

Helicobacter pylori promotes VEGF expression via the p38 MAPK-mediated COX-2-PGE₂ pathway in MKN45 cells

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Abstract. *Helicobacter pylori* has been suggested to be the major cause of gastric malignancy. However, the pathogenesis and molecular mechanisms of gastric tumorigenesis induced by *H. pylori* infection are yet to be elucidated. In the present study, the expression levels of vascular endothelial growth factor (VEGF), which has been suggested to promote angiogenesis in gastric cancer, were found to be elevated in *H. pylori*-infected MKN45 cells. Furthermore, it was demonstrated that the expression of VEGF was modulated by the p38 mitogen-activated protein kinases (MAPK) pathway via regulation of the cyclooxygenase (COX)-2 pathway. It was also found that prostaglandin E₂ (PGE₂) and its receptor EP2/EP4 may mediate the upregulation of VEGF in gastric cells exposed to *H. pylori*. In combination, these results suggest that VEGF expression is regulated by the p38 MAPK COX-2-PGE₂-EP2/EP4 pathway in gastric cancer cells induced by *H. pylori*. This provides a theoretical basis for the investigation of the pathogenesis of *H. pylori*-induced gastric cancer.

Introduction

Helicobacter pylori, a Gram-negative, spiral-shaped, micro-aerophilic bacterium found in the human stomach, is recognized as a major risk factor for chronic gastritis, peptic ulcers and gastric cancer (1). *H. pylori* infects approximately half of the world's population and has been classified as a carcinogen by the World Health Organization and the International Agency for Research on Cancer (2,3). Gastric cancer is the second

leading cause of cancer-associated mortality worldwide (4). Although considerable research has shown that *H. pylori* infection is closely associated with gastric cancer, the molecular mechanisms of gastric tumor initiation and development induced by *H. pylori* infection remain poorly understood (5).

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase, is a key rate-limiting enzyme responsible for the formation of prostanoids and thromboxanes (6,7). There are three COX isoenzymes, COX-1, COX-2 and COX-3 (a splice variant of COX-1). COX-1 is considered a constitutive enzyme that is expressed in nearly all mammalian cells, whilst COX-2 is an inducible enzyme that is undetectable in the majority of normal tissues (8,9). However, COX-2 expression is elevated in many types of cancer, including gastric cancer, and is closely correlated with the clinical outcome (10-15). Furthermore, a previous study demonstrated that COX-2 expression is associated with *H. pylori* infection in human gastric cancer, and the elevation of COX-2 expression may be mediated by the p38 mitogen-activated protein kinases (MAPK) pathway (16).

Prostaglandin E₂ (PGE₂) is the main product generated from arachidonic acid catalyzed by COX-2, and it exerts a wide range of pathological effects via receptors on the cell and nuclear membranes (17-19). Enhanced expression of PGE₂ is found in several types of cancer and is associated with tumor growth and angiogenesis (20,21). PGE₂ receptors (EPs) are types of G protein-coupled receptor (GPCR), and exist in at least four isoforms: EP1, EP2, EP3 and EP4 (22-25). PGE₂ binds to EPs to promote the expression of vascular endothelial growth factor (VEGF) in human prostate PC3 and gastric MKN28 cancer cells (20,26). However, whether EPs mediate VEGF expression in gastric cancer cells following *H. pylori* infection, and the type of EP involved in such regulation, have yet to be elucidated.

The MAPK family of proteins are involved in cell differentiation, migration, apoptosis and autophagy (27). p38 MAPK is a key member of this family and it is part of a signaling cascade that modulates cellular responses to cytokines and stresses, including inflammatory cytokines, osmotic shock, lipopolysaccharide, ultraviolet light and growth factors (26). Previous studies have demonstrated that p38 MAPK is also activated following *H. pylori* infection, and promotes COX-2

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expression in MKN45 gastric cancer cells, which has been found to be involved in mediating *H. pylori*-induced gastric tumorigenesis (16,28-30). Several studies have investigated the oncogenic potential of the p38 MAPK pathway, since p38 MAPK also has a critical role in regulating VEGF expression leading to angiogenesis (27,31). However, whether p38 MAPK is involved in regulating VEGF expression in *H. pylori*-infected gastric cells is yet to be elucidated.

To investigate the mechanism of *H. pylori*-induced gastric cancer, VEGF expression was analyzed in MKN45 gastric cells following *H. pylori* infection. It was found that p38 MAPK has a critical role in regulating VEGF expression in *H. pylori*-infected MKN45 cells, and this effect may be mediated via the COX-2-EP2/EP4 pathway.

Materials and methods

Cell culture and reagents. MKN45 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 (Invitrogen™ Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) bovine serum albumin (BSA; Invitrogen Life Technologies) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen Life Technologies). Cells were cultured in a humidified incubator containing 5% CO₂ at 37°C. The p38 MAPK inhibitor SB203580, the COX-2 inhibitor N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide (NS-398), the EP2 inhibitor AH6089 and the EP4 inhibitor AH23848 were all obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). For the inhibition treatment, confluent cells were treated with 20 µM SB203580, 50 µM NS-398, 50 µM AH6089 or 50 µM AH23848 for the indicated times.

***H. pylori* culture.** The *cagA*- and *vacA*-positive *H. pylori* strain (NCTC11637) was acquired and cultured as previously described (15). In brief, *H. pylori* were cultured on Columbia Agar plates (Oxoid, Thermo Fisher Scientific, Basingstoke, UK) containing 10% sheep blood and incubated at 37°C for 48-72 h, with 5% O₂, 10% CO₂ and 85% N₂. Prior to use, *H. pylori* were identified using Gram staining, colony morphology and positive oxidase, catalase and urease reactions. To prepare *H. pylori* for infection, the cells were suspended in phosphate-buffered saline (PBS) and cell density was determined using spectrophotometry (Eppendorf BioSpectrometer; Eppendorf, Hamburg, Germany). Confluent MKN45 cells were then incubated with *H. pylori* at a quantity of 100 bacteria per cell for the indicated times.

RNA isolation and quantitative polymerase chain reaction (qPCR). Total RNA was isolated from MKN45 cells using RNAisol Reagent kit (Takara Bio, Inc., Shiga, Japan) in accordance with the manufacturer's instructions. RNA quality was verified using spectrophotometry at an absorbance ratio of A260/280. Total RNA (1 µg) was used for reverse transcription into cDNA using Prime-Script™ RT-PCR kit (Takara Bio, Inc.) under the following conditions: 37°C for 15 min and 85°C for 5 sec. A total of 1 µl cDNA was then used for qPCR amplification using the ABI 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA)

under the following conditions: 95°C for 10 sec, 95°C for 5 sec, and 60°C for 31 sec, run for 40 cycles. The forward and reverse primers for VEGF, COX-2 and GAPDH were used at a final concentration of 200 nM and the sequences were as follows: VEGF, 5'-GGCCTCCGAAACCATGAAC-3' (forward) and 5'-CACTTGGCATGGTGGAGGTA-3' (reverse); COX-2, 5'-AATGAGTACCGCAAACGCTTCT-3' (forward) and 5'-TTCTGCAGCCATTTCTTCTC-3' (reverse); GAPDH, 5'-CCACTCCTCCACCTTTGAC-3' (forward) and 5'-ACCCTGTTGCTGTAGCCA-3' (reverse). The TaqMan® probes (Invitrogen Life Technologies) used were as follows: 5'-TGTCTTGGGTGCATTGGAGC-3' for VEGF, 5'-CCTGAAGCCGTACACATCATTTG-3' for COX-2 and 5'-TTGCCCTCAACGACCACTTTGTGTC-3' for GAPDH.

Lenti-virus RNA interference (RNAi) plasmid construction and virus infection. The plasmids of lenti-virus RNAi system were obtained from Shanghai Genechem Co., Ltd. (Shanghai, China). Four small interfering RNAs (siRNAs) against human COX-2 mRNA [National Center for Biotechnology Information (NCBI) GenBank, NM-000963.2] were designed using the siRNA Target Finder from GenScript (Piscataway, NJ, USA). The target sequences used were as follows: Clone 1, 5'-GCTGAATTTAACACCTCTAT-3' (1230-1250 bp); clone 2, 5'-CCATTCTCCTTGAAAGGACTT-3' (1677-1697 bp); clone 3, 5'-GCAGATGAAATACCAGTCTTT-3' (1463-1483bp); and clone 4, 5'-CATTCCTTCCTTCGAAAT-3' (407-425 bp). The clones were then inserted into a green fluorescent protein (GFP)-expressing pFU-GW-RNAi vector in accordance with the manufacturer's instructions. The pFU-GW-RNAi vector was co-transfected with pHelper 1.0 and pHelper 2.0 vectors into 293T cells using Lipofectamine® 2000 Transfection Reagent (Invitrogen Life Technologies). The virus was subsequently analyzed and amplified in 293T cells. The appropriate amount of virus was then used to infect MKN45 cells for 72 h in order to suppress the endogenous COX-2 expression.

Western blot analysis. Cells were lysed using lysis buffer solution (50 mM Tris-HCl, pH 7.5; 150 mM NaCl) containing 1% nonyl phenoxypolyethoxylethanol-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethanesulfonyl-fluoride, 10 nM microcystin, 1 µg/ml aprotinin and 1 µg/ml leupeptin (all constituents of the lysis buffer were purchased from Sangon Biotech Shanghai Co., Ltd., Shanghai, China). Following centrifugation at 14,000 x g for 20 min, the protein in the supernatant was quantified using the BCA Protein Assay Reagent (Merck Millipore, Billerica, MA, USA) and equal amounts of protein were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology, Shanghai, China), prior to being transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). Following blocking with 5% fat-free milk in Tris-buffered saline in 0.05% Tween 20 (TBST; Sangon Biotech Shanghai Co., Ltd.) for 1 h at room temperature, the membranes were then separately incubated overnight at 4°C with the following antibodies: Rabbit anti-human COX-2 monoclonal antibody and rabbit anti-human β-actin monoclonal antibody (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit anti-human EP-2 polyclonal antibody and rabbit anti-human EP-4 polyclonal antibody

(1:1,000; Abcam, Cambridge, UK). Membranes were rinsed three times with TBST (5 min each time) and then incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature prior to visualization using the Pierce enhanced chemiluminescence kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Results were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

ELISA. The cell culture medium was centrifuged at 3,000 x g for 5 min, and the supernatant was then used for further analysis. ELISA was performed in accordance with the manufacturer's instructions (Invitrogen™ Life Technologies). Briefly, the microtiter plates were incubated with 100- μ l samples at 37°C for 120 min. The plates were washed five times with 10 mM PBS, prior to incubation with 100 μ l VEGF and PGE₂ primary antibodies labeled with biotin at 37°C for 60 min. The plates were then rinsed five times with 10 mM PBS and 100 μ l avidin-biotin-peroxidase complex was added, prior to the plates being cultured at 37°C for 30 min. Following extensive rinsing, the plates were then filled with 100 μ l TMB Microwell substrate and incubated in darkness at 37°C for 15 min. The reaction was terminated using 100 μ l TMB stop solution and the optical density (OD) values were analyzed within 30 min using a microplate reader (Multiskan Spectrum; Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm. The OD values were subsequently converted into concentrations deduced from a calibration curve.

Statistical analysis. Statistical analysis was performed using the SPSS 19.0 software package (SPSS, Inc., Chicago, IL, USA). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls test. All results are presented as the mean \pm standard error (SE) for three independent experiments. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

***H. pylori* infection enhances expression levels of VEGF in MKN45 cells.** To determine whether *H. pylori* induced VEGF expression, confluent MKN45 cells were co-cultured with *H. pylori* for 0, 6, 12 and 24 h. Cells were then harvested to examine the relative expression levels of VEGF mRNA compared with GAPDH mRNA expression levels using qPCR. It was found that VEGF mRNA levels in cells treated with *H. pylori* for 6 h ($P < 0.05$), 12 h ($P < 0.01$) and 24 h ($P < 0.01$) were significantly elevated compared with cells not exposed to *H. pylori* (Fig. 1A). The VEGF mRNA expression was highest in cells treated with *H. pylori* for 12 h. VEGF mRNA expression in cells treated with *H. pylori* for 12 h was significantly higher than that in cells treated for 6 h ($P < 0.01$); however, expression appeared to decline after 24 h (Fig. 1A). The VEGF protein expression levels were analyzed using ELISA. MKN45 cells were incubated with *H. pylori* for 0, 12, 24, 36 and 48 h, and the supernatant was harvested and analyzed. It was found that VEGF protein levels increased with the length of incubation, and were significantly elevated in cells cultured with *H. pylori* for 36 h ($P < 0.05$) and 48 h ($P < 0.01$) (Fig. 1B).

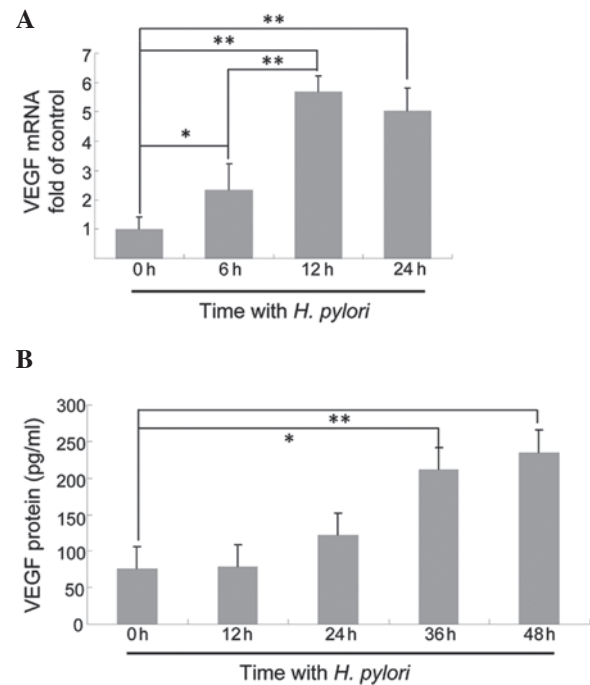


Figure 1. Time-dependent induction of VEGF expression by *H. pylori* in MKN45 cells. (A) Increase in VEGF mRNA expression in *H. pylori*-treated cells. Confluent MKN45 cells were incubated with *H. pylori* at a quantity of 100 bacteria per cell for 0, 6, 12 and 24 h. Cells were then harvested and analyzed using quantitative polymerase chain reaction to measure the relative expression levels of VEGF mRNA compared with GAPDH mRNA. (B) Increase in VEGF protein expression in *H. pylori*-treated cells. MKN45 cells were incubated with *H. pylori* for 0, 12, 24, 36 and 48 h and then harvested prior to analysis using ELISA to determine the VEGF protein expression levels. * $P < 0.05$ and ** $P < 0.01$. VEGF, vascular endothelial growth factor.

Inhibition of p38 MAPK attenuates the effects of H. pylori on VEGF expression. To examine whether p38 MAPK modulated VEGF expression, confluent MKN45 cells were pretreated with p38 MAPK inhibitor SB203580 for 2 h prior to incubation with or without *H. pylori* for 12 or 48 h. Cells were then harvested and the levels of VEGF mRNA expression relative to GAPDH mRNA expression were analyzed using qPCR (cells treated with *H. pylori* for 12 h), while VEGF protein expression levels were analyzed using ELISA (cells treated with *H. pylori* for 48 h). In cells that were not exposed to *H. pylori*, treatment with SB203580 did not affect the VEGF mRNA or protein expression levels (Fig. 2A and B). Without SB203580 pretreatment, VEGF mRNA and protein levels in *H. pylori*-treated cells were significantly increased compared with cells not treated with *H. pylori* ($P < 0.01$) (Fig. 2A and B). However, when pretreated with SB203580, VEGF mRNA and protein expression levels in *H. pylori*-treated cells significantly decreased compared with *H. pylori*-treated cells without incubation with SB203580 ($P < 0.01$) (Fig. 2A and B).

Blocking COX-2 with the inhibitor NS-398 attenuates the effects of H. pylori on VEGF expression. It was demonstrated in our previous study that p38 MAPK activity was essential for increased expression of COX-2 in MKN45 cells following *H. pylori* infection (15); therefore, in the present study, it was investigated whether COX-2 was involved in the p38 MAPK-mediated upregulation of VEGF

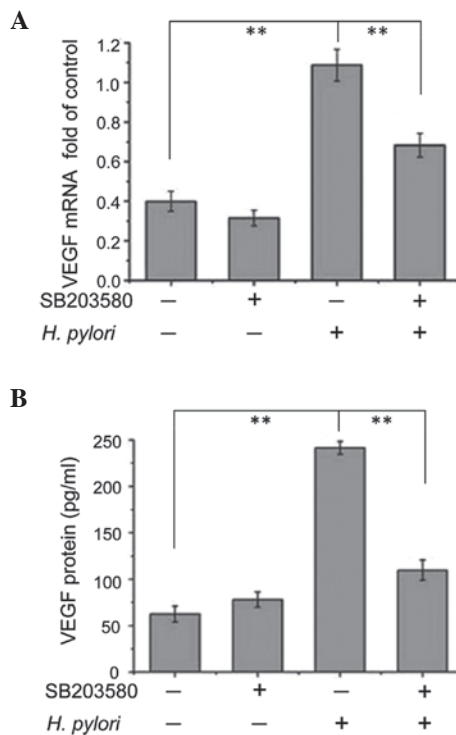


Figure 2. Effect of p38 MAPK inhibitor on VEGF expression following *H. pylori* infection in MKN45 cells. (A) p38 MAPK inhibitor SB203580 attenuates the effects of *H. pylori* on VEGF mRNA expression. Confluent MKN45 cells were pretreated with 20 μ M p38 MAPK inhibitor SB203580 for 2 h prior to co-culture with or without *H. pylori* for 12 h. The cells were then harvested to measure the relative expression levels of VEGF mRNA compared with GAPDH mRNA using quantitative polymerase chain reaction. (B) p38 MAPK inhibitor SB203580 attenuates the effects of *H. pylori* on VEGF protein expression. Confluent MKN45 cells were pretreated with 20 μ M p38 MAPK inhibitor SB203580 for 2 h prior to co-culture with or without *H. pylori* for 12 h. The cells were then harvested to measure the relative protein expression levels of VEGF using ELISA. ** $P < 0.01$. p38 MAPK, p38 mitogen-activated protein kinase; VEGF, vascular endothelial growth factor.

expression. Confluent MKN45 cells were pretreated with the COX-2 inhibitor NS-398 for 2 h prior to incubation with or without *H. pylori* for 12 h. The cells were then harvested to measure the expression levels of VEGF mRNA compared with GAPDH mRNA using qPCR. In cells not exposed to *H. pylori*, treatment with NS-398 did not affect the VEGF mRNA expression levels (Fig. 3A). Consistent with our previous results, VEGF mRNA levels in *H. pylori*-treated cells increased significantly ($P < 0.01$) (Fig. 3A). However, in *H. pylori*-infected cells pretreated with NS-398, VEGF mRNA expression levels were downregulated compared with *H. pylori*-infected cells not incubated with NS-398 ($P < 0.01$) (Fig. 3A). Our previous studies demonstrated that *H. pylori* increased COX-2 expression in MKN45 cells (16); therefore, it was hypothesized that the downstream products of COX-2 may have been elevated. The present study analyzed the protein expression levels of PGE₂, one such downstream product of COX-2, in MKN45 cells incubated with *H. pylori* for 0, 6, 12, 24 and 48 h using ELISA. PGE₂ protein levels in cells treated with *H. pylori* for 12, 24 and 48 h were significantly higher compared with cells not exposed to *H. pylori* ($P < 0.01$), with the highest expression observed in cells treated with *H. pylori* for 24 h (Fig. 3B).

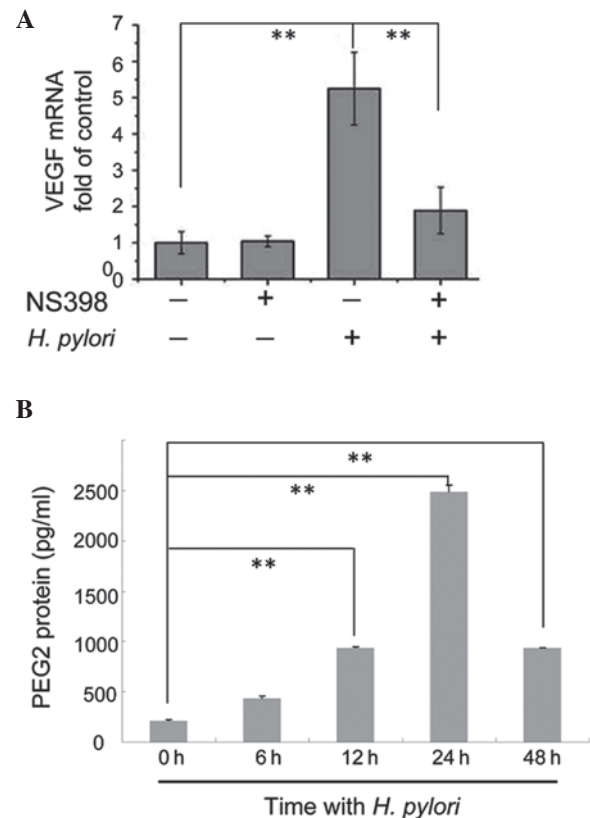


Figure 3. Effect of COX-2 inhibitor on VEGF expression following *H. pylori* infection in MKN45 cells. (A) COX-2 inhibitor NS-398 attenuates the effects of *H. pylori* on VEGF mRNA expression. Confluent MKN45 cells were pretreated with 50 μ M COX-2 inhibitor NS-398 for 2 h prior to co-culture with or without *H. pylori* for 12 h. Cells were then harvested and the relative levels of VEGF mRNA expression compared with GAPDH mRNA were analyzed using quantitative polymerase chain reaction. (B) Increase in PGE₂ protein expression in *H. pylori*-treated cells. MKN45 cells were incubated with *H. pylori* for 0, 6, 12, 24 and 48 h and supernatant was harvested for ELISA analysis to measure the protein expression of PGE₂, one downstream product of COX-2. ** $P < 0.01$. VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide.

RNAi-mediated suppression of COX-2 attenuates the effects of H. pylori on VEGF expression. In order to specifically suppress endogenous COX-2 expression, four lentiviral based RNAi clones targeted to different parts of the COX-2 gene were designed, and the inhibitory effect was analyzed by infecting MKN45 cells for 72 h with each clone separately. The cells were then harvested and the expression levels of COX-2 mRNA relative to GAPDH mRNA expression were analyzed using qPCR, while COX-2 protein expression was measured using western blot analysis. All four of the clones of lenti-viral RNAi targeted to COX-2 efficiently suppressed mRNA and protein expression levels of COX-2; however, clone 4 suppressed COX-2 mRNA and protein expression levels by ~95 and ~81%, respectively and, therefore, was used in the following experiments (Fig. 4A and B). To determine the effects of COX-2 RNAi on VEGF expression, confluent MKN45 cells were infected with control RNAi (GFP control), or COX-2 RNAi for 72 h, and then cells were incubated with or without *H. pylori* for 48 h. The supernatant was harvested to measure the protein expression of VEGF using ELISA. The results showed that background expression of VEGF in

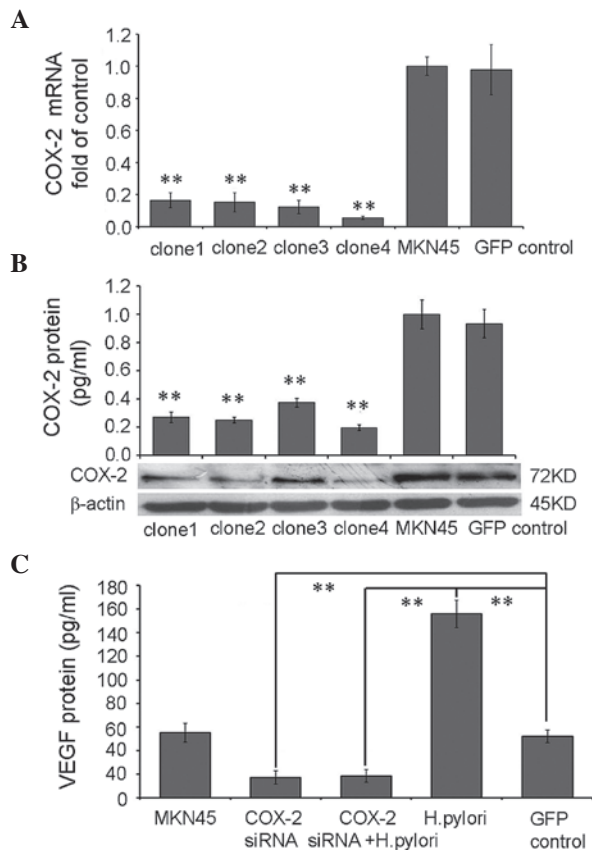


Figure 4. Effect of COX-2 RNAi on VEGF expression following *H. pylori* infection in MKN45 cells. (A and B) COX-2 RNAi largely suppresses endogenous COX-2 (A) mRNA and (B) protein expression levels. Confluent MKN45 cells were infected with control lenti-viral RNAi (GFP) or four clones of lenti-viral RNAi of COX-2 for 72 h. Cells were then harvested to measure the relative expression levels of COX-2 mRNA and protein using quantitative polymerase chain reaction and western blot analysis, respectively. ** $P < 0.01$, compared with the GFP control group. (C) RNAi-mediated inhibition of COX-2 aborts the upregulation of VEGF expression following *H. pylori* infection. Confluent MKN45 cells were infected with control lenti-viral RNAi (GFP) or clone 4 of the lenti-viral RNAi of COX-2 for 72 h and incubated with or without *H. pylori* for 48 h. The supernatant was harvested and the protein expression of VEGF was analyzed using ELISA. ** $P < 0.01$. COX-2, cyclooxygenase-2; RNAi, RNA interference; VEGF, vascular endothelial growth factor; GFP, green fluorescent protein.

MKN45 cells was significantly reduced in cells infected with COX-2 siRNA ($P < 0.01$), and the same effect was observed in cells also treated with *H. pylori*. This indicates that RNAi-mediated suppression of COX-2 inhibits the upregulation of VEGF expression in MKN45 cells following *H. pylori* treatment (Fig. 4C).

EP2/EP4-mediated regulation of VEGF expression upon *H. pylori* infection. Since it was found that COX-2 promotes VEGF expression in *H. pylori*-treated MKN45 cells and that the downstream product of COX-2, PGE_2 , was upregulated, further studies were performed to determine whether the PGE_2 receptors EP2/EP4 were also associated with COX-2-mediated upregulation of VEGF. Confluent MKN45 cells were incubated with *H. pylori* for 0, 2, 6, 12, 24 and 48 h and then harvested. Western blot analysis was performed to determine the protein expression levels of EP2 and EP4, using β -actin as the internal control. As shown in Fig. 5A,

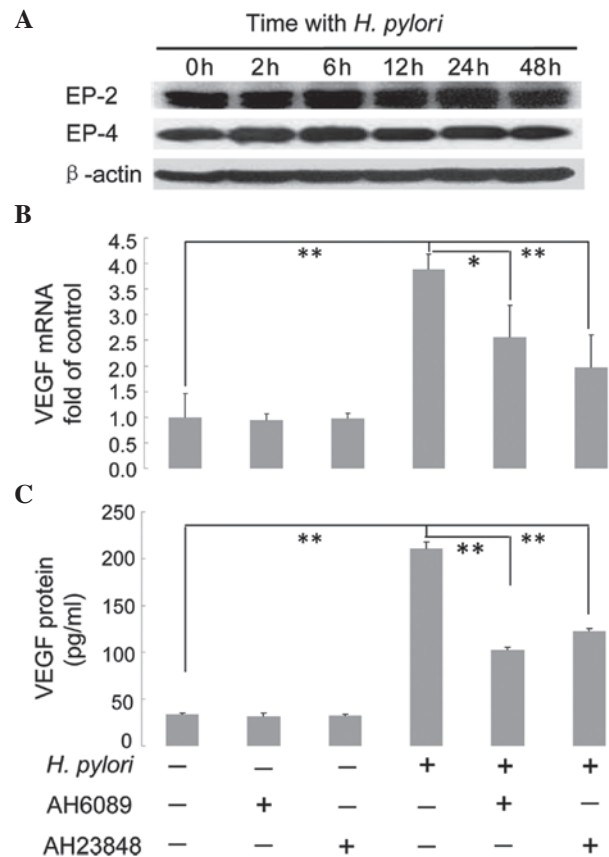


Figure 5. Effect of EP2/EP4 inhibitors on *H. pylori*-induced VEGF expression in MKN45 cells. (A) Protein expression of EP2 and EP4 does not change in *H. pylori*-treated MKN45 cells. Confluent MKN45 cells were co-cultured with *H. pylori* for 0, 2, 6, 12, 24 and 48 h, prior to western blot analysis to measure the expression of EP2 and EP4. β -actin was used as an internal control. (B) The inhibitors AH6089 and AH23848 attenuate the effects of *H. pylori* on VEGF mRNA expression. Confluent cells were pretreated for 2 h with 50 μ M specific inhibitors against EP2 or EP4, AH6089 and AH23848, respectively. Cells were then incubated with or without *H. pylori* for 12 h, and harvested to measure the relative expression levels of VEGF mRNA compared with GAPDH using quantitative polymerase chain reaction. (C) The inhibitors AH6089 and AH23848 attenuate the effects of *H. pylori* on VEGF protein expression. Confluent cells were pretreated for 2 h with 50 μ M specific inhibitors against EP2 or EP4, AH6089 and AH23848, respectively. Cells were then incubated with or without *H. pylori* for 48 h, and harvested to measure the expression levels of VEGF protein using ELISA. * $P < 0.05$ and ** $P < 0.01$. EP, prostaglandin E2 receptor; VEGF, vascular endothelial growth factor.

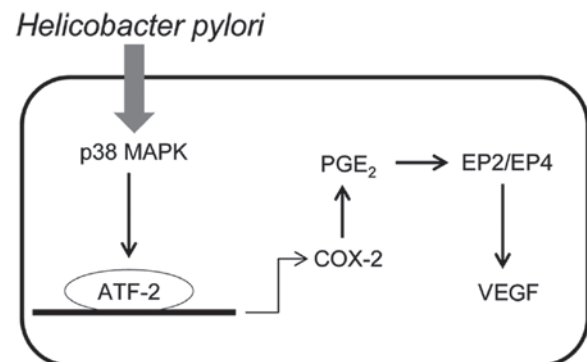


Figure 6. Model of the upregulation of VEGF expression via p38 MAPK-mediated COX-2- PGE_2 pathway following *H. pylori* infection in MKN45 cells. VEGF, vascular endothelial growth factor; p38 MAPK, p38 mitogen-activated protein kinase; ATF-2, activating transcription factor 2; COX-2, cyclooxygenase-2; PGE_2 , prostaglandin E2; EP, PGE_2 receptor.

no significant difference was observed in the protein expression levels of EP2 or EP4 during the continual induction of *H. pylori* (Fig. 5A). To examine whether EP2/EP4 expression was associated with VEGF expression in MKN45 cells, confluent cells were pretreated with specific inhibitors against EP2 and EP4, AH6089 and AH23848, respectively, for 2 h prior to incubation with or without *H. pylori* for 12 or 48 h. The cells were then harvested and the expression levels of VEGF mRNA relative to GAPDH mRNA were analyzed using qPCR (cells treated with *H. pylori* for 12 h), while expression levels of VEGF protein were analyzed using ELISA (cells treated with *H. pylori* for 48 h). In cells not exposed to *H. pylori*, treatment with AH6089 or AH23848 did not affect VEGF mRNA or protein expression levels ($P>0.05$) (Fig. 5B and C). In cells not treated with AH6089 or AH23848, VEGF mRNA and protein levels in *H. pylori*-infected cells increased significantly compared with cells not infected with *H. pylori* ($P<0.01$) (Fig. 5B and C). However, when cells were pretreated with AH6089, VEGF mRNA ($P<0.05$) and protein ($P<0.01$) expression levels in *H. pylori*-infected cells significantly decreased compared with *H. pylori*-infected cells without incubation of AH6089 (Fig. 5B and C). A similar result was observed in cells pretreated with the inhibitor AH23848: VEGF mRNA and protein expression levels in *H. pylori*-infected cells significantly decreased compared with *H. pylori*-infected cells without incubation of AH23848 ($P<0.01$) (Fig. 5B and C).

Discussion

VEGF, an oncogenic marker in cancer diagnosis, has potent angiogenic activity on endothelial cells and promotes tumor growth (32). Enhanced expression of VEGF is often observed in malignant tumors and is frequently used as a therapeutic target (32). A previous study demonstrated that *H. pylori* infection promoted gastric cancer cell invasion via upregulation of VEGF expression (33). However, the mechanism by which *H. pylori* induces VEGF expression in gastric cancer has yet to be elucidated. The p38 MAPK pathway has been found to be involved in tumor growth and metastasis through regulation of the production of VEGF in cancer. p38 MAPK activity also has a key role in increasing *H. pylori*-induced COX-2 expression in gastric cancer cells (16,27,31). Therefore, in the present study, the role of p38 MAPK in the regulation of VEGF expression in gastric cancer cells exposed to *H. pylori* was investigated. It was found that mRNA and protein expression levels of VEGF were significantly increased following *H. pylori* infection; however, the p38 MAPK-specific inhibitor, SB203580, significantly attenuated this effect, indicating that p38 MAPK was involved in promoting VEGF expression in *H. pylori*-infected MKN45 cells (Figs. 1 and 2).

COX-2 activity is associated with *H. pylori* infection in gastric cells, and may promote the production of PGE₂ (16,26). In a previous study, we demonstrated that *H. pylori* infection upregulates the expression of COX-2 via the p38 MAPK/activating transcription factor 2 pathway (16). In this present study, the expression of PGE₂ in *H. pylori*-infected cells was analyzed and it was found that PGE₂ levels were significantly increased, suggesting that COX-2 activity was upregulated following *H. pylori* infection (Fig. 3B). To investigate whether the p38 MAPK-associated upregulation of VEGF expression

was mediated by COX-2, the COX-2 specific inhibitor, NS-398, was used in cells exposed to *H. pylori*. The results show that VEGF expression significantly decreased in NS-398-treated cells (Fig. 3A). Endogenous COX-2 expression was also downregulated using RNAi and it was found that background expression levels of VEGF in MKN45 cells were significantly reduced, and the same effect was observed in *H. pylori*-treated cells, indicating that RNAi-mediated inhibition of COX-2 suppressed the upregulation of VEGF expression in MKN45 cells following *H. pylori* infection (Fig. 4C).

The results of the present study suggest that COX-2 is involved in the regulation of VEGF expression, downstream of p38 MAPK. EP2 and EP4 are PGE₂ receptors, and have been shown to have an important role in modulating VEGF production in prostate cancer cells (20,26). In the present study, the roles of EP2/EP4 in VEGF production in gastric cancer cells following *H. pylori* infection were investigated. The results demonstrated that inhibition of EP2 and EP4 with the specific inhibitors AH6089 and AH23848 significantly decreased *H. pylori*-induced VEGF levels in cells, indicating that EP2/EP4 mediate the upregulation of VEGF expression in *H. pylori*-infected gastric cancer cells (Fig. 5B and C). The protein expression of EP2/EP4 was then analyzed, and it was found that *H. pylori* infection did not alter the EP2/EP4 protein levels. This suggests that the EP2/EP4-associated upregulation of VEGF is not mediated by EP2/EP4 protein levels, but by increased levels of PGE₂ product, induced by enhanced COX-2 activity (Fig. 5A).

In combination, these results suggest a novel pathway of p38 MAPK-COX-2-PGE₂-EP2/EP4 for the regulation of VEGF expression in *H. pylori*-infected gastric cells (Fig. 6). Following *H. pylori* infection, p38 MAPK is activated and COX-2 expression levels are upregulated, the level of PGE₂ is therefore increased. PGE₂ then binds to EP2/EP4 to promote VEGF expression. In a previous study we demonstrated that Jianpi Jiedu, a formulation used in traditional Chinese medicine, was demonstrated to downregulate COX-2 expression via inhibition of the *H. pylori*-induced p38 MAPK pathway (34). Therefore, further studies may be performed to examine whether the Jianpi Jiedu recipe regulates VEGF expression via modulation of this pathway, as shown in Fig. 6.

In conclusion, the present study elucidated a p38 MAPK-mediated signaling pathway that regulates VEGF expression in *H. pylori*-infected gastric cancer cells. This contributes to the investigation into the pathogenesis of *H. pylori*-induced gastric cancer. Furthermore, this study may provide novel therapeutic targets for *H. pylori*-induced gastric cancer.

Acknowledgements

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