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Original Article

Ginsenoside Rg1 alleviates pulmonary inflammation caused by cigarette smoke-induced COPD

Yujing Tao[1], Xinyan Liu[1], Zhenhua Ni[2], Xuejun Yu[1]

[1]*Geriatrics Department, Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200062, China;* [2]*Central Laboratory, Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200062, China*

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Abstract: Ginsenoside Rg1, the major effective component in ginseng, has been reported to have potent anti-inflammatory properties. However, the effect of Rg1 on pulmonary inflammation caused by exposure to cigarette smoke (CS) has not been investigated. In this study, we examined the molecular mechanisms underlying the effect of Rg1 on CS-induced inflammation using a mouse model. We found that inflammatory cell numbers (total cells, macrophages and neutrophils) in the bronchoalveolar lavage (BAL) fluid of the CS-exposed (CSE) mice group were remarkably increased compared with air-exposed mice, and these changes were significantly ameliorated in Rg1-treated mice. Rg1 treatment also reduced the CS-induced increase in TNF-α, IL-6 and TGF-β in BAL supernatant and lung tissues using ELISA and qRT-PCR analysis. Moreover, the administration of Rg1 significantly attenuated the phosphorylation of mitogen-activated protein kinase (MAPK) and downregulated the activation of nuclear factor-κB (NF-κB) in CSE mice. These results suggested that Rg1 may effectively attenuate inflammatory responses induced by CS through the MAPK and NF-κB pathways, and thus maybe an ideal agent for treating inflammatory pulmonary diseases.

Keywords: Ginsenoside Rg1, inflammatory responses, COPD, MAPK, NF-κB

Introduction

Chronic obstructive pulmonary disease (COPD) is defined by a progressive airflow limitation due to an abnormal inflammatory response of the lung to noxious particles and gases [1]. Cigarette smoking (CS) is by far the most common risk factor for COPD, in addition to other risk factors such as genetic susceptibility and inhalation of occupational dusts, chemicals and pollutants [2]. CS exposure (CSE) induces inflammation in the lung and airway, characterized by marked recruitment of neutrophils to the site of inflammation [3]. The increased numbers of neutrophils recruited following CSE aggravate airway inflammation resulting instructural damage via increased levels of pro-inflammatory mediators [4]. The prevalence of COPD has markedly increased worldwide and has become a significant health problem [4]. Despite significant progress, there are few effective disease-modifying drugs for COPD, and there is an urgent need for more potent medicine for intervention in this disease.

Ginsenoside is a medicinal ingredient extracted from ginseng, and Rg1 is among the most important and active ingredients in various ginsenosides [5]. In the cardiomyocyte hypoxia-reoxygenation model, Rg1 has been shown to function as an antioxidant substance that can reduce the release of lactate dehydrogenase and intracellular ROS [6]. Rg1 has been reported to enhance immune responses induced by recombinant *Toxoplasmagondii* SAG1 antigen and may act as an adjuvant to promote both Thelper (Th) 1 and Th2 responses [7, 8]. Previous studies also reported that Rg1 restrained inflammatory responses via downregulation of NF-κB activity [6, 9-11]. Furthermore, ginsenoside Rg1 improved lipopolysaccharide (LPS)-induced acute lung injury by inhibiting inflammatory responses and modulating infiltration of M2 macrophages [12]. These evidences suggest the intriguing possibility that

Rg1 maybe useful for treating COPD by inhibiting inflammatory responses.

In our study, we established a mouse model of COPD induced by CSE and found that inflammatory cell numbers in the bronchoalveolar lavage (BAL) fluid of CSE mice were significantly enhanced compared with air-exposed mice, and these changes were ameliorated by Rg1 treatment. Furthermore, we revealed that Rg1 could attenuate the inflammatory cytokine levels in BAL fluid and lung tissues through inhibition of NF-κB and MAPK pathways, and suggest that Rg1 maybe an ideal agent for treating COPD via inhibition of inflammatory responses.

Materials and methods

Animals

C57BL/6J mice, 6-8 weeks old, were purchased from SLAC Lab Animal Co., Ltd (Shanghai, China), maintained in standard conditions under a 12 h light-dark cycle and provided a standard diet and chlorinated tap water ad libitum. All experimental procedures involving animals were approved by the Institutional Animal Use and Care Committee of Shanghai University of Traditional Chinese Medicine.

Cigarette smoke exposure (CSE)

Mice were exposed whole body to CS, as described previously [13]. Mice were divided into three groups of six mice per group as follows: air-exposed mice group (AE), CSE mice group and CSE+Rg1 group. Briefly, mice in the CSE group were exposed to the mainstream CS of 5 cigarettes, four times a day with 30 minutes smoke-free intervals, 5 days a week for 24 weeks. Rg1 (20 mg/kg; Sigma-Aldrich Corporation, St. Louis, MO, USA) was administered tomic eby oral gavage 1 h before CSE. The control groups were exposed to room air.

Bronchoalveolar lavage (BAL)

Male mice were sacrificed 24 h after the last CSE with an overdose of pentobarbital (Sanofi, Libourne, France), and a tracheal cannula was inserted. Lungs were lavaged via the cannula using a total of 300 μl HBSS, free of Ca^{2+} and Mg^{2+} and supplemented with 1% BSA, and this process was repeated three times, followed three times with 1 ml HBSS supplemented with 0.6 mM EDTA. The BAL fluid was pooled and centrifuged at 400×g for 10 min. The supernatant was stored at -70°C and used for determination of cytokine concentrations by enzyme-linked immunosorbent assay (ELISA). The cell pellet was resuspended in 200 μl buffer (PBS supplemented with 1% BSA, 5 mM EDTA and 0.1% sodium azide). Total cell counts were obtained in a Bürker chamber and differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations (Auto smear CF-120; Sakura Finetek, Tokyo, Japan) after May-Grünwald-Giemsa staining [14]. Flow cytometric analysis of BAL cells was performed to determine macrophages, neutrophils and lymphocytes.

Measurement of cytokines and myeloperoxidase (MPO) activity

The levels of IL-6 and TNF-α in BAL supernatant were measured using commercially available ELISA kits (R&DSystems, Minneapolis, MN, USA) according to the manufacturer's instructions. MPO activity in lung tissues was assessed using an MPO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Preparation of lung tissue

Lung tissue was either shock-frozen in liquid nitrogen to determine tissue mRNA expression or fixed in 10% (v/v) neutral buffered formalin for histological examination. Lung tissue was embedded inparaffin, cut into 4 μm-thick sections and stained by hematoxylin and eosin (HE) for histopathological examination.

Quantitative reverse transcription PCR (qRT-PCR)

Following recovery of BAL fluid, lung tissue was rapidly excised en bloc and frozen in liquid nitrogen. Gene expression levels in samples of lung tissue were determined by qRT-PCR. Total RNA was extracted using an RNeasy kit (Qiagen, Hamburg, Germany) and cDNA was prepared from total mRNA using the ImPromII reverse transcription system (Promega, Madison, WI, USA). All procedures were performed according to the manufacturers' instructions. Murine TNF-α, IL-6 and glyceraldehyde-3-phosphatede hydrogenase (GAPDH) were quantified by real-

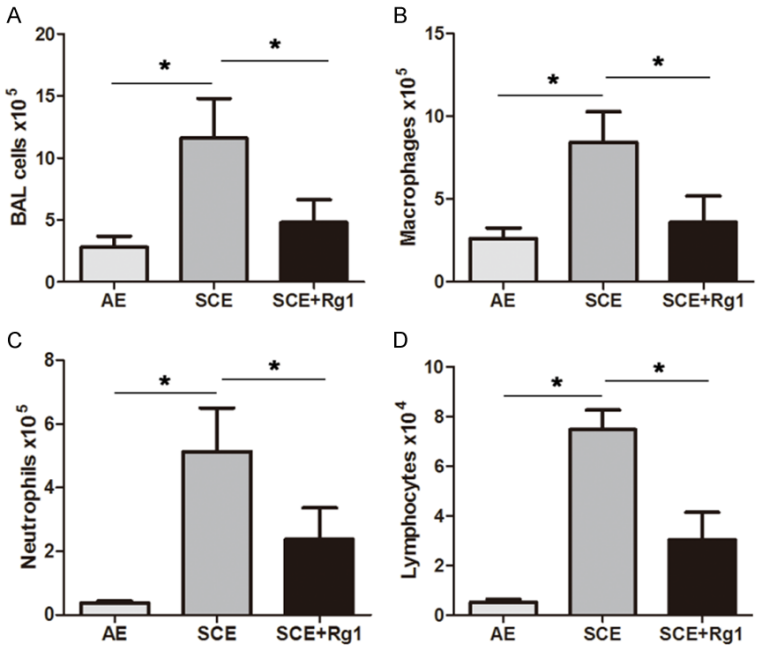

Figure 1. Effect of Rg1 on inflammatory cell numbers in BAL fluid of mice induced by CSE. A. Numbers of total lavage cells. B. Numbers of macrophages. C. Numbers of neutrophils. D. Numbers of lymphocytes. AE: air-exposed mice; CSE: cigarette smoke-exposed mice; CSE+Rg1: cigarette smoke-exposed mice treated with Rg1. Animals (at week 24, n=6) were exposed to five cigarettes per group per exposure, 4 experiments day^{-1}, 5 days $week^{-1}$. *P<0.05.

time PCR using are action mixture with SYBR Premix Ex Taq (Takara, Tokyo, Japan). The following primer sequences were used for qRT-PCR: TNF-a Forward 5'-GCCTCTTCTCATTCCTGCTC-3', Reverse 5'-CCC ATTTGG GAACTTCTCCT-3'; IL-6 Forward 5'-AGTCGGAGGCTTAATTACACATGTT-3', Reverse 5'-AAGTGCATCATCGTTGTTCATACA-3'; TGF-β Forward 5'-ATACGCCTGAGTGGCTGTCT-3', Reverse 5'-TCATGGATGGTGCCCAGGTC-3'; GAPDH, Forward: 5'-TGA AGG GTG GAG CCA AAA GG-3', Reverse: 5'-GAT GGC ATG GAC TGTGGT CA-3'. The mRNA expression level was normalized by GAPDH. The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Protein extracted from lung tissues using PIPA lysis buffer (Beyotime, Jiangsu, China) was measured with the bicinchoninic acid method (Pierce, Rockford, IL, USA). Equal amounts of protein were separated on 10% SDS acrylamide gels and transferred to PVDF membranes. Membranes were blocked for 1 h at room temperature with 5% BSA in TBS-Tween and incubated overnight at 4°C with the appropriate primary antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies, the immune complexes were detected with Super Signal West Pico chemiluminescent substrate. Band intensities were quantified using computerized image analysis (Quantity One sofware, Bio-Rad, Hercules, CA, USA) [15].

Statistical analysis

The data were expressed as means ± standards deviation (SD) from at least three independent experiments. The statistical significance between groups was evaluated using Student's *t*-test or one-way ANOVA analysis using Graphpad Prism 5.0. *P*-values <0.05 were considered to be significant.

Results

Effect of Rg1 on inflammatory cell recruitment in BAL fluid from CS-induced COPD mice

Lungs were lavaged 24 h post-last CSE, and differential cell counts were performed on BAL fluid to investigate the effect of Rg1 on inflammatory cell influx. We found that CSE mice developed a progressive biphasic increase in the number of total cells, neutrophils, macrophages and lymphocytes from BAL fluid compared with that in air-exposed mice, indicating that there was an accumulation of monocytes/macrophages, neutrophils and lymphocytes in BAL fluid (**Figure 1A-D**). Importantly, administration of Rg1 significantly ameliorated the CS-induced increase in inflammatory cells in BAL fluid. These results showed that Rg1 had potent anti-inflammatory properties on lung inflammation induced by CSE.

Effect of Rg1 on inflammatory cytokine levels in BAL fluid and lung tissues

In order to investigate the effects of Rg1 on lung inflammation during CSE *in vivo*, we first measured the inflammatory cytokine levels of

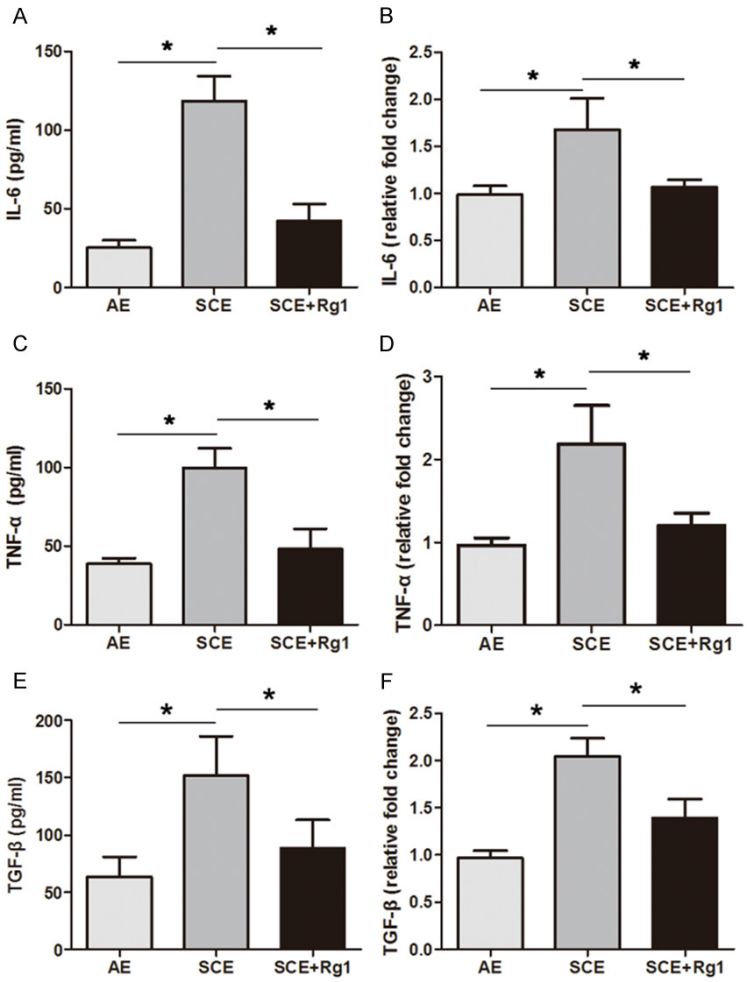

Figure 2. Effect of Rg1 on inflammatory cytokine levels in BAL fluid and lung tissues. Protein levels of (A) IL-6, (C) TNF-α and (E) TGF-β in BAL supernatant from mice exposed to air, CS and CS after Rg1 treatment. mRNA expression levels of (B) IL-6, (D) TNF-α and (F) in total lung tissue of mice exposed to air, CS and CS after Rg1 treatment. *P<0.05.

Effect of Rg1 on pulmonary histopathology changes induced by CS

Lung sections were prepared and HE staining was performed to investigate the effect of Rg1 treatment on cell infiltration. The lung section from the control group shows a normal bronchoalveolar structure with little inflammatory cell infiltration (**Figure 3A**). The lung sections from CSE mice showed markedly increased infiltration of inflammatory cells compared with air-exposed mice (**Figure 3B**). Treatment with Rg1 markedly attenuated inflammatory cell infiltration in lungs compared with the CSE mice group (**Figure 3C**). Accordingly, there was significantly increased MPO activity in lung tissue from CSE mice, while Rg1 treatment attenuated this increase (**Figure 3D**). These results suggested that Rg1 could suppress inflammatory cell influx in lungs in response to CSE in mice.

Effect of Rg1 on activation of NF-κBand MAPK signaling in CSE mice

TNF-α, IL-6 and TGF-β in centrifuged BAL supernatant. CSE mice showed significantly increased TNF-α, IL-6 and TGF-β content in the supernatant compared to clean air-exposed mice. The increased content of TNF-α, IL-6 and TGF-β in BAL supernatant induced by CS were significantly reduced by treatment with Rg1 (**Figure 2A**, **2C** and **2E**). Moreover, we assayed the levels of TNF-α, IL-6 and TGF-β in lung tissues. As shown in **Figure 2B**, **2D** and **2F**, the CS-induced COPD mice model group showed significant increases in mRNA levels of TNF-α, IL-6 and TGF-β in lung tissue compared with the air-exposed mice group. Compared with the CSE group, Rg1 reduced the mRNA levels of TNF-α, IL-6 and TGF-β in lung tissue samples.

It is well established that NF-κB and MAPK activation is implicated in the production of many inflammatory mediators present in the COPD lung or in CSE airway epithelial cells [16, 17]. To further investigate whether the anti-inflammatory effects of Rg1 were partly mediated through the NF-κB and MAPK pathways, we measured NF-κB and MAPK signaling-related protein expression by western blot. As shown in **Figure 4A** and **4B**, we determined the effects of Rg1 on the degradation of IκB-α and activation of NF-κB by western blot analysis, and found that the mice treated with CS exhibited significant degradation of IκB-α in lungs, whereas Rg1 treatment prevented the IκB-α degradation. In contrast, CS challenge promoted the

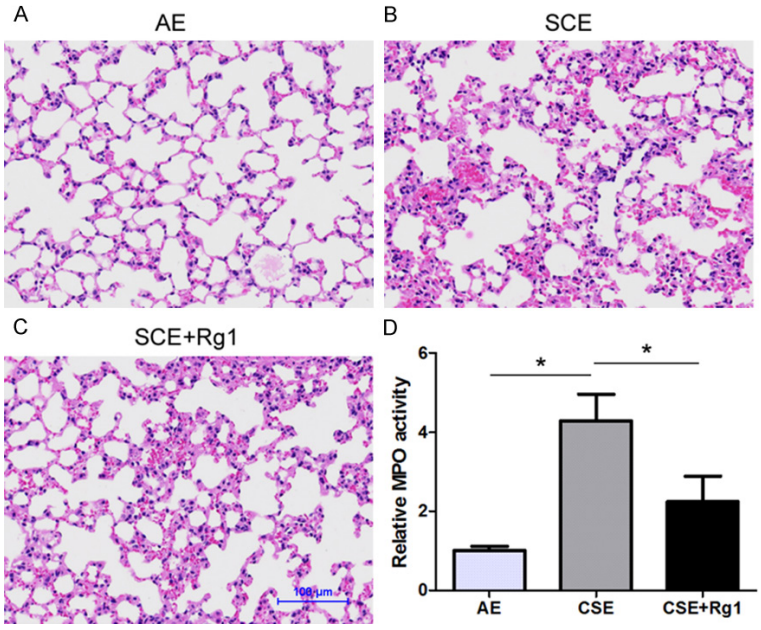

Figure 3. Effect of Rg1 on histological changes and MPO activity in lung tissues of COPD mice. A-C. Representative images of HE staining in lung sections from the indicated groups. D. MPO activity was measured in lung tissues. *P<0.05.

nuclear translocation of NF-κB p65 subunit, compared with air exposed group. Rg1 treatment suppressed the nuclear translocation of NF-κB p65. The MAPK signaling pathway was also activated by CS administration, while Rg1 pretreatment effectively blocked the phosphorylation of ERK and p38 in CS-induced COPD mice (**Figure 4C** and **4D**). These results indicated that Rg1 exerted a protective effect against COPD possibly involving the NF-κB and MAPK pathways.

Discussion

COPD is a common and serious respiratory disease with a high risk of mortality [18]. This disease is characterized by progressive airflow limitation and is considered to be closely related to pulmonary inflammatory responses [19]. CS-induced chronic inflammation has long been viewed as central to the pathogenesis of COPD [20]. Until now, various drugs, including 1,8-cineole and resveratrol, have been used for treating COPD [21, 22], but their therapeutic efficacy has been limited.

Rg1, the major effective component in ginseng, has been reported to have potent anti-inflammatory properties, shown by inhibiting the secretion of NO and TNF-α in LPS-stimulated macrophage cells [23], and Rg1 has shown excellent protective effects against sepsis-induced lung damage [9]. In particular, it has been demonstrated that Rg1 plays a protective role against LPS-induced acute lung injury by ameliorating inflammatory responses [12]. However, the effects of Rg1 on CS-induced COPD and its underlying mechanism have not yet been elucidated. In the present study, Rg1 was found to clearly ameliorate the CS-induced increase in inflammatory cell numbers (total cells, neutrophils, lymphocytes and macrophages) in BAL fluid, and to reduce the inflammatory cytokine levels of TNF-α, IL-6 and TGF-β induced by CSE in BAL supernatant and lung tissues through inhibition of NF-κB and MAPK pathways.

The innate defense system of the lung is provided by the epithelial barrier and the acute inflammatory response which follows tissue injury, including the recruitment and activation of neutrophils, eosinophils and macrophages [24]. Neutrophil sharkey mediators in the development of COPD, and toxic compounds present in cigarette smoke cause their infiltration into the airways, which eventually induce pathophysiological alterations in lung tissue [25]. Macrophages play a key role in orchestrating chronic inflammation in patients with COPD. Macrophage numbers are markedly increased in the airways, lung parenchyma, BAL fluid and sputum of patients with COPD [26, 27]. Previous studies also reported that there is an increase in the total number of lymphocytes in the lung parenchyma and peripheral and central airways of patients with COPD [28, 29]. In the present study, we found that CSE mice had a markedly increased number of total cells, neutrophils, lymphocytes and macrophages in BAL fluid. Rg1 treatment significantly reduced inflammatory cell numbers in BAL fluid compared with CSE mice without treatment. These

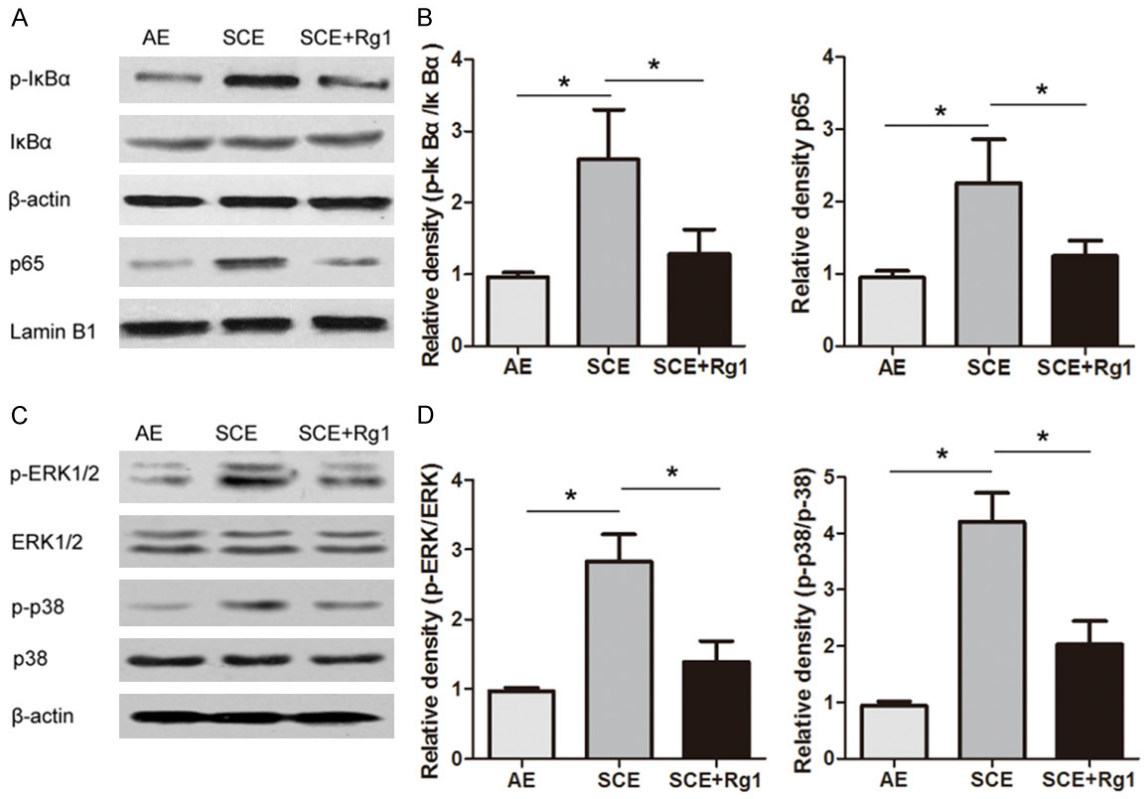

Figure 4. Effect of Rg1 on the activation of NF-κB and MAPK signaling in CS-induced COPD. A. Phosphorylated and total levels of IκBα in cytoplasm and p65 in nucleus were detected using western blot analysis. B. Densitometry was performed and the ratios of p-IκBα/IκBα and p65 were calculated and quantified. C. Phosphorylated and total levels of ERK and p38 were measured by western blot. D. Densitometry was performed and the ratios of p-p38/p38 and p-ERK/ERK were calculated and quantified. *P<0.05.

results suggest that Rg1 effectively suppressed there recruitment of inflammatory cells induced by CS.

Inflammatory cells secrete various stimulatory mediators, including proinflammatory cytokines and chemokines, resulting in aggravation of airway inflammation [26, 30]. Cytokines are mediators of chronic inflammation, and several have been implicated in patients with COPD [31]. TNF-α is implicated in inflammatory responses in COPD. Multiple cell types are able to release and respond to TNF-α, including macrophages. Several chemokines have also been implicated in patients with COPD and are of particular interest because chemokine receptors are G protein-coupled receptors for which small-molecule receptor antagonists have been developed [32]. It has been hypothesized that Rg1 ameliorates neutrophil recruitment to the lung, at least in part through inhibitory actions on proinflammatory cytokines in the lung. Here, we demonstrated that TNF-α, IL-6 and TGF-β levels were significantly increased in BAL fluid and lung tissues of COPD mice compared with air-exposed mice, and this increase reduced by Rg1.

Recent findings have demonstrated that activation of the NF-κB pathway is an important feature of COPD [33]. CS acts as a potent stimulator of NF-κB signaling in the lungs and provokes severe inflammation induced by NF-κB-dependent production of cytokines, including TNF-α and IL-6, and recruitment of neutrophils to lung tissue, causing acute lung injury or emphysema [16, 34]. It is well established that MAPK activation is implicated in the production of many inflammatory mediators present in the COPD lung or in CSE airway epithelial cells [35, 36]. In addition, Huang et al. have found that conjugated linoleic acids suppress proinflammatory cytokines and chemokines by blocking NF-κB transcriptional regulation and by attenuating MAPK signaling pathways. In our study, we showed that Rg1 suppressed phosphoryla-

tion of ERK and p38, inhibited the degradation of IkB-α and reduced nuclear translocation of NF-κB subunit p65 in CSE lung tissues.

In conclusion, our study demonstrated that Rg1 effectively inhibited the increased inflammatory cell numbers (total cells, neutrophils, lymphocytes and macrophages) and activation of proinflammatory mediators in BAL fluid and lung tissues, and also ameliorated inflammatory cell infiltration into lung tissue of CS-induced COPD mice. The effects of Rg1 were likely caused by blocking NF-κB transcriptional regulation and by attenuating MAPK signaling pathways. These results suggest that Rg1 may have therapeutic potential for the suppression of inflammation, which is a crucial step in the development of COPD.

Disclosure of conflict of interest

None.

Abbreviations

Rg1, Ginsenoside Rg1; CS, cigarette smoke; BAL, bronchoalveolar lavage; CSE, CS-exposed; COPD, Chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; MPO, myeloperoxidase; HE, hematoxylin and eosin; qRT-PCR, Quantitative reverse transcription PCR; GAPDH, glyceraldehyde-3-phosphatede hydrogenase.

Address correspondence to: Xinyan Liu, Geriatrics Department, Putuo Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, No.164 lanxi Road, Putuo District, Shanghai 200062, China. E-mail: sophiejacky@126.com

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