



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Celastror suppresses allergen-induced airway inflammation in a mouse allergic asthma model

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ABSTRACT

Celastror has anti-inflammatory and immunomodulatory activities, but its anti-allergic effects remain poorly understood. Therefore, we aimed to investigate the ability of celastror to inhibit asthmatic reactions in a mouse allergic asthma model. BALB/c mice were sensitized and challenged with ovalbumin to induce asthma. We measured the recruitment of inflammatory cells into the bronchoalveolar lavage fluid or lung tissues by Diff-Quik and hematoxylin and eosin staining, respectively, goblet cell hyperplasia by periodic acid-Schiff (PAS) staining, airway hyperresponsiveness by Flexvent system, mRNA and protein expression of cytokines, matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) by reverse transcriptase polymerase chain reaction and ELISA, respectively, and the activities of mitogen-activated protein (MAP) kinases and nuclear factor-kappa B (NF-κB) in the bronchoalveolar lavage cells and lung tissues by Western blot and electrophoretic mobility shift assay (EMSA), respectively. Celastror reduced the total number of inflammatory cells in the bronchoalveolar lavage fluid and in peribronchial areas, and decreased the airway hyperresponsiveness, mRNA and protein expression levels for inflammatory cytokines such as interleukin (IL)-4, IL-13, TNF-α and IFN-γ, and for MMPs and TIMPs, MAP kinases and NF-κB activities in the bronchoalveolar lavage cells and in the lung tissues increased in ovalbumin-induced allergic asthma in mice. Our data suggest that oral administration of celastror suppresses ovalbumin-induced airway inflammation, hyperresponsiveness, and tissue remodeling by regulating the imbalance of MMP-2/-9 and TIMP-1/-2 by inflammatory cytokines via MAP kinases/NF-κB in inflammatory cells. Based on our findings, we suggest that celastror may be used as a therapeutic agent for allergy-induced asthma.

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1. Introduction

Celastror is a pentacyclic-triterpene that is extracted from the traditional Chinese medicine, *Trypterygium Wilfordii* Hook, which belongs to the Celastraceae family (Zhou, 1991). It has traditionally been used for the treatment of arthritis and other inflammatory diseases, and celastror structure and IC₅₀ for various effects have already been elucidated (Jin et al., 2002). Although it has not yet been tested as a single agent in humans, celastror has shown promise as an anti-inflammatory compound in rat models and in human with collagen-induced arthritis, rheumatoid arthritis (Tao et al., 2002) or asthma (Liu et al., 2004). Celastror has been reported to inhibit the expression of pro-inflammatory cytokines (Calixto et al., 2004) and NF-κB activity that is related to inflammation and cellular proliferation (Jin et al., 2002; Lee et al., 2006; Sethi et al., 2007).

Allergic asthma is a chronic inflammatory disease of the lung that is characterized by the infiltration of inflammatory cells into the lung tissues, mucus hypersecretion by goblet cells, reversible obstruction of airway hyperresponsiveness, over-expressions of Th2-mediated cytokines including interleukin (IL)-4, IL-5 and IL-13, and chemokines such as eotaxin and RANTES in the airways of patients with allergic asthma (Zimmermann et al., 2003).

Matrix metalloproteinases (MMPs) mediate tissue remodeling in the airway by regulating the integrity of the basal lamina and the degree of infiltration by inflammatory cells (Kumagai et al., 1999; Lanone et al., 2002). MMPs are produced by structural cells such as endothelial cells, epithelial cells and fibroblasts and by inflammatory cells including macrophages, eosinophils, neutrophils and mast cells (Abel and Vliagoftis, 2008; Opdenakker et al., 2001; Wills-Karp and Karp, 2004). Expression of MMPs is modulated by a variety of stimuli such as cytokines (Girolamo et al., 2006), and MMP-9 expression is increased in the airways of asthmatic individuals (Kumagai et al., 1999). MMPs levels are also increased in the bronchoalveolar lavage (Henry et al., 2002; Locke et al., 2007), lung tissues (Gueders et al., 2008) and sputum (Hur et al., 2008; Vignola et al., 1998) of patients with allergic airway disease.

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As mentioned above, celastrol has been used to inhibit inflammatory responses in diverse origins (Calixto et al., 2004; Tao et al., 2002). Celastrol has also been shown to suppress airway inflammation in an asthmatic mouse model through inhibition of the production of histamine and eotaxin in mast cells (Liu et al., 2004). However, the molecular mechanism underlying the anti-asthmatic effects of celastrol is not fully known. Therefore, we aimed to investigate the inhibitory activities of orally administered celastrol in an ovalbumin-specific mouse allergic asthma model. In particular, we focused on the regulation of MMPs and tissue inhibitors of metalloproteinases (TIMPs) by inflammatory cytokines. We observed that celastrol suppresses the allergic asthma reaction by regulating the production of MMP-2/-9 and TIMP-1/-2 by inflammatory cytokines via the mitogen-activated protein (MAP) kinases/nuclear factor-kappa B (NF- κ B) pathway.

2. Materials and methods

2.1. Materials

We purchased celastrol from Cayman Chemical (Ann Arbor, MI), ovalbumin (Grade V), polyacrylamide, gelatin, sodium dodecyl sulphate, hematoxylin, aprotinin, leupeptin, dimethylsulfoxide (DMSO), 3,3'-diaminobenzidine (DAB), ethidium bromide (EtBr) and periodic acid-Schiff (PAS) from Sigma-Aldrich (St. Louis, MO), alum from Superfos Biosector (Vedaek, Denmark), phenylmethylsulphonyl fluoride (PMSF) from Roche (Mannheim, Germany), Diff-Quik from International Reagents Corp. (Kobe, Japan), human MMP-2 and -9 from Chemicon (Temecula, CA), antibodies against ERK, JNK and p38 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), p-ERK, p-JNK, and p-p38 antibody from Cell Signaling (Beverly, MA), IL-4, -5, -13, TNF- α and IFN- γ from R&D systems (San Diego, CA), streptavidin-horseradish peroxidase (HRP), chromogen substrate, and aqueous mounting medium from DakoCytomation (Carpinteria, CA), TRIZOL reagent and SuperScript One-step RT-PCR kit from Invitrogen (Carlsbad, CA), HRP-conjugated goat anti-mouse IgG, and HRP-conjugated rabbit anti-goat IgG from Zymed Laboratory Inc. (San Francisco, CA), electrogenerated chemiluminescent (ECL) solution, microspin G-25 column, nitrocellulose membrane, and 10 μ Ci γ -[^{32} P] ATP (specific activity, 3,000 Ci/mmol) from Amersham BioSciences (Buckinghamshire, UK) and the oligonucleotide of NF- κ B from Promega (Madison, WI).

2.2. Animals

We used specific pathogen-free female BALB/c mice (Oriental Ltd., Seoul, Korea) that were 6–8 weeks of age and weighed about 20 g. All animal experiments were conducted in the Laboratory Animal Research Center of Sungkyunkwan University and were approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and by the ethical committee.

2.3. Sensitization, challenge and experimental protocol

BALB/c mice were divided into eight groups (8 mice/group). Group A (negative control) mice were sensitized and nebulized with phosphate buffered saline (PBS); group B (general sensitization) mice were sensitized with ovalbumin and nebulized with PBS; group C (ovalbumin-challenged) mice were sensitized and nebulized with ovalbumin; group D, E and F (celastrol-treated) mice were ovalbumin-challenged and then treated with 10, 30 and 100 mg/kg of celastrol, respectively; group G, H, I [vehicle (DMSO)-treated] mice were ovalbumin-challenged and then treated with 2, 6 and 20 μ l DMSO, respectively (final administrated volumes, 200 μ l). All mice (excluding the negative control group) were sensitized with 10 μ g ovalbumin adsorbed in 250 μ g/200 μ l of alum (aluminum hydroxide, 2%

Alhydrogel) by i.p. injection on days 0, 5, 14, 21, and 28 (general sensitization). The negative control mice were sensitized with PBS. One week after the final injection, the mice were nebulized with 2% ovalbumin for 10 min once daily for 7 consecutive days from day 35 to 41 (local challenge) using a nebulizer (Mega Medical, Seoul, Korea), and then the mice were nebulized again with 2% ovalbumin for 10 min on day 49. Control and general sensitized mice were nebulized by PBS at the same times. All mice were sacrificed on the following day (day 50).

2.4. Preparation of and treatment with celastrol

Celastrol was prepared as a 100 mg/ml stock solution. Briefly, 100 mg of celastrol was dissolved in 1 ml of DMSO (vehicle). Celastrol (10, 30 or 100 mg/kg/200 μ l) was administered orally for 7 consecutive day, 30 min before every local challenge (from day 35 to day 41). The volume of the vehicle contained in each concentration of celastrol was 2, 6 or 20 μ l. In order to examine the effect of the vehicle, it was orally administrated instead of celastrol 30 min before every local challenge, after adjusting to 200 μ l with PBS. We examined three groups (group G, H, I) for the vehicle, but only the data for the high dose vehicle (group I) is shown in order to avoid the complication due to no influence on allergic responses. The data from mice treated with celastrol alone (10, 30 or 100 mg/kg) is not shown because this compound did not have any significant effects in these mice.

2.5. Bronchoalveolar lavage fluid

Thoracic cavities were carefully opened, tracheas were exposed, and bronchoalveolar lavage fluid was collected by the same methods previously described (Kim et al., 2007a). Lavage supernatants were used to determine the protein levels and activities of MMPs and inflammatory cytokines. After centrifugation using a Cytospin III (Shandon, Pittsburgh, PA), a portion of the cells pellet was stained with Diff-Quik for differential cell counting (details provided in Appendix A). The remnant bronchoalveolar lavage cells were examined for mRNA of cytokines, MMP-2/-9 and TIMP-1/-2, MAP kinase and NF- κ B activity.

Table 1

The effect of celastrol on inflammatory cell recruitment into bronchoalveolar lavage fluid in ovalbumin-challenged allergic mice^a.

	Total cells ($\times 10^4$)	Macrophages ($\times 10^4$)	Lymphocytes ($\times 10^4$)	Neutrophils ($\times 10^4$)	Eosinophils ($\times 10^4$)
A	2.3 $\pm$ 2.50	1.5 $\pm$ 0.03	0.8 $\pm$ 0.03	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
B	2.6 $\pm$ 0.82	1.7 $\pm$ 0.02	0.9 $\pm$ 0.02	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
C	17.4 $\pm$ 2.50 ^c	4.5 $\pm$ 0.04 ^c	3.2 $\pm$ 0.08 ^c	3.1 $\pm$ 0.03 ^c	6.6 $\pm$ 0.10 ^c
D	15.0 $\pm$ 2.20 ^c	3.8 $\pm$ 0.04 ^c	2.8 $\pm$ 0.03 ^c	2.8 $\pm$ 0.03 ^c	5.8 $\pm$ 0.10 ^c
E	10.5 $\pm$ 1.15 ^{c,d}	2.8 $\pm$ 0.03 ^{c,d}	1.9 $\pm$ 0.12 ^{c,d}	2.1 $\pm$ 0.23 ^{c,d}	3.6 $\pm$ 0.08 ^{c,e}
F	7.5 $\pm$ 2.32 ^{c,e}	2.1 $\pm$ 0.31 ^{b,e}	1.5 $\pm$ 0.01 ^{c,e}	1.3 $\pm$ 0.01 ^{c,e}	2.6 $\pm$ 0.05 ^{c,e}
I	17.4 $\pm$ 1.19 ^c	4.4 $\pm$ 0.04 ^c	3.6 $\pm$ 0.03 ^c	3.3 $\pm$ 0.03 ^c	6.1 $\pm$ 0.11 ^c

^aBALB/c mice were sensitized and challenged with ovalbumin, and then bronchoalveolar lavage fluid was collected as described in the section of Materials and methods. The bronchoalveolar lavage cells were separated using a Cytospin, and then stained with Diff-Quik. Differential cell counts were performed using standard morphological criteria. A (negative control), mice sensitized and nebulized with PBS; B (general sensitized mice), mice sensitized with ovalbumin and nebulized with PBS; C (ovalbumin-challenged mice), mice sensitized and nebulized with ovalbumin; D, E, F (celastrol-administrated mice), ovalbumin-challenged mice treated with 10, 30 or 100 mg/kg celastrol, respectively, 30 min before every local challenge; I (vehicle-administrated mice), ovalbumin-challenged mice treated with 20 μ l DMSO 30 min before every local challenge. The data for other vehicles (2 or 6 μ l) are not shown because the results are the same as with the high dose vehicle. Data are expressed as the mean $\pm$ S.E. (n = 8). ^b, P < 0.05; ^c, P < 0.01; ^d, P < 0.001 versus control or general sensitized mice; ^e, P < 0.01; ^f, P < 0.001 versus ovalbumin-challenged mice.

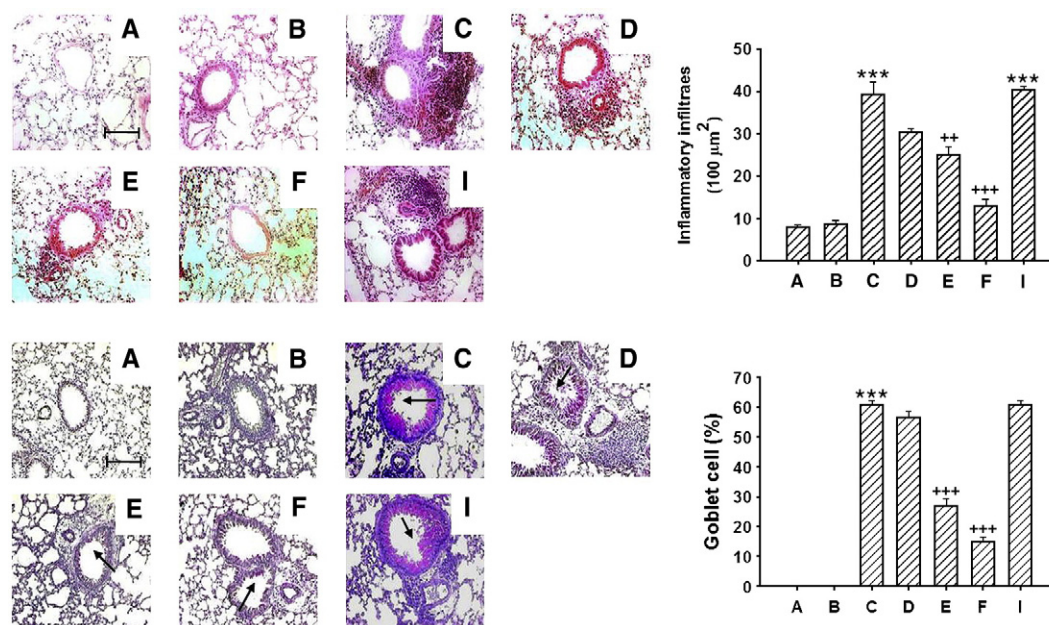


Fig. 1. The effect of celastrol on histopathologic changes in the lung tissues of ovalbumin-challenged allergic mice. BALB/c mice were sensitized and challenged with ovalbumin as described in the section of **Materials and methods**. Inflammation was evaluated in the lung tissues by analyzing inflammatory cell infiltration using H & E (upper panel) and goblet cell hyperplasia using PAS (lower panel). Inflammatory infiltrated cells were counted in a $100\ \mu\text{m} \times 100\ \mu\text{m}$ area under microscope (upper panel). The goblet cells were counted under a microscope and the ratio (percentage) of PAS-positive cells (indicated by arrows)/total cells in a $50\ \mu\text{m} \times 50\ \mu\text{m}$ area was recorded (lower panel) and the results were presented in histograms. The experimental details are described in the Materials and methods section. The symbols (A–F, I) are defined in Table 1. The data in the histograms are the mean $\pm$ S.E. of $n = 8$. The arrow in the lower panel indicates goblet cells. Bars in control group (group A) indicate $100\ \mu\text{m}$. *** $P < 0.001$ versus negative control or general sensitized mice; +++ $P < 0.001$ versus ovalbumin-challenged mice.

2.6. Lung histology

Lung tissues were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (H & E) to detect inflammatory infiltrates and with periodic acid-Schiff (PAS) for goblet cells (magnification 200X) (Kim et al., 2007a). The remnant lung tissues were stored at $-70\ ^\circ\text{C}$ for activity assays, RT-PCR and immunoblotting for various proteins (see experimental details provided in Appendix A).

2.7. Cytokine and MMPs levels in bronchoalveolar lavage fluid or lung tissues

Lung tissues (50 mg/500 μl) were homogenized in PBS using a Polytron (Kinematica, Littau, Switzerland) and then centrifuged ($770 \times g$, 10 min). The protein levels of IL-4, IL-5, IL-13, TNF- α , IFN- γ , MMP-2, and MMP-9 in the bronchoalveolar lavage fluid (50 μl) and lung

tissue homogenates (50 μl) were determined by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. Standard curves were generated using different concentrations of recombinant cytokines or MMPs. The limit of detection for cytokines, MMP-2 and MMP-9 were better than 7.8 pg/ml and 0.78 ng/ml, respectively.

2.8. Measurement of airway hyperresponsiveness

Airway hyperresponsiveness was assessed as previously described (Choi et al., 2006) by measuring changes in the airway resistance ($\text{cmH}_2\text{O}/\text{ml}/\text{s}$) and lung compliance ($\text{ml}/\text{cmH}_2\text{O}$) using the Flexivent system (SCIREQ, Montreal, Quebec, Canada) after mice were treated with methacholine. Anesthetized (pentobarbital sodium 70–90 mg/kg, i.p.) and tracheostomized (18G cannula) mice were mechanically ventilated (160 breaths/min, tidal volume of 10 ml/kg, positive end-expiratory pressure of 3 cmH_2O). Increasing concentrations (3–25 mg/

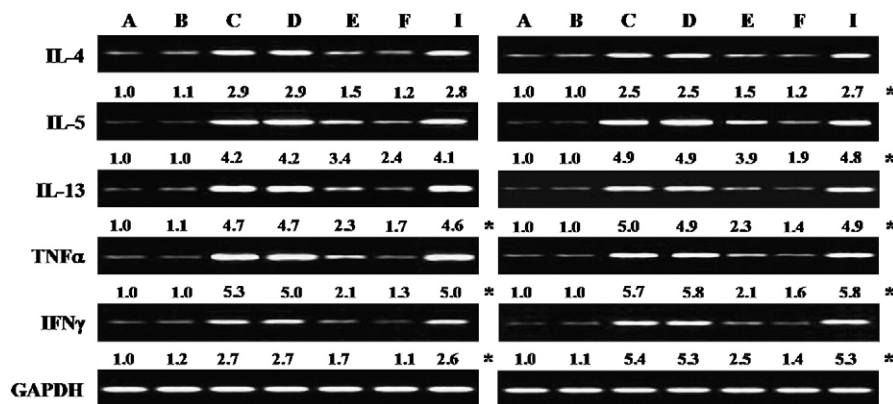


Fig. 2. The effect of celastrol on the expression of various cytokine mRNAs in the bronchoalveolar lavage cells and lung tissues of ovalbumin-challenged allergic mice. BALB/c mice were sensitized and challenged with ovalbumin as described in the section of **Materials and methods**. mRNA expression in the bronchoalveolar lavage cells (left panel) and lung tissues (right panel) was determined by RT-PCR analysis. The symbols (A–F, I) are defined in Table 1. *, Numbers below the bands are the ratio of band density of each group versus that of the negative control and the band density of the GAPDH mRNA. The bands for PCRs are representative of four independent experiments ($n = 4$).

Table 2

The effect of celastrol on cytokine protein in the bronchoalveolar lavage fluid or lung tissues of ovalbumin-challenged allergic mice^a.

	IL-4	IL-5	IL-13	TNF- α	IFN γ
BAL fluid (pg/ml)					
A	8.6 $\pm$ 0.2	36.2 $\pm$ 0.8	66.5 $\pm$ 3.4	92.0 $\pm$ 3.7	10.3 $\pm$ 0.3
B	8.9 $\pm$ 0.4	35.6 $\pm$ 1.7	68.7 $\pm$ 1.2	101.6 $\pm$ 5.1	10.7 $\pm$ 0.7
C	31.4 $\pm$ 1.4 ^b	66.1 $\pm$ 1.4 ^b	165.4 $\pm$ 6.1 ^b	212.7 $\pm$ 5.9 ^b	38.7 $\pm$ 0.6 ^b
D	31.1 $\pm$ 1.0	65.4 $\pm$ 2.3	156.8 $\pm$ 5.7	201.4 $\pm$ 6.4	37.8 $\pm$ 0.4
E	19.8 $\pm$ 0.2 ^c	46.8 $\pm$ 1.3 ^c	107.8 $\pm$ 2.4 ^d	157.4 $\pm$ 2.9 ^e	21.6 $\pm$ 1.1 ^c
F	12.0 $\pm$ 0.1 ^c	40.3 $\pm$ 0.7 ^c	87.2 $\pm$ 4.3 ^d	119.2 $\pm$ 3.1 ^c	14.8 $\pm$ 1.0 ^d
Lung tissues (pg/ml/50 mg)					
A	90.3 $\pm$ 2.4	249.0 $\pm$ 13.0	699.0 $\pm$ 20.8	740.5 $\pm$ 22.8	102.9 $\pm$ 3.3
B	93.3 $\pm$ 4.4	280.5 $\pm$ 8.4	680.5 $\pm$ 8.4	741.9 $\pm$ 8.7	106.7 $\pm$ 7.0
C	329.5 $\pm$ 14.7 ^b	694.2 $\pm$ 14.8 ^b	1294.2 $\pm$ 14.8 ^b	2233.9 $\pm$ 62.2 ^b	387.3 $\pm$ 6.0 ^b
D	326.1 $\pm$ 11.0	686.6 $\pm$ 24.3	1311.6 $\pm$ 7.6	2114.7 $\pm$ 66.9	377.8 $\pm$ 3.7
E	290.7 $\pm$ 09.9 ^c	526.6 $\pm$ 19.1 ^c	986.6 $\pm$ 21.1 ^d	1851.5 $\pm$ 36.0 ^e	216.3 $\pm$ 10.9 ^c
F	191.2 $\pm$ 12.6 ^d	357.8 $\pm$ 19.3 ^d	772.8 $\pm$ 21.8 ^d	1062.7 $\pm$ 40.8 ^d	155.9 $\pm$ 3.5 ^d

^a BALB/c mice were sensitized and challenged with ovalbumin as described in the section of **Materials and methods**. Individual cytokine levels in the bronchoalveolar lavage fluid and lung tissues were determined by ELISA ($n = 8$). The symbols (A–F) are defined in **Table 1**. The data for the vehicle (DMSO) alone are not shown (2, 6 or 20 μ l of DMSO) because no significant effects were observed. The data are expressed as the mean $\pm$ S.E. ($n = 8$).

^b $P < 0.001$ versus control or general sensitized mice.

^c $P < 0.01$.

^d $P < 0.001$ versus OVA-challenged mice.

^e $P < 0.05$.

ml) of methacholine aerosol were administered by nebulization (3–25.0 mg/ml). Airway resistance (R_L) and lung compliance (C_L) were measured at 60 and 120 s after the administration of methacholine.

2.9. Zymography

The activities of MMPs in the first bronchoalveolar lavage fluid supernatant (35 μ l) were analyzed by a previously described method (Kim et al., 2007a). Gelatinolytic activity was detected as a clear band against a blue background. Human MMP-2 and MMP-9 were used as gelatinase zymography standard markers (see experimental details provided in **Appendix A**).

2.10. RT-PCR

Total cellular RNA was isolated from bronchoalveolar lavage cells (1×10^6 cells/ml) or lung tissues (100 mg/ml) using Trizol reagent. RT-PCR was performed using the SuperScript One-step RT-PCR kit in an automated thermal cycler (XP cycler, Bioer Technology, Tokyo, Japan) (Picado et al., 2003). The experimental details for the measurement of mRNA levels of cytokines, MMPs and TIMPs provided in **Appendix A**.

2.11. Assay of MAP kinases by western blot

Bronchoalveolar lavage cells (1×10^6 cells/50 μ l lysis buffer) or lung tissues (50 mg/500 μ l lysis buffer) were homogenized in low-salt lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2.0 μ g/ml aprotinin, 2.0 μ g/ml leupeptin] using a Polytron (Kinematica), and immunoblotting was performed using a previously described method (details available in **Appendix A**) (Kim et al., 2007a).

2.12. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from bronchoalveolar lavage cells (1×10^6 cells/50 μ l lysis buffer) or lung tissues (50 mg/500 μ l lysis buffer) were prepared, and electromobility shift assays were performed using a modification of a previously described method (experimental details available in **Appendix A**) (Dedeoglu et al., 2004).

2.13. Statistical analysis

Data are presented as means $\pm$ S.E. Data were analyzed by ANOVA with multiple comparisons. An analysis of significance between each control group and experimental group was carried out with the SPSS statistic program (SPSS Inc., Chicago, IL). The PCR and blots are representative of four independent experiments ($n = 4$). A p -values of < 0.05 was regarded as statistically significant.

3. Results

3.1. Inflammatory cell recruitment into bronchoalveolar lavage fluid and lung tissues

Since inflammation inevitably occurs in allergic and asthmatic disorders, we first examined whether celastrol inhibited the infiltration of inflammatory cells into bronchoalveolar lavage fluid in ovalbumin-challenged mice. The elevated levels of total inflammatory cells and of each inflammatory cell type (i.e., eosinophils, neutrophils, lymphocytes and macrophages) in the bronchoalveolar lavage fluid of ovalbumin-challenged mice (versus the negative control or general

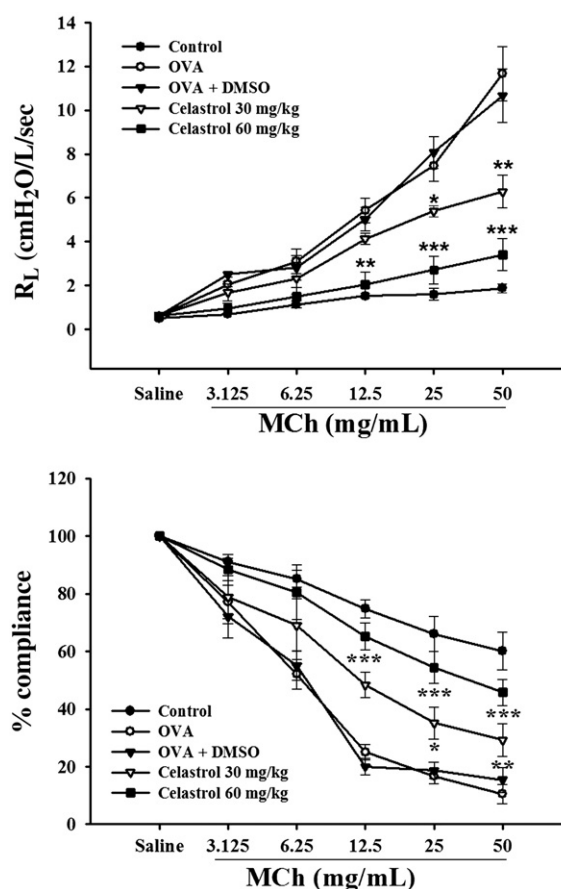


Fig. 3. The effect of celastrol on airway hyperresponsiveness of ovalbumin-challenged allergic mice. Airway hyperresponsiveness was assessed by measuring changes in airway resistance (R_L) (upper panel) and lung compliance (lower panel) using the Fexivent system as described in the Materials and methods section. After each methacholine nebulization, the lung resistance ($\text{cmH}_2\text{O/L/s}$) and compliance ($\text{ml/cmH}_2\text{O}$) were measured and expressed as $\text{cmH}_2\text{O/L/s}$ and percent of the saline-administered group, respectively. Sham (negative control) mice were sensitized and nebulized with PBS; ovalbumin (OVA) mice were sensitized and nebulized with ovalbumin (ovalbumin-challenged mice); celastrol–30 mg/kg, 100 mg/kg, or ovalbumin + DMSO mice were ovalbumin-challenged mice received an orally administration of 30 mg/kg, 100 mg/kg celastrol and 20 μ l DMSO 30 min before every local challenge. Data are presented as the mean $\pm$ S.E. of $n = 6$. $^+$, $P < 0.05$; $^{++}$, $P < 0.01$; $^{+++}$, $P < 0.001$ versus ovalbumin-challenged mice.

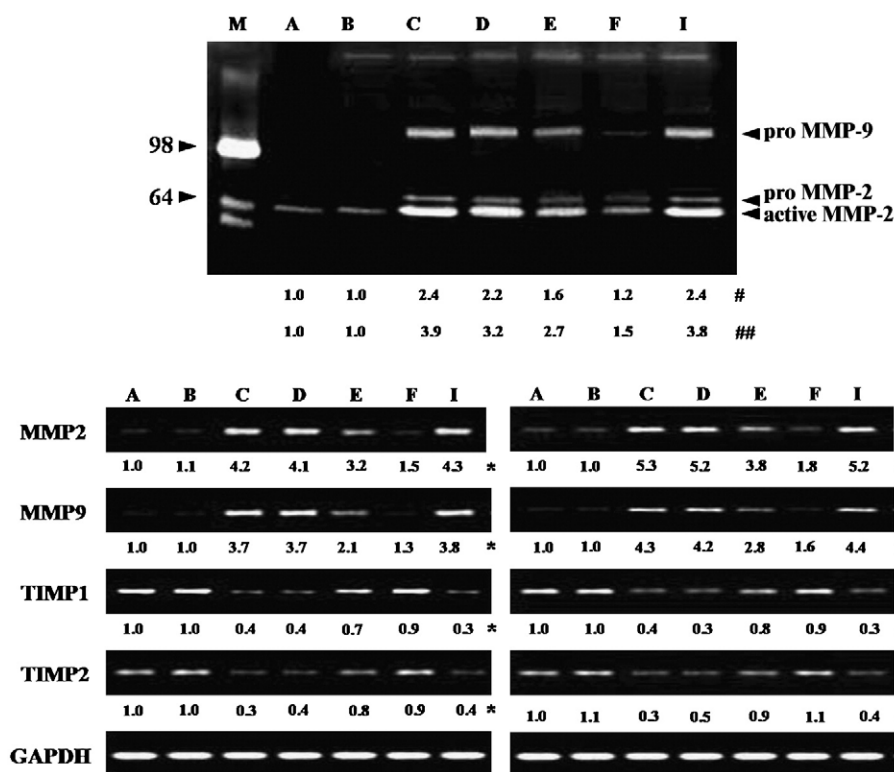


Fig. 4. The effect of celastrol on the MMPs activity in the bronchoalveolar lavage fluid and on MMPs and TIMPs mRNA expression in the bronchoalveolar lavage cells and lung tissues of ovalbumin-challenged allergic mice. BALB/c mice were sensitized and challenged with ovalbumin as described in the section of **Materials and methods**. Gelatinolytic activity (upper panel) was detected as a clear band against a blue background. Human MMP-2 and MMP-9 were used as the gelatinase zymography standard markers. The RT-PCR analysis of the mRNA expression of MMPs and TIMPs in the bronchoalveolar lavage cells (left panel) and lung tissues (right panel) collected from mice is described in the Materials and methods section ($n = 4$). The symbols (A–F, I) are defined in Table 1. M, Marker. #, Numbers below bands are the ratio of band density for MMP-9 versus that of negative control or ovalbumin-sensitized mice; ##, numbers below bands are the ratio of band density of for MMP-2 versus that of the negative control or ovalbumin-sensitized mice. *, Numbers below the bands are the ratio of band density of each group versus that of the negative control and the band density of the GAPDH mRNA. The bands for zymography and PCRs are representative of four independent experiments ($n = 4$).

sensitized mice) were reduced in a dose-dependent manner by celastrol (Table 1). The administration of a vehicle alone (2, 6 or 20 μ l DMSO) instead of celastrol did not affect inflammatory cell recruitment into the bronchoalveolar lavage fluid. Therefore, we only showed the data for the high dose vehicle (Table 1–I).

We further examined changes in inflammatory cell recruitment in lung tissues of ovalbumin-challenged mice by counting infiltrated cells in a $100 \times 100 \mu\text{m}$ area under a microscope. The recruitment of inflammatory cells in ovalbumin-challenged mice was observed in the peribronchial and perivascular areas (Fig. 1C, upper panel). Celastrol administration remarkably reduced the inflammatory cell infiltration increased in ovalbumin-challenged mice (Fig. 1D, E and F, upper panel).

The number of goblet cells containing mucus was quantified as the percentage of PAS-positive cells/total cells counted in a $50 \mu\text{m} \times 50 \mu\text{m}$ area under the microscope. The increase in the number of goblet cells (stained pink dot) in ovalbumin-challenged mice (Fig. 1, lower panel) was remarkably reduced in celastrol-treated mice. General sensitized mice showed no change in inflammatory cell infiltration or goblet cells in comparison to the negative control mice. The administration of a vehicle alone (high dose DMSO) instead of celastrol did not affect inflammatory cell recruitment into the lung tissues (Fig. 1–I).

3.2. Expressions of mRNA and proteins of cytokines

Th2 and Th1 cytokines have been known to induce allergic asthmatic inflammation and airway remodeling (Wills-Karp and Karp, 2004; Zimmermann et al., 2003) and to cause early phase asthma (Renauld, 2001), respectively. Therefore, we further examined whether celastrol affected the mRNA and protein levels of cytokines in

the bronchoalveolar lavage cells (Fig. 2, left panel) and in the lung tissues (Fig. 2, right panel) of ovalbumin-induced mice. Celastrol reduced the mRNA expressions of all cytokines that are normally elevated in ovalbumin-challenged mice (compared to negative control mice or general sensitized mice) in a dose-dependent manner (Fig. 2D, E and F).

Table 3

The effect of celastrol on protein production of MMPs in the bronchoalveolar lavage fluid or lung tissues of ovalbumin-challenged allergic mice^a.

		MMP2	MMP9
BAL fluid (ng/ml)	A	03.2 $\pm$ 0.07	0.08 $\pm$ 0.004
	B	03.3 $\pm$ 0.09	0.09 $\pm$ 0.013
	C	27.6 $\pm$ 1.25 ^b	2.69 $\pm$ 0.124 ^b
	D	27.4 $\pm$ 1.65 (0.7)	2.64 $\pm$ 0.084 (1.9)
	E	14.2 $\pm$ 0.95 (48.2) ^c	1.40 $\pm$ 0.096 (48.0) ^c
	F	06.7 $\pm$ 0.26 (75.7) ^d	0.78 $\pm$ 0.083 (71.0) ^d
Lung tissues (ng/ml/50 mg)	A	08.5 $\pm$ 2.96	01.4 $\pm$ 0.73
	B	08.8 $\pm$ 2.90	01.3 $\pm$ 0.44
	C	44.0 $\pm$ 7.69 ^b	26.0 $\pm$ 3.31 ^b
	D	42.9 $\pm$ 8.01 (2.5)	25.3 $\pm$ 7.34 (2.7)
	E	30.8 $\pm$ 2.57 (30.0) ^c	18.1 $\pm$ 3.35 (30.4) ^c
	F	16.7 $\pm$ 5.90 (62.0) ^d	11.8 $\pm$ 2.07 (54.6) ^d

^a BALB/c mice were sensitized and challenged with ovalbumin as described in the section of **Materials and methods**. MMP levels in the bronchoalveolar lavage fluid and lung tissues were determined by ELISA ($n = 8$). The symbols (A–F) are defined in Table 1. The data for the vehicle (DMSO) alone are not shown (2, 6 or 20 μ l of DMSO) because there were no significant effects. Parentheses designate a decreasing percentage evoked by celastrol treatment. Data are expressed as the mean $\pm$ S.E. ($n = 8$).

^b $P < 0.001$ versus control or general sensitized mice.

^c $P < 0.01$.

^d $P < 0.001$ versus ovalbumin-challenged mice.

Celastrol significantly reduced the production of cytokines in bronchoalveolar lavage fluid and lung tissues versus in ovalbumin-challenged mice (group A) (Table 2). In contrast, the vehicle alone (high dose DMSO) instead of celastrol had no effect on mRNA (Fig. 3-I) and protein expression of cytokines in ovalbumin-challenged mice (data not shown).

3.3. Effect of celastrol on airway hyperresponsiveness to methacholine

The findings presented in Fig. 1 and Table 1 suggest that celastrol has anti-allergic activity. Therefore, we examined the effect of celastrol on airway hyperresponsiveness since this is the most common symptom in asthmatic disorders. Airway hyperresponsiveness was assessed by changes in the airway resistance (R_L) and lung compliance (C_L) in response to increasing doses of methacholine administration. The R_L of ovalbumin-challenged mice was dramatically increased by methacholine administration compared to that of negative control mice (Fig. 3, upper panel). However, this methacholine-induced increase of R_L in ovalbumin-challenged mice was effectively reduced by celastrol (30 or 100 mg/kg) pretreatment. The maximum effect seemed to be attained at 100 mg/kg celastrol. The C_L was dramatically decreased in ovalbumin-challenged mice compared to in negative control mice. This decrease was also effectively prevented by celastrol (Fig. 3, lower panel). The administration of a vehicle (high dose DMSO) alone instead of celastrol did not affect the airway hyperresponsiveness.

3.4. Activities, proteins and mRNA of MMPs and TIMPs

MMPs, the extracellular matrix (ECM)-degrading enzymes, are up-regulated, and TIMPs are down-regulated in areas of inflammatory cell

accumulation and lung tissue remodeling, respectively (McMillan et al., 2004). Therefore, we examined the activity and protein levels of constitutive MMP-2 and inducible MMP-9 in the bronchoalveolar lavage fluid and lung tissues of ovalbumin-challenged mice. Celastrol administration reduced the gelatinolytic activities (Fig. 4, upper panel) in bronchoalveolar lavage fluid, and reduced the protein levels of MMP-2 and MMP-9 that are normally increased in the bronchoalveolar lavage fluids and lung tissues of ovalbumin-challenged mice (Table 3).

The mRNA expression of MMPs and TIMPs was examined in the bronchoalveolar lavage cells (Fig. 4, lower-left panel) and lung tissues (Fig. 4, lower-right panel). The mRNA levels of MMP-2 and MMP-9 were increased and levels of TIMP-1 and TIMP-2 were decreased in ovalbumin-challenged mice. Celastrol reduced MMP-2 and MMP-9 mRNA expression in a dose-dependent manner, but restored TIMP-1 and TIMP-2 mRNA expression in the bronchoalveolar lavage cells and lung tissues of ovalbumin-challenged mice (Fig. 4, lower panel). The administration of a vehicle alone (high dose DMSO) instead of celastrol did not affect the activity (Fig. 4, upper panel-I) and protein levels or mRNA expression level of MMPs and TIMPs in ovalbumin-challenged mice (Fig. 4-I).

3.5. Activities of MAP kinases

We examined the phosphorylation of the MAP kinases, which play a major role in the pathogenesis of allergic asthma remodeling (Blease, 2005), in the bronchoalveolar lavage cells and lung tissues of ovalbumin-challenged allergic mice. Celastrol inhibited the increased activities of phosphorylated MAP kinases in the bronchoalveolar lavage cells (Fig. 5, upper-left panel) and lung tissues (Fig. 5 upper-

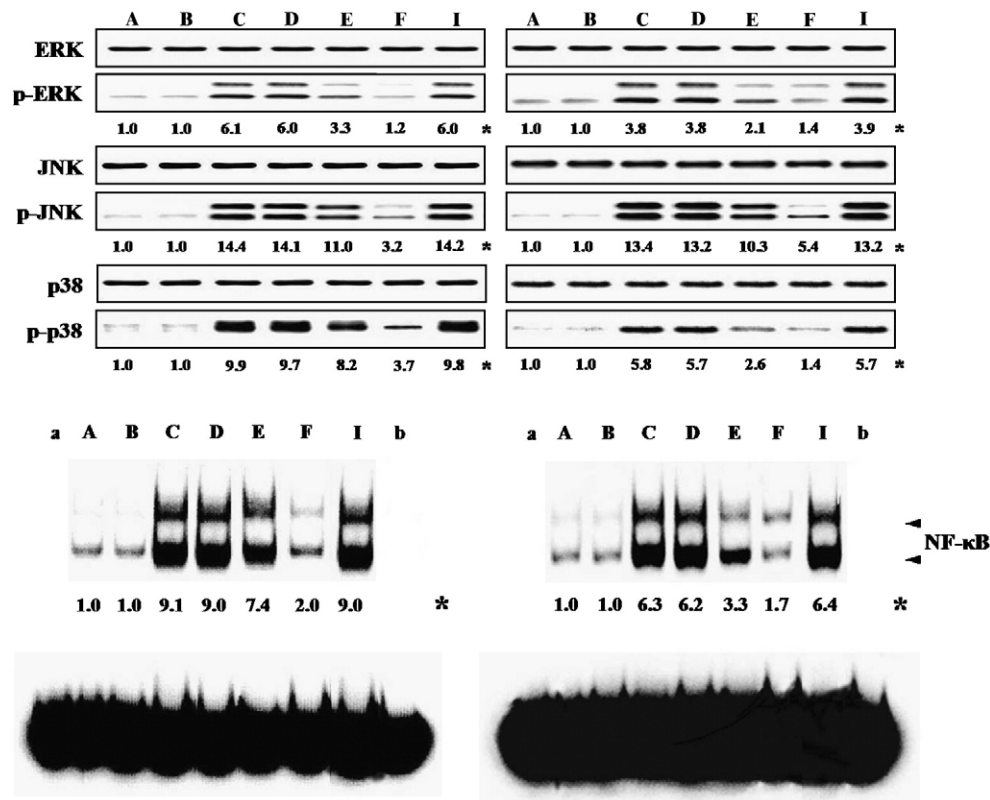


Fig. 5. The effect of celastrol on the activation of MAP kinases and NF- κ B in the bronchoalveolar lavage cells and lung tissues of ovalbumin-challenged allergic mice. BALB/c mice were sensitized and challenged with ovalbumin as described in the section of Materials and methods. The MAP kinase (upper panel) activities were determined in protein extracts prepared from bronchoalveolar lavage cells (left panel) and lung tissues (right panel) of ovalbumin-challenged mice by western blot. NF- κ B activities (lower panel) were assayed in nuclear extracts collected from bronchoalveolar lavage cells (left panel) and lung tissues (right panel) by the EMSA method ($n=4$), as described in the section of Materials and methods. a, negative control; b, competition assay. The symbols (A–F, I) are defined in Table 1. *, Numbers below the bands are the ratio of band density of each group versus that of negative control and each G protein or total protein. The bands for blots and EMSA are representative of four independent experiments ($n=4$).

right panel). The administration of a vehicle alone (high dose DMSO) instead of celastrol did not affect MAP kinase activity (Fig. 5-I).

3.6. Activity of NF- κ B or activator protein (AP)-1

We examined whether the activities of transcription factors NF- κ B and AP-1 were affected by celastrol using the electrophoretic mobility shift assay (EMSA) method. Nuclear extracts from bronchoalveolar lavage cells (Fig. 5, lower-left panel) and lung tissues (Fig. 5, lower-right panel) of ovalbumin-challenged mice showed increased NF- κ B DNA binding activity versus nuclear extracts from control mice or general sensitized mice. Celastrol administration inhibited these activities (Fig. 5D, E, and F). However, AP-1 transcription factor activity was not detected in any bronchoalveolar lavage cells and lung tissues (data not shown). The administration of a vehicle alone (high dose DMSO) instead of celastrol did not affect the activity of NF- κ B (Fig. 5-I).

4. Discussion

Allergic asthma is a complex inflammatory disorder characterized by several pathologic symptoms. Further, the drug development process is challenging because of the limited understanding of molecular pathogenesis of allergic asthma. Recently, celastrol has shown promise as an anti-inflammatory compound in animal models for asthma and in a few human diseases (Liu et al., 2004; Tao et al., 2002). However, the anti-asthmatic mechanism has not yet been investigated. Therefore, in this study, we obtained evidence of the anti-asthmatic activity of celastrol. Our data suggest that celastrol regulates MMP-2/-9 and TIMP-1/-2 production, and production of cytokines in relation to airway tissue remodeling via MAP kinases and NF- κ B, suggesting that celastrol has potential as a therapeutic agent in the oral treatment of allergic asthma.

The main goals of asthma treatment are to prevent bronchoconstriction, to decrease inflammatory reaction and to prevent lung tissue remodeling. The results obtained in this study indicate that these primary manifestations of allergic asthma are effectively inhibited by celastrol.

Th2-inflammatory cytokines such as interleukin-4 (IL-4), IL-5, IL-9 and IL-13 are increased in lung tissues of asthmatic human (Zimmermann et al., 2003) and animals (Kim et al., 2007a; Secor et al., 2008; Zosky et al., 2008). Th1-cytokines (TNF- α , IFN- γ) also cause early phase asthma (Renauld, 2001) and affect asthma severity (Cho et al., 2005; Kim et al., 2007b). Th1 and Th2 lymphocytes and the cytokines that they produce are key players in allergic asthma. Activation of the transcription factor NF- κ B is also linked to the expression of various proteins, including cytokines (Kim et al., 2007a; Penido et al., 2006). Celastrol has been shown to inhibit pro-inflammatory cytokines (Calixto et al., 2004) and NF- κ B activity (Lee et al., 2006) in other studies, as well as in our study (Figs. 2 and 5; Table 2, lower panel). Thus, it is expected that the inhibition of inflammatory action by celastrol (Table 1; Fig. 1) was caused by the suppression of the functional linkage, including NF- κ B and inflammatory cytokines.

Allergen-induced airway hyperresponsiveness is also regarded as a manifestation of a Th2-dependent allergic disorder. Th2-associated IL-4, IL-5, IL-9, and IL-13 are believed to be the key cytokines involved in the induction of airway hyperresponsiveness, lung eosinophil infiltration and mucus secretion (Nakae et al., 2007). Based on the finding of this study, it can be inferred that the inhibition of Th-2-associated cytokines by celastrol suppresses the airway hyperresponsiveness (Fig. 3), eosinophil infiltration (Table 1; Fig. 1, upper panel), and mucus secretion (Fig. 1, lower panel).

Airway tissue remodeling is thought to result from chronic repetitive injury to the airway wall caused by airway inflammation. It is characterized by increased goblet cell hyperplasia and the

production of matrix metalloproteinases (MMPs) (Locke et al., 2007; Wills-Karp and Karp, 2004). MMPs are also produced via various cytokines such as TNF- α , IL-1b, IL-13 and IL-17 (Abel and Vliagoftis, 2008; Bond et al., 1998; Girolamo et al., 2006; Sergejeva et al., 2005; Zheng et al., 2000) and are associated with tissue remodeling and processes of cellular infiltration to inflammatory sites (Kumagai et al., 1999). Overexpression and activation of MMPs or an imbalance in active MMPs and TIMPs has also been linked with a number of specific diseases including allergic asthma remodeling, due to their ability to cleave all proteins that contribute to the ECM (Kimata et al., 2006). Recently, it was reported that MMPs are up-regulated in the airway and serum of patients with asthmatic mucus hypersecretion (Ko et al., 2005) and by regulated inflammatory cytokines (Girolamo et al., 2006). MMP9 deficiency results in enhanced antigen-induced allergic asthma (McMillan et al., 2004), and alveolar macrophage-derived metalloproteinases and proteinases in the sputum lead to tissue remodeling in asthma (Hur et al., 2008; Ko et al., 2005). These reports support our results that celastrol controls goblet cell hyperplasia (Fig. 1, lower panel) in bronchial epithelium and the imbalance of MMP2/9 and TIMP1/2 in the bronchoalveolar lavage and lung tissues (Fig. 4; Tables 2 and 3). Therefore, it can be inferred that celastrol reduces airway tissue remodeling and cellular infiltration due to the regulation of MMPs and TIMPs by cytokines such as TNF- α and IL-13, etc.

MAP kinases have been reported to regulate the expression of inflammatory cytokines (Lee et al., 2004; Ray et al., 2007; Venuprasad et al., 2006) and MMPs (Liang et al., 2007). NF- κ B is also involved in the expression of pro-inflammatory cytokines (Kim et al., 2007a; Penido et al., 2006), and MMP-2/-9 and TIMP-1/-2 (Blease, 2005). These reports further support our findings that celastrol-induced inhibition of MAP kinases (Fig. 5, upper panel) and NF- κ B (Fig. 5, lower panel) inhibits the production of inflammatory cytokines and serves as a mechanism of its anti-allergic and anti-asthmatic activity.

In conclusion, we have demonstrated that celastrol effectively suppresses airway hyperresponsiveness and tissue remodeling by regulating the imbalance of MMP-2/-9 and TIMP-1/-2 that is induced by inflammatory cytokines produced via MAP kinases and NF- κ B in ovalbumin-induced allergic mice.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.03.078.

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