Helicobacter pylori cytotoxin-associated gene A protein upregulates α-enolase expression via Src/MEK/ERK pathway: Implication for progression of gastric cancer

SHUAIYIN CHEN[1], GUANGCAI DUAN[1], RONGGUANG ZHANG[1] and QINGTANG FAN[2]

[1]Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University;
[2]Henan Key Laboratory of Molecular Medicine, Zhengzhou, Henan, P.R. China

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Abstract. Persistent infection with *Helicobacter pylori* confers an increased risk for the development of gastric cancer. In our previous investigations, we found that ENO1 was overexpression in *cagA*-positive *H. pylori*-infected gastric epithelial AGS cells by proteomic method, in contrast to the isogenic *cagA* knock out mutant *H. pylori*-infected cells. ENO1 is a newly identified oncoprotein overexpressed in some cancer. However, the relationship between *H. pylori* infection and ENO1 expression still remains undefined. The AGS gastric cancer cells were transfected with WT-cagA plasmid and PR-cagA plasmids. Expression of ENO1 mRNA and protein were measured by real-time quantitative PCR and western blot analysis. Signal protein inhibitor treatment was used to investigate the signal pathways. It was found that the ENO1 mRNA and protein overexpression levels were dependent on *cagA* gene expression and CagA protein phosphorylation. Further analysis revealed that the Src, MEK and ERK pathway was involved in this upregulation effect. Our data suggest that ENO1 was upregulated by CagA protein through activating the Src and MEK/ERK signal pathways, thereby providing a novel mechanism underlying *H. pylori*-mediated gastric diseases.

Introduction

Infection with *Helicobacter pylori* is the strongest risk factor for the development of chronic gastritis, gastric ulcer and gastric carcinoma, which is the second most common cause of cancer-related death worldwide (1,2). Some *H. pylori* strains possess a cytotoxin-associated gene (cag) pathogenicity island (cag PAI) that is present in about half of the Western strains and most of the Eastern strains (3). One constituent of the cag PAI is cagA, which encodes a 120-140-kDa CagA protein. The CagA protein is directly injected into gastric epithelial cells by the type IV secretory system of *H. pylori*. Once inside host cells, CagA is tyrosine phosphorylated on conserved carboxyl-terminal Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs by Src family kinases (4,5). The phosphorylated and unphosphorylated CagA targets multiple enzymes to activate downstream signal pathways, such as the Ras/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, nuclear factor κB (NF-κB) pathway, Src kinase and the PI3 kinase pathway (6-9). Eventually, these events are important for cell function and are also implicated in carcinogenesis since they regulate fundamental cellular responses such as cell proliferation, cell morphology, cell motility and apoptosis. According to these observations, bacterial oncoprotein CagA participates in gastric epithelial cell injury caused by *cagA*-positive *H. pylori* infection. Epidemiological studies reveal that strains of *H. pylori* carrying the virulence factor CagA protein are associated with an increased risk of gastric mucosal inflammation as well as severe atrophic gastritis and gastric carcinoma compared to strains of *H. pylori* lacking CagA (10,11). However, details of the exact molecular mechanisms by which CagA promotes carcinogenesis are fragmentary.

In order to illuminate the pathogenesis of *H. pylori*-induced gastric diseases, our group has developed a *cagA* gene knock out isogenic mutant strain. In our previous study, an *in vitro* model was established to characterize proteins differentially expressed in *cagA*-positive *H. pylori*-infected gastric epithelial cells versus the *cagA* mutant strain. We found that α-enolase (ENO1) expression level was increased in *cagA*-positive *H. pylori*-infected cells as compared to mutant strain by two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis (12). ENO1 is an isoenzyme of enolase, a key protein in the glycolytic pathway. Overexpression of ENO1 is considered to take part in unlimited cellular proliferation of cancer. ENO1 has also been described as a stress protein induced by hypoxia (13,14).

As mentioned above, *H. pylori* infection could affect the expression level of gastric carcinogenic factors, such as Myc, cyclin D1 and COX-2 (15-18). However, up to now, the relation-

Correspondence to: Dr Guangcai Duan, Department of Epidemiology and Biostatistics, College of Public Health, Zhengzhou University, 100 Kexue Avenue, Zhengzhou, Henan 450001, P.R. China
E-mail: gcduan@zzu.edu.cn

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ship between *H. pylori* infection and ENO1 expression has not been reported. To figure out the effect of CagA on ENO1 may help us to understand the carcinogenic mechanism of *H. pylori* infection. In the present study, we designed CagA transfection in gastric epithelial cells and tried to investigate whether main virulence factor CagA of *H. pylori* could increase the expression of ENO1 *in vitro*. Furthermore, we used signal inhibitors to examine which pathway was involved in the CagA-mediated regulation of ENO1 expression.

Materials and methods

Materials

Bacterial strains and culture conditions. Hp27 (cagA$^+$) isolated from a patient with chronic atrophy gastritis in Zhengzhou, China, was grown on Brucella agar plates containing 10% sheep blood supplemented with 10 mg/l vancomycin, 2,500 U/l polymyxin B, 2 mg/l amphotericin and 5 mg/l trimethoprim under microaerophilic conditions at 37˚C for 3 days. Hp27 is the East Asia type strain, which has EPIYA-ABD type CagA.

Cell lines and culture conditions. The AGS gastric cancer cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in F12 medium (Gibco Life Technologies) with 10% FBS (Gibco Life Technologies) at 37˚C in 5% CO_2.

Methods

Construction of pcDNA3.1 (+) expression vectors containing cagA and of site-directed mutations. Full-length *cagA* DNA (QD306710) was amplified by high-fidelity PCR from the DNA of *H. pylori* strain *Hp27* using primers containing *Eco*RI/*Xho*I. The resultant PCR fragment was digested with *Eco*RI and *Xho*I, cloned into pcDNA3.1 (+) vector giving rise to wild-type *cagA*/pcDNA3.1 (+) plasmid (WT-*cagA*). *Hp27* has three copies of tyrosine phosphorylation site sequences which is the ABD type EPIYA.

We generated the CagA site-directed mutagenesis in which the tyrosine residues present in the B or D copies of the EPIYA sequences were replaced by cysteine by using splice overlap extension PCR. Specific primers were designed for B or D copies respectively. The first step involves synthesis of individual fragments containing mutant sites. Then the mutations were connected by overlap extension PCR. After the results were validated by gene sequencing, the mutated *cagA* gene was cloned into pcDNA3.1 (+) vector. The phosphorylation-resistant plasmids were named PRB-*cagA* and PRD-*cagA*, respectively. The specific primers used for the gene site directed mutations are listed as follows: P1, 5'-CATGAATT CGCCACCATGACTAACGAAAC-3'; P2, 5'-CGCCTCGA GTTAAGATTTCTGGAAAC-3'; B1, 5'-GCCCTGAAGAGC CCATTTGCGCTCAAGTTGCTAAAAAG-3'; B2, 5'-CTTT TTAGCAACTTGAGCGCAAATGGGCTCTTCAGGGC-3'; D1, 5'-CTAGTCCTGAACCCATTTGCGCTACAATTGATT TTGATG-3'; D2, 5'-CATCAAAATCAATTGTAGCGCAAAT GGGTTCAGGACTAG-3'.

Transient transfection. Lipofectamine 2000 reagent (Invitrogen) was used to transfect the plasmid WT-*cagA*, PR-*cagA* or blank vector into 5x10^5 AGS cells in 6-well plates. For transient transfection, 5 μg of the plasmid was transfected into cells with 10 μl transfection reagent according to the manufacturer's protocol and all experiments were repeated three times. After the transfection, cells were harvested and lysed with RIPA buffer supplemented with protease inhibitors before freezing at -80˚C. To investigate associated signal pathways involved in CagA-mediated ENO1 upregulation in the

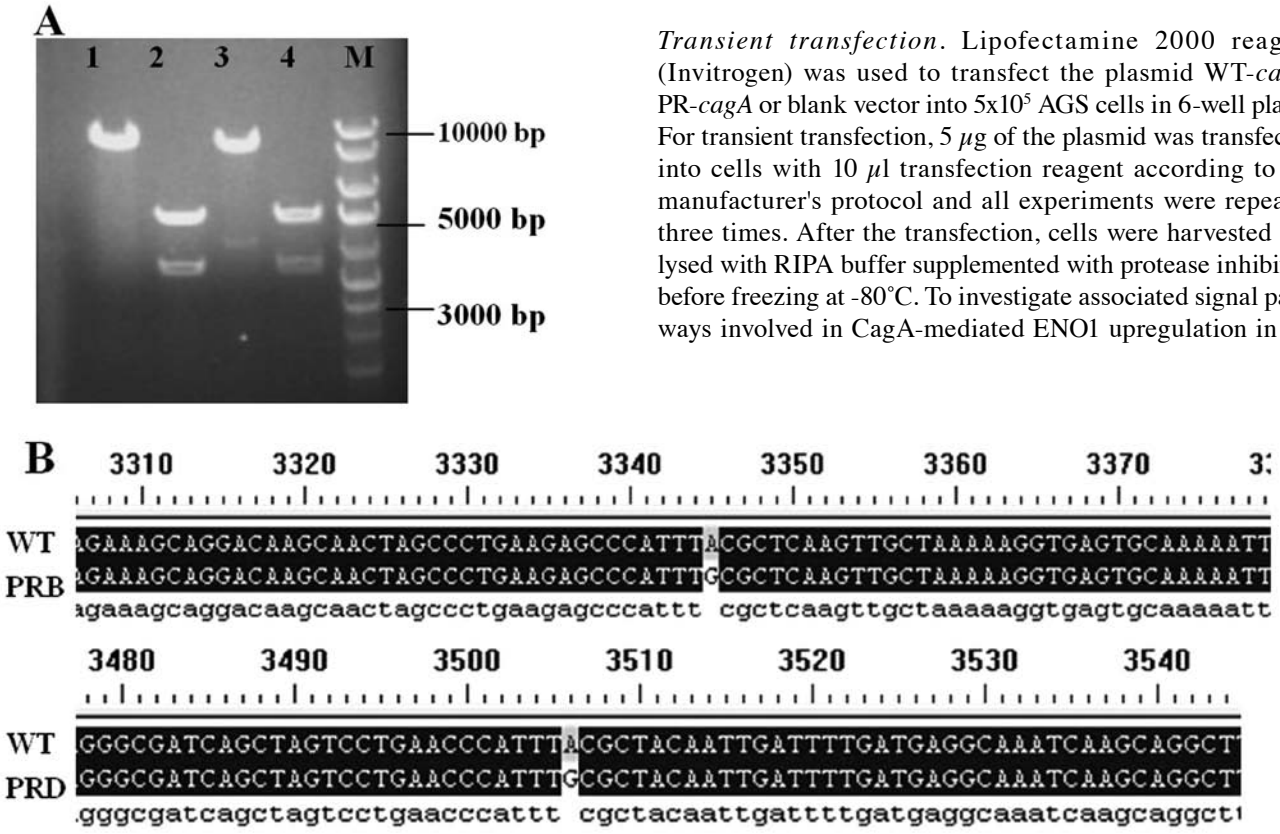

Figure 1. Identification of the recombinant vector. (A) Identification of the WT-cagA and PR-cagA vector by enzyme digestion. Lane 1, WT-cagA vector digested by *Eco*RI. Lane 2, WT-cagA vector digested by *Eco*RI and *Xho*I. Lane 3, PR-cagA vector digested by *Eco*RI. Lane 4, PR-cagA vector digested by *Eco*RI and *Xho*I digested by *Xba*I. PCR and enzyme digestion. The recombinant vector WT-cagA and PR-cagA. (B) The PR-cagA vectors were verified by sequencing. The DNA sequencing results revealed that the codon TAC which codes the tyrosine residues present in EPIYA sequences were replaced by cysteine (TGC).

epithelial cells, we used the following reagents: BAY 11-7082 (5 μM), LY294002 (50 μM), PP1 (10 μM), U0126 (10 μM) (all from Cell Signaling Technology). A respective reagent was used to pre-incubate cells for 1 h and then the plasmid was transfected into cells.

Quantitative detection of eno1 mRNA. The quantitative RT-PCR was designed to detect eno1 mRNA level in which *GAPDH* gene was used as the reference gene. Briefly, total RNA of AGS cells that were infected with different recombinant plasmids for desired time were extracted by using TRIzol reagent (Invitrogen) and then reverse transcribed to cDNA with random primers by using PrimeScript RT reagents kit (Takara). QRT-PCR was performed on an ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) using SYBR® Premix Ex Taq™ (Takara) according to the manufacturer's protocols. Relative gene expression values were obtained by normalization to the reference gene *GAPDH* using the $-2^{\Delta\Delta Ct}$ method, where $-2^{\Delta\Delta Ct}=\Delta Ct$ sample-ΔCt calibrator as described. The following primer pairs were used for PCR amplification: *eno1*-forward, 5'-TGTACCGCCACATCGC TGA-3'; *eno1*-reverse, 5'-TGAAGTTT-GCTGCACCG CTG-3'. *GAPDH*-forward, 5'-ACCACAGTCCATGCCATCAC-3'; *GAPDH*-reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Western blot analysis. Cells were lysed with RIPA buffer (pH 7.4, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% Na-deoxycholate, 1 mM PMSF, 1 mM orthovanadate, 1 μM leupeptin, 1 μM pepstatin) for 30 min at 4˚C. Cell lysates were centrifuged at 12,000 g for 20 min at 4˚C. After measuring the concentration of the protein using a BCA reagent kit (China), the lysates were boiled at 100˚C for 5 min in the presence of 2% β-mercaptoethanol. Equal amounts of protein were separated by SDS-PAGE (12% SDS-acrylamide gels), and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking with 1% bovine serum albumin in TBST buffer (pH 7.6, 20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20), membranes were incubated overnight with either mouse anti-CagA antibody (1:1,000, Abcam, USA), rabbit anti-ENO1 antibody (1:1,000, Abcam) or mouse anti-GAPDH antibody (1:3,000, Abcam) in TBST-milk. Blots were then incubated with the corresponding secondary horseradish peroxidase-conjugated antibody (1:5,000, Abcam, USA) for 1 h at room temperature. After washing the membranes, the reaction was visualized using the ECL Detection kit (GE Healthcare, Buckinghamshire, UK) and by exposing the blots to Fuji medical X-ray film. ImageJ software was used to analyse the western blot results.

Statistical analysis. SPSS17.0 software (SPSS Inc., USA) was adopted for statistical analysis. Statistical significance of the measured values was analyzed using the ANOVA. $P<0.05$ was considered statistically significant.

Results

Identification of recombinant vector. The recombinant vector WT-cagA and PR-cagA were detected as recombined properly with PCR and digestion experiments. Fig. 1A shows the *cagA* fragment and vector pcDNA3.1 analyzed by *Eco*RI and *Xho*I double digestion. By PCR amplification from recombinant vector, 3510 bp of *cagA* was produced and further verified by sequencing.

Mutation analysis was performed by direct sequencing. The DNA sequencing results revealed that the codon TAC which codes the tyrosine residues present in EPIYA sequences were replaced by cysteine (TGC). The CagA phosphorylation-resistant expression vector was constructed successfully (Fig. 1B).

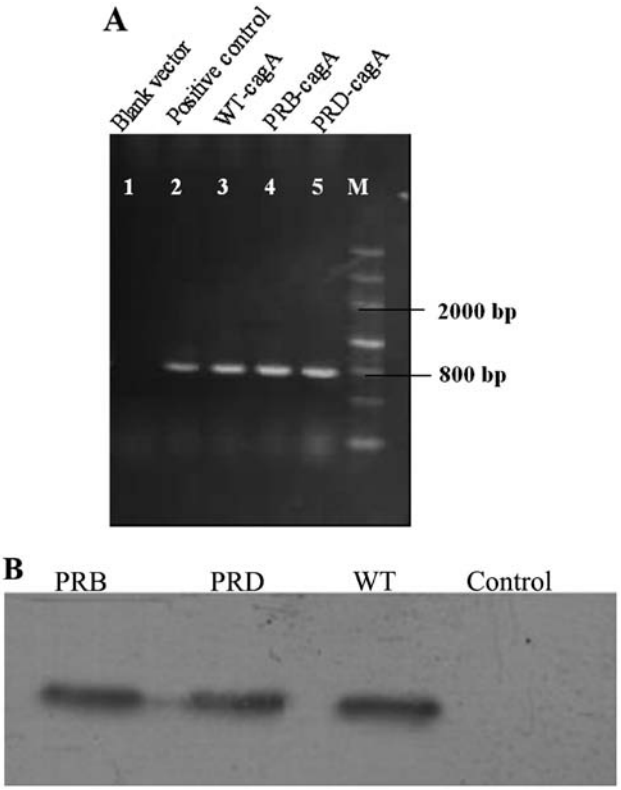

Figure 2. Analysis of CagA mRNA and protein expression after transfection. AGS cells were transfected with blank vector pcDNA3.1, WT-cagA plasmid and PR-cagA plasmids for 36 h, respectively. (A) The RT-PCR analysis was used to detect cagA mRNA in the transformed cells. (B) Western blot analysis was used to detect CagA protein expression after transfection.

Detection of CagA mRNA and protein expression in cell transfection. To confirm whether CagA protein was involved in cell transfection, the RT-PCR and western blot analysis were used to detect *cagA* mRNA and protein in the transformed cells. We designed the specific primers for *cagA* of ~780 bp in length. In the PCR results, we found a *cagA* cDNA band, 780 bp in length (Fig. 2A), confirming to the results of DNA sequencing, while it was absent in the negative control. Similarly, western blot results indicated that CagA protein band was clearly visible in the cagA-transformed cell group with molecular weight of 121 kDa, but not in the control group (Fig. 2B).

CagA-transformed cells promote the increase in eno1 mRNA expression. In order to evaluate the effect of CagA on eno1 expression level, AGS cells were transfected with WT-cagA or pcDNA3.1, respectively. Then we determined eno1 mRNA levels by quantitative RT-PCR analysis at 36 h after transfection. The results showed that eno1 expression at RNA level

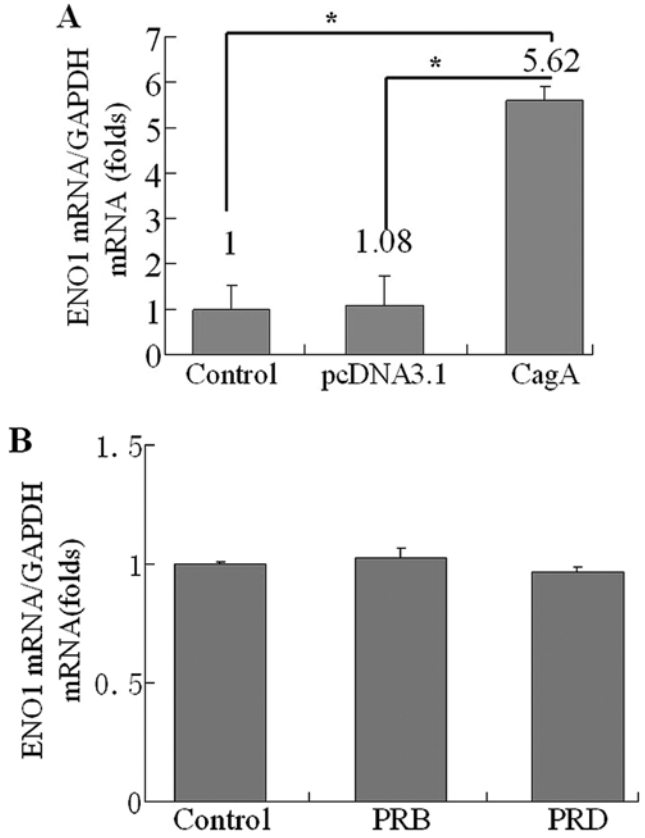

Figure 3. Analysis of WT-cagA and PR-cagA effects on ENO1 mRNA expression. (A) AGS cells were transfected with blank vector pcDNA3.1, WT-cagA plasmid and control for 36 h, respectively. (B) AGS cells were transfected with blank vector PRB-cagA plasmid, PRD-cagA plasmid and control for 36 h, respectively. After transfection, total RNA was isolated using TRIzol reagent. The relative level of ENO1 mRNA expression compared with GAPDH mRNA was measured by real-time quantitative PCR. Real-time quantitative PCR results are from three independent experiments, which are presented as the mean ± standard deviation (SD). *P<0.05.

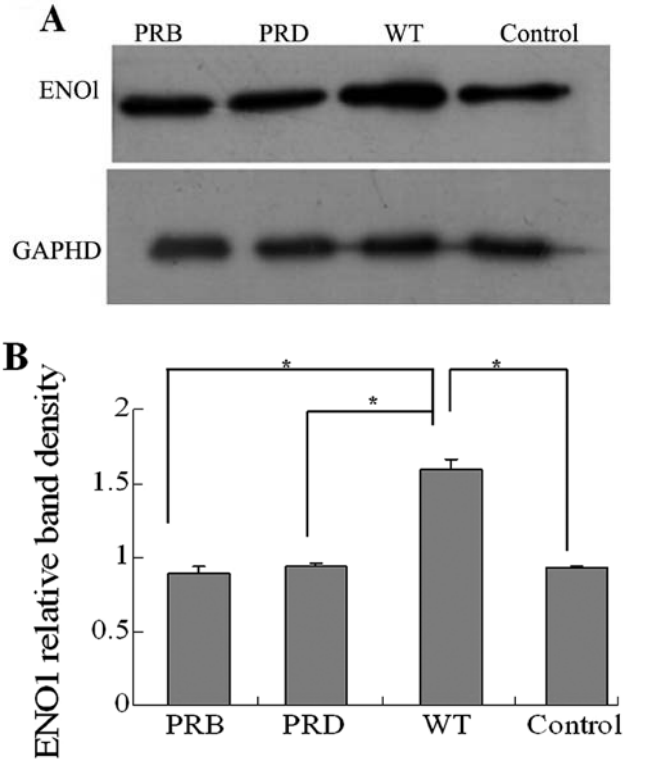

Figure 4. Analysis of WT-cagA and PR-cagA effects on ENO1 protein expression. AGS cells were transfected with WT-cagA, PR-cagA plasmids and control for 36 h, respectively. Total cellular protein was extracted for western blot analysis for the expression of ENO1 proteins. The blots were stripped and probed with GAPDH to document equal protein loading. The experiment was performed three times with similar results. *P<0.05.

was dramatically increased in WT-cagA overexpressing cells compared to non-induced control cells and blank vector transformed cells (Fig. 3A). Next, we investigated whether the upregulation is dependent on the CagA tyrosine phosphorylation. We used the PR-cagA plasmids to transtect AGS cells. The PR-cagA plasmid encodes a CagA protein with a mutation in the EPIYA B motif or D motif, required for CagA tyrosine phosphorylation. Thus the PR-CagA mutant protein cannot be phosphorylated nor can it transmit the signal pathway. After 36 h transfection of PR-cagA plasmid into AGS cells, eno1 mRNA levels were unchanged in PR-cagA overexpressing cells (Fig 3B).

ENO1 protein expression was upregulation by HP WT-CagA in AGS cells. As eno1 mRNA level upregulation was demonstrated to be associated with CagA, we next evaluated the relationship between CagA and ENO1 at the protein level. The WT-cagA or PR-cagA plasmid was transfected in AGS cells for 36 h. Total cellular protein analysed by western blot analysis indicated that the ENO1 expression level in AGS cells expressing WT-CagA was significantly greater than that in normal cells. In contrast, there was no increase in ENO1 expression level in AGS cells transfected with vector expressing PR-CagA compared with control cells. Taken together, bacterial oncoprotein CagA can upregulate the expression of ENO1 and this response is mediated specifically by tyrosine phosphorylate, but not unphosphorylated CagA (Fig. 4).

Signal inhibitor effects on ENO1 expression in AGS cells transfected with WT-cagA plasmid. We next wanted to determine the pathway(s) through which CagA affected ENO1. For this purpose, we used the MAP kinase inhibitor U0126 to block MEK1 and MEK2 kinase activity, thereby inhibiting ERK1/2 phosphorylation, BAY 11-7082 to inhibit the NF-κB pathway, PP1 to inhibit the Scr-family tyrosine kinase activity and LY294002 to block PI3 kinase activity. Each signal inhibitor was incubated with gastric cells for 1 h prior to plasmid transfection. After 36-h transfection, western blot analysis was used to detect the ENO1 expression level. Fig. 5 shows that ENO1 upregulation by CagA was attenuated by U0126 and PP1, ENO1 upregulation was maintained using BAY 11-7082 and LY294002. In total, the results indicated that MEK/ERK and Scr-family tyrosine kinase pathways participated in the upregulation of ENO1 by CagA.

Discussion

Infection by *H. pylori* is one of the major causes of chronic active gastritis and peptic ulcer and is closely related to the occurrence and progression of gastric cancer (19). CagA is one of the most important virulence factors encoded by *H. pylori*.

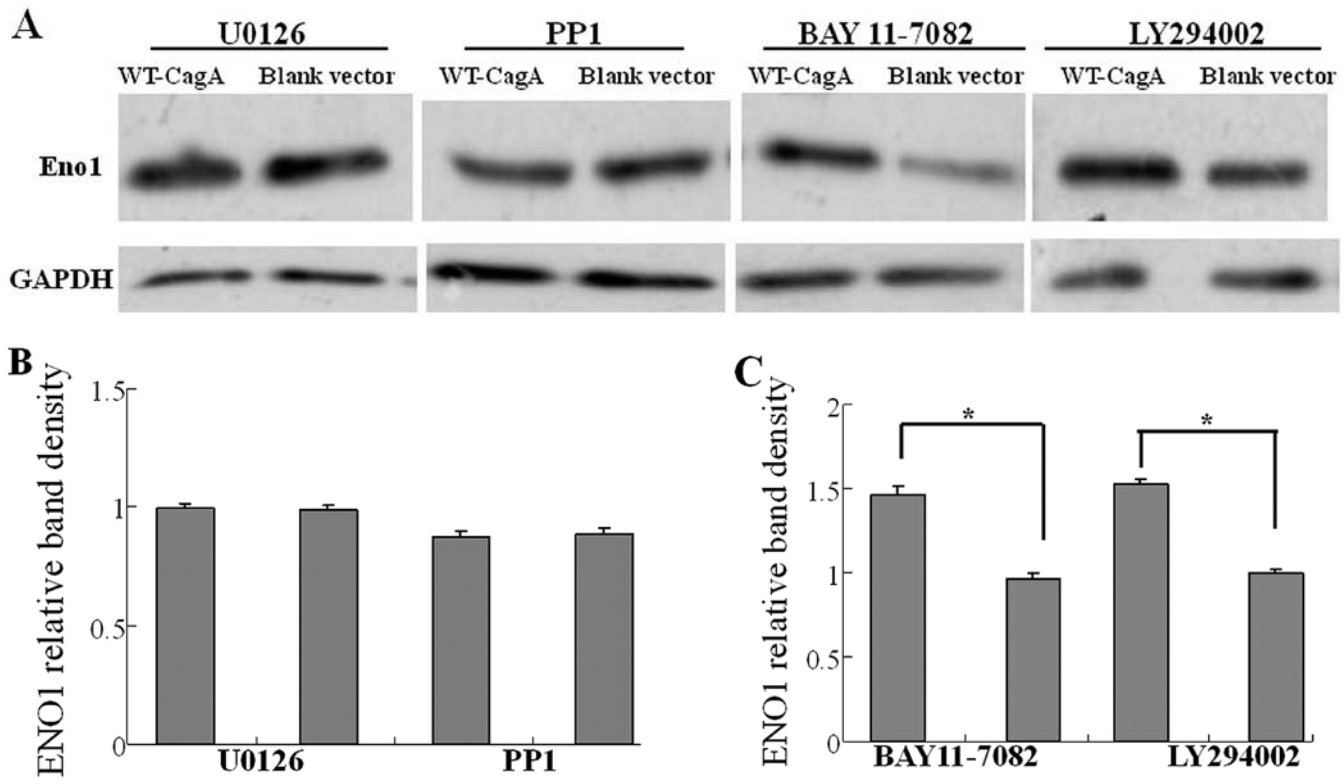

Figure 5. Effects of signal inhibitors on CagA-induced ENO1 upregulation in AGS cells. AGS cells were transfected with blank vector or WT-cagA plasmid. The AGS cells were incubated with MAP kinase inhibitor U0126 (10 μM), Scr-family tyrosine kinase inhibitor PP1 (10 μM), NF-κB pathway inhibitor BAY 11-7082 (5 μM) or PI3 kinase inhibitor LY294002 (50 μM). Signal inhibitors were used 1 h before plasmid transfection and total celluar protein was extracted for western blot analysis for the expression of ENO1 after transfection 36 h. *P<0.05.

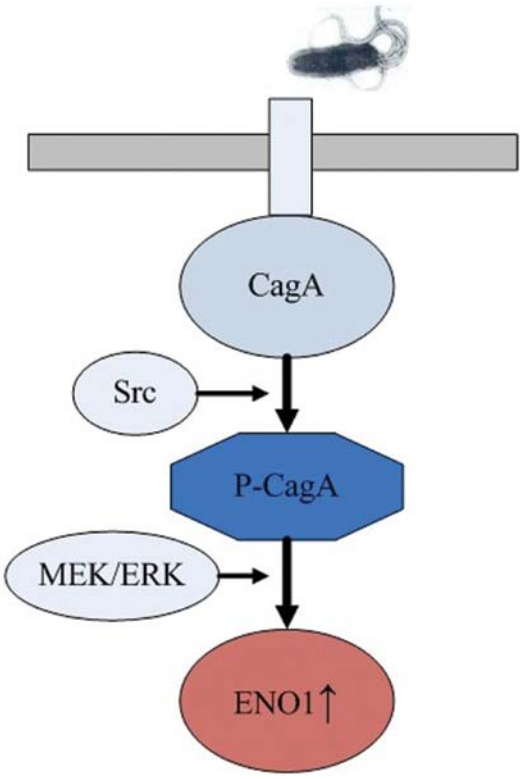

Figure 6. Schematic model of the proposed upregulation effect of *H. pylori* CagA on ENO1. Bacterial oncoprotein CagA was translocated into gastric epithelial cells through the TFSS, and then activated Src kinase and was phosphorylated by Src kinase. P-CagA activated the MEK/ERK pathway and upregulated gastric oncoprotein ENO1 expression.

H. pylori colonizing the gastric epithelial surface inject CagA into host cells via its type IV secretion system. CagA can interact with various host cellular proteins to trigger distinct signaling pathways in a tyrosine phosphorylation-dependent and -independent manner (9,20-22). The phosphorylation capability of a glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) motif in CagA is one of the major markers that correlates with *H. pylori* pathogenicity and the phosphorylation status is a key determinant of CagA-mediated signaling and transcriptional outcomes. The C-termini of the CagA proteins of different *H. pylori* strains exhibit considerable variations, which mainly include different numbers of EPIYA tandem repeats as well as different amino acid sequences flanking the motif (23). Higashi *et al* reported that most Asian *H. pylori* isolates express a highly pathogenic CagA protein, harboring the EPIYA-D tyrosine phosphorylation motif, which exhibits high affinity for binding to SHP-2 (24,25). In our study, *Hp27* is the East Asia type strain, which has EPIYA-ABD type CagA, thus we design the site-directed mutations at B and D motif (12).

In the former work of our laboratory (12), we had discovered ENO1 expression level was increased in wild-type *H. pylori* (*cagA*$^{+}$)-infected cells as compared to *cagA* mutant *H. pylori* (*cagA*$^{-}$). ENO1, a key glycolytic enzyme, belonging to a novel class of surface proteins. ENO1 serves as a plasminogen receptor on the surface of a variety of hematopoietic, epithelial and endothelial cells. It is involved in various pathological events such as tissue remodeling and the spread of transformed tumor cells induced by plasminogen activation (26). Some

evidence indicates a relation between ENO1 and the progression of tumors, such as neuroendocrine tumors, neuroblastoma, lung cancer and hepatocellular carcinoma (27). In cancer cells, the rate-determining enzyme for glycolysis is converted to an isoenzyme different from that in normal cells and the capability of glycolysis is increased because of increased cell proliferation. In response to upregulated ENO1 expression, the fibrinolytic system is inordinately accelerated. Consequently, increased local fibrinolysis may contribute to cancer cell invasion and metastasis. More recently, oncogenic AKT and Myc have been shown to stimulate anaerobic glycolysis directly and ENO1 is one of the Myc driven target genes (28,29). ENO1 has also been described as a stress protein induced by hypoxia. In large tumors, oxygen is relatively decreased in the central region. Thus, it is reasonable that ENO1 is upregulated in large tumors and it has been suggested to be a diagnostic marker for certain tumors.

However, up to now, the relationship between gastric carcinogenic pathogen *H. pylori* infection and gastric cancer oncoprotein ENO1 overexpression has not been reported. Therefore, we explored the relationship between *H. pylori* CagA, ENO1 overexpression and signaling pathways leading to gastric cancer.

After translocation into epithelial cells, *H. pylori* CagA protein was able to activate some major pathways, including the Src kinase, MEK/ERK pathway, PI3K/Akt pathway and NF-κB pathway. Kim *et al* reported that CagA transfection activated the Src kinase, and Zhu *et al* proved that CagA transfection and phosphorylated CagA protein activated the ERK1/2 pathway (30,31). It was previously shown that signal inhibitors could be used to elucidate which pathway was involved in the *H. pylori* infection-induced cyclooxygenase 2 upregulation (32) or the *H. pylori* CagA inhibition of Runx3 expression (33). To seach for the signal pathways involved in ENO1 regulation, we used four specific signal protein inhibitors to block corresponding signal transduction. We identified that Src and MEK/ERK pathways were involved in ENO1 upregulation by CagA. Taken together, CagA protein was tyrosine-phosphorylated by Src kinase, and thereafter activated the MEK/ERK pathway to upregulate ENO1 expression in AGS cells.

The combination of data from our study and others led us to propose the regulation mechanisms of *H. pylori* CagA on the expression of ENO1 (Fig. 6).

After translocating into gastric epithelial cells through the TFSS, bacterial oncoprotein CagA was tyrosine-phosphorylated by Src kinase. P-CagA activated the MEK/ERK pathway and upregulated ENO1 expression. Upregulation of ENO1 expression by *H. pylori* provided a further explanation for the gastric carcinogenic effects of *H. pylori* infection.

In conclusion, this study identifies the *H. pylori* effector protein CagA as potentially enhancing the risk for gastric cancer by increasing ENO1 expression in epithelial cells. This is the first time that the mechanism by which *H. pylori* infection and bacterial oncoprotein CagA upregulated ENO1 expression in gastric cell lines has been elucidated. CagA protein activated the Src and MEK/ERK signal pathways, resulting in the elevation of expression of ENO1 protein in AGS cells. These findings may be useful in identifying the mechanism by which gastric tumors are caused by *H. pylori* infection in humans.

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