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Tangeretin suppresses IL-1β-induced cyclooxygenase (COX)-2 expression through inhibition of p38 MAPK, JNK, and AKT activation in human lung carcinoma cells

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ABSTRACT

Tangeretin (5,6,7,8,4′-pentamethoxyflavone) is a polymethoxylated flavonoid concentrated in the peel of citrus fruits. Recent studies have shown that tangeretin exhibits anti-proliferative, anti-invasive, anti-metastatic, and antioxidant activities. However, the anti-inflammatory properties of tangeretin are unclear. In this study, we examine the effects of tangeretin and its structure-related compound, nobiletin, on the expression of cyclooxygenases-2 (COX-2) in human lung epithelial carcinoma cells, A549, and human non-small cell lung carcinoma cells, H1299. Tangeretin exerts a much better inhibitory activity than nobiletin against IL-1β-induced production of COX-2 in A549 cells, and it effectively represses the constitutively expressed COX-2 in H1299. RT-PCR was used to investigate the transcriptional inhibition of COX-2 by tangeretin. COX-2 mRNA was rapidly induced by IL-1β in 3 h and markedly suppressed by tangeretin. IL-1β-induced the activation of ERK, p38 MAPK, JNK, and AKT in A549 cells. COX-2 expression in response to IL-1β was attenuated by pretreatment with SB203580, SP600125, and LY294002, but not with PD98059, suggesting the involvement of p38 MAPK, JNK, and PI3K in this response. Pretreatment of cells with tangeretin inhibited IL-1β-induced p38 MAPK, JNK, and AKT phosphorylation and the downstream activation of NF-κB. These results may reveal that the tangeretin inhibition of IL-1β-induced COX-2 expression in A549 cells is, at least in part, mediated through suppression of NF-κB transcription factor as well as through suppression of the signaling proteins of p38 MAPK, JNK, and PI3K, but not of ERK.

1. Introduction

Lung cancer has the highest US and worldwide rate of cancer mortality, exceeding the mortality rates of colorectal, breast, and prostate cancers combined [1]. The high mortality of this disease is due to the difficulty of early diagnosis and its high potential to invade locally and metastasize to distant organs. Despite extensive research, the overall 5-year survival rate is only 8–14% and has improved only marginally during the past 25 years [2]. Lung cancer is highly resistant to conventional cancer therapy and although survival rates have improved slightly in recent years, more than 90% of patients diagnosed with lung cancer die from their disease [3,4]. Therefore, identification of the mechanisms preventing or controlling the development and progression of this deadly disease is urgently required because it could lead to more effective lung cancer management strategies [5].

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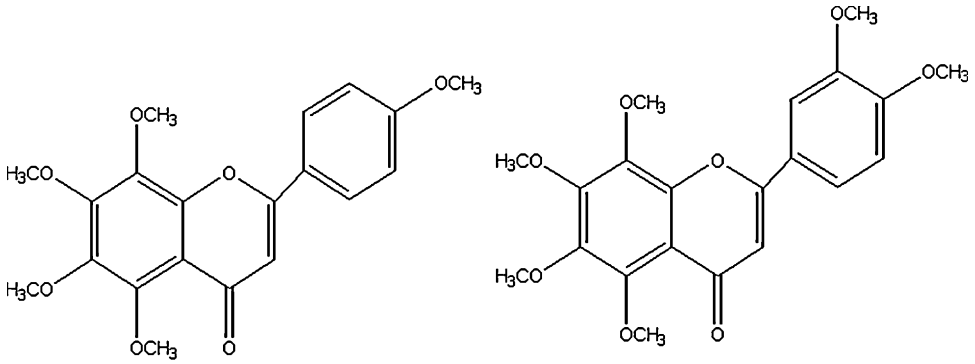
Recent research has shown that both human and experimental tumors contain increased amounts of prostaglandin (PG) E_2, which modulates cytokine balance, inhibits host immunity and may play an important role in carcinogenesis [6,7]. PGE_2 is produced from endogenous arachidonic acid by cyclooxygenase (COX). Two COX genes have been identified, COX-1 and COX-2. A COX-1-derived COX-3 transcript which is made from COX-1 gene but retain intron 1 in its mRNA is further determined in 2002 [8,9]. COX-1, the constitutive isoform, is present in most tissues and has been postulated to function as a "housekeeping" gene, responsible for physiological functions, particularly in the gastrointestinal tract. Conversely, inducible COX-2 is dramatically upregulated by a wide variety of stimuli such as interleukin (IL)-1, tumor necrosis factor α, platelet-derived growth factor, epidermal growth factor, lipopolysaccharide, and tumor promoters. COX-2 levels are elevated in gastric, hepatocellular, esophageal, pancreatic, head and neck, colorectal, breast, bladder, cervical, endometrial, skin, and lung cancers, when compared with non-malignant tissue controls [2,10]. In lung cancer, COX-2 expression is found at most stages of tumor progression. Almost all non-small cell lung cancer (NSCLC) preinvasive precursor lesions, as well as invasive lung carcinomas, express COX-2 at higher levels than normal lung tissue [11–13]. Also, markedly higher COX-2 expression has been observed in lung cancer metastatic to lymph nodes [11]. The prognostic significance of increased COX-2 expression has been reported by multiple groups. A number of studies have shown a correlation between COX-2 expression and poor prognosis: COX-2 protein levels are elevated in stage I disease and confer a poor prognosis [14]; and increased COX-2 mRNA levels portend a worse overall survival rate [7] and aggressive disease [15] in non-small cell lung cancer. These reports imply a potential role of COX-2 in the pathogenesis of lung cancer and that COX-2 is an independent poor prognostic indicator.

The levels of cytokines are increased in inflammatory airway diseases, such as asthma [16]. It has been observed that the concentration of IL-1β increased in humans with an asthmatic attack and its increase is related to the disease [17]. In rats, inhalation of IL-1β results in infiltration of neutrophils into the airways and increased airway responsiveness to inhaled bradykinin [18]. Lipopolysaccharide and certain cytokines, such as IL-1β, induced an increased expression of COX-2 in airway epithelial cells [19], airway macrophages [20], and monocytes [21]. In recent years, many studies have been carried out to explore the role of IL-1β in malignant diseases. These responses to IL-1β are mediated by a cascade of intracellular signaling events, including activation of p38 MAPK, c-Jun N-terminal kinase (JNK), phosphatidylinositol-3-kinase (PI3K), and nuclear transcription factor κB (NF-κB) [22–24].

Given the high cost of cancer therapy, significant emphasis has recently been placed on chemoprevention, especially with naturally occurring products. Flavonoids are widespread in fruits and vegetables. They are intensively studied for their role in human health, including cancer prevention. Citrus flavonoids have a broad spectrum of biological activity, including anticarcinogenic and antitumor activities [25]. It is suggested that cancer induction can be prevented by ingestion of certain food ingredients [26], and flavonoids in citrus fruits and juices are among the most prominent cancer-preventing agents. Polymethoxylated flavonoids are more potent inhibitors of tumor cell growth than free hydroxylated flavonoids. They possess potent anti-invasive and anti-metastatic activities [27] and were also reported to induce apoptosis in leukaemia cells [28]. Tangeretin and nobiletin are polymethoxylated flavones (5,6,7,8,4′-pentamethoxy- flavone) (Fig. 1) concentrated in the peel of citrus fruits [29] and probably act as natural resistance factors against pathogenic fungi [30]. Grapefruit and orange juice have been shown to inhibit human breast cancer cell proliferation [31] and to interact with drug administration [32]. Several biological activities have been shown for tangeretin itself, including the ability to enhance gap junctional intercellular communication [33], to counteract tumor promoter-induced inhibition of intercellular communication [34] and to inhibit cancer cell proliferation [35]. Previous study in our lab has also shown that tangeretin exerts its antitumor activity by growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins, such as Cdk2 and Cdk4, and mediates the increase of Cdk inhibitors p21 and p27 in human colorectal carcinoma cells [36].

In this report, we examine the inhibitory effects of tangeretin and nobiletin on pro-inflammatory protein expression. Our results provide evidence to demonstrate that tangeretin exerts a better activity than nobiletin on the



Tangeretin Nobiletin

Fig. 1 – The structure of polymethoxylated flavone, tangeretin and nobiletin.

inhibition of IL-1β-induced expression of pro-inflammatory protein, COX-2, in the A549 human lung carcinoma cells. Tangeretin can block IL-1β signaling by repressing the phosphorylation of AKT, p38 MAPK, and JNK, but not ERK, resulting in inhibition of NF-κB translocation. In addition, we also provide evidence that tangeretin can repress the constitutive expression of COX-2 in H1299 human non-small cell lung carcinoma cells.

2. Materials and methods

2.1. Drugs and materials

Tangeretin and nobiletin, isolated from orange peel extract rich in polymethoxyflavones, were purchased from Wako (Osaka, Japan). The purity of these two compounds is >98% as judged by HPLC as described previously [36]. Antibodies against phospho-AKT (p-AKT), AKT, ERK, phospho-p38 MAPK (p-p38 MAPK), p38 MAPK, and phospho-JNK (p-JNK) were obtained from Cell Signaling (Beverly, MA, USA). Antibodies against phospho-ERK (p-ERK), JNK 1, IκB-α, p65 NF-κB, COX-1, COX-2 and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against β-actin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Recombinant human IL-1β was purchased from R&D System (Minneapolis, MN, USA). MG 132 and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). PD98059, SB203580, and LY294002 were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and cytotoxicity assay

A549 cells, a human alveolar type II epithelial cell-like adenocarcinoma cell line, and H1299, a human non-small cell lung carcinoma cell line. A549 cells were cultured in DMEM medium supplemented with 10% FCS and 50 U/ml penicillin/streptomycin. H1299 cells were cultured in RPMI 1640 medium supplemented with 5% FCS and 50 U/ml penicillin/streptomycin. Both cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. All cells (1×10^5/well) were cultured in 24-well plates. After incubation for 24 h, cells were washed and treated with different concentrations of tangeretin or nobiletin to each well for 24 h. Fifteen-microliter MTT (3-(dimethyl-thiazol-2-yl)-2,5-diphenyl tetrasodium bromide) was added to each well and incubation was continued at 37 °C for 1 h. Then, remove the medium and add DMSO to each well in room temperature for 30 min. Absorbance values at 550 nm were measured with a microplate reader, and the reduction value was calculated. Each assay was performed in triplicate and the average absorbance was calculated. Data were expressed as the percentage inhibition by ratio of each absorbance relative to the absorbance in the control medium, multiplied by 100.

2.3. Measurements of PGE_2 release

A549 cells were cultured in 12-well plates. Cells were treated with the indicated concentrations of tangeretin for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. For experiments designed to measure the release of PGE_2 due to endogeneous arachidonic acid, the medium was collected, centrifuged, and stored at −80 °C until assay. The level of PGE_2 released into culture media was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham).

2.4. Trypan blue exclusion assay

Human A549 cells (2×10^5/well) were cultured in 6-well plates. The next day, the medium was changed and tangeretin or nobiletin was added. At the end of incubation, cells were collected by trypsin. Cells were then concentrated by centrifugation and were stained by trypan blue (Invitrogen, CA, USA) for 10 min. Viable cells were counted in a hemocytometer.

2.5. Western blot analysis

For the determination of the expressions of COX-2 and the phosphorylation of cytosolic signal transducers (ERK, p38 MAPK, JNK, and AKT) in A549 cells, the preparation of total/cytosolic proteins and Western blotting were performed and modified as described previously [23]. Briefly, A549 cells were cultured in 10 cm culture plates. After reaching confluence, cells were treated with vehicle, IL-1β (1 ng/ml), or pretreatment of drugs/inhibitors followed by IL-1β and incubated in a humidified incubator at 37 °C. After incubation, cells were washed with cold PBS (pH 7.4). The total cellular proteins were collected with Golden lysis buffer containing 10% glycerol, 1% Triton X100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml leupeptin. After vigorous vortexing, centrifuge at $12{,}000 \times g$ for 30 min. Cytosolic fraction was prepared in hypotonic buffer containing 10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1.5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin and incubated for 30 min on ice followed by vigorous vortexing for 10 s. The extracts were then centrifuged at $9000 \times g$ for 5 min, and the supernatant was obtained. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. Equal amounts of protein were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked in 1% bovine serum albumin. The membrane was further incubated with respective specific antibodies such as COX-2, p-ERK, ERK, p-JNK, JNK (1:2000), p-p38 MAPK, p38 MAPK, p-AKT, AKT, and β-actin. The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed in the ECL Western detection reagents.

2.6. RT-PCR

The expression value of COX-2 mRNA was quantified by semi-quantitative reverse transcription-polymerase chain reaction

(RT-PCR) analysis, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. Total cellular RNA was isolated from cells and reverse transcribed using a oligo(dT)$_{15}$ primer and reverse transcriptase (Promega, Madison, WI, USA) in 20 μl volume. PCR amplification was performed by using 0.1 μl MasterAmp™ *Taq* DNA polymerase (Epicentre, Madison, WI, USA), 0.8 μl of each 25 mM deoxyribonucleotide triphosphosphate, 1 μl MasterAmp™ *Taq* 10× PCR buffer (Epicentre, Madison, WI, USA), 0.4 μl of each specific sense and anti-sense primers at 100 μM, and 5.8 μl water with the hot-start method to enhance the sensitivity and specificity of amplification. The PCR products were analyzed on 1% agarose gel. The primer sequences and product sizes were as follows: COX-2 forward 5′-TCC CGG GGG ACA TTT AGC GTC CCT GCA-3′, reverse 5′-TAA GCT TGA GTT CCT GGA CGT GCT CCT-3′, 966 bp; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′, reverse 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′, 600 bp.

2.7. NF-κB p65 translocation and IκB-α degradation

To determine the effects of tangeretin on IL-1β-dependent NF-κB translocation and IκB-α degradation, nuclear and cytosolic protein fractions were separated. As described above, A549 cells were cultured in 10 cm culture plate. After reaching confluence, cells were treated with vehicle, IL-1β (1 ng/ml), or pretreatment of drugs/inhibitors followed by IL-1β and incubated in a humidified incubator at 37 °C. After incubation, cells were washed with cold PBS (pH 7.4) and collected by trypsin. The collected cells were suspended in ice-cold hypotonic buffer, and incubated for 30 min on ice followed by vigorous vortexing for 10 s. The extracts were then centrifuged at 9000 × *g* for 5 min, and the supernatant was obtained. The pellets (nuclear fraction) were then resuspended in Golden lysis buffer and incubated for 30 min on ice followed by vigorous vortexing each 10 min. The extracts were then centrifuged at 12,000 × *g* for 30 min, and the supernatants (nuclear fraction) were obtained. The protein levels of p65 NF-κB in the nuclear fraction and IκB-α in the cytosolic fraction were determined by Western blotting analysis performed as described.

2.8. Electrophoretic mobility shift assay (EMSA)

A549 cells were cultured in 10 cm culture plates. Nuclear and cytoplasmic extracts were prepared as described above. Each 2 μg of nuclear extract was mixed with the labeled double-stranded NF-κB oligonucleotide, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′, and incubated at room temperature for 20 min. The incubation mixture was included 1 μg of poly(dI–dC). The DNA–protein complex was electrophoresed on 6% non-denaturing polyacrylamide gels in 0.5× Tris/borate/EDTA buffer.

2.9. Statistical analysis

A one-way ANOVA was used for data statistical analysis. A Student's *t*-test was applied when multiple comparisons were performed.

3. Results

3.1. IL-1β induces COX-2 expression in A549 cells

Together with TNFα, IL-1β is defined as an ''alarm cytokine'', one that is secreted by macrophages and that initiates

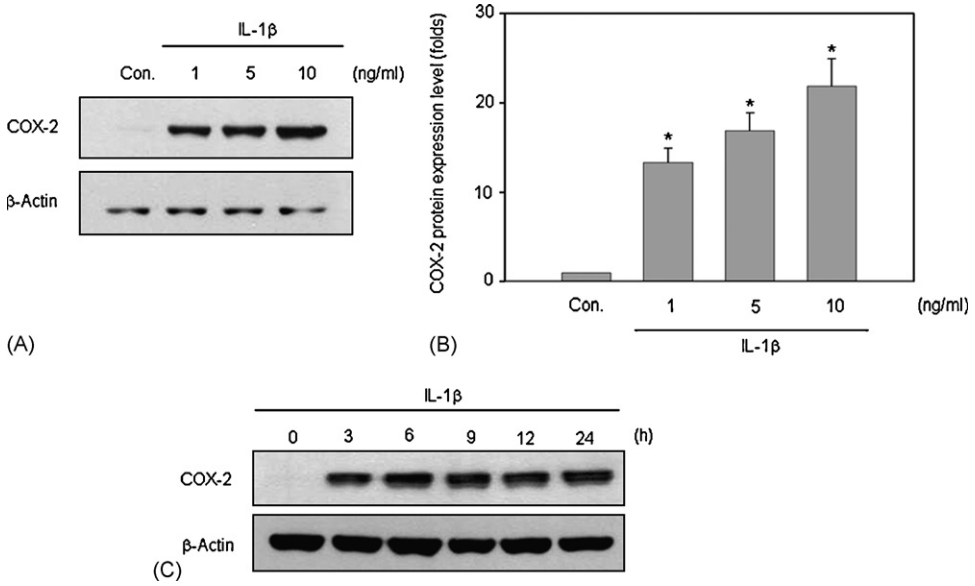


Fig. 2 – Concentration- and time-dependent IL-1β-induced stimulation of COX-2 protein expression in A549 cells. (A) Cells were incubated with the indicated concentrations (1, 5, and 10 ng/ml) of IL-1β for 24 h. Total cellular proteins were then immunodetected with COX-2 or β-actin-specific antibody as described in Section 2. Quantification of Western blot analysis was normalized by the similar intensities of β-actin (B). Data represent means ± S.D. (N = 3). *$p < 0.05$ vs. control. (C) Time-dependent stimulation of COX-2 expression was determined by incubating IL-1β (1 ng/ml) for the indicated time intervals (0, 3, 6, 9, 12, and 24 h).

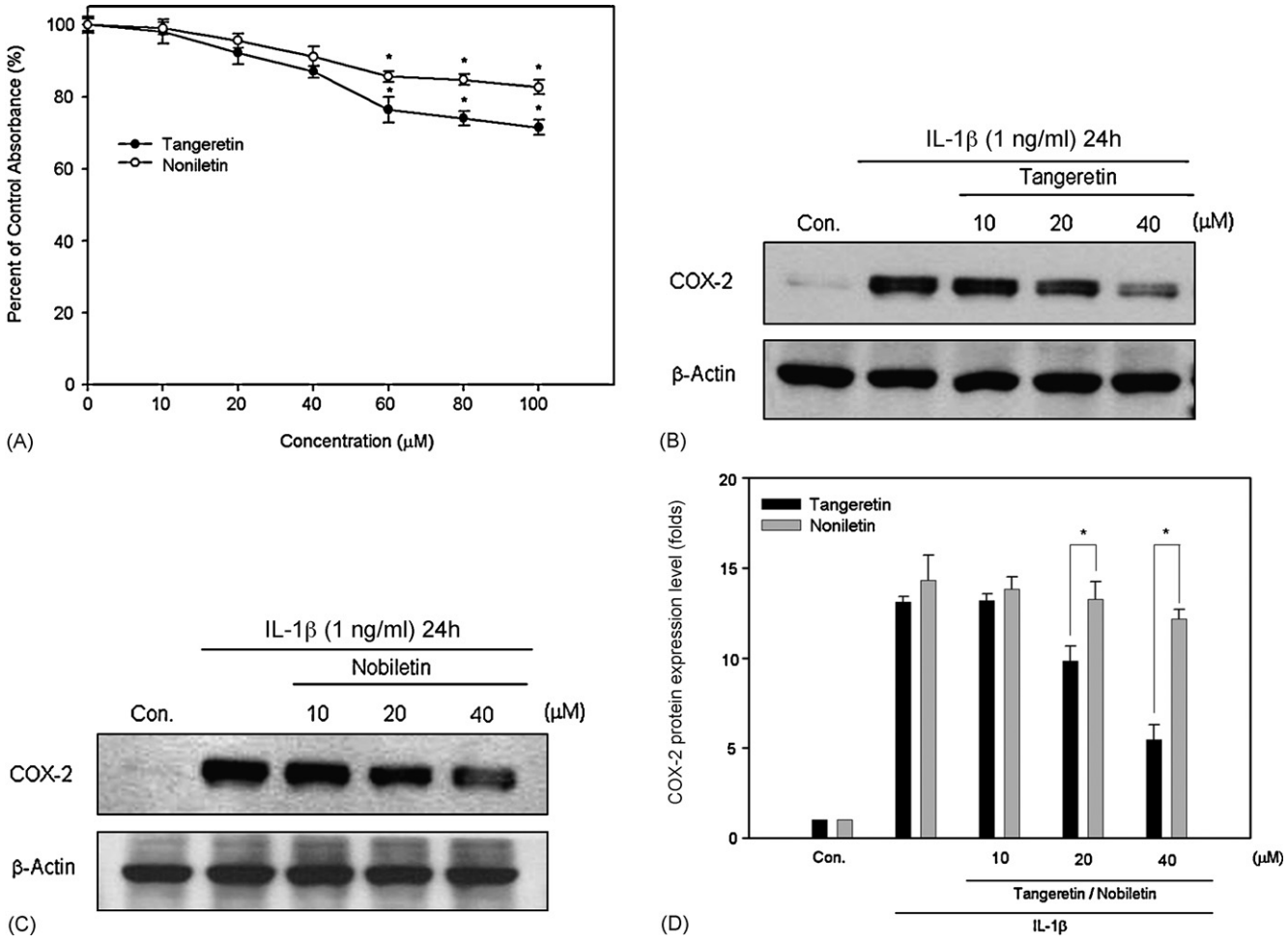


Fig. 3 – Effects of tangeretin and nobiletin on the inhibition of IL-1β-induced COX-2 protein expression in A549 cells. (A) Cells were maintained in 24-well plates at a density of 1×10^5 cells/well for 24 h. Cells were treated with tangeretin or nobiletin in various concentrations (0, 10, 20, 40, 60, 80, and 100) for 24 h. The MTT assay was performed as described in Section 2. The percent of absorbance was normalized with control medium. Cells were pretreated with various concentrations (10, 20, and 40 μM) of tangeretin (B) or nobiletin (C) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Total cellular proteins were collected. Western blot analysis was performed as described in Section 2. (D) Quantification of Western blot analysis was normalized with the similar intensities of β-actin. (E) Cells were pretreated with various concentrations (10, 20, and 40 μM) of tangeretin for 30 min followed by incubation with IL-1β (1 ng/ml) for 24 h, then the medium was collected, and the release of PGE_2 was measured. (F) A549 cells were pretreated with tangeretin (40 μM) for 30 min followed by incubation of IL-1β (1 ng/ml) for 24 h, total cellular proteins were collected and Western blot analysis was performed to detect the COX-1 expression. (G) Cells were treated with various concentrations (0, 10, 20, 40, 60, 80, and 100) of tangeretin for 24 h. The numbers of viable cells were determined by counting the trypan blue-excluding cells in a hemocytometer. Data represent means ± S.D. (N = 3). $^*p < 0.05$ vs. control.

inflammation [37]. IL-1β causes inflammation, but even more importantly, it induces the expression of pro-inflammatory genes. Of major importance are COX-2, inducible nitric oxide synthase (iNOS), IL-6, and other cytokines/chemokines. This accounts for the rapid activation of stromal and immune cells, which generate large amounts of IL-1β and TNFα-induced PGE_2, nitric oxide, and cytokines. IL-1β and TNFα stimulate their own and each other's production; and this represents an important amplification loop of the inflammatory response. Also, COX-2 expression has been reported to be found at most stages of lung cancer tumor progression [11–13]. We initially determined whether A549 cells express COX-2 in response to IL-1β. As shown in Fig. 2A, IL-1β-induced COX-2 protein expression in a concentration-dependent manner. For an exposure period of 24 h, semi-quantitative analysis in comparison with actin indicated that IL-1β significantly induced, at concentrations as low as 1 ng/ml, COX-2 protein expression in A549 cells, and the maximal induction in our experiment was observed at 10 ng/ml IL-1β (Fig. 2B). When cells were treated with 1 ng/ml IL-1β for various times, the earliest induction of COX-2 protein occurred at 3 h and was sustained for 24 h (Fig. 2C).

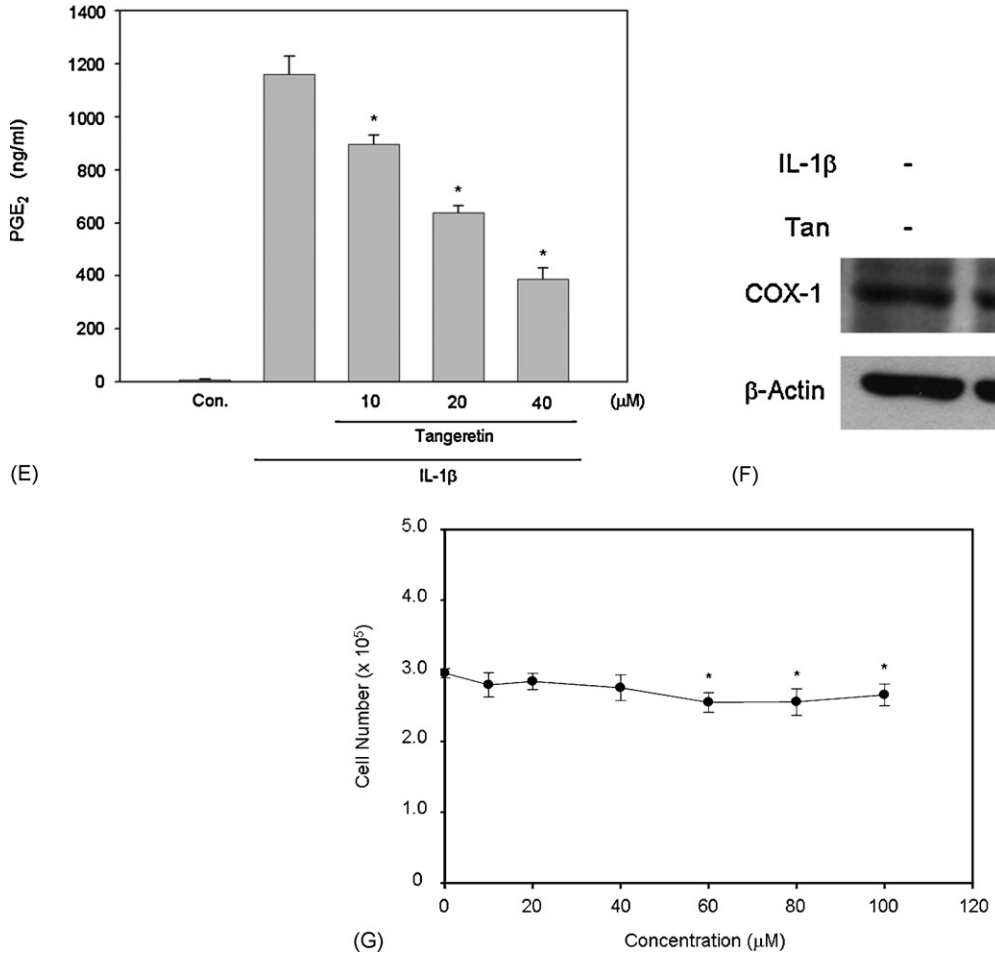


Fig. 3. (Continued).

3.2. *Tangeretin and nobiletin inhibit IL-1β-induced COX-2 protein expression in A549 cells*

Previous studies have shown that flavonoids are potent anti-proliferation [38,39] and anti-cancer agents [40]. Here, we investigated the anti-inflammation activities of the two structurally related polymethoxylated flavonoids: tangeretin and nobiletin. The structures of these two flavonoids are illustrated in Fig. 1. To assess the cytotoxic effects of selected polymethoxylated flavonoids on the growth of lung cancer cells, we first determined the growth condition of A549 lung cancer cells in the absence or presence with these two flavonoids. A549 cells were continuously cultured with different concentrations of tangeretin or nobiletin for 24 h and examined by MTT assay (Fig. 3A). Tangeretin or nobiletin induced a significant inhibition of cell proliferation in concentration more than 60 μM, assuming an IC_{50} value >100 μM. We used the concentration lower than 60 μM to evaluate the inhibitory effects on COX-2 expression in A549 lung cancer cells. To evaluate the inhibitory effects on pro-inflammatory gene expression, cells were pretreated with or without increasing concentrations of tangeretin or nobiletin (10–40 μM) for 30 min. Western blot analysis indicated that both polymethoxylated flavonoids were able to partially repress IL-1β-induced COX-2 protein expression and that tangeretin exerted a better inhibitory activity (Fig. 3B–D). Tangeretin also effectively suppressed IL-1β-induced PGE_2 production in a concentration-dependent manner (Fig. 3E). There is no significant different in the expression of COX-1 (Fig. 3F). To exclude the possibility that the down-regulation of COX-2 is due to unexpected cell death, the viable cells were determined by counting the trypan blue excluding cells in a hemocytometer. As shown in Fig. 3G, tangeretin did not cause a significant cell death under the concentration in use (40 μM) in A549 cells.

3.3. *Tangeretin inhibits IL-1β-induced COX-2 mRNA expression in A549 cells*

RT-PCR was further used to determine whether tangeretin's inhibition of COX-2 expression was due to the suppression of COX-2 transcripts. As shown in Fig. 4A, after treatment of 1 ng/ml IL-1β, COX-2 mRNA was rapidly and significantly induced in 3 h and sustained for 6 h before beginning to decrease. Following pretreatment of A549 cells with tangeretin for 30 min followed by incubation of 1 ng/ml IL-1β for 3 h, COX-2 mRNA expression was also downregulated in a concentration-dependent manner (Fig. 4B) consistent with the western blot data shown in Fig. 3B. Therefore, these results suggest that the inhibitory effect of tangeretin on IL-1β-induced COX-2 protein expression is due to the suppression of *COX-2 gene* expression.

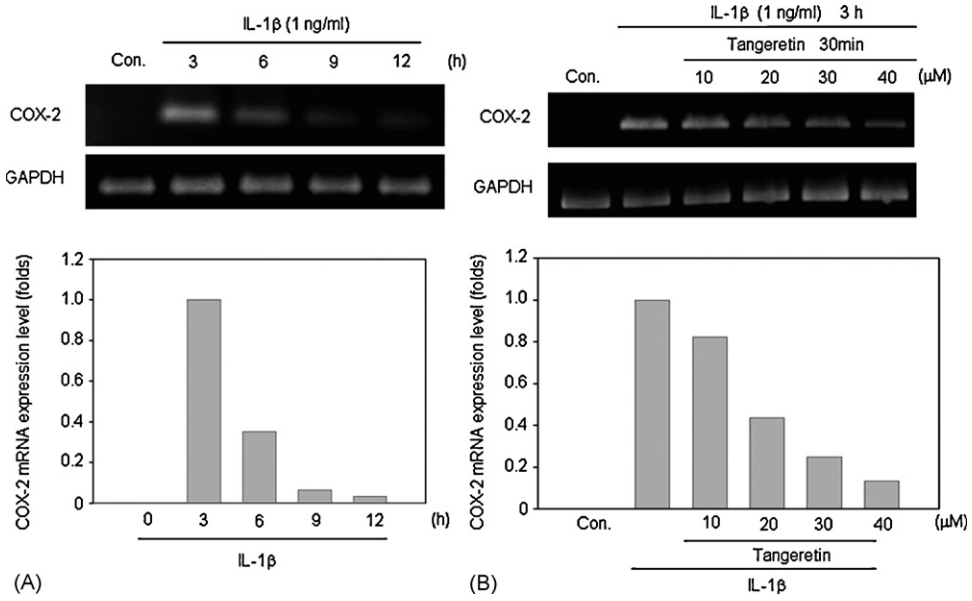


Fig. 4 – Effects of tangeretin on the inhibition of IL-1β-induced COX-2 mRNA expression in A549 cells. (A) A549 cells were treated with 1 ng/ml IL-1β for various time intervals (0, 3, 6, 9, and 12 h). Total RNA was extracted and analyzed by RT-PCR with specific primers for COX-2 and GAPDH. (B) A549 cells were pretreated with various concentrations of tangeretin (10, 20, 30, and 40 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 3 h. Total RNA was extracted and analyzed by RT-PCR with specific primers for COX-2 and GAPDH. The graph represents quantification results normalized to GAPDH levels and representative of an experiment performed in triplicate.

3.4. *Tangeretin suppresses IL-1β-induced activation of MAPKs signaling pathway in A549 cells*

The family of MAPKs has been shown to regulate COX-2 expression by various stimuli [41–43]. Therefore, we determined the effect of IL-1β on the activation of ERK, p38 MAPK, and JNK in A549 cells. As shown in Fig. 5A, treatment with 1 ng/ml IL-1β resulted in phosphorylation (activation) of these kinases. Activation of these kinases became apparent at 10 min and the maximal activation occurred at 15 min

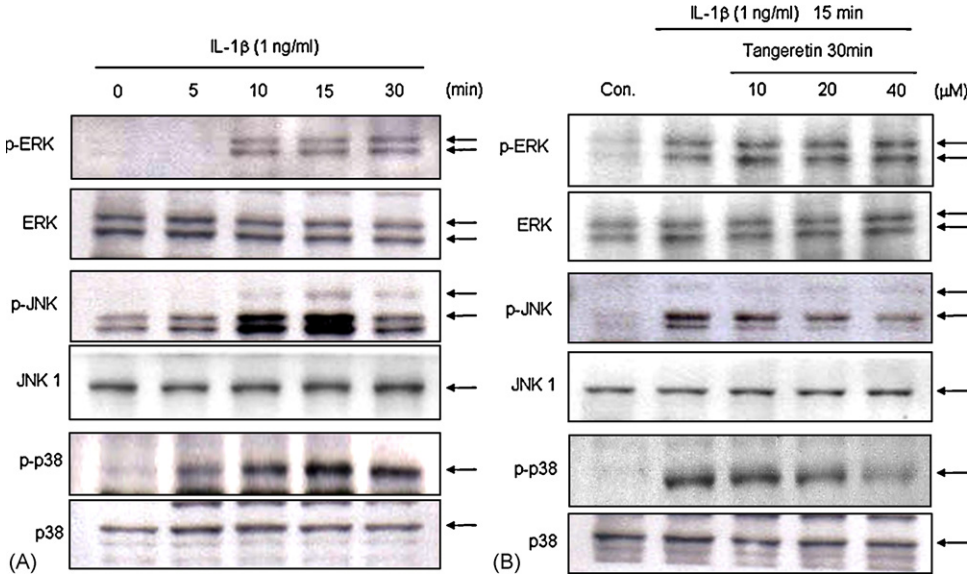


Fig. 5 – Tangeretin inhibited IL-1β-induced p38 MAPK and JNK but not ERK activation in A549 cells. (A) A549 cells were treated with IL-1β (1 ng/ml) for 0, 5, 10, 15, and 30 min. Each time, cytosolic proteins were prepared as described in Section 2 and used for Western blot analysis with respective p-ERK, ERK, p-p38 MAPK, p38 MAPK, p-JNK, or JNK antibodies. (B) A549 cells were pretreated with the indicated concentrations of tangeretin (10, 20, and 40 μM) for 30 min and then treated with IL-1β (1 ng/ml) for additional 15 min. Cytosolic proteins were prepared and used for Western blot analysis with respective p-ERK, ERK, p-p38 MAPK, p38 MAPK, p-JNK, or JNK antibodies.

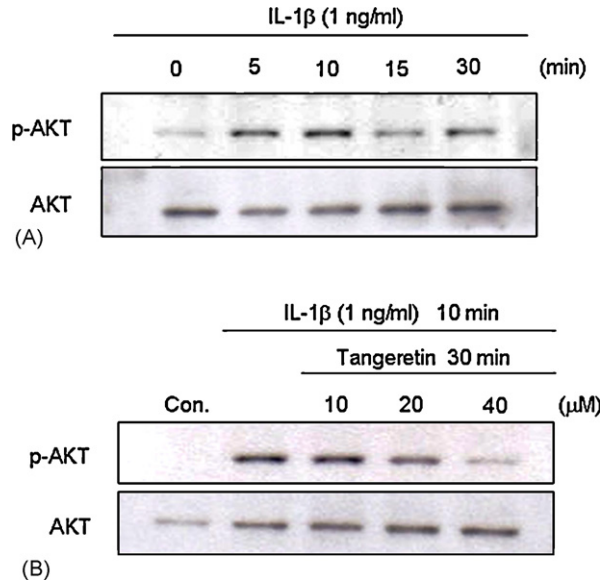


Fig. 6 – Tangeretin inhibited IL-1β-induced AKT activation in A549 cells. (A) A549 cells were treated with IL-1β (1 ng/ml) for 0, 5, 10, 15, and 30 min. Each time, cytosolic proteins were prepared and used for Western blot analysis with the respective p-AKT and AKT antibodies. (B) A549 cells were pretreated with the indicated concentrations of tangeretin (10, 20, and 40 μM) for 30 min and then treated with IL-1β (1 ng/ml) for additional 10 min. Cytosolic proteins were prepared and used for Western blot analysis with the respective p-AKT and AKT antibodies.

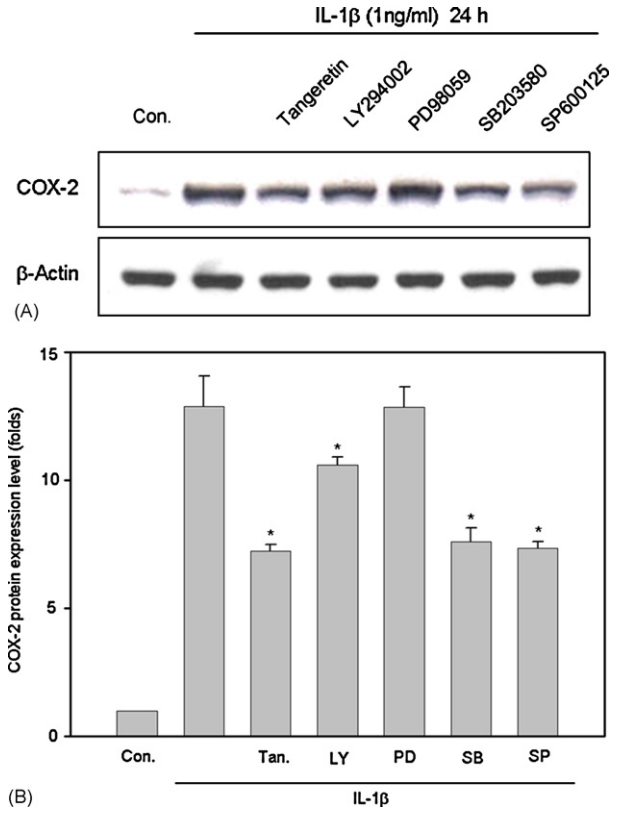


Fig. 7 – Effects of LY294002, PD98059, SB203580, and SP600125 on the COX-2 expression caused by IL-1β in A549 cells. (A) Cells were pretreated with LY294002 (25 μM), PD98059 (50 μM), SB203580 (50 μM), SP600125 (25 μM), or tangeretin (40 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 24 h. Whole cell lysates were prepared for Western blot analysis. (B) Quantitative analysis was normalized with the similar intensities of β-actin. Data represent means ± S.D. ($N = 3$). $^{*}p < 0.05$ vs. control. LY: LY294002. PD: PD98059. SB: SB203580. SP: SP600125.

following treatment with IL-1β. Compared with ERK, p38 MAPK and JNK revealed no change in total protein levels of each kinase, indicating IL-1β-induced activation of preexisting ERK, p38 MAPK, and JNK. We next examined whether tangeretin inhibits IL-1β signaling through suppression of MAPKs activation. A549 cells were pretreated with tangeretin (10–40 μM) for 30 min followed by IL-1β (1 ng/ml) for 15 min. Western blot analysis revealed that tangeretin was capable of repressing IL-1β-induced p38 MAPK and JNK phosphorylation in A549 cells while ERK phosphorylation was not affected (Fig. 5B).

3.5. *Tangeretin suppresses IL-1β-induced activation of PI3K/AKT signaling pathway in A549 cells*

To determine whether PI3K is also involved in IL-1β-induced COX-2 expression, we determined the effect of IL-1β on the activation of PI3K in A549 cells. As shown in Fig. 6A, treatment with 1 ng/ml IL-1β resulted in phosphorylation (activation) of AKT, which is known to be a downstream effector of PI3K. Activation of AKT became apparent at 5 min, and the maximal activation occurred 10 min following treatment with IL-1β. The total protein level of AKT was constant during treatment with IL-1β. Similar to the MAPKs inhibition, tangeretin also effectively inhibited the IL-1β-induced phosphorylation of AKT in A549 cells (Fig. 6B).

3.6. *Tangeretin inhibits COX-2 expression by blocking IL-1β signaling through p38 MAPK, JNK, and AKT in A549 cells*

In further studies, we examined whether inhibition of IL-1β-induced COX-2 expression in A549 by tangeretin is mediated through the suppression of MAPKs and PI3K/AKT activation. As shown in Fig. 7, pretreating the cells with the inhibitors of p38 MAPK (SB203580), JNK (SP600125), and PI3K/AKT (LY294002) for 30 min significantly reduced IL-1β-induced COX-2 protein expression. Interestingly, PD98059, the ERK inhibitor, had no inhibitory effect on IL-1β-induced COX-2 protein expression. Taken these together, it suggests that p38 MAPK, JNK, and PI3K/AKT are involved in IL-1β-induced COX-2 expression in A549 cells while ERK is not. Tangeretin inhibits IL-1β-induced COX-2 expression by repressing the phosphorylation of p38 MAPK, JNK, and AKT.

3.7. *Tangeretin blocks IL-1β-induced IκB-α degradation and transcription factor NF-κB translocation in A549 cells*

NF-κB has been shown to be involved in COX-2 transcriptional activation by extracellular stimuli including IL-1β [44–46]. As shown in Fig. 8A, IL-1β markedly increased NF-κB binding activity and tangeretin reduced it. Since it has been well documented that activation of NF-κB correlates with rapid nuclear translocation and proteolytic degradation of IκB, we next determined the activation of NF-κB by assessing the degree of nuclear translocation of p65 NF-κB and the degradation of IκB-α in cytosol. Treatment of A549 cells with 1 ng/ml IL-1β resulted in a marked translocation of p65 NF-κB from cytosol to the nucleus (Fig. 8B) as well as a strong degradation of IκB-α in the cytosol (Fig. 8C). After pretreatment of cells with tangeretin (40 μM) for 30 min, the translocation of p65 NF-κB and degradation of IκB-α stimulated by IL-1β was inhibited (Fig. 8C). As mentioned above, the p38 MAPK, JNK, and PI3K/AKT signal pathways are involved in IL-1β-induced COX-2 expression in A549 cells. To determine whether IL-1β-induced activation of NF-κB is mediated through the p38 MAPK, JNK, or PI3K/AKT

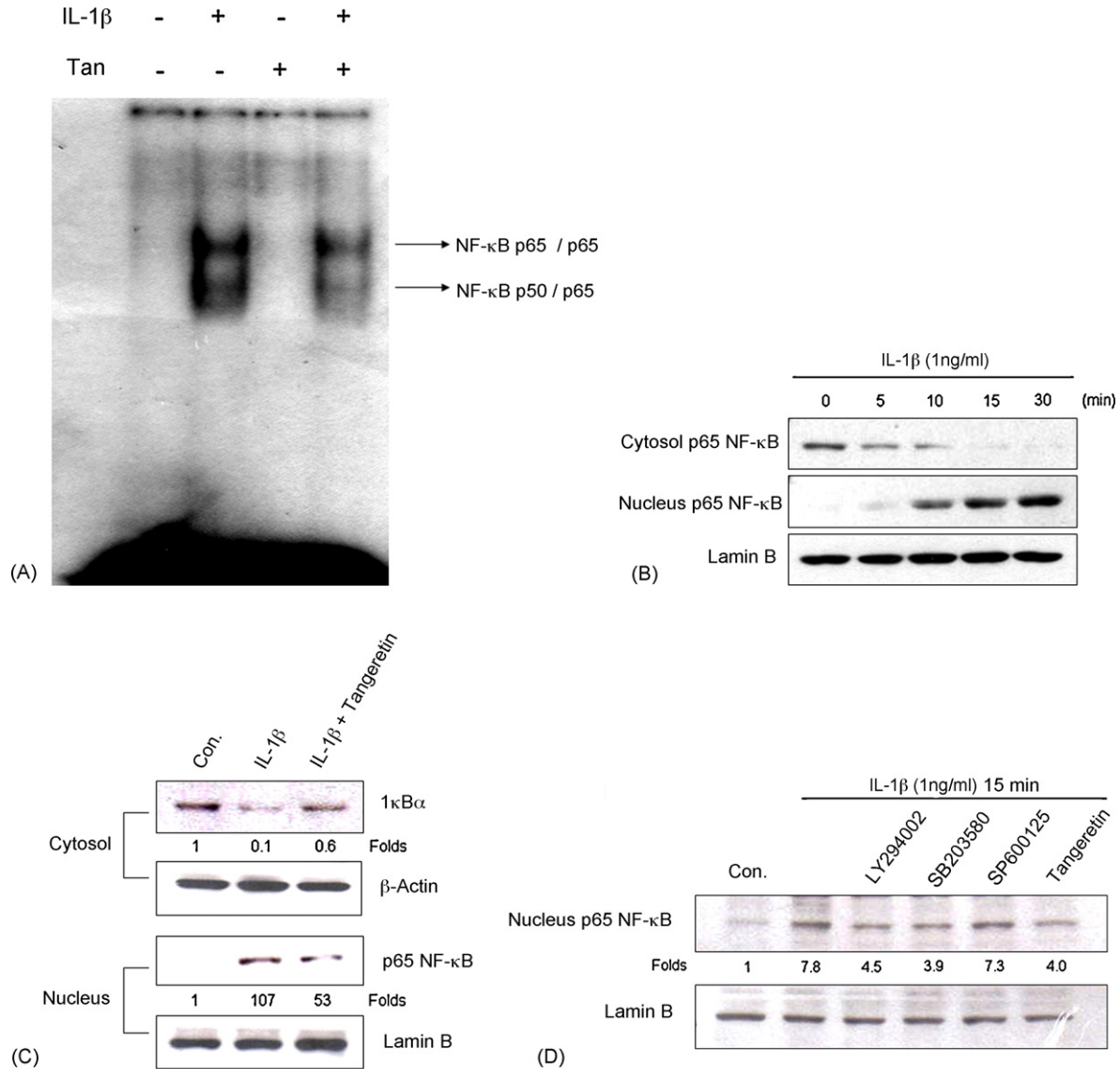


Fig. 8 – Tangeretin, LY294002, and SB203580, but not SP600125, inhibited IL-1β-induced p65 NF-κB translocation and IκB-α degradation in A549 cells. (A) A549 cells were pretreated with tangeretin (40 μM) for 30 min followed by incubation of IL-1β (1 ng/ml) for 15 min. Nuclear extracts were prepared and EMSA were carried out as described in Section 2. (B) Cells were treated with IL-1β (1 ng/ml) for 0, 5, 10, 15, and 30 min. Each time, cytosolic and nuclear proteins were prepared as described in Section 2 and immunodetected with p65 NF-κB and IκB-α specific antibodies. (C) A549 cells were pretreated with tangeretin (40 μM) for 30 min followed by incubation of IL-1β (1 ng/ml) for 15 min. The cytosolic levels of IκB-α and nuclear levels of p65 NF-κB were determined by Western blotting. (D) A549 cells were pretreated with LY294002 (25 μM), SB203580 (50 μM), SP600125 (25 μM), and tangeretin (40 μM) for 30 min before incubation with IL-1β (1 ng/ml) for 15 min. Nuclear fractions were prepared and immunodetected with p65 NF-κB specific antibodies. β-Actin and lamin B were used as cytosolic and nuclear internal control, respectively.

pathways, A549 cells were pretreated for 30 min with inhibitors of these kinases, and then treated with IL-1β for an additional 15 min, followed by measurement of the degree of nuclear translocation of p65 NF-κB. Interestingly, LY294002 and SB203580 partially suppressed nuclear translocation of p65 NF-κB in response to IL-1β treatment while SP600125 did not (Fig. 8D). These results suggest that the p38 MAPK, and PI3K/AKT pathways, but not the JNK pathway, seem to regulate NF-κB activation.

3.8. *Tangeretin inhibits constitutive COX-2 expression in H1299 cells*

Previous studies have demonstrated that COX-2 significantly overexpressed in human non-small cell lung carcinoma (NSCLC) compared with SCLC [47]. In order to investigate if tangeretin can also downregulate constitutively expressed COX-2, H1299 cells were used. H1299 is a human non-small cell lung carcinoma cell line which constitutively expresses COX-2 mRNA [48]. As shown in Fig. 9, treatment of tangeretin (40 μM) with cells resulted in obvious suppression of endogenous COX-2 expression in a time-dependent manner. We suggest that tangeretin is a potent COX-2 inhibitor of either IL-1β-induced or constitutively expressed COX-2.

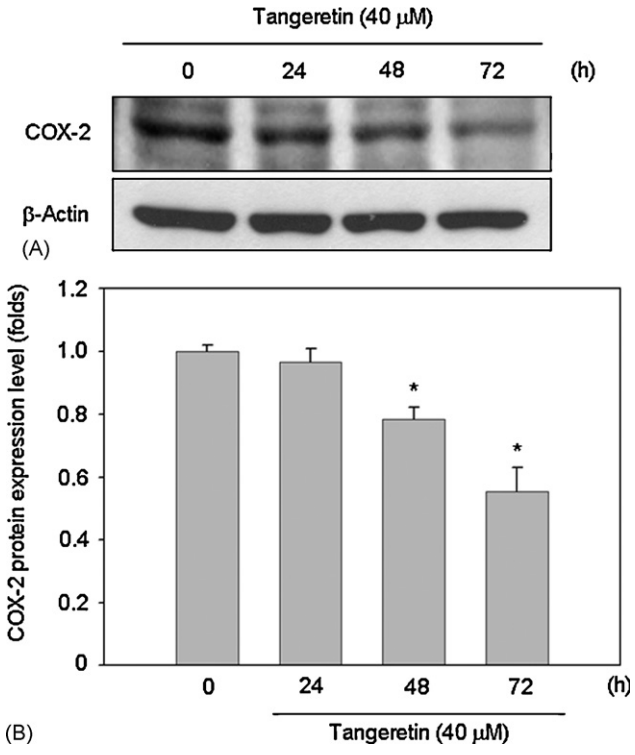


Fig. 9 – Tangeretin inhibited constitutive COX-2 expression in H1299 cells. (A) Cells were cultured for 24 h followed by treating with tangeretin (40 μM) for the indicated time intervals (0, 24, 48, and 72 h). Total cell lysates were prepared as previously described and used in Western blot analysis. (B) The graph represents quantification results normalized to β-actin levels. Data represent means ± S.D. (N = 3). *$p < 0.05$ vs. control.

4. Discussion

In this study, we assessed the ability of polymethoxylated flavonoids to inhibit COX-2 expression in human non-small cell lung cancer. We demonstrated that the downregulation of IL-1β-induced COX-2 production by tangeretin is a consequence of the inhibition of the activation of JNK, p38 MAPK, and AKT and indicates that IL-1β-stimulated NF-κB translocation is also blocked by tangeretin.

Several studies have shown that COX-2 is upregulated in lung cancer cells compared with normal lung tissue [5,11–15]. This suggests COX-2 may be a potential target for lung cancer chemoprevention. COX-2 is a rapid-responsive protein, and incubation of cells with 1 ng/ml IL-1β can induce maximum mRNA expression in only 3 h (Fig. 4A). In a study similar to ours, Lin et al. [49] demonstrated that nobiletin has an anti-inflammatory activity in downregulating COX-2 gene and protein expression in human synovial fibroblasts. In the present study, we compared these two structurally related flavonoids, tangeretin and nobiletin, and showed that tangeretin exerts a much better inhibitory effect on IL-1β-induced COX-2 protein expression than nobiletin (Fig. 3). Tangeretin also effectively suppresses endogenous expression of COX-2 in H1299 cells (Fig. 9). Furthermore, tangeretin exhibited a concentration-dependent inhibitory effect on COX-2 mRNA expression in A549 cells (Fig. 4B). However, the molecular mechanism of COX-2 inhibition by tangeretin was not completely understood. In this study, we investigated the inhibitory mechanism of tangeretin on IL-1β-induced COX-2 expression in A549 cells.

MAPKs are linked to the expression of COX-2 by various stimuli including IL-1β [41–43]. In present study, we have observed that IL-1β causes a marked increase in the level of phosphorylated ERK, p38 MAPK, and JNK in A549 cells. Incubation with tangeretin (40 μM) for 30 min significantly reduces the phosphorylation of p38 MAPK and JNK in A549 cells while the phosphorylation of ERK is not affected (Fig. 5). Notably, though phosphorylation of ERK, p38 MAPK, and JNK is induced by IL-1β, pretreatments with SB203580 and SP600125, but not PD98059, attenuate IL-1β-induced COX-2 expression in A549 cells (Fig. 7). These findings suggest that tangeretin effectively suppresses the phosphorylation of p38 MAPK and JNK to further block the downstream activation of IL-1β-stimulated COX-2 expression in A549 cells. It has previously been shown that tangeretin represses the phosphorylation of a number of cytoplasmic kinases and kinase substrates including ERK in T47D breast cancer cell [50]. The authors also found that polymethoxylated flavonoid congeners have different inhibitory effects on these cytoplasmic proteins as a result of the distinct position of methoxyl group. It may reveal that why tangeretin has a better inhibitory activity than nobiletin on the COX-2 expression. In our studies, tangeretin was not able to suppress the IL-1β-induced downstream activation of ERK in A549 cells. Simultaneously, data not shown experiments have demonstrated that tangeretin reduced the phosphorylation status of ERK, but not of p38 MAPK and JNK, in selected MCF7 breast cancer cells. Combined these results, we suggest that tangeretin is not a specific inhibitor of cytoplasmic MAPKs and has a broad spectrum of kinase inhibition.

Activation of the PI3K pathway by IL-1β has been well studied [51,52]. The regulatory subunit of PI3K is known to interact with IL-1β receptors and the subsequent association of the p110 catalytic subunit results in the full activation of PI3K [53]. In the present study, we have indicated that the PI3K pathway appears to be involved in IL-1β-induced COX-2 expression, and the downregulated COX-2 expression caused by tangeretin is at least in part mediated by PI3K inhibition. This notion is based on the facts that tangeretin represses the IL-1β-induced phosphorylation (activation) of AKT, a well-known downstream effector of PI3K, and inactivation of the PI3K pathway by LY294002 partially decreases IL-1β-induced COX-2 protein expression in A549 cells (Fig. 6).

The NF-κB signaling pathway has been implicated in the expression of COX-2 stimulated by various stimuli [44–46]. NF-κB is constitutively present in cells as a heterodimer, consisting of a p50 DNA-binding subunit and a p65 *trans*-activating subunit. NF-κB is normally held in cytoplasm in an inactivated state by the inhibitor protein IκB-α. After activation, the cytosolic NF-κB/IκB-α complex dissociates, and free NF-κB translocates to the nucleus [54,55]. In this study, we have demonstrated that IL-1β markedly induces nuclear translocation of p65 NF-κB and concomitantly the strong degradation of IκB-α, suggesting NF-κB activation by IL-1β in A549 cells. Furthermore, the translocation of p65 NF-κB stimulated by IL-1β is inhibited by tangeretin, SB203580, and LY294002. As mentioned above, tangeretin effectively suppresses the activation of p38 MAPK and AKT indicates that IL-1β may act through the p38 MAPK and PI3K pathways to induce NF-κB activation, and tangeretin inhibits these signaling pathways in A549 cells (Figs. 5 and 8). The promoter region of human COX-2 has been cloned and sequenced and shown to contain putative recognition sequences for a variety of transcriptional factors, including NF-κB and activator protein-1 (AP-1) [56,57]. Previous studies have demonstrated that JNK (c-Jun NH2-terminal kinase) is the upstream kinase of AP-1 [58]. Based on this, we propose that IL-1β can also stimulate COX-2 expression through JNK activation and other transcriptional regulation mechanisms, at least the AP-1 pathway, rather than NF-κB activation. Our studies have shown that tangeretin represses the phosphorylation of JNK, but the inhibitory effect of tangeretin on AP-1 activation is still unknown. On the other hand, SB203580, LY294002, and SP600125 are not able to completely block IL-1β-induced COX-2 expression, suggesting that other signal pathways may also be involved. Indeed, in the studies of Lin et al., the PKC pathway was demonstrated to be involved in IL-1β-induced COX-2 expression in A549 cells [44]. However, whether tangeretin could simultaneously inhibits PKC activation as well as through MAPKs and PI3K/AKT suppression to downregulate IL-1β-induced COX-2 expression in A549 cells is still unknown.

In conclusion, the findings of the present study indicate that tangeretin is able to inhibit IL-1β-induced COX-2 expression in A549 cells by suppression of p38 MAPK, JNK, and PI3K/AKT, as well as NF-κB. To our knowledge, it is the first report to show that tangeretin can repress p38 MAPK, JNK, and AKT activation. More importantly, tangeretin significantly represses induced and constitutively expressed COX-2 in human lung cancer cells, indicating polymethoxylated flavonoids may be a new and potent chemopreventive agent in cancer therapy.

REFERENCES

[1] Hirsch FR, Lippman SM. Advances in the biology of lung cancer chemoprevention. J Clin Oncol 2005;23:3186–97.
[2] Brown JR, DuBois RN. Cyclooxygenase as a target in lung cancer. Clin Cancer Res 2004;10:4266–9.
[3] Kim ES, Hong WK, Khuri FR. Prevention of lung cancer. The new millennium. Chest Surg Clin North Am 2000;10:663–90.
[4] Cancer facts and figures 2000. Atlanta, GA: American Cancer Society; 2000, http://www.cancer.org/dowloads/STT/F&F00.pdf.
[5] Petkova DK, Clelland C, Ronan J, Pang L, Coulson JM, Lewis S, et al. Overexpression of cyclooxygenase-2 in non-small cell lung cancer. Respir Med 2004;98:164–72.
[6] Sjodahl R. Nonsteroidal anti-inflammatory drugs and the gastrointestinal tract. Extent, mode, and dose dependence of anticancer effects. Am J Med 2001;110:66–9.
[7] Khuri FR, Wu H, Lee JJ, Kemp BL, Lotan R, Lippman SM, et al. Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin Cancer Res 2001;7:861–7.
[8] Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97–120.
[9] Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS, et al. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci 2002;99:13926–31.
[10] Koki A, Khan NK, Woerner BM, Dannenberg AJ, Olson L, Seibert K, et al. Cyclooxygenase-2 in human pathological disease. Adv Exp Med Biol 2002;507:177–84.
[11] Hida T, Yatabe Y, Achiwa H, Muramatsu H, Kozaki K, Nakamura S, et al. Increased expression of cyclooxygenase 2 occurs frequently in human lung cancers, specifically in adenocarcinomas. Cancer Res 1998;58:3761–4.
[12] Hosomi Y, Yokose T, Hirose Y, Nakajima R, Nagai K, Nishiwaki Y, et al. Increased cyclooxygenase 2 (COX-2) expression occurs frequently in precursor lesions of human adenocarcinoma of the lung. Lung Cancer 2000;30:73–81.
[13] Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, Ristimaki A. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res 1998;58:4997–5001.
[14] Achiwa H, Yatabe Y, Hida T, Kuroishi T, Kozaki K, Nakamura S, et al. Prognostic significance of elevated cyclooxygenase 2 expression in primary, resected lung adenocarcinomas. Clin Cancer Res 1999;5:1001–5.
[15] Brabender J, Park J, Metzger R, Schneider PM, Lord RV, Holscher AH, et al. Prognostic significance of cyclooxygenase 2 mRNA expression in non- small cell lung cancer. Ann Surg 2002;235:440–3.
[16] Barnes PJ. Cytokines as mediators of chronic asthma. Am J Respir Crit Care Med 1994;150(5 Pt 2):42–9.
[17] Mattoli S, Mattoso VL, Soloperto M, Allegra L, Fasoli A. Cellular and biochemical characteristics of bronchoalveolar lavage fluid in symptomatic nonallergic asthma. J Allergy Clin Immunol 1991;87(4):794–802.
[18] Tsukagoshi H, Sakamoto T, Xu W, Barnes PJ, Chung KF. Effect of interleukin-1 beta on airway hyperresponsiveness and inflammation in sensitized and nonsensitized Brown-Norway rats. J Allergy Clin Immunol 1994;93(2):464–9.
[19] Belvisi MG, Saunders MA, Haddad el-B, Hirst SJ, Yacoub MH, Barnes PJ, et al. Induction of cyclo-oxygenase-2 by cytokines in human cultured airway smooth muscle cells:

novel inflammatory role of this cell type. Br J Pharmacol 1997;120(5):910–6.
[20] Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, et al. Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J Biol Chem 1992;267(36):25934–8.
[21] Hempel SL, Monick MM, Hunninghake GW. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. J Clin Invest 1994;93(1):391–6.
[22] Dinarello CA. Biologic basis for interleukin-1 in disease. Blood 1996;87(6):2095–147.
[23] Jang BC, Lim KJ, Paik JH, Kwon YK, Shin SW, Kim SC, et al. Up-regulation of human beta-defensin 2 by interleukin-1beta in A549 cells: involvement of PI3K, PKC, p38 MAPK, JNK, and NF-kappaB. Biochem Biophys Res Commun 2004;320(3):1026–33.
[24] Chen P, Cai Y, Yang ZG, Zhou R, Zhang GS, Domann F, et al. Involvement of PKC, p38 MAPK and AP-2 in IL-1beta-induced expression of cyclooxygenase-2 in human pulmonary epithelial cells. Respirology 2006;11(1):18–23.
[25] Attaway JA. Citrus juice flavonoids with anticarcinogenic and antitumor properties. In: Huang MT, Osawa T, Ho CT, Rosen RT, editors. Food Phytochemicals for Cancer Prevention I. Washington, DC: American Chemical Society; 1994. p. 240–8.
[26] Stavric B. Antimutagens and anticarcinogens in foods. Food Chem Toxicol 1994;32:79–90.
[27] Bracke M, Bruyneel EA, Vermeulen SJ, Vennekens K, Marck VV, Mareel MM. Citrus flavonoid effect on tumor invasion and metastasis. Food Technol 1994;48:121–42.
[28] Hirano T, Abe K, Gotoh M, Oka K. Citrus flavone tangeretin inhibits leukaemic HL-60 cell growth partially through induction of apoptosis with less cytotoxicity on normal lymphocytes. Br J Cancer 1995;72(6):1380–8.
[29] Nelson EK. The occurrence of pentamethyl flavonol in tangerine peel. J Am Chem Soc 1934;56:1392–3.
[30] Ben-Aziz A. Nobiletin is main fungistat in tangerines resistant to mal secco. Science 1967;155:1026–7.
[31] So FV, Guthrie N, Chambers AF, Moussa M, Carroll KK. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer 1996;26(2):167–81.
[32] Fuhr U. Drug interactions with grapefruit juice. Extent, probable mechanism and clinical relevance. Drug Saf 1998;18(4):251–72.
[33] Chaumontet C, Bex V, Gaillard-Sanchez I, Seillan-Heberden C, Suschetet M, Martel P. Apigenin and tangeretin enhance gap junctional intercellular communication in rat liver epithelial cells. Carcinogenesis 1994;15(10):2325–30.
[34] Chaumontet C, Droumaguet C, Bex V, Heberden C, Gaillard-Sanchez I, Martel P. Flavonoids (apigenin, tangeretin) counteract tumor promoter-induced inhibition of intercellular communication of rat liver epithelial cells. Cancer Lett 1997;114(1/2):207–10.
[35] Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. Antiproliferative activity of flavonoids on several cancer cell lines. Biosci Biotechnol Biochem 1999;63(5):896–9.
[36] Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, Lin JK. Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinases 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. Carcinogenesis 2002;23(10):1677–84.
[37] Apte RN, Voronov E. Interleukin-1—a major pleiotropic cytokine in tumor–host interactions. Semin Cancer Biol 2002;12(4):277–90.
[38] Lin YL, Juan IM, Chen YL, Liang YC, Lin JK. Composition of polyphenols in fresh tea leaves and associations of their oxygen-radical absorbing capacity with antiproliferative actions in fibroblast cells. J Agric Food Chem 1996;44: 1387–94.
[39] Wenzel U, Kuntz S, Brendel MD, Daniel H. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res 2000;60(14):3823–31.
[40] Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. Eur J Cancer 1999;35(10):1517–25.
[41] Nieminen R, Leinonen S, Lahti A, Vuolteenaho K, Jalonen U, Kankaanranta H, et al. Inhibitors of mitogen-activated protein kinases downregulate COX-2 expression in human chondrocytes. Mediators Inflamm 2005;2005(5):249–55.
[42] Rosch S, Ramer R, Brune K, Hinz B. Prostaglandin E2 induces cyclooxygenase-2 expression in human non-pigmented ciliary epithelial cells through activation of p38 and p42/44 mitogen-activated protein kinases. Biochem Biophys Res Commun 2005;338(2):1171–8.
[43] Cho JW, Park K, Kweon GR, Jang BC, Baek WK, Suh MH, et al. Curcumin inhibits the expression of COX-2 in UVB-irradiated human keratinocytes (HaCaT) by inhibiting activation of AP-1: p38 MAP kinase and JNK as potential upstream targets. Exp Mol Med 2005;37(3):186–92.
[44] Lin CH, Sheu SY, Lee HM, Ho YS, Lee WS, Ko WC, et al. Involvement of protein kinase C-gamma in IL-1beta-induced cyclooxygenase-2 expression in human pulmonary epithelial cells. Mol Pharmacol 2000;57(1): 36–43.
[45] Shishodia S, Koul D, Aggarwal BB. Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates TNF-induced NF-kappa B activation through inhibition of activation of I kappa B alpha kinase and Akt in human non-small cell lung carcinoma: Correlation with suppression of COX-2 synthesis. J Immunol 2004;173(3):2011–22.
[46] Shishodia S, Aggarwal BB. Cyclooxygenase (COX)-2 inhibitor celecoxib abrogates activation of cigarette smoke-induced nuclear factor (NF)-kappaB by suppressing activation of IkappaBalpha kinase in human non-small cell lung carcinoma: correlation with suppression of cyclin D1, COX-2, and matrix metalloproteinase-9. Cancer Res 2004;64(14):5004–12.
[47] Petkova DK, Clelland C, Ronan J, Pang L, Coulson JM, Lewis S, et al. Overexpression of cyclooxygenase-2 in non-small cell lung cancer. Respir Med 2004;98(2):164–72.
[48] Jang MH, Shin MC, Lim S, Han SM, Park HJ, Shin I, et al. Bee venom induces apoptosis and inhibits expression of cyclooxygenase-2 mRNA in human lung cancer cell line NCI-H1299. J Pharmacol Sci 2003;91(2):95–104.
[49] Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, Yano M, et al. Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem Pharmacol 2003;65(12): 2065–71.
[50] Van Slambrouck S, Parmar VS, Sharma SK, De Bondt B, Fore F, Coopman P, et al. Tangeretin inhibits extracellular-signal-regulated kinase (ERK) phosphorylation. FEBS Lett 2005;579(7):1665–9.
[51] Sizemore N, Leung S, Stark GR. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. Mol Cell Biol 1999;19(7):4798–805.
[52] Teshima S, Nakanishi H, Nishizawa M, Kitagawa K, Kaibori M, Yamada M, et al. Up-regulation of IL-1 receptor through PI3K/Akt is essential for the induction of iNOS gene expression in hepatocytes. J Hepatol 2004;40(4):616–23.
[53] Reddy SA, Huang JH, Liao WS. Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFkappaB and AP-1 activation. J Biol Chem 1997;272(46):29167–73.

[54] Grimm S, Baeuerle PA. The inducible transcription factor NF-kappa B: structure–function relationship of its protein subunits. Biochem J 1993;290(Pt 2):297–308.
[55] Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–79.
[56] Ogasawara A, Arakawa T, Kaneda T, Takuma T, Sato T, Kaneko H, et al. Fluid shear stress-induced cyclooxygenase-2 expression is mediated by C/EBP beta, cAMP-response element-binding protein, and AP-1 in osteoblastic MC3T3-E1 cells. J Biol Chem 2001;276(10):7048–54.
[57] Bazan NG, Lukiw WJ. Cyclooxygenase-2 and presenilin-1 gene expression induced by interleukin-1beta and amyloid beta 42 peptide is potentiated by hypoxia in primary human neural cells. J Biol Chem 2002;277(33):30359–67.
[58] Minden A, Lin A, Smeal T, Derijard B, Cobb M, Davis R, et al. c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK sub-group of mitogen-activated protein kinases. Mol Cell Biol 1994;14(10):6683–8.