Brief Communication

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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

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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.[3,4]

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•The authors have no financial conflicts of interest.

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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 NH_2-terminal protein kinases (JNKs), and p38 MAPK. The cytotoxin-associated gene (*cagA*) pathogenicity island of *H. pylori* is involved in NF-κB and MAPK activation in gastric

epithelial cells.[3] 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.[3,4,7]

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.[10,11] α-LA showed beneficial effect on treating ROS-mediated diseases including diabetes, atherosclerosis, and hypertension.[12-15]

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.[3] *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).[3] 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 12 h (for IL-8 protein levels). None and control cells without α-LA received ethanol instead

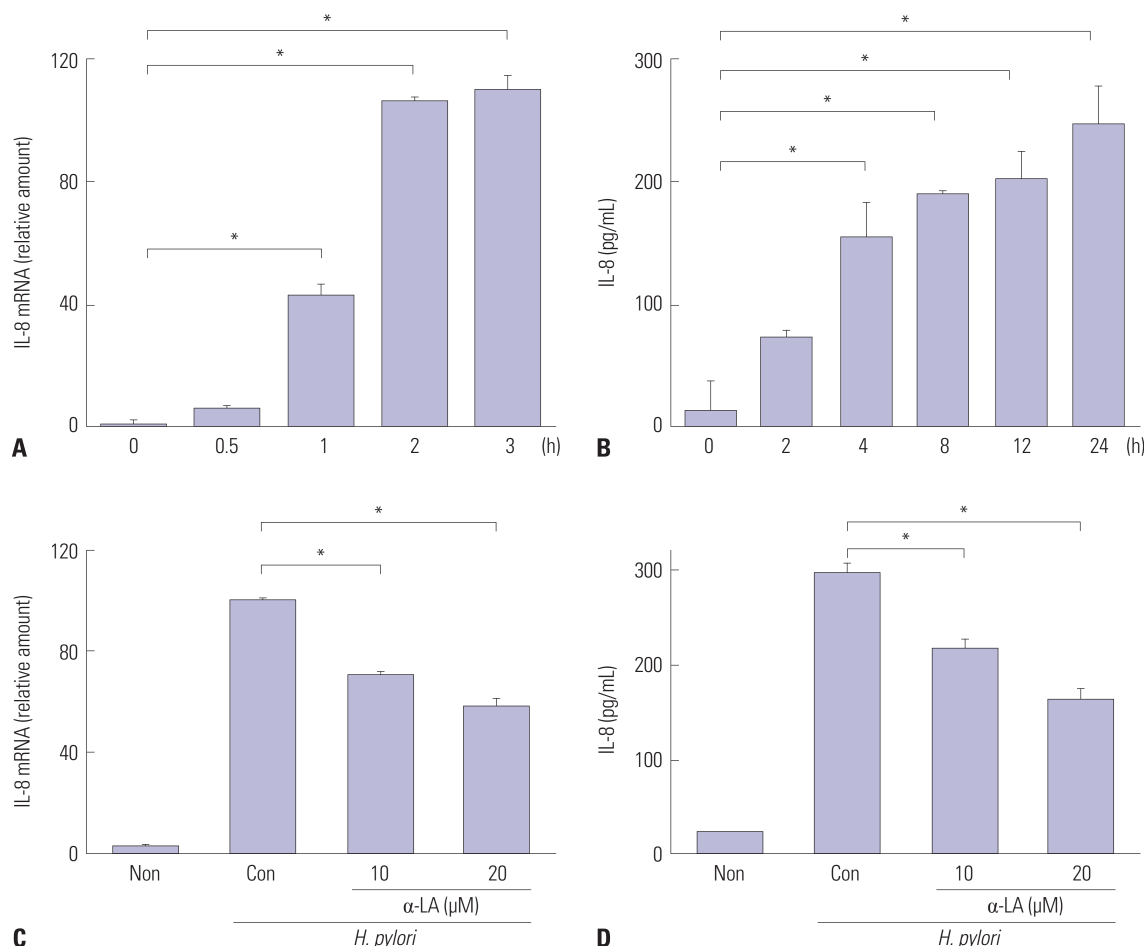

Fig. 1. mRNA and protein levels of IL-8 in *H. pylori*-infected AGS cells treated with or without α-LA. (A and B) The cells were cultured in the presence of *H. pylori* for indicated time points. mRNA levels of IL-8 were determined by real-time PCR. IL-8 mRNA levels were normalized to β-actin (A). IL-8 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 immunosorbent assay; IL, interleukin; SEM, standard error of means; AGS, gastric adenocarcinoma.

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'-dichlorodihydroflu-

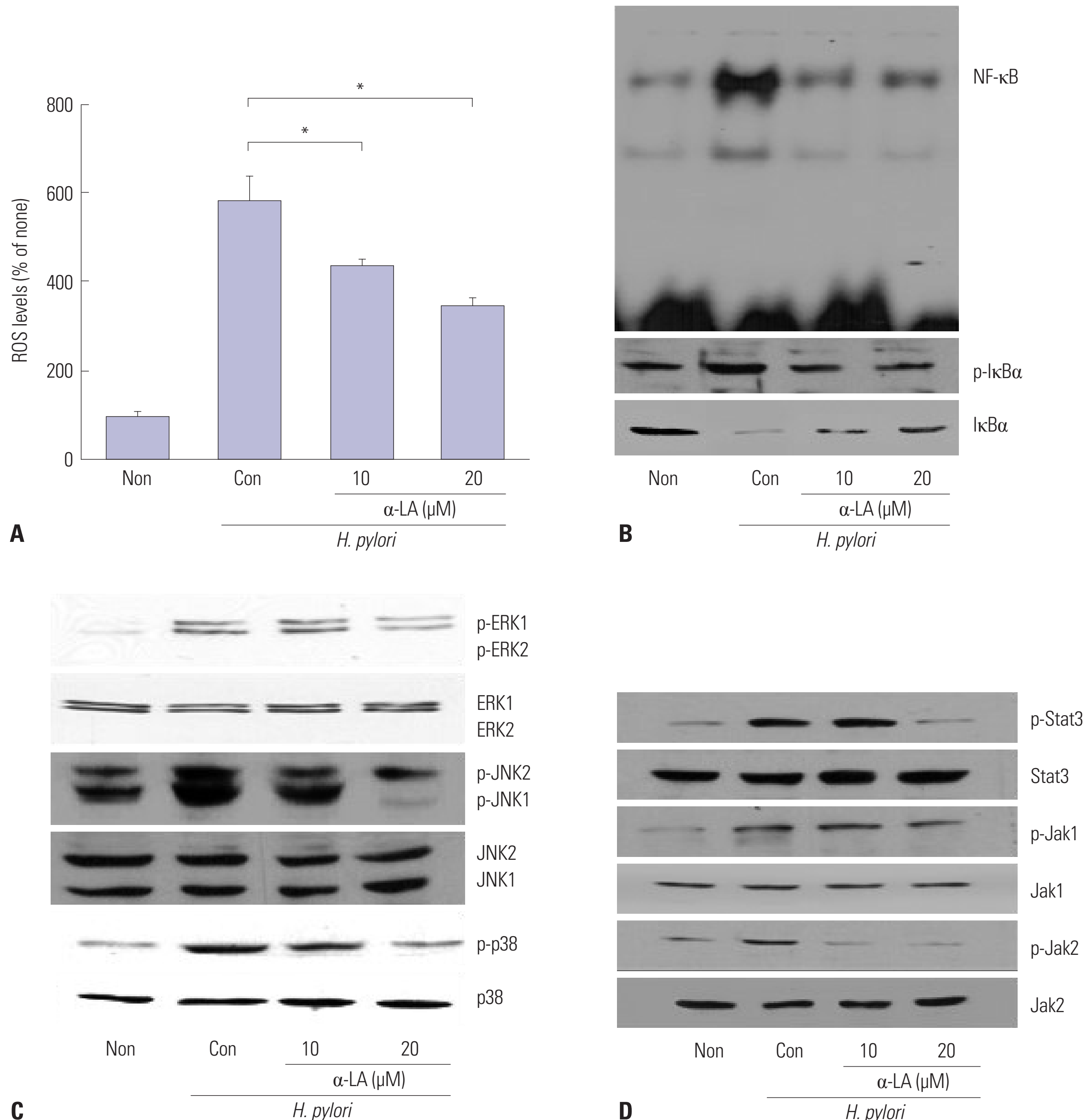

Fig. 2. ROS levels, activation of NF-κB, MAPK, and Jak/Stat in *H. pylori*-infected AGS cells treated with or without α-LA. The cells were pretreated with α-LA for 2 h and cultured in the presence of *H. pylori* for 30 min (ROS levels, activation of NF-κB, MAPK, and Jak/Stat) or 1 h (NF-κB). (A) ROS 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, C) 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 NH_2-terminal protein kinase; Jak/Stat, 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'-dichlorodihydrofluorescein.

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orescein diacetate (Invitrogen, Carlsbad, CA, USA) as previously described.[20] The amount of ROS trapped in the cells was expressed as the relative increase over the ROS level in cells cultured in the absence of *H. pylori*, which was set at 100.

NF-κB-DNA binding activity was determined by electrophoretic mobility shift assay (EMSA) as previously described.[4]

For Western blot analysis, proteins in whole cell extracts were subjected to 6% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes which was blocked using 3–5% nonfat dry milk in Tris-buffered saline and 0.2% Tween 20 (TBS-T) for 2 h at room temperature. The membranes were incubated with antibodies for total and phospho-specific forms of ERK1/2, JNK1/2, p38, Stat3, Jak1, and Jak2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBS-T containing 3% dry milk at 4°C for 16 h. After washing with TBS-T, primary antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (anti-goat or anti-rabbit), respectively, and visualized by the enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instruction.

The statistical differences were determined using one-way ANOVA and Newman-Keul's test. All values are expressed as mean±standard error of means (SEM) of four different experiments. A value of $p<0.05$ was considered statistically significant.

As shown in Fig. 1A and B, *H. pylori* infection induced mRNA expression of IL-8 time-dependently. IL-8 levels in the medium were also increased by *H. pylori* infection. *H. pylori*-induced increases in IL-8 mRNA levels were maximized at 3 h, while IL-8 protein levels in the medium stably increased from 8 h-culture. Therefore, to examine the effect of α-LA on *H. pylori*-induced expression of IL-8, AGS cells were pretreated with α-LA and cultured in the presence of *H. pylori* for 3 h (to assess mRNA levels) and 8 h (to assess protein levels in the medium) (Fig. 1C and D). α-LA showed inhibitory effect on *H. pylori*-induced IL-8 expression at both mRNA and protein levels in a dose-dependent manner.

To investigate whether α-LA inhibits *H. pylori*-induced increases in ROS levels and activation of NF-κB, intracellular ROS levels, NF-κB DNA binding activity, and levels of total and phospho-specific forms of IκBα were determined in the cells infected with *H. pylori* treated with or without α-LA. α-LA suppressed *H. pylori*-induced increases in ROS levels and NF-κB activation, accompanied with inhibition of phosphorylation of IκBα and a decrease in the total forms of IκBα (Fig. 2A and B). As shown in Fig. 2C, α-LA suppressed *H. pylori*-induced phosphorylation of ERK1/2, JNK1/2, and p38, while total forms of ERK1/2, JNK1/2, and p38 were not affected by *H. pylori* infection or α-LA treatment.

We investigated whether Jak-Stat signaling is involved in *H. pylori*-induced inflammation. As shown in Fig. 2D, *H. pylori* infection induced phosphorylation of Stat3, accompanied with phosphorylation of Jak1 and Jak2 in AGS cells, which was inhibited by α-LA dose-dependently. Total forms of Stat3, Jak1, and Jak2 were not changed by *H. pylori* infection or α-LA treatment. 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,[17,18] 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.[3] 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.[21] Bronte-Tinkew, et al.[22] 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.[23] 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 actvation 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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