International Immunopharmacology 30 (2016) 62–68

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Protective effect of EC-18, a synthetic monoacetyldiglyceride on lung inflammation in a murine model induced by cigarette smoke and lipopolysaccharide

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ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form 16 November 2015
Accepted 20 November 2015
Available online 1 December 2015

Keywords:
EC-18
Monoacetyldiaglyceride
Cigarette smoke
Lung inflammation
Chronic obstructive pulmonary disease

ABSTRACT

The antler of Sika deer (*Cervus nippon* Temminck) has been used a natural medicine in Korea, China and Japan, and a monoacetyldiaglyceride (1-palmitoyl-2-linoleoyl-3-acetylglycerol, PLAG) was found in the antler of Sika deer as a constituent for immunomodulation. In this study, we investigated protective effects of EC-18 (a synthetic copy of PLAG) on inflammatory responses using a cigarette smoke with lipopolysaccharide (LPS)-induced airway inflammation model. Mice were exposed to cigarette smoke for 1 h per day for 3 days. Ten micrograms of LPS dissolved in 50 μL of PBS was administered intra nasally 1 h after the final cigarette smoke exposure. EC-18 was administered by oral gavage at doses of 30 and 60 mg/kg for 3 days. EC-18 significantly reduced the number of neutrophils, reactive oxygen species production, cytokines and elastase activity in bronchoalveolar lavage fluid (BALF) compared with the cigarette smoke and LPS induced mice. Histologically, EC-18 attenuated airway inflammation with a reduction in myeloperoxidase expression in lung tissue. Additionally, EC-18 inhibited the phosphorylation of NF-κB and IκB induced by cigarette smoke and LPS exposure. Our results show that EC-18 effectively suppresses neutrophilic inflammation induced by cigarette smoke and LPS exposure. In conclusion, this study suggests that EC-18 has therapeutic potential for the treatment of chronic obstructive pulmonary disease.

1. Introduction

Chronic obstructive pulmonary diseases (COPD) prevalence has increased worldwide in recent decades, and COPD is considered to be a significant global health problem and an important cause of death in most countries [1]. COPD is characterized by persistent airflow limitations, which is related to the condition's mortality [2]. The development of COPD is associated with various risk factors, such as cigarette smoke, occupational dusts or chemicals, and bacterial infection. In particular, cigarette smoke is considered the most important risk factor and accounts for more than 80 to 90% of COPD cases [3]. Cigarette smoke induces harmful alterations in lung tissue because it contains a number of toxic chemicals [4]. Neutrophilic inflammation is considered a crucial feature of COPD. The toxic chemicals in cigarette smoke induce lung inflammation through infiltration of inflammatory cells including neutrophils and macrophages [5]. In a clinical trial, there were increased numbers of neutrophils and macrophages in bronchial biopsies from COPD patients [6]. These inflammatory cells, which are a source of reactive oxygen species, proinflammatory cytokines or chemokines, and tissue damaging enzymes, are believed to be important for the development of COPD [7,8]. Recently, phytochemicals showing anti-inflammatory activities in a cigarette smoke (CS) with lipopolysaccharide (LPS) treated murine model have reported as possible candidates for COPD treatment [9,10]. Considering these evidences, inhibition of the inflammatory responses is an attractive therapeutic option for controlling COPD.

Monoacetyldiaglyceride (MADG) is a constituent of antlers, and has many of their pharmacological properties [11]. EC-18 (1-palmitoyl-2-linoleoyl-3-acetylglycerol, PLAG) is a synthetic MADG that has been determined by many researchers to have pharmacological properties such as the hematopoietic and immunomodulatory effects [11–13]. In particular, our previous study demonstrated that EC-18 attenuates airway inflammation in an ovalbumin-induced murine asthma model [12]. However, there is no study on the beneficial effects of EC-18 on acute lung inflammation induced by cigarette smoke and lipopolysaccharide (LPS) so far.

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http://dx.doi.org/10.1016/j.intimp.2015.11.025
1567-5769/

In this study, we investigated the effects of EC-18 on CS + LPS induced lung inflammation by measuring neutrophilic infiltration, proinflammatory cytokines and protein expression to evaluate the beneficial effects of EC-18 against the inflammatory responses of COPD development.

2. Materials and methods

2.1. Animals

Specific pathogen-free male C57BL/6N mice (6 weeks old, weight 20–25 g) were purchased from Koatech Co. (Pyeongtaek, Republic of Korea) and used after 2 week of quarantine and acclimatization. The mice were given sterilized tap water and standard rodent chow. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology and were performed in compliance with the National Institutes of Health Guidelines for the care and use of laboratory animals and Korean national laws for animal welfare.

2.2. Experimental procedure

The mice were divided into five groups: normal control (NC), CS + LPS (cigarette smoke with intranasal LPS instillation), ROF (cigarette smoke with intranasal LPS instillation + roflumilast (10 mg/kg, p.o.)), and EC-18-30 or 60 (cigarette smoke with intranasal LPS instillation + EC-18 (30 mg/kg or 60 mg/kg, p.o.)). The smoke was generated from 3R4F research cigarette (Kentucky reference cigarette, University of Kentucky, USA), containing 11.0 mg of total particulate matter, 9.4 mg of tar, and 0.76 mg of nicotine per cigarette. Cigarette smoke exposure (one puff/min, 35 mL puff volume over 2 s, every 60 s, 8 cigarettes per day) was conducted using a cigarette smoke generator (Daehan Biolink, Republic of Korea). The mice were exposed to cigarette smoke at dose of 35 mL/min for 1 h in an exposure chamber (50 cm × 30 cm × 30 cm) on three consecutive days. Roflumilast and EC-18 were administered to the mice by oral gavage 1 h before each cigarette smoke exposure. EC-18 (purity: >98%) was obtained from ENZYCHEM Lifescience Co. (Daejeon, Republic of Korea). Ten micrograms of the lipopolysaccharide (LPS) dissolved in 50 μL of PBS was instilled intranasally under anesthesia 1 h after the final cigarette smoke exposure. A positive control, Roflumilast is a phosphodiesterase-4 (PDE-4) inhibitor and commercially prescribed for treating COPD (trade name Daxas) based on anti-inflammatory effect in airways [16,17].

2.3. Inflammatory cell count in bronchoalveolar lavage fluid (BALF)

Twenty four hours after the intranasal LPS instillation, the mice were sacrificed by intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharm. Co., Seoul, Korea), and a tracheostomy was performed according to described previously [14]. The trachea was cannulated and the bronchoalveolar lavage fluid (BALF) was collected by filling the lung three times with ice-cold PBS (0.5 mL) and withdrawing it for a total lavage volume of 1.5 mL. To determine the differential cell counts, 100 μL of the BALF was centrifuged onto slides using a Cytospin (Hanil Science Industrial, Seoul, Korea) (200 g, 4 °C, 10 min). The slides were dried, and the cells were fixed and stained using Diff-Quik® staining reagent (B4132-1A; IMEB Inc., Deerfield, IL), according to the manufacturer's instructions. The supernatant obtained from the BALF was stored at −70 °C for biochemical analysis.

2.4. Measurement of reactive oxygen species (ROS), neutrophil elastase activity and the BALF protein concentration

The induction of oxidative stress was monitored using 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich, Carlsbad, CA), which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. Briefly, the cells from the BALF were washed with PBS, and the total numbers of cells ($5x10^3$) was counted. The BALF cells were treated with 20 μM DCF-DA for 10 min at 37 °C. Intracellular ROS activity was determined by measuring the fluorescence at 488 nm excitation and 525 nm emission on a fluorescence plate reader (PerkinElmer, Waltham, MA).

To measure neutrophil elastase activity, the BALF was reacted with N-succinyl-(Ala)3-p-nitroanilide (Sigma-Aldrich) at 37 °C for 90 min as previously published [15]. The absorbance was measured at 405 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA). In addition, the BALF protein concentration was determined using a protein assay kit (Bradford assay, Bio-Rad, Hercules, CA). The absorbance was measured at 595 nm using an ELISA reader.

2.5. Measurement of the levels of proinflammatory cytokines in the BALF

The levels of IL-1β (R&D Systems, Minneapolis, MN), IL-6 and TNF-α (Invitrogen, Carlsbad, CA) in the BALF were quantified by ELISA according to the manufacturer's protocols. The absorbance was measured at 450 nm using an ELISA reader.

2.6. Histology

After the BALF samples were obtained, the lung tissue was fixed in 10% (v/v) neutral buffered formalin. The tissues were embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin to evaluate the inflammatory response.

For immunohistochemistry, paraffin sections were deparaffinized, dehydrated, washed in PBS with 0.3% Triton X-100, and pre-incubated for 10 min at room temperature with 10% goat serum to block nonspecific staining. Subsequently, the slides were incubated overnight at 4 °C with an MPO antibody (1:100 dilution; Santa Cruz, CA). After removing the primary antibodies, the sections were washed and incubated with a biotinylated secondary antibody at 37 °C for 1 h, followed by incubation with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The excess complex was removed and sections were washed with PBS, before incubating with 0.05% diaminobenzidine (1:200; Millipore, Billerica, MA) for a further 10 min. The sections were counterstained, rinsed with PBS to terminate the reaction, and protected with coverslips before microscopic examination. Quantitative analysis of inflammation was determined using an image analyzer (Molecular Devices, Sunnyvale, CA).

Photomicrographs were obtained using a Photometric Quantix digital camera running a Windows program, and montages were assembled in Adobe Photoshop 7.0. The images were cropped and corrected for brightness and contrast, but they were not otherwise manipulated.

2.7. Immunoblotting

Lung tissue was homogenized using a homogenizer with a tissue lysis/extraction reagent (1/10 w/v, Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich). Each protein concentration was determined using Bradford reagent (Bio-Rad) according to the manufacturer's instructions. Equal amounts of total cellular protein (30 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with blocking solution (5% skimmed milk), followed by an overnight incubation at 4 °C with the appropriate primary antibody. The following primary antibodies and dilutions were used: anti-β-actin (1:2000 dilution; Cell Signaling, Denver, MA), anti-p-p65 (1:1000 dilution; Cell Signaling), anti-p65 (1:1000 dilution; Cell Signaling), anti-p-IκB (1:1000 dilution; Santa Cruz, Dallas, TX) and anti-β-actin (1:2000 dilution; Cell Signaling). The blots were washed three times with Tris-buffered saline containing Tween 20 (TBST), and then incubated with a 1:3000 dilution of a horseradish peroxidase (HRP)-conjugated

secondary antibody (Jackson Immuno Research, West Grove, PA) for 30 min at room temperature. The blots were again washed three times with TBST, and then developed using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA). To determine the relative ratio of protein expression, we measured densitometric band values using ChemiDoc (Bio Rad).

2.8. Statistical analysis

The data are expressed as the means ± standard deviation (SD). Statistical significance was determined using analysis of variance (ANOVA) followed by a multiple comparison test with a Dunnett adjustment. The P value <0.05 was considered significant.

3. Results

3.1. EC-18 suppresses the increased neutrophils in the BALF

The CS + LPS group showed a significant increase in inflammatory cell counts in the BALF compared with the normal controls (Fig. 1). In contrast, the roflumilast-treated group exhibited a marked reduction in the inflammatory cell numbers in the BALF compared with the CS + LPS group. The EC-18-treated mice had reduced inflammatory cell numbers in the BALF in a dose-dependent manner. In particular, the 60 mg/kg EC-18 group showed a significant decrease in inflammatory cells including neutrophils compared with the CS + LPS group, and the reduction of cells was found to be very similar to the roflumilast-treated group.

3.2. EC-18 decreases ROS production, contents and neutrophil activity in the BALF

ROS production significantly increased in the CS + LPS group compared with the normal controls (Fig. 2A). However, the roflumilast-treated group exhibited a marked reduction in ROS production compared with the CS + LPS group. The EC-18 treated group also had significantly reduced ROS production compared with the CS + LPS group. As shown in Fig. 2B, the BALF contents significantly increased in the CS + LPS group, whereas this was markedly reduced in the roflumilast or EC-18 treated group (60 mg/kg) compared with the CS + LPS group. The neutrophil elastase activity was consistent with the ROS production and BALF contents. The CS + LPS group had significantly increased neutrophil elastase activity compared with the normal controls, whereas 60 mg/kg EC-18 group was significantly decreased it compared with CS + LPS group. 30 mg/kg EC-18 group showed the reduction of elastase activity, though the statistical difference was not observed. (Fig. 2C).

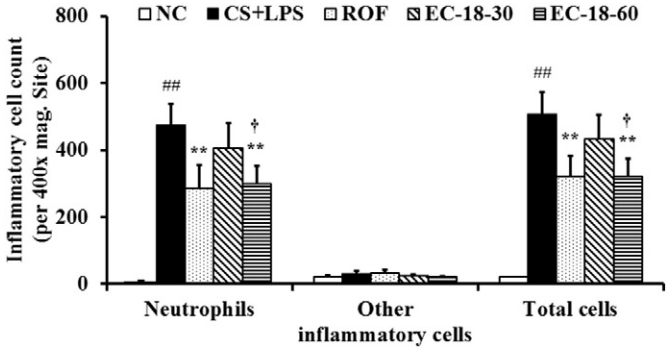

Fig. 1. EC-18 suppresses the increased neutrophil counts in bronchoalveolar lavage fluid (BALF) induced by CS + LPS challenge. Cells were isolated by centrifugation and stained with Diff-Quik® staining reagent. The cell numbers were counted at 400× magnification using a light microscope. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal lipopolysaccharide (LPS) instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and -60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation. Data are expressed as the means ± SD. Six animals were used in each group. ##Significantly different from the normal control group, $P < 0.01$; **significantly different from the CS + LPS group, $P < 0.01$; †significantly different from the EC-18-30 group, $P < 0.05$.

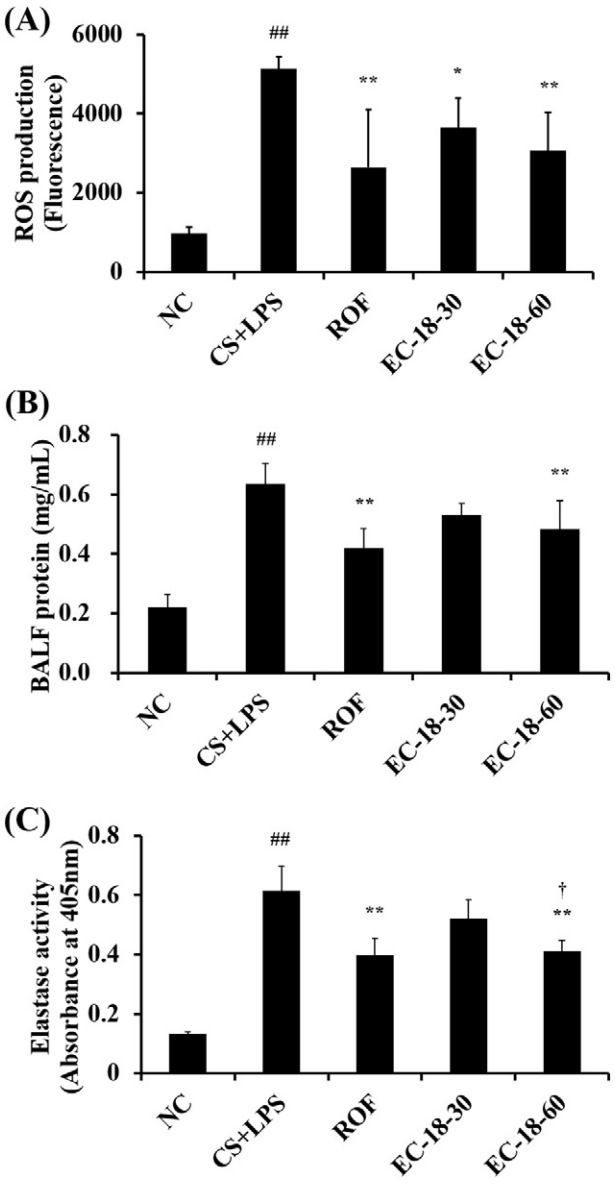

Fig. 2. EC-18 reduces reactive oxygen species production, contents and neutrophil elastase activity in BALF. (A) ROS production, (B) BALF protein concentration, (C) neutrophil elastase activity. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal LPS instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and 60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation. Data are expressed as the means ± SD. Six animals were used in each group. ##Significantly different from the normal control group, $P < 0.01$; *, **significantly different from the CS + LPS group, $P < 0.05$ and $P < 0.01$, respectively. †significantly different from the EC-18-30 group, $P < 0.05$.

3.3. EC-18 reduces proinflammatory cytokines levels in the BALF

The level of IL-6 in the BALF was significantly increased in the CS + LPS group compared with the normal controls (Fig. 3A). The roflumilast-treated mice showed markedly reduced IL-6 levels in the BALF compared with the CS + LPS group. Additionally, the EC-18-treated groups had significantly decreased IL-6 levels in the BALF in a dose-dependent manner compared with the CS + LPS group. The tendency of TNF-α concentration in the BALF were similar to those of IL-6; The

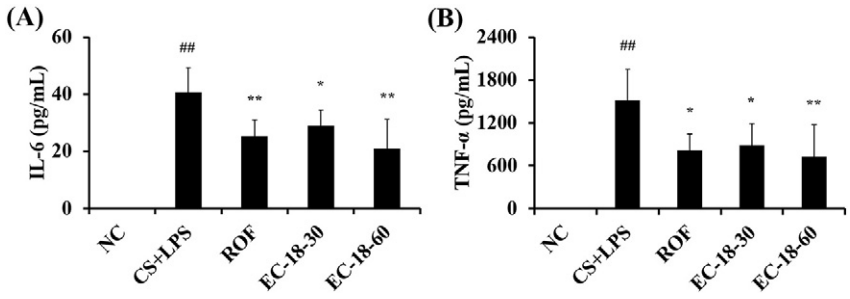

Fig. 3. EC-18 reduces the levels of IL-6 and TNF-α in the BALF. (A) IL-6, (B) TNF-α. The levels of cytokines were determined using an ELISA. The absorbance was measured at 450 nm using a microplate reader. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal LPS instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and 60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation. Data are expressed as the means ± SD. Six animals were used in each group. ##Significantly different from the normal control group, $P < 0.01$; *, **significantly different from the CS + LPS group, $P < 0.05$ and $P < 0.01$, respectively.

CS + LPS group had significantly increased TNF-α levels in the BALF compared with the CS + LPS group, however, the roflumilast- and EC-18-treated groups had markedly reduced TNF-α levels in comparison with the CS + LPS group (Fig. 3B).

3.4. EC-18 attenuates the inflammatory responses in lung tissue

The CS + LPS group had inflammatory cell infiltration into the lung tissue (Fig. 4A). This accumulation was observed extensively in perivascular and alveolar lesions. However, the roflumilast-treated group had reduced inflammatory cell infiltration. Additionally, the EC-18-treated groups had attenuated inflammatory infiltration in lung tissue. In addition, the CS + LPS group had a significantly increased inflammatory index compared with the normal controls, whereas the roflumilast- and EC-18-treated groups had a markedly decreased inflammatory index in comparison with the CS + LPS group (Fig. 4B).

3.5. EC-18 reduces myeloperoxidase expression in lung tissue

The CS + LPS group exhibited increased MPO expression in lung tissue compared with the normal controls (Fig. 5). However, the roflumilast-treated mice had reduced MPO expression compared with the CS + LPS group. The EC-18-treated groups also had decreased MPO expression in lung tissue compared with the CS + LPS group.

3.6. EC-18 suppresses the phosphorylation of NF-κB in lung tissue

The CS + LPS group showed increase IκB phosphorylation compared with the normal controls (Fig. 6A and B). However, EC-18 suppressed

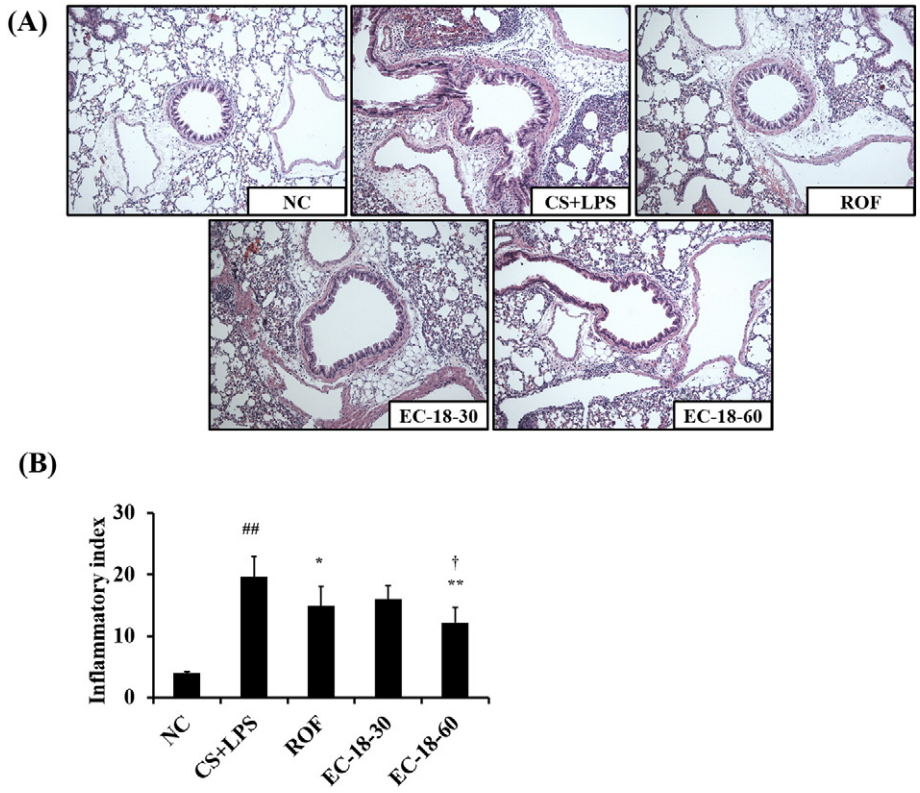

Fig. 4. EC-18 attenuates inflammatory responses in lung tissue. (A) Representative H&E stained histological section, (B) quantitative analysis of the inflammatory response. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal LPS instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and 60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation. Data are expressed as the means ± SD. Six animals were used in each group. ##Significantly different from the normal control group, $P < 0.01$; *, **significantly different from the CS + LPS group, $P < 0.05$ and $P < 0.01$, respectively. †significantly different from the EC-18-30 group, $P < 0.05$.

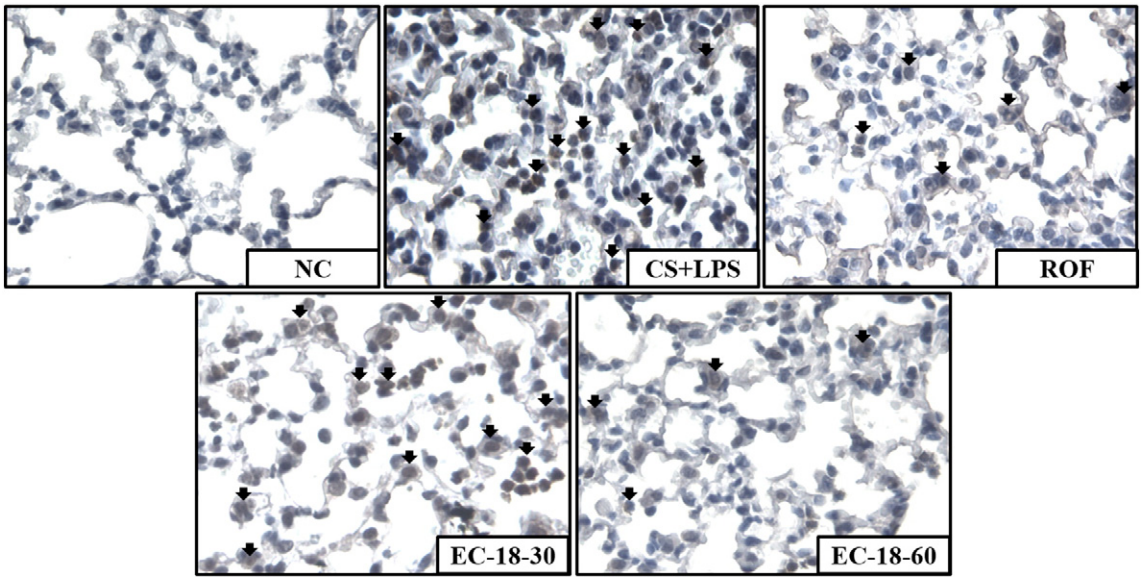

Fig. 5. EC-18 suppresses myeloperoxidase (MPO) expression in lung tissue. Black arrows indicate MPO positive cells. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal LPS instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and 60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation.

the IκB phosphorylation induced by cigarette smoke and LPS exposure. In addition, the phosphorylation of NF-κB was markedly increased in the CS + LPS group compared with the normal controls, whereas it was significantly reduced in the EC-18-treated group compared with the CS + LPS group.

4. Discussion

COPD is characterized by slowly progressive and largely irreversible airflow limitation due to chronic bronchitis, emphysema or both. It is a rapidly increasing global health problem and is predicted to be the third leading causes of morbidity and mortality throughout the world. In this study, we evaluated the inhibitory effects of EC-18, a synthetic MADG, on neutrophilic inflammation in lung tissue using a cigarette smoke and LPS induced airway inflammation model. EC-18-treated group showed a significant reduction in the number of neutrophils, ROS production, protein concentration, neutrophil elastase activity, and proinflammatory cytokines in the BALF compared with the CS + LPS group. Upon histological examination, the EC-18-treated group had markedly reduced inflammatory cell infiltration in lung tissue with a decrease in MPO expression compared with the CS + LPS group. In addition, the EC-18-treated mice had decreased the IκB and NF-κB phosphorylation induced by cigarette smoke and LPS.

Neutrophils are a crucial factor in the development of COPD [18]. Various toxic components from cigarette smoke induce the recruitment of neutrophils into lung tissue. Increased numbers of neutrophils have been observed in sputum from COPD patients [19]. In this study, the CS + LPS group had markedly increased numbers of neutrophils in the BALF. However, the EC-18-treated group had significantly decreased numbers of neutrophils in the BALF compared with the CS + LPS group. This indicates that EC-18 effectively suppressed the recruitment of neutrophils. Neutrophils contain various stimulatory mediators including ROS, proinflammatory cytokines, elastase and MPO [4]. These mediators not only aggravate neutrophilic airway inflammation but also destroy normal alveolar structure, resulting in emphysema [20]. ROS and proinflammatory cytokines activate inflammatory signaling such as the NF-κB pathway [21,22]. Additionally, these factors markedly elevated the neutrophil recruitment because they act as chemo-attractants for inflammatory cells [23]. In this study, EC-18 effectively suppressed the increased ROS production, proinflammatory cytokines and MPO induced by cigarette smoke and LPS exposure. This evidence supports that EC-18 has the potential to inhibit inflammatory responses induced by cigarette smoke and LPS exposure. In addition, neutrophil elastase destroys elastin that maintains the normal alveolar structure [24]. Eventually, an elevation of elastase causes structural alterations, resulting in emphysema [25]. EC-18 prominently suppressed the elevated neutrophil elastase

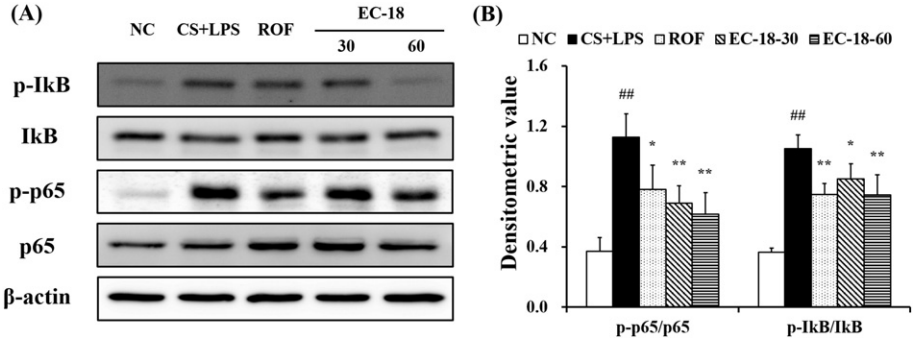

Fig. 6. EC-18 suppresses the phosphorylation of IκB and NF-κB induced by cigarette smoke and LPS. (A) Representative figure for protein expression, (B) relative ratio of protein expression. NC, normal control mice treated with PBS only; CS + LPS, cigarette smoke + intranasal LPS instillation; ROF, roflumilast (10 mg/kg) + cigarette smoke + intranasal LPS instillation; EC-18-30 and 60, EC-18 (30 mg/kg and 60 mg/kg) + cigarette smoke + intranasal LPS instillation. Data are expressed as the means ± SD. Six animals were used in each group. ##Significantly different from the normal control group, $P < 0.01$; *, **significantly different from the CS + LPS group, $P < 0.05$ and $P < 0.01$, respectively.

activity induced by cigarette smoke and LPS exposure. This finding indicates that EC-18 attenuates the development of neutrophilic airway inflammation via a reduction in elastase activity. These effects of EC-18 were strongly supported by a histological examination. The CS + LPS group had extensive inflammation in lung tissue. However, the EC-18-treated mice had markedly decreased airway inflammation that was associated with a reduction in MPO expression. Collectively, EC-18 may have therapeutic potential for suppressing neutrophilic inflammation, a crucial step in the development of COPD induced by cigarette smoke and LPS exposure.

NF-κB is closely associated with inflammatory responses. NF-κB modulates the amount of gene expression associated with an inflammatory response such as that of inflammatory cytokines and chemokines, matrix metalloproteinases (MMPs) and adhesion molecules [26]. LPS elicits NF-κB activation in various structural cells, and mediates inflammatory responses [27]. NF-κB activation is caused by IκB phosphorylation, resulting in its degradation and then NF-κB is translocated to the nucleus. NF-κB, once translocated binds to promoter regions of proinflammatory genes inducing the production of various inflammatory mediators including IL-6 and TNF-α [28]. In development of COPD, NF-κB phosphorylation is considered as an important step. According to previous study, cigarette smoke induced the phosphorylation of NF-κB in lung tissue, which eventually produced proinflammatory cytokines and chemokines associated with increasing the recruitment of inflammatory cells and aggravating inflammatory response in damaged lesions [29]. Also, increased NF-κB phosphorylation has been demonstrated in the airway, sputum and macrophages from COPD patients [30]. As shown in Fig. 6, CS + LPS group showed the significant increase in NF-κB phosphorylation in lung tissue compared with the normal controls, which was accompanied with the reduction in the proinflammatory cytokines in BALF as described previously. However, the EC-18-treated mice had attenuated IκB and NF-κB phosphorylation. These findings indicate that the anti-inflammatory effects of EC-18 could be associated with the suppression of NF-κB activation.

In Oriental herbal medicine, deer antlers is applied not only to increase body strength but also to cure inflammatory disease such as mastitis [31]. Previous studies have demonstrated that deer antler possesses anti-inflammatory properties to suppressed effectively inflammatory responses induced by formaldehyde, carrageenan and dimethylbenzene [31]. In this study, the effects of EC-18, an active component of deer antler were consistent with previous findings. In addition, EC-18 attenuated eosinophilic airway inflammation in an ovalbumin-induced asthma model in a previous study from this laboratory.

5. Conclusions

These data show that EC-18, a synthetic PLAG effectively suppressed increases in the number of neutrophils, proinflammatory cytokines, ROS production and MPO expression induced by cigarette smoke and LPS with a concomitant reduction in NF-κB phosphorylation. These findings indicate that EC-18 may be useful for the treatment of COPD because of its anti-inflammatory properties. Our results suggest that EC-18 has therapeutic potential for the treatment of COPD.

Conflicts of interest

All authors including researchers from the ENZYCHEM have no competing interests.

Acknowledgments

This study was supported by the KRIBB Research Initiative program (KGM1221521) and ENZYCHEM Life Sciences (IGM0071311) of Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.intimp.2015.11.025.

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