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Acacetin suppressed LPS-induced up-expression of iNOS and COX-2 in murine macrophages and TPA-induced tumor promotion in mice

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Lipopolysaccharide (LPS)
Mitogen-activated protein (MAPK)
Phosphatidylinositiol 3-kinase (PI3K/Akt)
Cyclooxygenase-2 (COX-2)

Abbreviations:
iNOS, inducible nitric oxide synthase
LPS, lipopolysaccharide
NO, nitric oxide
COX-2, cycoloxygenase-2
PGE_2, prostaglandin E_2
MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

ABSTRACT

Acacetin (5,7-dihydroxy-4′-methoxyflavone), a flavonoid compound, has anti-peroxidative and anti-inflammatory effects. In this study, we investigated the inhibitory effects of acacetin and a related compound, wogonin, on the induction of NO synthase (NOS) and COX-2 in RAW 264.7 cells activated with lipopolysaccharide (LPS). Acacetin markedly and actively inhibited the transcriptional activation of iNOS and COX-2. Western blotting, reverse transcription-polymerase chain reaction (PCR), and real-time PCR analyses demonstrated that acacetin significantly blocked protein and mRNA expression of iNOS and COX-2 in LPS-inducted macrophages. Treatment with acacetin reduced translocation of nuclear factor-κB (NFκB) subunit and the dependent transcriptional activity of NFκB. The activation of NFκB was inhibited by prevention of the degradation of inhibitor κB (IκB). Furthermore, acacetin inhibited LPS-induced phosphorylation as well as degradation of IκBα. We further investigated the roles of tyrosine kinase, phosphatidylinositiol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) in LPS-induced macrophages. We found that acacetin also inhibited LPS-induced activation of PI3K/Akt and p44/42, but not p38 MAPK. After initiation of 7,12-dimethlybene[*a*]anthracene (DMBA), applying acacentin topically before each 12-O-tetradecanoylphorbol 13-acetat (TPA) treatment was found to reduce the number of papillomas at 20 weeks. Taken together, these results show that acacetin down regulates inflammatory iNOS and COX-2 gene expression in macrophages by inhibiting the activation of NFκB by interfering with the activation PI3K/Akt/IKK and MAPK, suggesting that acacetin is a functionally novel agent capable of preventing inflammation-associated tumorigenesis.

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iκB, inhibitor κB
NF-κB, nuclear factor-κB
MAPK, mitogen-activated protien kinase

1. Introduction

Acacetin (5,7-dihydroxy-4′-methoxyflavone), present in safflower seeds, plants, flowers, *Cirisium rhinoceros* Nakai, has been reported to be able to exert anti-peroxidative, anti-inflammatory, anti-plasmodial [1–3], and anti-proliferative activities by inducing apoptosis and blocking the progression of cell cycles [4–6]. In our recent studies, we have shown that acacetin inhibits cell growth and induces apoptosis in human gastric carcinoma cells [7]. Although a broad range of biological and pharmacological activities of acacetin have been reported, the mechanism(s) behind its anti-inflammatory and anti-carcinogenic effects are not fully understood.

Cyclooxygenase-2 (COX-2) is an inducible enzyme catalyzing the conversion of arachidonic acid to prostaglandins. Recent studies have suggested that increased levels of prostaglandins and cyclooxygenase activity may play important roles in multiple epithelial cancers such as colon carcinoma [8,9]. COX-2 plays a critical role not only in maintaining the endometrium during the menstrual cycle, but also in promoting endometrial cancer. COX-2-derived bioactive lipids, including prostaglandin E_2, are potent inflammatory mediators that promote tumor growth and metastasis by stimulating cell proliferation, invasion, and angiogenesis [10]. Therefore, high levels of prostaglandins may promote the development of malignancy [11].

Nitric oxide (NO) is produced endogenously by a family of nitric oxide synthases (NOSs) with a wide range of physiological and pathophysiological actions [12,13]. NOS enzymes are classified into two groups. One group (cNOS), is constitutively present in several cell types (e.g. neurons and endothelial cells) and is regulated predominantly at the post-transcriptional level by calmodulin in a Ca^{2+}-dependent manner [14]. The other group, the inducible form (iNOS), which is expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes and astrocytes, is induced in response to proinflammatory cytokines and bacterial lipopolysaccharide (LPS) [15,16].

Increased NOS expression and/or activity has been reported in human gynecological [17], breast [18] and central nervous system [19] tumors. Also, the accumulation of nitrotyrosine in inflamed mucosa of patients with ulcerative colitis and gastritis indicates NO is being produced and may be involved in the pathogenesis of these diseases [20]. The mechanism of the signal transduction cascade involved in the induction of iNOS in response to LPS and cytokines is an active area of investigation [21]. Previous reports have shown a potential role for tyrosine kinase in LPS promoter that contain 24 transcriptional factor binding sites, including those for NFκB family, which appear to be essential for the enhanced iNOS gene expression seen in macrophages exposed to LPS [22]. The p65 NFκB also seems to be responsible for inducing iNOS in astrocytes [23]. Activation of NFκB by LPS is induced by a cascade of events leading to the activation of inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB and leads to the degradation of NFκB and its translocation to the nucleus [24]. These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase, mitogen-activate protein kinase, and protein kinase C [25,26]. In addition, many studies imply cytokine in the induction of transcription activity of NFκB through Erk1/2 (p42/44), p38 MAPK, and PI3K/AKT pathways [27–30]. Importantly, iNOS has been shown to be involved in regulating cycoloxygenase-2 (COX-2), which plays a pivotal role in colon tumorigenesis [31]. These observations clearly suggest that iNOS may exacerbate turmorigenesis. Collectively, suppression of enzyme induction and the activities of iNOS/COX-2 is an important approach to preventing carcinogenesis in several organs, including the stomach and colon [32].

In the present study, we examine the effect of acacetin and a related compound, wogonin, on the generation of NO and PGE_2, the expression of iNOS and COX-2, and activation of NFκB, p38, p44/42 MAPK, and PI3K/Akt in LPS-stimulated RAW 264.7 macrophage cells. Acacetin was found about to protect against LPS-induced inflammation by blocking the activation of NFκB, p44/42 MAPK, and PI3K/Akt, thereby inhibiting the expression of iNOS and COX-2. We also investigated the effect of acacetin on mouse skin tumors using a two-stage carcinogenesis model. Acacetin was found to be a potential functionally novel chemopreventive agent that might 1 day be used to treat inflammation-associated tumorigenesis.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS) (*Escherichia coli* 0127: E8), sufanilamide, naphthylethylenediamine dihydrochloride, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Acacetin (>99%) was purchased from Fluka (St. Gallen, Switzerland). Wogonin (>99%) was purchased from Wako Pure Chemical Industries Ltd. (Japan).

2.2. Cell culture

RAW 264.7 cells, derived from murine macrophages, were obtained from the American Type Culture Collection (Rockville, MD, USA). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin. When the cells reached a density of 2–3 × 10^6 cells/ml, they

were activated by incubation in medium containing *E. coli* LPS (100 ng/ml). Various concentrations of test compounds dissolved in dimethylsulfoxide were added together with LPS. Cells were either treated with 0.05% DMSO as vehicle control.

2.3. Determination of PGE2

The culture medium of control and treated cells was collected, centrifuged and stored at −70 °C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) according to the manufacturer's instructions (Amersham) [33].

2.4. Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [34]. One hundred microliters of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was measured with an enzyme-linked immunosorbent assay plate reader (Dynatech MR-7000; Dynatech Labs, Chantilly, VA, USA).

2.5. LPS-induced COX-2 and iNOS enzyme activities

The cells were plated in a 24-well plate and treated with LPS (50 ng/ml) for 6 h. The cells were washed three times with fresh medium and treated with one of the six flavonoids for 30 min. The cells were further incubated with 100 μM arachidonic acid for 15 min. The supernatants were removed and assayed for PGE_2 [33] as described above. For the iNOS enzyme activity assay, the cells were cultured in 100 mm tissue culture dishes and incubated with LPS (50 ng/ml) for 12 h. The cells were harvested and plated in a 24-well plate and treated with one of the six flavonoids for a further 12 h. The supernatants were removed and assayed for nitrite [34] as described above.

2.6. Cytotoxicity assay

The RAW 264.7 cells were cultivated at a density of 2×10^5 cells in a six-well plate. The polyphenols studied were added to the medium 18 h after inoculation. The cells were harvested after 18 h. Viability was determined by trypan blue exclusion and microscopy examination.

2.7. Western blotting

Equal amount of total cellular protein (50 μg) were resolved by SDS-polyacrylamide minigels and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) as described previously [35]. The membrane was then incubated with an anti-COX-2 or anti-macrophage iNOS (Transduction Laboratories, Lexington, KY) and cytosolic fraction (for IκBα, p65). The membrane was blocked overnight at room temperature with blocking solution (20 mM Tris–HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide), anti-phospho (Ser 32)-specific IκBα (New England Biolabs, Ipwich, MA, USA), or anti-β-actin monoclonal antibodies (Oncogene Science Inc., Uniondale, NJ) at room temperature for 1 h. The anti-phospho-Akt (Ser 473), anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), ERK, p38, Akt obtained from Cell Signaling Technology (Beverly, MA) were used to determine the level of phosphorylated proteins. The membranes were subsequently probed with anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (ECL, Amersham). The densities of the bands were quantitated with a computer densitometer (AlphaImager™ 2200 System).

2.8. RT-PCR

Total RNA was isolated from mouse macrophage RAW264.7 cell using Trizol Reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Changes in the steady-state concentration of mRNA in iNOS, COX-2 and G3PDH were assessed by reverse-transcription polymerase chain reaction (RT-PCR). Total RNA (2 μg) was converted to cDNA in a series of standard 10-μl reverse transcription reactions. DNA amplification was carried out in "Ready To Go" PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The initial conditions were 42 °C for 60 min. Amplification 30 cycles of iNOS were 95 °C for 40 s, 65 °C for 60 s, and 72 °C for 2 min, followed by a 10 min extension at 72 °C. The thermal cycle conditions of COX-2 were initiated at 42 °C for 60 min, then 30 cycles of amplification (94 °C for 45 s, 55 °C for 60 s, and 72 °C for 2 min) were performed followed by 10 min extension at 72 °C. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. Amplification of G3PDH served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and anti-sence primer: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ (2944–2968), reverse primer 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ (3416–3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094–1117), reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931–1954); G3PDH, forward primer 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, reverse primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

2.9. Quantitative real-time reverse-transcription polymerase chain reaction

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis for iNOS, COX-2 and G3PDH mRNA were performed using a Bio-Rad iCycler iQ Detection System instrument and software (Bio-Rad).The iCycler iQ™ system incorporates a gradient thermocycler and a 96-channel optical unit. For the quantitative analysis of mRNA expression, iCycler iQ Detection System was employed using DNA binding dye SYBR Green for the detection of PCR products. The melting point, optimal conditions and the specificity of the reaction were first determined using a standard procedure. The working stock solution of SYBR Green was 1:100 (Bio-Rad). Quantitative PCR was carried out in

96-well plate with 10 pmol forward and reverse primers, and the working solution SYBR green, using a customer PCR master mix, with the following conditions: 95 °C for 5 min, followed by 40 cycles at 95 °C for 1 min, 55 °C for 45 s, 72 °C for 30 s. G3PDH, a housekeeping gene, was chosen as an internal standard to control for variability in amplification because of differences in starting mRNA concentrations. The sequences of the PCR primers were as follows: iNOS, 5′-TCCTACACCACACCAAAC-3′ and 5′-CTCCAATCTCTGCCTATCC-3′; COX-2, 5′-CCTCTGCGATGCTCTTCC-3′ and 5′-TCACACTTATACTGGTCAAATCC-3′; G3PDH, 5′-TCAACGGCACAGTCAAGG-3′ and 5′-ACTCCACGACATACTCAGC-3′. The copy number of each transcript was calculated as the relative copy number normalized by GAPDH copy number.

2.10. Transient transfection and luciferase assay

The luciferase assay was performed as described by George et al. [36] with some modifications. RAW 264.7 cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Gibco). The cells were then transfected with the pNFκB-Luc plasmid reporter gene (Stratagene, Jalla, CA, USA) using LipofectAMINE™ reagent (Gibo, NRL, Life Technologies, Inc.). After another 24 h of incubation, the medium was replaced with complete medium. After 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well tissue culture plates for 18 h. The cells were then incubated with 100 ng/ml LPS and acacetin and selected compounds for 3 h. Each well was then washed twice with cold PBS and harvested in 150 μL of lysis buffer (0.5 M HEPES pH 7.8, 1% Triton N-101, 1 mM $CaCl_2$ and 1 mM $MgCl_2$). Luciferase activity was assayed by means of the LucLite™ luciferase reporter gene kit (Packard BioScience Company, Meriden, CT), with 100 μL of cell lysate used in each assay. Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.1 min/well, following a 5 min adaptation in the dark. Luciferase activities were determined and normalized to protein concentrations.

2.11. Two-stage tumorigenesis in mouse skin

The anti-tumor promoting activity of acacetin was examined by a standard initiation-promotion with DMBA and TPA, as reported previously [37]. One group was composed of 12 female ICR mice. These mice were given commercial rodent pellets and fresh tap water *ad libitum*, both of which were changed twice a week. The dorsal region of each mouse was shaved with an electric clipper 2 days before initiation. Mice at 6 weeks old were started on 200 nmol DMBA in 200 μL acetone; control mice received 200 μL acetone alone. One week after initiation, the mice were treated topically with 200 μL acetone or promoted with TPA (5 nmol in 200 μL acetone) twice a week for 20 weeks. In the other two groups, the mice were treated with acacetin (100 and 500 nmol in 200 μL acetone) 30 min before each TPA treatment. Tumors of at least 1 mm in diameter were counted and recorded every week. The results were expressed as the average number of tumors per mouse, percentage of tumor-bearing mice, and average tumor weight per mouse.

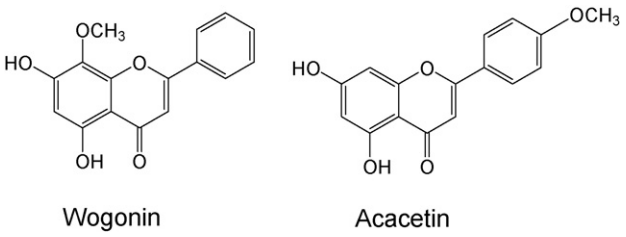

Fig. 1 – The chemical structures of wogonin and acacetin.

2.12. Statistical analysis

Data are presented as means ± S.E. for the indicated number of independently performed experiments. One-way Student's *t*-test was used to assess the statistical significance between the TPA- and acacetin plus TPA-treated groups. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. Inhibition of LPS-induced nitrite production by wogonin and acacetin in RAW 264.7 macrophages

To investigate the anti-inflammatory effects of wogonin and acacetin (Fig. 1), we tested their effect on nitrite and prostaglandin production in LPS-activated macrophages. Wogonin and acacetin at 40 μM did not interfere with the reaction between nitrite and Griess reagents (data not shown). As shown in Fig. 2, wogonin and acacetin inhibited nitrite production by >50% at 10 μM. Acacetin inhibited nitrite production in a dose-dependent manner with an IC_{50} of 13.78 μM. Inhibition of nitrite production was not toxic, as

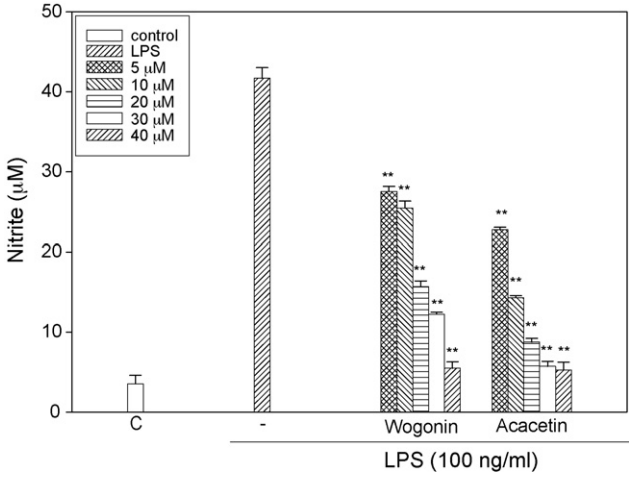

Fig. 2 – Effects of wogonin and acacetin on LPS-induced nitrite production in RAW 264.7 macrophage. The cells were treated with 100 ng/ml of LPS only or with different concentrations of wogonin and acacetin for 24 h. At the end of incubation time, 100 μl of the culture medium was collected for nitrite assay. The values are expressed as means ± SE of triplicate tests. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences from the LPS-treated group.

Table 1 – Effect of the four flavonoids on the activity of iNOS enzyme in RAW264.7 cells

LPS induction of cells	Treatment with flavonoid	Nitrite (μM)
None	DMSO (control)	3.12 ± 0.38
LPS (100 ng/ml)	DMSO (control)	42.45 ± 0.59
	Wogonin 10 μM	53.46 ± 2.31
	Wogonin 30 μM	50.33 ± 4.56
	Acacetin 10 μM	41.77 ± 0.26
	Acacetin 30 μM	37.81 ± 0.98

Effects of various flavonoid on LPS-induced NO synthesis in RAW 264.7 macrophages. Cells were stimulated with LPS (100 ng/ml) for 12 h, and cells were washed with PBS to remove LPS. The cells were then scraped and placed in a 24 well, and the indicated flavonoids were added and incubated at 37 °C for an additional 12 h. At the end of incubation time, the culture medium was collected for nitrite assay.

determined by the trypan blue exclusion assay. The main inhibitory effects of acacetin and wogonin did not come from intrinsic activity of NOS enzyme ($P > 0.05$, no significant difference from LPS alone, Table 1).

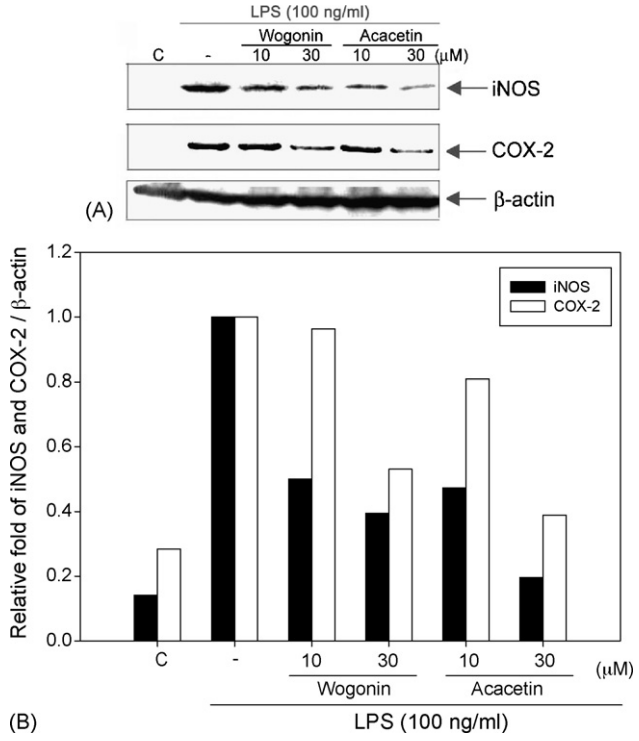

Fig. 3 – Effects of wogonin and acacetin on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. (A) The cells were treated with wogonin and acacetin (10 and 30 μM) for 24 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2 and β-actin protein was detected by Western blot using specific antibodies. (B) Quantification of iNOS and COX-2 protein expression was performed by densitometric analysis of the immunoblot. These experiments were repeated three times with similar results.

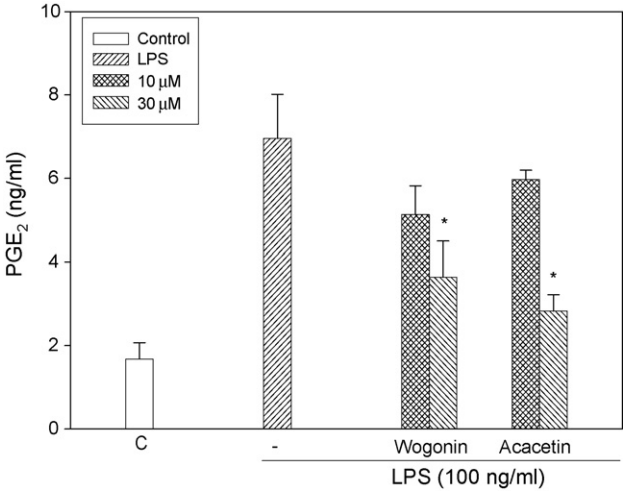

Fig. 4 – Effects of wogonin and acacetin on LPS-induced PGE_2 production in RAW 264.7 macrophage. The cells were treated with 100 ng/ml of LPS only or with different concentrations (10 and 30 μM) of wogonin and acacetin for 24 h. At the end of incubation time, 100 μl of the culture medium was collected for PGE_2 assay. The values are expressed as means ± S.E. of triplicate tests indicate statistically significant differences from the LPS-treated group ($^{*}P < 0.05$ and $^{**}P < 0.01$; Student's t-test).

3.2. *Wogonin and acacetin inhibition of LPS-induced iNOS and COX gene expression*

We next investigated whether acacetin and wogonin might affect levels of iNOS and COX-2 protein. As shown in Fig. 3, both acacetin and wogonin strongly reduced the protein levels of both iNOS and COX-2. These data suggest that transcriptional events are involved in acacetin's and wogonin's inhibition of LPS-induced expression of iNOS and COX-2. After treatment with LPS for 24 h, the medium concentration of PGE_2 had elevated significantly to 7 ng/mL. This increase was markedly inhibited by different concentrations of wogonin and acacetin (Fig. 4). Changes in amounts of iNOS and COX-2 enzyme could reflect altered protein synthesis or degradation. RT-PCR was done to investigate whether acacetin and wogonin suppressed LPS-mediated induction of iNOS and COX-2 via a pretranslational mechanism. The amplification of cDNA with primers specific for mouse iNOS and COX-2 (G3PDH as control gene) is shown in Fig. 5. Acacetin was the most potent inhibitor of expression of iNOS and COX-2 in LPS-activated macrophages, as measured by densitometer scans (Fig. 5, bottom). Similar levels were obtained from real-time PCR analysis of specific iNOS and COX-2 mRNA in cell extracts (Fig. 6A and B). There was an up-regulation of iNOS mRNA in RAW 264.7 cells exposed to LPS, whereas coincubation of macrophages with LPS plus acacetin decreased iNOS and COX-2 mRNA expression after 5 h induction. These data suggest that acacetin may inhibit the expression of iNOS and COX-2 at the transcription levels. Therefore, we wanted to study the inhibitory effect of acacetin on iNOS and COX-2 activities in LPS-activated macrophages in detail.

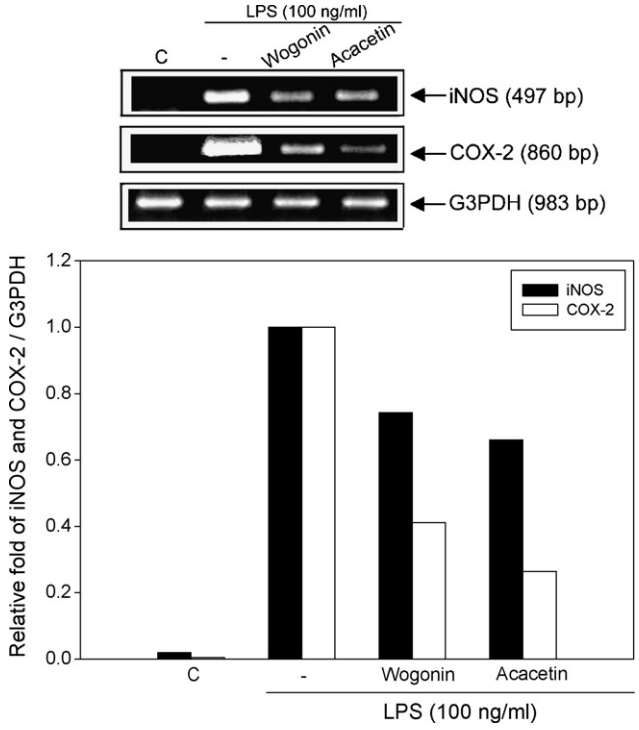

Fig. 5 – RT-PCR analysis of the expression of iNOS and COX-2 mRNA. Cells were treated with LPS (100 ng/ml) and wogonin and acacetin (30 μM) for 5 h, and total RNA was subjected to RT-PCR with the primers iNOS or COX-2 with G3PDH as internal control. The PCR product was resolved in 1.5% agarose gel. Quantification of iNOS and COX-2 RNA expression were performed by densitometric analysis of the agarose gel. This experiment was repeated three times with similar results.

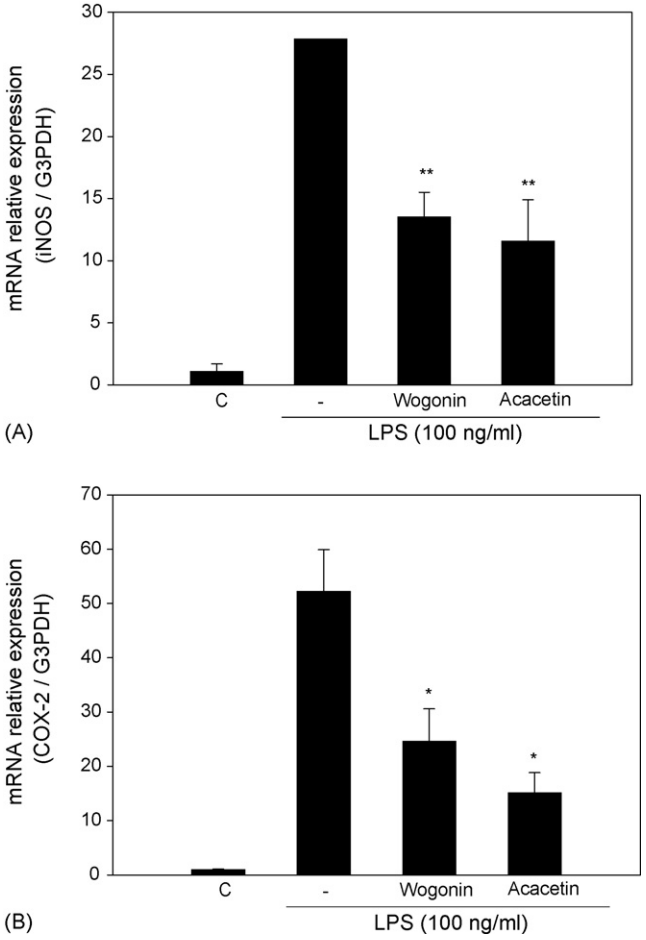

Fig. 6 – Real-time RT-PCR analysis of iNOS and COX-2 mRNA expression in RAW 264.7 cells. (A) The cells were treated with 100 ng/ml of LPS only or with 30 μM of wogonin and acacetin for 5 h, and total RNA was subjected to real time RT-PCR. Relative iNOS (B) and COX-2 (C) mRNAs expression ($2^{-\Delta C_t}$) was performed by real-time PCR and calculated by subtracting the C_t value for G3PDH from the C_t value for iNOS and COX-2 which were determined by real-time RT-PCR relative to G3PDH mRNA. $\Delta C_t = C_{tiNOS \text{ or } COX\text{-}2} - C_{tG3PDH}$. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences from the LPS-treated group. Each experiment was done in triplicate and repeated twice for reproducibility.

3.3. *Reduction of nuclear NFκB level and NFκB activation by acacetin treatment in LPS-stimulated macrophages*

Because activation of NFκB is critical for induction of both iNOS and COX-2 by LPS or other inflammatory cytokines [38,39], we used nuclear accumulation to test whether acacetin would perturb the distribution of NFκB subunits. Nucleus and cytosolic extracts were prepared and subjected to immunoblot analysis. As shown in Fig. 7A, co-incubation with LPS plus acacetin decreased NFκB proteins in the nucleus. PARP, a nuclear protein, and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction. In an additional study, transient transfection with a NFκB-dependent luciferase reporter plasmid was done to confirm whether acacetin and selected compounds inhibited NFκB binding activity in LPS-induced macrophages. As shown in Fig. 7B, acacetin strongly inhibited LPS-induced NFκB transcriptional activity. Wogonin significantly inhibited LPS-induced NFκB transcriptional activity as well.

3.4. *Inhibitory effects of acacetin on LPS-induced phosphorylation and degradation of IκBα*

Because the LPS-mediated the translocation of NFκB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκBα, we examined the phosphorylated and protein levels of IκBα by immunoblot analysis. Treatment with LPS was found to cause the serine-phosphorylation of IκBα protein, as evidenced by the presence of by anti-Ser32-phospho-specific IκBα antibody after 30–120 min and the degradation of IκBα after 45–60 min. Levels gradually recovered after 120 min. As shown in Fig. 8B, treatment with acacetin effectively sustained the IκBα protein content. The pattern of inhibition on IκBα phosphorylation by acacetin was paralleled to the pattern of inhibition on its degradation. These results suggest that blocking the phosphorylation and the degradation of IκBα protein, acacetin can inhibit the production of NO and PGE_2 (Fig. 8), thus preventing the translocation and activation of NFκB in the nucleus (Fig. 7).

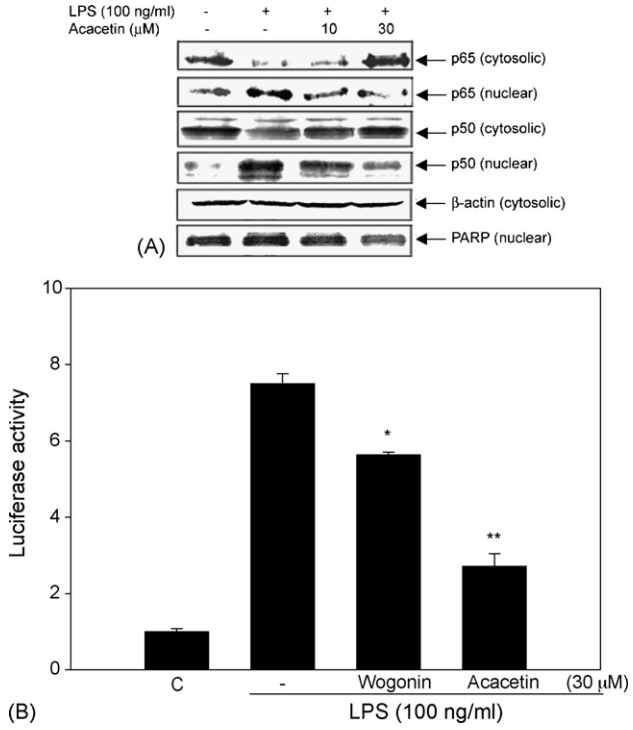

Fig. 7 – Effects of acacetin on LPS-induced p65 and p50 translocation and NF-κB activation in RAW264.7 cells. (A) Cells were treated with LPS (100 ng/ml) with or without acacetin (10 or 30 μM) for 30 min. Then cytosolic and nuclear fractions were prepared and analyzed by Western blotting. (B) The cells were transiently transfected with 2 μg of pNF-κB-Luc reporter gene, and then treated with LPS (100 ng/ml) with or without flavonoids for 12 h. Cells were harvested and the levels of luciferase activities were determined as described in Section 2. Results show the means ± S.E. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS treatment.

3.5. *Effects of acacetin on activation of p38, p44/42 MAP kinase, PI3K, and Akt*

Because p38 and p44/42 MAPK have been shown to be involved in the LPS-mediated induction of iNOS and COX-2 in mouse macrophages [40,41] and cytokine activation of PI3K/Akt pathway leads to the phosphorylation and activation of the NFκB [42], we investigated the effects of acacetin on the activation of p38, p44/42 MAPK and PI3K/Akt in LPS-stimulated macrophages. Activation of MAPK requires phosphorylation of threonine and tyrosine residues [43]. Using immunoblot analysis with anti-phospho-specific antibody, we found activation of p38 and p44/42 to peak after 20–40 min of treatment with LPS and return to basal level 60 min afterwards (data not shown). When the cells were co-treated both acacetin and LPS for 30 min, acecetin was found to attenuate the LPS-stimulated activation of p44/42 MAPK (Fig. 9A), but not affect LPS's activation of p38 (Fig. 9B). We used immunoblot analysis with anti-p85 antibody to investigate whether PI3K pathway was involved in the acacetin's inhibition of LPS-induced RAW 264.7 macrophages. We detected PI3K activity.

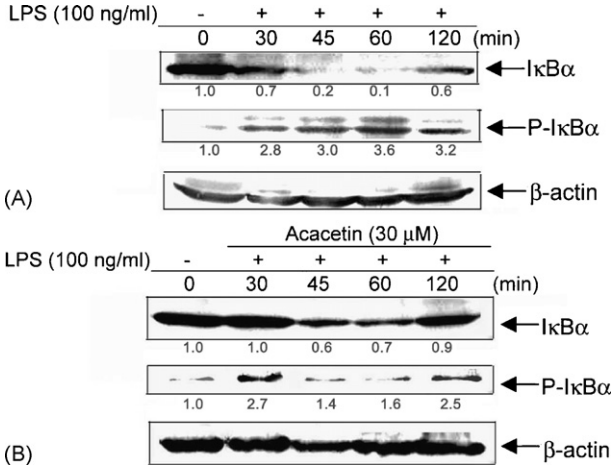

Fig. 8 – Effects of acacetin on LPS-induced phosphorylation and degradation of IκBα. (A) RAW 264.7 were treated with LPS (100 ng/ml) for different times. Total cellular lysates were prepared for Western blot analysis. (B) Cells were treated with LPS (100 ng/ml) and acacetin (30 μM) for different time, and the cellular lysates were prepared and analyzed for content of IκBα, P-IκBα and β-actin by Western blot. These experiments were repeated three times with similar results. The values below the figure represent change in protein expression of the bands normalized to β-actin.

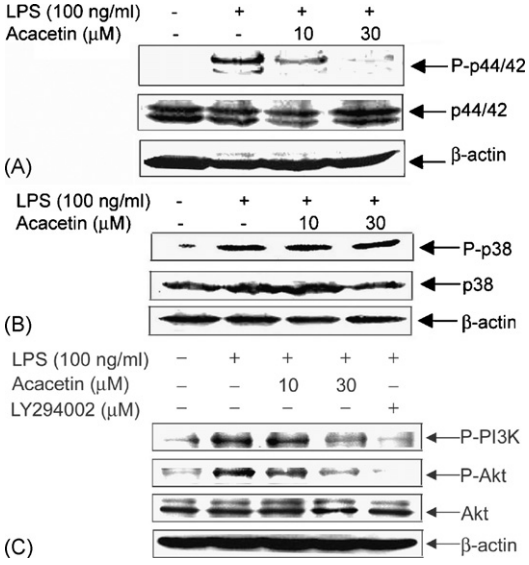

Fig. 9 – Inhibition of p44/42 MAPK and Akt by acacetin in LPS-activated RAW macrophages. RAW 264.7 cells were treated with LPS (100 ng/ml) with or without acacetin (10 or 30 μM) for 30 min. Cells extracts were then prepared and analyzed for (A) p44/42 and P -p44/42, (B) p38 and P-p38 or Akt and P-Akt by Western blot. (C) Cells were treated with LPS (100 ng/ml) with or without acacetin and LY294002 for 30 min and the lysates were analyzed by Western blotting using phospho-PI3K and phospho-Akt antibodies. These experiments were repeated three times with similar results.

The LPS-stimulated activation of PI3K was attenuated by acacetin (30 μM) and PI3K inhibitor, LY294002 (Fig. 9C). To further evaluate the involvement of Akt, a downstream target of PI3K, in LPS-induced responses, we used immunoblot analysis with anti-phospho-Akt antibody to examine Akt activity in macrophage cells. When the cells were co-treated with acacetin and LPS for 30 min, the LPS-stimulated activation of Akt was attenuated by acacetin and LY294002 (Fig. 9C). These results of our immunoblot analyses suggest that acacetin's inhibition of iNOS and COX-2 expression might block LPS-induced NFκB activation by inhibiting Erk1/2, but not p38, and PI3K/Akt/IKK pathway, which interrupts the degradation of IκBα.

3.6. *Inhibitory effect of acacetin on TPA-induced mouse skin tumor promotion*

Up-regulation of iNOS and COX-2 occurs in many pathological conditions, including tumorigenesis. We examined the inhibitory effects of a topical application of acacetin, at 100 and 500 nmol, on tumor formation in DMBA (200 nmol)-initiated and TPA (5 nmol)-promoted mouse skin. Papillomas were first observed in the DMBA and TPA group after 9 weeks of promotion and reached 100% incidence with ~8 papillomas per mouse at week 16 (Fig. 10). Throughout the experiment, there was no noticeable difference in weight gain in the mice treated with two doses of acecetin and those not treated with it, indicating that the topical application of acacetin was not toxic (Fig. 10A). The average number of tumors per mouse in the control group was 13 at the termination of the experiment at 20 weeks. In the treated groups, pretreatment with 100 and 500 nmol acacetin dose dependently reduced the number of tumors per mouse by 31% ($P < 0.01$) and 46% ($P < 0.01$), respectively (Fig. 10B), but it had very little influence on incidence of tumors (Fig. 10C). Comparing the weight of the tumors in all groups, tumor weight in the 100 and 500 nmol acacetin-treated groups were 69% and 89% lower per mouse than in the controls ($P < 0.01$, Student's test) (Fig. 10D). The animals started on DMBA and treated twice weekly with 500 nmol acacetin were devoid of any skin tumors throughout the experiment (data not shown), suggesting that acacetin itself is not a tumor promoter.

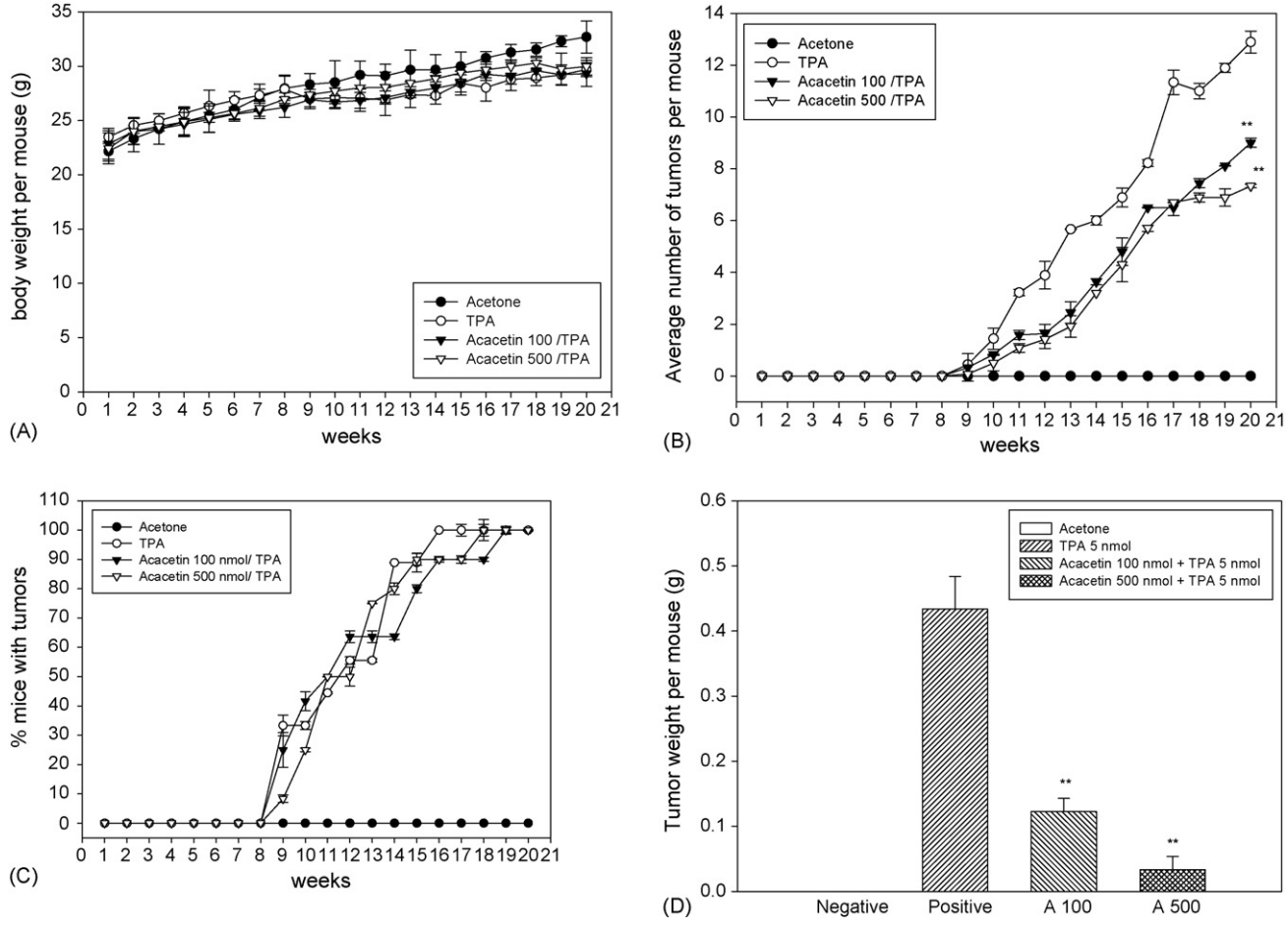

Fig. 10 – Effect of acacetin on TPA-promoted skin carcinogenesis. Tumor formation in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 week after initiation. (A) The body weight of mice during skin tumor promotion ($P > 0.05$). (B) Average number of papillomas/mouse ($P < 0.01$). (C) Percentage of mice with papillomas. (D) The tumor weight of per mouse (g). $^{**}P < 0.01$ indicates statistically significant differences from the TPA-treated group.

4. Discussion

In present investigation we examined the effects and mechanisms of acacetin and wogonin on LPS-induced expression of iNOS and COX-2. Recent findings wogonin and oroxylin A, contain a methoxyl (CH_3O) group on the A ring, showed significant inhibition on LPS-induced PGE_2 production and COX-2 expression. Several recent studies have demonstrated that, depending on their structure, flavonoids may be potent inhibitors of several kinase involved in signal transduction. Results of the present study is consistent with reports by others [44,45]. Acacetin, methoxyl group on the B ring, is structure similar to apigenin, which is a competitive inhibitor of ATP binding and inhibit protein kinase C activity in TPA-induced fibroblast cell [46]. Acacetin was stronger inhibitor of iNOS and COX-2 expression in LPS-activated macrophages than that of wogonin (Fig. 3). The results showed that the position of methoxyl on the B ring strongly influences the conformation of the molecule and modulates their inhibitory effect and provide new information for the design of anti-inflammatory agents and the study of these functional groups in the future.

Flavonoids are naturally occurring plant polyphenols found in abundance in diets rich in vegetables, fruits, and plant-derived beverages such as tea. Several flavonoids are biochemically active compounds with known anti-inflammatory, anti-carcinogenic and free radical scavenging properties. However, the anti-inflammatory functions of some flavonoids are not well established. Multiple-stage carcinogenesis involves aberrant alterations in intracellular signal transduction to initiate neoplastic conversion of cells. Abnormal upregulation or silencing of many of these intracellular signaling cascades is a reversible process and may be treated with dietary phytochemicals, which may block or reverse harmful changes in cellular signaling and prevent cancer [47]. Because there is a causal relationship between inflammation and cancer, iNOS and COX-2 are considered potential molecular targets for chemoprevention [47,48]. Our present results suggest that acacetin's inhibition of the p44/42 MAPK and PI3K/Akt signaling pathway may partially explain how it reduces the induction of iNOS and COX-2 protein. In contrast, acacetin did not affect the p38 MAPK signaling cascade elicited by LPS in macrophages. As predicted by the suppressive efficacies of biochemical markers related to inflammation, topical application of acacetin at doses of 100 and 500 nmol, when applied before TPA during promotion, significantly lowered the number and weight of papillomas. Acacetin inhibited TPA-induced formation of the average number of skin tumors per mouse, but not in a dose-dependant manner. The possible mechanism is that acacetin down regulates inflammatory iNOS and COX-2 gene expression in macrophages by inhibiting the activation NFκB by interfering with the activation of PI3K/Akt and MAPK. In the previous also reported that wogonin was strongly mutagenic, while acacetin was only weakly mutagenic activity when they are tested in Salmonella typhiurmium strains of TA-100 and TA-98 [49]. Therefore, this study is the first to demonstrate the chemopreventive ability of acacetin in an animal model.

Activation of NFκB is necessary for LPS-induction of the iNOS and COX-2 promoter [22,50,51]. NFκB is composed mainly of two proteins: p50 and p65. In resting cells, the NFκB heterodimer is held in the cytosol through interaction with IκB inhibitory proteins [52]. With exposure to proinflammatory stimuli, IκB becomes phosphorylated, ubiquitinated, and then degraded of IκB. Thus, the liberated NFκB dimers are translocated to the nucleus, where the transcription of target gene is induced. Our results show that acacetin reduces iNOS and COX-2 expression by blocking transcription of its gene, a conclusion supported by the observation that it reduced the steady state of iNOS mRNA levels, and promoter activity (as assessed by leuciferase activity assay). Phosphorylation plays an important role in activating protein tyrosine kinase. Many signaling pathways, including PI3K/Akt and mitogen-activated protein kinase, have been proposed to respond to LPS stimulation [53]. PI3K activation leads to phosphorylation of phosphatidylinositides, which then activate the downstream main target, Akt, which appears to play various important roles in regulating cellular growth, differentiation, adhesion, and the inflammatory reaction [54]. Activation of PI3K/Akt plays an important role in the expresseion of iNOS and COX-2 in vascular smooth muscle cells, peritoneal macrophages, and mesangial cells [55,56]. Our findings showed the PI3K/Akt pathway to be involved in acacetin's inhibition of expression of NFκB-related iNOS and COX-2. The p38 MAPK is an important mediator of stress-induced gene expression [43]. In particular, p38 MAPK is known to play a key role in the LPS-induced signal transduction pathway. In this study, we found that incubation of RAW 264.7 cells with LPS brought about the activation of p38 and p44/42 MAPK. We also found that co-treatment of acacetin only blocked the activation of p44/42 MAPK, not p38. These results suggest that acacetin suppresses LPS-induced NFκB translocation by inhibiting the activation of p44/42 MAPK and subsequently decreasing the protein levels of iNOS and COX-2.

Early studies in skin during chemically induced carcinogenesis already pointed to an important role of an interaction between inflammatory cells and epithelia cells in tumor initiation and progression [57]. During early stages of skin tumour development, mast cells, macrophages and neutrophils are recruited to the activated stroma. This inflammatory cell recruitment is highly similar to the initial inflammatory reaction during wound healing. Upon malignant progression induces by the expression of granulocyte-monocyte stimulating factor (GM-CSF) in the tumour cells macrophage recruitment is earlier and enhanced and macrophages now invade the tumour epithelium. Neutrophlis might contribute to a tumour-promoting inflammatory infiltrate by modulating the phenotype of the macrophages in the tumour vicinity via the secretion of cytokines or growth factors. Therefore, the studies suggest that the tumour-promoting micro-environment is indispensable for tumour formation and progression, mast cells might have an important role in the initial stages of tumour development, while the interaction of granulocytes and macrophages might be necessary for progression to a malignant tumour phenotype [58]. We suggest that acacetin could block macrophage infiltration in the skin tumor.

Based on our findings, acacetin shows great potential as a novel chemopreventive agent and may be used in the future to treat inflammation-associated tumorigenesis, for as we observed it was able to inhibit LPS-stimulated expression of

iNOS and COX-2 by regulating the signaling pathway, particularly affecting the activation of PI3K/Akt, p44/42 MAPK, the degradation and phosphorylation of IκBα, and the translocation of NFκB. Acacetin's inhibition of LPS-induced inflammation in RAW 264.7 cells resulted in reduced levels of iNOS and COX-2, suggesting that PI3K/Akt/IKK and p44/42 MAPK can be targeted by drug therapy. Both iNOS and COX-2 have been reported to contribute to tumor growth, and co-expression of two has been observed in malignancies [59]. In our study, acacetin significantly attenuated TPA-induced tumor promotion, suggesting that acacetin's anti-tumor promotion effect may have resulted from its ability to suppress the expression of iNOS and COX-2 and offer exciting information in prevention and treatment of epithelial skin cancers and it is of great clinical interest in prevention and treatment of epithelia skin cancers.

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