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Apigenin inhibits PMA-induced expression of pro-inflammatory cytokines and AP-1 factors in A549 cells

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Abstract Acute and chronic alveolar or bronchial inflammation is thought to be central to the pathogenesis of many respiratory disorders. Cytokines and granulocyte macrophage colony-stimulating factors (GM-CSF) play an important role in chronic inflammation. Activator protein-1 (AP-1) the superfamily of transcription factors is involved in proliferation, differentiation, apoptosis, and transformation including inflammation. Understanding the function and regulation of proinflammatory factors involved in inflammation may provide the novel therapeutic strategies in the treatment of inflammatory diseases. Our aim of the present study is to investigate the pro-inflammatory cytokines and pattern of AP-1 factors expressed during activation of lung adenocarcinoma A549 cells by Phorbol-12-myristate-13-acetate (PMA) and to understand the anti-inflammatory effect of apigenin. A549 cells were treated with and without PMA or apigenin, and the cell viability was assessed by MTT assay. Expressions of inflammatory mediators and different AP-1 factors were analyzed by semi-quantitative RT-PCR. IL-6 protein secreted was analyzed by ELISA, and expressions of IL-1β, c-Jun, and c-Fos proteins were analyzed by Western blotting. Activation of A549 cells by PMA, induced the expression of pro-inflammatory cytokine (IL-1β, IL-2, IL-6, IL-8, and TNF-α) mRNAs and secretion of IL-6 and the expression of specific AP-1 factors (c-Jun, c-Fos, and Fra-1). Treatment of cells with apigenin, significantly inhibited PMA-stimulated mRNA expression of above pro-inflammatory cytokines, AP-1 factors, cyclooxygenase-2, and secretion of IL-6 protein. Results suggested that the AP-1 factors may be involved in inflammation and apigenin has anti-inflammatory effect, which may be useful for therapeutic management of lung inflammatory diseases.

Keywords Phorbol-12-myristate-13-acetate · Apigenin · Cytokines · AP-1 · A549 · qRT-PCR

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Introduction

Inflammation is a protective physiological response of the body to activate the immune system in response to a variety of stimuli, the infections, and tissue injury. However, chronic inflammatory response may induce various diseases such as sepsis, rheumatoid arthritis, and cancer [1, 2]. In response to bacterial infection or stimuli the pulmonary epithelial cells present at the mucosal surface are capable of secreting chemoattractants and pro-inflammatory cytokines, the important mediators in both lung defense and inflammation. Thus, the response of epithelial cells to inflammation acts as an early warning system to other local immune and inflammatory cells [3]. Cytokines (IL-6, TNF-α, IL-8, and Granulocyte–macrophage colony-stimulating factor) are released in a coordinated network and among interleukins IL-6 acts as a pro-inflammatory cytokine and a potent mediator of inflammatory process [4]. In human respiratory disease, pulmonary epithelial cells are one of the major sources of prostaglandin E2 (PGE2) [5]. PGE2 are produced from arachidonic acid by inducible cyclooxygenase-2 (COX-2) [6, 7]. Induced expression of

COX-2 and increased production of PGE2 have been implicated in many inflammatory diseases and pulmonary disorders [8]. Nitric oxide (NO) a free radical mediates many physiological processes [9], and its production is controlled by inducible isoforms nitric oxide synthase (iNOS). Increased synthesis of NO in macrophages and lung cells also lead to many organ destruction and inflammatory autoimmune diseases.

The most studied transcription factors and most of the information exist during immune and inflammatory responses are nuclear factor-kappa-B (NF- kB) and activator protein-1 (AP-1) [10]. It is a well-known fact that NF-kB plays an important role in immune system [11] that regulate the expression of nearly all inflammatory mediators involved in inflammation [12]. AP-1 are involved in proliferation, differentiation, apoptosis, transformation, carcinogenesis, tumorigenesis, inflammation, and defense reactions [13]. AP-1 is a dimeric transcription factor comprising of proteins derived from two super families the Jun (c-Jun, JunB, and JunD) and the Fos (c-Fos, FosB, Fra-1, and Fra-2) proteins. Different combinational AP-1 dimers integrate numerous signaling pathways stimulated by great variety of extracellular cues and hence synthesis turnover of subunits of AP-1 transcription factors regulate many physiological or pathological status of an organism. Literature on AP-1 is very large, and the regulation and expression of different AP-1 subunits during differentiation, cell cycle, other pathophysiological processes, inflammation in macrophages and lungs are well defined [13, 14]. However, the expression pattern of different AP-1 factors in PMA-induced inflammation is less understood in lungs.

Long-term administration of non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of inflammatory diseases may result in severe adverse effects [15]. Hence, many natural products particularly flavonoids with an alleged anti-inflammatory activity have been used in the traditional medicine for treatment. Apigenin (4′,5,7- trihydroxyflavone), is a flavone that is the aglycone found mainly in fruit, vegetables, nuts, and plant-derived beverages, shown to have non-toxic, non-mutagenic [16], blocks the development of mouse skin tumors [17], inhibits the proliferation of human breast-cancer cells [18] and is a potent antioxidant [19], apigenin is known to inhibit high glucose levels and tumor necrosis factor-α (TNF-α)-induced adhesion molecule levels in human endothelial cells. Although large bodies of evidence exist in the use of apigenin in the treatment of inflammation, not much is known about its mechanism of anti-inflammatory action on AP-1 factors and pro-inflammatory cytokines in lungs. Hence in the present study, we aimed to demonstrate the expression pattern of different AP-1 factors on PMA-induced inflammation and the effect of apigenin on PMA-induced pro-inflammatory cytokines and AP-1 factors in human lung adenocarcinoma A549 cells.

Materials and methods

Materials

Lung adenocarcinoma alveolar type II A549 cells were purchased from NCCS (Pune, India), Phorbol 12-myristate 13-acetate (PMA), Apigenin, Griess reagent, oligos forward and reverse primers for different cytokines and AP-1 factors, cell cycle regulators, and apoptosis gene sequences were designed (Table 1) [20] and were purchased from Sigma-Aldrich (St Louis, USA). Fetal bovine serum (FBS), penicillin, streptomycin, glutamine, RPMI 1640 media, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), phenylmethanesulfonyl fluoride (PMSF), and trypan blue were purchased from Himedia (Mumbai, India). Superscript reverse transcriptases for qRT-PCR and IL-6 ELISA Kit were purchased from Invitrogen (CA, USA). Antibodies to c-Jun, c-Fos, and IL-1β were purchased from Neo-Biolab (MA, USA), and antirabbit antibody HRP conjugate was purchased from Imgenex India Pvt. Ltd. (Bhubaneswar, India). Taq DNA polymerase (1 U/μl) and Luminata Forte Western HRP substrate was procured from Merck-Millipore (Mumbai, India). Griess reagent was prepared in distilled water containing 1 % sulfanilamide in 5 % phosphoric acid and 0.1 % N-l-naphthylethylenediamine dihydrochloride. The cell lysis buffer was prepared in distilled water containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1.0 % NP-40, and 100 μM PMSF.

Culturing of A549 cells

A549 cells were grown in 75 cm^2 culture flask in RPMI 1640 media with 10 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37 °C by passing 5 % CO_2 in an incubator [21]. Flask containing 90–100 % confluent cells were sub-cultured in 96-well plate (3×10^3 cells/well) or in 6-well plate (5×10^5cells/well) for the treatment. (Whereever apigenin was used, the cells were preincubated 1 h either with two different concentrations of apigenin (20 or 40 μM), and then stimulated with PMA (10nM) for 2 and 24 h).

MTT assay for cell viability

MTT assay for cell viability was carried out as per the protocol described earlier [22]. MTT is a pale yellow substrate taken up and reduced by active live cell

Table 1 Sequence of primers (F: forward and R: reverse) used for the amplification of different cytokines, inflammatory enzymes, AP-1 factors, cell cycle regulators, and β-actin

Gene	Primer sequence	Annealing temp. (°C)	Product size (bp)
Cytokines and inflammatory enzymes			
IL-1β	F: AAACAGATGAAGTGCTCCTTCCAGG	62	388
	R: TGGAGAACACCACTTGTTGCTCCA		
IL-2	F: CCAAAGAGTCATCAGAAGAGG	64	470
	R: GCACTTCCTCCAGAGGTTTGAG		
IL-6	F: CCACTCACCTCTTCAGAACG	58	564
	R: GACAGGTTTCTGACCAGAAG		
IL-8	F: AGATATTGCACGGGAGAA	55	671
	R: AACTAGGATTGTTAGTTC		
IL-10	F: CCA AGA CCC AGA CAT CAA GGC	64	309
	R: GAG CCC CAG ATC CGA TTT TGG		
TNF-α	F: CAAGCCTGTAGCCCATGTTGTAGC	58	430
	R: ATCCCAAAGTAGACCTGCCCAGAC		
iNOS	F: TCACCTACCACACCCGAGAT	57	384
	R: CATAGACCTTGGGCTTGCCA		
COX-2	F: AGTCCCTGAGCATCTACGGT	58	352
	R: AAAGGTGTCAGGCAGAAGGG		
AP-1 factors			
c-Jun	F: GCCTACAGATGAACTCTTTCTGGC	64	525
	R: CCTGAAACATCGCACTATCCTTTG		
Jun-D	F: CGCAGCCTCA AACCCTGCCT TTCC	64	500
	R: AAACAGGAATGTGGACTCGTAG		
Jun-B	F: CCAGTCCTTCCACCTCGACGTTTACAAG	58	257
	R: GACTAAGTGCGTGTTTCTTTTCCACAGTAC		
c-Fos	F: TCTTCCTTCGTCTTCACC	58	577
	R: AATCAGAACACACTATTGCC		
Fra-1	F: AGGAAGGAACTGACCGAC	60	497
	R: GAAGGGGAGGAGACATTG		
Fra-2	F: AGGAGGAGAGATGAGCAG	60	518
	R: GGATAGGTGAAGACGAGG		
Cell cycle regulators			
Cyclin D1	F: AGACCTGCGCGCCCTCGGTG	58	574
	R: GTAGTAGGACAGGAAGTTGTTG		
Cyclin E1	F: GTCCTGGCTGAATGTATACATGC	60	415
	R: CCCTATTTTGTTCAGACAACAT		
CDK 4	F: AGTGGCGGATCCATGGCTACCTCTCGATAT	60	912
	R: TCTCGGAAGCTTTCACTCCGGATTACCTTCA		
P53	F: GAGCCCCCTCTGAGTCAG	56	375
	R: GCAAAAACATCTTGTTGAG		
Bcl-2	F: AGATGTCCAGCCAGCTGCACCTGAC	62	365
	R: AGATAGGCACCCAGGGTGATGCAAGCT		
Bax	F: AAGCTGAGCGAGTGTCTCAAGCGC	61	366
	R: TCCCGCCACAAAGATGGTCACG		
β-actin	F: TACCACTGGCATCGTGATGGACT	62	516
	R: TCCTTCTGCATCCTGTCGGCAAT		

mitochondria to yield a dark blue formazan product. A549 cells ($3x10^3$cells/well) in 200 µl of RPMI-1640 medium were seeded into 96-well culture plate and incubated overnight at 37 °C with the supply of 5 % CO_2. Cells were treated with or without various concentrations of PMA or apigenin, incubated for 24 h, washed with PBS, treated with 20 µl of MTT (5 mg/ml), and incubated further for 4 h at 37 °C in a CO_2 incubator. Blue formazan products formed inside the cells were dissolved in DMSO (100 µl), and absorbance was measured at 540 nm using spectrophotometer. The effect of PMA and apigenin on cell viability was calculated and represented graphically as % of viable cells compared to control.

Nitric oxide measurement

NO produced by A549 cells was assayed by the measurement of nitrite, a stable NO oxidation product as described earlier [23]. Cells (5×10^5cells/well) were plated in 6-well culture plate and incubated at 37 °C for 24 h in a humidified 5 % CO_2 incubator. Later, the cells were treated with PMA (10nM) or apigenin (40 µM) alone or PMA with apigenin (20 or 40 µM) for 24 h. Spent culture medium aspirated from wells of culture plate (100 µl) was mixed with 100 µl of Griess reagent, and the absorbance of the mixture was measured in a microplate reader at 540 nm. Concentration of nitrite in µM was calculated using standard curve developed with known concentration of sodium nitrite.

RNA isolation and semi-quantitative RT-PCR analysis

A549 cells (5×10^5cells/well) were seeded into 6-well plate and incubated with or without PMA (10nM) or PMA and apigenin (20 µM or 40 µM) or apigenin alone (40 µM) for 2 and 24 h. Total RNA was isolated from each of the samples using ''Trizol reagent'' as per the instructions provided by the manufacturer. Reverse transcription of RNA and PCR analysis was carried out as per the protocol described earlier [24]. In brief, total RNA (2 µg) was reverse transcribed using Oligo (dT) primers and superscript reverse transcriptase. The cDNA was subjected to 30 cycles of PCR using different forward and reverse primers of inflammatory mediators or AP-1 factors or cell cycle regulators or apoptosis genes using appropriate annealing temperatures as indicated in Table 1 [20] in a gradient Eppendorf thermocycler. Amplified PCR products were analyzed on 1 % agarose gel using 1X TAE buffer. Relative mRNA levels were quantified using image analysis software (ImageJ). The expression of β-actin mRNA was used as a positive control and for normalization.

Enzyme-linked immunosorbent assay (ELISA)

A549 cells (5×10^5 cells/well) were seeded into six-well plate and incubated with or without PMA (10nM) or PMA and apigenin (20 µM or 40 µM) or apigenin alone (40 µM) for 24 h. Cell-free culture supernatants (spent medium) were collected and stored at −80 °C until further used. The amount of IL-6 protein present in spent medium was measured using Invitrogen ELISA kit. Standard (100 µl) or diluted control or treated samples (100 µl) were added into wells of ELISA 96-well plate. The Biotin conjugate (100 µl) was added, and incubated for 2 h at room temperature. The wells were washed four times with wash buffer, and 100 µl of Streptavidin–HRP solution was added, incubated further for 30 min at room temperature. The wells are washed thrice with wash buffer, 100 µl of Chromogen was added, incubated for 20 min at room temperature, and 100 µl of stop-solution was added to all the wells. Absorbance was measured at 450 nm using the PerkinElmer multimode plate reader (MA, USA). Concentrations of IL-6 in each samples were calculated using standard curve drawn with known concentration of recombinant cytokines and represented graphically.

Western blotting of IL-1β, c-Jun, and c-Fos

The total cell protein extracts of A549 cells from control and treated samples were prepared and analyzed by Western blotting as per the protocol described earlier [24]. The control and treated cells present in 6-well plate were washed thrice with PBS, and 0.2 ml of cold cell lysis buffer was added. Cells were scraped, and suspension was gently transferred to a pre-cooled Eppendorf tube, mixed gently by swirling on ice and centrifuged at 15,000 x g for 20 min at 4 °C. Supernatant was used to determine protein concentration by Bradford's method [25]. The cell lysates containing equal amount of protein (40 µg) of different samples were separated on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred on to PVDF membrane. The membrane was blocked with 5 % Carnation fat-free milk at room temperature for 1 h. Antibody (anti IL-1β or c-Jun or c-Fos) in blocking solution (1:500) was added and incubated for 1 h, washed and incubated with anti-rabbit antibody-HRP (1:500), and further incubated for 1 h at room temperature. Immunoreactive proteins were visualized using Luminata Forte Western HRP substrate and as per the specifications provided by the supplier in a Syngene Gel Documentation system (MD, USA). Immunoreactive bands were quantified using image analysis software (ImageJ). GAPDH was used as a positive control and for normalization.

Statistical analysis

The MTT assay, nitric oxide production, the images of semi-quantitative RT-PCR gels, ELISA, and Western blotting data were analyzed by one-way ANOVA followed by post-hoc tukey test. Values were considered statistically significant if $*P < 0.05$, $**P < 0.005$ compared to control and if $^{\#}P < 0.05$ compared to PMA-treated sample.

Results

Effect of PMA and apigenin on A549 cells viability

The PMA used between 1-25nM concentration show no effect on the cell viability, while at 50 nM PMA show marginal decrease (5 %) in cell viability (Fig. 1a). Similar results were also obtained by counting the number of viable cells by trypan blue dye exclusion method using Neubauer counting chamber (data not shown). To study the cytotoxic effect of apigenin, A549 cells were treated with or without different concentrations of apigenin (10-100 μM) for 24 h, and the cell viability was determined by MTT assay. Results show that the apigenin up to 60 μM concentration show no cytotoxic effect. However, compared to control a marginal decrease in cell viability at 80 and 100 μM concentrations of apigenin was observed (Fig. 1b).

Effect of PMA and apigenin on Nitric Oxide production in A549 cells

The A549 cells were treated with LPS or PMA (10nM) or PMA and apigenin (20 or 40 μM) or apigenin (40 μM) alone for 24 h, and the concentration of nitrite in spent medium was determined using Griess reagent. The results show that compared to control PMA alone has no effect on nitric oxide production, while PMA with apigenin at 20 and 40 μM concentration show marginal decrease in nitric oxide production. Apigenin alone also significantly inhibited NO production (18 %) compared to control (Fig. 2). The positive control LPS found to induce significantly the nitric oxide production by more than 30 % compared to control (Fig. 2).

Effect of PMA and apigenin on iNOS and COX-2 mRNA Expression

To assess the effect of PMA and apigenin on NO and PGE2 production in A549 cells, the mRNA levels of iNOS and COX-2 were analyzed by semi quantitative RT-PCR. In our study, PMA almost has no effect on iNOS expression, but induced the COX-2 mRNA expression by more than 3.3-fold compared to control (Fig. 3a). The cells treated with PMA with apigenin decreased the PMA-induced mRNA levels of COX-2 in a dose-dependent manner. Cells treated with apigenin (40 μM) alone also show more than 30 % decrease in the expression of iNOS and 69 % decrease in COX-2 mRNA levels compared to control (Fig. 3a). However, the positive control LPS induced the expression of iNOS by 34 % compared to control (Fig. 3b).

Apigenin inhibits the PMA-induced mRNA expression of pro-inflammatory cytokines

Compared to control the treatment of A549 cells with PMA both at 2 and 24 h significantly induced the expression of all the pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α). However, IL-2 was found to be induced only at 24 h but not at 2 h. Among the pro-inflammatory cytokines

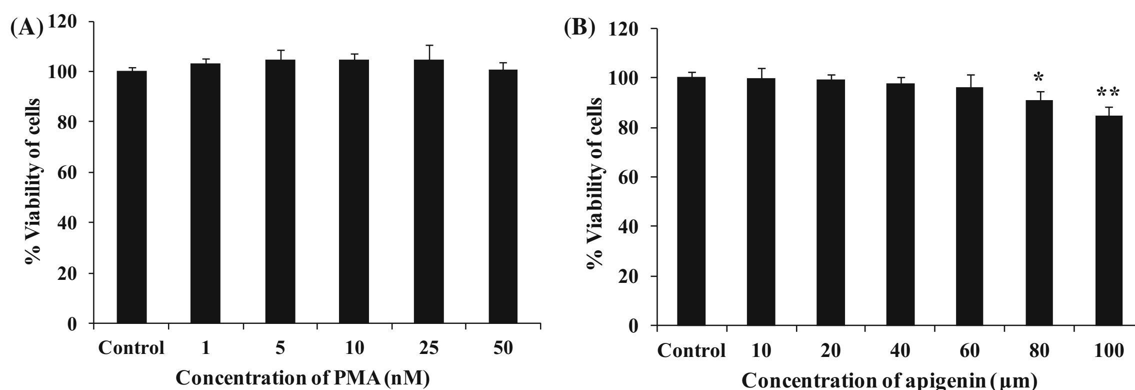

Fig. 1 Effect of different concentrations of (A) PMA and (B) apigenin on the viability of A549 cells. Cells were treated with or without different concentrations of PMA (1–50 nM) or apigenin (10-100μM) in a 96-well plate for 24 h, and the cell viability was measured by MTT assay. Results were expressed as % viability of cells as compared to control (mean ± SD, n=8). Values are significantly different from control if $*P<0.05$, $**P<0.005$ using one-way ANOVA followed by Post-hoc Tukey test. The results are shown as a representative of three independent experiments

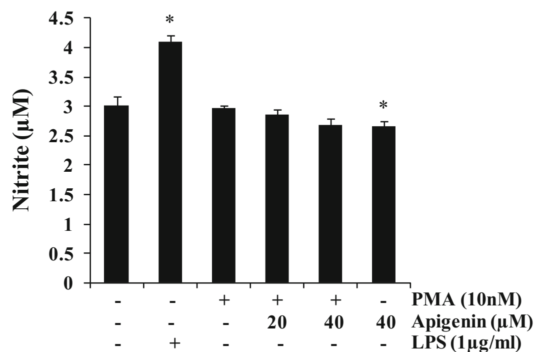

Fig. 2 Effect of PMA and apigenin on nitric oxide production. A549 cells were treated with LPS or PMA (10nM) or PMA with apigenin (20 or 40μM) or apigenin (40μM) alone for 24 h, and the concentration of nitrite in the spent medium was determined using Griess reagent using sodium nitrite as standard. Data presented as mean ± SD, n = 3. Values are significantly different from the control if $*P < 0.05$ using one-way ANOVA followed by Post-hoc Tukey test. The results are shown as representative of three independent experiments

studied, at 24 h maximum induction of more than 5.8-fold was observed for IL-6, 2.3-fold was observed for IL-2, and twofold was observed for TNF-α mRNA levels. At 2 h maximum induction of 2.7-fold was observed for IL-1β, 1.8-fold was observed for IL-8, and 1.7-fold was observed for TNF-α mRNA levels compared to control. The cells treated with apigenin (at 20 μM or 40 μM concentration) for 24 h significantly inhibited the PMA-induced expression of mRNA levels of all the above pro-inflammatory cytokines in a dose-dependent manner and almost reached to control levels (Fig. 4b). At 2 h apigenin with 20 and 40 μM, significantly inhibited PMA induced the expression of mRNA levels of IL-1β and TNF-α while marginal decrease was observed for IL-6. However, apigenin at both the concentrations found to have no effect on IL-8 and IL-2 mRNA levels (Fig. 4a). The cells treated with apigenin (40 μM) alone show no effect on IL-6, while significantly decreased the expression of IL-1β, IL-2 IL-8, and TNF-a mRNA levels compared to control (Fig. 4b). Further, PMA and apigenin (20 and 40 μM) show no effect on anti-inflammatory IL-10 cytokine mRNA levels compared to control.

Apigenin inhibits the PMA-Induced synthesis and secretion of IL-6 cytokine

Treatment of A549 cells with PMA compared to control show more than fivefold increase in the secretion of IL-6

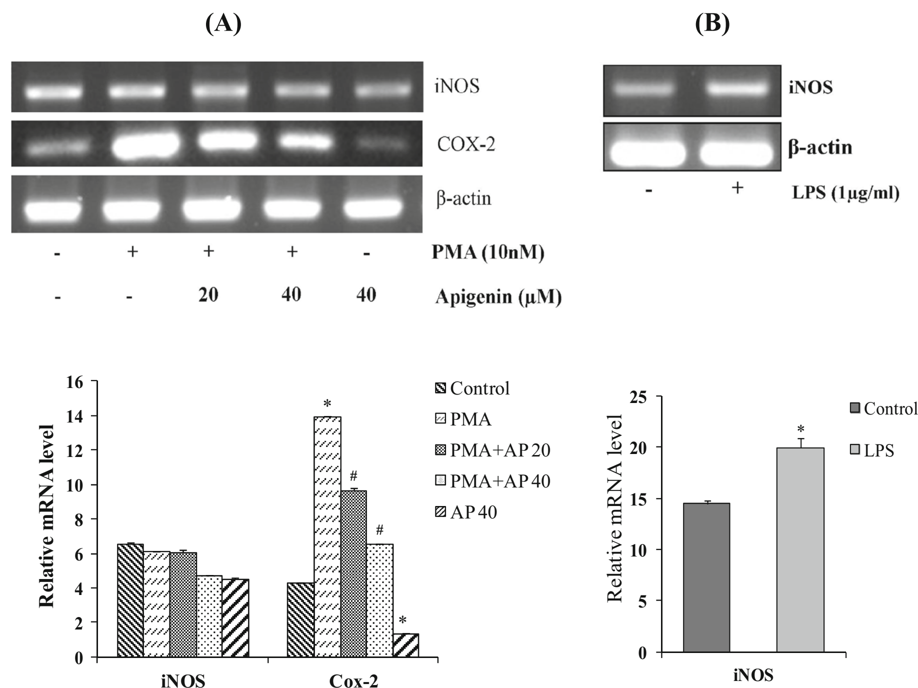

Fig. 3 Effect of (A) PMA or apigenin or PMA with apigenin (B) LPS alone on the mRNAs of iNOS and COX-2 in A549 cells. A549 cells (5×10^5 cells/well) were treated with or without PMA or PMA with apigenin (20 or 40μM) or apigenin (40μM) alone or LPS alone for 24 h. The mRNA levels of amplified genes of qRT-PCR were analyzed on 1 % agarose gel, and the band intensity of the experimental samples was compared with the control and PMA treatment alone. β-actin was used as a positive control and for normalization. Data shown are means ± SD from 3 independent experiments. Differences in inflammatory mediators mRNA levels are statistically significant, if $*P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA-stimulated values using one-way ANOVA followed by Post-hoc Tukey test. The *bar graphs* present below the respective figures are the densitometric analysis of mRNA levels

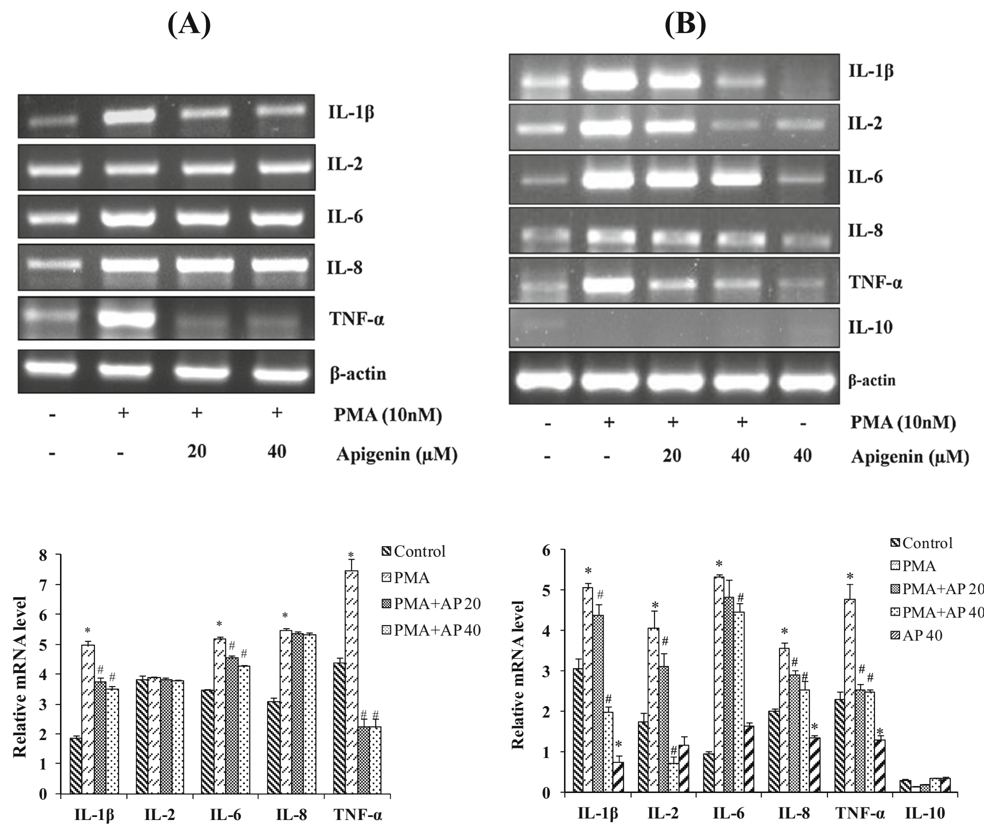

Fig. 4 Effect of apigenin on PMA-induced mRNA expression of pro-inflammatory cytokines (A) for 2 h and (B) for 24 h in A549 cells. A549 cells (5×10^5cells/well) were treated with or without PMA or PMA with apigenin (20 or 40μM) or apigenin (40μM) alone for 2 and 24 h in 6-well plate. The mRNA levels of amplified genes of qRT-PCR were analyzed on 1 % agarose gel and the band intensity of the experimental samples was compared with the control and PMA treatment alone. β-actin was used as a positive control and for normalization. Data shown are means ± SD from 3 independent experiments. Differences in proinflammatory cytokines mRNA levels are statistically significant, if $*P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA-stimulated values using 1-way ANOVA followed by Post-hoc Tukey test. The *bar graphs* present below the respective figures are the densitometric analysis of mRNA levels

protein into the medium. However, the cells treated with PMA and apigenin significantly decreased the PMA-induced IL-6 release in a dose-dependent manner. Cells treated with apigenin (40 μM) alone show marginal decrease in the secretion of IL-6 protein levels compared to control (Fig. 5).

Apigenin inhibits the PMA-induced expression of mRNAs of c-Jun and c-Fos AP-1 factors

A549 cells, except FosB, show the expression of mRNAs of Jun (c-Jun, JunB, and JunD) and Fos (Fra-1, Fra-2, and c-Fos) family members at different levels. Compared to control the cells treated with PMA induced the expression of c-Jun significantly by fourfold and fivefold, c-Fos by 2.6-fold and fourfold by 2 h and 24 h, respectively (Fig. 6a, b). However, PMA marginally increased the expression of Jun-D and Fra-1 mRNA transcripts by 0.2- and 0.6-fold, respectively, at 24 h (Fig. 6b). At 2 h

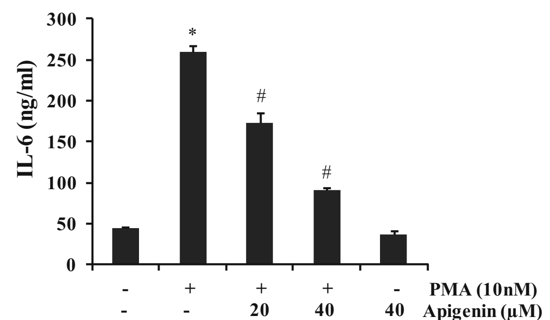

Fig. 5 Effect of apigenin on PMA-induced synthesis and secretion of IL-6 cytokine in to media from A549 cells. Cells (5×10^5cells/well) were treated with or without PMA or PMA with apigenin (20 or 40μM) or apigenin (40μM) alone for 24 h in a 6-well plate. The amount of IL-6 released in the spent medium was determined by ELISA. *Each bar* represents the mean ± SD, n=3, and significant at the level of $*P< 0.05$ compared to control, $^{\#}P< 0.05$ compared to PMA-stimulated values using one-way ANOVA followed by Post-hoc Tukey test. The results are shown as a representative of three independent experiments

PMA significantly increased the expression of Jun-B by 1.7-fold and marginally increased the expression of Fra-1 by 0.2-fold and Fra-2 by 0.27-fold (Fig. 6a). The treatment of cells with apigenin at 20 or 40 μM significantly decreased the PMA-induced mRNA levels of c-Jun and c-Fos in a dose-dependent manner. All most no inhibition of PMA-induced Fra-1 mRNA levels at 20 and 40 μM concentration of apigenin was observed. Cells treated with apigenin (40 μM) alone showed marginal decrease in mRNA level of Jun-B and significant increase in mRNA transcripts of Fra-2 compared to control (Fig. 6b). The results suggested that the c-Jun and c-Fos may be involved in inflammatory reaction and apigenin show anti-inflammatory activity in lung cells.

Apigenin inhibits PMA-Induced expression of IL-1β, c-Jun and c-Fos proteins in A549 cells

Our study also show that PMA induce significantly, the IL-1β protein by twofold and c-Jun proteins by more than 2.9-fold while marginal of 0.57-fold increase was observed for c-Fos. Apigenin at 20 μM and 40 μM concentrations show significant decrease in PMA-induced IL-1β protein, c-Jun, and c-Fos proteins in a dose-dependent manner (Fig. 7).

PMA induces the cell cycle regulators

Cells treated with PMA induced the expression of mRNA levels of p53, CDK4, cyclin D1, and cyclin E1 transcripts at different levels. Results show that the PMA induces p53 mRNA by 2 and CDK4 mRNA by more than twofolds and marginal increase in 43 % of cyclin D1 mRNA levels (Fig. 8). Apigenin treatment show the decreased PMA-induced expression of p53, cyclin D1 mRNA levels in a dose-dependent manner. However, apigenin treatment, either with PMA or alone show increased mRNA levels of CDK4 (Fig. 8), while show decreased levels of cyclin E1 mRNAs suggested that the apigenin has varied effect on cell cycle regulators.

Effect of PMA and apigenin on the expression of apoptotic genes

A549 cells treated with PMA induced the expression of mRNAs of Bcl-2 an anti-apoptotic gene, while decreased

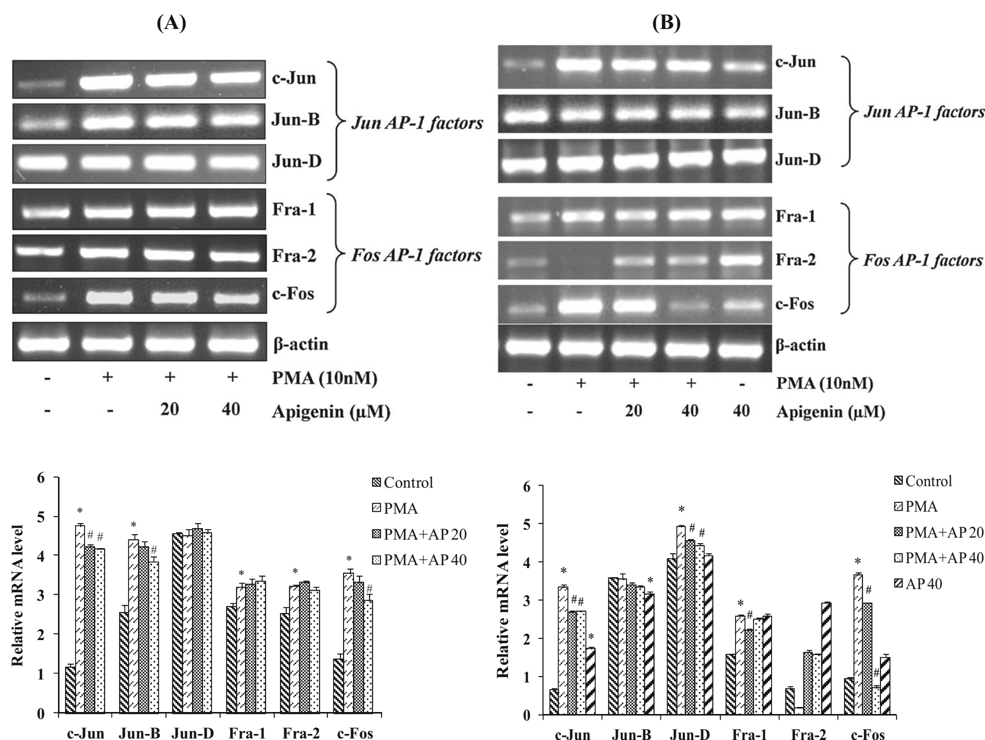

Fig. 6 Effect of apigenin on the PMA-induced expression of mRNAs of AP-1 factors (A) for 2 h and (B) for 24 h in A549 cells. Cells were treated with or without PMA or PMA with apigenin (20 or 40μM) or apigenin (40μM) alone for 2 and 24 h in 6-well plate. The mRNA levels of amplified genes of qRT-PCR were analyzed on 1 % agarose gel, and the band intensity of the experimental samples was compared with the control and PMA treatment alone. β-actin was used as a positive control and for normalization. Data shown are means ± SD from 3 independent experiments. Differences in AP-1 factors mRNA levels are statistically significant: if $^{*}P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA-stimulated values using one-way ANOVA followed by Post-hoc Tukey test. The *bar graphs* present below the respective figures are the densitometric analysis of mRNA levels

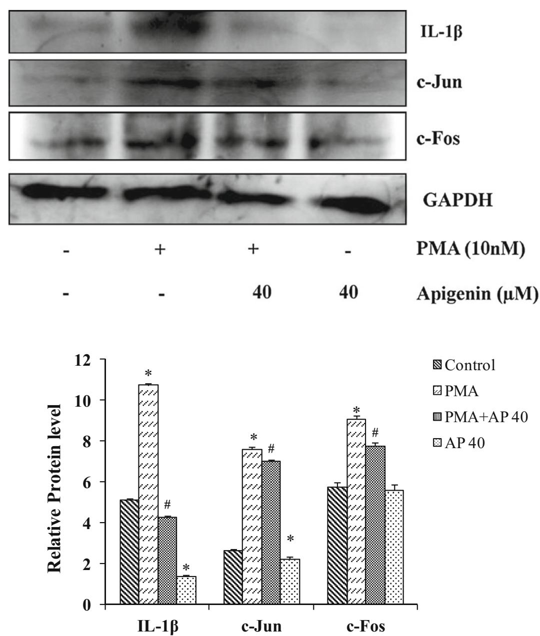

Fig. 7 Effect of apigenin on PMA-induced expression of IL-1β, c-Jun, and c-Fos Protein levels in A549 cells. Cells were pretreated with or without 40μM concentration of apigenin for 1 h and later treated with or without PMA (10 nM) for 24 h. Cell lysates were prepared with lysis buffer and equal amount of protein (40μg) of cell lysates were analyzed on SDS PAGE, transferred on to PVDF membrane. Expression levels of IL-1β, c-Jun, and c-Fos proteins in PVDF membranes were analyzed using Western blotting technique, and GAPDH was used as a positive control and for normalization. Differences in protein levels are statistically significant: if $*P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA treatment using one-way ANOVA followed by Post-hoc Tukey test. The *bar graph* present below the respective figures is the densitometric analysis of protein levels

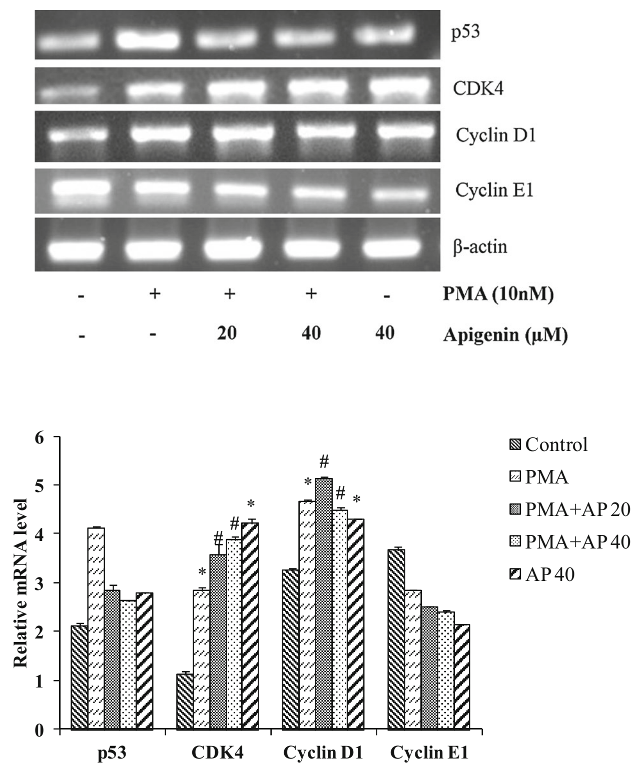

Fig. 8 Effect of PMA on mRNA levels of cell cycle regulators. Cells were treated with or without PMA (10 nM) or PMA with apigenin (20 or 40 μM) or apigenin (40μM) alone for 24 h in 6-well plate. The mRNA levels of amplified genes of qRT-PCR were analyzed on 1 % agarose gel, and the band intensity of the experimental samples was compared with the control and PMA treatment alone. β-actin was used as a positive control and for normalization. Differences in cell cycle regulators mRNA levels are statistically significant: if $*P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA-stimulated values using one-way ANOVA followed by Post-hoc Tukey test. The results are shown as a representative of three independent experiments. The *bar graph* present below the figure is the densitometric analysis of mRNA levels

the pro-apoptotic Bax mRNA expression compared to control (Fig. 9). However, treatment of cells with apigenin (20 and 40 μM) decreased the PMA-induced Bcl-2 mRNA expression and increased the Bax expression to control mRNA levels. Apigenin alone found to have no effect on Bcl-2 and Bax gene expressions.

Discussion

Phytochemicals are important and promising group of compounds acts as a potential anti-inflammatory agents because of their low toxicity and apparent benefit in the treatment of acute and chronic diseases. Recently, the new approaches on the use of herbal products for the treatment of inflammatory diseases are being practiced in traditional medicine but without understanding their associated complications. Hence, our study focuses on the mechanism of action of bioflavonoid the apigenin, which is extensively used in alleviating the inflammatory diseases by inhibiting cytokines and TNF-α [26]. Apigenin is an anti-inflammatory molecule found in fruits and vegetables. Studies show that the apigenin inhibits the production of cytokines [27] and suppresses the LPS-induced NO production in macrophages [28]. Apigenin also found to inhibit the LPS-induced pro-inflammatory cytokine production in monocytes, mouse macrophages [29] and blocks the collagenase activity that is involved in rheumatoid arthritis. Apigenin exhibit an anti-inflammatory activity in a mechanism involving the action of heme oxygenese (HO-1) enzyme in hPDL cells. [30]. In the present study, we demonstrate the anti-inflammatory effect of apigenin on PMA-activated human pulmonary epithelial cells. Apigenin up to 60 μM concentration show no effect on the reduction in cell viability as measured by MTT assay and suggested that the flavonoid does not exhibit cytotoxic effect on lung cells.

The enzyme Nitric oxide synthase (NOS) is involved in the synthesis of nitric oxide (NO). Although, NO plays a

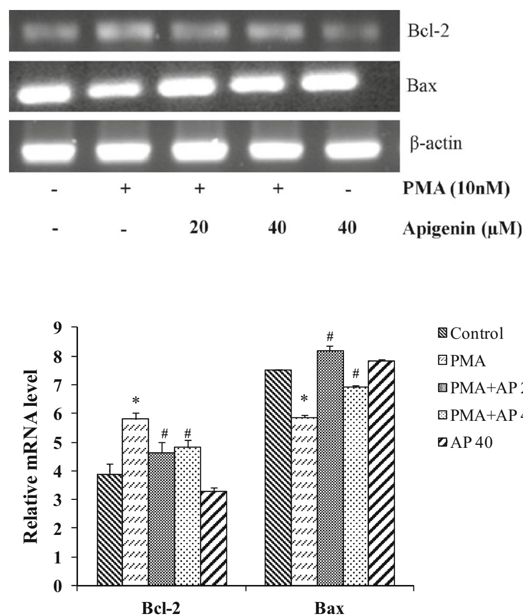

Fig. 9 Effect of PMA/apigenin on mRNA levels of Apoptotic genes. Cells were treated with or without PMA (10 nm) or PMA with apigenin (20 or 40 µM) or apigenin (40µM) alone for 24 h in 6-well plate. The mRNA levels of amplified genes of qRT-PCR were analyzed on 1 % agarose gel, and the band intensity of the experimental samples was compared with the control and PMA treatment alone. β-actin was used as a positive control and for normalization. Differences in Apoptotic genes mRNA levels are statistically significant: if $*P < 0.05$ compared with controls, $^{\#}P < 0.05$ compared with PMA-stimulated values using one-way ANOVA followed by Post-hoc Tukey test. The results are shown as representative of three independent experiments. The *bar graph* present below the figure is the densitometric analysis of mRNA levels

beneficial role as anti-cancer and anti-viral molecule [31], its extensive activation may lead to various inflammatory diseases [32]. Hence, the decreased expression of inducible nitric oxide synthase (iNOS) is regarded as a therapeutic target for inflammation [33]. In our study A549 cells treated with PMA has no effect on NO production. However, apigenin alone or PMA with apigenin marginally inhibited NO production compared to control. The inhibitory activity of apigenin on NO production was also reflected in the suppression of iNOS mRNA levels. Another important major mediator of inflammation is COX-2 enzyme, which catalyzes the biosynthesis of prostaglandin-E_2 from arachidonic acid [34]. In A549 cells, COX-2 mRNA was expressed at low levels, but rapidly and transiently induced by PMA. Apigenin significantly decreases the PMA-induced mRNA levels of COX-2 in a dose-dependent manner which suggested the anti-inflammatory activity of flavonoid. Similarly in many tissues COX-2 is undetectable, but was found to be transiently induced by pro-inflammatory cytokines, growth factors, oncogenes, and PMA [35].

In many tissues, the production of TNF-α and pro-inflammatory interleukins (IL-1β and IL-12) takes part in immune response to many inflammatory stimuli and overproduction of these mediators are detected in acute septic hemorrhagic shock [36], and in chronic (rheumatoid arthritis, atherosclerosis) inflammatory disorders [37, 38]. Our results show that within 2 h of treatment of lung cells with PMA induced the expression of IL-1β, IL-6, IL-8, and TNF-α, while at 24 h IL-2 the pro-inflammatory cytokine was also induced. At 2 h of treatment of cells with apigenin significantly inhibited the PMA induced expression of mRNA levels of IL-1β and TNF-α while marginal decrease was observed for IL-6. However, apigenin at 24 h significantly decreased the expression of all the above pro-inflammatory cytokines studied in a dose-dependent manner. Treatment of A549 cells with PMA or PMA plus apigenin show no effect on anti-inflammatory IL-10 mRNA levels, suggested the specificity of PMA as well as apigenin action on pro-inflammatory cytokines. The mRNA level of IL-6 present in the cell was also reflected in the protein secretion. Treatment of cells with PMA shows significant induction in the secretion of IL-6 protein into the medium. However, the cells treated with PMA with apigenin significantly decreased the PMA-induced IL-6 release in a dose-dependent manner. Cells treated with apigenin (40 µM) alone show marginal decrease in the secretion of IL-6 protein levels compared to control. Western blotting result shows that treatment of cells with PMA significantly induced expression of IL-1β. However, PMA with apigenin significantly decreased the PMA-induced IL-1β expression. Cells treated with apigenin (40 µM) alone show decreased IL-1β protein expression compared to control. IL-8, acts as an important mediator of inflammation of CXC chemokine family recruits neutrophils into inflamed tissue [39]. Enhanced synthesis of IL-8 has been shown in the intestinal mucosa of patients suffering from inflammatory bowel disease (IBD) [37]. In our study, apigenin also suppress the PMA-induced IL-8 mRNA expression levels suggesting the anti-inflammatory effect of flavonoid in A549 cells.

In the present study, we also reported the expression pattern of all the AP-1 factors (c-Jun, JunB, JunD and c-Fos, Fra-1, and, Fra-2) except FosB in PMA-induced inflammation and the effect of apigenin on the induced Jun/Fos proteins. The cells treated with PMA induced the expression of c-Jun, JunB, Fra-1, Fra-2, and c-Fos by 2 h and c-Jun and c-Fos by 24 h. However, PMA marginally increased the expression of Jun-D and Fra-1 at 24 h, confirms Jun and Fos family of AP-1 factors are early expressing genes. The treatment of cells with apigenin significantly decreased the PMA-induced mRNA levels of c-Jun and c-Fos in a dose-dependent manner. All most no inhibition of PMA-induced Fra-1 mRNA levels by apigenin was

observed. Cells treated with apigenin alone showed marginal decrease in Jun-B and significant increase in mRNA transcripts of Fra-2 compared to control. The results suggested that the c-Jun and c-Fos may be involved in inflammatory reaction and apigenin show anti-inflammatory activity in lung cells and this needs further investigation.

Although all the Jun and Fos proteins are expressed in A549 cells, only c-Jun, c-Fos, and Fra-1 were induced by PMA. In normal lobular and ducts, the epithelial cells show higher expression of FosB mRNA and protein, whereas in majority of carcinomas and MCF-7 cells less of FosB protein was expressed [40], and this is in agreement with our study using A549 cells where little or no FosB mRNA expression was observed.

Western blotting also confirms the induced expression of c-Jun and c-Fos by PMA and apigenin decreased the expression by 24 h. Further, our study also show that PMA-induced p53 and cyclin D1 mRNA expression modulated to control levels by certain extent by increased concentration of apigenin. However, apigenin has stimulatory effect on CDK4 expression that needs to be investigated. Apigenin alone significantly decreased the cyclin E1 mRNA levels suggested that the flavonoids may have anti-proliferative and anti-cancerous effect on the lung cells challenged with the phorbol esters. Apigenin also show inhibitory effect on PMA-induced anti-apoptotic Bcl2 gene, confirms the therapeutic property of flavonoid [41] and probably has protective effect.

The anti-inflammatory effect of apigenin is probably based on the modulation of pro-inflammatory gene expression of cyclo-oxygenase, lipoxygenase, nitric oxide synthases, and several inflammatory cytokines and AP-1 factors mainly acting through NF-κB and MAPK signaling [42]. Because of their essential role in intracellular signaling network, MAPK pathways and the connected transcription factors are appropriate targets for pharmacological treatment of inflammatory disorders [43]. The flavonoids especially apigenin is required to be extensively investigated as possible regulatory molecule(s) in inflammatory processes.

Conclusion

Our study demonstrate that the treatment of apigenin inhibits and modulate the PMA-induced mRNA expression of cyclooxygenase-2 (COX-2) and pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, and tumor necrosis factor-α (TNF-α). Our study also confirms that apigenin decreased the expression of IL-1β protein and synthesis and secretion of IL-6. Apigenin also inhibited the PMA-induced expression of AP-1 factors (c-Jun and c-Fos) both at mRNA and protein levels. Hence, apigenin is a valuable source of new potential anti-inflammatory compound that may have therapeutic potential for the modulation and regulation of lung epithelial cell activation, and may provide safe and effective treatment options for variety of lung inflammation-mediated diseases.

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References

1. Dinarello CA (1997) Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest 112:321s–329s
2. Palladino MA, Bahjat FR, Theodorakis EA, Moldawer LL (2003) Anti-TNF-alpha therapies: the next generation. Nat Rev Drug Discov 2:736–746. doi:10.1038/nrd1175
3. Levine SJ (1995) Bronchial epithelial cell-cytokine interactions in airway inflammation. J Investig Med 43:241–249
4. Ershler WB, Keller ET (2000) Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. Annu Rev Med 51:245–270. doi:10.1146/annurev.med.51.1.245
5. Arnold R, Humbert B, Werchau H, Gallati H, Konig W (1994) Interleukin-8, interleukin-6, and soluble tumour necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. Immunology 82:126–133
6. Esposito E, Cuzzocrea S (2007) The role of nitric oxide synthases in lung inflammation. Curr Opin Investig Drugs 8:899–909
7. Murakami A, Ohigashi H (2007) Targeting NOX, INOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. Int J Cancer 121:2357–2363. doi:10.1002/ijc.23161
8. Mitchell JA, Larkin S, Williams TJ (1995) Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 50:1535–1542
9. Nathan C, Xie QW (1994) Nitric oxide synthases: roles, tolls, and controls. Cell 78:915–918
10. Papavassiliou AG (1995) Transcription factors: structure, function, and implication in malignant growth. Anticancer Res 15:891–894
11. Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2:725–734. doi:10.1038/nri910
12. Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25:280–288. doi:10.1016/j.it.2004.03.008
13. Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. Nat Cell Biol 4:E131–E136. doi:10.1038/ncb0502-e131
14. Guo RF, Lentsch AB, Sarma JV, Sun L, Riedemann NC, McClintock SD, McGuire SR, Van Rooijen N, Ward PA (2002) Activator protein-1 activation in acute lung injury. Am J Pathol 161:275–282. doi:10.1016/s0002-9440(10)64179-x
15. Bjarnason I, Hayllar J, MacPherson AJ, Russell AS (1993) Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology 104:1832–1847
16. Maruta A, Enaka K, Umeda M (1979) Mutagenicity of quercetin and kaempferol on cultured mammalian cells. Gann 70:273–276
17. Wei H, Tye L, Bresnick E, Birt DF (1990) Inhibitory effect of apigenin, a plant flavonoid, on epidermal ornithine decarboxylase and skin tumor promotion in mice. Cancer Res 50:499–502

18. Hirano T, Oka K, Akiba M (1989) Antiproliferative effects of synthetic and naturally occurring flavonoids on tumor cells of the human breast carcinoma cell line, ZR-75-1. Res Commun Chem Pathol Pharmacol 64:69–78
19. Nielsen SE, Young JF, Daneshvar B, Lauridsen ST, Knuthsen P, Sandstrom B, Dragsted LO (1999) Effect of parsley (Petroselinum crispum) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. Br J Nutr 81:447–455
20. Babu RL, Naveen Kumar M, Patil RH, Devaraju KS, Ramesh GT, Sharma SC (2013) Effect of estrogen and tamoxifen on the expression pattern of AP-1 factors in MCF-7 cells: role of c-Jun, c-Fos, and Fra-1 in cell cycle regulation. Mol Cell Biochem 380:143–151. doi:10.1007/s11010-013-1667-x
21. Sharma CS, Sarkar S, Periyakaruppan A, Barr J, Wise K, Thomas R, Wilson BL, Ramesh GT (2007) Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. J Nanosci Nanotechnol 7:2466–2472
22. Periyakaruppan A, Kumar F, Sarkar S, Sharma CS, Ramesh GT (2007) Uranium induces oxidative stress in lung epithelial cells. Arch Toxicol 81:389–395. doi:10.1007/s00204-006-0167-0
23. Eigler A, Moeller J, Endres S (1995) Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in the murine macrophage cell line RAW 264.7. J Immunol 154:4048–4054
24. Sharma SC, Clemens JW, Pisarska MD, Richards JS (1999) Expression and function of estrogen receptor subtypes in granulosa cells: regulation by estradiol and forskolin. Endocrinology 140:4320–4334. doi:10.1210/endo.140.9.6965
25. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
26. Kim SJ, Jeong HJ, Moon PD, Lee KM, Lee HB, Jung HJ, Jung SK, Rhee HK, Yang DC, Hong SH, Kim HM (2005) Anti-inflammatory activity of gumiganghwaltang through the inhibition of nuclear factor-kappa B activation in peritoneal macrophages. Biol Pharm Bull 28:233–237
27. Kowalski J, Samojedny A, Paul M, Pietsz G, Wilczok T (2005) Effect of apigenin, kaempferol and resveratrol on the expression of interleukin-1beta and tumor necrosis factor-alpha genes in J774.2 macrophages. Pharmacol Rep 57:390–394
28. Lee JH, Zhou HY, Cho SY, Kim YS, Lee YS, Jeong CS (2007) Anti-inflammatory mechanisms of apigenin: inhibition of cyclooxygenase-2 expression, adhesion of monocytes to human umbilical vein endothelial cells, and expression of cellular adhesion molecules. Arch Pharm Res 30:1318–1327
29. Nicholas C, Batra S, Vargo MA, Voss OH, Gavrilin MA, Wewers MD, Guttridge DC, Grotewold E, Doseff AI (2007) Apigenin blocks lipopolysaccharide-induced lethality in vivo and proinflammatory cytokines expression by inactivating NF-kappaB through the suppression of p65 phosphorylation. J Immunol 179:7121–7127
30. Jeong GS, Lee SH, Jeong SN, Kim YC, Kim EC (2009) Anti-inflammatory effects of apigenin on nicotine- and lipopolysaccharide-stimulated human periodontal ligament cells via heme oxygenase-1. Int Immunopharmacol 9:1374–1380. doi:10.1016/j.intimp.2009.08.015
31. MacMicking J, Xie QW, Nathan C (1997) Nitric oxide and macrophage function. Annu Rev Immunol 15:323–350. doi:10.1146/annurev.immunol.15.1.323
32. Bogdan C (2001) Nitric oxide and the immune response. Nat Immunol 2:907–916. doi:10.1038/ni1001-907
33. Hobbs AJ, Higgs A, Moncada S (1999) Inhibition of nitric oxide synthase as a potential therapeutic target. Annu Rev Pharmacol Toxicol 39:191–220. doi:10.1146/annurev.pharmtox.39.1.191
34. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK (1999) Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20:1945–1952
35. Vane SJ (1998) Differential inhibition of cyclooxygenase isoforms: an explanation of the action of NSAIDs. J Clin Rheumatol 4:s3–s10
36. Jachak SM (2006) Cyclooxygenase inhibitory natural products: current status. Curr Med Chem 13:659–678
37. Ohshima H, Bartsch H (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat Res 305:253–264
38. Krakauer T (2004) Molecular therapeutic targets in inflammation: cyclooxygenase and NF-kappaB. Curr Drug Targets Inflamm Allergy 3:317–324
39. Mazzucchelli L, Hauser C, Zgraggen K, Wagner H, Hess M, Laissue JA, Mueller C (1994) Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. Am J Pathol 144:997–1007
40. Milde-Langosch K, Kappes H, Riethdorf S, Loning T, Bamberger AM (2003) FosB is highly expressed in normal mammary epithelia, but down-regulated in poorly differentiated breast carcinomas. Breast Cancer Res Treat 77:265–275
41. Morrissey C, O'Neill A, Spengler B, Christoffel V, Fitzpatrick JM, Watson RW (2005) Apigenin drives the production of reactive oxygen species and initiates a mitochondrial mediated cell death pathway in prostate epithelial cells. Prostate 63:131–142. doi:10.1002/pros.20167
42. Yoon JH, Baek SJ (2005) Molecular targets of dietary polyphenols with anti-inflammatory properties. Yonsei Med J 46:585–596
43. Lewis TS, Shapiro PS, Ahn NG (1998) Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49–139