International Immunopharmacology 18 (2014) 116–123

Contents lists available at ScienceDirect

International Immunopharmacology

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

EC-18, a synthetic monoacetyldiglyceride (1-palmitoyl-2-linoleoyl-3-acetylglycerol), attenuates the asthmatic response in an aluminum hydroxide/ovalbumin-induced model of asthma

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ARTICLE INFO

Article history:
Received 3 June 2013
Received in revised form 24 September 2013
Accepted 8 November 2013
Available online 21 November 2013

Keywords:
EC-18
Monoacetyldiacylglyceride
Cytokines
Inducible nitric oxide synthase
Asthma

ABSTRACT

EC-18 is a synthetic monoacetyldiaglyceride that is a major constituent in antlers of Sika deer (*Cervus nippon* Temmenick). In this study, we evaluated the protective effects of EC-18 on Th2-type cytokines, eosinophil infiltration, and other factors in an aluminum hydroxide/ovalbumin (OVA)-induced murine asthma model. Mice were sensitized on days 0 and 14 by intraperitoneal injection of OVA with aluminum hydroxide. On days 21, 22 and 23 after the initial sensitization, the mice received an airway challenge with OVA for 1 h using an ultrasonic nebulizer. EC-18 was administered to mice by oral gavage at doses of 30 mg/kg and 60 mg/kg once daily from day 18 to 23. Methacholine responsiveness was measured 24 h after the final OVA challenge, and the bronchoalveolar lavage fluid (BALF) was collected 48 h after the final OVA challenge. EC-18 significantly reduced methacholine responsiveness, T helper type 2 (Th2) cytokines, eotaxin-1, immunoglobulin (Ig) E, IgG, and the number of inflammatory cells. In addition, EC-18-treated mice exhibited the reduction in the expression of inducible nitric oxide synthase (iNOS) in lung tissue. In the histological analysis using hematoxylin–eosin stain and periodic acid–Schiff stain, EC-18 attenuated the infiltration of inflammatory cells into the airway and reduced the level of mucus production. Our results showed that EC-18 effectively suppressed the asthmatic response induced by OVA challenge. These effects were considered to be associated with iNOS suppression. In conclusion, this study suggests that EC-18 may be a therapeutic agent for allergic asthma.

1. Introduction

Eosinophil-rich airway inflammation is an important feature of allergic asthma. Worldwide, the prevalence of allergic asthma has increased substantially, particularly in children [1]. Exposure to various irritants, such as house dust, smoke, and volatile organic compounds, induces allergic responses in the airway. T-helper type 2 (Th2) cytokines serve as crucial mediators in the progression of allergic asthma [2]. Th2 type cytokines, such as interleukin (IL)-4, IL-5, and IL-13, were shown to increase the inflammatory cell infiltration into the airway and to stimulate the release of inflammatory mediators, thereby causing airway inflammation, methacholine responsiveness, and mucus overproduction [3,4]. Many researchers have sought to develop therapeutic agents for treating allergic asthma with a focus on reducing the release of Th2 cytokines.

In Oriental traditional medicine, antlers of Sika deer (*Cervus Nippon* Temminck) are believed to have beneficial therapeutic properties for the treatment of various diseases, including mammary hyperplasia, mastitis, uterine fibroids, malignant sores and children's mumps [5,6]. Several studies have demonstrated that antlers possess immunomodulatory, anti-cancer, anti-fatigue, anti-inflammatory, anti-bacterial, and anti-oxidant activities [6–8]. The antler consists of various constituents. Of its constituents, monoacetyldiaglyceride (MADG) has also been shown to have pharmacological properties, such as hematopoietic, anti-tumor, and immunomodulatory effects in *in vitro* and *in vivo* experiments [9–12]. EC-18 is a 1-palmitoyl-2-linoleoyl-3-acetylglycerol as synthetic MADG (Fig. 1). Kim et al. [11] demonstrated that EC-18 possesses hematopoietic and immunomodulatory properties. It was reported that EC-18 improves survival in a murine model of abdominal sepsis *via* immunomodulation and enhancement of stem cell factor and stromal cell-derived factor [12]. However, only limited information is available about the immunomodulatory effects of EC-18. In particular, there has been no study on the effects of MADG on allergic asthma until now.

In this study, we investigated the effects of EC-18, a synthetic MADG on allergic asthma by measuring Th2-type cytokines, eosinophil

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http://dx.doi.org/10.1016/j.intimp.2013.11.006

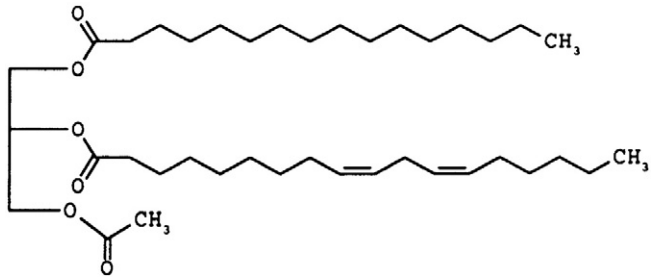

Fig. 1. The chemical structure of EC-18.

infiltration, and other factors in an aluminum hydroxide/ovalbumin (OVA)-induced murine asthma model.

2. Materials and methods

2.1. Animals

Specific pathogen-free female BALB/c mice (6 weeks old) were purchased from Koatech Co. (Pyeongtaek, Korea) and used after 2 weeks 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 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. Aluminum hydroxide/ovalbumin-induced allergic asthma

The mice were divided into five groups (n = 7). To induce allergic asthma, the mice were sensitized on days 0 and 14 by intraperitoneal injection of 20 μg ovalbumin (OVA) emulsified in 2 mg aluminum hydroxide (Alum) in 200 μL PBS buffer (pH 7.4). On days 21, 22, and 23 after the initial sensitization, the mice received an airway challenge with OVA (1%, w/v, in PBS) for 1 h using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan). EC-18 was obtained from Enzychem Lifesciences Co. (Daejeon, Korea). EC-18 was prepared fresh daily before each treatment and was administered to the mice by gavage at doses of 30 mg/kg or 60 mg/kg once daily from day 18 to 23. Negative and positive control mice were administered PBS or montelukast (30 mg/kg in PBS) by oral gavage, respectively.

2.3. Measurement of methacholine responsiveness

Twenty-four hours after the final OVA challenge, methacholine responsiveness was assessed in conscious and unrestrained mice by means of whole-body plethysmography (OCP3000; Allmedicus, Seoul, Korea). Each mouse was placed in a plastic chamber and exposed to methacholine aerosols in increasing concentrations (12.5–50 mg/mL in PBS) for 3 min. After each methacholine challenge, the penh values were measured for 3 min.

2.4. Measurement of inflammatory cell counts in bronchoalveolar lavage fluid

The mice were sacrificed by intraperitoneal injection of pentobarbital (50 mg/kg; Hanlim Pharm. Co., Seoul, Korea) 48 h after the last challenge, and a tracheostomy was performed. To obtain the bronchoalveolar lavage fluid (BALF), ice-cold PBS (0.5 mL) was infused into the lung and withdrawn *via* tracheal cannulation three times (total volume 1.5 mL). The total inflammatory cell numbers were assessed by the counting cells in at least five squares of a hemocytometer after exclusion of dead cells by Trypan blue staining. To determine differential cell counts, 100 μL of 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.

2.5. Measurement of the levels of IL-4, IL-5, IL-13, and eotaxin-1 in BALF

The levels of IL-4 (R&D System, MN, USA), IL-5 (R&D System), IL-13 (R&D System) and eotaxin-1 (eBioscience, CA, USA) in BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, CA, USA). Absolute concentrations were obtained by running standard curves on the same ELISA plates.

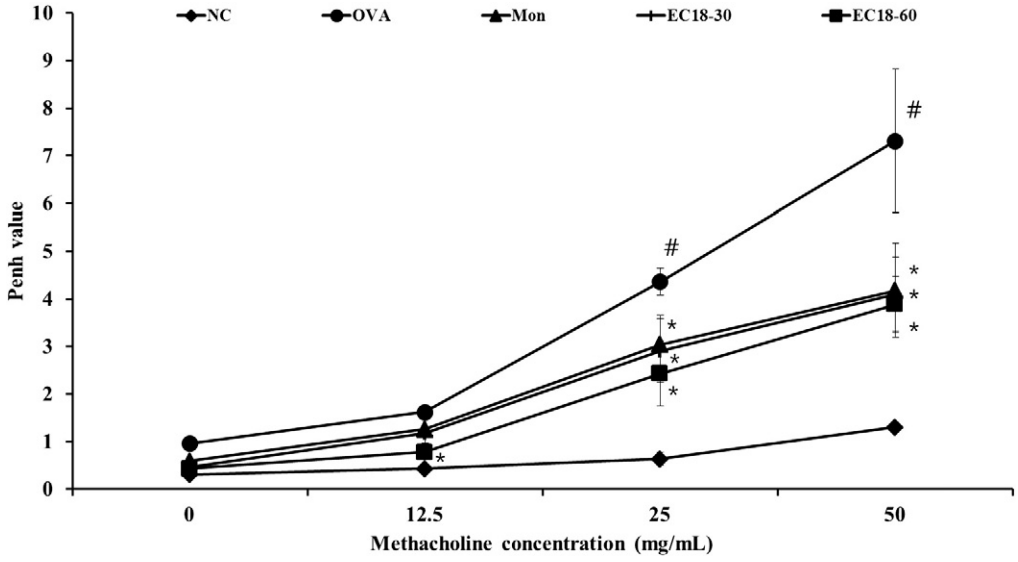

Fig. 2. EC-18 suppresses the elevated methacholine responsiveness induced by OVA challenge. Methacholine responsiveness was assessed 24 h after the last challenge by indirectly using single-chamber, whole body plethysmography (Allmedicus, Seoul, Korea). The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

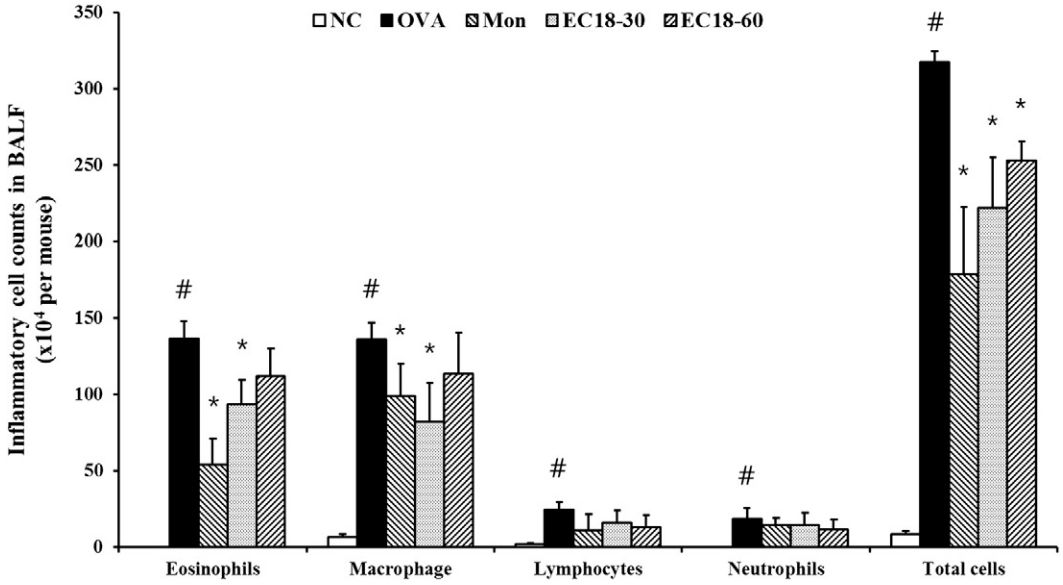

Fig. 3. EC-18 reduces the recruitment of inflammatory cells in the bronchoalveolar lavage fluid (BALF). Cells were isolated by centrifugation and stained with Diff-Quik® staining reagent. The cell numbers were counted using a light microscope by counting the cells in at least five squares of a hemocytometer after excluding the dead cells using Trypan blue. The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

2.6. Measurement of the IgE and IgG in serum

Total IgE (Biolegend, CA, USA) and IgG (Bethyl Laboratories, TX, USA) were measured by commercial ELISA kits. In OVA-specific IgE and IgG, 96-well microtiter plates were coated overnignt with 10 μg/mL OVA in PBS-Tween 20. After washing and blocking of each plate, the samples were incubated for 2 h. Subsequently, 96-well plates were washed, and HRP-conjugated goat anti-mouse IgE and anti-mouse

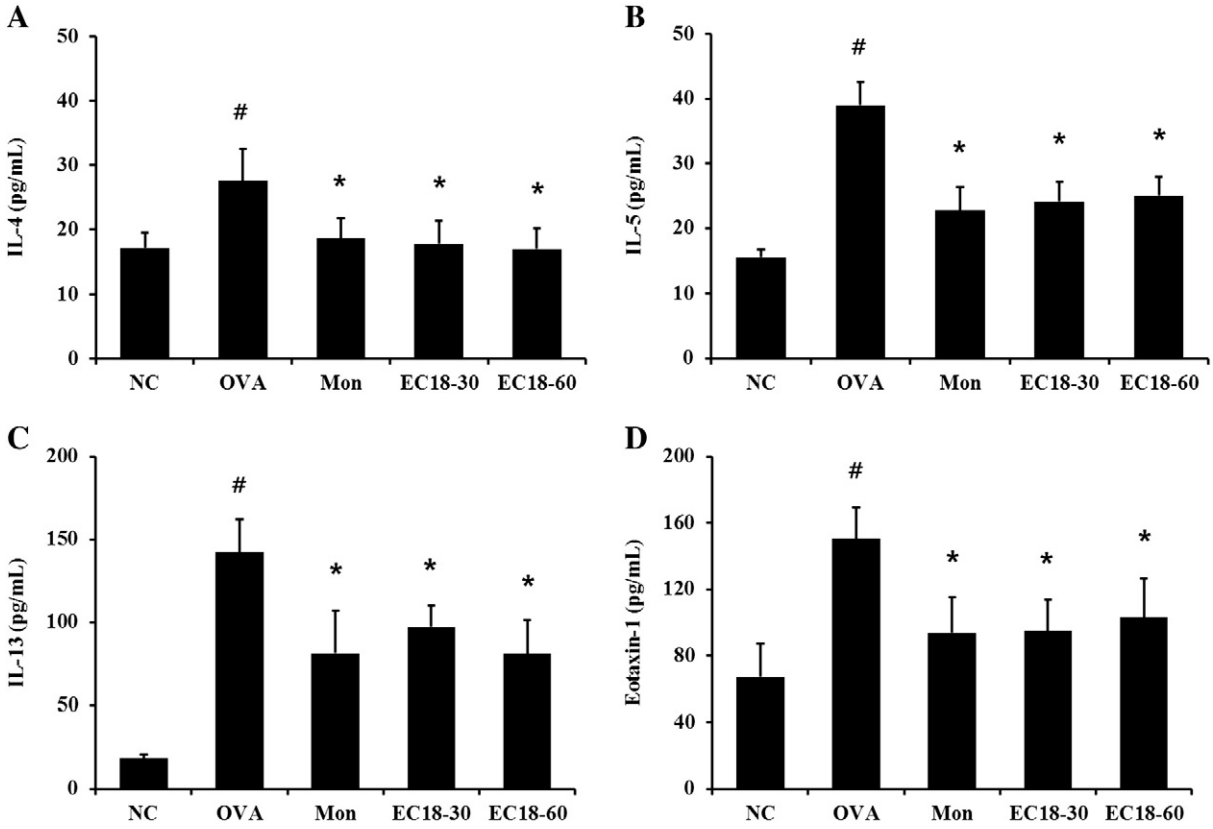

Fig. 4. EC-18 reduces the levels of IL-4, IL-5, IL-13, and eotaxin-1 in the BALF. (A) IL-4 level, (B) IL-5 level, (C) IL-13 level, (D) eotaxin-1 level. The levels of Th2 cytokines and eotaxin-1 were determined using an ELISA. The absorbance was measured at 450 nm using microplate reader. The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

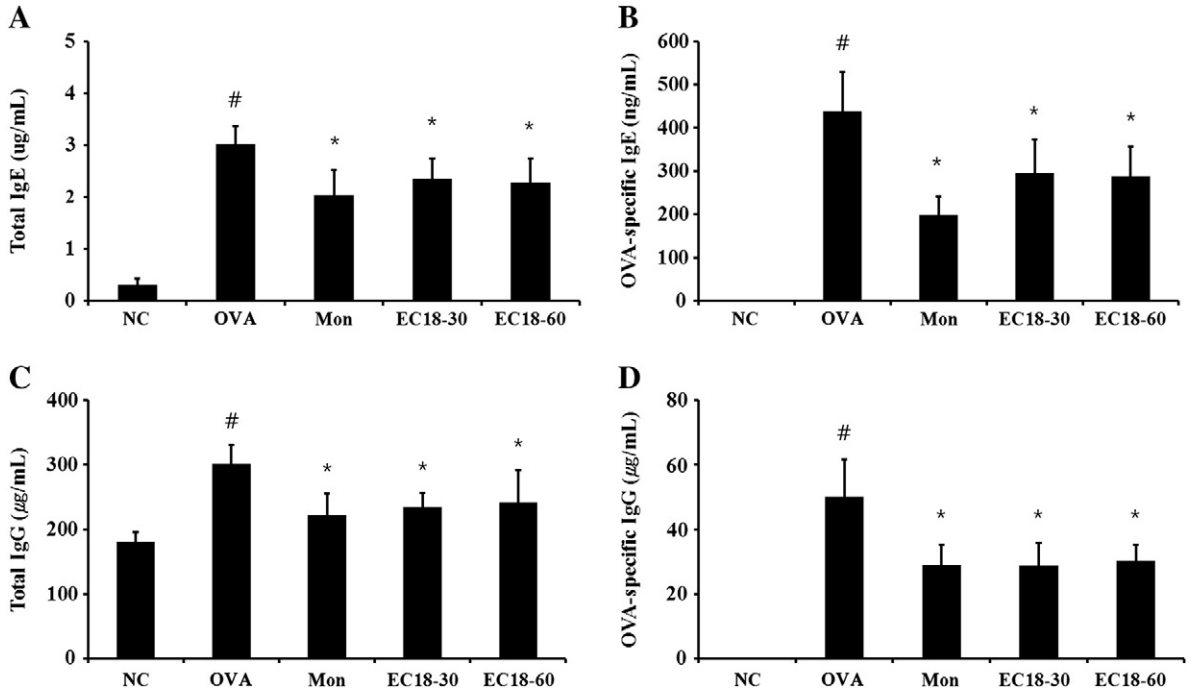

Fig. 5. EC-18 decreases the levels of IgE and IgG in the serum. (A) Total IgE (B) OVA-specific IgE, (C) Total IgG, (D) OVA-specific IgE. The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

IgG antibodies were added. After washing for four times, 200 μL of o-phenylenediamine dihydrochloride (Sigma-Aldrich, CA, USA) was added to each well. Each plate was incubated for 10 min in the dark and then the absorbance was determined at 450 nm using a microplate ELISA reader (Bio-Rad Laboratories).

2.7. Histology

After the BALF samples were obtained, 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 H&E solution (hematoxylin, Sigma MHS-16, and eosin, Sigma HT110-1-32) and periodic acid–Schiff (PAS) (IMEB Inc., San Marcos, CA) to estimate inflammation and mucus production, respectively. 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. Quantitative analysis of inflammation and mucus production was determined using an image analysis (Molecular Devices, Inc., Sunnyvale, CA). Slides were prepared for two per animal (n = 7/group). In quantitative analysis, we randomly chosen five histological fields per slide focused on bronchial (×200 magnification). Quantitative index was expressed as the ratio (%) of selected area to whole histological field.

2.8. Immunoblotting

Lung tissue was homogenized (1/10 w/v) using a homogenizer with a tissue lysis/extraction reagent (Sigma-Aldrich, CA, USA) containing a protease inhibitor cocktail (Sigma-Aldrich). Each protein concentration was determined using Bradford reagent (Bio-Rad Laboratories) 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% skim 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, MA, USA) and anti-iNOS (1:200 dilution; Abcam, MA, USA). The blots were washed for 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, PA, USA) for 30 min at room temperature. The blots were again washed three times with TBST, and then developed using an enhanced chemiluminescence kit (Thermo, CA, USA). To determine relative ratio of iNOS to β-actin, we measured densitometric band values using ChemiDoc (Bio-Rad Laboratories). The relative ratio was expressed as the density ratio of iNOS to β-actin.

2.9. Statistical analysis

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

3. Results

3.1. EC-18 reduces methacholine responsiveness induced by OVA challenge

The methacholine responsiveness significantly increased in the Alum/OVA-sensitized/challenged mice (Fig. 2) with increasing methacholine concentrations. By contrast, the montelukast-treated mice presented a marked decrease in the methacholine responsiveness with increasing methacholine concentrations. The EC-18-treated mice also had significantly decreased methacholine responsiveness compared with the Alum/OVA-sensitized/challenged mice, which was similar to results obtained for the montelukast-treated mice.

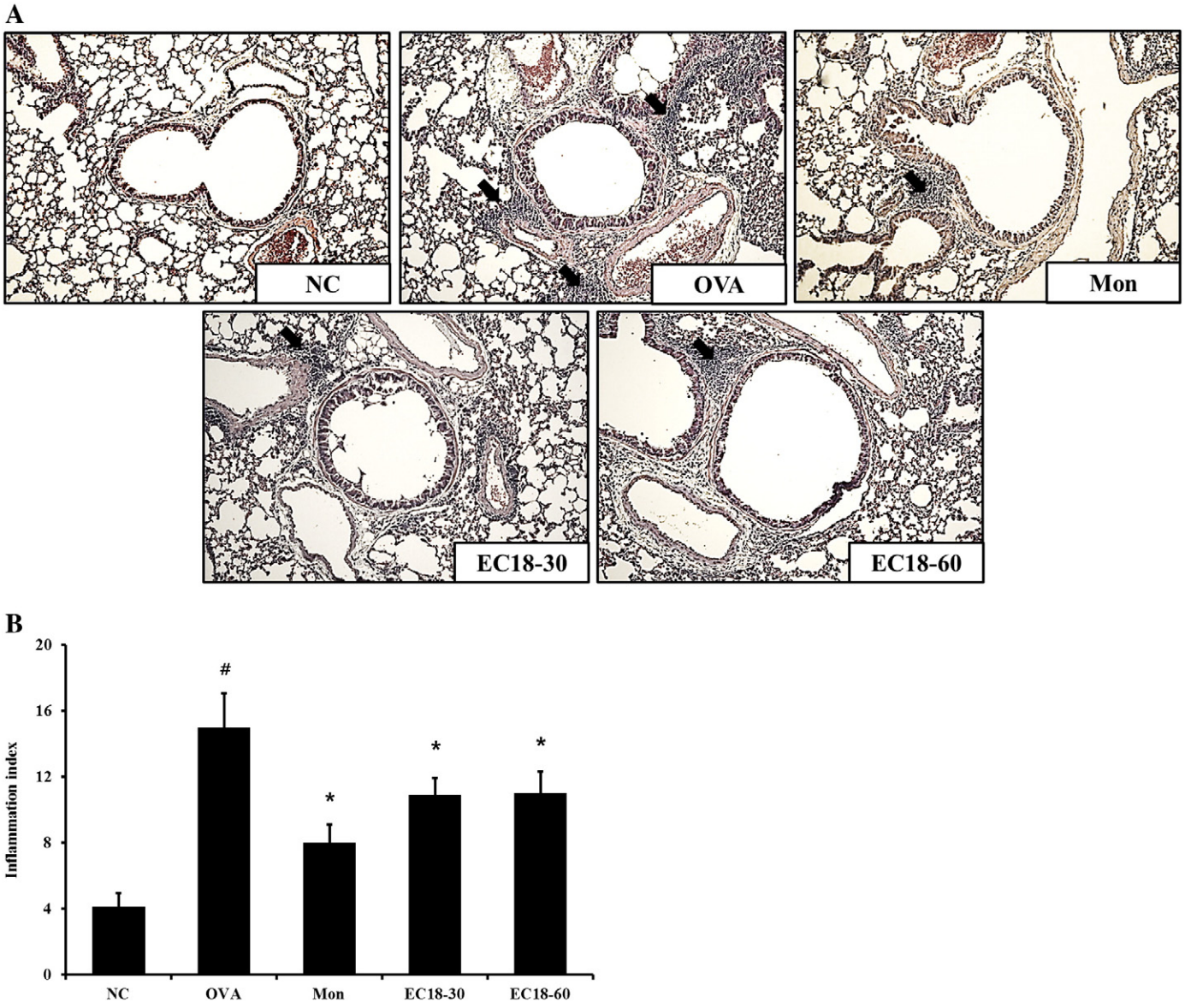

Fig. 6. EC-18 inhibits the recruitment of inflammatory cells to lung tissue in mice 48 h after the final OVA challenge. (A) Lung tissue with H&E staining (magnification ×200), (B) quantitative analysis for airway inflammation. Arrows indicated the lesions of inflammatory cell infiltration. The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

3.2. EC-18 suppresses the recruitment of inflammatory cells in BALF induced by OVA challenge

Alum/OVA-sensitized/challenged mice had significantly more eosinophils, macrophages, neutrophils, and lymphocytes in the BALF compared with the negative control mice (Fig. 3). The montelukast-treated mice had significantly fewer inflammatory cells than the Alum/OVA-sensitized/challenged mice. Similarly, the EC-18-treated mice had significantly fewer inflammatory cells, particularly eosinophils, compared with the Alum/OVA-sensitized/challenged mice.

3.3. EC-18 decreases the elevated levels of IL-4, IL-5, IL-13, and eotaxin-1 in BALF induced by OVA challenge

The levels of IL-4, IL-5, and IL-13 in the BALF were significantly higher in the Alum/OVA-sensitized/challenged mice compared with the negative controls (Fig. 4A, B and C). By contrast, the montelukast-treated mice had significantly lower levels of these cytokines in the BALF compared with the Alum/OVA-sensitized/challenged mice. The EC-18-treated mice had significantly lower levels of these cytokines compared with the Alum/OVA-sensitized/challenged mice. The level of eotaxin-1 was also higher in the Alum/OVA-sensitized/challenged mice. The reduction in cytokines and eotaxin-1 in the EC-18 treated mice was similar to that in the montelukast-treated mice (Fig. 4D).

3.4. EC-18 reduces the increased IgE and IgG in serum induced by OVA challenge

The level of total IgE and IgG in the serum was much higher in the Alum/OVA-sensitized/challenged mice than in the negative controls, and it was significantly lower in the montelukast-treated mice compared with the Alum/OVA-sensitized/challenged mice. The total IgE

and IgG level in serum was significantly lower in mice treated with EC-18 compared with the Alum/OVA-sensitized/challenged mice (Fig. 5A and C). As observed for the total IgE and IgG levels, the level of OVA-specific IgE and IgG in the serum was significantly lower in the mice treated with EC-18 or montelukast compared with the Alum/OVA-sensitized/challenged mice (Fig. 4B and D).

3.5. EC-18 attenuates airway inflammation induced by OVA challenge

We observed a marked infiltration of inflammatory cells into the peribronchiolar and perivascular lesions in the lung tissues sections from Alum/OVA-sensitized/challenged mice compared with the negative control mice (Fig. 6A). By contrast, EC-18 treated mice exhibited less infiltration of inflammatory cells into the peribronchiolar and perivascular lesions compared with the Alum/OVA-sensitized/challenged mice. In quantitative analysis for airway inflammation, EC-18 exhibited the significant reduction in the inflammatory index compared with the Alum/OVA-sensitized/challenged mice (Fig. 6B).

3.6. EC-18 suppresses mucus production induced by OVA challenge

Lung sections from Alum/OVA-sensitized/challenged mice stained with PAS exhibited overproduction of mucus (Fig. 7A). Sections from the montelukast-treated mice showed mild mucus production in the bronchial airway compared with the Alum/OVA-sensitized/challenged mice. Similar to those of the montelukast-treated mice, the EC-18 treated mice showed reductions in the mucus production in the airways compared with the Alum/OVA-sensitized/challenged mice. In quantitative analysis for mucus production, EC-18 exhibited the significant decrease in the mucus production index compared with the Alum/OVA-sensitized/challenged mice (Fig. 7B).

3.7. EC-18 reduces the expression of iNOS in lung tissue challenged with OVA

The expression of iNOS was significantly higher in the Alum/OVA-sensitized/challenged compared with the negative controls (Fig. 8A and B). The EC-18-treated mice had significantly lower the expression

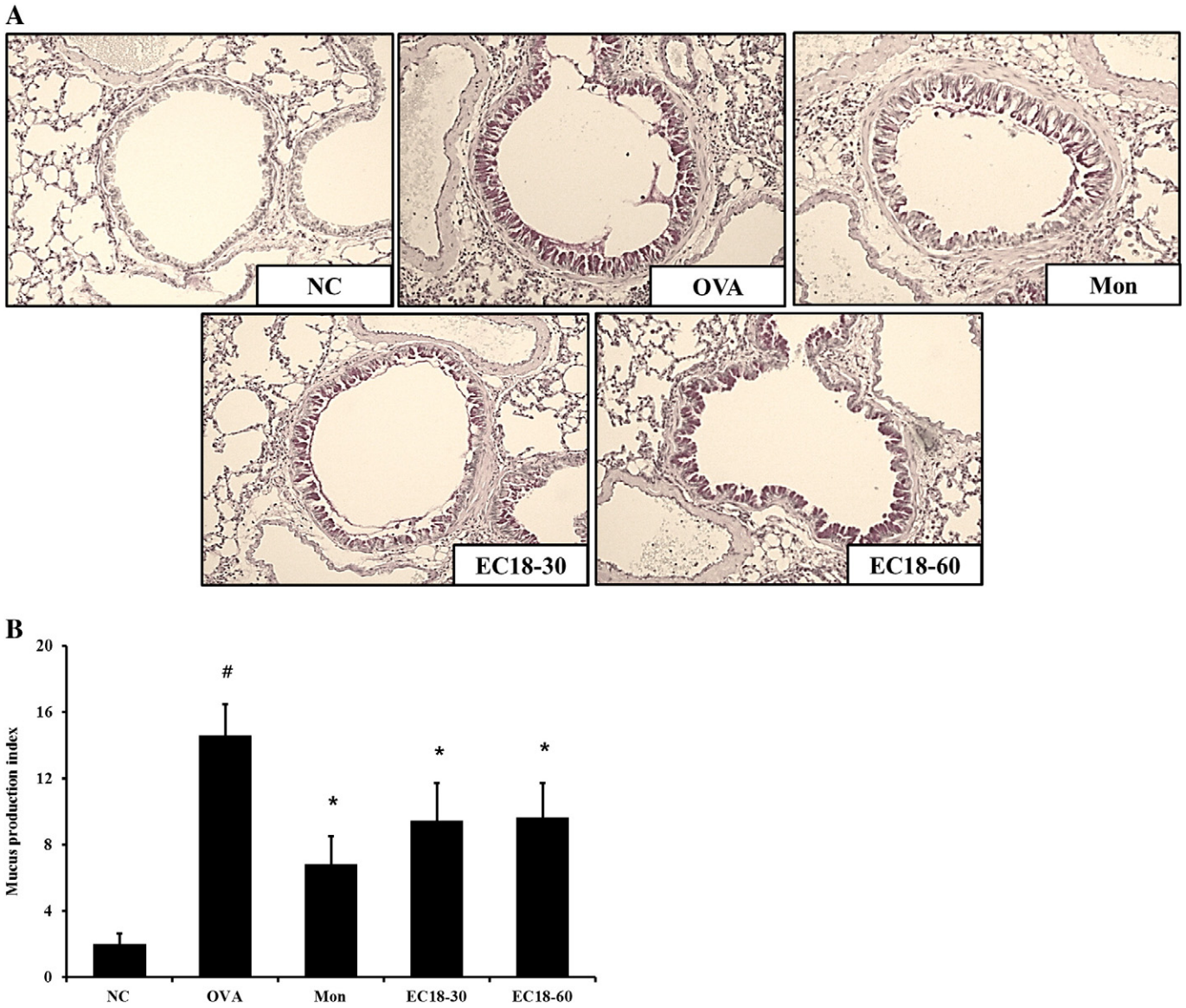

Fig. 7. EC-18 reduces mucus production in lung tissues of mice 48 h after the final OVA challenge. (A) Lung tissue with PAS staining (magnification ×200), (B) quantitative analysis for mucus production. The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

of iNOS compared with the Alum/OVA-sensitized/challenged mice. The level of eotaxin-1 was also higher in the Alum/OVA-sensitized/challenged mice.

4. Discussion

Allergic asthma is a common chronic airway disease and its prevalence has continued to increase. In this study, we evaluated the inhibitory effects of EC-18, a synthetic MADG, on asthmatic responses using an OVA-induced murine asthma model. The OVA-challenged asthmatic mice showed eosinophilia, airway inflammation, mucus hypersecretion, and elevated levels of IL-4, IL-5, and IL-13 in the BALF. By contrast, the EC-18 treated mice had fewer inflammatory cells, particularly eosinophils, in the BALF and lung tissue, and lower levels of IL-4, IL-5, IL-13, and eotaxin-1 in the BALF compared with the Alum/OVA-sensitized/challenged mice. In addition, EC-18 significantly decreased the expression of iNOS in lung tissue. According to the histological analysis, EC-18 attenuated inflammatory cell infiltration into the airway and suppressed the mucus overproduction that is induced by OVA challenge.

Th2 cytokines play an important role in the initiation and progression of allergic asthma. Th2 cytokines, such as IL-4, IL-5, and IL-13 lead to eosinophil-rich airway inflammation, methacholine responsiveness, and mucus overproduction [3]. Of the Th2 cytokines, IL-4 causes Ig isotype switching to IgE and promotes differentiation of native T lymphocytes to the Th2 lineage, which further increases Th2 cytokine production. IL-5 is closely associated with eosinophil-rich airway inflammation [2]. IL-5 is also critical for the differentiation, maturation, and survival of eosinophils, leading to overproduction of IgE and mucus [13]. IL-13 induces the production of chemoattractants for eosinophils and stimulates pulmonary epithelial cells, fibroblasts and airway smooth muscle cell, resulting in airway inflammation and methacholine responsiveness [14]. Taken together, these previous results strongly suggest that Th2 cytokines play a crucial role in the pathogenesis of asthma. Therefore, the reduction in Th2 cytokines production could provide therapeutic benefits to asthmatic patients. As described in previous studies, Alum/OVA-sensitized/challenged mice exhibited marked increases in the number of eosinophils in the BALF, the serum IgE, and methacholine responsiveness with elevation of IL-4, IL-5, IL-13, and eotaxin-1 [3,4]. By contrast, the EC-18-treated mice showed the reverse effects with a reduction in Th2 cytokines compared with the Alum/OVA-sensitized/challenged mice. These findings are consistent with the histological analyses. EC-18 decreased inflammatory cell infiltration into the airway and suppressed the production of mucus compared with the Alum/OVA-sensitized/challenged mice. Based on our results, EC-18 appears to effectively attenuate the asthmatic response caused by the downregulation of Th2 cytokines.

Overexpression of iNOS is an important finding in the development of asthma. iNOS is responsible for overproduction of nitric oxide that induced the stimulation of inflammatory signaling and the recruitment of eosinophils [15,16]. Previous studies have demonstrated that asthmatic responses such as eosinophilia, elevation of Th2 cytokines and IgE, and airway inflammation are markedly suppressed in iNOS-deficient animals or iNOS inhibitor-treated animals [17,18]. On the other hand, Th2 cytokines such as IL-13, induced increases in the expression of iNOS, which caused the production of nitric oxide to increase [19,20]. These findings indicated that iNOS plays a crucial mediator in aggravating asthma. In this study, Alum/OVA-sensitized/challenged mice increase iNOS expression in lung tissue compared with the negative controls. However, EC-18 treatment decreased iNOS expression with a reduction of inflammatory cell count and Th2 cytokines. These results suggest that suppression of iNOS caused by EC-18 administration is a possible mechanism for attenuating asthmatic responses.

A recent study demonstrated that a constituent of antlers exhibits immunomodulatory effects on the immunocytes of mice *via* the upregulation of Th1 cytokines and downregulation of Th2 cytokines [21].

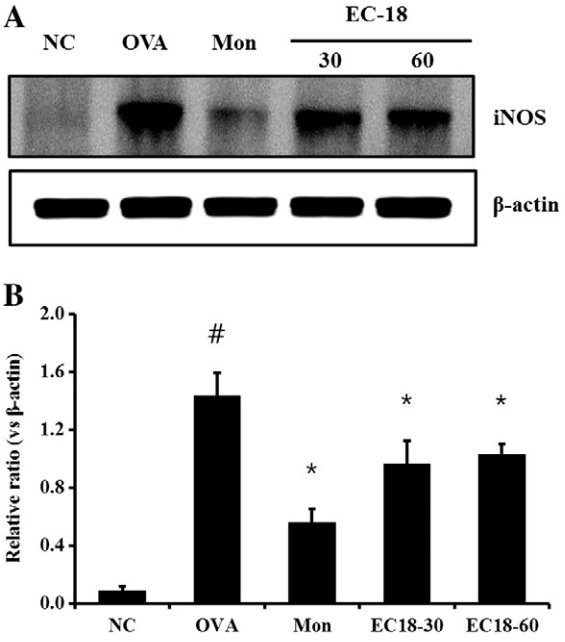

Fig. 8. EC-18 decreases the expression of iNOS in lung tissue. (A) Western blot, (B) relative ratio (vs β-actin). The values expressed as mean ± SD (n = 7). NC, normal control mice treated with PBS only; OVA, Alum/OVA-sensitized/challenged mice; Mon, montelukast (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-30, EC-18 (30 mg/kg) + Alum/OVA-sensitized/challenged mice; EC18-60, EC-18 (60 mg/kg) + Alum/OVA-sensitized/challenged mice. #Significantly different from normal control, $P < 0.05$; *Significantly different from cells treated with OVA, $P < 0.05$.

These previous findings were supported by the results of our study. In conclusion, EC-18 attenuated the asthmatic response induced by OVA challenge, which resulted in a suppression of Th2 cytokine release and iNOS expression. These findings indicate that EC-18 may be useful for the treatment of allergic disease due to its immunomodulatory effects. Therefore, our results suggest that EC-18 can be developed as a useful therapeutic for treating allergic asthma.

Acknowledgements

This study was supported by the KRIBB Research Initiative program (KGM1221312) and ENZYCHEM Lifesciences (IGM0071311) of the Republic of Korea.

Conflicts of interest

The authors declare that they have no competing interests.

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