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SHORT COMMUNICATION

Astaxanthin Prevents Decreases in Superoxide Dismutase 2 Level and Superoxide Dismutase Activity in *Helicobacter pylori*-infected Gastric Epithelial Cells

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Background: *Helicobacter pylori* increases production of reactive oxygen species (ROS), which activates inflammatory and carcinogenesis-related signaling pathways in gastric epithelial cells. Therefore, reducing ROS, by upregulating antioxidant enzyme, such as superoxide dismutase (SOD), may be a novel strategy to prevent *H. pylori*-associated gastric diseases. Astaxanthin is an antioxidant carotenoid that prevents oxidative stress-induced cell injury. The present study was aimed to determine whether *H. pylori* decreases SOD activity by changing the levels of SOD1/SOD2 and whether astaxanthin prevents changes in SOD levels and activity in *H. pylori*-infected gastric epithelial AGS cells.
Methods: AGS cells were pre-treated with astaxanthin for 3 hours prior to *H. pylori* infection and cultured for 1 hour in the presence of *H. pylori*. SOD levels and activity were assessed by Western blot analysis and a commercial assay kit, respectively. Mitochondrial ROS was determined using MitoSOX fluorescence.
Results: *H. pylori* decreased SOD activity and the SOD2 level, but increased mitochondrial ROS in AGS cells. The SOD1 level was not changed by *H. pylori* infection. Astaxanthin prevented *H. pylori*-induced decreases in the SOD2 level and SOD activity and reduced mitochondrial ROS in AGS cells.
Conclusions: Consumption of astaxanthin-rich food may prevent the development of *H. pylori*-associated gastric disorders by suppressing mitochondrial oxidative stress.
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Key Words: Astaxanthin, *Helicobacter pylori*, Gastric diseases, Reactive oxygen species, Superoxide dismutase

INTRODUCTION

Helicobacter pylori infection increases oxidative stress in the infected cells, which contributes to the development of gastric diseases including gastric cancer [1-4]. Mitochondria are the center of energy production and generate reactive oxygen species (ROS) from electron transport respiratory chain [5]. Our recent study has demonstrated that interluekin-8 expression is highly related to mitochondrial ROS in gastric epithelial cells infected with *H. pylori* [6].

Superoxide dismutases (SODs) are important antioxidant enzymes, which catalyze superoxide anion dismutation on hydrogen peroxide and oxygen. Therefore, expression and activation of SODs have great impact on cellular oxidative response. SODs have three isoforms; cytosolic SOD (SOD1, Cu/Zn SOD), mitochondrial SOD (SOD2, Mn SOD) and extracellular SOD (SOD3, ecSOD) [7]. Because ROS activate signaling pathways related to inflammation, immune system, and cancer development, regulating ROS levels in the cells is important for maintaining normal physiology of the cells [8]. Therefore, it is essential to investigate whether *H. pylori* affects SOD1/SOD2 levels and SOD activity in gastric epithelial cells to better understand pathologic mechanisms of *H. pylori*-induced gastric diseases. Because SOD3 is a secretory extracellular Cu/Zn-containing SOD and primarily

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located in the extracellular matrix and extracellular fluids [9], we determine the changes in SOD1 and SOD2 levels in the present study.

Astaxanthin, a potent antioxidant xanthophyll, contributes to red-orange color of aquatic animals including shrimp, salmon, and lobster [10]. We previously demonstrated that astaxanthin acted as a PPAR-γ agonist and increased the catalase level through PPAR-γ activation in gastric epithelial cells infected with *H. pylori* [6]. Some evidence showed that SODs are PPAR-γ target genes and SOD activity was positively correlated with PPAR-γ activity in aorta and cardiomyocytes [11,12]. Therefore, we hypothesize that astaxanthin may decrease ROS levels by increasing SOD activity in gastric epithelial cells infected with *H. pylori.*

The present study is aimed to determine whether *H. pylori* changes the levels of SOD1 and SOD2 and SOD activity, and whether *H. pylori*-induced changes in SOD levels and activity are inhibited by astaxanthin in gastric epithelial AGS cells

MATERIALS AND METHODS

1. Reagents

Astaxanthin (Sigma-Aldrich, St. Louis, MO, USA), dissolved in dimethyl sulfoxide and stored under nitrogen gas at −80°C, was thawed and added to fetal bovine serum to achieve the desired concentrations.

2. Cell line and culture condition

The human gastric epithelial AGS cells (gastric adenocarcinoma, ATCC CRL 1739; American Type Culture Collection, Rockville, MD, USA) were grown in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) and cultured at 37°C under 95% air and 5% CO_2.

H. pylori bacteria (NCTC 11637; American Type Culture Collection) were grown on chocolate agar plates (Becton Dickinson Microbiology Systems, Cockeysvile, MD, USA) at 37°C, under microaerophilic conditions, using an anaerobic chamber (BBL Campy Pouch® System; Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA).

AGS cells were seeded overnight to reach 80% confluency. The *H. pylori* was harvested and suspended in antibiotic-free RPMI 1640 medium supplemented with 10% FBS, and then added to the AGS cell culture at the ratio of bacterium: AGS cells, 50 : 1. AGS cells (1.0×10^5/mL) were pre-treated with astaxanthin (at final concentration of 1 and 5 μM) for 3 hours before adding the *H. pylori* and cultured for 1 hour.

3. Preparation of cell extracts and Western blot analysis for superoxide dismutase 1 and superoxide dismutase 2

Preparation of cell extracts and mitochondrial ROS levels were determined by the method described previously [10]. Western blotting was performed by the method previously described [13,14]. Briefly, whole-cell extracts were loaded onto 8% to 10% SDS PAGE (6-40 μg protein/lane) and separated by electrophoresis under reducing conditions. The proteins were verified using reversible staining with Ponceau S. The membranes were blocked using 3% non-fat dry milk in TBS and 0.2% Tween 20 (TBS-T). The proteins were detected using antibodies for SOD1 (sc-11407; Santa Cruz Biotechnology, Dallas, TX, USA), SOD2 (sc-30080; Santa Cruz Biotechnology), and actin as a loading control (sc-1615; Santa Cruz Biotechnology) in TBS-T solution containing 3% dry milk, and incubated overnight at 4°C. After washing with TBS-T, the primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-mouse, anti-rabbit, anti-goat), and visualized using the enhanced chemiluminescence detection system (Santa Cruz Biotechnology). Protein levels of SOD1 and SOD2 were compared to that of actin and expressed as the percentage density ratio.

4. Determination of mitochondrial reactive oxygen species levels and superoxide dismutase activity

Mitochondrial ROS levels were determined by the method described previously [15]. SOD activity level was measured using SOD assay kit (19160-1KT-F; Sigma-Aldrich). SOD activity was defined in unit/mg protein.

5. Statistical analysis

Statistical analysis was performed using one-way ANOVA, followed by Newman–Keul's tests. The results were expressed as the mean ± SE of three different experiments. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

As shown in Figure 1A, AGS cells with *H. pylori* significantly increased the levels of mitochondrial ROS (column "None" vs. column "Control"). Astaxanthin (5 μM) reduced mitochondrial ROS levels in *H. pylori*-stimulated AGS cells (column "AST" vs. column "Control"). Figure 1B showed that *H. pylori* reduced the protein level of SOD2, but not the SOD1 level in AGS cells (column "None" vs. column "Control"). Astaxanthin dose-dependently

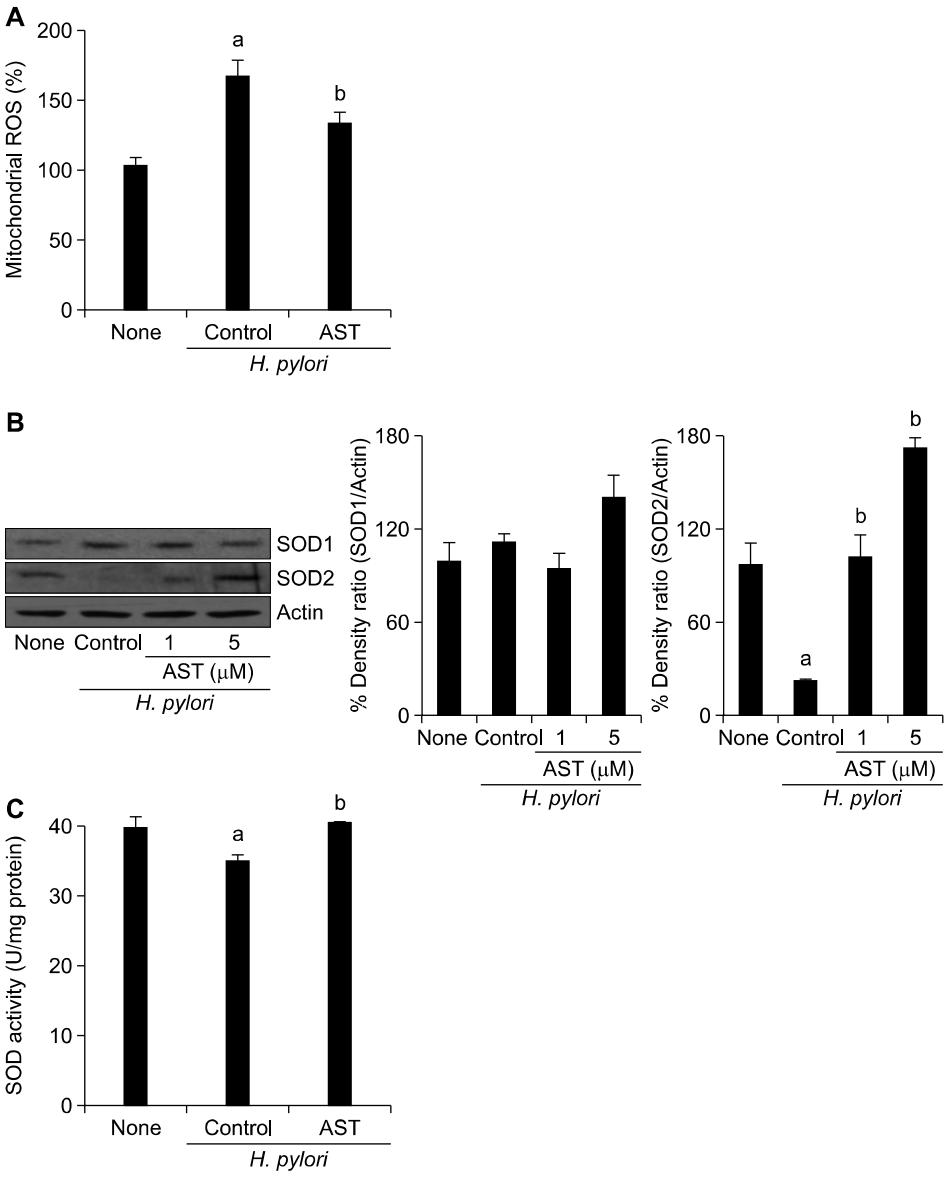

Figure 1. Effect of astaxanthin (AST) on mitochondrial reactive oxygen species (ROS), superoxide dismutase (SOD) levels and SOD activity in *Helicobacter pylori*-infected AGS cells. The cells were pre-treated with 1 or 5 μM of AST for 3 hours and then stimulated with *H. pylori* for 1 hour. (A) Mitochondrial ROS levels were measured by MitoSOX fluorescence. "None" corresponds to uninfected cells; "Control" corresponds to the cells infected with *H. pylori* alone; "AST" corresponds to the cells infected with *H. pylori* with treatment of 5 μM AST. $^{a}P < 0.05$ vs. "none"; $^{b}P < 0.05$ vs. "control". (B) The levels of SOD1 and SOD2 were measured by Western blot analysis for uninfected AGS cells (none), those infected with *H. pylori* (control), AGS cells infected with *H. pylori* and treated with 1 or 5 μM AST (left panel). The protein level of SOD1 and SOD2 were compared to that of actin and expressed as the percentage density ratio. All values were expressed as mean ± SE of three different experiments. $^{a}P < 0.05$ vs. "none"; $^{b}P < 0.05$ vs. "control" (right panel). (C) SOD activity was measured by a commercial assay kit. The description of the columns is the same as in (A). $^{a}P < 0.05$ vs. "none"; $^{b}P < 0.05$ vs. "control".

prevented loss of SOD2 level in *H. pylori*-infected cells (column "AST 1" vs. column "Control") (column "AST 5" vs. column "Control"). Astaxanthin (5 μM) tended to increase the SOD1 level in *H. pylori*-infected cells. However, there are no significant differences among experimental groups. SOD activity in AGS cells decreased by *H. pylori* infection (Fig. 1C). Astaxanthin (5 μM) prevented a decrease in SOD activity in *H. pylori*-infected cells (column "AST" vs. column "Control").

The results demonstrate that *H. pylori* infection reduces the SOD2 level and SOD activity, which may increase mitochondrial ROS in AGS cells. *H. pylori*-induced oxidative stress may be caused by decreased mitochondrial antioxidant capacity in the infected cells. Astaxanthin inhibits *H. pylori*-induced mitochondrial SOD loss and mitochondrial oxidative stress in the infected cells.

DISCUSSION

In the present study, decreased SOD2 levels and total SOD activity were found in *H. pylori*-infected gastric epithelial cells. Therefore, loss of SOD2 may increase mitochondrial ROS in *H. pylori*-infected gastric epithelial cells. Interestingly, the level of cytosolic SOD1 was not changed by *H. pylori* infection. Smoot et al. [16] demonstrated that *H. pylori* had no effect on SOD1 levels in AGS cells at 24 hour-culture. However, they showed that *H. pylori* increased SOD2 activity and catalase activity in AGS cells. In that study, AGS cells were cultured in the medium containing 1×10^7 bacteria/mL for 24 hours. In the present study, we used the ratio of bacterium : AGS cells (1.0×10^5/mL), 50 : 1. Therefore, they used the higher bacterial infection ratio and cultured 24

hours. Since we used 1 hour-culture, different infection ratio and culture time may cause different result on SOD2 activity induced by *H. pylori* infection.

We previously demonstrated that astaxanthin activated PPAR-γ to induce catalase expression in *H. pylori*-infected gastric epithelial cells [6]. PPAR-γ has transcriptional activity towards a number of genes involved in metabolic processes as well as antioxidant genes [17,18]. PPAR-γ increases expression of catalase [19] and SOD1 expression is dependent on PPAR-γ activation [11]. In the present study, the SOD2 level increased by astaxanthin treatment in *H. pylori*-infected cells. SOD1 level was tended to be increased by a high concentration of astaxanthin even though there was no significant difference between groups. For the further study, various concentrations of astaxanthin and various infection rate should be used to assess inhibitory effect of astaxanthin on decreases in SOD levels in *H. pylori*-infected cells. Since we only used one cell line in the present study, it will be necessary to use more gastric epithelial cell lines to confirm the effect of astaxanthin for the further study.

Regarding the studies of astaxanthin on mitochondrial function, astaxanthin prevented loss of mitochondrial respiration in cardiac and neuronal damage [20-23]. These studies suggest the possible protective effect of astaxanthin against mitochondrial malfunction.

Here we found that *H. pylori* decreased SOD activity and the SOD2 level, but increased mitochondrial ROS in AGS cells. the SOD1 level was not changed by *H. pylori* infection. Astaxanthin prevented *H. pylori*-induced decreases in SOD2 level and SOD activity and reduced mitochondrial ROS in AGS cells. Therefore, consumption of astaxanthin-rich food may prevent the development of *H. pylori*-associated gastric disorders by suppressing mitochondrial oxidative stress.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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