) non-infected control (None) group comprising mice administered sterile PBS and fed standard chow (AIN-76A; Research Diets, New Brunswick, NJ, USA); (2) infected control (H. pylori control) group comprising mice inoculated with H. pylori and fed standard chow; (3) astaxanthin (H. pylori + ASX) group comprising mice infected with H. pylori and fed an AIN-76A diet supplemented with 0.005% astaxanthin (Sigma-Aldrich, St. Louis, MO, USA). The daily dose of astaxanthin was set at 5 mg/kg body weight, based on previous studies demonstrating that astaxanthin decreased the incidence of colonic adenocarcinoma in a mouse colitis model and hepatic cholesterol levels in mice fed a high-fat diet [18,19]. Mice were weighed weekly during the study period, while food intake was evaluated three times a week. Mice were killed by carbon dioxide inhalation at the end of the 7th week. Upon death, gastric mucosal tissues were collected. One half of the tissues were subjected to histological analysis. The remaining tissues were homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 25 mM Tris pH 7.4) and used for the determination of LPO abundance, MPO activity, levels of total protein (using the Bradford assay; Bio-Rad Laboratories, Hercules, CA, USA), and protein and mRNA levels of IFN-γ, c-myc, and cyclin D1.

Assessment of LPO level and MPO activity
For evaluating the oxidative damage, the level of LPO was assessed by measuring the gastric mucosal concentration of malondialdehyde according to the method of Ohkawa et al. [20]. Results were expressed as nmol/mg protein. For the determination of the gastric mucosal accumulation of neutrophilic components, the activity of MPO, a peroxidase enzyme abundantly expressed in neutrophils, was quantified according to the modified method of Krawisz et al. [21] and expressed in U/mg protein. One unit of MPO indicates the enzymatic activity needed to reduce 1 µmol of hydrogen peroxide per minute at 25°C.

Real-time PCR analysis
The mRNA levels of IFN-γ, c-myc, and cyclin D1 were assessed using real-time PCR. Complementary DNA (cDNA) was generated from total RNA by reverse transcription using random hexamers and MuLV reverse transcriptase (Promega, Madison, WI, USA) using the following protocol: 23°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes. The cDNA was amplified with the specific primers and FAM-BHQ-labeled probes described in Table 1 under the following conditions: 45 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 30 seconds. In the first cycle, the 95°C step was extended to 3 minutes. GAPDH was used as the reference gene for data normalization.
Western blot analysis

Western blot analysis was conducted as previously described [22]. After electro-blotting, nitrocellulose membranes were probed using antibodies for c-myc (sc-40), cyclin D1 (sc-8396), IFN-γ (sc-52673), and β-actin (sc-47778; all purchased from Santa Cruz Biotechnology, Dallas, TX, USA) diluted in TBS-Tween solution containing 2% dry milk, and incubated overnight at 4°C. After washing three times with TBS-Tween, the primary antibodies were detected using horseradish peroxidase-conjugated anti-mouse secondary antibody and visualized with the enhanced chemiluminescence detection system (Santa Cruz Biotechnology). β-Actin was used as a loading control. Results were expressed as the percentage density ratio of each protein to β-actin.

Histological analysis

Half of the stomach tissues dissected from mice were fixed in freshly prepared 10% neutral-buffered formalin, embedded in paraffin, cut into 4 µm slices, stained with hematoxylin and eosin, and subjected to morphological observation under an optical microscope. To evaluate pathological changes, microscopic images (200 ×) were examined by a single investigator who was blind to the group assignments.

Statistical analysis

All values were expressed as means ± SEM. One-way ANOVA was carried out to assess statistical significance, followed by Newman-Keul’s post-hoc test. Differences with P < 0.05 were considered statistically significant.

RESULTS

Astaxanthin does not change body weight gain and daily food intake in H. pylori-infected mice

Body weight gain in the infected control group (H. pylori control) tended to increase compared to the ASX group (H. pylori + ASX) (Fig. 1A). However, the changes in the body weight among the three groups at the end of the experiment were not significantly different. The average daily food intake did not show significant differences among the three groups (Fig. 1B).

Table 1. Mouse primers for RT-PCR

| Gene     | Primer | Sequence (5’-3’)          | Gene     | Primer | Sequence (5’-3’)          |
|----------|--------|---------------------------|----------|--------|---------------------------|
| GAPDH    | Probe  | FAM-TGCTCTTCTCATGTTGACACATCACG-BHQ1 |          |        |                           |
|          | Forward| TGCTCAGAATCCCTTGAAGGAGTT  | IFN-γ    | Probe  | FAM-CTTCAGAGCTTCCAGT-BHQ1 |
|          | Reverse| CTGCTGATGCCCATGT          |          | Forward| TGCATCCATCGCGAAA           |
|          |        |                           |          | Reverse| AGCGGGAAGAACACTCCCTTC     |
| c-myc    | Probe  | FAM-ACCTCCCAGAGCCACTTGGT-BHQ1 | Cyclin D1| Probe  | FAM-TACATCTTCTCATGACACAGG-BHQ1 |
|          | Forward| AGCGACTCTGAAAGAGGCA       |          | Forward| TCAAATGCGATAGATGTTGGAAGAA |
|          | Reverse| CGTAGTGTTGCTGAGTGTG       |          | Reverse| TGCTCCTGAGGATTTCTCG       |

RT-PCR, real-time PCR.

Figure 1. Effects of astaxanthin on body weight gain and daily food intake of mice infected with H. pylori. (A) Graph showing the weekly changes in body weight of each group. (B) Graph showing the average daily food intake of each group as measured three times a week. Values are presented as mean ± SEM (n = 15 for each group). None, non-infected mice fed standard chow; H. pylori control, H. pylori-infected mice fed standard chow; H. pylori + ASX, H. pylori-infected mice fed chow supplemented with astaxanthin (ASX).
Astaxanthin decreases the level of LPO and MPO activity in H. pylori-infected gastric mucosa
The level of LPO reflects the oxidative stress to lipids, and, by extension, is indicative of the oxidative injury to cells. On the other hand, MPO activity is used as an index for H. pylori-induced accumulation of neutrophils because of the abundance of this enzyme in the infected tissues. Comparing the values of the non-infected (None) and the infected control (H. pylori control) groups (Fig. 2) revealed that H. pylori infection significantly increased both LPO level and MPO activity in gastric mucosal tissues. Notably, astaxanthin supplementation attenuated the infection-induced elevation of LPO production and MPO activity in mouse gastric mucosal tissues. These results demonstrate that astaxanthin may inhibit H. pylori-induced oxidative stress and neutrophil influx to mouse gastric mucosal tissues.

Astaxanthin suppresses the expression of IFN-γ, c-myc, and cyclin D1 in H. pylori-infected gastric mucosa
H. pylori infection significantly increased both the mRNA and protein levels of IFN-γ, whereas astaxanthin supplementation attenuated these effects (Fig. 3). The same changes, i.e., increases in the infected controls and attenuation by astaxanthin, were observed in the two examined oncogenes, c-myc and cyclin D1 (Fig. 4). These results indicate that astaxanthin can suppress H. pylori-mediated gastric carcinogenesis by attenuating the expression of inflammatory mediators and oncogenes in gastric mucosa.

Astaxanthin prevents H. pylori-induced alterations in gastric mucosa
The gastric mucosa of the uninfected (None) group displayed normal morphology, i.e., lack of inflammation or hyperplasia

Figure 2. Effect of astaxanthin on lipid peroxide (LPO) level and myeloperoxidase (MPO) activity in the gastric mucosal tissues of H. pylori-infected mice. (A) Graph showing the level of LPO in each of the three groups, expressed as nmol/mg of protein. (B) Graph showing MPO activity in each of the three groups, expressed as units/mg protein. Values are presented as mean ± SEM (n = 5 for each group). None, non-infected mice fed standard chow; Control (H. pylori control), H. pylori-infected mice fed standard chow; ASX (H. pylori + ASX), H. pylori-infected mice fed chow supplemented with astaxanthin (ASX). *P < 0.05 vs. None, **P < 0.05 vs. infected controls (H. pylori control).

Figure 3. Effect of astaxanthin on the expression of IFN-γ in the gastric mucosal tissues of H. pylori-infected mice. (A) IFN-γ mRNA levels in the gastric mucosal tissues were assessed by real-time PCR. GAPDH mRNA was used to normalize the results. (B) Upper: Western blot analysis of IFN-γ. Lower: graph showing the levels of each protein as a density ratio relative to the density of the loading control (β-actin). The density ratio of the non-infected control (None) group was set as 100%. None, non-infected mice fed standard chow; Control (H. pylori control), H. pylori-infected mice fed standard chow; ASX (H. pylori + ASX), H. pylori-infected mice fed chow supplemented with astaxanthin (ASX). *P < 0.05 vs. non-infected controls (None), **P < 0.05 vs. infected controls (H. pylori control).
In contrast, the infected control (H. pylori control) group had mild inflammation and hyperplasia, as indicated by the presence of focal hyperplastic glands and the infiltration of neutrophils in the gastric mucosa (Fig. 5B). Infected mice fed an astaxanthin-supplemented diet (H. pylori + ASX) exhibited reduced neutrophil infiltration and hyperplasia compared to those of the infected control group (Fig. 5C). These results suggest that astaxanthin supplementation might exert a preventive effect against H. pylori-induced gastric alterations.

**DISCUSSION**

Oxidative stress in the gastric mucosa resulting from H. pylori infection has a crucial role in gastric carcinogenesis.
infection is highly correlated with increased oxidative damage to the gastric mucosa [1]. In particular, the elevated production of ROS enhances the generation of LPO, as they react with polyunsaturated fatty acids in the cellular membrane and promote the oxidative degradation of the lipids [23]. Therefore, the increased LPO level observed in this study after the inoculation with *H. pylori* may be attributed to infection-induced oxidative damage to gastric mucosa cells. Since astaxanthin treatment reverses this effect, we conclude that its antioxidative activity might protect gastric tissues from the oxidative damage induced by *H. pylori* infection. Our results concur with those of previous studies in which the oral administration of astaxanthin was shown to prevent the elevation of the LPO level in the gastric tissues in a mouse model of gastric ulceration [24,25].

*H. pylori*-induced inflammation is characterized by the persistent infiltration of neutrophils in gastric mucosa, which contributes to the pathologies of gastric disorders, particularly by releasing inflammatory mediators and ROS [26]. This was confirmed in our study, as MPO, an indicator of neutrophil influx in tissues, was elevated by *H. pylori* infection. Importantly, this increase was mitigated by astaxanthin treatment.

Increased expression of IFN-γ after *H. pylori* infection has been observed in the stomach tissues of Mongolian gerbils [27] and humans [3]. *H. pylori* is known to stimulate the production of IFN-γ from peripheral blood mononuclear cells and lymphocytes in gastric tissue [28]. A role for IFN-γ in *H. pylori*-induced gastric inflammation is supported by the finding that infected IFN-γ−/− mice develop less severe gastritis compared with their wild-type counterparts [29]. In the current study, *H. pylori* infection increased the mRNA and protein levels of IFN-γ in mouse gastric mucosa, whereas astaxanthin intake attenuated this increase. These findings are consistent with the previous observation that the dietary intake of astaxanthin can reduce the release of IFN-γ in splenocytes extracted from *H. pylori*-infected mice [30], and suggest that astaxanthin supplementation might alleviate the gastric inflammation by reducing *H. pylori*-induced IFN-γ expression.

Several studies have shown that *H. pylori* infection increases the expression of c-myc and cyclin D1 and induces hyperproliferation in gastric epithelial AGS cells [14,31]. Since the transcription factor c-myc suppresses the cell cycle arrest and enhances cell proliferation, the deregulation of its expression is a pivotal event in the pathological process leading to cancer [32]. On the other hand, cyclin D1 plays a major role in cell-cycle control as it allows the cycle to progress from the G1 to the S phase. Abnormal cyclin D1 expression is known to participate in cancer development and progression by increasing the rate of cellular proliferation [33]. Our data show that astaxanthin supplementation attenuates the increases in the mRNA and protein levels of c-myc and cyclin D1 in mouse gastric tissues that would otherwise result from *H. pylori* infection. These results are in line with previous findings illustrating that astaxanthin decreases the expression of both proteins and inhibits the proliferation of human gastric cancer and colon cancer cells [34,35].

We have previously demonstrated that activation of NF-κB and AP-1 mediates hyperproliferation by inducing β-catenin and c-myc in *H. pylori*-infected gastric epithelial cells [14]. Therefore, NF-κB and β-catenin signaling may mediate hyper-proliferation via induction of c-myc and cyclins. *H. pylori* infection increased expression of c-myc and cyclin D1 through activation of NF-κB [14,36]. We also showed that astaxanthin inhibited *H. pylori*-induced NF-κB and c-myc and cyclin D1 signaling via the reduced levels of β-catenin in gastric epithelial cells [8]. The antioxidant β-carotene inhibited *H. pylori*-induced cell proliferation and the expression of c-myc and cyclin E by reducing the levels of β-catenin and phosphorylated glycogen synthase kinase 3β [37]. Lycopene inhibited oxidative stress-mediated activation of Wnt/β-catenin signaling including alteration of Wnt/β-catenin multiprotein complex molecules and attenuated hyper-proliferation in gastric epithelial cells [38]. These studies suggest that astaxanthin supplementation may suppress *H. pylori*-induced expression of c-myc and cyclin D1 through inhibition of NF-κB activation and Wnt/β-catenin signaling in gastric mucosal tissues.

Jin et al. [39] and Luo et al. [40] demonstrated that 6- to 7-week-treatment of *H. pylori* induced erosions and infiltration of inflammatory cells, and hyperplasia in gastric mucosal tissues of mice. Our results showed that 7-week-treatment of *H. pylori* caused inflammation and hyperplasia of gastric tissues of the infected mice.

In conclusion, astaxanthin may suppress oxidative gastric tissue damage and the expression of the inflammatory cytokine, IFN-γ, and the oncogenes, c-myc and cyclin D1, in *H. pylori*-infected gastric mucosal tissues. These results suggest that astaxanthin supplementation might have a preventive effect against *H. pylori*-associated gastric carcino genesis.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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