Research Article

Wogonin, a bioactive flavonoid in herbal tea, inhibits inflammatory cyclooxygenase-2 gene expression in human lung epithelial cancer cells

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Wogonin, a naturally occurring plant flavonoid, is isolated from Chinese herbal plants *Scutellaria baicalensis* Georgi and *S. barbata* D. Don. The extract of *S. baicalensis* Georgi has been added to an assortment of health drinks or food supplements. Wogonin has been reported to exhibit anticancer and anti-inflammatory properties. Cyclooxygenase-2 (COX-2) is a key enzyme in the production of prostaglandins in inflammatory conditions. In this study, the effect of wogonin on phorbol 12-myristate 13-acetate (PMA)-induced COX-2 expression was investigated. It showed that wogonin inhibited PMA-induced COX-2 protein and mRNA levels in human lung epithelial cancer cells, and the mechanism of this inhibition was at the transcriptional level by using COX-2 gene promoter assay. Among various signal inhibitors, the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 also inhibited PMA-induced COX-2 expression and COX-2 promoter activation. The activity of AP-1-driven promoter, but not nuclear factor-kappa B (NF-κB), was inhibited by U0126. The data indicated that MEK1/2-AP-1 is very important for PMA-induced COX-2 expression. Wogonin also inhibited PMA-induced AP-1 activation and the expression of c-Jun, a key component of AP-1. Taken together, it is suggested that wogonin inhibits PMA-induced COX-2 gene expression by inhibiting c-Jun expression and AP-1 activation in A549 cells.

Keywords: Cyclooxygenase-2 / c-Jun / Lung cancer / Phorbol 12-myristate 13-acetate / Wogonin

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1 Introduction

Wogonin (5,7-dihydroxy-8-methoxyflavone) (Fig. 1A) is found in the traditional Chinese herb *Scutellaria baicalensis* Georgi [1], which has been used to treat allergic and inflammatory diseases by its traditional indications such as "cleansing heat" and "removing toxins." Wogonin is also found in another Chinese medicinal plant *S. barbata* D. Don [2], which has usually been brewed in water and used as a health drink in Taiwan. Wogonin has been reported to have anticancer [3], anti-inflammation [4, 5], and anti-angiogenesis activities [6]. Because of these beneficial bioactivities, *S. baicalensis* extract has been used as an additive to drinks or food supplements for health benefit. According to the pharmacokinetics study, it has been shown that wogonin is absorbed into plasma by oral administration of *S. baicalensis* extract [7, 8].

Cyclooxygenase (COX) is a key enzyme in prostaglandins (PGs) biosynthesis. PGs are synthesized in various tissues and cells, and are regulators of inflammation, fever,

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Abbreviations: Abs, antibodies; **COX-2,** cyclooxygenase-2; **CRE,** cAMP response element; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **MAPK,** mitogen-activated protein kinase; **MCP-1,** monocyte chemotactic protein-1; **MEK1/2,** mitogen-activated protein kinase kinase 1/2; **MMP-9,** matrix metalloproteinase-9; **NF-κB,** nuclear factor-kappa B; **PMA,** phorbol 12-myristate 13-acetate; **PG,** prostaglandin; **RLU,** relative luciferase unit

and pain. There are two isoforms of COX, COX-1, and COX-2 [9]. COX-1 is a housekeeping enzyme, while COX-2 expression is induced by various stimuli including cytokines, growth factors, hormones, and environmental carcinogens [10]. Based on the facts that the PG products of COX-2 pathway, especially PGE_2, are very effective in enhancing proliferation of cancer cells, and that the expression of COX-2 gene is associated with a variety of malignancies, COX-2 could be a good target for anti-cancer therapy and cancer prevention [11 – 13].

Because COX-2 gene is inducible, its gene promoter is an important region for gene activation. COX-2 promoter region contains several potential transcription regulatory elements, including Sp1, nuclear factor-kappa B (NF-κB), AP-2, NF-IL-6, and cAMP response element (CRE)/E-box binding sites [10]. It was reported that IL-1β induced COX-2 gene expression *via* NF-κB pathway in human lung carcinoma cells [14]. In murine NIH3T3 cells, c-Jun is activated by platelet-derived growth factor (PDGF) and binds to the CRE binding site of COX-2 promoter, which stimulates COX-2 gene expression [15]. Epidermal growth factor (EGF) also activates COX-2 gene by enhancing c-Jun binding to the CRE site of COX-2 promoter in human epidermoid carcinoma cells [16].

Besides IL-1β, PDGF, and EGF, some environmental carcinogens are also important factors for COX-2 induction. Phorbol 12-myristate 13-acetate (PMA), found in croton oil of *Croton tiglium* [17], functions as an environmental tumor promoter and has been reported to modulate diverse cellular responses such as gene transcription, proliferation, differentiation, programmed cell death, immune response, and receptor desensitization through protein kinase C signaling pathway [18, 19]. PMA has been studied to clarify the role of environmental factors involved in the progression of mutations to malignant transformation [20]. PMA had been reported to upregulate COX-2 expression in Madin-Darby Canine kidney cells [21], mouse skin [22], and human lung cancer cells [23]. Due to the possible correlation between malignancy and COX-2, inhibition of PMA-induced COX-2 expression is an option for cancer prevention and tumor treatment.

Some reports indicated that wogonin inhibited PMA-induced COX-2 expression in mouse ears [24], mouse skin fibroblasts [25], and mouse dorsal skin [22]. Since wogonin is one of the bioactive flavonoid in *S. baicalensis* extract, and PMA-induced COX-2 expression may facilitate tumor formation and progression, wogonin-inhibited PMA-induced COX-2 expression merits a better investigation especially in human cancer cells. Among the statistic of cancer-related death, lung cancer is the leading cause of cancer-related deaths in the world [26, 27] and in Taiwan [28], and inhibiting COX-2 has a strong potential for the chemoprevention of human lung cancer [29]. Therefore, we studied whether wogonin inhibited PMA-induced COX-2 gene expression in human lung epithelial cancer cells, and the molecular mechanism of wogonin-inhibited COX-2 expression was elucidated by gene promoter analysis and signal pathway study.

2 Materials and methods

2.1 Cell culture

Human lung carcinoma A549 cells, obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), were cultured in F-12 Kaighn's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained at 37°C in humidified atmosphere with 5% CO_2.

2.2 Cell viability assay

Wogonin was prepared as described by Chen *et al*. [30]. The purity of wogonin was determined by RP-HPLC. The HPLC was conducted on a LiChrospher 100 RP-18e column (5 μm, 4 mm id × 250 mm, Merck, Darmstadt, Germany) eluted with 0.05% TFA/CH_3CN (60:40 v/v). The flow rate was 1.0 mL/min with detection at 280 nm and the oven temperature was maintained at 40°C. The retention time of wogonin was 14.7 min and the purity was shown to exceed 99.6%. The stock solution of wogonin and PMA (Sigma – Aldrich, St. Louis, MO, USA) were prepared by dissolving them in DMSO (Sigma – Aldrich) and then storing them at −20°C until use. A549 cells were culture in 24-well plate (1.2×10^5 cells/well) 1 day before wogonin treatment. DMSO or wogonin was added into A549 cells 1 h before PMA stimulation, and cells were incubated for another 6 h. Cells were collected by trypsin treatment and cell numbers were counted by using a hemocytometer and trypan blue exclusion method.

2.3 Nuclear extract preparation

Cell nuclear extracts were prepared as described by Andrews and Faller [31] with slight modification. Briefly, cells from 10 cm dishes were washed twice with PBS and scraped in 1 mL of PBS. They were then collected by centrifuging at $7500 \times g$ for 30 s, re-suspended in 0.4 mL of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM EDTA) at 4°C for 10 min. Nuclei were pelleted by centrifugation at $7500 \times g$ for 30 s. Pellets were resuspended in 0.1 mL of buffer C (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol v/v) at 4°C for 20 min. The suspension was centrifuged at $7500 \times g$ for 2 min. Supernatants were collected and stored at −80°C until use. Both buffers A and C contain the following protease and phosphatase inhibitors: 0.5 mM phenylmethylsulfonyl, 1 mM orthovanadate, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin.

2.4 Western blotting

Analytical 10% SDS-polyacrylamide slab gel electrophoresis was performed. The total cell lysates and nuclear extracts were prepared and 30 μg of each protein extract was analyzed. For immunoblotting, proteins in the SDS-PAGE gels were transferred to a polyvinylidene difluoride membrane by an electroblot apparatus. Antibodies (Abs) against COX-2 (LAB Vision, Fremont, CA, USA), β-actin (Sigma–Aldrich), mitogen-activated protein kinases (MAPKs) (ERK, JNK, and p38), and phospho-MAPKs (Cell Signaling Technology, Beverly, MA, USA), NF-κB p65 (Abcam, Cambridge, UK), c-Jun (BD Biosciences, San Jose, CA, USA) and Sp1 (Upstate, Charlottesville, VA, USA) were employed as the primary Abs. Immunoblot analysis was carried out with mouse or rabbit IgG Abs coupled to horseradish peroxidase. The enhanced chemiluminescence kit and VL Chemi-Smart 3000 were used for detection.

2.5 Reverse transcription-PCR (RT-PCR)

Total cellular RNA was extracted by the TRIZOL Reagent (Invitrogen) according to the supplier's instructions. For RT-PCR, a cDNA was synthesized from 2 μg of total RNA using SuperScript II (Invitrogen) with reaction at 42°C for 50 min and 95°C for 10 min in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). The cDNA was amplified by PCR using Taq polymerase (MDBio, Taipei, Taiwan) with following specific primers: human COX-2, 5′-ATTGCCCGACTCCCTTGG-3′ (sense) and 5′-TGGCCCAGCCCGTTG-3′ (antisense), PCR reaction for 25 cycles at 94°C, 1 min; 55°C, 30 s; 72°C, 1 min; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGGATTTGGTCGTATTGG-3′ (sense) and 5′-AGATGGTGATGGGATTTC-3′ (antisense), PCR reaction for 25 cycles at 95°C, 40 s; 55°C, 1 min; 72°C, 90 s; human c-Jun, 5′-CCTGCGGACTCCGAGGA-3′ (sense) and 5′-TGTAGCCATAAGGTCCGCTCTC-3′ (antisense), PCR reaction for 25 cycles at 95°C, 40 s; 55°C, 1 min; 72°C, 90 s. PCR products were analyzed by agarose gel electrophoresis and visualized by treatment with ethidium bromide. The length of PCR products were 205, 203, and 401 bp for human COX-2, human GAPDH, and human c-Jun, respectively.

2.6 Transfection and luciferase assay

Plasmids pAP1-Luc and pNFκB-Luc (BD Biosciences Clontech, Palo Alto, CA), and pXC918 (a gift from Dr. Wen-Chang Chang [16]) were transiently transfected into A549 cells by Lipofectamine™ 2000 (Invitrogen) according to supplier's instruction. Cells were subcultured at a density of 9000 cells in 1 mL of culture medium in a 12-well plastic dish for 16 h. In transfection, Lipofectamine 2000 was incubated with plasmid (1 μL Lipofectamine 2000/0.5 μg DNA/well) in 500 μL of OPTI-MEM (Invitrogen) for 20 min at room temperature. The mixture was added dropwise to cells and then incubated for 6 h. After DNA/Lipofectamine 2000 incubation, the medium was changed to DNA-free medium for 18 h. The medium was replaced by serum-free F12K medium for 24 h, 100 μM wogonin were added for 1 h before 10 nM PMA treatment for 6 h. The cells were lysed and cell lysates were prepared for luciferase assay (Promega, Madison, WI, USA) and Bradford protein concentration assay with BioRad Protein Assay (BioRad, Hercules, CA, USA). The relative luciferase activities were normalized to the same protein concentration.

3 Results

3.1 Wogonin inhibits PMA-induced COX-2 protein and mRNA expression

In order to determine the most efficient concentration of wogonin in inhibiting COX-2 protein expression, A549 cells were pretreated with various dosages of wogonin for 1 h followed by a PMA treatment for 6 h. There was no significant cytotoxicity in the presence of 10 nM PMA and/or 100 μM wogonin in A549 cells (Fig. 1B). In Fig. 2A, wogo-

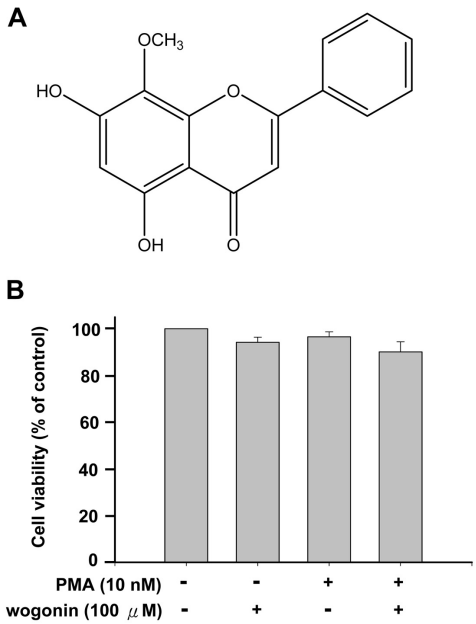

Figure 1. (A) Molecular structure of wogonin. (B) Cell viability after treatment with PMA and/or wogonin in A549 cells. A549 cells were cultured in a 24-well plate 24 h before the addition of wogonin and/or PMA. The cells were pretreated with or without 100 μM wogonin for 1 h and then treated with or without 10 nM PMA for 6 h. The cells were harvested and cell number was determined by using hemocytometer and trypan blue exclusion method. The results were presented as mean ± SD of three independent experiments.

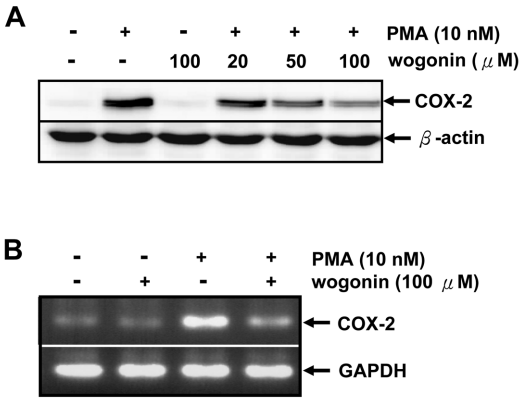

Figure 2. Effect of wogonin on PMA-induced COX-2 protein and mRNA expression. (A) Wogonin -inhibits PMA-induced COX-2 protein expression. A549 cells were pretreated with different concentration of wogonin from 0 to 100 μM for 1 h and then treated with or without 10 nM PMA for 6 h. Total cell lysates were analyzed by Western blotting with anti-COX-2 and anti-β-actin (internal control) Abs. This experiment was repeated three times. (B) Wogonin inhibits PMA-induced COX-2 mRNA expression. A549 cells were pretreated with or without 100 μM wogonin for 1 h before being treated with or without 10 nM PMA for 2 h. Total cellular RNA was isolated for mRNA level measurement by RT-PCR with specific primers for COX-2 and GAPDH (internal control). This experiment was repeated three times.

nin treatment markedly inhibited the PMA-induced COX-2 protein expression in a dose-dependent manner from 20 to 100 μM. Because the increase of COX-2 protein is usually based on transcriptional activation, we next identified whether the inhibitory effect of wogonin on PMA-induced COX-2 was transcriptional inhibition. COX-2 mRNA expression was detected by RT-PCR, which showed that 100 μM wogonin also markedly inhibited PMA-induced COX-2 mRNA expression (Fig. 2B). It suggested that wogonin transcriptionally inhibited COX-2 gene expression.

3.2 Wogonin inhibits PMA-induced COX-2 promoter activity

In order to confirm the inhibitory effect of wogonin on PMA-induced COX-2 gene expression by transcriptional inhibition, we analyzed the transcriptional activity of human COX-2 gene promoter which contains Sp1, NF-κB, AP-2, NF-IL-6, CRE/E-box, and TATA sites (Fig. 3A) [16]. As shown in Fig. 3B, after transient transfection of COX-2 promoter-driven luciferase vector pXC918, PMA significantly enhanced the luciferase activity and wogonin completely inhibited this induction. This reconfirmed that wogonin transcriptionally inhibited COX-2 gene expression and its inhibitory target site was within COX-2 promoter from −918 to+49 bp.

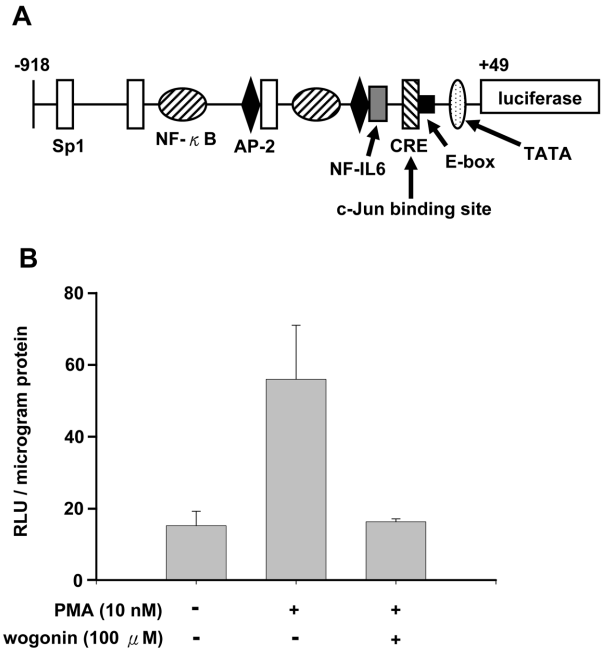

Figure 3. Effect of wogonin on PMA-induced COX-2 promoter activity. (A) The promoter and transcription factor binding sites of pXC918 containing human COX-2 gene promoter and luciferase gene [16]. (B) Effect of wogonin on PMA-induced COX-2 promoter activity. COX-2 promoter reporter gene pXC918 was transfected into A549 cells. After treatment with or without wogonin for 1 h, cells were treated with or without PMA for another 6 h. Total cell lysates were analyzed by luciferase activity assay and protein concentration assay. The luciferase activity was presented as relative luciferase unit (RLU) *per* μg protein. Each value represents the mean ± SD of three determinations.

3.3 Effect of MAPK inhibitors and NF-κB inhibitor on PMA-induced COX-2 expression

It has been reported that PMA activates COX-2 gene expression through MAPK ERK and NF-κB pathways in A549 cells [23]. We then analyzed whether wogonin inhibited PMA-induced MAPK phosphorylation and NF-κB nuclear translocation. The data show that wogonin did not inhibit PMA-induced phosphorylation of ERK, JNK, and p38 (Fig. 4A), and did not inhibit PMA-induced NF-κB p65 nuclear translocation (Fig. 4B). In spite of these results, the downstream molecule of MAPK or NF-κB might be affected by wogonin. In order to identify whether ERK, JNK, p38, or NF-κB is important for PMA-induced COX-2 expression, we compared the inhibitory effect of individual MAPK inhibitors and NF-κB inhibitor on PMA-induced COX-2 expression. The data show that only U0126 (an MEK inhibitor) inhibited PMA-induced COX-2 expression (Fig. 5A), and U0126 also inhibited PMA-induced COX-2 promoter pXC918 activation (Fig. 5B). It suggested that ERK, not JNK, p38 or NF-κB, is the key pathway in PMA-induced COX-2 gene expression. Because transcription fac-

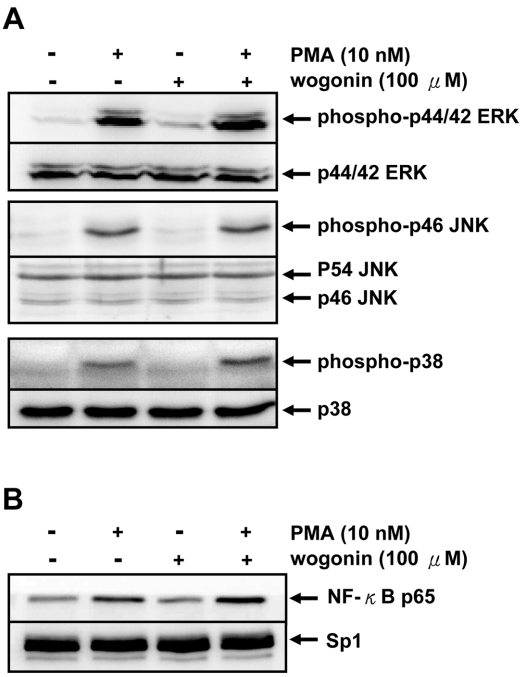

Figure 4. Effect of wogonin on PMA-induced MAPKs phosphorylation and NF-κB p65 nuclear translocation. (A) Wogonin does not inhibit PMA-induced MAPKs phosphorylation. A549 cells were pretreated with or without 100 μM wogonin for 1 h and then treated with or without PMA for 30 min. The phosphorylation level of ERK, JNK, and p38 were measured by Western blotting with antiphospho-ERK, antiphospho-JNK, antiphospho-p38, and antitotal ERK, JNK and p38 (internal control) Abs. This experiment was repeated three times. (B) Wogonin does not inhibit PMA-induced nuclear translocation of NF-κB p65. After incubating with or without 100 μM wogonin for 1 h, cells were treated with or without PMA for another 2 h. Cell nuclear extracts were collected and analyzed by Western blotting with anti-NF-κB p65 and anti-Sp1 (internal control) Abs. This experiment was repeated three times.

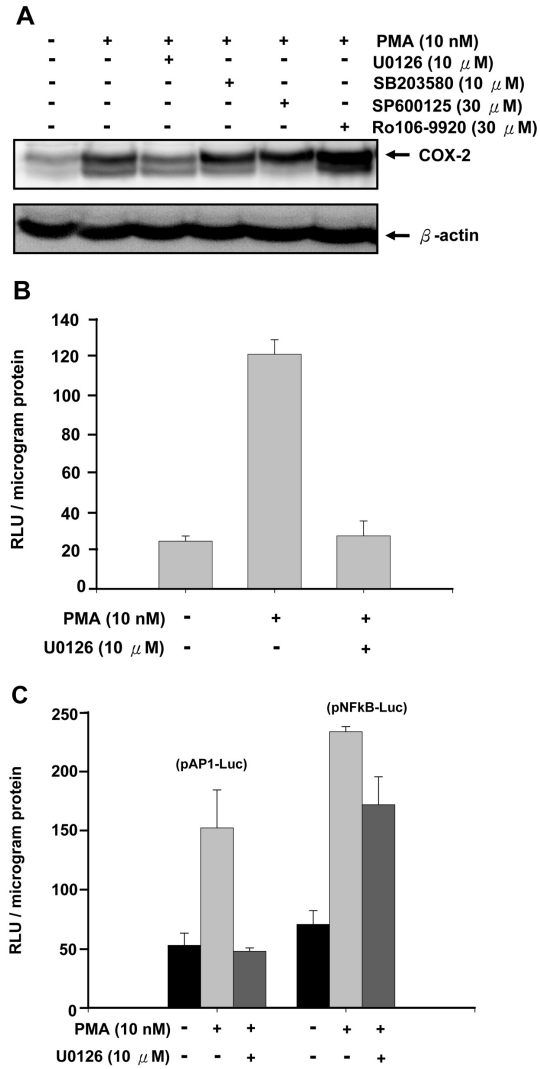

Figure 5. Effect of inhibitor(s) on PMA-induced COX-2 protein expression and transcription factor activities. (A) Effect of various inhibitors on PMA-induced COX-2 protein expression. Cells were pretreated with or without various inhibitors 1 h before being treated with or without PMA for 6 h. Total cell lysates were analyzed by Western blotting with anti-COX-2 and anti-β-actin (internal control) Abs. This experiment was repeated five times. (B) Effect of U0126 on PMA-induced COX-2 promoter activity. COX-2 promoter reporter gene pXC918 was transfected into A549 cells. After treatment with or without U0126 for 1 h, cells were treated with or without PMA for another 6 h. Total cell lysates were analyzed by luciferase activity assay and protein concentration assay. The luciferase activity was presented as RLU *per* μg protein. Each value represents the mean ± SD of three determinations. (C) Effect of U0126 on PMA-induced AP-1-driven reporter gene and NF-κB-driven reporter gene activities. Plasmid DNA pAP1-Luc or pNFκB-Luc was transfected into A549 cells. After treatment with or without U0126 for 1 h, cells were treated with or without PMA for another 6 h. Total cell lysates were analyzed by luciferase activity assay and protein concentration assay. The luciferase activity was presented as RLU *per* μg protein. Each value represents the mean ± SD of three determinations.

tor AP-1 and NF-κB have been reported to be the downstream elements of ERK [23, 32], we further analyzed the effect of U0126 on PMA-induced AP-1 and NF-κB activation. The data show that U0126 completely inhibited PMA-induced AP-1 activation, and slightly inhibited PMA-induced NF-κB activation (Fig. 5C). It suggested that ERK-AP-1 is the main pathway in PMA-induced COX-2 gene expression.

3.4 Wogonin inhibits PMA-induced transcriptional activity of AP-1

According to the results of Fig. 5, PMA-induced COX-2 expression was mainly mediated through ERK-AP-1 pathway, and wogonin did not affect the phosphorylation of ERK (Fig. 4A). Therefore, the effect of wogonin on PMA-induced AP-1 activation was studied. The data show that wogonin completely inhibited PMA-induced AP-1 activa-

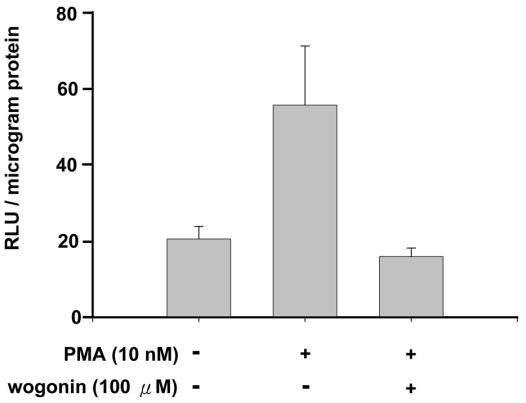

Figure 6. Effect of wogonin on PMA-induced AP-1-driven reporter gene activity. A549 cells were transfected with AP-1-driven reporter gene plasmid, pAP1-Luc. After pretreatment with or without wogonin for 1 h, A549 cells were treated with or without PMA for another 6 h. Total cell lysates were analyzed by luciferase activity assay and protein concentration assay. The luciferase activity was presented as RLU *per* μg protein. Each value represents the mean ± SD of three determinations.

tion (Fig. 6). It suggested that the inhibition of PMA-induced AP-1 activation by wogonin contributed to the inhibition of PMA-induced COX-2 expression.

3.5 Wogonin inhibits PMA-induced c-Jun protein and mRNA expression

AP-1 heterodimer consists of c-Jun/c-Fos or c-Jun/ATF2, and AP-1 homodimer consists of c-Jun dimer. It has been reported that c-Jun protein is increased in, and important for, stimulator-induced COX-2 gene expression *via* CRE binding site [15, 16]. Therefore, we analyzed the effect of PMA and wogonin on c-Jun protein expression. The data show that PMA induced c-Jun protein expression in A549 cells, and wogonin inhibited PMA-induced c-Jun expression in protein (Fig. 7A) and mRNA level (Fig. 7B). Next, we also examined the effect of U0126 on PMA-induced c-Jun expression. As shown in Fig. 7C, U0126 also inhibited PMA-induced c-Jun protein expression. It was suggested that inhibition of PMA-induced COX-2 expression by wogonin was mainly mediated by inhibiting PMA-induced c-Jun protein expression.

3.6 Overexpression of c-Jun induces COX-2 promoter activity

In order to confirm the transcript factor c-Jun is important for COX-2 gene expression, the effect of c-Jun overexpression on the promoter activity of COX-2 was studied. A549 cells were transfected with 0.4 μg of c-Jun expression plasmid pRSVjun together with plasmid pXC918 for 48 h. The data show that overexpression of c-Jun significantly enhanced the COX-2 promoter activity (Fig. 8).

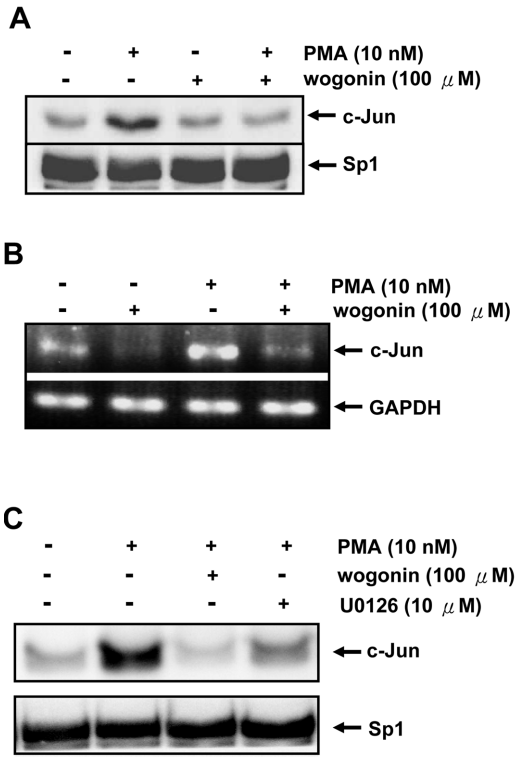

Figure 7. Effect of wogonin on PMA-induced c-Jun protein and mRNA expression. (A) Wogonin inhibits PMA-induced c-Jun protein expression. After incubating with or without 100 μM wogonin for 1 h, cells were treated with or without PMA for another 2 h. Cell nuclear extracts were collected and analyzed by Western blotting with anti-c-Jun and anti-Sp1 (internal control) Abs. This experiment was repeated three times. (B) Wogonin inhibits PMA-induced c-Jun mRNA expression. A549 cells were pretreated with or without 100 μM wogonin for 1 h, and treated with or without 10 nM PMA for another 2 h. Total cellular RNA were isolated and analyzed by RT-PCR with specific primers for c-Jun and GAPDH (internal control). This experiment was repeated three times. (C) U0126 inhibits PMA-induced c-Jun protein expression. After incubating with or without 100 μM wogonin or 10 μM U0126 for 1 h, cells were treated with or without PMA for another 2 h. Cell nuclear extracts were collected and analyzed by Western blotting with anti-c-Jun and anti-Sp1 (internal control) Abs. This experiment was repeated three times.

3.7 Effect of wogonin on PMA-induced ERK activity

It is known that c-Jun is an autoactivated gene by MAPK pathway and AP-1 binding site [33, 34], and PMA-induced ERK activation is required for PMA-induced AP-1 activation [32]. Because wogonin inhibited PMA-induced c-Jun expression (Fig. 7A) without inhibiting PMA-induced ERK phosphorylation (Fig. 4A), we then analyzed the effect of wogonin on PMA-activated ERK activity. When ERK is activated in response to stimulators, it phosphorylates certain downstream factors including a 90 kDa ribosomal S6

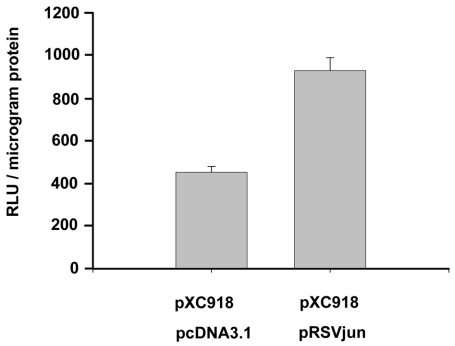

Figure 8. Overexpression of c-Jun protein enhanced COX-2 promoter activity. A549 cells were transfected with pXC918 and c-Jun overexpressed plasmid pRSVjun [16] or control vector pcDNA3.1. Total cell lysates were harvested 48 h after transfection and analyzed by luciferase activity assay and protein concentration assay. The luciferase activity was presented as RLU *per* μg protein. Each value represents the mean ± SD of three determinations.

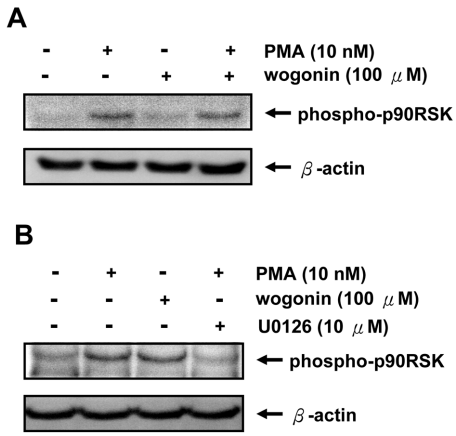

Figure 9. Effect of wogonin and U0126 on PMA-induced ERK activity. (A) Effect of wogonin on PMA-induced ERK activity. A549 cells were pretreated with or without 100 μM wogonin for 1 h and then treated with or without PMA for 40 min. Total cell lysates were analyzed by Western blotting with antiphospho-p90RSK and anti-β-actin (internal control) Abs. This experiment was repeated three times. (B) Effect of U0126 on PMA-induced ERK activity. A549 cells were pretreated with or without 100 μM wogonin or 10 μM U0126 for 1 h and then treated with or without PMA for 40 min. Total cell lysates were analyzed by Western blotting with anti-phospho-p90RSK and anti-β-actin (internal control) Abs. This experiment was repeated three times.

kinase (p90RSK). PMA enhanced p90RSK phosphorylation after the cells had been stimulated for 40 min, and wogonin did not inhibit PMA-induced p90RSK phosphorylation (Fig. 9A). In contrast to wogonin, U0126 inhibited PMA-induced p90RSK phosphorylation (Fig. 9B) because it inhibited mitogen-activated protein kinase kinase 1/2 (MEK1/2)-induced ERK phosphorylation. This data suggested that the inhibition of PMA-induced AP-1 activation by wogonin was not mediated by directly inhibiting ERK activity.

4 Discussion

The overexpression of COX-2 in premalignant and malignant tissues provided substantial evidence that connected COX-2 to the development of cancer. Hence, many researches are being done in order to find the chemicals that inhibit COX-2 expression and can be used for cancer prevention or treatment [10 – 13]. As previous reports have shown that natural herb extract wogonin exhibited inhibitory effect on COX-2 expression [4, 5, 22, 24, 25, 30] in various cells, we also identified that wogonin inhibited PMA-induced COX-2 expression in A549 cells. This is the first time that the cellular and molecular mechanism of wogonin inhibition of COX-2 gene promoter in human cells was demonstrated.

In the present study, we found that ERK pathway played an important role in the inhibitory effect of wogonin on PMA-induced COX-2 expression. Initially, we showed that wogonin had an inhibitory effect on PMA-induced COX-2 expression in a dose-dependent manner (Fig. 2A), and wogonin also significantly inhibited PMA-induced COX-2 mRNA expression (Fig. 2B) and promoter activity (Fig. 3) at 100 μM. In addition, MEK inhibitor U0126 also inhibited PMA-induced COX-2 expression (Fig. 5A). It suggested that PMA-PKC-MEK-ERK was essential in COX-2 induction and wogonin might inhibit this pathway, too. Although PMA also activates JNK, p38, and NF-κB pathways (Fig. 4), they were not important in COX-2 induction according to their specific inhibitor study (Fig. 5A). The importance of ERK pathway in PMA-induced COX-2 expression has also been reported by others [23]. Besides, there was other report indicating that NF-κB had been regarded as the important upstream modulators in the transcriptional control of COX-2 gene expression by IL-1β [14]. However, the NF-κB inhibitor Ro106-9920 had no effect on PMA-induced COX-2 expression in our study. In order to check the efficacy of Ro106-9920 in inhibiting NF-κB activation, we examined the effect of Ro106-9920 on PMA-induced NF-κB transcriptional activity and p65 nuclear translocation. Ro106-9920 (30 μM) completely inhibit PMA-induced luciferase activity of pNFκB-Luc and PMA-induced p65 nuclear translocation (data not shown). It suggested that NF-κB was not essential for PMA-induced COX-2 expression in A549 cells.

Transcriptional factor c-Jun also carries an important role in regulating the expression of COX-2 [15, 16]. Our data showed that wogonin not only inhibited PMA-induced c-Jun protein and mRNA expression (Fig. 7), but also inhibited the AP-1-driven luciferase activity (Fig. 6). Because the c-Jun proto-oncogene is positively autoregulated by its product c-Jun [34], and ERK is an essential pathway for

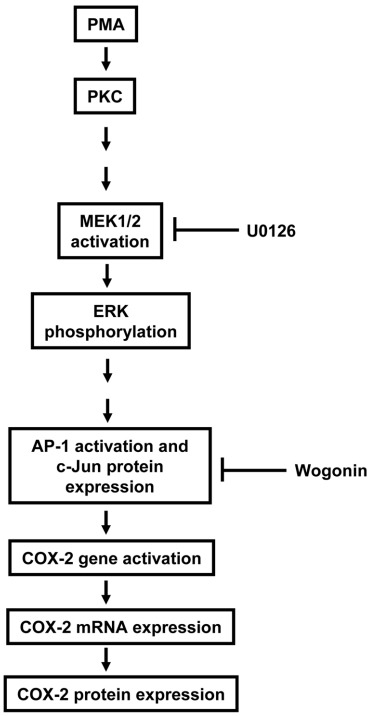

Figure 10. Schematic illustration of proposed intracellular signaling of PMA-induced COX-2 expression and the inhibitory site of wogonin and U0126.

PMA-induced AP-1 activity [32], wogonin's inhibition of PMA-induced COX-2 expression is at least in part through the inhibition of the ERK-AP-1-c-Jun pathway.

In COX-2 promoter assay, wogonin showed a complete inhibition of PMA-induced pXC918 (Fig. 3B). In Western blot and RT-PCR analysis, wogonin inhibited PMA-induced COX-2 protein (Fig. 2A) and mRNA expression (Fig. 2B) but the inhibition was not complete. The difference of these results might be due to the limited length of COX-2 promoter of pXC918. There might be other regulatory sites located in the distant 5′ upstream of COX-2 promoter –918 or other post-transcriptional regulation involved.

In the regulation of PG production, it has been reported that wogonin also directly inhibits COX-2 activity with an IC_{50} of 46 μM [4]. The dual function of wogonin in direct enzymatic inhibition and gene expression inhibition of COX-2 provides more effectiveness in blocking PG biosynthesis. A wogonin-related compound acacetin was also shown to have an inhibitory effect in PMA-induced papilloma formation in mice, *via* inhibition of COX-2 and iNOS gene expression [35]. Besides the inhibition of COX-2, another wogonin-related compound 2′,4′,7-trimethoxyflavone inhibits PG production by inhibiting phospholipase A2 activity [36]. This information suggests that wogonin and its related flavonoid derivatives provide the potential for new anti-inflammatory agent.

Besides the inhibition of COX-2 gene expression, wogonin was also reported to inhibit tumor necrosis factor-α-induced matrix metalloproteinase-9 (MMP-9) expression by inhibiting the NF-κB pathway in human aortic smooth muscle cells [37], PMA-induced monocyte chemotactic protein-1 (MCP-1) gene expression *via* downregulation of JNK, ERK, and AP-1 activity in human endothelial cells [38], and LPS-induced angiogenesis in human umbilical endothelial cells [6]. COX-2 and MCP-1 are the important factors for inflammatory reaction, and MMP-9 plays a major role in the pathogenesis of atherosclerosis. Therefore, inhibition of COX-2, MCP-1, and MMP-9 expression by wogonin could be the key mechanism for its anti-inflammation and anti-angiogenesis.

In conclusion, wogonin, a major flavonoid in *S. baicalensis* extract, inhibited PMA-induced COX-2 gene expression *via* the inhibition of c-Jun protein and mRNA expression, and AP-1 promoter activity (Fig. 10). Transcription factor c-Jun is an inducible factor that plays a role in the expression of over 100 genes involved in immunity, inflammation, and proliferation. Because wogonin inhibits c-Jun expression and AP-1 transcriptional activity, this might provide one of the mechanisms of *S. baicalensis* extract in its traditional indication "cleansing heat" and "removing toxins."

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