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# Induction of Cyclooxygenase-2 Overexpression in Human Gastric Epithelial Cells by *Helicobacter pylori* Involves TLR2/TLR9 and c-Src Dependent NF-κB Activation

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**Running Title:** *H. pylori* induced COX-2 expression via TLR2/TLR9.

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**Abstract: 250**

**Introduction: 714**

**Discussion: 1549**

**Abbreviations:** *H. pylori*, *Helicobacter pylori*; GC, gastric cancer; COX, cyclooxygenase; CRE, cAMP response element; HC, *H. pylori* isolate from gastric cancer; HU, *H. pylori* isolate from gastric ulcer; HD, *H. pylori* isolate from duodenal ulcer; HS, *H. pylori* isolate from gastritis; PCR, polymerase chain reaction

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**Abstract**

Gastric epithelial cells were incubated with a panel of clinical isolates of *H. pylori*, including non-ulcer dyspepsia with gastritis (HS, n=20), gastric ulcer (HU, n=20), duodenal ulcer (HD, n=21), and gastric cancer (HC, n=20). HC strains induced a higher COX-2 expression than those from HS, HD and HU. The bacterial virulence factors and the host cellular pathways were investigated. Virulence genes of iceA, vacA, babA2, cagA 3' repeat region and hrgA failed to show any association with the disease status and COX-2 expression. Methylation-specific PCR revealed HC strains not affecting the methylation status of COX-2 promoter. NF-κB, NF-IL6 and CRE were found to involve in the COX-2 induction. We explored a novel NF-κB activation pathway. The mutants of TLR2 and TLR9, not TLR4, inhibited *H. pylori*-induced COX-2 promoter activity, and neutralizing antibodies for TLR2 and TLR9 abolished *H. pylori*-induced COX-2 expression. COX-2 induction was inhibited by the PI-PLC, PKC and Src inhibitors. The dominant negative mutants of NIK and various IKKs including IKKβ (Y188F), IKKβ (Y199F) and IKKβ (FF), inhibited the COX-2 promoter activity. Phosphorylation of GST-IKKβ (132-206) at Tyr188 and Tyr199 by c-Src was found after *H. pylori* infection. In summary, *H. pylori* induces COX-2 expression via activations of NF-κB, NF-IL6 and CRE. In NF-κB activation, *H. pylori* acts through TLR2/TLR9 to activate both the cascade of PI-PLCγ/PKCα/c-Src/ IKKα/β and the cascade of NIK/IKKα/β, resulting in the IκBα degradation and the expression of COX-2 gene. The COX-2 overexpression may contribute to the carcinogenesis in patients colonized with these strains.

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*Helicobacter pylori* (*H. pylori*) have been identified as a major pathogen leading to the development of a wide range of gastroduodenal diseases (Passaro et al., 2002). However, only a small portion of infected patients suffered from the more severe gastric pathology such as gastric malignancy (Peek and Blaser, 2002). Evidence has emerged that the inappropriate inflammation of gastric mucosa would dictate the clinical outcomes after exposure to *H. pylori* (Bodger and Crabtree, 1998). Clinical outcomes associated with *H. pylori* infection include gastritis, duodenal ulcer, gastric ulcer, gastric adenocarcinoma, and gastric mucosa associated lymphoid tissue lymphoma (Parsonnet et al., 1991). Both host factors and the characteristics of infecting strains have been postulated to contribute to the variable outcome and have been the focus of intensive investigations (Blaser, 2002). The reported strain-specific virulence factors for gastric cancer (GC) include cagA, vacA, iceA, babA2, and hrgA (Yamaoka et al., 1998; Kidd et al., 2001; Prinz et al., 2001; Ando et al., 2002; Nogueira et al., 2001; Bravo et al., 2002).

Cyclooxygenase (COX)-2 is the key enzyme responsible for the prostaglandin production during gastric inflammation and ulcer healing (Jackson et al., 2000). The overexpression of COX-2 has been implicated in the development and progression of GC (van Rees et al., 2002). *H. pylori* infection is the most important factor for the induction of COX-2 in the stomach. Both animal study and human samples have confirmed that *H. pylori*-induced inflammation is linked to COX-2 expression (Takahashi et al., 2000). The degree of COX-2 expression strongly correlated with the extent of inflammation and the severity of gastric disease (Sung et al., 2000). In addition, the COX-2 protein was significantly higher in *H. pylori*-infected patients with GC than those with non-ulcer dyspepsia (Wambura et al., 2002), and the upregulation of COX-2 in *H. pylori*-associated GC is related to vascular invasion (Chen et al., 2001). Therefore, COX-2 expression was assumed to play a crucial role

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in *H. pylori*-associated GC in addition to gastric inflammation. Recently, *in vitro* studies demonstrated the modulation of COX-2 expression by *H. pylori* (Romano et al., 1998). It is of interest to investigate whether *H. pylori* isolates from patients with different disease status vary in their capabilities to induce COX-2 expression, as such data are limited. Furthermore, the roles of hrgA, iceA, vacA, babA2, and cagA genotypes in relation to COX-2 expression and disease status are also determined.

The induction of COX-2 expression requires the *de novo* mRNA and protein synthesis (Kosaka et al., 1994), indicating the regulation at transcriptional level. The promoter region of human COX-2 gene has been cloned and sequenced, and shown to contain the putative recognition sequences for several transcriptional factors, including two NF-κB sites, a nuclear factor for IL-6 expression (NF-IL6)/CEBP, an AP-1 and a cAMP response element (CRE) (Kosaka et al., 1994). Although NF-κB activation by *H. pylori* has previously been reported (Keates et al., 1997), whether it is involved in the *H. pylori*-induced COX-2 expression and whether other transcriptional factor is also activated are not explored. These issues are addressed in the present study.

It has been reported that epithelial cells and macrophages recognize microbial infections via Toll-like receptors (TLRs) (Rock et al., 1998). These receptors are oligospecific and recognize conserved motifs on pathogens. To date, 10 human TLRs have been identified (Rock et al., 1998), and their ligands are beginning to be unraveled. TLR4 recognizes lipopolysaccharide (LPS), which is the major outer membrane component of gram-negative bacteria (Poltorak et al., 1998). TLR2 is involved in the recognition of gram-positive bacteria (Takeuchi et al., 1999). TLR9 recognizes bacterial DNA (Hemmi et al., 2001). In this study, which types of TLRs and their role in *H. pylori*-regulated COX-2 expression are evaluated.

The phosphorylations of $Ser^{177}$ and $Ser^{181}$ on IKKβ by the upstream MAP3K

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leading to NF-κB activation are well-recognized (Malinin et al., 1997). However, our recent studies found the additional phosphorylations of $Tyr^{188}$ and $Tyr^{199}$ by c-Src through PKC activation resulting in COX-2 and ICAM-1 expressions (Huang et al, 2003a; Huang et al, 2003b). The upstream signaling molecule involving in *H. pylori*-induced NF-κB activation is investigated in the present study. Whether PKC/c-Src/IKKβ pathway also involves in the *H. pylori*-induced NF-κB activation leading to COX-2 expression is examined to demonstrate if this pathway exists in different types of cells despite of different stimuli.

## Materials and Methods

**Materials.** The rabbit polyclonal antibodies specific to TLR2, TLR4, IκBα, IKKβ and c-Src, and the goat polyclonal antibodies specific to COX-2, COX-1, TLR9 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant TNF-α was purchased from R&D Systems (Minneapolis, MN). RPMI, fetal calf serum (FCS), penicillin, and streptomycin were obtained from GIBCO BRL (Gaithersburg, MD). Staurosporine was obtained from Sigma (St. Louis, MO). U73122, U73343 and PP2 were obtained from Calbiochem (San Diego, CA). Reagents for SDS-PAGE were from Bio-Rad. T4 polynucleotide kinase from New England Biolabs (Beverly, MA), poly(dI-dC) from Pharmacia Biotech, [$\gamma$-$^{32}$P] ATP (3,000 Ci/mmol) from Dupont-New England Nuclear, the SuperFect reagent from QIAGEN and the luciferase assay kit from Promega (Madison, WI).

**Bacterial Strains and Growth Conditions.** We studied *H. pylori* strains from patients undergoing gastroscopy for the evaluation of upper gastrointestinal symptoms. At the time of gastroscopy, two biopsy specimens were taken from antrum for bacterial culture. A total of 81 clinical isolates from patients with nonulcer dyspepsia with gastritis (HS, n=20), gastric ulcer (HU, n=20), duodenal ulcer (HD, n=21), and

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gastric cancer (HC, n=20, 11 from intestinal type and 9 from diffuse type) respectively were collected. Columbia agar with 5% sheep blood (Gibco, Rockville, Md.) was used for *H. pylori* culture. The bacterial cells were cultured at 37°C in a microaerophilic chamber (Don Whitley, West Yorkshire, England) containing 10% $CO_2$, 5% $O_2$, and 85% $N_2$. Bacterial cells were grown to 48 h on Columbia agar plates, collected, washed with PBS buffer (pH 7.4), and pelleted. Cell pellets were then resuspended in PBS buffer (pH 7.4) and used for infection experiment (Wang et al., 1998).

**Cell Culture and *H. pylori* Infection Experiments.** The human gastric cancer epithelial cell lines, AGS and MKN45, were obtained from the ATCC and RIKEN (Japan) respectively. Both of them cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml of penicillin, and 100 ug/ml of streptomycin. AGS cells and *H. pylori* were co-cultured in antibiotics-free RPMI 1640 supplemented with 10% FCS. Bacteria were resuspended in PBS (pH 7.4) and diluted corresponding to the multiplicity of infection (MOI) as 150:1. Cells were incubated in the absence (controls) or in the presence of bacteria for 16 h.

**Preparation of Cell Extracts and Western Blot Analysis.** After 16 h incubation with indicating *H. pylori* strains, AGS or MKN45 cells were rapidly washed with PBS to remove bacteria and then lysed with the ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM NaF, 150 mM NaCl, 1 mM PMSF, 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM $Na_3VO_4$, 10mM β-glycerophosphate, 5mM Na-pyrophosphate, 1% Triton X-100). The cell lysate was subjected to SDS-PAGE using 10 % running gels. The proteins were transferred to the nitrocellulose paper, and the Western blot was performed as described previously (Huang et al, 2003b). The quantitative data were obtained using a computing densitometer with ImageQuant software and normalized by the actin expression

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(Molecular Dynamics, Sunnyvale, CA, U.S.A).

**Immunofluorescence Staining.** AGS or MKN45 cells grown on coverslips were co-cultured for 16 h with *H. pylori* in antibiotic-free growth medium, rapidly washed with PBS, then fixed at room temperature for 30 min with 3.7% paraformaldehyde. After washing with PBS, the cells were blocked for 30 min with 3% BSA in TTBS containing 0.1% Triton X-100, then incubated with anti-COX-2 Ab (1:100) for 1 h, washed extensively, and stained for 30 min with anti-goat IgG-fluorescein (1:2000). After further washes, the coverslips were mounted on glass slides using mounting medium (2% *n*-propyl gallate in 60% glycerol, 0.1 M PBS, pH 8). Optical sections of the immunostained cells were observed and photographed using a Zeiss Axiovert inverted microscope equipped with a photoMicroGraph Digitized Integration System (Zeiss, Oberkochen, Germany).

**RT-PCR.** Total RNA was isolated from AGS cells using TriZol$^{TM}$ Reagent (Life technology). The reverse transcription reaction was performed using 2 μg of total RNA which was reverse transcribed into cDNA using the oligo dT primer, then the cDNA was amplified for 30 cycles using two oligonucleotide primers derived from a published COX-2 sequence (5'-CAGCACTTCACGCATCAGTT-3' and 5'-TCTGGT CAATGGAAGCCTGT-3') and two oligonucleotide primers from a β-actin sequence (5'-TGAC GGGGTCACCCACACTGTGCCCATCTA-3' and 5'-CTAGAAGCATTT GCGGGGACGATGGAGGG-3'). For COX-2, a PCR cycle consisted of denaturation step (94$^{o}$C, 1 min), an annealing step (60$^{o}$C, 1 min), and an elongation step (72$^{o}$C, 1.5 min). There were a total of 35 cycles, which was followed by an additional extension step (72$^{o}$C, 7 min). For β-actin, PCR cycle was carried out for 30 sec at 94$^{o}$C, 30 sec at 65$^{o}$C, and 1 min at 70$^{o}$C. The PCR products were subjected to electrophoresis on a 1.5 % agarose gel. Quantitative data was obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

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**Detection of iceA, cagA 3' repeat region, hrgA, babA2, and vacA genotypes.** Polymerase chain reaction (PCR) with specific primers was utilized to detect genotypes of *H. pylori* as described previously (Yamaoka et al., 1998; Kidd et al., 2001; Ando et al., 2002; Wang et al, 1998; Sheu et al., 2003). The primers used are 5'-GTTGGGTATATCACAATTAT-3'/5'-TTACCCTATTTTCTAGTAGGT-3' for ice A (Kidd et al., 2001), 5'-ACCCTAGTCGGTAATGGGTTA-3'/5'-GTAATTGTCTAG TTTCGC-3' for CagA (Yamaoka et al., 1998), 5'-TCTCGTGAAAGAGAATTTCC-3'/5'-TAAGTGTGGGTATATCAATC-3' for hrgA, and 5'-CTCATTGCTGTGAGGG-AT-3'/5'-TCTTGATAGGATCTTGCG-3' for hpyIIIR (Ando et al., 2002), 5'-CCAA-ACGAAACAAAAAGCGT-3'/5'-GCTTGTGTAAAAGCCGTCGT-3' for babA2 (Sheu et al., 2003), 5'-GTCAGCATCACACCGCAAC-3'/5'-CTGCTTGAATGCGC-CAAACTTTATC-3' for vacA s1a, 5'-GCTAACACGCCAAATGATCC-3'/5'-CTGC-TTGAATGCGCCAAACTTTATC-3' for vacA s2, 5'-GGCCACAATGCAGTCATG-G-3'/5'-CTCTTAGTGCCTAAAGAAACA-3' for vacA m1, and 5'-GGAGCCCCA-GGAAACATTG-3'/5'-CATAACTAGCGC-CTTGCAC-3' for vacA m2 (Wang et al, 1998). The amplification condition was denatured at 94□ for 5 min, followed by 35 cycles of 94□ for 1 min, 50□ for 1 min and 72□ for 1 min, and a final extension at 72□ for 5 min (for cagA, hrgA and iceA). The annealing temperature was changed to 55□ for vacA and 45□ for babA2. All samples with negative results were tested at least twice. The strains from randomly selected samples with positive PCR results were subjected for sequencing using an ABI PRISM$^{TM}$ 377 DNA sequencer (Applied Biosystems, Foster, CA, USA).

**Methylation status of the COX-2 promoter.** The methylation status of the COX-2 promoter was determined by methylation-specific PCR as detailed previously (Akhtar et al., 2001). In brief, 1μg of genomic DNA isolated from AGS cells was bisulfite modified. PCR was performed with unmethylated primers:

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5’-ATAGATTATATGGTGGTGGTGGT-3’ / 5’-CACAATCTTTACCCAAACACTTC C-3’ (171bp product), and methylated primers: 5’-TTAGATACGGCGGCGGCGGC-3’/5’-TCTTTACCCGAACGCTTCCG3’ (161bp product). The PCR condition was 30s at 95□, 45s at 65□, 45s at 72□ for 35 cycles followed by a final 5 min extension at 72□.

**Preparation of nuclear extracts and the electrophoretic mobility shift assay (EMSA).** AGS cells were co-cultured with *H. pylori* isolate from GC patient for 30, 60 or 120 min, and then nuclear extracts were prepared as described previously (Huang et al, 2003a). Oligonucleotides corresponding to the downstream κB (5’-GAGTG**GGGACTACCC**CCTC-3’), NF-IL6 5’-CGGC**TTACGCAAT**TTTT-3’), or CRE (5’-TCAT**TTCGTCA**CATG-3’) consensus sequence in the human COX-2 promoter were synthesized, annealed, and end labeled with [γ-$^{32}$P] ATP using T4 polynucleotide kinase, and EMSA was performed as described previously (Huang et al, 2003b).

**Plasmids.** The COX-2 promoter constructs pGS-459/+9, -327/+59, CRM, ILM or KBM (Luc) were generous gifts from Dr. L.H. Wang (University of Texas, Houston, TX). NF-κB luciferase reporter (κB-Luc) was from Stratagene. The PLC-γ2 wild type and the mutant SH2(N), which the Arg at position 564 is replaced by Ala, and the PKC-αconstitutively active mutant (PKC-α AE) and the PKC-α Dominant negative mutant (PKCα/KR) were gifts from Dr. T. Kurosaki (Kansai Medical University, Japan) and Dr. A. Altman (La Jolla Institute for Allergy and Immunology, San Diego, CA) respectively. The dominant-negative mutants of NIK (KKAA), IKKα (KM), and IKKβ (KM) were gifts from Signal Pharmaceuticals (San Diego, CA). The dominant negative mutant of IKKβ (AA) was from Dr. Karin (UCSD, CA). pGEX-I*κ*Bα□ (1-100) was a gift from Dr. Nakano (University of Juntendo, Tokyo). pGEX-IKKβ (132-206) was a gift from Dr. Nakanishi (University of Nagoya, Nagoya). TLR2 and

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TLR4 plasmids including wt TLR2, wt TLR4, mutants of TLR2 (P/H) and TLR4 (P/H) were as described previously (Muta and Takeshige, 2001). TLR9 plasmids including wt TLR9 and mutant of TLR9 (ICD) from Ken J. Ishii (FDA, Bethesda) were as described previously (Takeshita et al., 2001). The dominant negative mutants of c-Src (K295M), IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) were prepared as described previously (Huang et al, 2003b).

**Transient Transfection and Luciferase Activity Assay.** AGS cells grown to 60% confluence in 12-well plates were transfected with either the human COX-2 promoter construct or kB-Luc using SuperFect$^{TM}$ (Qiagen) according to the manufacturer's recommendations. Briefly, reporter DNA (0.3 μg) and β-galactosidase DNA (0.15 μg; pRK plasmid containing the β-galactosidase gene driven by the constitutively active SV40 promoter was used to normalize the transfection efficiency) were mixed with 0.45 μl of SuperFect in 0.4 ml of serum-free RPMI 1640. After 10-15 min incubation at room temperature, the mixture was applied to the cells. 6 h later, 0.4 ml of RPMI 1640 with 20% FCS was added. 24 h after transfection and change to an antibiotic-free medium, the cells were treated with inhibitors (as indicated) for 30 min and incubated with *H. pylori* isolate from GC patients for 6 h. Cell extracts were then prepared, and the luciferase and β-galactosidase activities measured. The luciferase activity was normalized to the β-galactosidase activity. In experiments using dominant-negative mutants, cells were co-transfected with reporter (0.3 μg) and β-galactosidase (0.15 μg) and either the dominant-negative TLRs, PLCγ2, PKCα, NIK, IKKα, IKKβ, and c-Src mutants or the empty vector (0.6 μg). In experiments using wt plasmids, cells were co-transfected with 0.3 μg of reporter plasmid, 0.15 μg of β-galactosidase plasmid, 0.45 μg of the wt PLCγ2, constitutively active PKCα (A/E) plasmid, or wt c-Src plasmid, or empty vector, and 0.6 μg of the dominant-negative PLCγ2, PKCα, NIK, IKKα, IKKβ, or c-Src mutant or empty

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vector.

**Immunoprecipitation and Kinase Activity Assay.** Following incubation with *H. pylori* isolate from GC patient, with or without 30 min pretreatments with PI-PLC, PKC, and Src kinase inhibitors, AGS cells were rapidly washed with PBS and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 5 μg/ml of leupeptin, 20 μg/ml of aprotinin, 1 mM NaF, and 1 mM $Na_3VO_4$), then either IKK or c-Src was immunoprecipitated. For the *in vitro* kinase assay, 100 μg of total cell extract was incubated for 1 h at 4°C with either 0.5 μg of rabbit anti-IKKβ or anti-c-Src Ab. The protein A-Sepharose CL-4B beads were then added to the mixture and incubation continued for 4 h at 4°C. The immunoprecipitates were collected by centrifugation, washed three times with lysis buffer without Triton X-100, then they were incubated for 30 min at 30°C in 20 μl of kinase reaction mixture (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM MnCl2, 0.1 mM $Na_3VO_4$, 1 mM DTT) containing 10 μM [$\gamma$-$^{32}$P]ATP and either 1 μg of bacterially expressed GST-IκBα (1-100) as IKK substrate, 1 μg of acidic denatured enolase or 6 μg of bacterially expressed GST-IKKβ (132-206) as c-Src substrate. The reaction was stopped by the addition of an equal volume of Laemmli buffer, the proteins were separated by electrophoresis on 10% SDS polyacrylamide gels, and the phosphorylated-GST-IκBα (1-100), phosphorylated-enolase, and phosphorylated-GST-IKKβ (132-206) were visualized by autoradiography. Quantitative data were obtained using a densitometer with ImageQuant software and normalized by the protein expression.

**Statistical Analysis.** To establish the significance of the results, the Student's t test was used for numerical data. Fisher's exact test or chi-square test was used for categorical data as appropriate. A p value less than 0.05 were considered statistically significant.

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## Results

**COX-2 Protein Expression Induced by *H. pylori* Isolates from Patients with Different Disease Status.** Comparing to the basal levels, overexpression of COX-2 protein was seen in the AGS cells treated with either TNF-α or HC at a bacterium/cell ratio of 50:1, 150:1 or 350:1. In contrast, HS and HD did not show obvious COX-2 overexpression (Fig. 1A-a). At a bacterium/cell ratio of 150:1, the capabilities of representative HS, HU, HD and HC to induce COX-2 expression were shown in Fig. 1A-b. The clinical isolate from GC had a greater capability to induce COX-2 protein expression, while relatively lower induction of COX-2 protein among isolates of non-ulcer dyspepsia, gastric ulcer, and duodenal ulcer was seen. To determine whether *H. pylori*-induced COX-2 expression occurred at the transcriptional level, the induction of COX-2 mRNA expression stimulated by HC in AGS cells was examined by RT-PCR and found the time-dependent increase (Fig.1A-c). The Box plot of COX-2 protein expression in AGS cells induced by different clinical isolates was shown in Fig. 1B. The fold of increase induced by these 20 isolates from gastric cancer (6.19±0.86, mean±S.E.) was significantly higher than that from non-ulcer dyspepsia (1.48±0.16, $p<0.001$), gastric ulcer (1.92±0.38, $p<0.001$), and duodenal ulcer (1.97±0.46, $p<0.001$) (Fig. 1B). Compared to HS, HU and HD, HC also exhibited a stronger capability to induce the COX-2 expression in MKN45 cells, another gastric adenocarcinoma cell line (Fig. 1C).

The induction of COX-2 by HC in AGS and MKN45 cells was further demonstrated by immunofluorence staining. As shown in Fig.1D, no COX-2 expression was seen in the basal state, but was apparent in the nuclear envelope of AGS cells and in the cytosol of MKN45 cells after co-cultured with HC.

**Genotypes of *H. pylori* Isolates from Patients with Different Disease Status.** Table 1 showed the genotypes of hrg, iceA, babA2, cagA, and vacA from 20 patients

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of HS, HU, and HC, and 21 patients of HD. There was no difference in the genotypes among the *H. pylori* isolates from patients with different disease status despite of a higher COX-2 induction capability of HC. Furthermore, no difference in COX-2 induction capability was seen among HC isolates from 11 intestinal and 9 diffused subtypes of GC. Regardless of the difference in induction capacity, all *H. pylori* strains can induce COX-2 expression in MKN45 cells (Fig. 1C), suggesting that *H. pylori*-induced COX-2 expression is a common phenomenon. To further study the mechanism of this effect, *H. pylori* isolate from GC patient which exhibited stronger ability of inducing COX-2 overexpression was chosen for the following studies using AGS cells.

***H. pylori*-induced COX-2 Promoter Activity via NF-κB, C/EBP and CREB Activation without Change in the Methlyation Status.** To analyze the transcriptional regulation, methylation status of COX-2 promoter in AGS cells before and after incubation with *H. pylori* was examined. By methylation-specific PCR, both the unmethylated and methylated products were shown before the incubation with *H. pylori* (Fig.2A, lanes 1-2). The incubation with *H. pylori* did not affect the methylation status (Fig.2A, lanes 3-4).

To identify which cis-acting element was involved, the COX-2 promoter-Luc constructs including -327/+59, KBM with κB site (-223/-214) mutation, ILM with NF-IL6 site (-132/-124) mutation, and CRM with CRE site (-59/-53) mutation were used (Huang et al, 2003b). Our results showed a decrease in the induction of COX-2 promoter activity by *H. pylori* using KBM, ILM, and CRM (Fig. 2B), demonstrating that NF-κB, NF-IL6, and CRE elements contribute to the *H. pylori*-induced COX-2 transcription.

Since NF-κB, NF-IL6, and CRE elements were involved in the COX-2 gene transcription following *H. pylori* infection, the DNA-protein complexes formation

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was examined by EMSA. An increase in NF-κB DNA-protein binding was seen after co-culture with *H. pylori* for 30 min and reached maximum at 60 min (Fig 2C). Similar induction of C/EBP and CREB DNA-protein complexes formation was also seen after 60 and 120 min of co-culture (Fig. 2C).

**Involvement of Toll Like Receptors In *H. pylori*-mediated COX-2 Expression.** Several studies have shown the involvement of TLRs in bacterial infections (Rock et al.,1998), whether TLR is involved in the *H. pylori*-induced NF-*k*B activation leading to COX-2 expression is elucidated. The expressions of TLR2, TLR4 and TLR9 were found in AGS and MKN45 cells (Fig. 3A), we examined their role in the *H. pylori*-induced COX-2 promoter activity. As shown in Fig. 3B, the induction of COX-2 promoter activity by *H. pylori* was inhibited in a dose-dependent manner by the mutant of TLR2 (P/H) and TLR9 (ICD), but not TLR4 (P/H), indicating the involvements of TLR2 and TLR9, not TLR4. The neutralizing antibody for TLR2 and TLR9 also attenuated the *H. pylori*-induced COX-2 expression (Fig. 3C).To further identify the TLRs-mediated COX-2 promoter activity and its downstream signaling, we overexpressed the wild type TLRs in AGS cells. The wt TLR2, not the wt TLR4, increased the COX-2 promoter activity (Fig. 3D). Although TLR9 mutant blocked the induction of *H. pylori*-mediated COX-2 promoter activity, the wt TLR9 was unable to induce the COX-2 promoter activity (Fig. 3D). Combination of TLR2 or TLR9 with *H. pylori* by transfected cells with TLR2 or TLR9 followed by *H. pylori* co-culture showed a synergistic effect on the COX-2 promoter activity (Fig. 3D), confirming their involvements in *H. pylori*-mediated COX-2 induction. Although HS alone had no COX-2 induction, the increases of COX-2 promoter activity were seen in the presence of overexpression of TLR2 and TLR9, but not TLR4 (Fig. 3D).

Since TLR2 and TLR9 were demonstrated to be involved in *H. pylori*-induced

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COX-2 promoter activity (Fig. 3E), their role in *H. pylori*-induced NF-κB activity was examined. As shown in Fig. 3E, HC, but not HS, HU or HD induced NF-κB activation, and this effect was inhibited by the dominant negative mutants of TLR2 and TLR9.

**Involvements of PLCγ, PKC, and c-Src in *H. pylori*-mediated COX-2 Expression.** The COX-2 expression induced by *H. pylori* was inhibited by either 10 μM U73122 (PI-PLC inhibitor) or 1 μM Ro 31-8220 (PKC inhibitor), whereas 10 μM U73343 (an inactive analogue of U73122) had no effect (Fig. 4A, lanes 3, 5 and 4), indicating the involvement of PI-PLC/PKC pathway. The Src inhibitor PP2 also abolished the *H. pylori*-induced COX-2 expression (Fig. 4A, lane 6), suggesting the role of Src kinase in this regulation. To further confirm the involvement of the PI-PLC/PKC pathway in. *pylori*-induced COX-2 expression (Fig. 4A), co-transfections of the PLCγ mutant with reporter, the dominant negative mutants of PKCα (K/R) with reporter, and the c-Src (K/M) with reporter were performed. The induction of COX-2 promoter activity by *H. pylori* was attenuated by PLCγ2 (SH2(N)), PKCα (K/R), and c-Src (KM) in a dose-dependent manner, confirming the involvement of these signaling molecules in the COX-2 expression.

**Demonstration of TLR2/PLCγ/PKC/c-Src/IKK Pathway in *H. pylori*-mediated COX-2 promoter activity.** Involvements of NIK and IKKα/β in the *H. pylori*-induced COX-2 promoter activity were demonstrated using the dominant negative mutants of NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (AA), IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) (Fig. 5A-a). wt TLR2-induced COX-2 promoter activity was inhibited by either the PLCγ2 mutant or the dominant negative mutants of PKCα (K/R), c-Src (KM), NIK(KKAA), IKKα (KM) and IKKβ (KM), (Y188F), (Y199F) and (YYFF) (Fig. 5A-b), suggesting that *H. pylori*-induced COX-2 expression through the activations of PLCγ2, PKCα, c-Src, NIK, IKKα, and IKKβ.

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To characterize the relationship between PLCγ2, PKCα, c-Src and IKKβ, overexpressions of the constitutively active PKCα(A/E), wt PLCγ2, and wt c-Src were performed. wt PLCγ2, PKCα (A/E), and wt c-Src increased the COX-2 promoter activity by 7.7-fold, 2.5-fold, and 42-fold respectively (Fig. 5B). The COX-2 promoter activity induced by either wt PLCγ2 or PKCα (A/E) was inhibited by the dominant-negative PKCα (K/R), c-Src (KM), IKKα (KM), and IKKβ (KM) mutants (Fig. 5B-a and 5B-b), while that induced by wt c-Src was inhibited by the dominant-negative IKKα (KM) and IKKβ (KM), IKKβ (YF) and IKKβ (YYFF), but not IKKβ (AA) and PLCγ2 (SH2(N)) mutants (Fig. 5B-c). These results indicated the involvement of PI-PLCγ/PKC/c-Src/IKKα/β pathway in *H. pylori*-induced COX-2 expression.

Our recent studies find that phosphorylations of IKKβ at $Tyr^{188}$ and $Tyr^{199}$ are required for the TNF-α-induced ICAM-1 and COX-2 expressions in the lung epithelial cells, and also demonstrated that these two tyrosine residues are the targets of c-Src (Huang et al., 2003a; Huang et al., 2003a). Overexpressions of the dominant-negative tyrosine mutants IKKβ (Y188F), IKKβ (Y199F), and IKKβ (YYFF) attenuated the *H. pylori*-induced, the wt TLR2-induced, and the wt c-Src-induced COX-2 promoter activity. The dominant-negative IKKβ (KM) mutant with $Lys^{44}$ mutated to methionine, had a similar inhibitory effect (Fig. 5A and 5B). On the other hand, IKKβ (AA) with $Ser^{177}$ and $Ser^{181}$ mutated to alanine, had no effect on the wt c-Src-induced COX-2 promoter activity (Fig. 5B-c), but was as effective as IKKβ (Y188F) and IKKβ (Y199F) in inhibiting the *H. pylori*-induced COX-2 promoter activity (Fig. 5A-a).

**Induction of c-Src Activation by *H. pylori* Infection and the Inhibitory Effect of PI-PLC, PKC or Src Kinase Inhibitor.** To further demonstrate that *H. pylori*-activated PKCα/c-Src/IKKβ pathway induced tyrosine phosphorylation of IKKβ, the

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*in vitro* c-Src activity was measured. c-Src was isolated by immunoprecipitation using anti-c-Src Ab, and activity was measured using enolase as the substrate. As shown in Fig. 6A, the maximal c-Src activity (enolase phosphorylation) was seen after 30 min co-culture with *H. pylori*, and this effect declined after 60 min (Fig. 6A, lanes 3-5). Marked autophosphorylation of c-Src was also seen (Fig. 6A). The *H. pylori*-induced c-Src activation was inhibited by 10 μM U73122, 1 μM Ro 31-8220 and PP2 at 1 and 10 μM (Fig. 6B, lanes 3-6).

**Induction of IKK Activation and IκBα Degradation by *H. pylori* Infection, and the Inhibitory Effect of PI-PLC, PKC or Src Kinase Inhibitor.** Since the dominant negative IKKα/β mutant inhibited *H. pylori*-induced COX-2 promoter activity, the endogenous IKK was immunoprecipitated with anti-IKKβ antibody and its activity measured. When cells were co-cultured with *H. pylori* for periods of either 10, 30, 60, or 120 min, a significant IKK activity was measured after 30 min (Fig. 7A-a, lane 3), which was paralleled with the degradation of IκBα (Fig. 7A-b, lane 3). IκBα levels were restored to the resting level after co-cultured with *H. pylori* for 24 h (data not shown). *H. pylori*-induced IKK activation was inhibited by the PI-PLC, PKC, and Src kinase inhibitors in a dose-dependent manner (Fig. 7B-a, lanes 3-8), which was paralleled with the recovery of IκBα degradation (Fig. 7B-b, lanes 3-8).

**Involvement of c-Src-dependent Tyrosine Phosphorylation of IKKβ by *H. pylori* Infection.** Because $Tyr^{188}$ and $Tyr^{199}$ of IKKβ were found to be critical in the PKCα/c-Src/IKKβ pathway eliciting NF-κB activation and inducing COX-2 promoter activity (Fig. 5A and 5B), the tyrosine phosphorylation of IKKβ by c-Src was examined further. c-Src was immunoprecipitated using anti-c-Src antibody, and its ability to phosphorylate IKKβ was measured using GST-IKKβ-(132-206) as an *in vitro* substrate. When cells were co-cultured with *H. pylori*, IKKβ was phosphorylated by c-Src in a time-dependent manner with the maximal effect being seen at 30 min

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(Fig. 8A, lane 3), and this effect was inhibited by 1 and 10 μM PP2 (Fig. 8B, lanes 3 and 4).

## Discussion

In the present study, we have demonstrated different capabilities among clinical isolates of *H. pylori* to stimulate COX-2 expression in vitro. Our results demonstrated that *H. pylori* strains isolated from GC patients induced higher expression of COX-2 protein *in vitro*. It is likely that overexpression of COX-2 by *H. pylori* isolates from GC patients may contribute to the carcinogenesis in host induced by these strains. This phenomenon is also in agreement with the *in vivo* observation that COX-2 overexpression is found in 50~80% of gastric cancer patients (Sung et al., 2000; Wambura et al., 2002; Chen et al., 2001). The bacterial virulence factors and host cellular pathways of *H. pylori*-mediated COX-2 expression were further investigated.

The clinical outcome of *H. pylori* infection is determined by a complex interaction of host, environmental influences and microbial virulence factors. The relevance of several specific *H. pylori* genes has been studied in the past. Although cagA, vacA, iceA, babA2, and hrgA genotypes have been reported to associate with GC (Yamaoka et al., 1998; Kidd et al., 2001; Prinz et al., 2001; Ando et al., 2002; Nogueira et al., 2001; Bravo et al., 2002), our data show no difference in these virulence genes among *H. pylori* isolates from patients with different disease status. The significances of VacA and CagA in *H. pylori*-induced COX-2 expression have been reported (Caputo et al., 2003; Juttner et al., 2003 ). Although we failed to show correlation of cagA and vacA genotypes with COX-2 expression in various clinical isolates, our data could not exclude their crucial roles in *H. pylori*-induced COX-2 expression. It is also probable that *H. pylori* might have cagA and vacA-independent pathways to induce COX-2 expression since all experimental strains are live bacteria

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and contain such toxins. The high induction of COX-2 expression by the HC strains might be exerted through other novel factors, such as gamma-glutamyl transpeptidase (Busiello et al., 2004). It is also possible that some strains express virulence factors only at the gene level but not at the protein level. Further exploration of the molecular mechanisms involved in the enhancing effects of *H. pylori* on COX-2 expression *in vitro* and *in vivo* is warranted to provide deep insights into the role of *H. pylori* and COX-2 in the gastric carcinogenesis.

Methylation of the gene promoter DNA at the areas of CpG islands has been linked to the silencing of gene expression (Song et al., 2001). The induction of COX-2 expression in AGS cells by *H. pylori* was not through its demethylation of gene promoter, since the methylated and unmethylated status of COX-2 promoter in AGS cells was not changed after co-cultured with HC. Akhtar et al also found that *H. pylori* did not change the methylation status of COX-2 promoter in AGS cells (Akhtar et al., 2001), and an increase in COX-2 protein expression was also seen after cells were co-cultured with *H. pylori* (Akhtar et al., 2001, Fig. 3C, compare lanes 1 and 2).

In addition to the methylation status, transcription factor-binding sites on the COX-2 promoter and their individual role as cis-acting elements regulating the transcription are of particular interest. Rodents have only one NF-κB site (-401/-393 bp in mouse), which has been shown to be involved in the TNF-α-induced COX-2 induction in a mouse osteoblast cell line (Yamamoto et al., 1995). The NF-κB-3' site (-223/-214 bp) on the human COX-2 promoter, in concert with the NF-IL6 and CRE sites, may play a role in facilitating the induction of COX-2 by LPS and phorbol ester (Inoue et al., 1995). Our results clearly showed the indispensable role of the downstream NF-κB site (-223/-214) in the TNF-α-induced COX-2 expression in human alveolar epithelial cells (Huang et al., 2003b). NF-κB, NF-IL6 and CRE are firstly demonstrated to be involved in the *H. pylori*-induced COX-2 expression in the

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present study. Activation of CRE site has also been reported to be involved in the *H. pylori*-stimulated COX-2 gene transcription (Juttner et al., 2003). However, Juttner et al used mouse, but not human COX-2 promoter construct to transfect the human AGS cells (Juttner et al., 2003, Fig 2). In addition to the differences in the number of NF-κB site, there are still some differences in the cis-acting element on the COX-2 promoter between these two species (Inoue et al., 1995). For instance, the relative position and sequences of CRE site (5'-CGTCACGTG-3' at –56 to –48 bp) on the mouse promoter are different from those on the human (5'-TTCGTCA-3' at –59 to -53). Using consensus sequences containing human CRE site, we found the bindings of CREB-1, ATF-2 and c-jun to this site. TLR2 and TLR9 also initiated CRE activation (unpublished data). C/EBPβ and C/EBPδ were found to bind *H. pylori*-activated NF-IL6 site (unpublished data), and the involvements of TLRs in this signaling are under-investigation.

All TLRs activate a common signaling pathway that culminates into the activation of NF-κB, as well as the MAPKs (Barton et al., 2003). Expression of three TLRs (TLR2, TLR4, and TLR9) in both AGS and MKN45 cells was found in the present study. Although Smith et al (2003) demonstrated the involvements of TLR2 and TLR5 in *H. pylori*-induced chemokine releases, we firstly revealed the roles of TLR2/9 in COX-2 induction and further explored their role in the *H. pylori*-mediated signaling pathway. Our data showed the involvements of TLR2 and TLR9, but not TLR4. When wild type of TLRs was overexpressed, TLR2 and TLR9, not TLR4, synergistically increased the *H. pylori*-induced COX-2 promoter activity. The neutralizing antibody for TLR2 or TLR9 also inhibited the *H. pylori*-induced COX-2 expression. The mutant and neutralizing antibody of TLR9 blocked the induction of *H. pylori*-mediated COX-2 promoter and expression, respectively, suggesting that *H. pylori* might mediate signaling via its CpG DNA (Hemmi et al., 2001). The finding

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that gram-negative bacterial LPS stimulated TLR2 leading to NF-κB activation had been reported (Yang et al., 1998). Our data demonstrated that TLR2/9 are the major receptors mediating *H. pylori*-induced NF-κB activation leading to the COX-2 expression. This is the first report that links TLR2/9, NF-κB, and COX-2. In addition, the expression level of TLR2/9 might be a sensitive factor. HS alone did not induce COX-2 promoter activity, but was enhanced in the presence of overexpression of these receptors (Fig. 3D). Recently, TLR2, but not TLR4 was also found to mediate *H. pylori*-induced NF-κB activation in MKN45 cells (Smith et al., 2003), and *Candida Albicans* also acted through TLRs to activate NF-κB and induce COX-2 expression in Hela cells (Deva et al., 2003).

Since *H. pylori* induced NF-κB activation through TLR2/TLR9 in AGS cells is demonstrated, the existence of PKC/c-Src/IKKβ pathway downstream of TLR2/9 is examined. Several lines of evidence showed that gastric epithelial cells also exist this pathway. First, both *H. pylori*- and wt TLR2-induced COX-2 promoter activities were inhibited by the dominant negative tyrosine IKKβ (Y188F), IKKβ (Y199F) or IKKβ (FF) mutants. Second, wt c-Src-induced COX-2 promoter activity was inhibited by the dominant negative tyrosine mutants, but not by the IKKβ (AA) mutant, in which $Ser^{177}$ and $Ser^{181}$ are mutated. Third, *H. pylori* induced c-Src activation as well as IKK activation and IκBα degradation, and these effects were inhibited by the PI-PLC, PKC and Src inhibitors. Fourth, an in vitro kinase assay demonstrated that *H. pylori*-stimulated c-Src phosphorylates IKKβ at $Tyr^{188}$ and $Tyr^{199}$, and this effect was inhibited by PP2. It is already known that these two tyrosine residues in IKKβ are conserved with other Ser/Thr kinases such as Akt1 and PKCδ (Huang et al., 2003a; Huang et al., 2003b). Therefore, two signal pathways are involved in the *H. pylori*-induced NF-κB activation leading to COX-2 expression. One is the activation of NIK/IKK pathway which was already recognized (Malinin et al., 1997), and the

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other is the activation of PKC-dependent c-Src pathway demonstrated here. These two pathways converge at IKKα/β. The PKC/c-Src/IKK pathway, shown to be involved in the induction of COX-2 expression here, might be a common pathway for the inducible gene expression, since the TNF-α-, IL-1β- and IFN-γ-induced COX-2 or ICAM-1 expression in lung epithelial cells also involved the PKC-dependent activation of c-Src (Huang et al., 2003a; 2003b; Chang et al., 2002; 2004). Although the *H. pylori*-upregulated COX-2 mRNA expression and PGE2 synthesis were already found (Romano et al., 1998), we are the first to identify the involvements of NF-κB, NF-IL6 and CRE sites in *H. pylori*-induced COX-2 expression and further explore the existence of a novel TLR2/9/c-Src/NF-κB/COX-2 pathway.

In summary, the identification of clinical isolates of *H. pylori* from GC patients induces high levels of COX-2 expression *in vitro*. Although the genetic bases for this phenomenon are not known at present, these clinical isolates may represent a group of *H. pylori* strains harboring novel virulence factors that could be pursued in the future. Involvements of NF-κB, NF-IL6, and CRE sites in the regulation of *H. pylori*-induced COX-2 expression are demonstrated. Mechanism of TLR2/TLR9 mediated *H. pylori*-induced NF-κB activation is further examined. *H. pylori* acts through the TLR2/TLR9 to activate both the PI-PLCγ/PKCα/c-Src/IKKα/β and NIK/IKKα/β pathways, resulting in the phosphorylation and degradation of IκBα, which in term leads to the stimulation of NF-κB and COX-2 gene expression. A schematic presentation of the involvements of these pathways in the AGS cells is shown in Fig. 9. This COX-2 expression may contribute to the carcinogenesis in patients colonized with these cancer strains.

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## Legends for Figures

**Fig. 1** COX-2 expression in AGS and MKN45 cells induced by *Helicobacter pylori* isolates from patients with gastritis (HS), gastric ulcer (HU), duodenal ulcer (HD) and gastric cancer (HC) and TNF-α. Cells were treated with *H. pylori* isolates from different patients at indicating bacterium/cell ratio in (A-a) or at a bacterium/cell ratio of 150:1 in (A-b) and (C) or 10 ng/ml of TNF-α for 16 h. Whole cell lysates were prepared and subjected to Western blotting using antibody specific for COX-2, COX-1 or Actin. In (A-c), AGS cells were co-cultured with HC for the indicated time. Total RNA (2 μg) was used for RT-PCR as described under " Materials and Methods". In (B), box plot of COX-2 expression in AGS cells summarized from different clinical isolates of *H. pylori*. Cells were co-cultured with *H. pylori* strains from patients with gastritis (HS) (n=20), gastric ulcer (HU) (n=20), duodenal ulcer (HD) (n=21) and gastric cancer (HC) (n=20). The quantitative data were normalized by the actin level. In (D), COX-2 is located around the nuclear envelope or cytosol. Immunofluorescence staining of AGS or MKN45 cells with affinity-purified anti-COX-2 Ab (1:100) were performed as described under "Materials and methods". Control AGS and MKN45 cells or co-cultured with HC for 16 h were shown.

**Fig. 2.** Methylation status and activation of the COX-2 promoter, and kinetics of DNA-protein complex formation induced by *Helicobacter pylori*. In (A), methylation-specific PCR was performed after bisulfite modification of DNA as described under "Materials and Methods". M indicates methylated COX-2 and U indicates unmethylated COX-2. In (B), upper diagram represents the schematic diagram of 5' regulatory region of the human COX-2 gene.

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Rectangles indicate the location of the NF-κB, NF-IL6 and CRE sites. Cells were transfected with the pGS459/+9, -327/+59, KBM, ILM, or CRM luciferase expression vector, then infected with *H. pylori*. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity. The luciferase activity was normalized using the β-galactosidase activity and expressed as the mean±SEM of three independent experiments performed in triplicate. *, $p < 0.05$ as compared with -327/+59. In (C), cells were co-cultured with *H. pylori* (MOI: 150) for 30 min, 1 h, or 2 h, then nuclear extracts were prepared. NF-κB, NF-IL6 or CRE oligonucleotide probe was used to measure the DNA-protein complex formation by EMSA as described under "Materials and Methods". *, free probe; **, NF-κB DNA-protein complex ; ***, NF-IL6 DNA-protein complex ; #, CRE DNA-protein complex.

**Fig. 3** Effects of TLRs mutant or neutralizing antibody on COX-2 promoter activity or COX-2 expression induced by *H. pylori*. In (A), cell lysates were prepared and 100 μg total proteins were subjected to Western blot analyses using anti-TLR2, TLR4, or TLR9 Ab. In (B) and (D), AGS cells were co-transfected with pGS459 and the mutant of TLR2 (P/H), TLR4 (P/H) or TLR9 (ICD) (B), or co-transfected with pGS459 and the wild-type TLR2, TLR4, TLR9 or the respective empty vector, or following by co-cultured with *H. pylori* (D). In (E), cells were transfected with the κB-Luc plasmid, then infected with HS, HU, HD, HC or treated with 10 ng/ml of TNF-α for 6 h or co-transfected with κB-Luc plasmid and TLR2 (P/H) or TLR9 (ICD) mutant, or the respective empty vector, then co-cultured with *H. pylori* for 6 h. Luciferase activity was measured as described under "Materials and Methods". The results were

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normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone in (B) and (E), or with empty vector (D). **, $p < 0.05$ compared with the respective wt TLR alone. #, $p < 0.05$ compared with wt TLR2+wt TLR9 (D). In (C), AGS cells were incubated with anti-TLR2, TLR9 or control Ab for 3 h, then co-cultured with *H. pylori* (MOI: 150) for 16 h. Whole cell lysates were prepared and subjected to Western blotting using anti-COX-2, or anti-Actin Ab.

**Fig. 4** Effects of various inhibitors or PLCγ2 mutant or dominant-negative mutants on *H. pylori*-induced COX-2 expression or promoter activity in AGS cells. In (A), cells were pretreated with U73122 (10 μM), U73343 (10 μM), Ro 31-8220 (1 μM) or PP2 (10 μM) for 30 min before co-cultured with *H. pylori* for 16 h. Whole cell lysates were prepared and subjected to Western blotting using Ab specific for COX-2, COX-1 or actin. In (B), cells were co-transfected with pGS459 and the PLCγ2 (SH2(N)) mutant or the dominant-negative mutants of PKCα (K/R) or c-Src (KM), or the respective empty vector, then co-cultured with *H. pylori* (MOI:150) for 6 h. Luciferase activity was measured as described under "Material and Method".The results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone.

**Fig. 5** Effect of PLCγ2 mutant or various dominant-negative mutants on wild-type or constitutive active plasmid-induced COX-2 promoter activity. In (A-a), AGS cells were co-transfected with the dominant negative NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (Y188F), IKKβ (Y199F) or IKKβ (FF) mutant or

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the respective empty vector, then co-cultured with *H. pylori* (MOI:150) for 6 h. In (A-b and B), cells were co-transfected with wild-type TLR2 and PLCγ2 (SH2(N)) mutant or the dominant negative mutants of PKCα (K/R), c-Src (KM), NIK (KKAA), IKKα (KM), IKKβ (KM), IKKβ (Y188F), IKKβ (Y199F) or IKKβ (FF), or the respective empty vector (A-b), or co-transfected with PLCγ2 (wt) and the dominant negative PKCα (K/R), c-Src (KM), IKKα (KM) or IKKβ (KM) mutant (B-a), or co-transfected with PKCα (A/E) and the dominant negative c-Src (KM), IKKα (KM) or IKKβ (KM) mutant (B-b), or co-transfected with c-Src (wt) and the PLCγ2 (SH2(N)) mutant or the dominant negative mutants of IKKα (KM), IKKβ (KM), IKKβ (AA), IKKβ (Y188F), IKKβ (Y199F) or IKKβ (FF), or the respective empty vector (B-c). Luciferase activity was measured as described under "Material and Method". The results were normalized to the β-galactosidase activity and expressed as the mean ± S.E. for three independent experiments performed in triplicate. *, $p < 0.05$ compared with *H. pylori* alone (A-a), wt TLR2 alone (A-b), wt PLCγ2 alone (B-a), PKCα (A/E) alone (B-b) or c-Src (wt) alone (B-c).

**Fig. 6** Time-dependent activation of c-Src by *H. pylori* and effects of various inhibitors. AGS cells were co-cultured with *H. pylori* for 10, 30, 60 or 120 min (A) or pretreated with 10 μM U73122, 1 μM Ro 31-8220, 1 or 10 μM PP2 for 30 min before co-cultured with *H. pylori* for 60 min (B). Whole cell lysates were prepared and immunoprecipitated with anti-c-Src antibody. The kinase assay (KA) and autoradiography for phosphorylated enolase were performed on the precipitates as described under "Material and Method". Levels of immunoprecipitated c-Src were estimated by Western blotting (WB) using anti-c-Src antibody.

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**Fig. 7** Kinetics of *H. pylori*-induced IKK activation and IκB-α degradation and effects of various inhibitors. AGS cells were co-cultured with *H. pylori* for 10, 30, 60 or 120 min (A), or pretreated with 1 or 10 μM U73122, 0.1 or 1 μM Ro 31-8220, 1 or 10 μM PP2 for 30 min before co-cultured with *H. pylori* for 60 min (B), then whole cell lysates were prepared. Cell lysates were immunoprecipitated with anti-IKKβ antibody, then kinase assay (KA) and autoradiography for phosphorylated GST-IκBα (1-100) were performed on the precipitates as described under "Material and Method". Levels of immunoprecipitated IKKβ protein were estimated by Western blotting (WB) using anti-IKKβ antibody (A-a and B-a). Cytosolic levels of IκB-α and actin were measured using anti-IκB-α and actin antibody, respectively (A-b and B-b).

**Fig. 8** c-Src-dependent phosphorylation of IKKβ induced by *H. pylori* and the inhibition by PP2. AGS cells were co-cultured with *H. pylori* for 10, 30, 60 or 120 min (A) or pretreated with 1 or 10 μM PP2 for 30 min before co-cultured with *H. pylori* for 60 min (*B*). Whole cell lysates were prepared and immunoprecipitated with anti-c-Src antibody, then kinase assay (KA) and autoradiography for phosphorylated GST-IKKβ (132-206) were performed. The amount of immunoprecipitated c-Src was detected by Western blotting (WB) using anti-c-Src antibody.

**Fig. 9** Schematic representation of the signaling pathways involved in *H. pylori*-induced COX-2 expression in the AGS epithelial cells. The κB, NF-IL6 and CRE elements are involved in *H. pylori*-induced COX-2 expression. *H. pylori* acts through TLR2 and TLR9, then activates PI-PLCγ to induce PKCα and c-Src activation, leading to tyrosine phosphorylation of

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IKKα/β. *H. pylori* also activates NIK, leading to serine phosphorylation of IKKα/β. These two pathways converge at IKKα/β, resulting in the phosphorylation and degradation of IκBα, stimulation of NF-κB in the COX-2 promoter, and finally initiation of COX-2 expression.

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Table 1. The relationship between the extent of cyclooxygenase-2 expression in AGS cells and genotypes in different clinical isolates of *Helicobacter pylori*

| | HS (n=20) | HU (n=20) | HD (n=21) | HC (n=20) |
|---|---|---|---|---|
| COX-2 expression | | | | |
| Mean±SE | 1.48±0.16 | 1.92±0.38 | 1.97±0.46 | 6.19±0.89* |
| Restriction endonuclease-replacing gene | | | | |
| hrgA | 7 | 8 | 7 | 7 |
| hpy□R | 13 | 12 | 14 | 13 |
| iceA | | | | |
| A1A1 | 17 | 18 | 18 | 17 |
| A1A2 | 2 | 1 | 2 | 2 |
| A2A2 | 1 | 1 | 1 | 1 |
| babA2 | | | | |
| positive | 20 | 20 | 21 | 20 |
| negative | 0 | 0 | 0 | 0 |
| cagA 3' repeat region | | | | |
| A | 19 | 18 | 21 | 19 |
| B,C,D | 1 | 2 | 0 | 1 |
| vacA | | | | |
| s1a/m1 | 3 | 6 | 8 | 6 |
| s1a/m2 | 17 | 14 | 13 | 14 |

HS: strain from nonulcer dyspepsia; HU: strain from gastric ulcer

HD: strain from duodenal ulcer; HC: strain from gastric cancer

*: $p<0.001$ versus HS, HU, or HD.

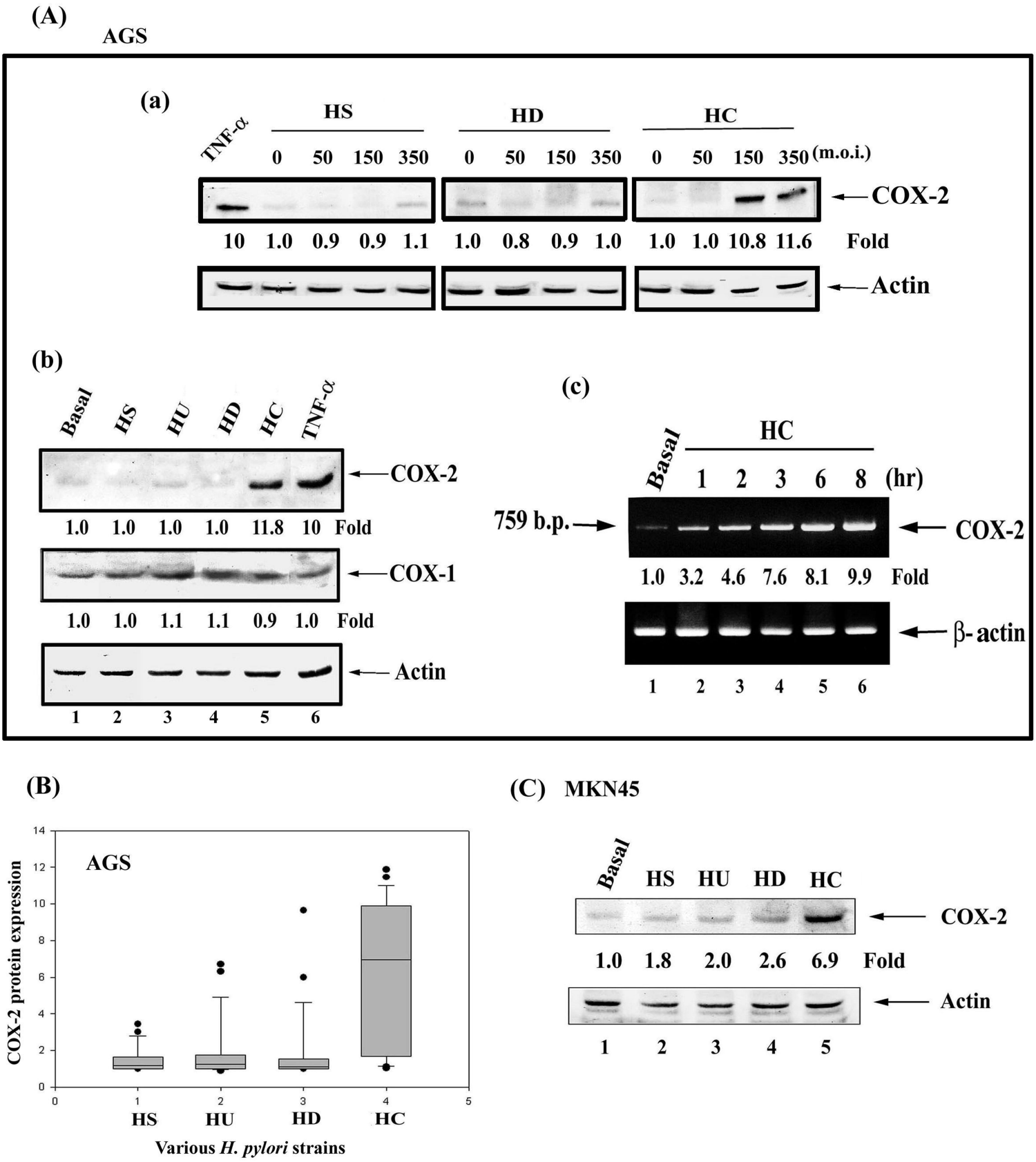

(A)
AGS
(a)
TNF-α
HS
HD
HC
0 50 150 350 0 50 150 350 0 50 150 350 (m.o.i.)
COX-2
10 1.0 0.9 0.9 1.1 1.0 0.8 0.9 1.0 1.0 1.0 10.8 11.6 Fold
Actin
(b)
Basal HS HU HD HC TNF-α
COX-2
1.0 1.0 1.0 1.0 11.8 10 Fold
COX-1
1.0 1.0 1.1 1.1 0.9 1.0 Fold
Actin
1 2 3 4 5 6
(c)
Basal
HC
1 2 3 6 8 (hr)
759 b.p.
COX-2
1.0 3.2 4.6 7.6 8.1 9.9 Fold
β-actin
1 2 3 4 5 6
(B)
AGS
COX-2 protein expression
0 2 4 6 8 10 12 14
1 2 3 4 5
HS HU HD HC
Various H. pylori strains
(C) MKN45
Basal HS HU HD HC
COX-2
1.0 1.8 2.0 2.6 6.9 Fold
Actin
1 2 3 4 5

Fig. 1

(D)

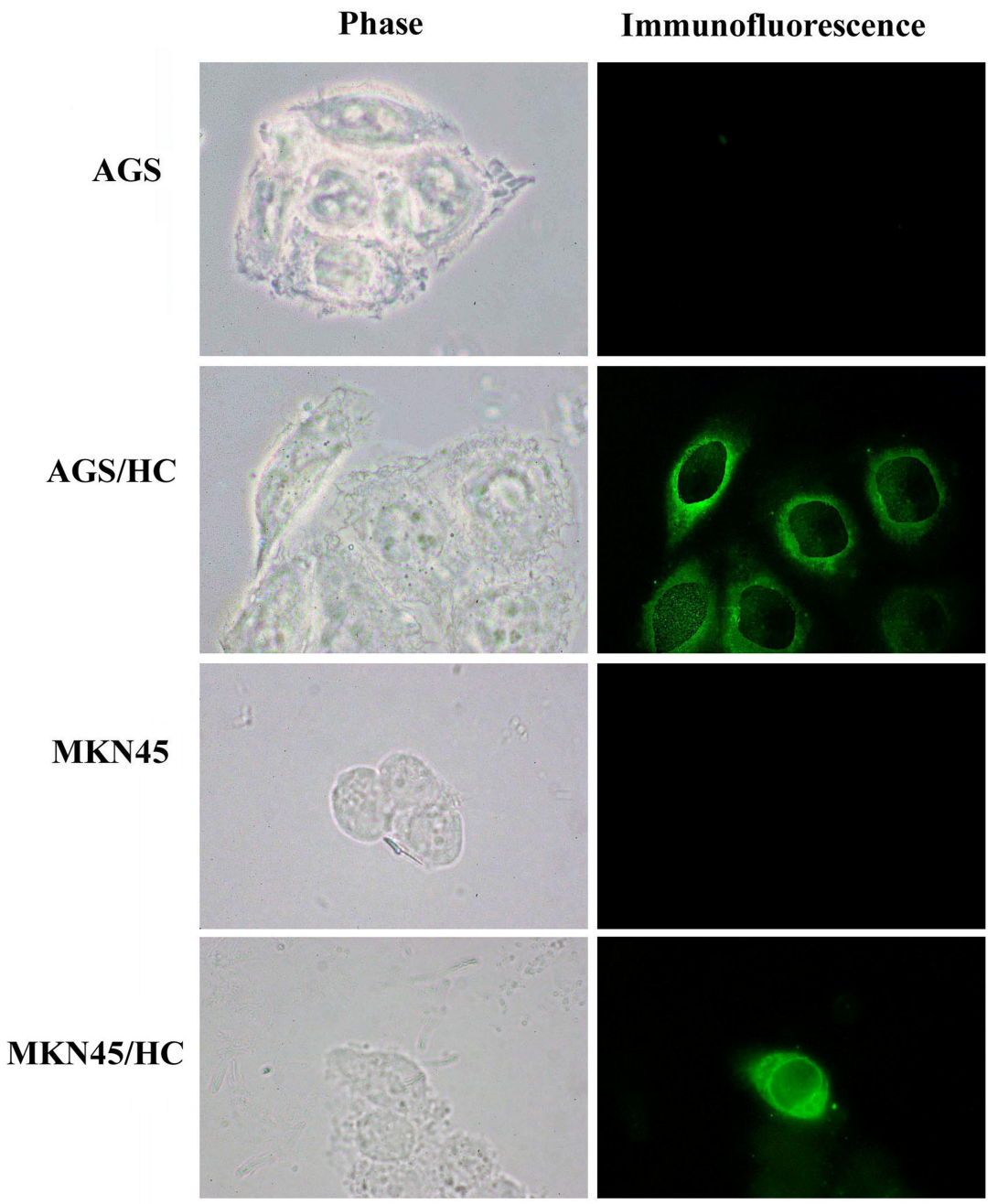



Fig. 1

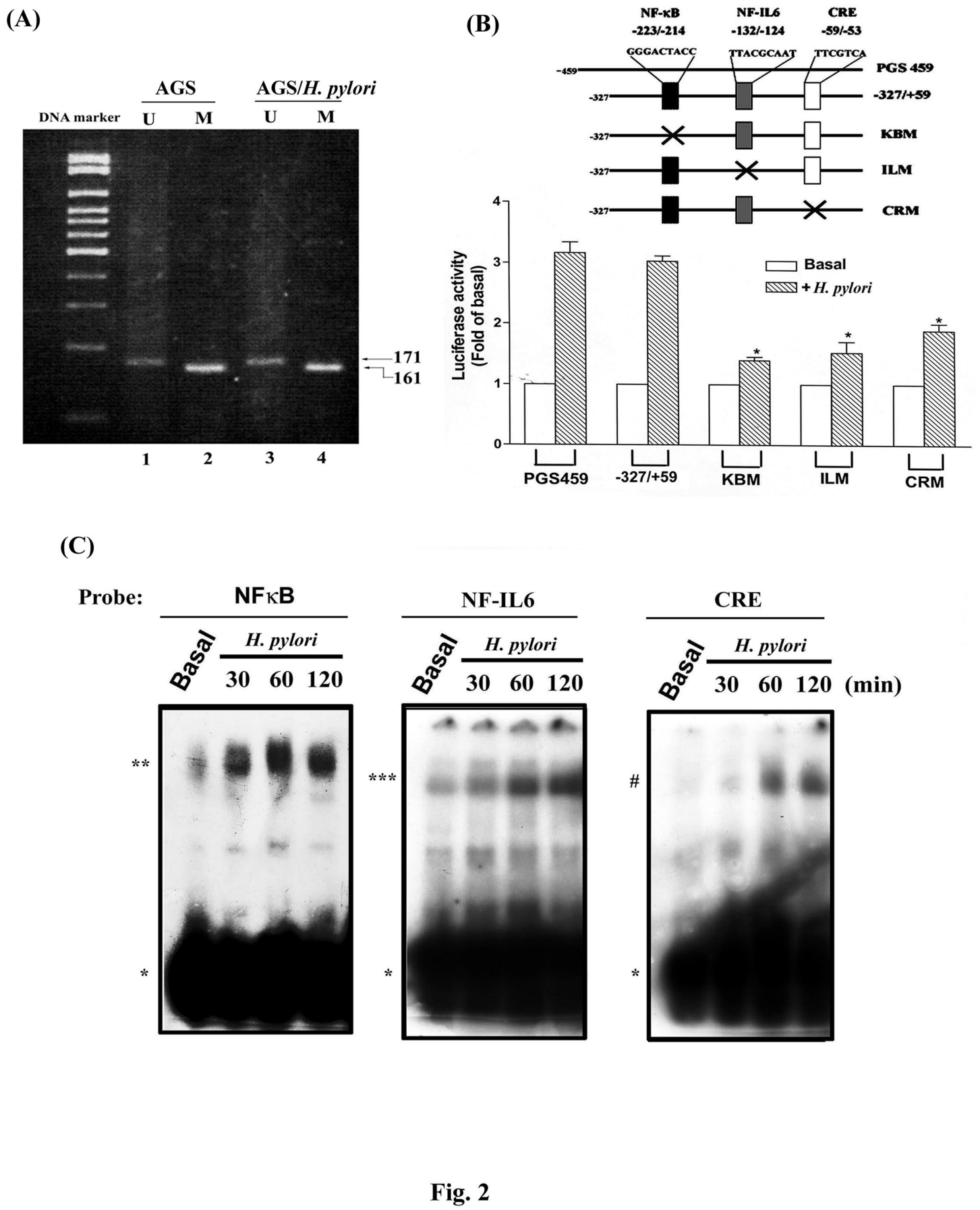

(A)
AGS
AGS/H. pylori
DNA marker
U
M
U
M
171
161
1
2
3
4
(B)
NF-κB
-223/-214
NF-IL6
-132/-124
CRE
-59/-53
GGGACTACC
TTACGCAAT
TTCGTCA
-459
PGS 459
-327
-327/+59
KBM
ILM
CRM
Luciferase activity
(Fold of basal)
Basal
+ H. pylori
PGS459
-327/+59
KBM
ILM
CRM
(C)
Probe:
NFκB
NF-IL6
CRE
Basal
H. pylori
30
60
120
(min)
**
*
***
#

Fig. 2

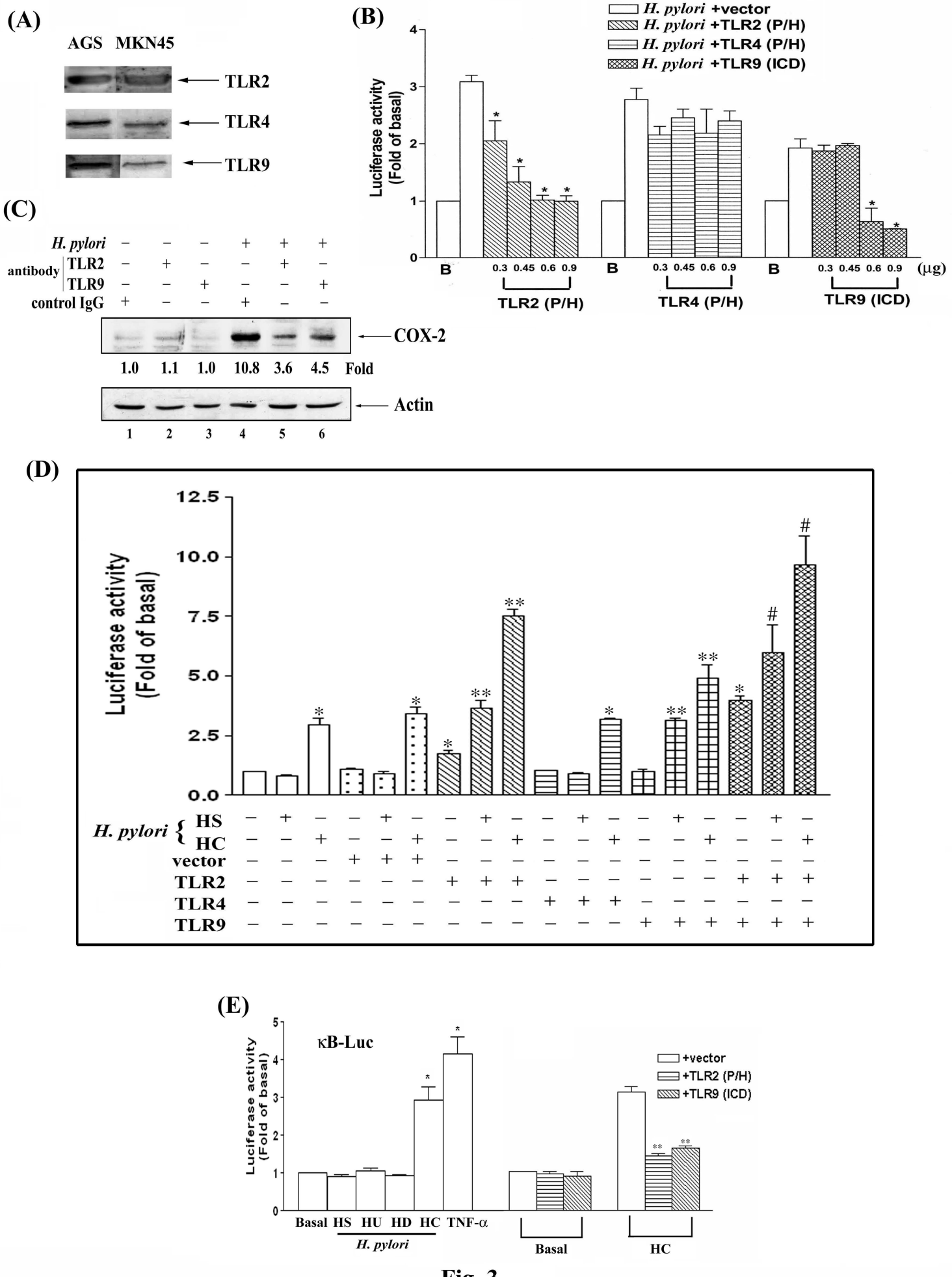
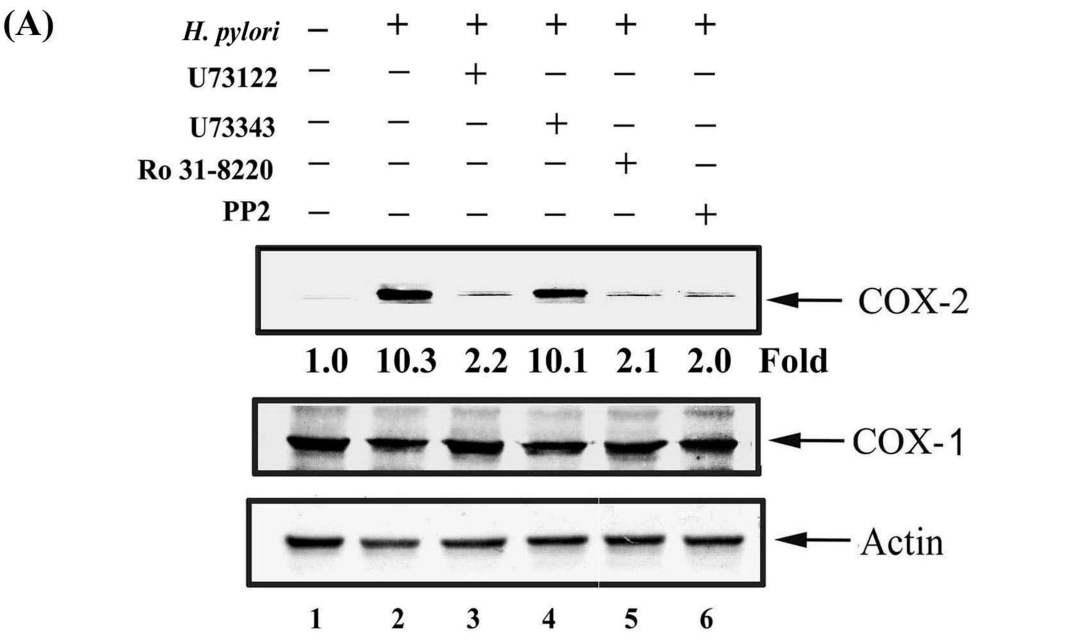

(A)
AGS MKN45
TLR2
TLR4
TLR9
(B)
H. pylori +vector
H. pylori +TLR2 (P/H)
H. pylori +TLR4 (P/H)
H. pylori +TLR9 (ICD)
Luciferase activity (Fold of basal)
B 0.3 0.45 0.6 0.9 (μg)
TLR2 (P/H)
TLR4 (P/H)
TLR9 (ICD)
(C)
H. pylori
antibody TLR2
TLR9
control IgG
COX-2
1.0 1.1 1.0 10.8 3.6 4.5 Fold
Actin
1 2 3 4 5 6
(D)
Luciferase activity (Fold of basal)
H. pylori HS
HC
vector
TLR2
TLR4
TLR9
(E)
κB-Luc
Luciferase activity (Fold of basal)
+vector
+TLR2 (P/H)
+TLR9 (ICD)
Basal HS HU HD HC TNF-α
H. pylori
Basal
HC

**Fig. 3**


(A)
H. pylori – + + + + +
U73122 – – + – – –
U73343 – – – + – –
Ro 31-8220 – – – – + –
PP2 – – – – – +
COX-2
1.0 10.3 2.2 10.1 2.1 2.0 Fold
COX-1
Actin
1 2 3 4 5 6

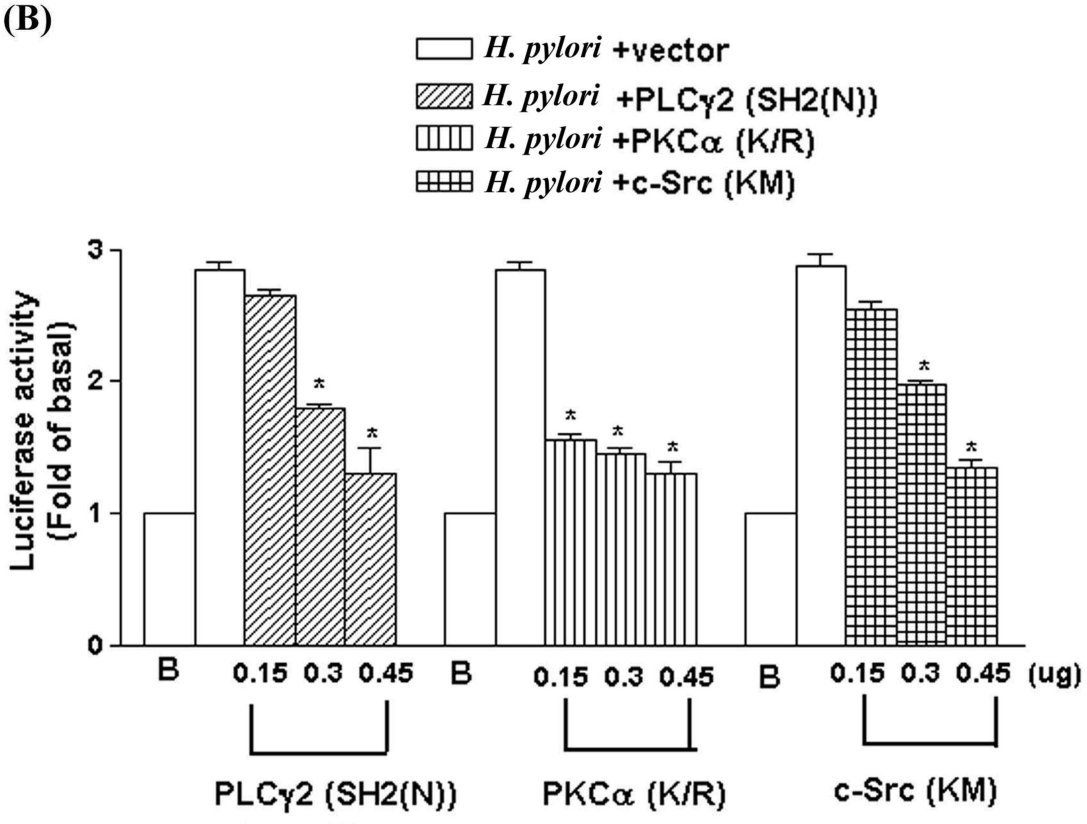

(B)
H. pylori +vector
H. pylori +PLCγ2 (SH2(N))
H. pylori +PKCα (K/R)
H. pylori +c-Src (KM)
Luciferase activity (Fold of basal)
0
1
2
3
B 0.15 0.3 0.45 B 0.15 0.3 0.45 B 0.15 0.3 0.45 (ug)
PLCγ2 (SH2(N))
PKCα (K/R)
c-Src (KM)

**Fig. 4**

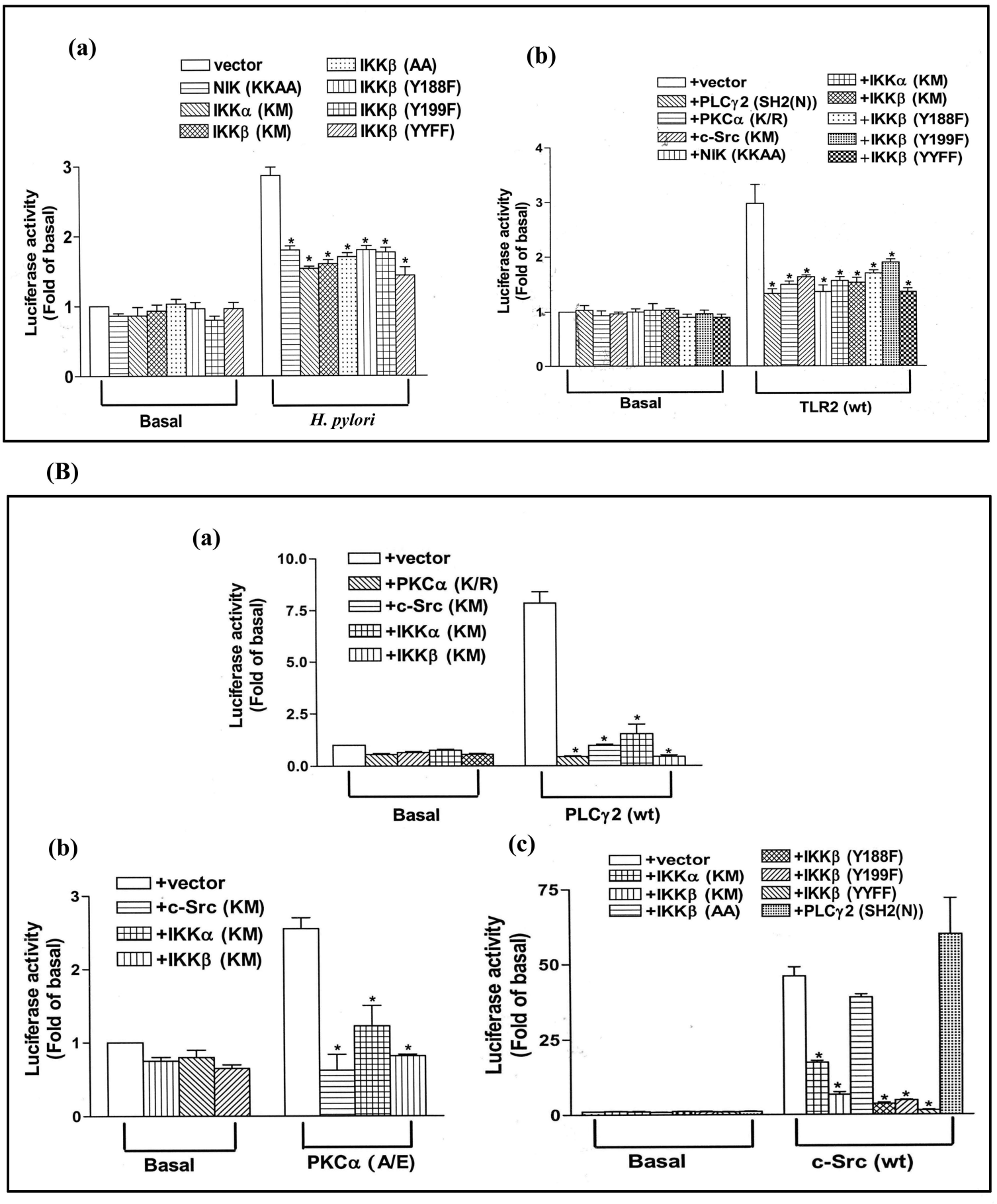

(A)
(a)
vector
NIK (KKAA)
IKKα (KM)
IKKβ (KM)
IKKβ (AA)
IKKβ (Y188F)
IKKβ (Y199F)
IKKβ (YYFF)
Luciferase activity (Fold of basal)
Basal
H. pylori
(b)
+vector
+PLCγ2 (SH2(N))
+PKCα (K/R)
+c-Src (KM)
+NIK (KKAA)
+IKKα (KM)
+IKKβ (KM)
+IKKβ (Y188F)
+IKKβ (Y199F)
+IKKβ (YYFF)
Luciferase activity (Fold of basal)
Basal
TLR2 (wt)
(B)
(a)
+vector
+PKCα (K/R)
+c-Src (KM)
+IKKα (KM)
+IKKβ (KM)
Luciferase activity (Fold of basal)
Basal
PLCγ2 (wt)
(b)
+vector
+c-Src (KM)
+IKKα (KM)
+IKKβ (KM)
Luciferase activity (Fold of basal)
Basal
PKCα (A/E)
(c)
+vector
+IKKα (KM)
+IKKβ (KM)
+IKKβ (AA)
+IKKβ (Y188F)
+IKKβ (Y199F)
+IKKβ (YYFF)
+PLCγ2 (SH2(N))
Luciferase activity (Fold of basal)
Basal
c-Src (wt)

Fig. 5

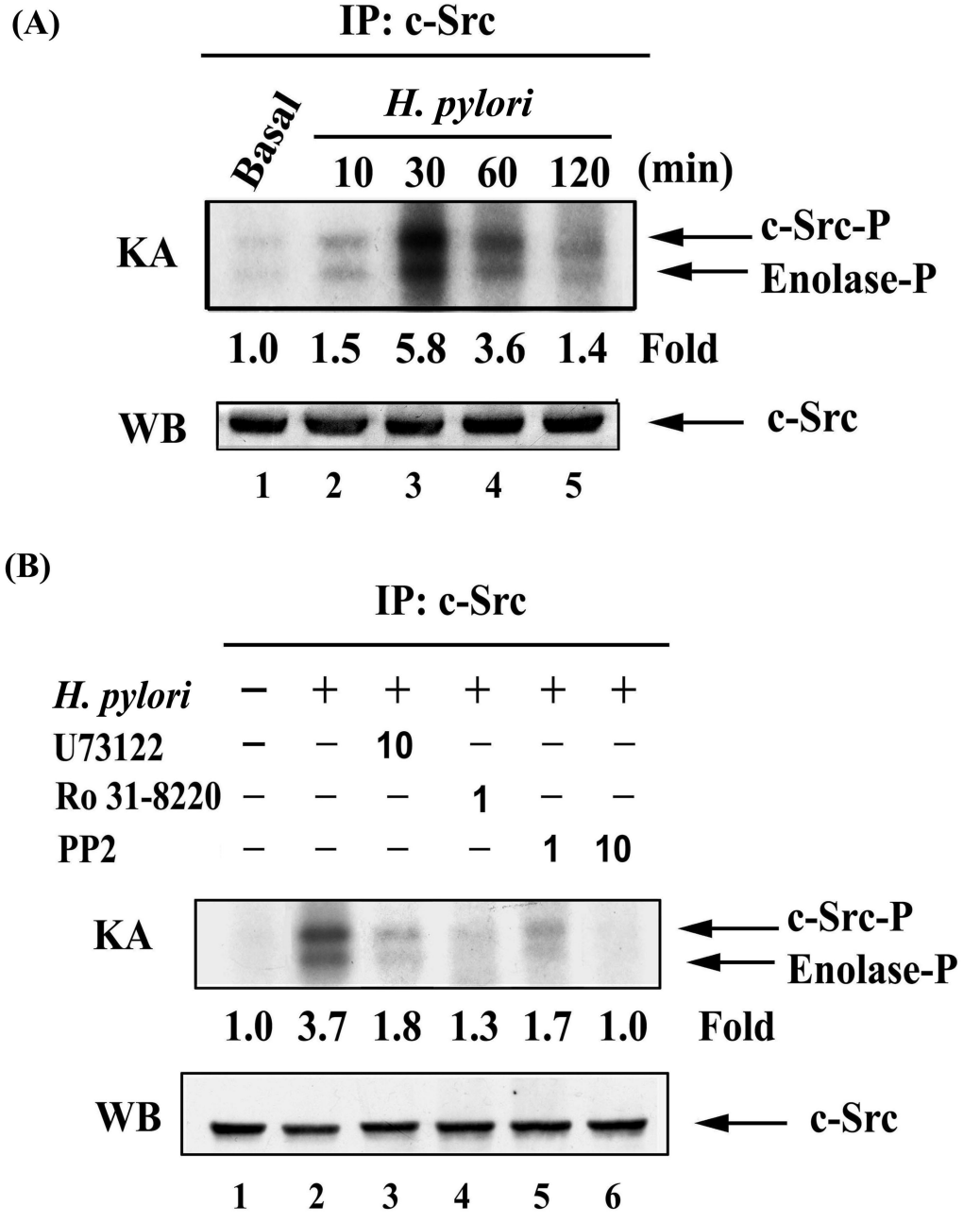

(A)
IP: c-Src
Basal
H. pylori
10 30 60 120 (min)
KA
c-Src-P
Enolase-P
1.0 1.5 5.8 3.6 1.4 Fold
WB
c-Src
1 2 3 4 5
(B)
IP: c-Src
H. pylori − + + + + +
U73122 − − 10 − − −
Ro 31-8220 − − − 1 − −
PP2 − − − − 1 10
KA
c-Src-P
Enolase-P
1.0 3.7 1.8 1.3 1.7 1.0 Fold
WB
c-Src
1 2 3 4 5 6

**Fig. 6**

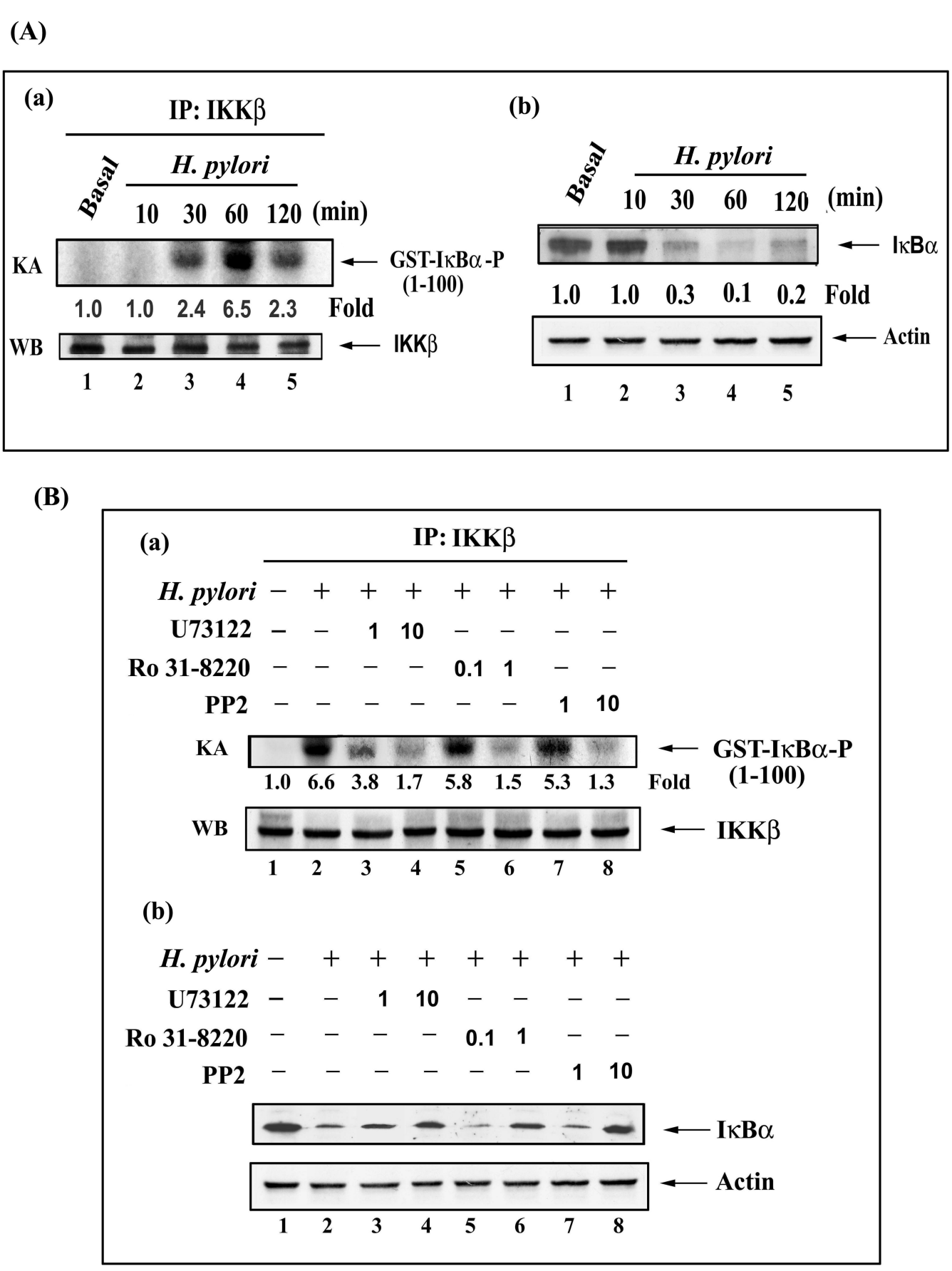

(A)
(a)
IP: IKKβ
Basal
H. pylori
10 30 60 120 (min)
KA
GST-IκBα-P
(1-100)
1.0 1.0 2.4 6.5 2.3 Fold
WB
IKKβ
1 2 3 4 5
(b)
Basal
H. pylori
10 30 60 120 (min)
IκBα
1.0 1.0 0.3 0.1 0.2 Fold
Actin
1 2 3 4 5
(B)
(a)
IP: IKKβ
H. pylori – + + + + + + +
U73122 – – 1 10 – – – –
Ro 31-8220 – – – – 0.1 1 – –
PP2 – – – – – – 1 10
KA
GST-IκBα-P
(1-100)
1.0 6.6 3.8 1.7 5.8 1.5 5.3 1.3 Fold
WB
IKKβ
1 2 3 4 5 6 7 8
(b)
H. pylori – + + + + + + +
U73122 – – 1 10 – – – –
Ro 31-8220 – – – – 0.1 1 – –
PP2 – – – – – – 1 10
IκBα
Actin
1 2 3 4 5 6 7 8

Fig. 7

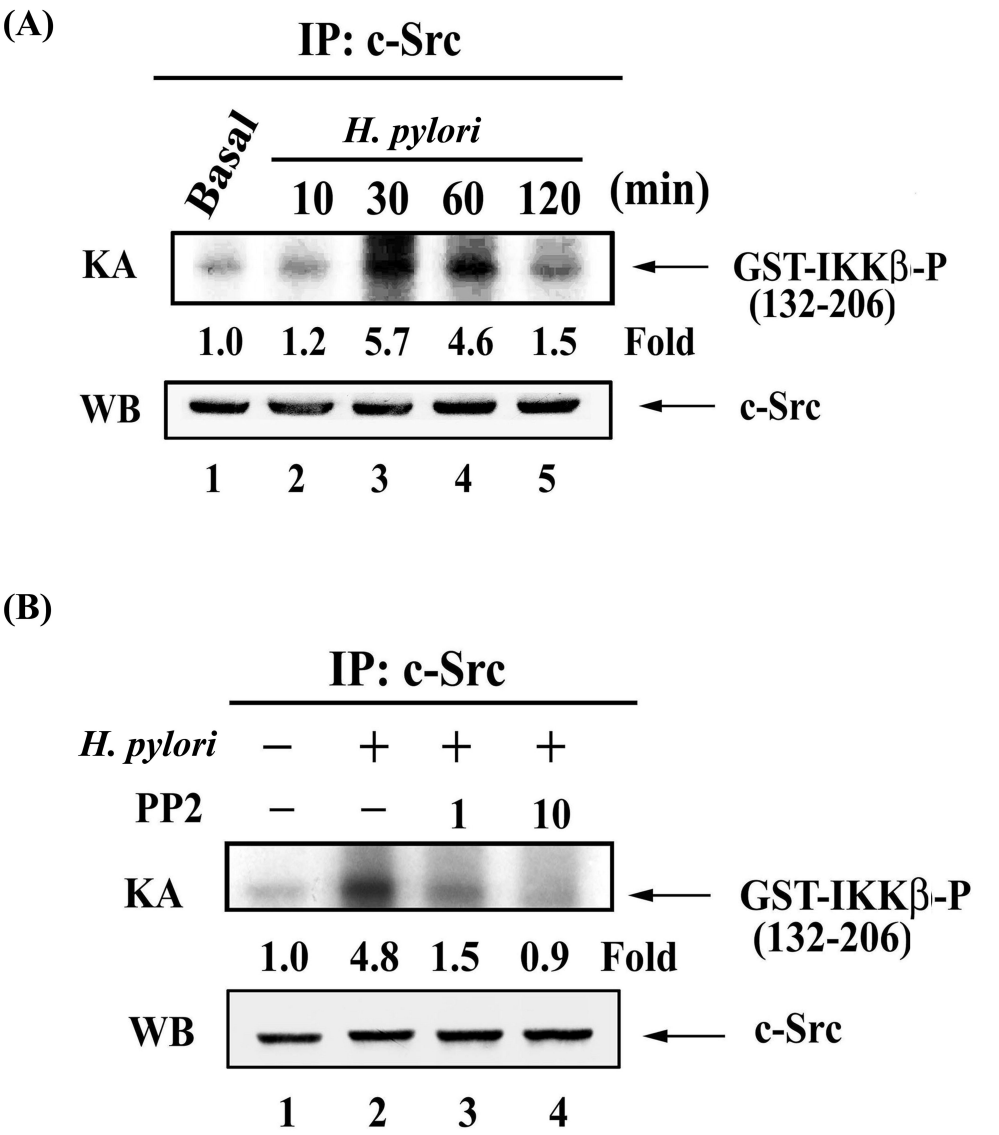

(A)
IP: c-Src
Basal
H. pylori
10 30 60 120 (min)
KA
GST-IKKβ-P
(132-206)
1.0 1.2 5.7 4.6 1.5 Fold
WB
c-Src
1 2 3 4 5
(B)
IP: c-Src
H. pylori − + + +
PP2 − − 1 10
KA
GST-IKKβ-P
(132-206)
1.0 4.8 1.5 0.9 Fold
WB
c-Src
1 2 3 4

**Fig. 8**

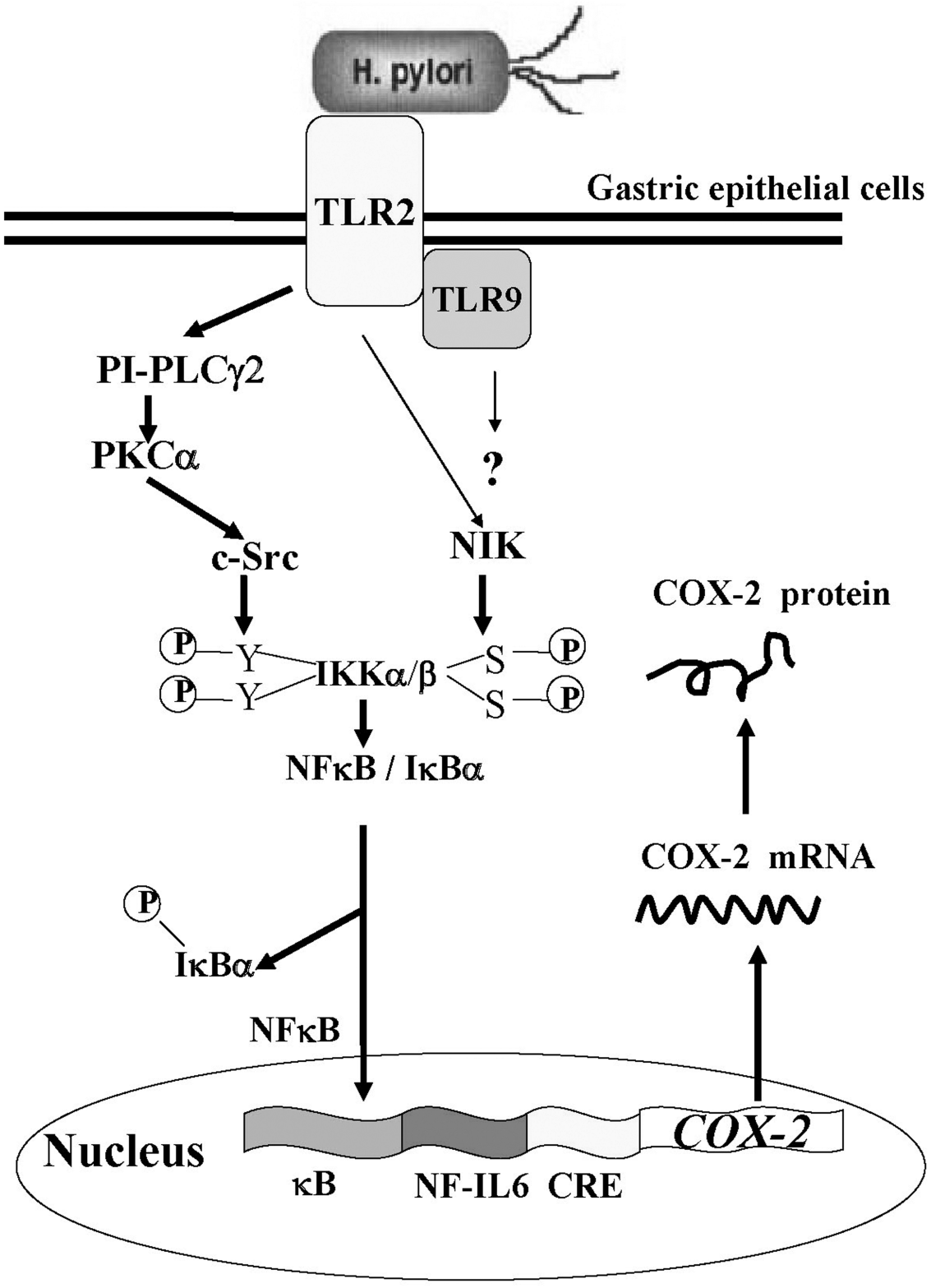

H. pylori
TLR2
TLR9
Gastric epithelial cells
PI-PLCγ2
PKCα
c-Src
?
NIK
P
Y
IKKα/β
S
NFκB / IκBα
COX-2 protein
COX-2 mRNA
IκBα
NFκB
Nucleus
κB
NF-IL6
CRE
COX-2

Fig. 9