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Curcumin induces expression of 15-hydroxyprostaglandin dehydrogenase in gastric mucosal cells and mouse stomach *in vivo*: AP-1 as a potential target

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Abstract

15-Hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the conversion of oncogenic prostaglandin E_2 to non-tumerigenic 15-keto prostaglandin E_2. In the present study, we found that curcumin, a yellow coloring agent present in the rhizome of *Curcuma longa* Linn (*Zingiberaceae*), induced expression of 15-PGDH at the both transcriptional and translational levels in normal rat gastric mucosal cells. By using deletion constructs of 15-PGDH promoter, we were able to demonstrate that activator protein-1 (AP-1) is the principal transcription factor responsible for regulating curcumin-induced 15-PGDH expression. Curcumin enhanced the expression of c-Jun and c-Fos that are functional subunits of AP-1, in the nuclear fraction of cells. Silencing of c-*Jun* suppressed curcumin-induced expression of 15-PGDH. Moreover, the chromatin immunoprecipitation assay revealed curcumin-induced binding of c-Jun to the AP-1 consensus sequence present in the 15-PGDH promoter. Curcumin increased phosphorylation of ERK1/2 and JNK, and pharmacologic inhibition of these kinases abrogated the curcumin-induced phosphorylation of c-Jun and 15-PGDH expression. In contrast, tetrahydrocurcumin which lacks the α,β-unsaturated carbonyl group failed to induce 15-PGDH expression, suggesting that the electrophilic carbonyl group of curcumin is essential for its induction of 15-PGDH expression. Curcumin restored the expression of 15-PGDH which is down-regulated by *Helicobacter pylori* through suppression of DNA methyltransferase 1. In addition, oral administration of curcumin increased the expression of 15-PGDH and its regulators such as p-ERK1/2, p-JNK, and c-Jun in the mouse stomach. Taken together, these findings suggest that curcumin-induced upregulation of 15-PGDH may contribute to chemopreventive effects of this phytochemical on inflammation-associated gastric carcinogenesis.

Keywords: Curcumin; 15-Hydroxyprostagladin dehydrogenase; AP-1; Anti-inflammation; Stomach

1. Introduction

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the arachidonic acid cascade, catalyzes the biosynthesis of prostaglandin E_2 (PGE_2) upon inflammatory insults [1,2]. Overproduction of PGE_2 stimulates proliferation of various cancer cells, confers resistance to apoptosis of cancerous cells, and accelerates metastasis and angiogenesis [3]. An intracellular level of PGE_2 is regulated not only by COX-2 but also by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). 15-PGDH oxidizes the 15-hydroxyl group of PGE_2 to generate 15-keto prostaglandin E_2 (15-keto PGE_2) [4].

A low level of 15-PGDH has been observed in various tumors, including those of colon, gastric, lung, bladder and breast [5–9]. One of the molecular mechanisms underlying down-regulation of 15-PGDH

Abbreviations: AP-1, Activator protein-1; COX-2, Cyclooxygenase-2; CDDO-Me, 2-cyano-3,12-dioxooleana-1,9(11)-dien-C28-Methyl ester; DCF-DA, Dichlorofluorescein diacetate; DNMT1, DNA methyltransferase 1; 15d-PGJ_2, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2; ERK, Extracellular signal-regulated kinases; HDAC, Histone deacetylase; *Helicobacter pylori*, *H. pylori*; JNK, c-Jun N-terminal kinase; 15-keto PGE_2, 15-keto prostaglandin E_2; MAP kinases, Mitogen-activated protein kinases; NAC, *N*-Acetyl-L-cysteine; NF-κB, Nuclear factor-κB; 15-PGDH, 15-Hydroxyprostaglandin dehydrogenase; PGE_2, Prostaglandin E_2; RGM, Rat gastric mucosal; ROS, Reactive oxygen species

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is attributed to hypermethylation of its promoter region [5,6]. Loss of 15-PGDH provoked increased susceptibility to tumor induction through aberrant accumulation of PGE_2 [8,10]. In contrast, overexpression of 15-PGDH in various cancer cells suppressed proliferation, invasion, metastasis, angiogenesis, and tumor-induced immune suppression while promoting apoptosis [11–14]. Furthermore, 15-PGDH also sensitizes the cancer cells to chemotherapeutic agents [15].

Some peroxisome proliferator-activated receptor γ (PPARγ) ligands, histone deacetylase (HDAC) inhibitors, and non-steroidal anti-inflammatory drugs have been reported to increase 15-PGDH expression/activity [16–18]. Therefore, 15-PGDH has been considered as a molecular target for cancer chemoprevention and therapy [19]. In our previous study, an endogenous PPARγ ligand, 15-deoxy $\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) induced the expression of 15-PGDH in breast [16] and colon [20] cancer cells. Treatment of MDA-MB-231 cells with 15d-PGJ_2 induced demethylation of CpG island of 15-PGDH promoter through suppression of DNA methyltransferase (DNMT), leading to reduced migration of these cells [16,21].

The synthetic chemopreventive triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-C28-methyl ester (CDDO-Me) upregulated the expression of 15-PGDH, which was associated with suppression of proinflammatory cytokine production and proliferation of colon epithelial cells [22]. Expression of 15-PGDH is also regulated by transforming growth factor-β [23]. Knock out of Smad3, a downstream target of transforming growth factor-β signaling, abolished the CDDO-Me-induced 15-PGDH expression [22]. In addition, elevated expression of HDAC correlated with down-regulation of 15-PGDH, which was attributable to interaction of Snail with the promoter of *15-PGDH* [17]. In contrast, HDAC inhibitors such as scriptaid, apicidin, sodium butyrate and oxamflatin induced the expression of 15-PGDH in lung adenocarcinoma by augmenting acetylation of histones H3 and H4 in the 15-PGDH promoter [23]. In addition, 15-PGDH is speculated to function as an antagonist of COX-2, and there is a reciprocal relationship between COX-2 and 15-PGDH [13]. Some natural compounds exert their chemopreventive effects through up-regulation of 15-PGDH, while down-regulating COX-2 expression [24].

Curcumin, a yellow coloring ingredient of turmeric (*Curcuma longa* L., Zingiberaceae), has anti-oxidative and anti-inflammatory properties, which contribute to majority of its health beneficial effects [25,26]. One of the well-defined molecular mechanisms by which curcumin exerts anti-inflammatory and anti-carcinogenic effects is suppression of intracellular signaling responsible for COX-2 expression [27]. Curcumin has been shown to inhibit the expression of COX-2 in various preclinical studies by blocking the activation of the transcription factor NF-κB [28–30]. By down-regulating COX-2 expression, curcumin also inhibited production of PGE_2 induced by lipopolysaccharide in Raw264.7 murine macrophages [31].

If curcumin exerts its anti-inflammatory effect by reducing the tissue or circulating level of PEG_2, this may be mediated by stimulating the catabolism of PGE_2 as well as reducing its biosynthesis. In this study, we investigated the effects of curcumin on 15-PGDH expression and underlying molecular mechanisms in cultured rat gastric mucosa (RGM)-1 cells and mouse stomach *in vivo*.

2. Materials and methods

2.1. Materials

Curcumin was purchased from the Sigma-Aldrich (St. Louis, MO, USA). Tetrahydrocurcumin prepared by the catalytic hydrogenation of curcumin was generously supplied by Prof. Jeewoo Lee, College of Pharmacy, Seoul National University. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin and Trizol® reagent were products of GIBCO BRL (Grand Island, NY, USA). U0126 and SP600125 were purchased from TOCRIS (Ellisville, MO, USA). Protease inhibitor cocktail was provided from Boehringer Mannheim (Mannheim, Germany). Bicinchoninic acid protein assay reagent was a product of Pierce Biotechnology (Rockford, IL, USA). Polyvinylidene difluoride membranes were supplied from Gelman Laboratory (Ann Arbor, MI, USA). The ECL chemiluminescent detection kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). The rat-specific c-Jun siRNA and Stealth™ RNAi negative control duplexes were provided by Invitrogen (Carlsbad, CA, USA). Dichlorofluorescein diacetate (DCF-DA) was obtained from Invitrogen (Carlsbad, CA, USA). *N*-Acetyl-L-cysteine (NAC) and carboxymethylcellulose (CMC) sodium salt were obtained from Sigma-Aldrich (St Louis, MO, USA). All other chemicals used were of analytical or the highest purity grade available. Curcumin was dissolved in dimethyl sulfoxide (DMSO) and was diluted further in culture medium.

2.2. Cell culture

The rat gastric mucosal RGM1 cells were kindly given by Prof. Ki Baik Hahm (CHA University Bundang Medical Center, Seongnam, Korea) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and cultured in DMEM containing 10% (v/v) fetal bovine serum and 100 U/ml penicillin. The cells were treated with vehicle (DMSO) or curcumin.

2.3. Antibodies

Antibodies to the following proteins were used: β-actin, ERK (Extracellular signal- regulated kinase)-1/2 (sc-135,900), phospho-ERK (sc-101,760) and lamin B (SC-374015) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phospho-c-Jun (#2361), c-Jun (#9165), c-Fos (#2250), p38 Mitogen-activated protein kinase (MAPK) (#9212), phospho-p38 MAPK (#9215), SAPK/JNK (c-Jun N-terminal kinase) (#9252), phospho-SAPK/JNK (#4668) from Cell Signaling (Denver, MA, USA); 15-PGDH (160615) from Cayman (Ann Arbor, MI, USA).

2.4. Western blot analysis

RGM-1 cells (1.5×10^5 cells/ml) were plated in a 60 mm dish and treated with curcumin when the cells were grown to approximately 60–70% confluence. After rinse with phosphate-buffered saline (PBS), the cells were exposed to the lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP-40, and protease inhibitors] in the ice for 30 min. After centrifugation at 13,000×*g* for 15 min, the supernatant was collected and stored at−70°C until use. The protein concentration was determined by using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane at 300 mA for 3 h. The membrane was blocked in 5% non-fat dry milk reconstituted in 0.1% Tween 20 in PBS (PBST) for 1 h, followed by incubation with the indicated antibodies in PBST with 3% non-fat dry milk. The membrane was then rinsed three times with PBST buffer for 10 min each. The washed membrane was incubated with 1:3000 dilution of the horseradish peroxidase-conjugated secondary antibody in PBST with 3% non-fat dry milk for 1 h at room temperature. The membrane was washed again three times in PBST buffer, and transferred proteins were detected with an enhanced Peroxidase Detection Western blot Detection Kit (ELPIS-BIOTECH, Daejeon, Korea) or ECL Plus Western Blotting Reagent Pack (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions and visualized with an X-ray film or the Imagequant™ LAS 4000 (Fujifilm Life Science).

2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from RGM-1 cells using Trizol® (Invitrogen; Carlsbad, CA, USA). One microgram of total RNA was used for the complementary DNA synthesis using random primers. PCR was performed following standard procedures. PCR conditions for *15-PGDH* and the house keeping gene *GAPDH* were as follows: *15-PGDH*, 26 cycles of 94°C for 1 min; 60°C for 1 min and 72°C for 1 min. The primer pairs and the size of the expected products were as follows (forward and reverse, respectively): *15-PGDH*, 5′-CAGGCAGAGAATGCTGAGTTC-3′ and 5′-GATGTTGAGCAG-GAACGCAGT-3′, 555 bp and *GAPDH*, 5′-TGAAGGTCGGTGT-CAACGGATTTGGC-3′ and 5′-CATGTAGGCCATGAGGTCCACCAC-3′, 983 bp. Amplification products were resolved by 1.0% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light. Primers were purchased from Bionics (Seoul, South Korea).

2.6. 15-PGDH promoter activity assay

The cultured cells were seeded at 60–70% confluence in 12-well plates for 24 h before transfection. The cells were transiently co-transfected with the 15-PGDH promoter construct ligated to the luciferase gene (15-PGDH-2368/luc) or its deletion constructs (15-PGDH-1024/luc, 15-PGDH-388/luc, and 15-PGDH-203/luc), and 0.5 μg of the pCMV-β galactosidase control vector using lipofectamine RNAiMAX reagents (Thermo Fisher Scientific; Waltham, MA, USA) according to the instructions supplied by the manufacturer. After 24 h of transfection, the cells were treated with curcumin for additional 6 h and lysed in reporter lysis buffer (Promega; Madison, WI, USA). The activity of firefly luciferase in the cell lysates was measured using the luciferase reporter assay system according to the manufacturer's instructions. The β-galactosidase assay was conducted according to the supplier's instructions (Promega β-galactosidase Enzyme Assay System) for normalizing the luciferase assay.

2.7. Transient transfection

RGM-1 cells were seeded at a density of 1.5×10^5 cells in 60 mm dish and grown to 50% confluence. c-*Jun* siRNA (20 nM) or negative control siRNA (20 nM) was transfected into RGM-1 cells with lipofectamine RNAiMAX reagents according to the manufacturer's instructions. After 24 h transfection, cells were treated with curcumin for additional 6 h and the cell lysis was carried out with the lysis buffer. The target sequence for c-*Jun* siRNA was 5′-GGCATAATCGGATTCA-CACG-3′ (forward) and 5′-CGAGGCCGTGATCTTCATAA-3′ (reverse) (Genolution Pharmaceuticals, Seoul, South Korea).

2.8. Chromatin immunoprecipitation (ChIP) assay

RGM-1 cells were treated with curcumin for 6 h, followed by fixation in 1% formaldehyde at 37°C for 10 min to cross link histones to DNA. After rinse with PBS, the cells were incubated in SDS lysis buffer [50 mM Tris–HCl (pH 8.1), 1% SDS, 10 mM EDTA and protease inhibitors] for 10 min on ice, sonicated to generate DNA fragments of 200–500 bp in length, and centrifuged at 13,000×*g* for 10 min. ChIP dilution buffer [16.7 mM Tris–HCl (pH 8.1), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100 and protease inhibitors] was added to each tube containing 100 μl of cross-linked fractions. Samples were pre-immunoprecipitated with Protein G agarose beads to preclear the chromatin for 1 h at 4°C. After centrifugation at 7000×*g* for 1 min, supernatant was transferred into new tubes. Each sample was immunoprecipitated with 5 μg of c-Jun antibody or normal mouse IgG overnight at 4°C. Immune complexes were precipitated with Protein Agarose G beads for 4 h at 4°C with rotation. Precipitates were washed once with low salt immune complex wash buffer [20 mM Tris–HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100], once with high salt immune complex wash buffer [20 mM Tris–HCl (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100], once with LiCl immune complex wash buffer [10 mM Tris–HCl (pH 8.1), 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% NP-40, 1% IGEPAL-CA630, 1% deoxycholic acid and 0.25 M LiCl] and twice with TE buffer [10 mM Tris–HCl (pH 8.1) and 1 mM EDTA]. After extraction of antigen/antibody complexes with elution buffer [0.1 M $NaHCO_3$ and 1% SDS], cross-linking was reversed by incubating at 65°C overnight. DNA was extracted using the AccuPrep Genomic DNA extraction kit (Bioneer; Daejeon, South Korea) according to the manufacturer's protocol. Washes and eluted DNA were used for PCR analysis using specific primers for the AP-1 binding sites at the 15-PGDH promoter region. The primers employed are as follows (forward and reverse, respectively): c-*Jun*; 5'-CCAGACAATCGTCTCGCCCA-3′ and 5′-GGCTAGGTAACAGTTTAGCGAGGA-3′. Amplification products were resolved by 3.0% agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet light.

2.9. Preparation of nuclear extracts

After treatment with curcumin, cells were washed with ice-cold PBS, scraped in 1 ml PBS and centrifuged at 13,000×*g* for 15 min at 4°C. Pellets were suspended in 100 μl of hypotonic buffer A [10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF] for 15 min on ice, and 1 μl of 10% Nonidet P-40 solution was added for 5 min. The mixture was centrifuged at 12,000×*g* for 7 min at 4°C. The pellets were washed with hypotonic buffer and resuspended in hypertonic buffer C [20 mM HEPES (pH 7.8), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] for 30 min on ice and centrifuged at 12,000×*g* for 7 min at 4°C. The supernatant containing nuclear proteins was collected and stored at −80°C. The protein concentration of the nuclear extract was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories; Hercules, CA, USA).

2.10. Immunocytochemistry

RGM-1 cells were seeded at 3×10^4 cells/well in an 8 chamber slide. RGM-1 cells were pretreated with MAPK inhibitors for 2 h, followed by treatment with curcumin 10 μM for additional 6 h. After fixation with 10% neutral-buffered formalin solution for 30 min at room temperature, RGM-1 cells were incubated with blocking agents [0.1% Tween-20 in PBS containing 5% bovine serum albumin], washed with PBS and then incubated with a diluted (1:100) p-c-Jun antibody for overnight at 4°C. After washing with PBS (twice for 5 min each), samples were incubated with a diluted (1:1000) FITC-goat anti-rabbit IgG secondary antibody for 1 h. This was followed by washing cells with PBS (twice for 5 min each), and incubation with propidium iodide (for 1 min). The signals were detected using a confocal microscope (Leica, Model: TCS SP, Germany).

2.11. Bacteria strain and infection condition

Helicobacter pylori (*H. pylori*, ATCC 43504) with the typical shape, gram-negative rods, possessing the CagA and VacA, was provided in a frozen state by ATCC. The bacteria were grown on tryptic soy agar with 5% sheep blood agar (BD Diagnostics) and Dent antibiotics supplement (Oxoid) at 37°C under microaerophilic conditions (Campy-Pak System; BBL). RGM-1 cells were incubated overnight in fresh serum- and antibiotic-free DMEM and incubated with *H. pylori* at multiplicities of infection (MOI) of 100:1 for 24 h.

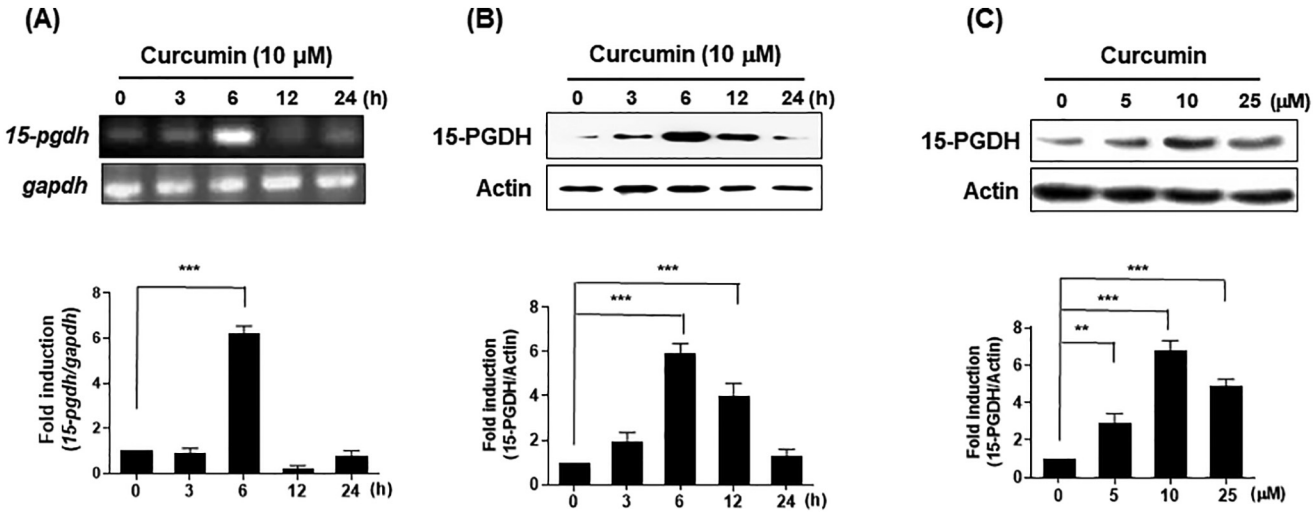

Fig. 1. **Effects of curcumin on 15-PGDH expression.** (A) RGM-1 cells were treated with curcumin (10 μM) for indicated periods of time. The expression of 15-*PGDH* mRNA was determined by RT-PCR. *GAPDH* was used as an internal control. (B) The cell lysates were subjected to Western blot analysis. Actin was used as an equal loading control for normalization. (C) RGM-1 cells were treated with concentration (5, 10, or 25 μM) of curcumin for 6 h. The cell lysates were subjected to Western blot analysis. Actin was used as an equal loading control for normalization. Significantly different between the groups compared (**P<.01; ***P<.001).

2.12. Measurement of intracellular reactive oxygen species (ROS) accumulation

The fluorescent probe DCF-DA was used to monitor the net intracellular accumulation of ROS. The RGM-1 cells were treated with curcumin for 6 h prior to *H. pylori* infection. The cells were rinsed with PBS and loaded with 10 mM DCF-DA for 30 min at 37°C to assess ROS-mediated oxidation of DCF-DA to the fluorescent DCF. The intracellular ROS accumulation was determined by fluorescent microscopy set at 488 nm for excitation and 530 nm for emission.

2.13. Animals study

Female C57BL/6 mice (5 weeks of age) were purchased from the Central Lab. Animal (Seoul, South Korea) and were housed in a climate-controlled quarters (24±1°C at 50% humidity) with a 12-h light/12-h dark cycle and with free access to food and water. Animals were handled in an accredited animal facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International policies (SNU-110511-4). After 1 week of adaptation, 6-week old mice weighing 18–22 g were randomly assigned to

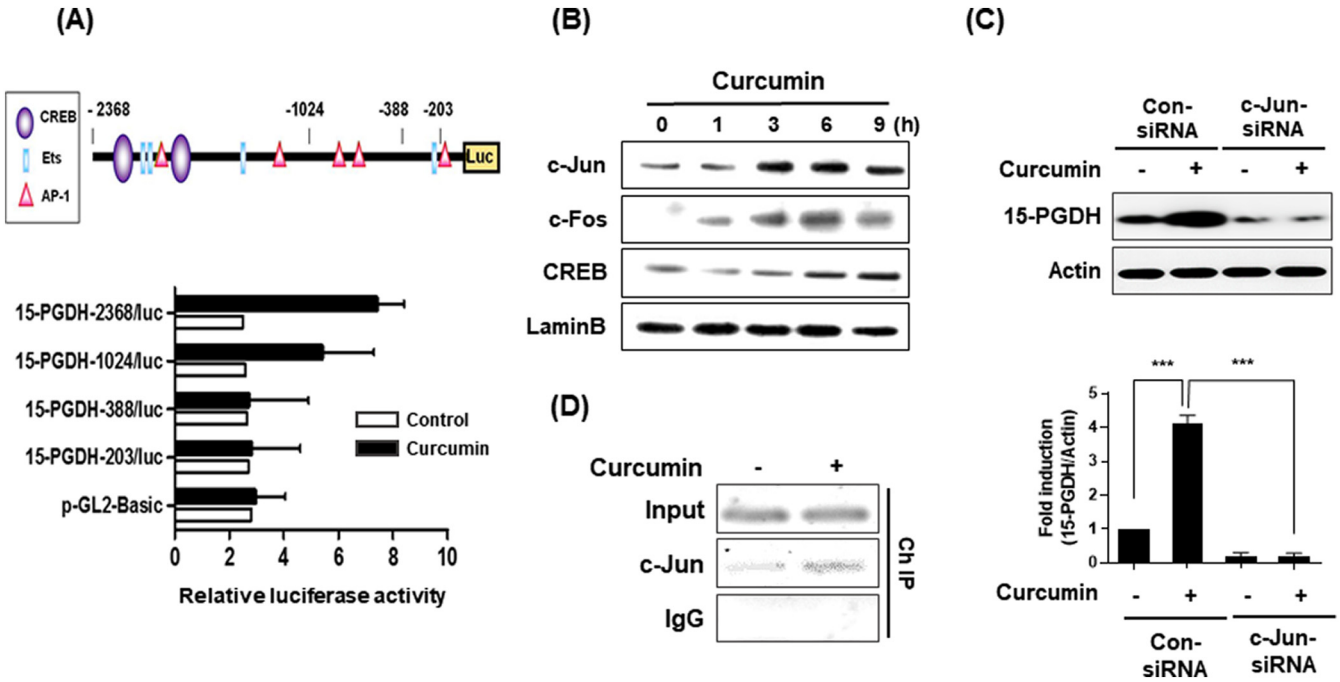

Fig. 2. **AP-1 plays an important role in induction of 15-PGDH by curcumin** (A) A schematic representation of the 15-PGDH promoter. RGM-1 cells were co-transfected with 1 μg of 15-PGDH promoter deletion constructs (−2368, −1024, −388 and −203) ligated to the luciferase gene and the pCMV-ß-galactosidase vector for 24 h. After incubation with curcumin for 6 h, and the cells were lysed for the measurement of luciferase activity. The luciferase activity was normalized to ß-galactosidase activity. (B) RGM-1 cells were treated with 10 μM of curcumin for indicated time periods. The nuclear fraction and whole lysates were subjected to Western blot analysis. Lamin B was used as a nuclear protein marker. (C) RGM-1 cells were transfected with scrambled or c-Jun siRNA for 48 h and then treated with 10 μM of curcumin for 6 h. The expression of 15-PGDH was measured by Western blot analysis. ***Significantly different between the groups compared (P<.001). (D) RGM-1 cells were treated with curcumin (10 μM) for 6 h. Total lysates were subjected to the ChIP assay using an antibody against c-Jun. ChIP-enriched DNA was amplified by PCR with specific primers for determining the AP-1 binding site present in the 15-PGDH promoter.

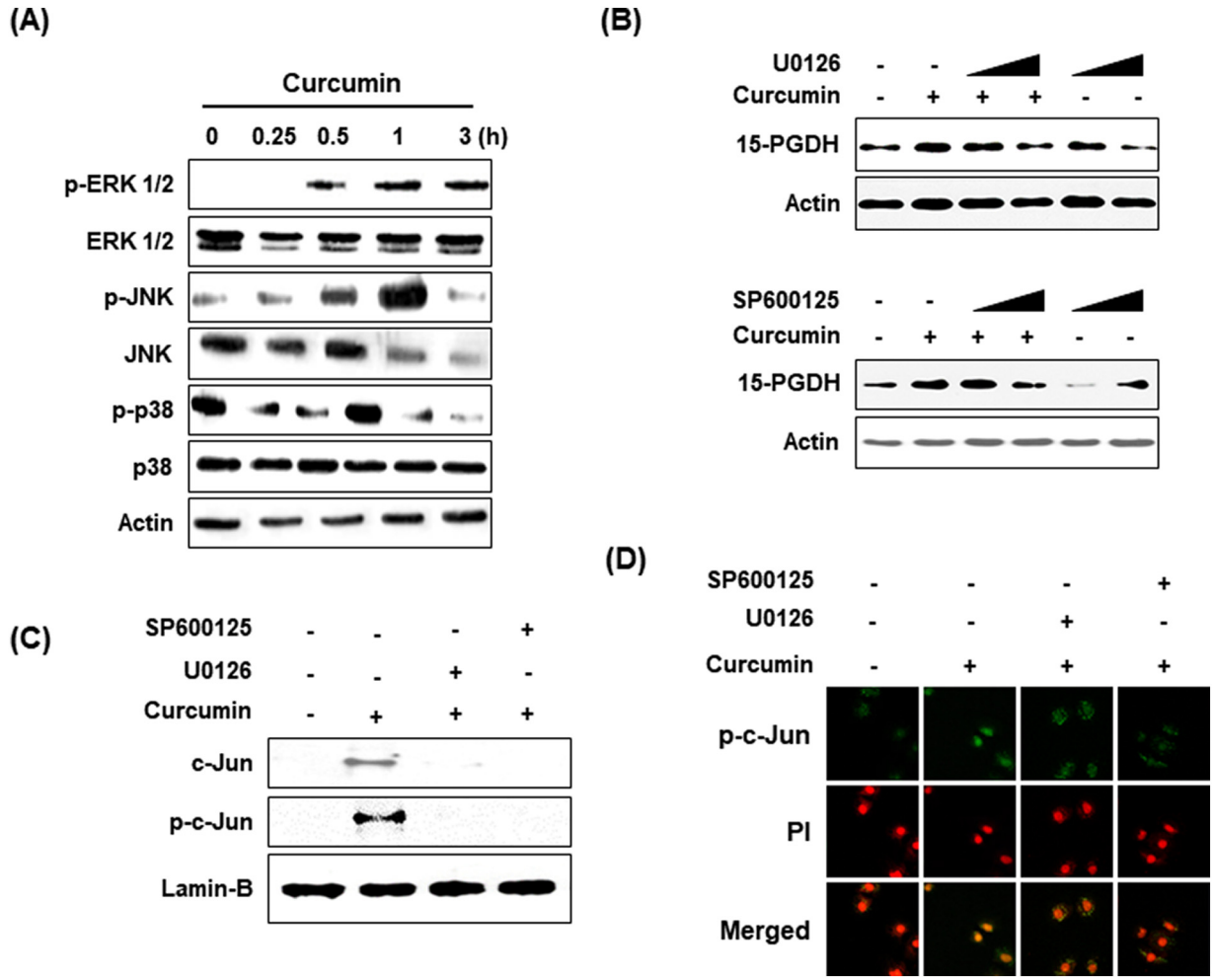

Fig. 3. **Role of ERK1/2 and JNK in curcumin-induced expression of 15-PGDH.** (A) RGM-1 cells were treated with curcumin (10 μM) for indicated time periods, and then the cell lysates were subjected to Western blot analysis using antibodies against ERK, pERK, JNK, p-JNK, p38MAPK, p-p38MAPK and actin. (B) RGM-1 cells were treated with the MEK1/2 inhibitor, U0126 (0.1 or 0.5 μM) or the JNK inhibitor, SP600125 (0.1 or 0.5 μM) for 2 h, followed by treatment with curcumin (10 μM) for additional 6 h. Total cell lysates were subjected to Western blot analysis with 15-PGDH antibody. The same blot was reprobed with anti-β-actin as an internal control. (C) RGM-1 cells were pretreated with U0126 or SP600125 for 2 h, followed by treatment with curcumin (10 μM) for additional 6 h. The nuclear fraction was subjected to Western blot analysis with c-Jun and p-c-Jun antibodies. Lamin B was used as a nuclear protein marker. (D) Immunocytochemical analysis was conducted to detect the nuclear localization of p-c-Jun (serine 63). RGM-1 cells were pretreated with MAPK inhibitors for 2 h, followed by treatment with curcumin 10 μM for additional 6 h. After fixation with 10% neutral-buffered formalin solution, the cells were immunoblotted with p-c-Jun antibody (serine 63) and FITC-goat anti-rabbit IgG secondary antibody. The signals were detected using a confocal microscope after incubation with propidium iodide.

two groups (five mice per group). Curcumin was freshly prepared with 0.05% CMC solution on each treatment day. Based on the previous *in vivo* study with curcumin [32,33], the mice were treated with vehicle (0.05% CMC) or curcumin (25 mg/kg) daily by gavage using oral Zonde needle and sacrificed 2 weeks later. Mice were weighed three times a week. Following euthanasia, stomach was resected.

2.14. Immunohistochemical staining

The dissected stomach tissues were prepared for immunohistochemical analysis of the expression patterns of 15-PGDH. The sections of 10% formalin-fixed, paraffin-embedded tissues were placed on glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated using microwave and boiled twice for 6 min in 10 mmol/l citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with 15-PGDH antibody at room temperature for 40 min in Tris-buffered saline containing 0.05% Tween 20 and then developed using respective horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit). The peroxidase binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin.

2.15. Statistical analysis

When necessary, data were expressed as means±S.D. of at least three independent experiments. The data were analyzed by one-way ANOVA, and the statistical significance between groups was determined by Duncan's multiple range test. Statistical significance was accepted at $P<.05$ and statistical analysis for single comparison was performed using the Student's *t*-test.

3. Results

3.1. Curcumin induces 15-PGDH expression in RGM-1 cells

We first examined the effects of curcumin on the expression of 15-PGDH. When RGM-1 cells were treated with curcumin, 15-PGDH expression was transiently induced at both transcriptional (Fig. 1A)

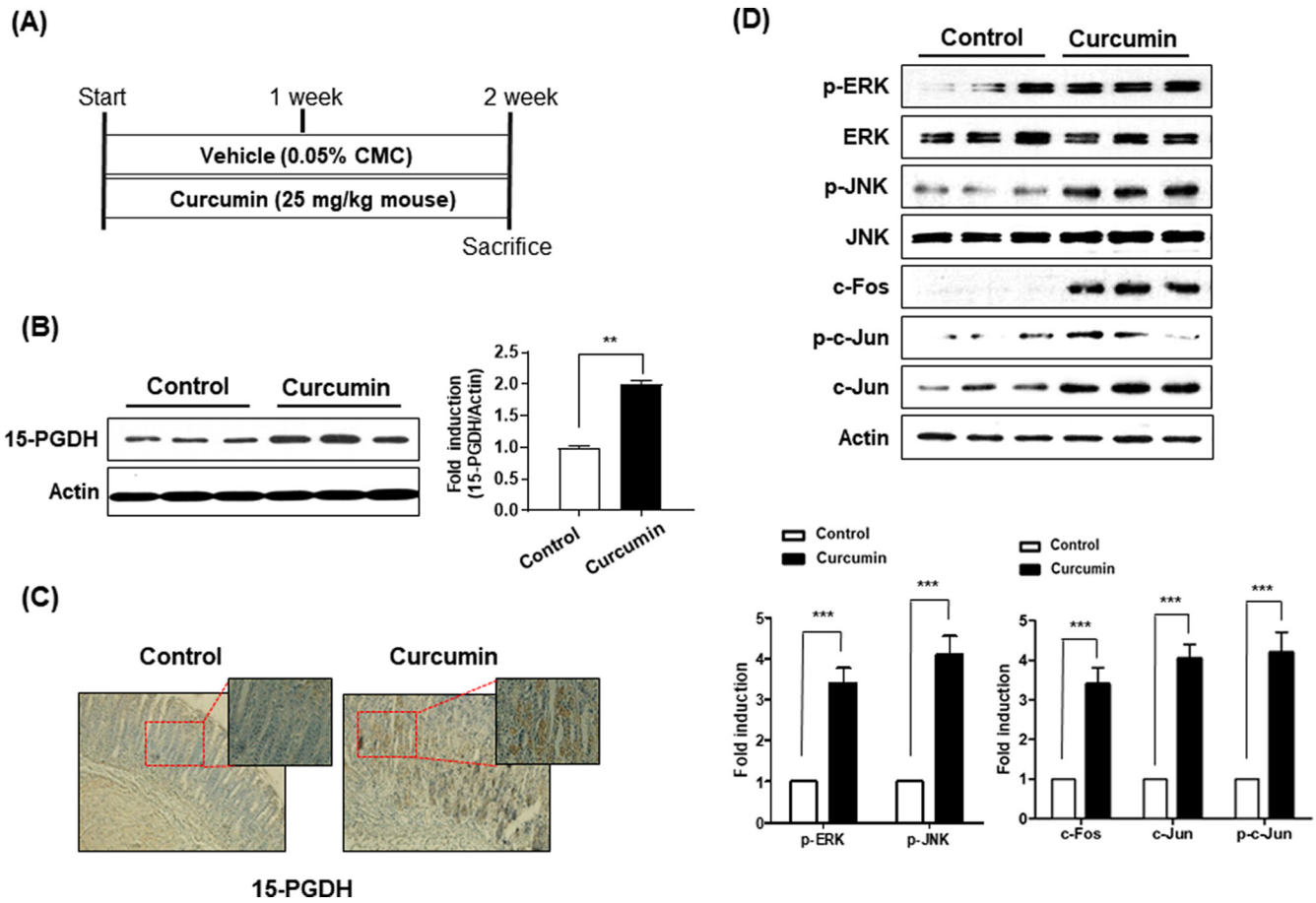

Fig. 4. **Curcumin induced 15-PGDH expression in mouse stomach *in vivo*.** (A) The schematic overview of experimental protocol. Mice in the control group were fed normal diet and received 0.05% CMC, *p.o.*, for 2 weeks (Group 1). Mice in Group 2 received a daily dose of 25 mg/kg curcumin by using oral zonde needle for 2 weeks. (B) Each gastric tissue was lysed and prepared for Western blot analysis to assess the 15-PGDH expression. (C) Immunohistochemical detection of 15-PGDH in mouse stomach. Magnifications: x200 and x400. (D) Effects of curcumin on phosphorylation of ERK and JNK and activation of AP-1 in mouse stomach *in vivo*. Significantly different between the groups compared (**P<.01; ***P<.001).

and translational (Fig. 1B) levels. The expression of 15-PGDH was also concentration-dependent (Fig. 1C).

3.2. Induction of 15-PGDH is mediated through AP-1 activation

There are several binding sites of transcription factors present in the promoter region of 15-PGDH [4]. The putative transcription factors speculated to bind to these sites include CREB, Ets and AP-1 (Fig. 2A). To investigate which transcription factor is essential for the regulation of *15-PGDH* expression, RGM-1 cells were transfected with 15-PGDH promoter deletion constructs tagged with the luciferase reporter gene (15-PGDH-Luc). The cells were then treated with curcumin for 6 h and the luciferase activity was measured. As illustrated in Fig. 2A, curcumin significantly enhanced the 15-PGDH-Luc activity. Compared to other constructs, the cells transfected with 15-PGDH-2368 and 15-PGDH-1024 constructs, which contain the AP-1 binding site, showed the most pronounced promoter activity induced by curcumin (Fig. 2A). Thus, AP-1 appears to be the key transcription factor involved in curcumin-induced expression of 15-PGDH in RGM-1 cells. We also measured the nuclear localization of c-Jun and c-Fos, key functional subunits of AP-1, in curcumin-treated RGM-1 cells. As shown in Fig. 2B, c-Jun was accumulated in nucleus of these cells treated with curcumin. The expression of c-Fos that forms a heterodimer with c-Jun was also elevated upon curcumin treatment. Curcumin also induced expression of CREB. To determine whether 15-PGDH upregulation by curcumin is mediated *via* c-Jun activation, RGM-1 cells were transiently transfected with c-*Jun* siRNA or its non-specific siRNA, and the expression of 15-PGDH was analyzed by Western blot analysis. Knockdown of *c-Jun* by siRNA abrogated the expression of 15-PGDH induced by curcumin (Fig. 2C). In order to determine whether curcumin-induced 15-PGDH expression is mediated by direct binding of c-Jun to the 15-PGDH promoter, the ChIP assay was carried out. As illustrated in Fig. 2D, curcumin stimulated interaction of c-Jun with the AP-1 binding site present in the 15-PGDH promoter. This finding lends further support to the notion that AP-1 is essential for curcumin-induced upregulation of 15-PGDH expression in RGM-1 cells.

3.3. Curcumin-induced expression of 15-PGDH is regulated by ERK1/2 and JNK

To elucidate the upstream signaling events that lead to activation of AP-1 and subsequent induction of 15-PGDH expression, we examined the phosphorylation of representative signal transducing MAPKs known to be involved in activation of AP-1 signaling. We found that curcumin treatment induced phosphorylation of ERK1/2 and JNK, but p38 MAPK was not affected (Fig. 3A). The curcumin-induced 15-PGDH expression was effectively suppressed by U0126 (MEK1/2-ERK1/2 inhibitor) and the JNK inhibitor, SP600125 (Fig. 3B). Moreover, pharmacologic inhibition of ERK and JNK abrogated curcumin-induced nuclear translocation of total and phosphorylated c-Jun at serine 63 as determined by Western blot analysis (Fig. 3C) and immunofluorescent staining (Fig. 3D). These findings suggest that curcumin-induced upregulation of 15-PGDH expression is mediated *via* activation of the ERK1/2 and JNK-AP-1 axis.

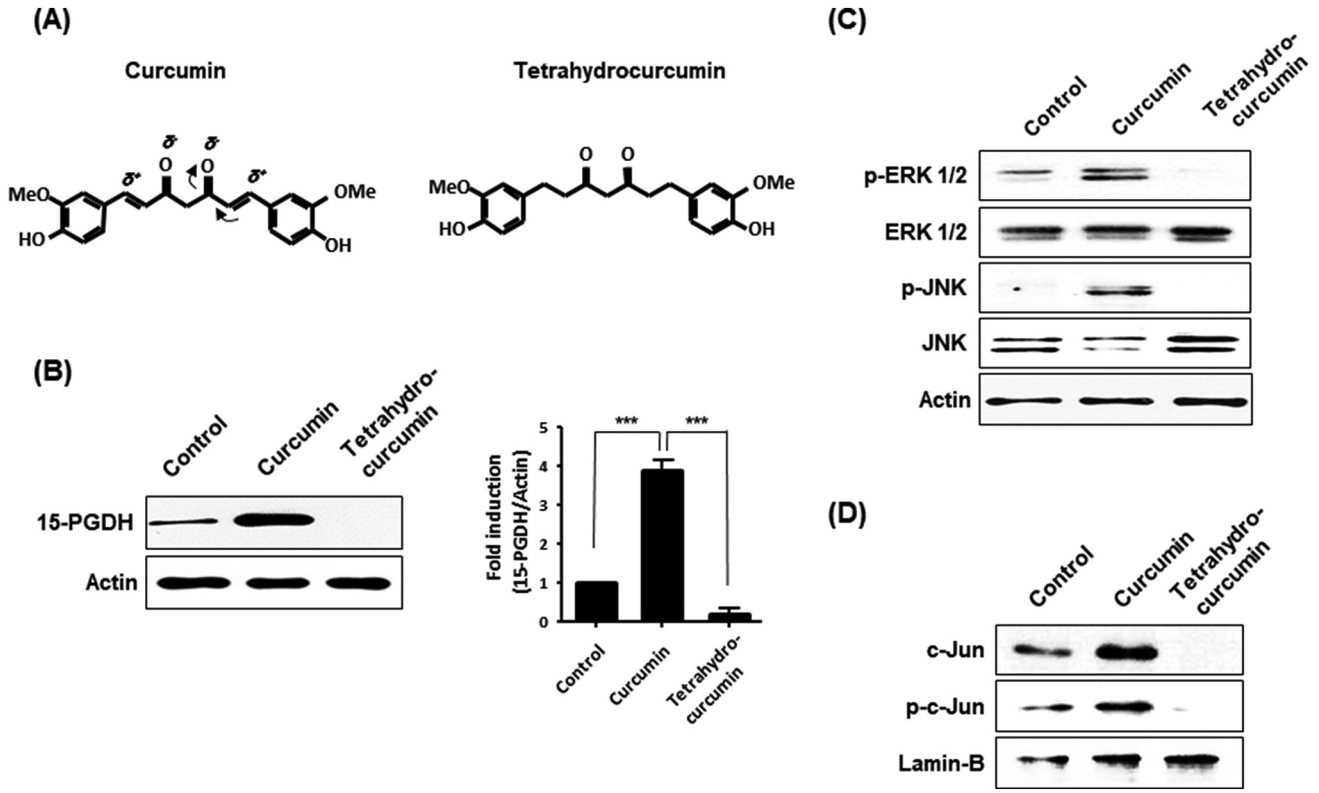

Fig. 5. **Comparative effects of curcumin and tetrahydrocurcumin on expression of signaling molecules involved in 15-PGDH expression.** (A) The chemical structures of curcumin and tetrahydrocurcumin. (B) RGM-1 cells were treated with curcumin (10 μM) or tetrahydrocurcumin (10 μM) for 6 h, and the whole lysates were subjected to Western blot analysis using 15-PGDH antibody. ***Significantly different between the groups compared ($P<.001$). (C) RGM-1 cells were treated with curcumin (10 μM) or tetrahydrocurcumin (10 μM) for 1 h and then the whole lysates were subjected to Western blot analysis using ERK, p-ERK, JNK, p-JNK antibodies. (D) RGM-1 cells were treated with 10 μM each of curcumin or tetrahydrocurcumin for 6 h, and then the nuclear fraction was subjected to Western blot analysis with c-Jun and p-c-Jun antibodies. Lamin B was used as a nuclear protein marker.

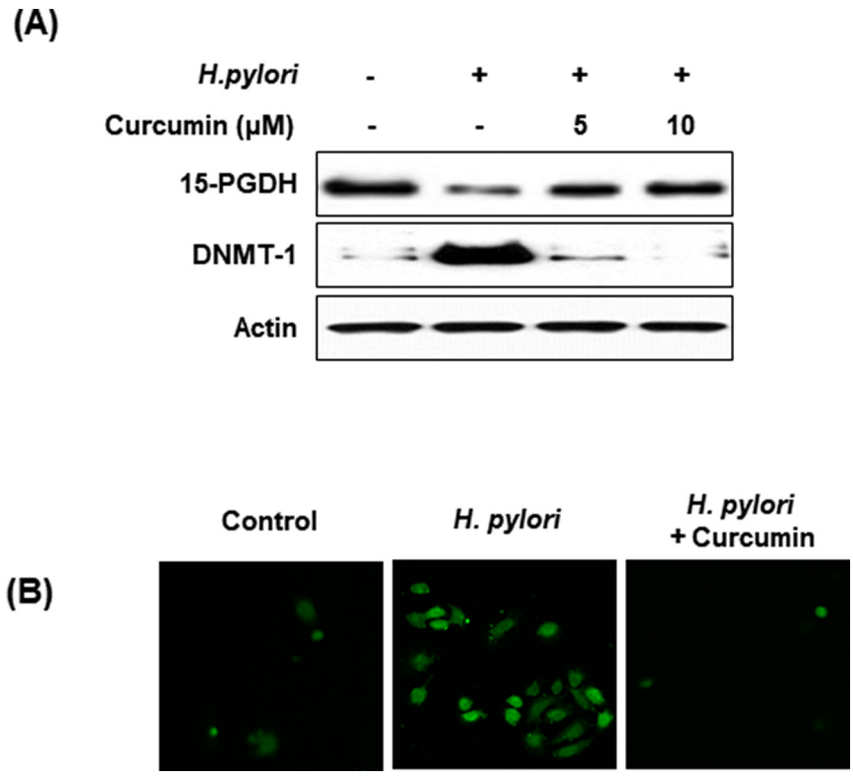

Fig. 6. **Effect of curcumin on *H. pylori*-induced expression of DNMT-1 and 15-PGDH in RGM-1 cells** (A) The RGM-1 cells were treated with curcumin (10 μM) for 6 h prior to *H. pylori* infection (24 h), and the whole lysates were subjected to Western blot analysis with antibodies against 15-PGDH and DNMT1. (B) Effect of curcumin on generation of ROS in *H. pylori*-infected RGM-1 cells. RGM-1 cells were treated with curcumin (10 μM) for 6 h prior to *H. pylori* infection (24 h), and the intracellular ROS level was measured by DCF-DA staining.

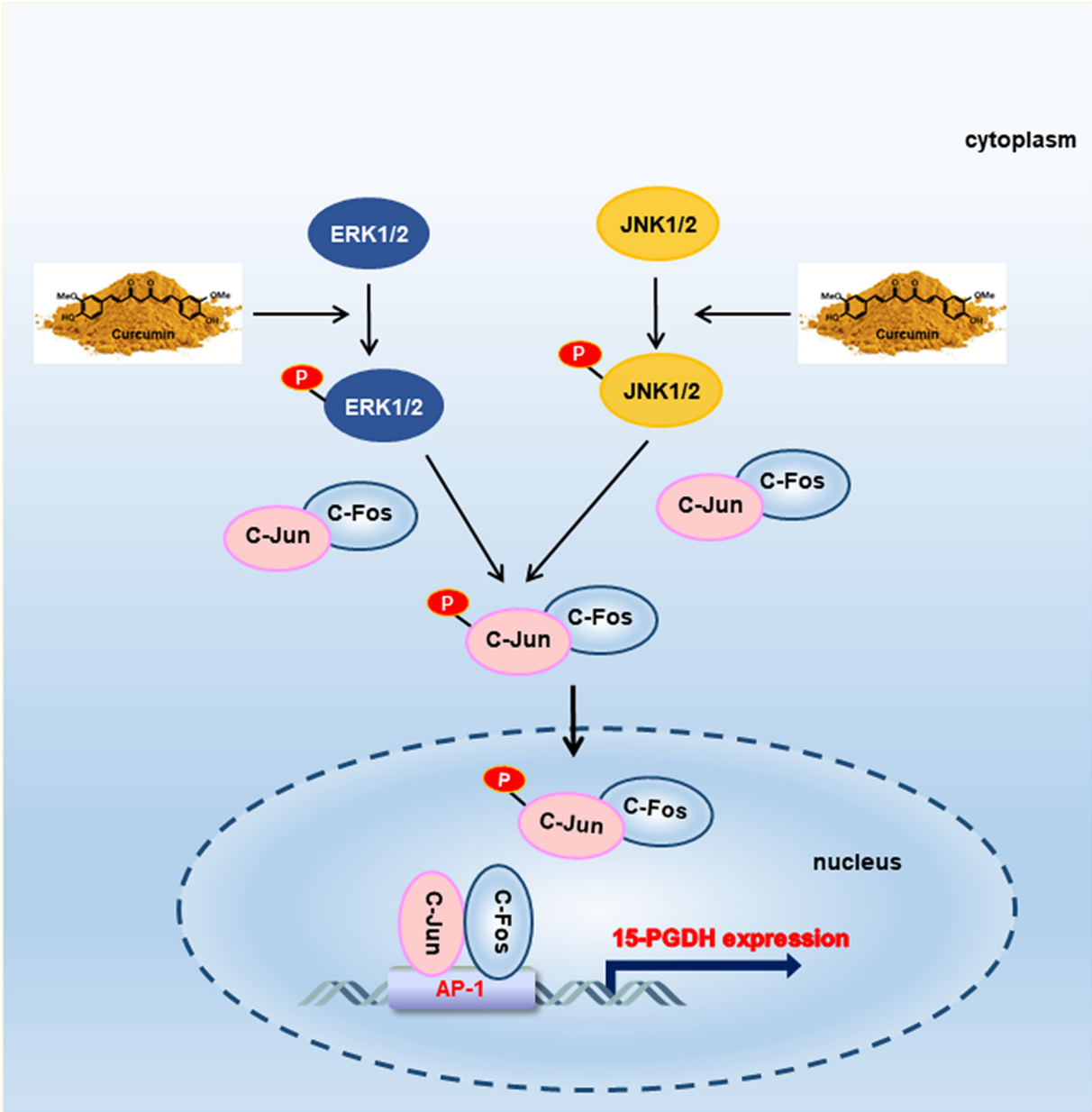

Fig. 7. **Proposed scheme for induction of 15-PGDH by curcumin.** Curcumin activates the ERK and JNK, which induces phosphorylation of c-Jun. c-Jun binds to the AP-1 binding sites located in the promoter region of 15-PGDH, leading to induction of 15-PGDH *via* activation of transcriptional activity of AP-1.

3.4. Oral administration of curcumin induces the expression of 15-PGDH in mouse stomach

To examine whether curcumin could induce 15-PGDH expression in gastric mucosa *in vivo*, C57BL/6 mice were treated with curcumin by gavage for 2 weeks (Fig. 4A). Administration of 25 mg/kg curcumin by oral zonde needle resulted in a marked induction of 15-PGDH expression (Fig. 4B). The expression of 15-PGDH was observed in the mucosal layer as determined by immunohistochemical analysis (Fig. 4C). In addition, curcumin administration resulted in significant increases in the phosphorylation of ERK and JNK in the stomach of mice (Fig. 4D). Under the same experimental conditions, there was a significant increase in the expression of c-Jun and c-Fos in the stomach of curcumin-treated mice (Fig. 4D). We also noted that phosphorylation of c-Jun was increased following treatment with curcumin (Fig. 4D).

3.5. The α,β-unsaturated carbonyl moiety of curcumin is critical for its induction of 15-PGDH expression

A compound bearing an α,β-unsaturated carbonyl moiety can act as a Michael reaction acceptor capable of covalently modifying proteins [34]. This can modulate biological function of the target proteins [35]. Curcumin has such a moiety, which has been known to contribute to its biological activities. To determine whether an electrophilic carbon center in the α,β-unsaturated carbonyl moiety of curcumin plays an important role in induction of 15-PGDH, we utilized tetrahydrocurcumin which lacks such moiety (Fig. 5A). Unlike curcumin, its non-electrophilic analogue, tetrahydrocurcumin failed to induce 15-PGDH expression (Fig. 5B). Moreover, tetrahydrocurcumin also could not induce phosphorylation of JNK and ERK (Fig. 5C) and subsequent nuclear translocation and phosphorylation of c-Jun in the nucleus of RGM-1 cells (Fig. 5D).

3.6. H. pylori-induced down-regulation of 15-PGDH expression is restored by curcumin treatment

H. pylori has been known to cause gastritis and gastric cancer [36]. The expression of 15-PGDH was found to be suppressed in the *H. pylori* infected gastric cancer specimens, which was associated with hypermethylation of CpG island in the promoter region of 15-PGDH [37]. We observed that *H. pylori* infection suppressed the expression of 15-PGDH in the RGM-1 cells, and this was restored by curcumin pretreatment (Fig. 6A). *H. pylori* infection has been known to induce expression of DNMT1 [38]. Curcumin suppressed the *H. pylori*-induced expression of DNMT1 (Fig. 6A). *H. pylori* has been known to generate ROS [39]. Curcumin attenuated the generation of ROS induced by *H. pylori* infection (Fig. 6B). These finding suggest that the antioxidant activity of curcumin may account for its induction of 15-PGDH through suppression of DNMT1 as well as generation of ROS in RGM-1 cells infected by *H. pylori*.

4. Discussion

Anti-inflammatory and anti-carcinogenic activities of curcumin have been associated with down-regulation of COX-2-catalyzed PGE_2 synthesis by blocking the activation of the proinflammatory transcription factor, NF-κB [28]. In this study, we found that curcumin induced the 15-PGDH expression in rat gastric mucosal RGM-1 cells and in mouse stomach *in vivo*. Curcumin induced activation of ERK1/2 and JNK, and consequent enhancement of the phosphorylation and nuclear translocation of c-Jun, which led to its binding to the promoter region of 15-PGDH (Fig. 7). We also observed that *H. pylori*-induced down-regulation of 15-PGDH was mitigated by curcumin pretreatment. These findings suggest that anti-inflammatory effects of curcumin are hence likely to be attributable to not only suppression of COX-2-catalyzed PGE_2 synthesis but also stimulation of 15-PGDH-mediated catabolism of PGE_2.

We attempted to elucidate mechanisms responsible for upregulation of 15-PGDH expression by curcumin. There are several binding sites located in the 15-PGDH promoter such as AP-1, Ets and CRE [4]. The AP-1 family transcription factors consist of dimers of Jun, Fos or activating transcription factor (ATF) [40]. Jun proteins form heterodimers with Fos- and ATF-family members, but can also homodimerize. The Jun-Fos heterodimer has higher the stability than the Jun homodimer [41]. The Jun-Fos dimer preferentially binds to the AP-1 element, whereas the Jun-ATF heterodimers or ATF homodimers prefers to bind to the cAMP-responsive element [42]. c-Jun is phosphorylated at serine/threonine of its amino-terminal activation domain by JNK, which suppresses its proteasomal degradation *via* ubiquitination [43]. Moreover, phosphorylation of serines 63 and 73 residues of c-Jun by JNK enhanced the binding affinity for the transcriptional coactivator CREB-binding protein (CBP), thereby stimulating its transcriptional activity [44,45]. However, c-Fos is degraded by the 26S proteasomes in a ubiquitin-dependent pathway, which was accelerated by its association with c-Jun and the coordinated actions of the protein kinases such as MAPKs [46]. We found that curcumin-induced activation of ERK and JNK was involved in phosphorylation at Ser63 of c-Jun, which may account for enhancement of stability and transcriptional activity of c-Jun, leading to upregulation of 15-PGDH.

Chemically, curcumin is a bis-α,β-unsaturated β-diketone composed of two aromatic rings linked together by two carbonyl groups. Such peculiar structural feature of curcumin is essential for its exerting a wide array of pharmacological activities [47]. Tetrahydrocurcumin is formed by reductive metabolism after intraperitoneal administration of curcumin [48]. Notably, tetrahydrocurcumin which lacks the α,β-unsaturated carbonyl group failed to induce expression of 15-PGDH as well as activation of AP-1. Therefore, it might be recommended that curcumin should be administered orally rather than intraperitoneally to retain its biological activity [48]. The α,β-unsaturated carbonyl moiety seems to play an important role in induction of 15-PGDH by curcumin. In line with this finding, the electrophilic cyclopentenone prostaglandin 15d-PGJ_2 induced the expression of 15-PGDH, while 9,10-dihydro-prostaglandin J_2, which lacks the α,β,-unsaturated carbonyl moiety, was unable to induce 15-PGDH expression in breast cancer cells [21].

Curcumin has anti-inflammatory effects against gastric diseases. Curcumin suppressed water immersion and restraint stress-induced mucosal damage and gastric blood flow in rats [49]. Moreover, curcumin inhibited secretion of the pentagastrin or histamine-stimulated gastric acid in rats [49]. *H. pylori* infection is one of the causes of gastritis and stomach cancer [36]. Although the results of clinical intervention studies are controversial, curcumin administration ameliorated dyspeptic symptoms with significant reduction of serologic signs of gastric inflammation in *H. pylori*-infected gastritis patients [50,51]. Curcumin suppressed the expression and production of IL-17 by Th17 cells through induction of indoleamine 2,3-dioxygenase in the *H. pylori*-infected human gastric mucosa [52].

Induction of COX-2 and down-regulation of 15-PGDH were observed in *H. pylori*-positive subjects and gastric cancer specimens [37,53]. In *H. pylori*-infected subjects, the suppression of 15-PGDH expression was reversed by *H. pylori* eradication therapy [37]. In *H. pylori*-positive subjects and gastric cancer patients, the 15-PGDH promoter exhibited increased methylation, and this was attenuated by a demethylation agent, 5-aza-2′-deoxycytidine [6]. We observed that curcumin restored the expression of 15-PGDH which was down-regulated by *H. pylori* infection. Curcumin has also been known to function as a potential DNMT inhibitor, thereby inducing hypomethylation of various genes [54,55]. For instance, curcumin significantly induced demethylation of proximal promoter of p21 *via* suppression of DNMT1, 3A and 3B, resulting in enhancement of p21 expression and inhibition of growth of lung cancer cells [54]. In our study, curcumin suppressed the expression of DNMT1, and this was attributable to reduction of hypermethylation of CpG island induced by *H. pylori* infection, which may involve anti-inflammatory effects of curcumin. In addition, *Fat-1* mice capable of constitutively producing ω-3 polyunsaturated fatty acids were less susceptible to the *H. pylori* induced-down-regulation of 15-PGDH and stomach cancer development [56].

Then the question is how upregulation or restoration of 15-PGDH confers protective effects against *H. pylori*. 15-PGDH oxidizes the 15-hydroxy group of PGE_2 to generate 15-keto PGE_2. In our previous study, 15-keto PGE_2 induced hemeoxygenase-1 through Nrf2 activation and whereby it exerted cytoprotective and anti-inflammatory effects [57]. 15-Keto PGE_2 suppress the growth of breast cancer cells and xenograft tumor growth through blockage of STAT3 activation [58]. Therefore, upregulation of 15-PGDH or restoration of 15-PGDH by curcumin may lead to accumulation of its end product 15-keto PGE_2, which may contribute to the protective effects against *H. pylori* infection.

In conclusion, curcumin induces expression of 15-PGDH in cultured RGM-1 cells and mouse stomach *in vivo* through activation of the ERK-JNK-AP-1 axis, which may account for new molecular mechanism underlying its chemopreventive effects against inflammation-associated carcinogenesis.

Conflicts of interest

All the authors declared no financial conflicts of interests.

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Ji-Hye Jang; Methodology, Investigation.

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Young-Joon Surh; Resources, Writing - Review & Editing.

Hye-Kyung Na; Conceptualization, Project administration, Funding acquisition, Writing - Review & Editing, Supervision.

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