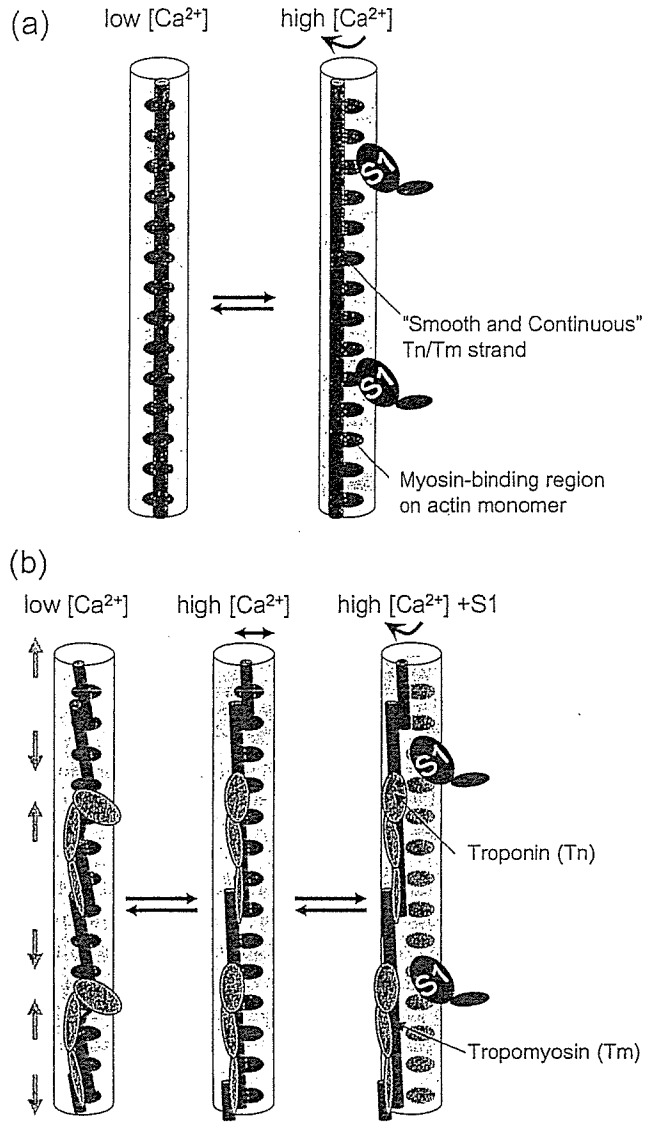

Fig. 5 The steric blocking model (a) and a model proposed in the present study (b). The steric blocking model assumes a smooth and continuous troponin/tropomyosin (Tn/Tm) strand. The new model assumes that the alternations in the strain imposed on the tropomyosin strand caused by the Ca^{2+}-dependent conformational changes in Tn, modify the accessibility of the myosin head to binding sites on the actin filament. Myosin heads (S1) are schematically drawn.

mechanism of muscle regulation. Ongoing efforts by our laboratory include solving the crystal structures of larger complexes, such as, Tn-Tm.

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Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity

Kyung Jin Woo[a,b], Yong-Jin Jeong[b], Hiroyasu Inoue[c], Jong-Wook Park[a], Taeg Kyu Kwon[a,*]

[a] *Department of Immunology, School of Medicine, Keimyung University, 194 DongSan-Dong Jung-Gu, Taegu 700-712, South Korea*
[b] *Department of Food Science and Technology, Keimyung University, Taegu, South Korea*
[c] *Department of Food Science and Nutrition, Nara Women's University, Nara, Japan*

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Abstract **Chrysin is a natural, biologically active compound extracted from many plants, honey and propolis. It possesses potent anti-inflammation, anti-cancer and anti-oxidation properties. The mechanism by which chrysin suppresses COX-2 expression remains poorly understood. In the present report, we investigated the effect of chrysin on the expression of COX-2 in lipopolysaccharide (LPS)-activated Raw 264.7 cells. Chrysin significantly suppressed the LPS-induced COX-2 protein and mRNA expression in a dose-dependent manner. The ability of chrysin to suppress the expression of the COX-2 was investigated using luciferase reporters controlled by various *cis*-elements in COX-2 promoter region. Mutational analysis and electrophoretic mobility shift assay verified that nuclear factor for IL-6 was identified as responsible for the chrysin-mediated COX-2 downregulation. These results will provide new insights into the anti-inflammatory and anti-carcinogenic properties of chrysin.**

Keywords: Chrysin; Cyclooxygenase-2; Nuclear factor for IL-6; Macrophage; Lipopolysaccharide

1. Introduction

Prostaglandins play important roles in many cellular responses including cell growth, ovulation, and immune functions, and inhibition of their synthesis is the target of most known nonsteroidal anti-inflammatory drugs [1,2]. Prostaglandins synthesis involve phospholipase catalyzed release of arachidonic acid from membranes phospholipids and its conversion by the two cyclooxygenase (Cox) enzymes to prostaglandins [2,3]. Cox-1 is constitutively expressed in tissues and is thought to be involved in homeostatic prostanoid biosynthesis [2–4]. COX-2 is thought to be the predominant isoform involved in the inflammatory responses [2–4]. Multiple lines of evidence suggest that COX-2 has a significant role in carcinogenesis. COX-2 is overexpressed in transformed cells as well as in various forms of cancer [5–7], because the targeted inhibition of COX-2 is a promising approach to inhibiting inflammation and carcinogenesis as well as to prevent cancer.

*Corresponding author. Fax: +82 53 250 7074.
E-mail address: kwontk@dsmc.or.kr (T.K. Kwon).

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones, flavanols and flavans. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid contained in many plant extracts, honey and propolis [8,9]. Recent studies have shown that chrysin inhibits activation of human immunodeficiency virus in models of latent infection [10]. Several studies in recent years have shown that chrysin has multiple biological activities, such as anti-inflammation, anti-cancer and anti-oxidation effects [11–14]. Recently, some flavonoids including chrysin exhibited weak peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist activities in an in vitro competitive-binding assay [15]. The PPAR-γ agonists have been considered to inhibit production of monocyte inflammatory cytokines and expression of inducible nitric oxide synthase and COX-2 [16]. However, the cellular and molecular mechanisms underlying chrysin-induced inhibition of COX-2 expression in macrophages are not clear.

In this study of chrysin anti-inflammatory properties, chrysin inhibited LPS-induced COX-2 expression in Raw 264.7 cells, apparently through blockading NF-IL6 binding to the COX-2 promoter, thereby inhibiting COX-2 expression.

2. Materials and methods

2.1. Cells and materials

All reagents were purchased from Sigma–Aldrich unless otherwise stated. Chrysin [(5,7-dihydroxyflavone) (Fig. 1)] was dissolved in dimethyl sulfoxide and freshly diluted in culture media for all in vitro experiments. The macrophage cell line Raw 264.7 was obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 2 mM of L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were subcultured twice weekly and grown on 6-well plates at 1×10^6cells/well, at 37 °C in fully humidified 5% CO_2 air. Anti-COX-2 and anti-NF-IL6 were purchased from Santa Cruz Biotechnology Inc. Anti-phospho-ERK, anti-phospho-JNK, and anti-phospho-p38 MAPK were purchased from New England Biolabs Inc. The inhibitors PD98059, SB203580, SP600125, genistein, and tyrphostin A 23 were purchased from Biomol.

2.2. Western blotting

Cellular lysates were prepared by suspending 1.5×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS (4-morpholinepropane-sulfonic acid), 100 μM phenylmethylsulfonyl fluoride,

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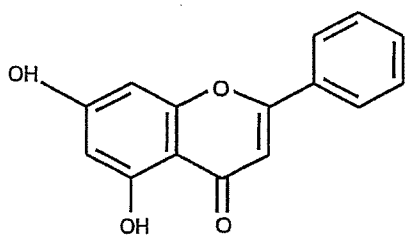

Fig. 1. Chemical structure of chrysin (5,7-dihydroxyflavone).

and 20 μM leupeptin, adjusted to pH 7.2), disrupted by sonication and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes and detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

2.3. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated according to Chomczynski and Sacchi [17]. Single-strand cDNA was synthesized from 2 μg of total RNA using Moloney–Murine leukemia virus reverse transcriptase. The cDNA for COX-2 and actin were PCR amplified using the following specific primers: COX-2 (sense) 5′-CCGTGGTGAATGTATGAGCA-3′ and (antisense) 5′-CCTCGCTTCTGATCTGTCTT-3′. PCR amplification was carried out as follows: 1× (94 °C, 3 min); 30× (94 °C, 45 s; 59 °C, 45 s; and 72 °C, 1 min); and 1× (72 °C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

2.4. DNA transfection and luciferase assay

Nuclear factor-κB (NF-κB) reporter constructs were purchased from Clontech. The human COX-2 promoter-containing plasmids have been described previously [18,19]. NF-κB reporter plasmids or COX-2 promoter plasmids were transfected into Raw 264.7 cells using the Lipofectamine reagent according to the manufacturer's instructions. To assess COX-2 promoter driving of the luciferase gene, cells were collected and disrupted by sonication in lysis buffer (25 mM tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of the supernatants were analyzed with the luciferase assay according to the manufacturer's instructions.

2.5. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously [20]. The sequences of the double-stranded oligonucleotides used to detect the DNA-binding activities of NF-κB, cAMP response element-binding protein (CREB), and NF-IL6 (CCAAT/enhancer-binding protein, C/EBP) are as follows: NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; CREB, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′; and NF-IL6 (C/EBP), 5′-TGC AGA TTG CGC AAT CTG CA-3′. The reaction mixture for EMSA contained 20 mM Tris–HCl, pH 7.6, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 μg of poly(dI-dC) and 5 μg nuclear proteins. Unlabeled wild type oligonucleotide was added into the reaction mixture and incubated for 10 min at room temperature. [^{32}P]-labeled probe DNA (300000 cpm) was added, and the binding reaction was allowed to proceed for another 20 min. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

3. Results

3.1. Chrysin inhibition of LPS-induced COX-2 mRNA and protein expression in RAW 264.7 cells

Unstimulated Raw 264.7 cells do not contain COX-2, whereas addition of 50 ng/ml LPS induced COX-2 synthesis in these cells (Fig. 2A). To investigate whether chrysin can inhibit LPS-induced COX-2 expression, Raw 264.7 cells were pretreated for 30 min with various concentrations of chrysin and subsequently treated with 50 ng/ml LPS. Cells pretreated

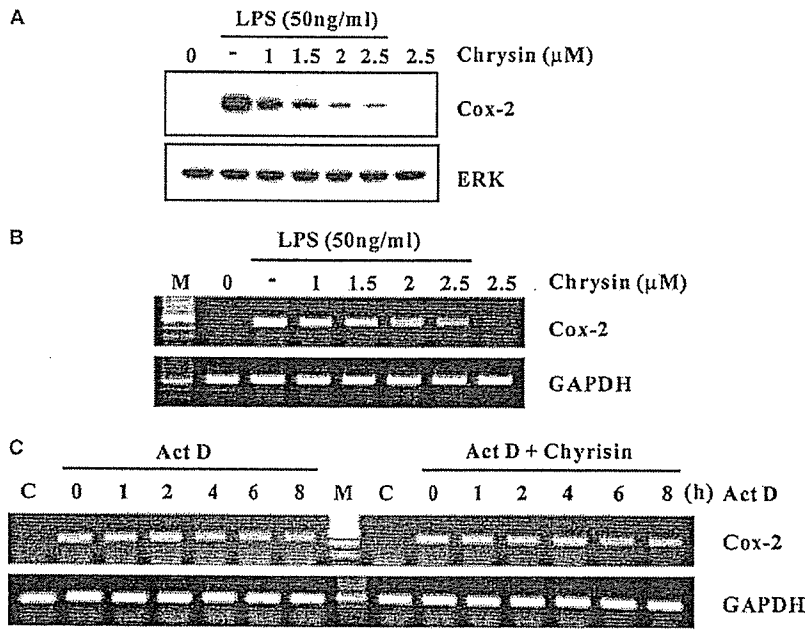

Fig. 2. Effect of chrysin on LPS-induced expression of COX-2 protein and mRNA. (A) Raw 264.7 cells were treated with indicated concentrations of chrysin in the presence of LPS (50 ng/ml) for 12 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-COX-2. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. (B) Total RNA was prepared and RT-PCR analysis was performed as described in Section 2. (C) Raw 264.7 cells were treated with 50 ng/ml LPS for 8 h and then with or without chrysin (2.5 μM) in the presence of actinomycin D (5 μg/ml) for the indicated times. Total RNA was isolated and RT-PCR analysis was performed. A representative result is shown; two additional experiments yielded similar results.

with chrysin showed a dose-dependent inhibition of COX-2 protein expression following LPS stimulation. To assess the effect of chrysin on COX-2 mRNA expression, we measured mRNA levels using RT-PCR. The expression of COX-2 mRNA was hardly detectable in unstimulated cells. Raw 264.7 cells that were not treated with chrysin expressed high level of COX-2 mRNA when stimulated with 50 ng/ml LPS for 12 h. Addition of chrysin inhibited this LPS-stimulated COX-2 mRNA production in a dose-dependent manner (Fig. 2B). We also investigated whether chrysin stabilized COX-2 mRNA. Raw 264.7 cells were treated with vehicle or LPS for 8 h to induce COX-2 transcription, and then transcription was stopped with the addition of actinomycin D. RNA was isolated at different time points after treatment with actinomycin D and subjected to RT-PCR analysis. As shown in Fig. 2C, treatment with chrysin did not cause a significant change in the half-life of COX-2 mRNA.

3.2. *Effect of chrysin on phosphorylation state of MAPKs and tyrosine kinase activated by LPS*

To investigate whether the ERK, JNK, and p38 MAPK pathways are involved in LPS stimulation of Raw 264.7 cells, we examined whether selective MAPK inhibitors could affect LPS-stimulated COX-2 expression. SP600125 (a JNK inhibitor) profoundly inhibited LPS-mediated COX-2 expression, however, treatment with PD98059 (a MEK inhibitor) and SB203580 (a p38 MAP kinase inhibitor) slightly decreased COX-2 expression (Fig. 3A). To investigate whether the inhibition of COX-2 expression by chrysin is mediated through the modulation of the MAPK pathways, we examined the activation of the three MAPKs by detecting their dually phosphorylated forms in Western blots probed with specific anti-phosphokinase antibodies (Fig. 3B). LPS treatment induced a strong transient increase in phosphorylated MAPKs levels, which peaked at 30 min and declined thereafter. Overall, LPS treatment of macrophages stimulated these three MAPKs with similar kinetics. The phosphorylation of MAPKs was not suppressed by cotreatment with LPS and chrysin (Fig. 3B).

Genistein and tyrphostin A23, inhibitors of tyrosine kinase, were used to determine whether LPS-induced tyrosine phosphorylation participated in the signaling pathways for the COX-2 expression. Pretreatment of genistein and tyrphostin A23 inhibited COX-2 expression by Raw264.7 cells following stimulation with LPS (Fig. 3C). To investigate whether the tyrosine phosphorylation is involved in the chrysin-mediated inhibition of LPS-induced COX-2 expression,

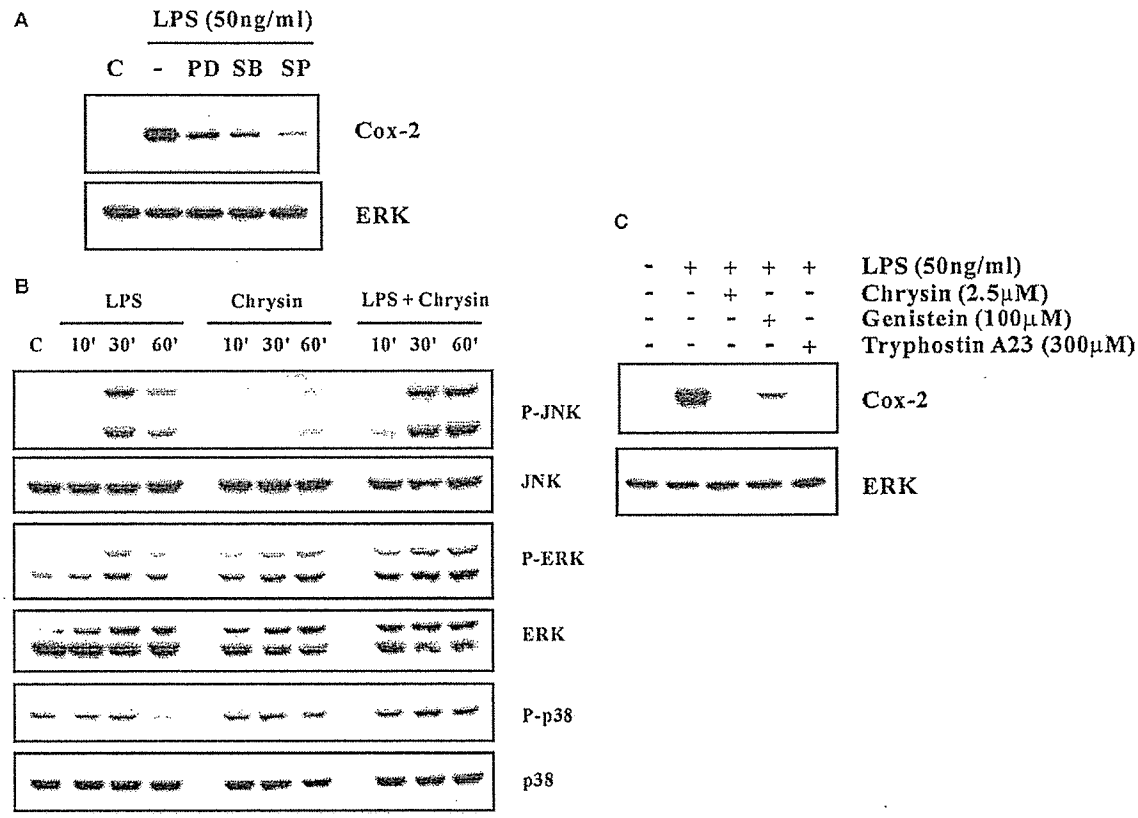

Fig. 3. Effect of chrysin on LPS-induced phosphorylation of MAPKs and tyrosine kinase in Raw 264.7 cells. (A) Raw 264.7 cells were pretreated with PD98059 (50 μM), SB203580 (10 μM) and SP600125 (20 μM) for 30 min followed by stimulation with LPS (50 ng/ml) for 12 h. COX-2 expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading. (B) Raw 264.7 cells were treated with vehicle, LPS (50 ng/ml), chrysin (2.5 μM) and LPS plus chrysin for indicated times. Equal amounts of lysates (40 μg) were subjected to electrophoresis and analyzed by immunoblotting using phosphorylation state-specific antibodies. To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPK. The results presented are representative of three independent experiments. (C) Raw 264.7 cells were pretreated with genistein (100 μM), chrysin (2.5 μM) and tyrphostin A23 (300 μM) for 30 min followed by stimulation with LPS (50 ng/ml) for 12 h. COX-2 expression was determined by Western blot analysis. The blot was stripped of the bound antibody and reprobed with anti-ERK antibody to confirm equal loading.

the effect of chrysin on phosphotyrosine accumulation in stimulated cells exposed to LPS was studied. Chrysin did not affect in LPS-induced phosphotyrosine accumulations (negative data not shown).

3.3. Chrysin inhibits COX-2 promoter activity

To investigate the mechanism underlying the chrysin-mediated inhibition of LPS-induced COX-2 expression, we used phPES2 (−327/+59), a plasmid that expresses firefly luciferase under the control of the human COX-2 gene promoter (−327/+59). The COX-2 promoter region (−327/+59) contains three *cis*-acting elements, namely, an NF-κB binding site, an NF-IL6 (C/EBP) binding sites, and a CREB, all of which have been shown to be involved in the regulation of COX-2 gene transcription. To identify which *cis*-acting elements play a critical role in LPS-mediated COX-2 gene promoter (−327/+59) activity, mutants of the three *cis*-acting element were made and tested in transfection assay (Fig. 4A). A mutation in CREB or NF-IL6 significantly reduced the basal and LPS-induced promoter activities. Mutation at the NF-κB site also inhibits basal and LPS-induced promoter activities, but it show less inhibitory effect than that of CREB or NF-IL6. To examine the effect of chrysin on LPS-induced COX-2 promoter activities, a reporter assay was performed using phPES2 (−327/+57), phPES2 (CRM; mutant for CREB), phPES2 (ILM; mutant for NF-IL6) and phPES2 (KBM; mutant for NF-κB). As shown in Fig. 4B, chrysin inhibits twofold in LPS-induced COX-2 promoter activities in the phPES2 (−327/+57), phPES2 (CRM) and phPES2 (KBM) constructs transfected cells. However, chrysin-mediated COX-2 promoter inhibition measured by LPS-driven luciferase activity is significant less in the phPES2 (ILM) construct than in the constructs with the wild type, CREB-mutated promoter and NF-κB-mutated promoter. These data suggest that NF-IL6 is mainly involved in the chrysin-mediated inhibition of LPS-induced COX-2 promoter activity.

3.4. Inhibition of NF-IL6 binding activity by chrysin in LPS-induced Raw 264.7 cells

It is well known that NF-IL6 is an important transcription factor for the expression of COX-2 gene by LPS [21,22]. To determine whether these transcription factors are an important target for chrysin in Raw 264.7 cells, we performed an EMSA. Treatment of Raw 264.7 cells with 50 ng/ml LPS caused a significant increase in the DNA-binding activity of NF-IL6 within 4 h. To confirm that increasing bands were indeed NF-IL6 specific DNA–protein complexes, we tested binding of wild-type oligonucleotides against that of a mutant oligonucleotide lacking the NF-IL6 site. The wild-type competitor inhibited LPS-induced NF-IL6 binding activity,

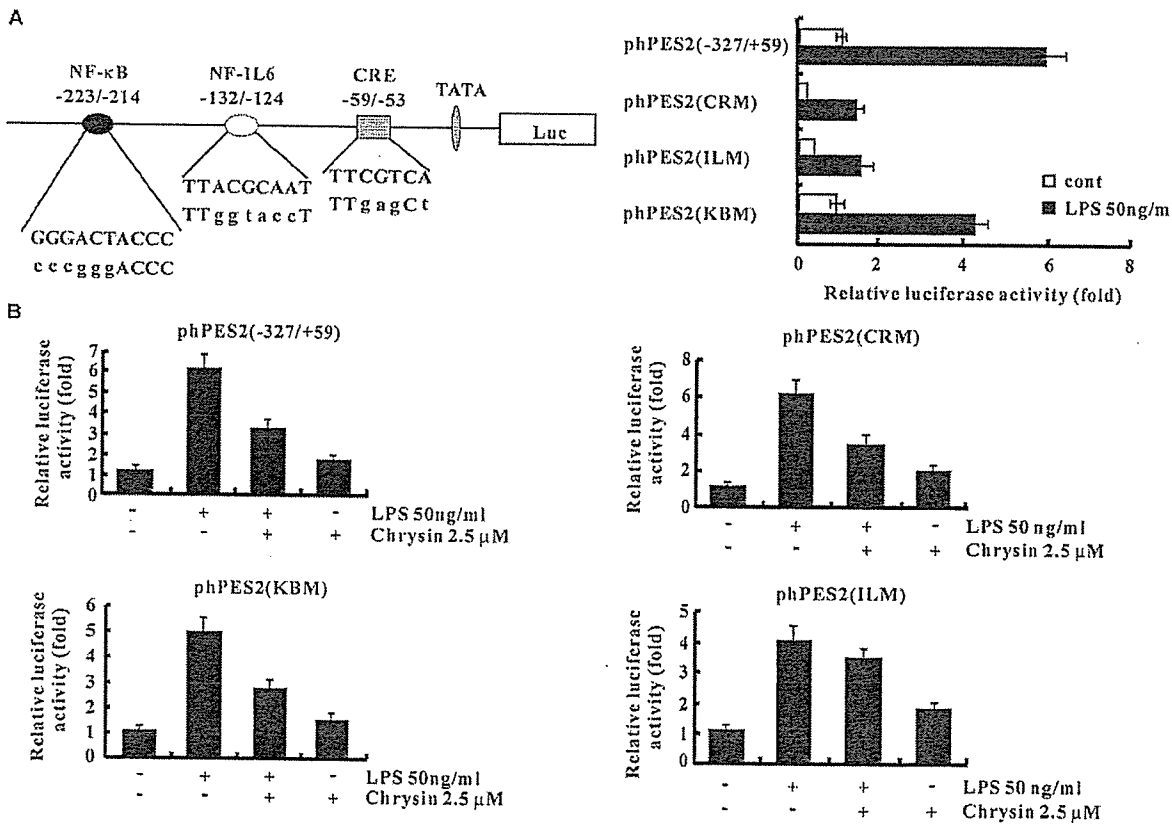

Fig. 4. Site-specific mutation of the COX-2 promoter region in response to chrysin. (A) The COX-2 gene promoter (−327/+59) was mutated at each putative transcriptional regulatory element. Lowercase letters in the lower sequence of each promoter indicate mutated bases, and the upper sequence shows wild-type bases. Wild type or mutant COX-2 promoters were transfected and treated with or without LPS (50 ng/ml) for 24 h and luciferase activity measured. Data represent the means ± S.D. of at least three independent experiments. (B) Wild type or mutant COX-2 promoters were transfected, and treated with or without chrysin in the presence of LPS (50 ng/ml). Data represent the means ± S.D. of at least three independent experiments.

whereas the mutant type competitor had no effect on the binding. The identity of the NF-IL6 protein in this complex was further confirmed by a super-shift assay using an antibody specific to NF-IL6 (Fig. 5A). LPS-induced NF-IL6 binding was markedly decreased at 2.5 μM chrysin treatment (Fig. 5A). The Western blot data suggest that NF-IL6 activity is not regulated NF-IL6 expression levels, because NF-IL6 protein expression of NF-IL6 remain unchanged regardless of whether the Raw 264.7 cells were treated with LPS and chrysin or not (Fig. 5B).

To determine whether chrysin inhibits activation of CREB and NF-κB through the inhibition of DNA binding of CREB and NF-κB, we examined the effect of chrysin on LPS-induced binding of CREB and NF-κB by EMSA. Chrysin did not affect the intensity of the CREB DNA complex induced by LPS or its migration in Raw 264.7 cells (negative data not shown). In the presence of chrysin, LPS-induced NF-κB binding was markedly increased in a dose-dependent manner (Fig. 6A). To determine the effect of chrysin on LPS-stimulated NF-κB-dependent reporter gene expression, we used a pNFκB-Luc plasmid, which was generated by inserting four spaced NF-κB binding sites into the pLuc-Promoter vector. Raw 264.7 cells were transiently transfected with the pNFκB-Luc plasmid and then stimulated with 50 ng/ml LPS either in the presence or absence of chrysin. Chrysin treatment significantly increased the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (Fig. 6B). These results suggest that chrysin-mediated inhibition of LPS-induced COX-2 expression is associated with inhibition of NF-IL6 DNA-binding activity, but not in CREB and NF-κB DNA-binding activity.

4. Discussion

Chrysin is known to have anti-inflammatory and anti-cancer effects. Here, we demonstrate that chrysin inhibits COX-2 gene expression in LPS-stimulated cultured macrophages, and that this effect is mediated through the inhibition of NF-IL6 DNA-binding activity.

The regulation of COX-2 gene transcription is complex and varies according to the cell type and the stimulus applied. Studies have shown that CREB, NF-κB, and NF-IL6 (C/EBP) were commonly or individually involved in the regulation of the COX-2 gene [21–26]. NF-κB exerts as a positive regulator of several gene expressions involved in chronic inflammatory diseases [27]. NF-κB signaling has been implicated variously in the expression of COX-2 stimulated by several factors including LPS [28–30]. Our findings indicate that NF-κB binding activity and NF-κB luciferase activity are increased by treatment with chrysin (Fig. 6A and B). This renders NF-κB as an unlikely target for the pharmacological actions of chrysin. In addition, the mitogen-activated protein kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses [31]. Studies using pharmacological inhibitors of MAPKs or their upstream protein kinase activators and dominant-negative mutant forms of protein kinases demonstrated the role of ERK, JNK, and p38 MAPK in transcriptional activation of COX-2 [32–35]. Moreover, MAP kinases are involved in the signaling pathway for LPS-induced COX-2 expression [22,36]. Hwang et al. [37] reported that blockade of ERK and p38 MAPK activities by PD98059 and SB203580, respectively, resulted in partial suppression of LPS-induced expression of COX-2 in Raw 264.7

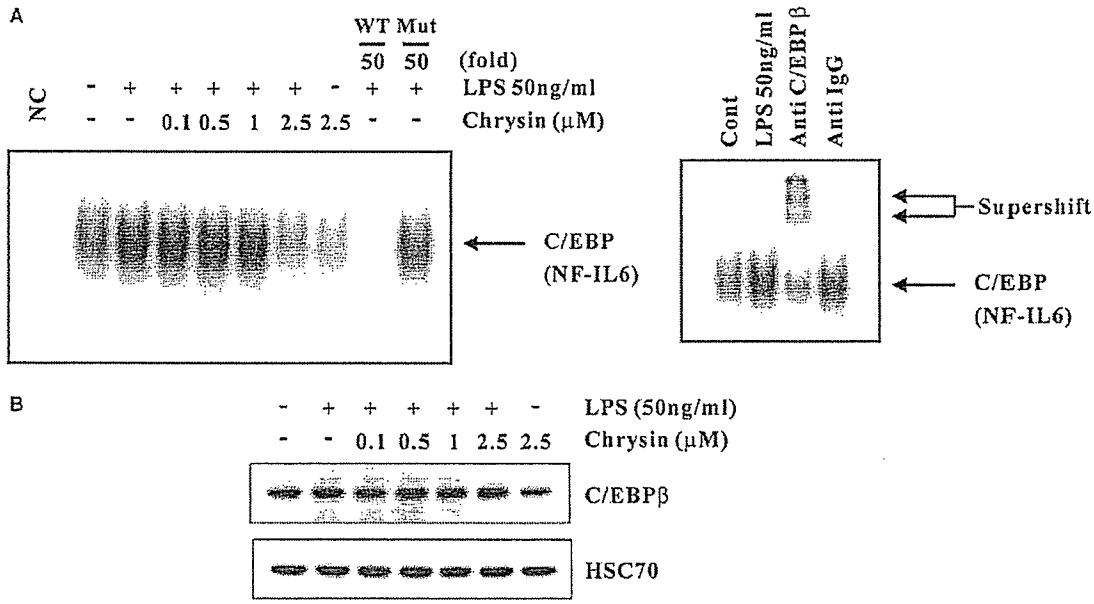

Fig. 5. Effect of chrysin on NF-IL6 DNA-binding activity in LPS-stimulated cells. (A) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. EMSA analysis of the nuclear extracts was conducted using a [^{32}P]-labeled NF-IL6 oligonucleotide probe. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the NF-IL6 binding sequence (50-fold in excess) to compete with the labeled oligonucleotide. To identify whether the bound proteins contain NF-IL6, super-shift analysis was performed using an antibody specific to NF-IL6. Super-shift with a rabbit IgG was used as a negative control. (B) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. Expression levels of NF-IL6 were determined by Western blot analysis.

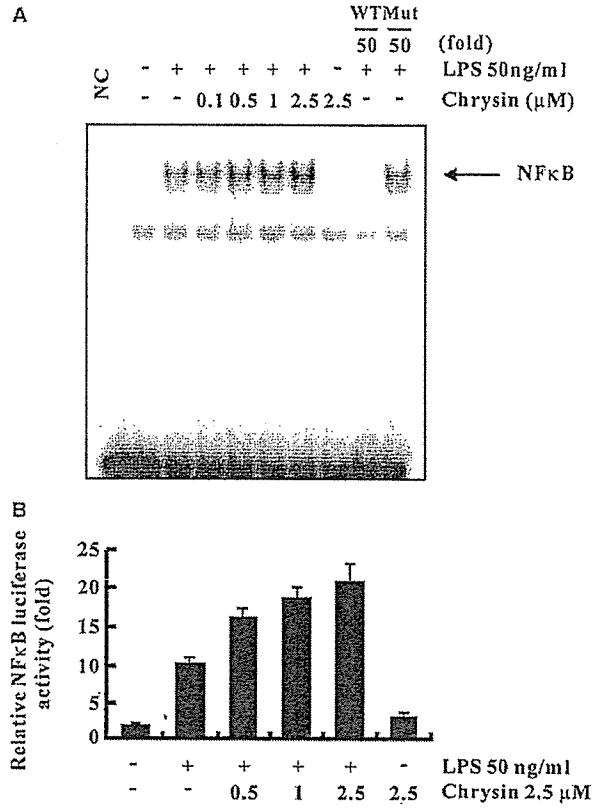

Fig. 6. Effect of chrysin on NF-κB DNA-binding activity and NF-κB promoter activity. (A) Raw 264.7 cells were pretreated with the indicated concentrations of chrysin for 30 min before incubation with LPS (50 ng/ml) for 4 h. EMSA analysis of the nuclear extracts was conducted using a [^{32}P]-labeled NF-κB oligonucleotide probe. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the NF-κB binding sequence (25-fold in excess) to compete with the labeled oligonucleotide. (B) Cells were transiently transfected with a pNFκB-Luc plasmid containing four copies of the NF-κB binding site, treated with the indicated concentrations of chrysin and LPS for 24 h. The cells were lysed and luciferase activity was measured. Data represent the means ± S.D. of at least three independent experiments.

cells. To determine the specific signal transduction pathway that is involved in the chrysin-mediated inhibition of LPS-induced COX-2 expression, we examined the effects of chrysin on the activation of MAPKs induced by LPS, finding that LPS-induced MAPKs activations are not inhibited by chrysin. Therefore, the effect of chrysin on inhibition of LPS-induced COX-2 expression is probably not associated with MAP kinase activation.

NF-IL6, a member of the C/EBP family of transcription factors, is involved in inducing several acute-phase protein genes in response to immune and inflammatory stimulation. NF-IL6 also plays a major role in inducing the expression of COX-2 by cytokines (TNF-α and IL-1 β) and endotoxin [23,24,32]. Recently, Gorgoni et al. [38], reported that COX-2 induction by LPS was profoundly defective in C/EBP$^{-/-}$ macrophages, essentially due to impaired transcriptional induction via C/EBP promoter element. Here, we demonstrate that chrysin inhibits COX-2 gene expression in LPS-stimulated cultured macrophages, and we propose that the actions of chrysin are mediated by inhibition of NF-IL6 binding and transactivation. Several pieces of evidence support this proposal: (1) mutation of the NF-IL6 site inhibits the stimulatory effect of LPS, and the inhibitory effect of chrysin; (2) chrysin inhibited the DNA-binding activity of NF-IL6; and (3) chrysin had not inhibit in LPS-induced NF-κB binding and transactivation.

In summary, chrysin inhibits LPS-induced COX-2 expression in macrophages. These effects are mediated, at least in part, by inhibition of NF-IL6 activation. The fact that NF-IL6 is negatively regulated by chrysin is important because this transcription factor plays a critical role in the regulation of a variety of genes involved in inflammatory responses. In view of the facts that COX-2 plays an important role in mediating inflammatory responses and that chrysin may be an important determinant of clinical response in inflammatory diseases, further efforts to explore this therapeutic strategy appear warranted.

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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• *GASTRIC CANCER* •

Helicobacter pylori promote gastric cancer cells invasion through a NF-κB and COX-2-mediated pathway

Chun-Ying Wu, Chau-Jong Wang, Chi-Chuan Tseng, Hsiao-Ping Chen, Ming-Shing Wu, Jaw-Town Lin, Hiroyasu Inoue, Gran-Hum Chen

Chun-Ying Wu, Jaw-Town Lin, Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taiwan, China
Chun-Ying Wu, Hsiao-Ping Chen, Gran-Hum Chen, Division of Gastroenterology, Taichung Veterans General Hospital, Taiwan, China
Chun-Ying Wu, College of Public Health, China Medical University, Taiwan, China
Chau-Jong Wang, Hsiao-Ping Chen, Institute of Biochemistry, College of Medicine, Chung-Shan Medical University, Taiwan, China
Chi-Chuan Tseng, Section of Gastroenterology, Boston University, School of Medicine, Boston, USA
Ming-Shing Wu, Jaw-Town Lin, Division of Gastroenterology, Department of Internal Medicine, National Taiwan University Hospital, Taiwan, China
Hiroyasu Inoue, Department of Pharmacology, National Cardiovascular Center Research Institute, Japan
Supported by the Taichung Veterans General Hospital Research Grant: TCVGH-933308C
Co-first-author: Chun-Ying Wu
Co-correspondent: Chi-Chuan Tseng
Correspondence to: Dr. Gran-Hum Chen, Division of Gastroenterology, Taichung Veterans General Hospital, 160, Section 3, Taichung-Kang Rd, Taichung, 407, Taiwan, China. chun@vghtc.gov.tw
Telephone: +886-2359-2525-3306 Fax: +886-2374-1331
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Abstract

AIM: To examine the effects of *Helicobacter pylori* (*H pylori*) infection on the invasiveness of gastric cancer cells, and to elucidate its mechanism.

METHODS: Gastric carcinoma cells, MKN-45, were incubated with CagA-positive *H pylori*, and cell invasion was determined by Matrigel analysis. The expression of matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) were assessed by Western-blot analysis, and transcriptional activation of the COX-2 promoter was examined by measuring luciferase and β-galactosidase activities. Lastly, the protein-DNA interaction was confirmed by an electrophoretic mobility shift assay.

RESULTS: The current studies showed that: (1) incubation of CagA-positive *H pylori* with MKN-45 cells significantly promotes gastric cancer cells invasion, and this effect is attenuated by pre-treatment with NS-398, a COX-2 inhibitor, or PDTC, a nuclear factor κB (NF-κB) inhibitor; (2) the induction of MKN-45 cells invasion by *H pylori* is associated with increases in COX-2, MMP-9, and VEGF protein expression, and co-incubation of NS-398 or PDTC significantly reduces these effects; (3) *H pylori* infection transactivates COX-2 promoter activity and increases the binding of NF-κB to this promoter.

CONCLUSION: Our data demonstrate that *H pylori* infection promotes gastric epithelial cells invasion by activating MMP-9 and VEGF expression. These effects appear to be mediated through a NF-κB and COX-2 mediated pathway, as COX-2 or NF-κB inhibitor significantly attenuate the invasiveness of gastric cancer cells and the expressions of MMP-9 and VEGF protein.

Key words: *H pylori*; Gastric cancer; Invasion; MMP-9; VEGF; COX-2; NF-κB

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a spiral, microaerophilic, neutralophilic gram-negative bacterium that colonizes the gastric mucosa in 25-50% and 70-90% of the population in the developed and developing countries, respectively[1]. *H pylori* is believed to be the major contributing factor to the development of chronic gastritis and peptic ulcer diseases in human, and epidemiological and interventional studies in human as well as in experimental animals strongly suggest that *H pylori* infection increases the risk of adenocarcinoma in the distal stomach[2,3]. Although *H pylori* has been demonstrated to be associated with gastric cancer occurrence, whether *H pylori* promote gastric cancer cells invasion is still unknown.

Upon bacterial infection, host effectors induced by *H pylori* are likely to contribute to gastric carcinogenesis and tumor invasion. Matrix metalloproteinases (MMPs), a family of closely related enzymes that degrade extracellular matrix (ECM), are considered to be important factors in facilitating tumor invasion and spread[4]. MMPs displays broad and overlapping substrate specificity and collectively and they are capable of degrading the major components of ECM. Furthermore, MMPs are found to play major roles in connective tissue remodeling during pathologic conditions, such as cancer and inflammatory disease. Among these MMPs,

matrix metalloproteinase-9 (MMP-9) has been considered to be an important factor in facilitating lymphatic invasion and metastases in early gastric carcinoma[5], and the level of tissue MMP-9 has been shown to be related to the overall survival of patients with gastric carcinoma[6]. Recently, MMP-9 has been reported to be induced by *H pylori* through activation of nuclear factor κB (NF-κB)[7]. Whether *H pylori* can promote gastric cancer cell invasion through MMP-9 is unknown.

Vascular endothelial growth factor (VEGF), the most well-characterized angiogenic factor, is known to play a major role in the multistep process leading to the reconstruction of normal mucosa architecture. This process is believed to be mediated through angiogenesis, ensuring an adequate supply of nutrients to the healing tissue[8]. Moreover, VEGF also plays a vital role in tumor-associated microvascular invasion[9]. In human gastric cancers, VEGF has been found to be over-expressed[10,11], and in a recent study, VEGF expression has been reported to be upregulated by *H pylori* through a cyclooxygenase-2 (COX-2) dependent mechanism [12]. Whether VEGF contributes to gastric cancer invasion induced by *H pylori* infection remains unknown.

Cyclooxygenase (COX), the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandin H_2, is the main target of non-steroid anti-inflammatory drugs (NSAIDs). Two isoforms of this enzyme have been identified: COX-1 is constitutively expressed in most tissues and is involved in the production of prostaglandins to maintain normal physiological functions; and COX-2 is involved in inflammation and has been shown to be induced by mitogens, cytokines, hormones, and growth factors. Several recent studies suggested that COX-2 might be an important factor in carcinogenesis, and COX-2 inhibitors were shown to possess anticancer effects. These properties were mediated through the inhibition of prostaglandins production by COX-2, leading to decreases in angiogenic factors, and changes in MMP activity[13]. In human gastric cancer cells, NF-κB mediated COX-2 expression is associated with cell proliferation[14]. Furthermore, *H pylori* activates NF-κB expression in gastric cancer cells[15].

The present study was undertaken to examine the effect of *H pylori* infection on gastric cancer cells invasiveness and to elucidate its mechanism. Our results suggest that *H pylori* may induce the expression of MMP-9 and VEGF and promote gastric cell invasion through a NF-κB-and COX-2-mediated pathway.

MATERIALS AND METHODS

Cell line

Human gastric carcinoma cell line, MKN-45, was obtained from American Type Culture Collection (Manassas, VA, USA). MKN-45 cells were maintained in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. On the day of experiment, cells were refed with fresh medium and co-cultured with *H pylori*.

Bacterial strain

Cag pathogenicity island-positive *H pylori* (ATCC 43504) strain was used in experiments described in this study. Stock cultures were maintained at -70 ℃ in brucella broth supplemented with 30% glycerol. The bacteria were grown at 37 ℃ in 5% horse blood agar plates and in a microaerobic condition. Cultures were routinely screened for urease activity. For co-infection studies, *H pylori* were harvested between 48 and 72 h after inoculation of agar plates, resuspended in sterile phosphate buffered saline (PBS), and enumerated by absorbance at 600 nm (1 optical density (A) at 600 nm = 2.4×10^8 colony-forming units/mL). MKN-45 cells were seeded into 65 mm dishes, and *H pylori* at a multiplicity of infection 80 were added in the culture.

Cell migration assays

The effect of *H pylori* infection on cell migration was examined using the Matrigel Invasion chamber, as suggested by the manufacturer (BD Bioscience). The lower surface of the chamber contained a transwell filter (8-μm pores), coated with fibronectin, and vitronectin. MKN 45 cells (1×10^5) and *H pylori* were added to the upper chamber, in the presence or absence of NS-398, and incubated overnight in a humidified tissue culture incubator, at 37 ℃, 50 mL/L CO_2 atmosphere. The next day, a cotton tipped swab was inserted into chambers to remove non-invading cells by applying gentle but firm pressure while moving the tip around the membrane surface. The cells on the lower surface of insert chambers were stained with hematoxylin for 10 min, and the cell number was counted under a microscope (40 to 200× magnifications). The extent of cell invasion was expressed as fold increases of total number of cells on the lower surface of chambers in treated over untreated samples.

Western blot analysis

To obtain whole-cell extracts, cells were washed twice with ice-cold phosphate-buffer saline (PBS), and pelleted by centrifugation (200 r/min). Cell pellets were then lysed in a standard RIPA buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), containing protease inhibitors. Protein concentrations were determined by Bio-Rad assays. Protein samples were dissolved in the loading buffer (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 100 mmol/L dithiothreitol, and 0.01% bromophenol blue), heated to 100 ℃ for 3 min, and loaded onto the gel in an electrophoresis buffer containing 25 mmol/L Tris-HCl, pH 8.3, 250 mmol/L glycine, and 0.1% SDS. At the completion of electrophoresis, protein was transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Life Science). The membrane was incubated in the blocking buffer (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20), containing 5% nonfat powdered milk for 2 h. The membrane was immunoblotted with COX-2, MMP-9, or VEGF antiserum (obtained from Santa Cruz Biotech, Santa Cruz, CA, USA). After incubation with the secondary antibody, the membrane was visualized with Enhanced Chemiluminescence kit from Amersham.

Transfection and luciferase assay

To examine transcriptional regulation of COX-2 promoters by NF-κB, MKN-45 cells were transiently transfected with pMT2-LacZ and COX-2-Luc or phPES2 (KBM)-Luc DNAs, in the presence of NF-κB p65, p50, or control pMT2 plasmid (kindly supplied by Dr. Gail Sonenshein, Boston Medical

Center, Boston, MA, USA). The phPES2 (KBM) plasmid contains a full-length COX-2 promoter in which a putative NF-κB binding site is mutated, as described previously[16]. For luciferase assays, cells were washed twice with PBS and then lysed in 500 μL of lysis buffer following the manufacturer's instructions (Analytical Luminescence, San Diego, CA, USA). To assay luciferase activity, cell lysate (100 μL) was mixed with 100 μL of luciferase substrate solution A (Analytical Luminescence). Using a luminometer with automatic injection, 100 μL of luciferase solution B was added (Analytical Luminescence) and luciferase activity was detected as the light emission over a 30-s period.

The β-galactosidase activity in 40 μL of the cell lysate was determined after a 5-30-min incubation at 37 ℃ with 2 mmol/L chlorophenol red β-galactopyranoside (Boehringer Mannheim) in 2 mmol/L $MgCl_2$ 0.1 mmol/L $MnCl_2$ 45 mmol/L 2-mercaptoethanol, and 100 mmol/L $NaHPO_4$, pH 8.0. The reactions were terminated by adding 500 μL of 0.5 mol/L EDTA, pH 8.0, and the absorbance at 570 nm was measured using a spectrophotometer. With each experiment, the luciferase activity was determined in duplicate and normalized to the β-galactosidase activity for each dish.

Electrophoretic mobility shift assay

Nuclear extracts were prepared using Nuclear Extract kit (Active Motif, Carlsbad, CA, USA). A double-strand oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'), corresponding to the putative NF-κB binding domain on the COX-2 promoter was synthesized and was labeled with [γ-^{32}P] ATP (3 000 Ci/mmol at 10 mCi/mL) using a T4 polynucleotide kinase. Nuclear protein (1 μg) was incubated in a buffer containing 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25 mg/mL poly (dI-dC)·poly (dI-dC), for 10 min at room temperature, and ^{32}P-labeled NF-κB oligo was added to each reaction and incubated for additional 20 min. Samples were subjected to electrophoresis at room temperature on a 4% acrylamide gel at 25 mA using 0.5× TBE buffer. The gels were dried at 80 ℃ for 2 h and exposed to radiography film at -70 ℃.

Immunohistochemical studies

To further investigate the role of MMP-9 and VEGF in gastric cancer invasion, the expression of these two proteins were examined in *H pylori*-positive gastric cancer tissues. Tissue sections from gastric cancer were de-paraffinized, rehydrated, and immersed in 3% hydrogen peroxide–methanol solution for 10 min at room temperature to inhibit endogenous peroxidase activity. The sections were then incubated with unmasking solution (0.01 mol/L citrate buffer pH 6.0), heated for 10 min. The sections were allowed to cool down to room temperature and washed twice in PBS buffer for 5 min. The sections were pre-incubated with diluted normal serum for 10 min, and then incubated with 1:40 mouse anti-MMP-9 monoclonal antibody (Novocastra Lab. Ltd, Newcastle, UK), 1:100 rabbit anti-VEGF polyclonal antibody (Zymed Lab. Inc., South San Francisco, CA, USA), or 1:40 mouse anti-COX-2 monoclonal antibody (Cayman Chemical Co., Ann Arbor, MI, USA) for 1 h at room temperature. The sections were washed in PBS for 5 min twice, and incubated with appropriate biotinylated secondary antibody. After washing in PBS for 5 min twice, the slides were incubated with ABC reagents, followed with DAB or other suitable peroxidase substrates. The sections were counterstained with hematoxylin for 30 s, washed, dried, and mounted.

RESULTS

H pylori infection promotes gastric cancer cells invasion

In vivo tumor invasion includes not only migration process, but also adhesion, proliferation and angiogenesis, *etc.*, dissociation of *in vitro* assay for tumor cell invasion (migration) with *in vivo* tumor invasion might exist. However, similar migration assay to assess tumor cell invasion has been used by several articles published recently[17].

We used Matrigel invasion chamber to examine whether *H pylori* infection induces gastric cancer cells invasion and to determine the effect of COX-2 inhibitor on this process. MKN-45 cells were incubated with *H pylori* in the presence or absence of COX-2 inhibitor, NS-398 (100 ng/mL). As illustrated in Figure 1A co-infection with *H pylori* induced a 2.5 fold increase in MKN-45 cells migrated through Matrigel-coated filters, indicating that *H pylori* promoted gastric cancer cells invasion. The COX-2 inhibitor, NS398, significantly reduced cell invasion in *H pylori* stimulated, but not in untreated cells. This result suggested that *H pylori* induced gastric cancer cells invasion was, in part, mediated through a COX-2-dependent mechanism.

To examine the effect of NF-κB on the migration of MKN-45 cells, cells were incubated with *H pylori* in the presence or absence of NF-κB inhibitor, (pyrrolidine dithiocarbamate (PDTC), 0.1 μmol/L, purchased from Sigma Chem, St. Louis, MO, USA). As illustrated in Figure 1B the effect of *H pylori* infection on gastric cancer cells migration was completely abolished by NF-κB inhibitor, suggesting a potential involvement NF-κB in this process. The representative microscopic photos of stained cells treated with *H pylori* in the presence or absence of COX-2 inhibitor were shown in Figures 1C-E.

H pylori infection induces MMP-9 and VEGF expression

Several proteins, including MMP-9 and VEGF, have been reported to play an important role in tumor invasion. The effects of *H pylori* on the expression level of MMP-9 and VEGF were examined in MKN 45 cells after co-infection with *H pylori* for a different period of time. The induction of VEGF protein expression was noticeable within an hour after *H pylori* infection and reached the highest level in 24 h (Figure 2A). Although the increment of MMP-9 level after *H pylori* infection was smaller than that of VEGF, a significant increase was observed at 24 h (Figure 2A).

COX-2 and NF-κB inhibitors reduce MMP-9 and VEGF expressions

Recent studies showed that COX-2 inhibitor reduced the release of MMP and COX-2-induced MMP-9 expression[18,19]. Therefore, we examined whether the activation of MMP-9 and VEGF by *H pylori* infection was also dependent on COX-2 expression. The MKN-45 cells were co-cultured

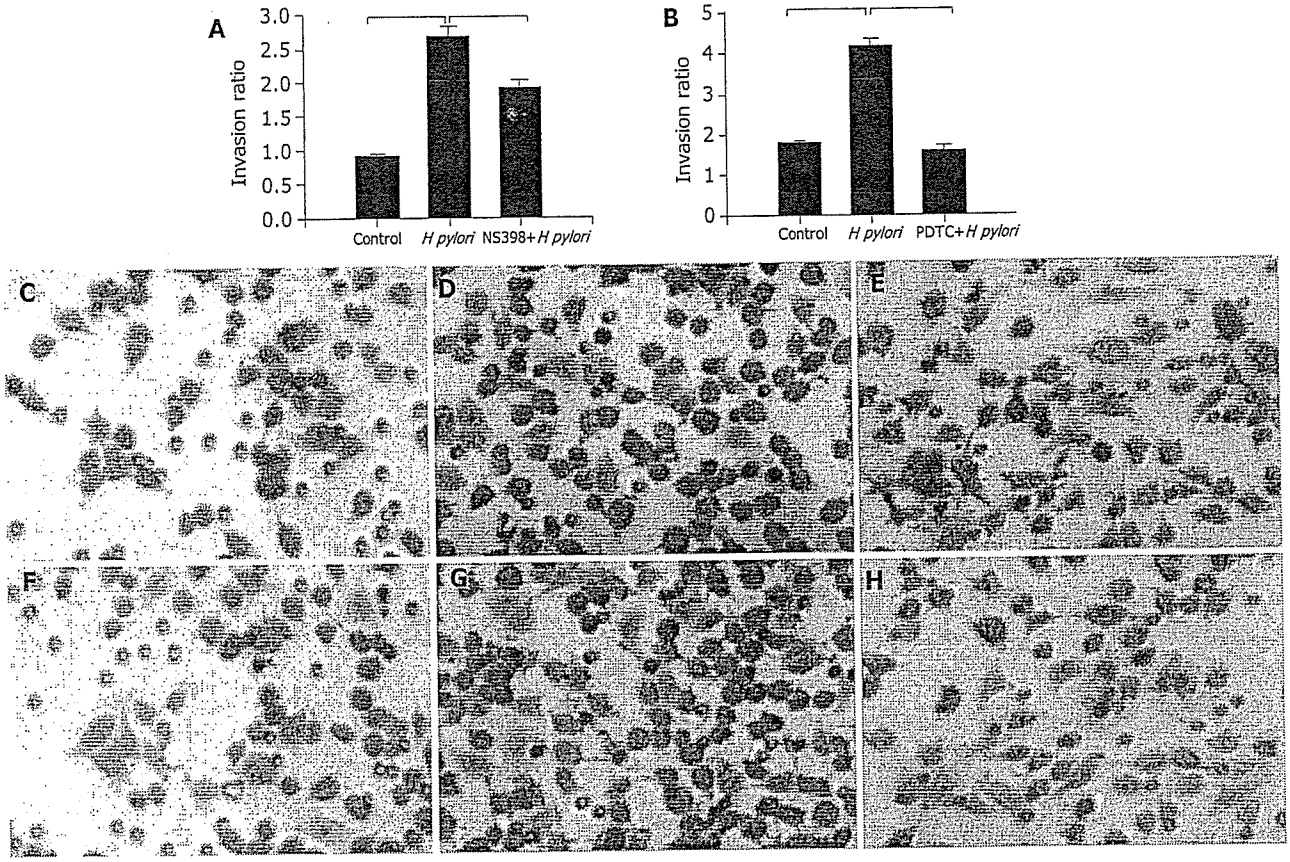

Figure 1 Effects of *H pylori* infection, a cox-2 (NS398), or a NF-κB inhibitor (PDTC) on gastric cancer cell invasion. **A:** MKN-45 cells were treated with *H pylori* in the presence or absence of NS398. Cells on the lower surface of insert chamber were stained with hematoxylin for 10 min and counted under microscope with 200× magnifications. Data are presented as mean±SD of three separate experiments ($P<0.05$); **B:** MKN-45 cells were treated with *H pylori* in the presence or absence of PDTC ($P<0.05$); microscopic photos of stained migration cells: **C:** control; **D:** with *H pylori*; **E:** with *H pylori* and NS-398; **F:** control; **G:** with *H pylori*; **H:** with *H pylori* and PDTC.

with *H pylori* for 24 h in the presence or absence of a COX-2 inhibitor, NS-398, and our results showed that co-culture with *H pylori* resulted in a time-dependent increase in COX-2 protein concentration in MKN-45 cells (Figure 2B). Moreover, NS398 significantly reduced the expression levels of COX-2, MMP-9, and VEGF, induced by *H pylori* in MKN-45 cells at 24 h (Figure 3A). These data suggest that the induction of MMP-9 and VEGF by *H pylori* is mediated through a COX-2-dependent mechanism.

To examine whether activations of MMP-9 and VEGF by *H pylori* infection were also dependent on NF-κB expression, MKN-45 cells were co-cultured with *H pylori* for 24 h in the presence or absence of a NF-κB inhibitor, PDTC. As demonstrated in Figure 3B, the effects of *H pylori* on COX-2, MMP-9, and VEGF expression were significantly reduced by PDTC. These results suggest that the induction of COX-2, MMP-9, and VEGF by *H pylori* is also NF-κB-dependent.

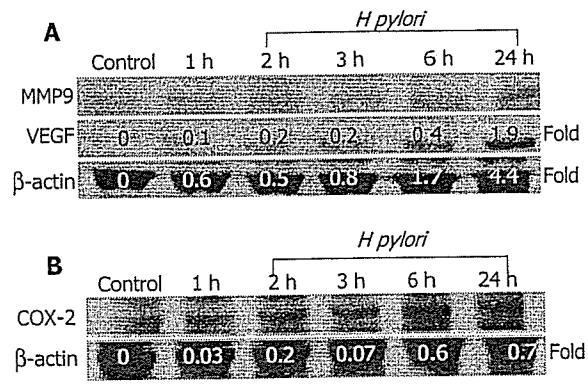

Figure 2 *H pylori* infection increases MMP-9, VEGF, and COX-2 expressions in gastric epithelial cell. MKN-45 gastric cancer cells were incubated with *H pylori* for 0-24 h and total cellular protein was extracted for Western blot analysis for the expression of **A:** MMP-9 and VEGF and **B:** COX-2 proteins. The blots were stripped and probed with β-actin to document equal protein loading. The experiment was performed for thrice with similar results.

Induction of MMP-9 and VEGF by H pylori infection depends on a NF-κB-mediated COX-2 activation

A previous study has shown that NF-κB regulated COX-2 expression and affected cell proliferation in human gastric cancer cells[14]. We hypothesize that the induction of MMP-9 and VEGF by *H pylori* is associated with NF-κB mediated COX-2 expression. To investigate this hypothesis, we first examined the effect of NF-κB on COX-2 promoter activities in MKN45 cells. As illustrated in Figure 4A co-transfection with NF-κB p65 or p50 plasmid DNA significantly enhanced COX-2 promoter activity, and the induction was completely

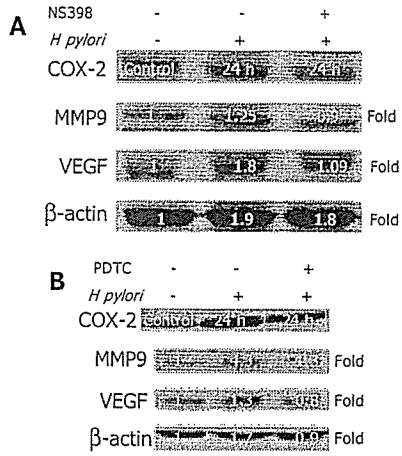

Figure 3 Effect of COX-2 or NF-κB inhibitor on COX-2, MMP-9, or VEGF protein level in MKN-45 cells. **A:** Cells were cultured in the presence or absence of *H pylori* and NS398 for 24 h, and cellular protein was extracted and subjected to Western blot analysis; **B:** MKN-45 cells were treated with or without PDTC and in the presence or absence of *H pylori* for 24 h. The experiments were repeated on at least three occasions, and the results were identical to these presented here. All blots were stripped and probed with β-actin to check for equal protein loading.

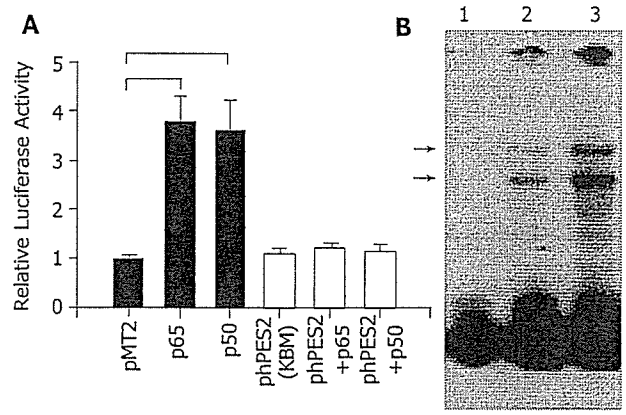

Figure 4 **A:** Transactivation of COX-2 promoter by NF-κB. MKN-45 cells were transiently transfected with full-length COX-2 promoter (Black bar), or mutated COX-2 construct, phPES2 (KBM), where the putative NF-κB binding domain was mutated (open bar), in the presence of p65, p50, or control vector (pMT2) plasmid. Luciferase and β-galactosidase activities were performed 48 h after transfection. ($n = 4$, $P<0.05$); **B:** Binding of nuclear NF-κB to COX-2 promoter DNA in MKN-45 cells. Nuclear protein was extracted from cells cultured in the absence (lane 2), or presence (lane 3) of *H pylori*. The protein-DNA interaction bands were shown as (→). Lane 1 showed free probe only.

abolished in phPES2 (KBM) construct where a putative NF-κB binding domain was mutated. Furthermore, the interaction between NF-κB and COX-2 promoter was also enhanced by *H pylori* infection in MKN-45 cells (Figure 4B).

High level of MMP-9 and VEGF expression in gastric cancer tissues with H pylori infection

To investigate whether these observations were also present *in vivo*, we randomly selected six gastric cancer patients (three cases with *H pylori* infection, confirmed by Giemsa stain and CLO test; three cases without *H pylori* infection) and examined the expression of COX-2, MMP-9, or VEGF protein in the surgical specimens. The immunostain of COX-2 (Figures 5A and B), MMP-9 (Figures 5C and D), or VEGF (Figures 5E and F) was located predominantly on the surface epithelial cells, and the intensity was more abundant in *H pylori*-positive than *H pylori*-negative tissue samples (Figure 5).

DISCUSSION

Gastric cancer is one of the most common malignancies in the world, especially in Eastern Asia. Although the incidence of gastric carcinoma is declining in Western countries, gastric

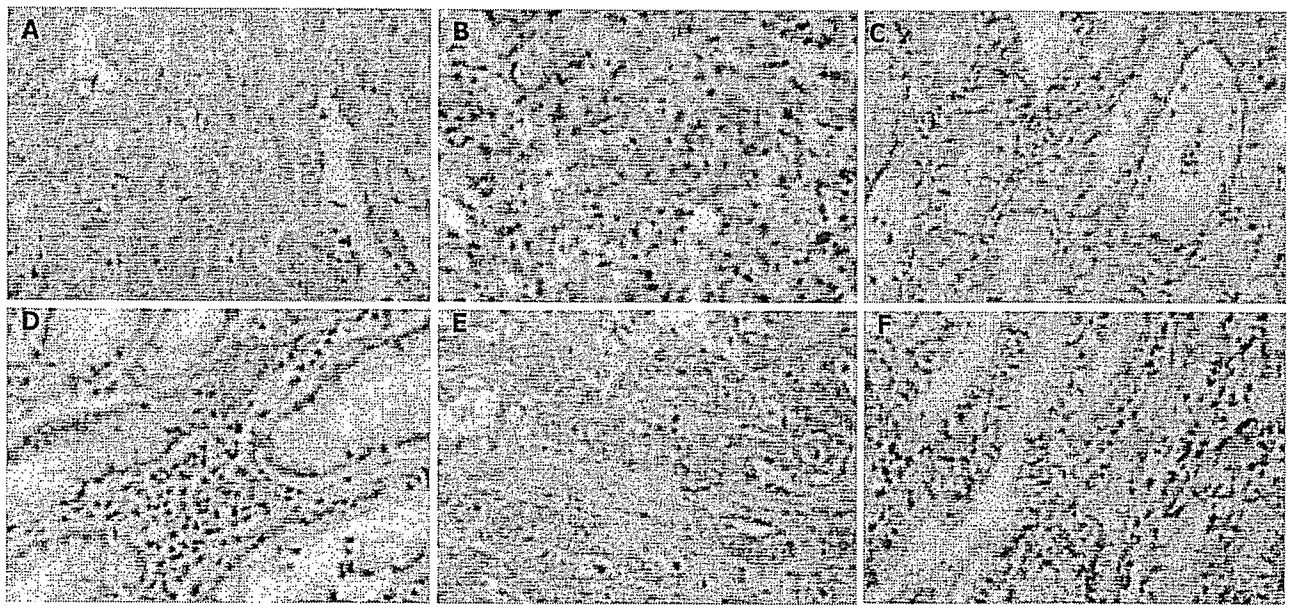

Figure 5 Immunohistochemical detection of COX-2, MMP-9, and VEGF in gastric cancer tissues. The serial sections of gastric cancer surgical specimens were stained with anti-COX-2 (**A** and **B**), anti-MMP-9 (**C** and **D**) , and anti-VEGF (**E** and **F**) antibodies. Sections were counterstained with hematoxylin. The immunoreactivities of COX-2, MMP-9 and VEGF are predominantly detected in the cytoplasm of the tumor cell. Tissues in A, C, and E are from *H pylori*-positive patients, whereas, sections in B, D, and F are from *H pylori*-negative individuals. (Magnification: **A-F**: 400×).

cancer remains the leading cause of cancer death worldwide. Current strategies to reduce mortality from this disease focus on early detection of gastric cancer or its precursor lesions by endoscopic screening. There is an increasing interest in the use of drugs to prevent the occurrence or the invasion of gastric cancers. Epidemiological studies have shown that NSAIDs decrease the risk of gastrointestinal carcinomas[20]. However, the mechanisms by which NSAIDs inhibit neoplastic growth are not fully elucidated[21,22].

The involvement of COX-2 in carcinogenesis has been shown in many epidemiological, animal and clinical studies. Individuals who took NSAIDs regularly had a markedly reduced risk of developing colon cancer[13], and COX-2 inhibitors have been proved to be effective in suppressing tumor progression both *in vitro* and *in vivo* in nude mice[21]. The anti-tumor effect of COX-2 inhibitors was attributed to their ability to induce apoptosis and inhibit tumor cell proliferation and angiogenesis. COX-2 inhibitors have also been illustrated to prevent tumor invasion in colon cancer, hepatocellular carcinoma[22], and lung cancer[23]. There is little evidence that COX-2 inhibitors may prevent gastric cancer invasion, although gastric cancers have been shown to over-express COX-2 protein[24].

In the present study, we found that *H pylori* infection promoted gastric cancer cells invasion and a COX-2 specific inhibitor significantly attenuated this process. Furthermore, the induction of gastric cancer cells invasion is associated with an increase in COX-2, MMP-9, or VEGF protein level, and these effects were also attenuated by a COX-2 inhibitor, suggesting a potential role of MMP-9 or VEGF in this process. In MKN-45 cells, *H pylori* infection enhanced nuclear NF-κB activity and transactivated COX-2 promoter. In addition, the induction of MMP-9 and VEGF by *H pylori* was suppressed by a NF-κB inhibitor. These data indicate that *H pylori*-induced MMP-9 and VEGF expressions in MKN-45 cells are mediated through the interaction of NF-κB on the COX-2 promoter. These results also support an important role of COX-2 in gastric cancer cells invasion.

Lim *et al*., have recently shown that inhibition of NF-κB results in inhibition of COX-2 expression and proliferation of gastric cancer cells[14]. These data suggest NF-κB may play an important role in gastric cancer proliferation via COX-2 expression. Recently, Callejas *et al*., reported that COX-2 expression promotes the release of MMP-9 in fetal rat hepatocytes[17], and Caputo *et al*., also revealed that *H pylori* induce VEGF expression in MKN-28 gastric epithelial cells through a COX-2 dependent mechanism[12]. Furthermore, Li *et al*., reported that COX-2 increased the angiogenic and metastatic potential of tumor cells by activation of VEGF in human transitional cell carcinoma cell line, and the effect on invasiveness could be reversed by COX-2 inhibitors[25-27].

Compared with previous studies, we have found several interesting points. In Figure 1 PDTC attenuated cell invasion completely, NS-398 only partially inhibited, suggesting that NF-κB could induce cell invasion not only through COX-2, but also through other pathways. One of the possibilities was through direct activation of VEGF expression. In Figure 3 we found that the inhibition of COX-2 by NS-398 attenuated MMP-9 expression to the control level, but it did not attenuate VEGF expression completely. On the other hand, the inhibition of NF-κB by PDTC inhibited both MMP-9 and VEGF expression to the control levels. These results suggest that MMP-9 expression is dependent on COX-2 pathway, while VEGF expression might be independent of COX-2 pathway. These observations might explain why NS-398 only partially attenuated cell invasion.

The activation of NF-κB by *H pylori* has been described by several groups[15,28,29]. Mori *et al*., reported that *H pylori* induced NF-κB activation and stimulated MMP-9 expression[7]. In the present study, we have observed that *H pylori* infection in gastric cancer cells induced MMP-9 protein level and the increase was attenuated by either a NF-κB inhibitor or a COX-2 inhibitor. These data suggested that the expression of MMP-9 in *H pylori*-infected cells is mediated by a direct activation NF-κB, or through a COX-2 mediated pathway. This conclusion is supported by a recent report showing that MMP-9 promoter contains several putative NF-κB binding sites, and its transcription requires the activation of NF-κB.

Infection with *H pylori* affects more than 50% of the world population; some patients exhibit a progression through chronic atrophic gastritis to cancer, others develop peptic ulcer, but most do not exhibit either disease[30]. It is believed that different pathogens, host and environmental factors may lead to variable outcomes. In this study, we suggest that the induction of MMP-9 and VEGF proteins by *H pylori* can be considered part of a host response to accelerate an oncogenic progression via disruption of epithelial organization or increased invasion. The identification of *H pylori*-specific signaling pathways leading to the gastric cancer cells invasion will add to our understanding of the mechanism of *H pylori*-associated gastric carcinogenesis and the potential use of therapeutic agents in preventing *H pylori*-associated gastric cancer.

In summary, we have demonstrated that *H pylori* promote gastric epithelial cells invasion by activating the expression of MMP-9 and VEGF, and these effects are attenuated by a COX-2 or a NF-κB inhibitor. Moreover, *H pylori* infection induces nuclear NF-κB binding activity to the COX-2 promoter, and the activation of COX-2 promoter is abolished when the NF-κB binding site is mutated. These data suggest that the promotion of gastric cancer cells invasion by *H pylori* infection appears to be mediated through a NF-κB and COX-2 mediated pathway. Therefore, we proposed a model of *H pylori*-induced gastric cancer cell invasion as shown in Figure 6.

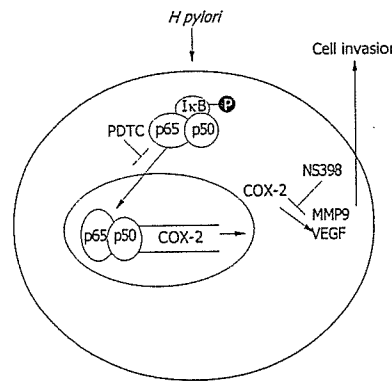

Figure 6 The schematic presentation of proposed mechanism of *H pylori* promote gastric epithelial cells invasion.

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PRECLINICAL RESEARCH

Beneficial Effect of Hydroxyfasudil, a Specific Rho-Kinase Inhibitor, on Ischemia/Reperfusion Injury in Canine Coronary Microcirculation In Vivo

Toyotaka Yada, MD, PHD,* Hiroaki Shimokawa, MD, PHD,† Osamu Hiramatsu, PHD,* Tatsuya Kajita, MD, PHD,* Fumiyuki Shigeto, MD, PHD,* Etsuro Tanaka, MD, PHD,‡ Yoshiro Shinozaki, BS,§ Hidezo Mori, MD, PHD,‖ Takahiko Kiyooka, MD,# Masashi Katsura, PHD,¶ Seitaro Ohkuma, MD, PHD,¶ Masami Goto, MD, PHD,* Yasuo Ogasawara, PHD,* Fumihiko Kajiya, MD, PHD#

Kurashiki, Fukuoka, Tokyo, Isehara, Suita, and Okayama, Japan

OBJECTIVES We examined whether hydroxyfasudil, a specific Rho-kinase inhibitor, exerts cardioprotective effect on coronary ischemia/reperfusion (I/R) injury and, if so, whether nitric oxide (NO) is involved.

BACKGROUND Recent studies have demonstrated that Rho-kinase is substantially involved in the pathogenesis of cardiovascular diseases; however, it remains to be examined whether it is also involved in ischemia/reperfusion (I/R) injury.

METHODS Canine subepicardial small arteries (SA, ≥100 μm) and arterioles (A, <100 μm) were observed by a charge-coupled device intravital microscope during I/R. Coronary vascular responses to endothelium-dependent (acetylcholine, intracoronary [IC]) and -independent (papaverine, IC) vasodilators were examined after I/R under the following four conditions: control (n = 7), NO synthase inhibitor alone (N^G-monomethl-L-arginine [L-NMMA], IC, n = 4), hydroxyfasudil alone (IC, n = 7), and hydroxyfasudil plus L-NMMA (n = 7).

RESULTS Hydroxyfasudil significantly attenuated serotonin (IC)-induced vasoconstriction of SA (−7 ± 1% vs. 2 ± 1%, $p < 0.01$). Coronary I/R significantly impaired coronary vasodilation to acetylcholine after I/R (SA, $p < 0.05$; and A, $p < 0.01$ vs. before I/R) and L-NMMA further reduced the vasodilation, whereas hydroxyfasudil completely preserved the responses. The vasoconstriction by L-NMMA after I/R was significantly improved by hydroxyfasudil in both-sized arteries (both $p < 0.01$). Expression of endothelial nitric oxide synthase (eNOS) protein in the ischemic endocardium of left anterior descending coronary artery area (as determined by Western blotting) significantly decreased (79 ± 4%) compared with the nonischemic endocardium of LCX area (100 ± 7%), which was improved by hydroxyfasudil (105 ± 6%, $p < 0.01$). Hydroxyfasudil significantly reduced myocardial infarct size, and hydroxyfasudil with L-NMMA also reduced the infarct size compared with L-NMMA alone.

CONCLUSIONS Hydroxyfasudil exerts cardioprotective effects on coronary I/R injury in vivo, in which NO-mediated mechanism may be involved through preservation of eNOS expression. (J Am Coll Cardiol 2005;45:599–607) © 2005 by the American College of Cardiology Foundation

Ischemia-reperfusion (I/R) injury attenuates endothelium-dependent dilation of large coronary arteries both in vitro (1,2) and in vivo (3,4). Endothelial dysfunction causes adverse outcome in the coronary circulation (5). Reperfusion injury is caused by direct myocardial injury through coronary vasospasm, free radicals, and inflammatory responses (6,7). Furthermore, local coronary vasoconstrictions in response to vasoconstrictors (e.g., serotonin) are enhanced (8,9). However, the mechanism of I/R-induced vascular injury remains to be clarified.

Recent studies have demonstrated that Rho-kinase, an effector of the small guanosine triphosphatase Rho, is substantially involved in the pathogenesis of cardiovascular diseases (10). Shimokawa et al. (10,11) have recently found that hydroxyfasudil is a potent and specific inhibitor of Rho-kinase and markedly inhibits coronary hyperconstriction and macrophage migration. They also demonstrated that intracoronary serotonin induces coronary hypercontractions at the inflammatory coronary lesions both in vitro and in vivo, in which up-regulated Rho-kinase is substantially

From the *Department of Medical Engineering and Systems Cardiology, Kawasaki Medical School, Kurashiki, Japan; †Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan; ‡Faculty of Applied Bioscience, Tokyo University of Agriculture, Tokyo, Japan; §Department of Physiology, Tokai University School of Medicine, Isehara, Japan; ‖Department of Cardiac Physiology, National Cardiovascular Center Research Institute, Suita, Japan; ¶Department of Pharmacology, Kawasaki Medical School, Kurashiki, Japan; and #Department of Cardiovascular Physiology, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan. This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, Culture, and Technology, Tokyo, Japan (Nos. 13307024, 13557068, 14657178, and 16300164); and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan.

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Abbreviations and Acronyms
I/R = ischemia-reperfusion
LAD = left anterior descending coronary artery
LCX = left circumflex artery
NO = nitric oxide

involved (12). Recent studies demonstrated that endothelial expression and activity of Rho-kinase are enhanced by hypoxia, with a resultant down-regulation of endothelial nitric oxide synthase (eNOS) expression and reduced nitric oxide (NO) production (13), and that Rho-kinase is also involved in a canine model of cerebral infarction associated with superoxide production and neutrophil infiltration (14).

It is conceivable that Rho-kinase is involved in the mechanisms of I/R injury associated with reduced endothelial NO production. In this study, we thus examined whether hydroxyfasudil exerts protective effect on coronary I/R injury in vivo and, if so, whether NO is involved.

METHODS

Animal preparation. This study conformed to the Guideline on Animal Experiments of Kawasaki Medical School and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Mongrel dogs (15 to 25 kg, n = 31) of either gender were anesthetized with morphine (3 mg/kg, intramuscular) and sodium pentobarbital (25 mg/kg, intravenous). After intubation, each animal was ventilated with a high-frequency jet ventilator (model VS600, IDC, Pittsburgh, Pennsylvania) with room air supplemented by 100% oxygen. Aortic pressure and left ventricular pressure were continuously monitored with an 8-F pigtail double manometer catheter (SPC-784A, Millar, Texas). The proximal portion of the left anterior descending coronary artery (LAD) was isolated and a transonic flow probe (T206, Transonic Systems, Ithaca, New York) was placed around the vessel.

Needle-probe intravital microscope. The needle-probe (4.5 mm in diameter, VMS 1210, Nihon Kohden, Tokyo, Japan) contains a gradient index lens (with a magnification of 200) surrounded by light guide fibers and a double lumen sheath. A doughnut-shaped balloon on the tip avoids direct compression of the vessels by the needle tip (15).

Measurements of coronary diameters. We placed the needle probe gently on subepicardial microvessels. When a clear vascular image was obtained, end-diastolic vascular images were taken with 30 pictures/s (15).

Measurements of regional myocardial blood flow. Regional myocardial blood flow was determined by the nonradioactive microsphere (Sekisui Plastic Co, Ltd, Tokyo, Japan) technique, as previously described in detail (16). Briefly, 1 ml of the microspheres suspension (2 to 4 × 10^6 spheres) was injected into the left atrium 85 min after the onset of coronary occlusion. Just before microsphere administration, a reference blood flow sample was drawn from the femoral artery at a constant rate of 8 ml/min for 2 min. The X-ray fluorescence of the stable heavy elements was measured by a wavelength-dispersive spectrometer (model PW 1480, Phillips Co., Ltd., Eindhoven, the Netherlands) (16). Myocardial blood flow was calculated according to the formula: time flow = tissue counts × (reference flow/reference counts) and was expressed in ml/g per minute (16).

Western blotting. Proteins were separated on sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis as previously described (17). The tissues were homogenized in a sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% glycerol). The tissue lysate was centrifuged and the supernatant collected. Protein concentration was quantified by a bicinchoninate (BCA) protein assay kit (Pierce Chemical, Rockford, Illinois). An aliquot of 10 μg of protein from each sample was electrophoresed on a 7.5% SDS-polyacrylamide gel. Proteins were subsequently transferred to polyvinylidine difluoride membrane (Immobilon-P membrane, Millipore, Bedford, Massachusetts) electrophoretically (100 V for 1 h) and membranes were incubated with antibody. The antibodies used in this study were rabbit anti-phosphorylated ezrin/radixin/moesin (ERM) family, total ERM. The antibody against phosphorylated ERM recognizes human moesin (phosphorylated at Thr558), which also binds to the phosphorylated ezrin (Thr567) and radixin (Thr564). Therefore, we used the extent of phosphorylation of ERM as a marker of Rho-kinase activity. The levels of Western blot for phosphorylated ERM were normalized to those for total ERM as a control. Membranes were then incubated with a horseradish peroxidase-conjugated horse anti-rabbit immunoglobulin G antibody (1:5,000). Immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL Western blotting detection kit; Amersham Pharmacia Biotechnology, United Kingdom).

The obtained samples were washed with ice-cold Tris-HCl buffer (pH 7.4), mixed with the sample buffer (4% sodium lauryl sulfate, 12% beta-mercaptoethanol, and 20% glycerol in 100 mM Tris-HCl [pH 6.8]), sonicated (1 min), boiled (3 min), and finally centrifuged (10,000 g, 60 min, 4°C). The resultant supernatant was stored at −80°C until use. The separation of proteins was carried out according to the previous study (18), with a minor modification. The relative intensity of immunoreactive bands was quantified by Image Master 1D Elite software (Amersham Biotech, Buckinghamshire, United Kingdom), and the data were estimated as percentage of each control.

Experimental protocols. After the surgical procedure and instrumentation, at least 30 min were allowed for stabilization while hemodynamic variables were monitored. The following protocols were examined.

1. We infused graded doses of hydroxyfasudil (10, 30, and 100 μg/kg, IC), and coronary vascular responses were

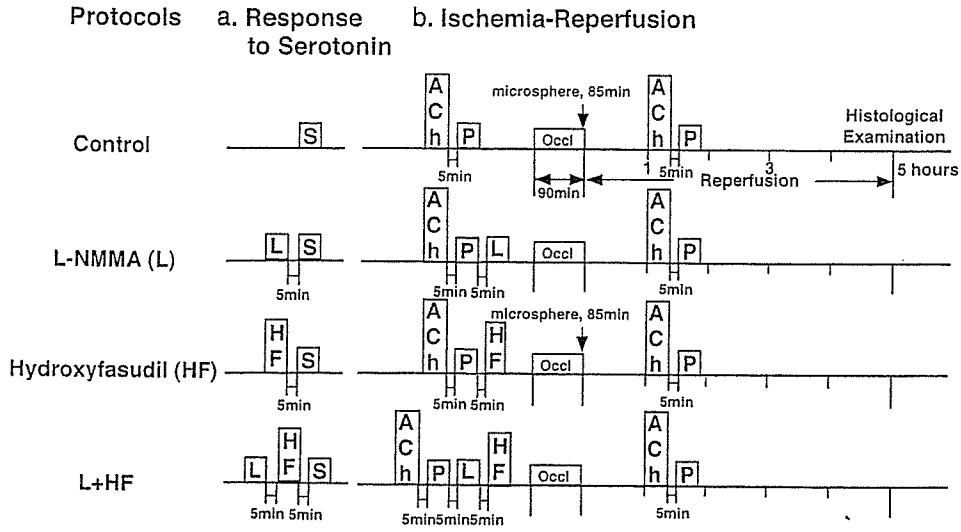

Figure 1. Experimental protocol. S = serotonin; L = L-NMMA; HF = hydroxyfasudil; Ach = acetylcholine; P = papaverine; Occl = coronary occlusion.

analyzed for 4 min by measuring end-diastolic vascular diameters and flows of the LAD.

2. The arteriolar vasoconstrictor response to serotonin before and after hydroxyfasudil (100 μg/kg, IC) was examined with or without inhibition of NO synthase (L-NMMA, 2 μmol/min for 20 min, IC) (Fig. 1). Hydroxyfasudil or L-NMMA was administered at 5 min before infusion of serotonin. The time interval between L-NMMA and hydroxyfasudil was also 5 min.
3. The arteriolar vasodilator responses to endothelium-dependent (acetylcholine, 1 μg/kg IC) and -independent (papaverine, 1 mg IC) vasodilators were examined before and after coronary I (90 min)/R (60 min) under the following four conditions separately in different animals: 1) control conditions, 2) L-NMMA alone, 3) hydroxyfasudil alone (100 μg/kg IC), and 4) hydroxyfasudil plus L-NMMA (Fig. 1). The time interval between each treatment was also 5 min. The basal coronary diameter is before administration of acetylcholine or papaverine either before or after I/R. Hydroxyfasudil and L-NMMA were administered at 5 min after administration of acetylcholine or papaverine. Microspheres were administered at 85 min after the onset of coronary occlusion.
4. After 5 h of reperfusion, LAD and the left circumflex artery (LCX) and myocardial tissue of LAD and LCX area were obtained for Western blotting. We reoccluded the LAD and injected Evans blue dye into a systemic vein. Then myocardial slices (5 mm) were incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma, Japan) solution to detect the infarct area. Infarct size was expressed as percentage of the infarct area that was contiguous with area at risk (19).

Drugs. We used the following drugs: hydroxyfasudil (Asahi Kasei Pharma, Tokyo, Japan), acetylcholine (Daiichi-Seiyaku, Tokyo, Japan), papaverine (Dainihon-Seiyaku, Tokyo, Japan), and N^G-methyl-L-arginine (L-

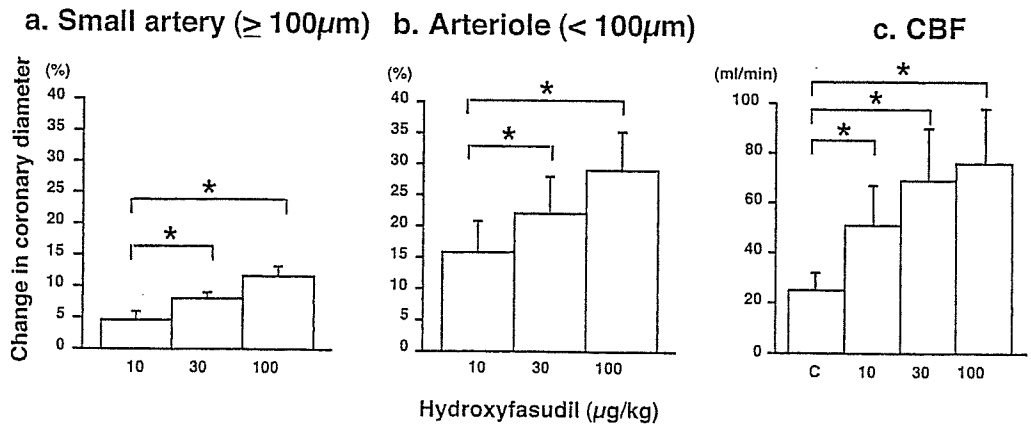

Figure 2. Coronary vasodilator effects of hydroxyfasudil in dogs in vivo. Hydroxyfasudil (10, 30, and 100 μg/kg, IC) caused coronary vasodilation, in a dose-dependent manner, under normal conditions in both small arteries (**a**) and arterioles (**b**). Number of vessels per animal used was 5/3 in small arteries and 7/4 in arterioles, respectively. Hydroxyfasudil also increased coronary blood flow (CBF) in a dose-dependent manner (**c**). *$p < 0.05$.

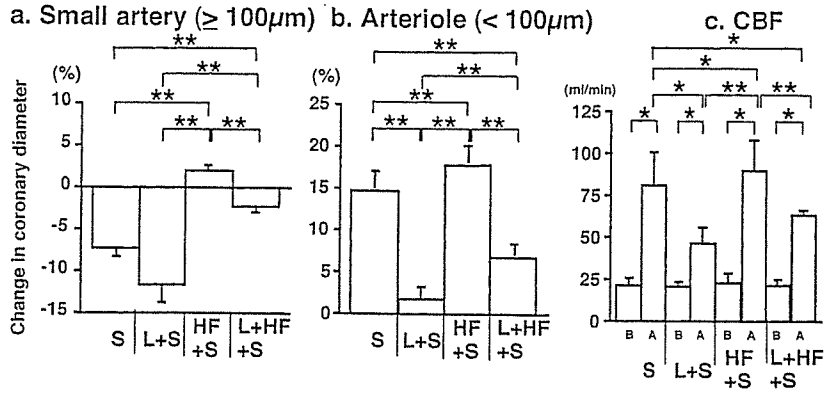

Figure 3. Effects of hydroxyfasudil on serotonin-induced coronary vascular responses in dogs in vivo. Hydroxyfasudil converted the serotonin-induced vasoconstriction of small arteries to vasodilation (a) and significantly enhanced the serotonin-induced vasodilation of arterioles (b). L-NMMA significantly attenuated the serotonin-induced vasodilation, which was counteracted by hydroxyfasudil. Number of vessels per animal used was 18/6 for S, L + S and HF + S, 13/4 for L + HF + S. *$p < 0.05$, **$p < 0.01$. S = serotonin; L = L-NMMA; HF = hydroxyfasudil; B = before drug; A = after drug.

NMMA, Sigma). All drugs were diluted in a physiologic saline immediately before use.

Statistical analysis. Results are expressed as means ± SEM. Vascular responses (Figs. 2a to 2c, 3c, 4c, 6c, 7a to 7c, 8a) were analyzed by one-way analysis of variance followed by Scheffe's post-hoc test for multiple comparisons. Difference in the effects of serotonin, acetylcholine, and papaverine on subepicardial microvessels before and after I/R (Figs. 3a, 3b, 4a, 4b, 5a to 5d, 6a, and 6b), and difference between infarct size/risk area and transmural collateral flow with or without hydroxyfasudil (Fig. 8b) were examined by a multiple regression analysis using a model in which the change in coronary diameter was set as a dependent variable (y) and vascular size as an explanatory variable (x) while the statuses of hydroxyfasudil and hydroxyfasudil plus L-NMMA were set as dummy variables (D_1, D_2) in the following equation; $y = a_0 + a_1x + a_2D_1 + a_3D_2$, where a_0 through a_3 are partial regression coefficients. The criterion for statistical significance was at $p < 0.05$.

RESULTS

Coronary vasodilator effects of hydroxyfasudil. Intracoronary administration of hydroxyfasudil caused a significant coronary vasodilation of both small arteries and arterioles (Figs. 2a and 2b, both $p < 0.05$, 10 μg/kg vs. 30 and 100 μg/kg) in a dose-dependent manner under control conditions with a resultant increase in CBF (Fig. 2c, $p < 0.05$, C vs. 10, 30 and 100 μg/kg). Intracoronary hydroxyfasudil did not significantly alter mean aortic pressure or heart rate (Table 1).

Hemodynamics and blood gases during I/R injury. In each experimental condition, mean aortic pressure and heart rate at baseline were constant and comparable (Table 1), and oxygen partial pressure (Po_2), carbon dioxide partial pressure (Pco_2), and pH were maintained within the physiologic ranges (pH 7.35 to 7.45, Pco_2 25 to 40 mm Hg, Po_2 >70 mm Hg) throughout the experiments. Hemodynamic

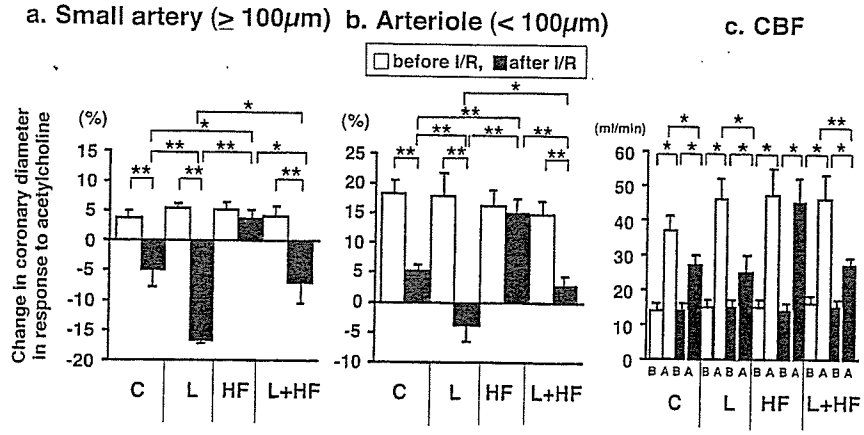

Figure 4. Endothelium-dependent coronary vasodilation before and after coronary ischemia/reperfusion (I/R) injury in dogs in vivo. Coronary I/R significantly impaired coronary vasodilation to acetylcholine under control conditions (C) and L-NMMA (L) further suppressed the vasodilation, whereas hydroxyfasudil (HF) completely preserved the responses. The vasoconstriction induced by L-NMMA after I/R was significantly improved by hydroxyfasudil in small arteries. Hydroxyfasudil also prevented the decrease in coronary blood flow (CBF) after I/R, which effect was attenuated by L-NMMA. Number of vessels per animals used was 7/6 for control (mean diameter 120 ± 7 μm), 5/4 for L-NMMA (123 ± 8 μm), 6/4 for hydroxyfasudil (118 ± 8 μm), and 5/4 for hydroxyfasudil plus L-NMMA (125 ± 9 μm) in small arteries, and 12/6 for control (70 ± 6 μm), 8/4 for L-NMMA (69 ± 7 μm), 8/5 for hydroxyfasudil (68 ± 7 μm), and 11/6 for hydroxyfasudil plus L-NMMA (71 ± 5 μm) in arterioles. I/R = ischemia/reperfusion; B = before acetylcholine; A = after acetylcholine. *$p < 0.05$; **$p < 0.01$.