α-Lipoic Acid Inhibits Expression of IL-8 by Suppressing Activation of MAPK, Jak/Stat, and NF-κB in *H. pylori*-Infected Gastric Epithelial AGS Cells

Ji Hyun Choi¹, Soon Ok Cho², and Hyeyoung Kim¹, ²

¹Department of Food and Nutrition, Brain Korea 21 PLUS Project, College of Human Ecology, Yonsei University, Seoul; ²Department of Pharmacology, Yonsei University College of Medicine, Seoul, Korea.

The epithelial cytokine response, associated with reactive oxygen species (ROS), is important in *Helicobacter pylori* (*H. pylori*)-induced inflammation. *H. pylori* induces the production of ROS, which may be involved in the activation of mitogen-activated protein kinases (MAPK), janus kinase/signal transducers and activators of transcription (Jak/Stat), and oxidant-sensitive transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and thus, expression of interleukin-8 (IL-8) in gastric epithelial cells. α-lipoic acid, a naturally occurring thiol compound, is a potential antioxidant. It shows beneficial effects in treatment of oxidant-associated diseases including diabetes. The present study is purposed to investigate whether α-lipoic acid inhibits expression of inflammatory cytokine IL-8 by suppressing activation of MAPK, Jak/Stat, and NF-κB in *H. pylori*-infected gastric epithelial cells. Gastric epithelial AGS cells were pretreated with or without α-lipoic acid for 2 h and infected with *H. pylori* in a Korean isolate (HP99) at a ratio of 300:1. IL-8 mRNA expression was analyzed by RT-PCR analysis. IL-8 levels in the medium were determined by enzyme-linked immunosorbent assay. NF-κB-DNA binding activity was determined by electrophoretic mobility shift assay. Phospho-specific and total forms of MAPK and Jak/Stat were assessed by Western blot analysis. ROS levels were determined using dichlorofluorescein fluorescence. As a result, *H. pylori* induced increases in ROS levels, mRNA, and protein levels of IL-8, as well as the activation of MAPK [extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase 1/2 (JNK1/2), p38], Jak/Stat (Jak1/2, Stat3), and NF-κB in AGS cells, which was inhibited by α-lipoic acid. In conclusion, α-lipoic acid may be beneficial for prevention and/or treatment of *H. pylori* infection-associated gastric inflammation.

**Key Words:** α-lipoic acid, *Helicobacter pylori*, IL-8, NF-κB, MAPK, Jak/Stat

*Helicobacter pylori* (*H. pylori*) infection mediates gastritis and gastric adenocarcinoma.¹ Interleukin-8 (IL-8) contributes to gastric inflammation.² IL-8 levels are found to be elevated in gastric mucosal tissues of the patients infected with *Helicobacter pylori*² and *H. pylori*-infected gastric epithelial cells.⁴

Reactive oxygen species (ROS) mediate the expression of IL-8 by activating oxidant-sensitive transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), janus kinase/signal transducers, and activators of transcription (Jak/Stat).³⁻⁵ *H. pylori* exhibits chemotactic activity by inducing neutrophil activation, and these activated neutrophils induce ROS production.⁶⁻⁷ It was recently reported that ROS is involved in Jak/Stat signal molecules in inflammatory signaling pathway of non-phagocytic cells, as well as phagocytic cells. Jak/Stat signaling mediates activation of cytokine signaling.⁸⁻⁹ There are three subfamilies of mitogen-activated protein kinases (MAPKs); extracellular signal-regulated kinases (ERKs), c-Jun NH2-terminal protein kinases (JNKs), and p38 MAPK. The cytotxin-associated gene (*cagA*) pathogenicity island of *H. pylori* is involved in NF-κB and MAPK activation in gastric

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**Corresponding author:** Dr. Hyeyoung Kim, Department of Food and Nutrition, College of Human Ecology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea. Tel: 82-2-2123-3125, Fax: 82-2-364-5781, E-mail: kim626@yonsei.ac.kr

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epithelial cells. Transcription of IL-8 gene requires NF-κB activation and NF-κB is indispensable for the enhanced IL-8 mRNA transcription in *H. pylori*-infected gastric epithelial cells.

α-lipoic acid (α-LA) is supplied from diets such as spinach and broccoli and from a supplement. α-LA and its active reduced counterpart dihydrolipoic acid (DHLA) reduce oxidative stress by chelating transition metals, recycling endogenous antioxidants, and scavenging ROS. α-LA showed beneficial effect on treating ROS-mediated diseases including diabetes, atherosclerosis, and hypertension.

Therefore, we investigated whether α-LA reduces levels of ROS produced in *H. pylori*-infected gastric epithelial cells, thereby suppressing the activation of inflammatory signaling molecules, such as MAPK (ERK1/2, JNK1/2, p38), Jak/Stat (Jak1, Jak2, Stat3), transcription factor NF-κB, and IL-8 expression in *H. pylori*-infected gastric epithelial cells.

A human gastric epithelial cell line AGS (gastric adenocarcinoma, ATCC CRL 1739) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as previously described. *H. pylori* strain in a Korean isolate (HP99; cagA+, vacA s1b, m2, iceA genotype) was inoculated onto chocolate agar plates at 37°C under microaerophilic conditions using GasPak™ EZ Gas Generating Pouch Systems (BD Biosciences, San Jose, CA, USA). Prior to infection, *H. pylori* were harvested, and then resuspended in antibiotic-free cell culture medium. *H. pylori* was added to cultured cells at a bacterium/cell ratio 300:1.

For time-course experiment for IL-8 levels, cells were infected with *H. pylori* for several time points. α-LA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in ethanol. The cells were pretreated with α-LA (final concentrations of 10 and 20 μM) for 2 h and then infected with *H. pylori* for 30 min (for ROS levels, NF-κB, p-IκBα, IkBα, MAPK, Jak/Stat), 3 h (for IL-8 mRNA) or 8 h (for IL-8 protein levels). None and control cells without α-LA received ethanol instead of means; AGS, gastric adenocarcinoma.

**Fig. 1.** mRNA and protein levels of IL-8 in *H. pylori*-infected AGS cells treated with or without α-LA. (A and B) The cells were cultured in the presence of *H. pylori* for indicated time points. mRNA levels of IL-8 were determined by real-time PCR. IL-8 mRNA levels were normalized to β-actin (A). IL-8 levels in the medium were assessed by ELISA (B). (C and D) The cells were pre-treated with α-LA for 2 h, and cultured in the presence of *H. pylori* for 3 h (IL-8 mRNA level, C) or 8 h (IL-8 level in the medium, D). All values are expressed as mean±SEM of four different experiments. *p<0.05 vs. 0 h (A and B) or control (C and D). Non (none), the cells cultured in the absence of *H. pylori* without treatment of α-LA; Con (control), the cells cultured in the presence of *H. pylori* without treatment of α-LA. *H. pylori*, *Helicobacter pylori*; α-LA, α-lipoic acid; ELISA, enzyme linked immunoassortment assay; IL, interleukin; SEM, standard error of means; AGS, gastric adenocarcinoma.

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of α-LA. The time points for determining ROS, NF-κB, p-IκBα, IκBα, MAPK, and Jak/Stat, as well as 2 h-pretreatment of α-LA, were adapted from our previous studies.16–18

IL-8 levels in the medium were determined by using enzyme linked immunosorbent assay (ELISA) kits (Biosource International, Inc., San Diego, CA, USA) following the manufacturer’s instructions. For real-time PCR analysis, total RNA in cells were isolated and converted into cDNA by reverse transcription process using a random hexamer and virus reverse transcriptase (Promega, Madison, WI, USA). Sequences of IL-8 primers and β-actin were adapted from our previous study.19 cDNA was added in a SYBR Green Realtime PCR Master Mix (TOYOBO Co., Osaka, Japan) containing 10 pg/mL of forward and reverse primers for IL-8. cDNA was amplified by 40 cycles, denaturation at 95°C for 15 sec, annealing at 60°C for 5 sec, and extension at 72°C for 30 sec. β-actin gene was amplified in the same reaction to serve as the reference gene.

ROS levels were determined using 2',7'-dichlorodihydrofluoroscein (DCF) fluorescence. All values are expressed as mean±SEM of four different experiments. (B) NF-κB activation was determined using EMSA, performing western blotting for phospho- and total forms of IκBα. (C and D) The levels of phospho-specific and total forms of MAPK (ERK1/2, JNK1/2, p38) and Jak1, Jak2, Stat3 (D) in whole cell lysates were determined by Western blot analysis. Non (none), the cells cultured in the absence of Helicobacter pylori, Con (control), the cells cultured in the presence of Helicobacter pylori without treatment of α-LA; Con (control), the cells cultured in the presence of Helicobacter pylori without treatment of α-LA. *p<0.05 vs. control. H. pylori, Helicobacter pylori; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinases; α-LA, α-lipoic acid; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal protein kinase; Jak/Janus kinase/signal transducers and activators of transcription; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AGS, gastric adenocarcinoma; DCF, 2',7'-dichlorodihydrofluoroscein.

**Fig. 2.** ROS levels, activation of NF-κB, MAPK, and Jak/Stat in H. pylori-infected AGS cells treated with or without α-LA. The cells were pretreated with α-LA for 2 h and cultured in the presence of H. pylori for 30 min (ROS levels, activation of NF-κB, MAPK, and Jak/Stat) or 1 h (NF-κB). (A) ROS levels were determined using DCF fluorescence. All values are expressed as mean±SEM of four different experiments. (B) NF-κB activation was determined using EMSA, performing western blotting for phospho- and total forms of IκBα. (C and D) The levels of phospho-specific and total forms of MAPK (ERK1/2, JNK1/2, p38) and Jak1, Jak2, Stat3 (D) in whole cell lysates were determined by Western blot analysis. Non (none), the cells cultured in the absence of H. pylori without treatment of α-LA; Con (control), the cells cultured in the presence of H. pylori without treatment of α-LA. *p<0.05 vs. control. H. pylori, Helicobacter pylori; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinases; α-LA, α-lipoic acid; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal protein kinase; Jak/Janus kinase/signal transducers and activators of transcription; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; AGS, gastric adenocarcinoma; DCF, 2',7'-dichlorodihydrofluoroscein.
Infection induced phosphorylation of Stat3, accompanied with infection or of ERK1/2, JNK1/2, and p38 were not affected by phosphorylation of ERK1/2, JNK1/2, and p38, while total forms of IκB were also increased by infected with H. pylori. As shown in Fig. 2C, H. pylori-induced increases in IL-8 mRNA levels were maximized at 3 h, accompanied with inhibition of phosphorylation of IκBα. The amount of ROS trapped in the cells was measured as the relative increase over the ROS level in cells expressing of IL-8 time-dependently. IL-8 levels in the media were increased by H. pylori infection, accompanied with inhibition of phosphorylation of IκBα and a decrease in the total forms of IκBα.

The results suggest that MAPK and Jak1/2-Stat3 pathways mediate H. pylori-induced IL-8 expression, which is inhibited by α-LA.

In the present study, we found that H. pylori, Korean isolate (HP99), induces IL-8 expression and activation of MAPK, Jak/Stat, and NF-κB, which were inhibited by α-LA. Since ROS mediates activation of MAPK, Jak/Stat, and NF-κB cells, the inhibitory effect of α-LA on ROS production may suppress H. pylori-induced signaling for IL-8 expression in AGS cells. Several studies have reported that MAPK inhibitors, U0126 (an ERK inhibitor), and SB203580 (a p38 inhibitor) suppressed NF-κB activation in H. pylori-infected AGS cells. These results indicate that NF-κB activation acts as a downstream of ERK and/or p38 signaling in H. pylori-infected AGS cells. Therefore, α-LA may inhibit H. pylori-induced IL-8 expression through suppression of MAPK-mediated NF-κB activation in AGS cells.

Additionally, we found that α-LA inhibits H. pylori-induced Stat3 activation in AGS cells. Jak-Stat signaling is responsible for various cellular responses to cytokines, growth factors, and hormones. Bronte-Tinkew, et al. demonstrated that H. pylori activates Stat3 in gastric epithelial cells. Inhibition of Jak/Stat activation with chemical inhibitors suppresses phosphorylation of ERK, indicating that ERK/NF-κB signaling acts as a downstream of Jak2 activation. For phosphorylation of Stat3, activation of Jak is required. Therefore, Jak may be phosphorylated prior to activation of Stat3 by infection of H. pylori. In the present study, both Jak1 and 2 were phosphorylated along with phosphorylation of Stat3 in AGS cells. Since H. pylori induces activation of MAPK and Jak/Stat at 30 min and NF-κB activation at 1 h-culture, Jak/Stat and MAPK may be upstream signaling of NF-κB in HP99-infected AGS cells. Since α-LA reduces ROS levels and activation of MAPK, Jak1/2-Stat3, and NF-κB, and thus, IL-8 expression, α-LA may have a therapeutic potential for H. pylori infection-associated inflammation.

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