CagA\textsuperscript{+} \textit{H.pylori} Induces Akt1 Phosphorylation and Inhibits Transcription of p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} via PI3K/Akt1 Pathway\textsuperscript{1}

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\textbf{Objective} Cytotoxin-associated protein (CagA) of \textit{H. pylori} has been confirmed to be closely associated with gastric inflammation and tumorigenesis, but the mechanism behind it is little understood. In this study, we try to determine roles of CagA\textsuperscript{+} strain in activating PI3K/Akt signaling pathway, and affecting expression of p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1}, and also in releasing IL-8 in host cells. \textbf{Methods} Akt1 phosphorylation and IL-8 levels of CagA\textsuperscript{+} and CagA\textsuperscript{−} strain infected AGS cells were detected by ELISAs. Two quantitative RT-PCRs were established to measure p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} mRNA levels in the CagA\textsuperscript{+} and CagA\textsuperscript{−} strain infected cells. LY294002, an inhibitor of PI3K/Akt pathway, was used to define effect of the pathway in IL-8 release. \textbf{Results} CagA\textsuperscript{+} strain could induce an obvious elevation of Akt1 phosphorylation in the infected AGS cells while CagA\textsuperscript{−} strain failed to do so. The CagA\textsuperscript{+} \textit{H. pylori} strain infected AGS cells showed significant drops both in p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} mRNA levels, whereas the CagA\textsuperscript{−} \textit{H. pylori} strain caused a remarkable increase in p21\textsuperscript{WAF1/CIP1} mRNA without affecting p27\textsuperscript{KIP1} gene transcription in the AGS cells. Both the CagA\textsuperscript{+} and CagA\textsuperscript{−} \textit{H. pylori} strains enabled AGS cells to produce close elevated levels of IL-8, and the LY294002 block resulted in unexpected elevations of IL-8 levels. \textbf{Conclusion} CagA can activate PI3K/Akt1 pathway that plays an inhibitory role in IL-8 release in \textit{H. pylori} infected AGS cells. Activation of PI3K/Akt1 pathway and subsequent negative regulation of p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} expression might be involved in CagA-associated carcinogenesis.

\textbf{Key words}: Helicobacter pylori; CagA; PI3K; Akt1; p21\textsuperscript{WAF1/CIP1}; p27\textsuperscript{KIP1}; IL-8

\textbf{INTRODUCTION}

\textit{Helicobacter pylori}, a Gram-negative bacterium that colonizes in human gastric epithelia, is the primary cause of human gastritis\textsuperscript{[1]}. Infection with \textit{H. pylori} will increase the risk to peptic ulceration and gastric adenocarcinoma\textsuperscript{[2-3]}. In the \textit{H. pylori} infected gastric mucosa, infiltration with a large number of neutrophil, a key inflammatory cell, could be found\textsuperscript{[4]}. Interleukin 8 (IL-8) is a powerful chemokine and activator of neutrophil\textsuperscript{[5]}. Furthermore, recent data have suggested that IL-8 might contribute to gastric carcinogenesis\textsuperscript{[6]}

Cytotoxin-associated protein (CagA), a key virulent factor of \textit{H. pylori}, has been confirmed to play an important role in pathogenicity by translocating CagA into host cells and inducing gastric inflammation and gastric tumors\textsuperscript{[7-8]}. However, so far the specific nature of CagA of \textit{H. pylori} to induce gastric carcinogenesis has been little understood.

Phosphoinositide 3-kinase (PI3K) pathway is an intracellular phosphatidylinositol-associated signaling route that mediates extensive important biological activities in cells\textsuperscript{[9]}. Akt1 (protein kinase B\textsubscript{a}) is located at the downstream of PI3K, and PI3K/Akt signaling pathway plays an important role in tumorigenesis\textsuperscript{[10]}. p21\textsuperscript{WAF1/CIP1} as part of cyclin-dependent kinase inhibitors (CDKIs), serves as a substrate of Akt1\textsuperscript{[11]}, and negatively regulates cell cycle progression resulting in an inhibition of tumorigenesis\textsuperscript{[12]}. p27\textsuperscript{KIP1}.
another member of CDKIs family, is a tumor depression and cell-cycle regulation gene. On the other hand, recent data revealed that \textit{H. pylori} could induce an increase in inositol phosphates in cultured epithelial cells, and PI3K/Akt pathway inhibitor LY294002 could block IL-8 mediated neutrophil adhesion. All these data imply that PI3K/Akt signaling pathway may play a role in \textit{H. pylori}-associated inflammation and tumorigenesis.

To make certain the role of PI3K/Akt signaling pathway in interaction between \textit{H. pylori} strains with or without CagA and host cell derived from human gastric mucosa, the effect and diversity of CagA-positive (CagA+) strain and CagA-negative (CagA-) strain of \textit{H. pylori} on Akt1 activation, p21\textsuperscript{WAF1/CIP1} regulation and p27\textsuperscript{KIP1} expressions, as well as on IL-8 release induction in infected cells are determined in this study.

MATERIALS AND METHODS

Cell Line and Cell Culture

Human gastric cell line (AGS) was offered by Cell Bank of the Institute of Cell Biology in Shanghai, Chinese Academy of Science, and was routinely grown in Ham’s F12 medium (Gibco BRL, Gaithersburg MD, USA) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified 5% CO\textsubscript{2} atmosphere.

\textit{H. pylori} Strains and Growth

One hundred and eight \textit{H. pylori} strains isolated from clinical gastric biopsy specimens of patients with either gastritis or peptic ulcer were identified by Gram-staining microscopy, urease assay (bioMérieux sa, France) and oxidase test (bioMérieux sa, France). CagA gene and its product CagA of the isolates were characterized by PCR using the reported D008/R008 primers, and by Western Blot using rabbit anti-rCagA1 serum as the primary antibody and sheep anti-rabbit HRP-labeling IgG (Jackson Immuno Research, West Grove, USA) as the secondary antibody. According to the results, one CagA+ strain and one CagA- strain used in this study were screened for the subsequent experiments. The two strains were cultured on Columbia agar (bioMérieux sa, France) plates supplemented with 5% sheep’s blood (Curtin Matheson, Jessup, MD), \textit{H. pylori} selective supplement (Oxoid, Basingstock, England), and antibiotic mixture (10 mg/L vancomycin, 5 mg/L trimethoprim, and 5 mg/L amphotericin-B) (Sigma, MO, USA). The plates were incubated at 37 °C in a microaerobic atmosphere (10% CO\textsubscript{2}, 5% O\textsubscript{2}, 85% N\textsubscript{2}).

Cell Infection Model

\(1 \times 10^5\) AGS cells were seeded per well in 12-well culture plates and incubated at 37 °C in a 5% CO\textsubscript{2} atmosphere for 24 h. Each of the well-grown \textit{H. pylori} strains was harvested from plates, and washed with autoclaved phosphate buffered saline (PBS), and then re-suspended in FCS- and antibiotics-free F12 medium of desired concentration. The medium in plates was removed, and then plates were washed twice with antibiotics-free Ham’s F12 medium. After washing, 2 mL per well of each the \textit{H. pylori} suspensions was added and then co-incubated at 37 °C in a 5% O\textsubscript{2} atmosphere for desired time.

MTT Assay

According to the manufacturer’s instruction, a MTT test kit (Sigma) was used to examine possible cytotoxicity of PI3K/Akt pathway inhibitor LY294002 (Sigma) in AGS cells. In this test, the used LY294002 dosages were 10, 30, and 50 µmol/L, and the cell was incubated at different dosages of LY294002 for 24 h.

Detection of Akt1 Phosphorylation

\(1 \times 10^5\) AGS cells were seeded per well in 12-well culture plates and then pre-cultured and infected with either CagA+ or CagA- \textit{H. pylori} strain as described above. After being rinsed with ice-cold PBS, the cells were lysed with Cell Lysis Buffer plus 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling Technology, MA, USA). And then, they were scraped off the plates, transferred into a tube, sonicated on ice-water, and centrifuged for 10 min. Finally, the supernatant was collected and Lowry assay was performed for equal loading. A PathScan Phospho-Akt1 (ser473) Sandwich ELISA Kit (Cell Signaling Technology) was used to detect the level of serine 473 phosphorylated Akt1 protein.

Quantitative Detection of p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} mRNAs

Based on a previous report, two separated quantitative RT-PCR were designed to detect p21\textsuperscript{WAF1/CIP1} and p27\textsuperscript{KIP1} mRNA levels in which \(\beta\)-actin gene was used as the housekeeping control. Briefly, total RNA of the AGS cells that were infected with either CagA+ or CagA- \textit{H. pylori} strain for desired time were extracted by using TRIzol reagent (Gibco BRL), and then were reversely transcribed to cDNA with random primers by using ThermoScript RT-PCR System Kit (Promega, WI,
USA). In PCRs, both the ratios of p21\textsuperscript{WAF1/CIP1} to β-actin primer-sets and p27\textsuperscript{KIP1} to β-actin primer-sets were 2.0/0.25. The products were firstly examined by electrophoresis in 1.2% agarose gel pre-stained with ethidium bromide, and then were quantitatively measured by a Gel Image Analyzor (Bio-Rad, USA). Information about primers and quantitative RT-PCR were presented in table 1\cite{12, 20}.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Annealing Temperature</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21\textsuperscript{WAF1/CIP1} F</td>
<td>ACTGTGATGCGCTAATGGC</td>
<td>58 °C</td>
<td>232 bp</td>
</tr>
<tr>
<td>p21\textsuperscript{WAF1/CIP1} R</td>
<td>ATGGCTTCTCTGCTGTCC</td>
<td>58 °C</td>
<td>232 bp</td>
</tr>
<tr>
<td>p27\textsuperscript{KIP1} F</td>
<td>AGGTGCCCTGCAAGGTGCCGC</td>
<td>58 °C</td>
<td>258 bp</td>
</tr>
<tr>
<td>p27\textsuperscript{KIP1} R</td>
<td>CTCCACAGAAACCGGCATTGG</td>
<td>58 °C</td>
<td>258 bp</td>
</tr>
<tr>
<td>β-actin F</td>
<td>TGACGGGGTCACCCACACTGCTCA TCTA</td>
<td>58 °C</td>
<td>661 bp</td>
</tr>
<tr>
<td>β-actin R</td>
<td>CTAGAAGCATTTGCGGTGGACGATGGAGGG</td>
<td>58 °C</td>
<td>661 bp</td>
</tr>
</tbody>
</table>

Note. *F and R mean the Forward and Reverse Primers, respectively.

**IL-8 Measurement**

AGS cells grown in 24-well plates were infected with either CagA\textsuperscript{+} or CagA\textsuperscript{−} H. pylori strain for desired time as described above and supernatants were then collected by centrifugation at 1 000 rpm at 4 °C for 10 min. IL-8 levels in the supernatants were determined by a commercial ELISA kit (TPI, USA).

**Statistical Analysis**

Data were presented as means±SD from at least three independent experiments. Statistical analysis was performed with t-test (SPSS software).

**RESULTS**

**Characteristics of H. pylori Strains**

Each of the two H. pylori isolates showed Gram-negative curved bacillus, and presented positive results in both urease assay and oxidase test. The CagA\textsuperscript{+} strain presented a 298 bp target amplification segment from cagA gene and a positive Western hybridization signal to rCagA1 antiserum, while CagA\textsuperscript{−} strain failed to do so (Fig. 1).

**Akt1 Activation Induced by CagA\textsuperscript{+} H. Pylori Strain**

While AGS cells were respectively co-incubated with the CagA\textsuperscript{+} and CagA\textsuperscript{−} strains, only the former was able to induce an obvious elevation of Akt1 phosphorylation after co-incubation for 1 h, and then the phosphorylation level gradually decreased (Fig. 2). Furthermore, the elevation of Akt1 phosphorylation caused by CagA\textsuperscript{+} H. pylori displayed a bacterial concentration dependent manner (Fig. 3).

**Cytotoxicity of LY294002**

After 24 h co-incubation, 10 and 30 μmol/L LY294002 had no impacts on viability of the treated AGS cells, while 50 μmol/L LY294002 treated cells...
showed a slight drop in its cell growth curve (data not shown).

### Alteration of p21\(^{\text{WAF1/CIP1}}\) and p27\(^{\text{KIP1}}\) mRNA Levels

The CagA\(^{+}\) \textit{H. pylori} strain infected AGS cells showed a lowest p21\(^{\text{WAF1/CIP1}}\) mRNA level after co-incubation for 30 min, and then the mRNA approached to the original level (Fig. 4). In contrast, the CagA\(^{-}\) \textit{H. pylori} strain induced a significant increase in p21\(^{\text{WAF1/CIP1}}\) mRNA level after co-incubation for 1 h, and then the mRNA level was gradually retrieved. For alteration of p27\(^{\text{KIP1}}\) transcription, the CagA\(^{+}\) \textit{H. pylori} strain infected AGS cells presented a remarkable drop in p27\(^{\text{KIP1}}\) mRNA level after co-incubation for 3 h, whereas no obvious alteration of p27\(^{\text{KIP1}}\) mRNA levels in AGS cells throughout the co-incubation with CagA\(^{-}\) \textit{H. pylori} strain could be found (Fig. 5). However, the CagA\(^{+}\) or CagA\(^{-}\) \textit{H. pylori} strain infected AGS cells did not show marked alterations of p21\(^{\text{WAF1/CIP1}}\) or p27\(^{\text{KIP1}}\) mRNA levels if cells were pre-treated with LY294002.

### IL-8 Release Induced by \textit{H. Pylori}

Both CagA\(^{+}\) and CagA\(^{-}\) \textit{H. pylori} strains could efficiently induce IL-8 release of the infected AGS cells in a time-dependent manner, but the IL-8 levels were close to each other (Figs. 6 and 7). When LY294002 (30 μmol/L), a common inhibitor of PI3K/Akt pathway, was used to pre-treat AGS cells for 30 min, unexpected elevations of IL-8 levels were presented (Figs. 6 and 7).

### DISCUSSION

PI3K/Akt signaling pathway is becoming significant in interaction between bacteria and host cells. However, the role of this pathway regarding \textit{H. pylori} infected cells has not been given sufficient attention\cite{21}. Related articles drew controversial
conclusions; some revealed that PI3K inhibition could block NF-κB-dependent gene expression\textsuperscript{[22]}, while others suggested that inhibition of PI3-kinase in HT-29 cells resulted in an induction of NF-κB binding activity\textsuperscript{[23]}. Since IL-8 encoding gene is a prominent target of NF-κB\textsuperscript{[24–25]}, and \textit{H. pylori} could induce IL-8 expression in gastric epithelial cells\textsuperscript{[26]}, PI3K/Akt signaling pathway might be involved in IL-8 expression in \textit{H. pylori} infected cells.

Our results showed that the CagA\textsuperscript{+} \textit{H. pylori} strain was able to induce an obvious transient elevation of Akt1 phosphorylation in the infected AGS cells, but the CagA\textsuperscript{−} \textit{H. pylori} strain was otherwise. In addition, the elevation of Akt1 phosphorylation caused by CagA\textsuperscript{+} \textit{H. pylori} displayed a bacterial concentration dependent nature. These data indicated that PI3K/Akt1 signaling pathway in the host cells was involved in \textit{H. pylori} infection, and activation of the pathway might be related to CagA-induced Akt1 phosphorylation. Furthermore, in this study we found that the p21\textsuperscript{WAF1/CIP1} and p2\textsuperscript{7KIP1} mRNA levels in CagA\textsuperscript{+} \textit{H. pylori} infected AGS cells decreased, while the p21\textsuperscript{WAF1/CIP1} mRNA level in CagA\textsuperscript{−} \textit{H. pylori} infected AGS cells increased. As described above, evidence predicted a close correlation between CagA\textsuperscript{+} \textit{H. pylori} infection and gastric cancer generation\textsuperscript{[2–3]}, and both p21\textsuperscript{WAF1/CIP1} and p2\textsuperscript{7KIP1} had potentials to inhibit tumorigenesis\textsuperscript{[12]}. So we suggested that cancerogenesis of CagA might be at least partially dependent on the elevation of Akt1 phosphorylation level and down regulation of p21\textsuperscript{WAF1/CIP1} and p2\textsuperscript{7KIP1} expressions.

Accumulated evidence showed that \textit{H. pylori} infection could induce IL-8 release in AGS cells and induction of IL-8 produce in gastric epithelial cells dependent on the presence of CagA protein\textsuperscript{[27–28]}. However, our results confirmed that both CagA\textsuperscript{+} and CagA\textsuperscript{−} \textit{H. pylori} strains induced similar levels of IL-8 in the infected AGS cells, which was different from the reports mentioned above. Besides, block of PI3K pathway in this study produced an unexpected elevation of IL-8 levels. Although in previous reports IL-8 gene was proved to be a unique target of activated NF-κB\textsuperscript{[6,13]} and DEP (diesel exhaust particle) was activated NF-κB through PI3K/Akt signaling pathway\textsuperscript{[29]}, our results suggested that PI3K/Akt1 pathway, at least in \textit{H. pylori} infected AGS cells, might play an inhibitory role in IL-8 release.

REFERENCES


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