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Title: Naringenin attenuates inflammation in chronic obstructive pulmonary disease in cigarette smoke induced mouse model and involves suppression of NF-κB

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Running title: Effect of Naringenin on cigarette smoke-induced COPD
Abstract

Inflammation of lungs and airways is a primary symptom of the Chronic Obstructive Pulmonary Disease (COPD). The COPD inflammation is known to be mediated by several factors such as pro-inflammatory cytokines, glucocorticoid receptor (GR), NF-κB. Patients with COPD respond very poorly to the corticosteroid therapy. Naringenin is a flavonoid known to possess anti-inflammatory properties. We studied the effect of naringenin on COPD induced due to cigarette smoke (CS) in BALB/c mouse model and in vitro in A549 cells. COPD was induced in Mice by exposing them to CS for 90 days. The animals were pre-treated with naringenin in 20, 40 and 80 mg/Kg concentration. Whereas, the in vitro cells were treated with naringenin along with CS extract exposure. Naringenin was found to significantly improve the pulmonary function, decreased inflammatory cells, and inhibited the production of pro-inflammatory cytokines such as IL-8, TNF-α, and MMP9 in the BALF and serum of CS animal group. Naringenin also appeared to inhibit the NF-κB pathway as revealed by reduced phosphorylation of NF-κB and IκB in western blot test. Moreover, the levels GR mRNA and protein were also significantly increased upon treatment with naringenin to CS-exposed animals and cell culture. It could be concluded that naringenin can be a potential therapeutic agent for the treatment of COPD-related inflammation.

Keywords: COPD, Flavonoids, Inflammation, Cigarette smoke, Glucocorticoid receptor

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by inflammation of the lungs and obstruction of airways. The Inflammation of Lungs is caused due to the influx of inflammatory cells, for example, macrophages, neutrophils, epithelial cells and lymphocytes [1, 2]. COPD is also associated with up-regulation of a cascade of inflammatory mediators including IL-8 and pro-inflammatory cytokines such as Interleukin-1β (IL-1β), Tumor...
Necrosis Factor-α, TNF-α and IL-6 [3, 4]. IL-1β activates macrophages which in turn elevate the levels of Matrix Metalloproteinase-9 (MMP9) [3]. MMP9 has also been shown to have higher levels in COPD as compared to non-COPD samples [5]. World Health Organization (WHO) has predicted COPD to be among the top five chronic diseases in the world [6, 7]. The response of COPD to anti-inflammatory therapies for example corticosteroids such as dexamethasone is very poor [8]. This suggests that COPD inflammation is specifically resistant to corticosteroids [9, 10]. The glucocorticoid resistance in COPD is poorly understood. However, several possible explanations have been given such as reduced glucocorticoid receptor (GR) binding ability [11]. The GR translocates the glucocorticoid signal to glucocorticoid response elements (GRE) by binding to it. GR is also known to bind NF-κB and AP-1 causing reduction gene expression related to inflammation [12, 13]. A reduction in GR concentration causes a reduced response to the presence of glucocorticoids causing increased concentration of NF-κB in the nucleus. NF-κB binds to open promoters and causes an early cigarette smoke response [14]. In view of glucocorticoid resistance in COPD, new anti-inflammatory molecules are needed.

Several flavonoids are known to have a beneficial effect on COPD [15]. Naringenin is a plant-derived flavone found in tomato, grapefruit and citrus fruits [16]. Naringenin is known for its antioxidant and anti-inflammatory properties [17]. Naringenin has also been shown to have a role in the modulation of MMP2 and MMP9 via inhibition of NF-κB pathway [18]. In this study, we determined the anti-inflammatory properties of naringenin. We used the corticosteroid dexamethasone as a negative control as it has extensively been reported to ineffective against COPD [19]. We also investigated the in vivo role of naringenin on NF-κB and GR levels in CS-induced lung-inflammation in BALB/c mice and in vitro on the epithelial cell line.

Materials and Methods
Animal experiments

BALB/c mice (male, 6 weeks old) were obtained from the institutional animal house. The animals were kept under a 12 by 12-hour light and dark circadian cycle and the under controlled conditions of temperature and humidity. The animals were fed with the standard rat diet and water ad libitum. All the animals’ up-keeping and experimental procedures were carried out in accordance with the ethical standards given in the Declaration of Helsinki. The animal experiments were conducted with prior approval from the institutional review board with approval number 2018-IRB6955 and institutional animal care and use committee (IACUC) with approval number 2018-IACUC6953-R. All the All efforts were made to minimize the number of animals and their suffering.

Marlboro brand Cigarettes were purchased from local market. Mice (n=15/group) were exposed to cigarette smoke (CS) of 15 cigarettes for one hour per day for 90 days [20, 21]. Naringenin (PubChem SID: 319221668) was procured from Sigma-Aldrich (U.S.A.) and dissolved in 5% dimethyl sulfoxide (DMSO). Naringenin (20, 40, 80 mg/Kg) or dexamethasone (2 mg/Kg) was orally administered to the animals by gavage in 300μl volume two hours before the CS exposure. The dose of dexamethasone was chosen on the basis of previous studies on cigarette smoke-induced COPD [22-24]. The control group animals were exposed to fresh air and were not given any treatment. The optimal doses of the drug were selected by a preclinical toxicity testing study. The animals were euthanized at the end of treatment using the CO₂ overdose method 48 hours after the commencement of the experiment. Lung tissue, serum and bronchoalveolar lavage (BALF) were obtained from the sacrificed animals. After the measurement of lung mechanics, the separation of serum and BALF was performed by centrifuging at 2000g for 15 min at 4 °C.

Analysis of Pulmonary function parameters
The basal function of lungs was determined using whole-body plethysmography (WBP) system (Data Sciences International). Pulmonary parameters such as minute ventilation (MV), peak expiratory flow (PEF) and, peak inspiratory flow (PIF) were measured. The WBP method allowed continuous data collection of the above parameters.

**Extraction of Bronchoalveolar lavage fluid**

Broncho-alveolar lavage (BAL) was done by lavaging with 5 ml of PBS and a gentle aspiration. Approximately 90% of the BALF was recovered. Centrifugation of the BALF was carried out at 1000g for 30 minutes at 4 °C temperature. The obtained BALF was centrifuged at 2000g for 15 min at 4 °C and the supernatants were obtained.

**Analysis of Pulmonary Histopathology**

The upper lobes of the right lungs were excised from all the six experimental groups. The lung tissues were fixed in 8% paraformaldehyde for 24 hours, dehydrated in ethanol and sliced into 4 µm sections after embedding them in paraffin. Post sectioning, the tissues were deparaffinized and subsequently stained using the hematoxylin-eosin (H&E) stain. Histopathological variations in lungs were analyzed under a light microscope. The histopathological assessment of lung parenchyma and bronchus was performed as a blind test. The severity of inflammation was estimated on a four-point scale (0-3). Where 0=normal histopathology with no inflammation, 1=mild inflammation with observation inflammatory cells in alveolar septa or vascular and bronchial walls, 2=moderate inflammation or inflammation localized on the bronchial walls, alveolar septum and blood vessels; one third or less lung cross-section area affected with these observations, 3=severe inflammation in lung tissue with diffused inflammatory cells in the bronchi walls or alveoli septum and blood vessels; one third or less lung cross-section area affected with these observations[25].
The variation in air spaces was determined by employing a modified procedure by Sato T, et al. [26]. This procedure determines the mean-linear-intercept (MLI). We randomly selected ten fields per section and the count of alveolar sections per unit area was determined. The destruction of alveolar wall was estimated using the destructive index (DI) as described by Saetta M, et al. [27].

**Preparation of cigarette smoke extract for in vitro experiments**

The aqueous extract of cigarette smoke (CS) was prepared for in vitro experiments. The filter of cigarette was cut off using a surgical blade and the cigarette was lighted and the smoke was sucked using a peristaltic pump. The smoke thus produced was passed through 10 ml of culture medium in form of bubbles [28-30]. Subsequently, the medium was filter sterilized and pH was readjusted to 7.4. This preparation was considered to be 10% and was then quickly applied for in vitro experiments.

**Cell-culture and naringenin treatment**

The A549 cell lines were purchased from Sigma Aldrich (USA). The cells were maintained in high glucose DMEM medium and supplemented with 12% fetal-bovine-serum (FBS), 75 Units/ml of penicillin and 150 mg/ml of streptomycin at 37 ºC under a humidified atmosphere containing 5% CO₂. The cells were cultured up to 80% of confluency in culture plates (100 mm) containing 10 ml DMEM medium and 12% FBS. The cells were serum starved for 10 h. Subsequently, the cells were washed with sterile ice-cold PBS then the cells were incubated with 2.5% CSE. The cells were treated with naringenin at a concentration of 2mM, 20mM and 50 mM for 2 h. Naringenin treatment was followed by treatment with CSE for 12 h.

**Analysis of pro-inflammatory cytokine levels**
The levels of MMP9, tumor necrosis factor-α (TNF-α), Interleukin (IL)-8, in the serum and BALF, and TNF-α and IL-8 in cultured A549 cell supernatants were determined using ELISA. The ELISA kits were purchased from Abcam, USA and ELISA was performed as per the manufacturer’s instructions.

**Immuno-histochemical Analysis for glucocorticoid receptor levels**

The expression of the glucocorticoid receptor (GR) in mice lung tissues was determined using immunohistochemistry (IHC). The assay was performed using the BioModule™ Immuno-histochemical (IHC) Staining kit (ThermoFisher, USA) following the manufacturer's protocol. Anti-GR polyclonal antibodies were procured from Abcam (USA). We read three slides at high power in 10 fields. The negative control was created by the addition of PBS instead of primary antibody solution. The cytoplasmic region stained in brown was considered positive. The quantitative expression of GR was determined using Nikon SMZ18 stereomicroscope. The area under GR-positive cells and their optical-density (OD) was evaluated by randomly selecting three fields per slide. The IHC-index was calculated on the basis of average-integral optical-density (AIOD) where AIOD=GR-positive area ×OD/total sample area.

**Western Blot densitometry**

Antibodies for NF-κB p65, p-NF-κB p65 and GR were procured from the Santa Cruz Biotechnology, Inc. (USA). The anti-IκB-α and anti-p-IκB-α antibodies were procured from the Cell Signaling Technology, Inc. (USA). The lung tissue was homogenized and the nuclear proteins were isolated using the NE-PER™ kit (ThermoFisher, USA). The isolated protein concentration was estimated using Bradford’s method. 15 μg of the estimated protein was loaded per well on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel. The proteins were electrophoretically transferred on a polyvinylidene difluoride (PVDF) membrane after completion of gel run. Blocking of the membranes was performed for
1.5 hours in Tris-buffered saline which contained 0.2% Tween 20 (TBST) and 2% non-fat dry milk. Binding of primary antibodies onto the membranes was performed by incubating it with primary antibody solution in TBST and 2% non-fat dry milk for 8 hours at 4°C. The membrane was washed thrice with TBST. Finally, the membranes incubated for 1 h with horse radish peroxidase (HRP) conjugated secondary antibodies and developed using an ECL detection system.

**qRT-PCR analysis for glucocorticoid receptor mRNA levels**

Total RNA was isolated from A549 cells using the RNeasy Mini Kit (Qiagen, USA) subsequently qRT-PCR was performed using One-Step qRT-PCR Kit (Qiagen, USA) following the manufacturer's protocol. The designing of qRT-PCR primers was done using the primer blast [31]. The melting temperature (T<sub>m</sub>) of primers was taken between 57°C and 63°C. The size of the amplification product was taken between 90 and 120 bp along with a maximum difference in Tm of 3°C. We took three technical replicates for every biological replicate. The quality of RNA was assessed by aQIAxpert<sup>TM</sup> microfluidic UV/VIS spectrophotometer (Qiagen, USA). The PCR master mix included 5 μl DyNAmo Flash SYBR Green (Thermo) (2X), 1.5 μl cDNA, 1 μl (5 pm/μl) each primer. The following cycling conditions were used for qRT-PCR: denaturation at 95°C for 10 min, 40 denaturation cycles at 95°C for 22 s, annealing with extension at 60°C for 60 s. The qRT-PCR amplification was performed in ABI 7500 system (Applied Biosystem). The threshold cycle value (Ct) for the genes were quantified and normalized by the Ct value of the β-actin expression. The following primers were used for qRT-PCR: GR (5′-CCAAAAGTACGTATGCGCGA -3′, 5′-GTGTCACTTGCAGGGCTGTA -3′). β-actin (5′-AGAGCTACGAGCTGCCTGAC -3′; 5′-AGCAGTCGTGTGGCGAG- 3′) was taken as an internal control. The relative expression was calculated using the 2<sup>-ΔΔCt</sup> method [32].
Statistical Analysis

The individual averages were expressed in the form of mean ± SEM. The variance between the experimental groups was estimated using one way ANOVA with Bonferroni's multiple corrections for post-hoc analysis. Significance values of P<0.05 were considered significant. All the statistical estimations were performed in STATISTICA v8.0.

Results

Effect of naringenin on the pulmonary function of CS-exposed animals

The parameters of breathing in mice exposed to CS were determined using whole-body plethysmography. The test was performed after 90 days exposure to fresh air or CS. There was a significant and consistent decrease in the peak expiratory flow (PIF), peak inspiratory flow (PEF), and minute ventilation (MV). The animals treated with naringenin showed a significant improvement in these parameters in a dose dependent manner when compared to the animals exposed to CS (Figure 1A, 1B, and 1C respectively). The animals treated with dexamethasone did not show any improvement of breathing parameters as compared to the CS group.

Effect of naringenin on the lung histopathology

Histopathological examination of the animal groups was performed after commencement of the experiment. Lung tissue stained with hematoxylin and eosin were examined. There were marked changes in animals exposed to CS. Exposure to CS caused massive infiltration of inflammatory cells, interstitial edema, and severe damage to the pulmonary architecture (Figure 2). Whereas, the fresh air-exposed animals showed normal histopathology. Treatment with naringenin (40 mg/Kg and 80 mg/Kg) caused significant attenuation of inflammation in lung tissue as characterized by a marked decrease in the
inflammatory cells (Figure 2 and Figure 3A). However, the reduction in inflammation was not observed in the dexamethasone-treated animals.

The mean linear intercepts (MLI) and destructive index (DI) showed a significant increase in CS animals as compared to the control group. The treatment with naringenin caused a significant reduction in these parameters in a drug dependent manner (Figure 3B and 3C respectively). Moreover, dexamethasone treatment showed no difference in from the animals exposed to CS.

**Effect of naringenin on pro-inflammatory cytokine levels**

The levels of inflammatory cytokines in the lung tissue (*in vivo*) and the A549 cell supernatants (*in vitro*) were determined to identify the effect of naringenin on inflammatory response due to CS exposure. In both serum and BALF, a significant increase in the levels of IL-8, TNF-α, and MMP9 was observed upon treatment with CS for 90 days as compared to fresh air treatment (Figure 4A and B respectively). Naringenin treatment at concentrations of 20 mg/kg, 40 mg/kg, and 80 mg/kg caused significant inhibition of IL-8, TNF-α, and MMP9 in comparison to the CS-exposed group. However, treatment with corticosteroid dexamethasone showed no reduction in the levels of IL-8, TNF-α, and MMP9. Naringenin also showed a significant decrease in BALF concentrations of IL-8, TNF-α, and MMP9 in concentrations of 20mM and 50mM in the A549 cells treated with CS extract (Figure 4C).

**Effect of naringenin on NF-κB levels**

On the basis of previous studies on the down-regulation of the NF-κB pathway in LPS induced acute lung injury (ALI) by naringenin [33, 34], we attempted to identify a similar role in the CS-exposed animals. The phosphorylation of IκB-α and NF-κB p65 was estimated using immunological (western blot) analysis (Figure 5A). These phosphorylations reflected the NF-
κB activation in the tissues of the lung. As illustrated in (Figure 5B and 5C), exposure to CS resulted in 17 and 11 fold increase (P-value < 0.01) in the phosphorylation of NF-κB p65 and IκB-α respectively in comparison to the fresh air treated group. Whereas, a significantly large and dose dependent decrease in the NF-κB p65 and IκB-α phosphorylation was observed upon treatment with naringenin. These results indicated that naringenin could inhibit the NF-κB activated by CS extract.

**Effects of naringenin on GR protein and mRNA expression**

The glucocorticoid receptor is known to act as an important co-factor for the activation of the inflammatory response. Thus, we determined the effect of naringenin treatment. Immuno-histochemical analysis showed that the IHC index of GR reduced significantly in the CS group of animals as compared to the fresh air (control) group. Treatment with naringenin at 20, 40 and 80 mg/kg caused a significant increase in the IHC index of GR (Figure 6). These results of immuno-histochemistry were verified using western blot analysis. This also showed that the CS-exposed group had decreased levels of GR protein as compared to the control group. Moreover, the naringenin treatment markedly improved the GR levels (Figure 7A and 7B).

To further verify this observation, we performed qRT-PCR to determine the GR mRNA expression in A549 cells treated with CS extract. The results of qRT-PCR revealed that GR expression was significantly less in CS extract treated the group as compared to that of the control group. Moreover, naringenin (50 mM) pre-treatment significantly increased the GR expression (Figure 7C). Whereas, the protein levels of GR showed no significant difference between the animals treated with dexamethasone and CS. These results suggested that naringenin mediated in the up-regulation of GR expression.

**Discussion**
COPD is a chronic disease involving lung inflammation and involves several inflammatory pathways, transcription factors such as NF-κB and receptors such as GR. COPD is known to be resistant to anti-inflammatory therapy involving corticosteroids such as dexamethasone. Therefore, natural alternatives to attenuation of inflammation are warranted.

Naringenin is a plant-derived flavonoid known to possess anti-inflammatory properties. In this study, we for the first time report the effect of naringenin treatment on the pulmonary function, pro-inflammatory cytokine levels, NF-κB levels, GR and histopathology of lung tissue in mice model. Previously only a few studies have shown the effect of naringenin on CS-induced COPD [35]. To study the effect of naringenin on COPD, we employed to different approaches viz. in vivo and in-vitro. In the in vivo approach, COPD was induced in BALB/c mice by exposing them to cigarette smoke for 90 days along with pre-treatment with naringenin. In the in vitro approach, A549 cells were treated with CS extract. Human lung epithelial cell line from human A549 cells are commonly used in studies involving the effect of CS on lungs [36]. This cell line has previously been used for the studies such as the effect of oxidative stress and pro-inflammatory cytokine release [29], COPD [37] and innate immune responses to bacteria [38]. Therefore, we selected this cell line for in vitro experiments with CSE.

Naringenin pre-treatment to BALB/c mice caused marked improvement in the pulmonary function of animals and inhibited the cytokines such as IL-8, TNF-α, MMP9, and NF-κB. The expression of the Glucocorticoid receptor was also induced by naringenin. In vitro, naringenin treatment of A549 cells exposed to CS extract showed suppression of IL-8 and TNF-α. Moreover, the transcription of GR was also significantly upregulated. Histopathological analysis of lung tissue showed marked improvement in the inflammatory symptoms. The reduction in pulmonary function parameters such as PIF, PEF, and MV were markedly alleviated with naringenin treatment indication a reduction of inflammation in the airways. Moreover, the immunohistochemical analysis showed that naringenin significantly
inhibited CS-induced degradation of GR. Overall the naringenin treatment showed significant anti-inflammatory properties leading to marked alleviation of COPD symptoms.

Cigarette smoke causes induction of oxidative stress and inflammatory response leading to the development of COPD [39]. Cigarette smoke causes inflammation of airways by the activation of neutrophils, T lymphocytes, and macrophages. These inflammatory mediators mediate the release of reactive oxygen species (ROS) and proteases which lead to cellular injury [27, 40]. Transcription factors such as AP-1 and NF-κB are released in response to oxidative damage leading to up-regulated expression of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α [41, 42]. The corticosteroid dexamethasone was used as a control treatment for COPD in mice. We found that dexamethasone failed to alleviate the pulmonary function parameters in addition to being ineffective in alleviation other in vivo inflammation parameters. These results indicated an emergence of corticosteroid insensitivity in CS indicated COPD in mice model.

The transcription factor NF-κB is central to the regulation of expression of genes of pro-inflammatory cytokines, chemokines, adhesion factors, growth mediators and several enzymes [43]. GR plays an important role in the inhibition of NF-κB induced gene expression of pro-inflammatory cytokines [12]. GR forms a complex with NF-κB, this complex fails to bind with promoters leading to down-regulation of gene expression of pro-inflammatory cytokines [14].

In the present study, naringenin caused significant up-regulation of the expression of IκB-a protein in COPD mice. Moreover, the phosphorylation of p65 protein was also significantly decreased indicating inhibition of the NF-κB pathway. From this result, it could be inferred that naringenin exercises its anti-inflammatory properties by modulation the NF-κB signaling pathway.
Impairment of corticosteroid sensitivity has been reported in CS-induced inflammatory models and patients suffering from COPD [10]. GR is known to play an important role in glucocorticoid responsiveness and the connection between low corticosteroid activity and COPD is based on GR and NF-κB in the lungs. Previous studies clearly show that the expression of GR is significantly down-regulated in CS-exposed animals as compared to normal controls, causing up-regulation of pro-inflammatory cytokines [44].

GR plays a key role in the translocation of pro-inflammatory signal and corticosteroid sensitivity. CS exposure and COPD are known to cause inhibition of GR expression which leads to up-regulation of pro-inflammatory genes and amplification of pro-inflammatory signal [44, 45]. Therefore, we studied the expression of GR in vivo in CS-exposed mice and in vitro in CS extract A549 cells. GR expression was studied using western blot, immunohistochemical analysis, and qRT-PCR. CS-exposure for 90 days caused a significant reduction in GR level as shown by western blot, immune-histochemistry, and qRT-PCR. Moreover, dexamethasone treatment did not show any attenuation of CS-induced down-regulation of GR as compared to naringenin. The ineffectiveness of dexamethasone towards COPD is consistent with previous findings [19] and indicates glucocorticoid insensitivity towards the disease. In all the three experiments the GR expression was found to be up-regulated due to naringenin treatment. This increased expression of GR might help in the inhibition of CS-induced inflammatory symptoms.

COPD patients exhibit elevated concentration of pro-inflammatory cytokines such as TNF-α and IL-8 in the blood plasma [3, 4]. Under inflammation, GR inhibits genes related to inflammatory responses through disruption of p-65 interferon regulatory-factor complex, [46] signaling pathways and pro-inflammatory mediators. In this study, we found that naringenin treatment leads to reduced expression of TNF-α, IL-8, and MMP9 leading to their diminished inflammatory response.
In conclusion, the plant flavonoid naringenin showed protective properties against cigarette smoke-induced COPD. All the parameters for measuring pulmonary function showed significant improvement of COPD symptoms. The decreased expression of cytokines, IκB, and NF-κB upon treatment with naringenin clearly indicated its anti-inflammatory role on the NF-κB signaling. Moreover, increased expression of GR *in vivo* and *in vitro* indicated that suppression of NF-κB pathway was mediated by complex formation between GR and NF-κB. Although this study highlights naringenin as a potential therapeutic agent for the treatment of COPD, further experimental studies would be required to investigate the role of NF-κB and GR in anti-inflammatory response related to COPD.

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Figure Legends:

Figure 1. Effects of naringenin on pulmonary function in CS-exposed mice. (A) Peak
inspiratory flow (PIF); (B) Peak expiratory flow (PEF); (C) Minute ventilation (MV), were
measured in unrestrained mice using a whole-body plethysmograph (WBP) system. Data are
mean ± SEM (n = 10). * P<0.05, ** P<0.01 vs. CS-exposed mice. Dex = dexamethasone.

Figure 2. Effects of naringenin on lung histopathology. Mice were exposed to CS for 90 days
and treated with naringenin (20, 40 and 80 mg/kg) or dexamethasone (2 mg/kg). Lung
parenchyma and bronchus was stained by H&E before being examined. Magnification bar
represents 200µm.

Figure 3. Histopathological assessment of lung parenchyma and bronchus. The (A)
Inflammation score (B) MLI (mean-linear-intercept) (C) and DI (destructive index) were
scored as described in methods. Data are mean ± SEM (n = 10). * P<0.05, ** P<0.01 vs. CS-
exposed mice.

Figure 4. Effects of naringenin on CS-induced TNF-α, IL-8 and MMP9 levels in vivo and in
vitro. The levels of cytokines were measured in (A) serum, (B) BALF and (C) culture
supernatants were measured by ELISA. Data are mean ± SEM (n = 10). * P<0.05, ** P<0.01
vs. CS-exposed mice or CSE-exposed A549 cells. CS = cigarette smoke; CSE = cigarette
smoke extract; DEX = dexamethasone; BALF = bronchoalveolar lavage fluid.
Figure 5. Effects of naringenin on NF-κB activation in CS-induced inflammation. Mice were exposed to CS for 90 days and treated with naringenin (20, 40 and 80 mg/kg) or dexamethasone (2 mg/kg). The levels of (A) p65, p-p65 and IκB-α, (B) phosphorylation of p65, (C) degradation of IκB-α (C) were determined using western blot densitometry using β-actin as internal control. Data are mean ± SEM (n = 10).* P<0.05, ** P<0.01 vs. CS-exposed mice.

Figure 6. Estimation of glucocorticoid levels in mice lung tissues using immunohistochemistry. Micrographs of GR immunostaining in mice (A) Control (B) CS (D) Naringenin 20 mg/Kg (E) Naringenin 40 mg/Kg (F) Naringenin 80 mg/Kg (G) Immuno-histochemical index of GR. Positive immunostaining is indicated by arrows. Data are mean ± SEM (n = 10).* P<0.05, ** P<0.01 vs. CS-exposed mice. Dex = dexamethasone. All magnifications are at 200x.

Figure 7. Effect of naringenin on CS-mediated reduction in expression of GR protein and mRNA in vivo and in vitro. (A) Western blot of GR in control, CS and treatment groups, (B) GR protein expression levels determined by western blot densitometry, (C) GR mRNA levels in control, CS and treatment group determined by qRT-PCR. Data are mean ± SEM (n = 10).* P<0.05, ** P<0.01 vs. CS-exposed mice or CSE-exposed cells. CS = Cigarette smoke; CSE = Cigarette smoke extract; Dex = dexamethasone.
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