

Jak1/Stat3 Is an Upstream Signaling of NF- κ B Activation in *Helicobacter pylori*-Induced IL-8 Production in Gastric Epithelial AGS Cells

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Helicobacter pylori (*H. pylori*) induces the activation of nuclear factor- κ B (NF- κ B) and cytokine expression in gastric epithelial cells. The Janus kinase/signal transducers and activators of transcription (Jak/Stat) cascade is the inflammatory signaling in various cells. The purpose of the present study is to determine whether *H. pylori*-induced activation of NF- κ B and the expression of interleukin-8 (IL-8) are mediated by the activation of Jak1/Stat3 in gastric epithelial (AGS) cells. Thus, gastric epithelial AGS cells were infected with *H. pylori* in Korean isolates (HP99) at bacterium/cell ratio of 300:1, and the level of IL-8 in the medium was determined by enzyme-linked immunosorbent assay. Phospho-specific and total forms of Jak1/Stat3 and I κ B α were assessed by Western blot analysis, and NF- κ B activation was determined by electrophoretic mobility shift assay. The results showed that *H. pylori* induced the activation of Jak1/Stat3 and IL-8 production, which was inhibited by a Jak/Stat3 specific inhibitor AG490 in AGS cells in a dose-dependent manner. *H. pylori*-induced activation of NF- κ B, determined by phosphorylation of I κ B α and NF- κ B-DNA binding activity, were inhibited by AG490. In conclusion, Jak1/Stat3 activation may mediate the activation of NF- κ B and the expression of IL-8 in *H. pylori*-infected AGS cells. Inhibition of Jak1/Stat3 may be beneficial for the treatment of *H. pylori*-induced gastric inflammation, since the activation of NF- κ B is inhibited and inflammatory cytokine expression is suppressed.

Key Words: *Helicobacter pylori*, Jak1/Stat3, IL-8, NF- κ B, gastric epithelial cells

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Helicobacter pylori (*H. pylori*) is involved in the pathogenesis of gastric diseases including gastritis, peptic ulcer, and gastric adenocarcinoma.¹⁻⁵ Interleukin-8 (IL-8) is an activator of neutrophils which migrate into the infected gastric mucosa.^{6,7} Up-regulation of IL-8 was found in gastric mucosa isolated from the patients infected with *H. pylori*,⁶ and the expression of IL-8 was mediated with the activation of transcription factor NF- κ B in gastric epithelial cells.⁸⁻¹⁰ Furthermore, signal transducers and activators of transcription (Stat) regulates IL-8 transcription.^{11,12}

Janus kinase (Jak)/Stat signaling is one of the important immune and cytokine

signals. Various subtypes of Jak and Stat molecules are activated by specific inflammatory stimuli.^{13,14} The binding of inflammatory ligand such as cytokine to its receptor induces the assembly of an active receptor associated Jak. Phosphorylated Jak leads to the activation of neighboring Jak, which provides the docking sites for Stat. Stat is phosphorylated on tyrosine and serine residues which is required for full Stat activity. Phosphorylated Stat forms dimers and translocate to the nucleus where they bind directly to the promoter region of specific target genes involved in immune responses.¹⁵⁻¹⁸ In the present study, we determined whether *H. pylori*-induced activation of NF- κ B and IL-8 production are mediated by the activation of Jak1/Stat3 in gastric epithelial AGS cells using a Jak/Stat3 specific inhibitor AG490.

A gastric epithelial cell line AGS (gastric adenocarcinoma, ATCC CRL 1739, American Type Culture Collection, Manassas, VA, USA) and *H. pylori* strain HP99 in Korean isolates were cultured as previously described.¹⁹ HP99 was identified as *cagA+*, *vacA* s1b, m2, *iceA1* *H. pylori* strain.¹⁹ Tyrphostin AG490 (a Jak/Stat3 specific inhibitor) was purchased from Calbiochem (La Jolla, CA, USA). AG490 was dissolved in dimethylsulfoxide (DMSO). The cells were pre-treated with AG490 (10, 20, or 40 μ M final concentration) for 2 h and cultured in the presence of *H. pylori* for 30 min (for Jak1/Stat3 activation) and 24 h (for IL-8 levels in the medium). The control group received DMSO instead of AG490. The concentration of DMSO did not exceed 1%. In other set of experiment, AGS cells were pretreated at a final concentration of 40 μ M AG490 2 h prior to *H. pylori* infection. The cells were cultured in the presence of *H. pylori* for 1 h (for NF- κ B-DNA binding activity and protein levels of I κ B α). IL-8 level in the medium was determined by enzyme-linked immunosorbent assay kits (Invitrogen Corporation, Carlsbad, CA, USA). For determination of protein levels of Jak1, p-Jak1, Stat, p-Stat3, I κ B α , and p-I κ B α , whole cell extracts (50 μ g protein) were subjected to 6% SDS-PAGE and the proteins were detected with polyclonal antibodies against Jak1 (1:500, Cat. No. 3332, Cell Signaling, Beverly, MA, USA), Stat3 (1:500, Cat. No. 06-596, Upstate Biotechnology, Lake Placid, NY, USA), phospho-Jak1 (1:500, Cat. sc-16773, Santa Cruz Biotechnology, Dallas, TX, USA), phospho-Stat3 (1:500, Cat. No. 9131, Cell Signaling), pan-I κ B (1:500, Cat. sc-371, Santa Cruz Biotechnology) and phospho-I κ B α (1:500, Cat. No. 9241, Cell Signaling), followed by goat anti-rabbit secondary antibodies (1:2000, Cat. No. sc-2004, Santa Cruz Biotechnology) conjugated to horseradish peroxidase, which was followed by

enhanced chemiluminescence (Santa Cruz Biotechnology).¹⁹ Actin served as a loading control. Electrophoretic mobility shift assay (EMSA) was performed for NF- κ B-DNA binding activity using nuclear extracts as described previously.¹⁹ The statistical differences were determined using one-way ANOVA, followed by a Newman-Keul's test. All values are expressed as mean \pm S.E. of four different experiments. A value of $p < 0.05$ was considered statistically significant.

Fig. 1A shows that *H. pylori*-induced increase in IL-8 level in the medium was inhibited by AG490 in AGS cells dose-dependently. Phospho-specific forms of Jak1 and Stat3 were increased by *H. pylori* which was inhibited by AG490 treatment (Fig. 1B). Total forms of Jak1 and Stat3 were not changed by *H. pylori* infection and AG490 treatment. These results show that AG490 efficiently inhibits Jak1/Stat3 activation in *H. pylori*-infected gastric epithelial AGS cells. NF- κ B activation was determined by NF- κ B-DNA binding activity and phosphorylation of I κ B by EMSA and Western blot analysis (Fig. 1C and D). AG490 suppressed phosphorylation of I κ B α and NF- κ B-DNA binding activity in *H. pylori*-infected AGS cells. Since I κ B α is phosphorylated to p-I κ B α by *H. pylori*, total I κ B α was decreased in the infected cells, which was inhibited by AG490.

H. pylori-induced inflammatory signaling has been known to focus on the activation of NF- κ B with IL-8 expression.^{8,9,19} In the present study, we found that Jak1/Stat3 activation mediated the production of IL-8 in *H. pylori*-infected cells. Even though Jak/Stat signaling has been widely studied on inflammatory response in brain,^{20,21} relatively little studies on Jak/Stat activation in relation to gastric diseases have been reported. Lee, et al.²² reported that the phosphorylation status of *H. pylori* protein CagA affects the signal switch between the SHP2 (Src homology 2 domain-containing Src homology tyrosine phosphatase 2)/ERK and Jak2/Stat3 pathways through glycoprotein 130 in gastric epithelial cells. Stat3 activation mediated by non-phosphorylated CagA is dependent on Jak2 activation in AGS cells. In the present study, AG490, an inhibitor of Jak/Stat3, suppressed *H. pylori*-induced activation of NF- κ B and IL-8 expression in AGS cells, and our recent study showed that HP99-induced activation of Jak2/Stat3 mediates the expression of monocyte chemoattractant protein-1 (MCP-1) and inducible nitric oxide synthase (iNOS) in AGS cells.²³ Since we did not determine phosphorylation status of *H. pylori* CagA in the present study, further study should be performed to investigate the involvement of phosphorylated or non-phosphorylated CagA in Jak/Stat3-mediated NF- κ B

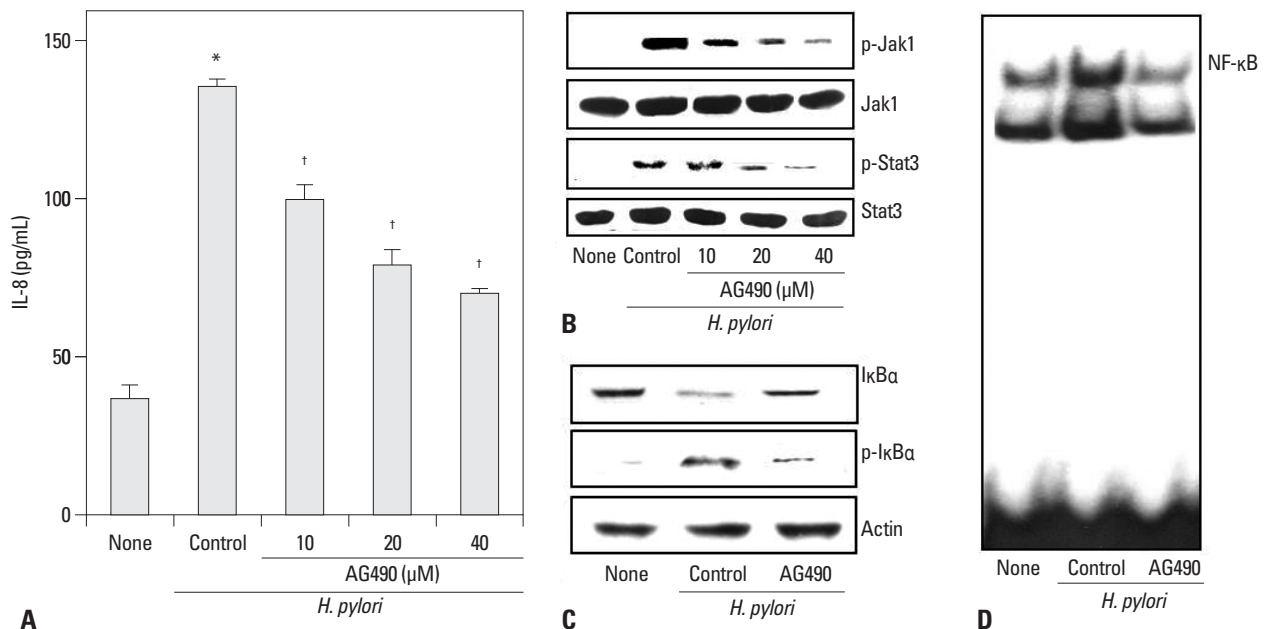


Fig. 1. Effect of AG490 on *H. pylori*-induced increase of IL-8 levels in the medium, activation of Jak1/Stat3, phosphorylation of IκBα, and NF-κB-DNA binding activity in AGS cells. The cells were pre-treated with AG490 for 2 h and cultured in the presence of *H. pylori* for 24 h (for IL-8 levels in the medium, A), 30 min (for Jak1/Stat3 activation, B), and 1 h [for protein levels of IκBα and p-IκBα (C) and NF-κB-DNA binding activity (D)]. IL-8 level in the medium was determined by enzyme-linked immunosorbent assay. Protein levels of Jak1, p-Jak1, Stat3, p-Stat3, IκBα, and p-IκBα were determined by Western blot analysis. Actin was used for protein loading control. Electrophoretic mobility shift assay was performed for NF-κB-DNA binding activity. All values are expressed as mean±S.E. of four different experiments. A value of $p < 0.05$ was considered statistically significant. * $p < 0.05$ vs. none (the cells cultured in the absence of *H. pylori* without treatment of AG490), † $p < 0.05$ vs. *H. pylori* control (the cells cultured in the presence of *H. pylori* without treatment of AG490). *H. pylori*, *Helicobacter pylori*; IL-8, interleukin-8; Stat, signal transducers and activators of transcription; Jak, Janus kinase; AGS, gastric adenocarcinoma.

activation in gastric epithelial cells. The present results demonstrate that Jak1/Stat3 activation is an upstream signaling for NF-κB activation to induce IL-8 expression in *H. pylori*-infected gastric epithelial cells.

In the present study, AGS cells were infected with HP99 at bacterium/cell ratio of 300:1. Our previous study showed that Jak1/Stat3 and Jak2/Stat3 were activated at multiplicity of infection (MOI) of 300 bacteria per AGS cells,^{23,24} and Ritter, et al.²⁵ demonstrated that IL-8 expression is differentially regulated by MOI of *H. pylori* per gastric epithelial cells; IL-8 mRNA level is elevated in response to high MOI independent of *cagA* and *vacA* gene characteristics, and only *cagA*⁺ strains significantly induce IL-8 expression by lower MOI.²⁵ Previously, we found that both 100 and 300 MOI induced COX-2 expression^{26,27} whereas 300 MOI showed the activation of NF-κB and Jak1/Stat3 in HP99-infected AGS cells.²⁴ Therefore, 100 MOI may activate Jak/Stat3 in HP99-infected cells. Further study with lower MOI is needed to determine whether Jak/Stat3 is differentially activated by MOI of *H. pylori* per gastric epithelial cells.

Since NF-κB activation is followed by phosphorylation and proteosomal degradation of IκBα, we determined the effect of AG490 on phosphorylation of IκBα in *H. pylori*-infected AGS cells. Inhibition of Jak1/Stat3 by AG490 in-

hibited phosphorylation of IκBα thus suppressing NF-κB activation in *H. pylori*-infected AGS cells. Novel finding of the present study is that the Jak1/Stat3 activation mediates the activation of NF-κB and the expression of IL-8 in *H. pylori*-infected AGS cells. Recently, we showed that AG490 inhibited the activation of Jak2 which is activated in HP99-infected cells.²³ Therefore, Jak2 may regulate NF-κB activation in HP99-infected AGS cells. Further study should be performed to elucidate the involvement of Jak2 on *H. pylori*-induced NF-κB activation in gastric epithelial cells.

We earlier showed that *H. pylori* induced translocation of heat shock protein 90β (Hsp 90β) from cytosol to membrane in *H. pylori*-infected gastric epithelial cells.²⁸ In the membrane, Hsp 90β interacts with Rac1, which activates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) to produce reactive oxygen species (ROS). An NADPH oxidase inhibitor diphenyleneiodonium (DPI) suppressed *H. pylori*-induced activation of Jak1/Stat3 in AGS cells, suggesting that ROS may activate Jak1/Stat3, determined by phosphorylation of Jak1/Stat3.²⁴ ROS are known activators for NF-κB by inducing phosphorylation of inhibitory subunit IκBα through activation of IκB kinase,²⁹ resulting in NF-κB binding to the promoter region of IL-8 gene.³⁰ Taken together, *H. pylori* induces activation of NADPH ox-

dase and produces ROS, resulting in the activation of both NF-κB and Jak1/Stat3 in gastric epithelial cells.

In addition, ROS activates Src kinase,³¹ which controls phosphorylation of *H. pylori* CagA³² in the infected cells. CagA activates focal adhesion kinase and Src.³³ Src-dependent activation of Stat3 by tyrosine phosphorylation has been observed in renal cyst-lining cells, independently of Jak family kinases.³⁴ Therefore, dual inhibition of Janus and Src family kinases blocks constitutively-activated Stat3 in pancreatic cancer cells.³⁵ Even though Src kinase was not determined in the present study, Src kinase may be involved in *H. pylori*-induced activation of Stat3 in gastric epithelial cells. Recent study showed that thrombin-induced NF-κB activation and IL-8 release are mediated by c-Src-dependent Shc, Raf-1, and ERK pathways in lung epithelial cells.³⁶ Therefore, Src kinase may mediate phosphorylation of IκBα and NF-κB activation in *H. pylori*-infected cells, which should further be studied to clarify pathologic mechanism of *H. pylori*-associated gastric diseases.

In the present study, we found that Jak1/Stat3 is an upstream signaling for NF-κB activation in *H. pylori*-infected gastric epithelial cells. Therefore, inhibition of Jak/Stat3 may be beneficial for the treatment of *H. pylori*-induced gastric inflammation by inhibiting the activation of NF-κB and suppressing inflammatory cytokine expression.

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