

Inhaled KMUP-1 Prevents Allergic Pulmonary Vascular Inflammation and Remodeling via NO and Suppressed MMP-9 and ICAM-1/VCAM-1

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Abstract: *Aims:* This study determines whether KMUP-1 inhalation suppresses ovalbumine (OVA)-sensitized and -challenged peri-bronchial vascular inflammation and remodeling in mice.

Methods and Results: After short-term KMUP-1 (1-5 mM, 30 min)-nebulization and L-NAME (12 mM, 15 min)-pretreatment, endothelial nitric oxide synthase (eNOS) and matrix metalloproteinases-9 (MMP-9) expression in lung were measured by Western blotting analysis. In 28-days experiment, mice were sensitized with intraperitoneal OVA on day 1 and day 8, challenged with OVA nebulization and treated with KMUP-1 nebulization (5 mM, 30 mins) on day 21-27. Expression of eNOS, inducible nitric oxide synthase (iNOS), soluble guanylyl cyclase (sGC), protein kinase G (PKG), MMP-9, VCAM-1 and ICAM-1 were measured by Western blotting analysis. eNOS- and MMP-9-immunostaining were used for peri-vascular or peri-bronchial localization. Hematoxylin and eosin staining was used to show the vascular and bronchial wall thickness and infiltration of inflammatory cells. Cell counting and measurement of NO metabolite (NOx) in bronchoalveolar lavage fluid (BALF) were used to examine the NO production. KMUP-1 increased eNOS and decreased MMP-9 expression. L-NAME-pretreatment reversed these changes. KMUP-1 reduced OVA-sensitized vascular and bronchial wall thickening, eNOS-immunostaining at the alveolar septa, MMP-9-immunostaining in the bronchioles and infiltrated inflammatory cells in the peri-vascular and peri-bronchiolar regions. The OVA-sensitized decrease of sGC and PKG and increase of iNOS, ICAM-1/VCAM-1 and plasma cytokines IL-5/IL-13 were reversed; cell count, NOx and MMP-9-activity in BALF were decreased by KMUP-1.

Conclusions: Inhaled KMUP-1, preventing allergic pulmonary vascular inflammation and remodeling, would be useful for the treatment of asthma and respiratory obstruction disease.

Keywords: Adhesion molecules, allergen, cytokines, MMP-9, NO donor inhalation, pulmonary vascular inflammation.

1. INTRODUCTION

This study investigated ovalbumine (OVA)-sensitized and -challenged peri-bronchial vascular inflammation and remodeling and associated protein expression with and without KMUP-1 inhalation in mouse lung tissues. Bringing to our notice, chronic obstructive pulmonary disease (COPD), a chronic inflammatory lung disease associated with progressive airflow obstruction, is characterized by significant changes in peri-bronchial blood vessels which contribute to pulmonary vascular remodeling [1]. Inflammatory processes are prominent in COPD and hypoxic pulmonary artery hypertension (PAH) and are increasingly recognized as major pathologic components of pulmonary vascular remodeling [2]. Despite the clinical and functional consequences of peri-bronchial micro-vascular remodeling in asthmatic lungs, up to now, there is little data on therapeutic approaches to this phenomenon [3, 4].

Anti-inflammatory therapies in the airway are rather ineffective for improving chronic symptoms and reducing inflammation, or reversing the lung function decline and airway remodeling that accompanied a less function in pulmonary vascular system. Specific drug directed against vascular remodeling and chronic inflammation are needed to prevent lung tissue damage and progressive lung function decline [1, 3, 4].

Endothelial and epithelial nitric oxide synthase (eNOS)/guanosine 3',5'-cyclic monophosphate (cGMP)-signaling is involved in the control of lung function and cell growth. Rapid release of nitric oxide (NO) from the endothelium and epithelium may influence adjacent smooth muscle cell contractility and growth. Endogenous NO produced by activation of eNOS can act as a signaling molecule in several processes, including regulation of pulmonary vascular and airway smooth muscle tone [5]. Overexpression of eNOS suppresses the features of allergic asthma, originally characterized by expression of inflammatory inducible nitric oxide synthase (iNOS) and exhalation of large NO from airway [6]. In addition, bronchial constriction can cause a pulmonary hypoxic state,

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reducing vascular endothelial eNOS and decreased vascular density [7].

Matrix metalloproteinases (MMPs) are a large family of endopeptidases that degrade components of the extracellular matrix in vascular and airway system. Of the MMPs family, MMP-9 is involved in extracellular matrix turnover because of its ability to cleave proteins constituting the extracellular matrix. Notably, MMP-9 induces migration of eosinophils, lymphocytes and neutrophils across basement membranes in response to inflammatory mediators [8, 9].

Very late antigen-4 (VLA-4), a counter-receptor of vascular cell adhesion molecule 1 (VCAM-1) on endothelium, plays an important role in the migration of eosinophils and lymphocytes to inflammation sites [10]. Lymphocyte function associated antigen (LFA-1) on neutrophils and monocytes interacts with intercellular-associated molecule (ICAM-1), a counter receptor for LFA-1 on endothelium in inflamed areas [11]. Inhibition of VCAM-1/ICAM-1 expression has the potential to interfere with migration of eosinophils and lymphocytes. In this regard, MMP inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression [12, 13].

Sildenafil has shown anti-inflammatory action against COPD and hypoxic PAH, including reducing airway hyper-reactivity, leukocyte influx and elevated NO; its effectiveness was confirmed by measuring elevated S-nitroso-N-acetylpenicillamine-induced cGMP generation [14, 15]. The benefits of sildenafil encouraged us to search for an even more effective eNOS/cGMP-enhancer to inhibit COPD and hypoxic PAH.

Previously, xanthine-based KMUP-1 (Fig. 1A) has been proved to relax vascular and airway smooth muscle contractions by enhancing eNOS/cGMP-pathway, including increasing soluble guanylyl cyclase $\alpha 1$ (sGC $\alpha 1$) and protein kinase G (PKG) expression [16, 17]. In contrast, inhibition of MMP-9 by sildenafil improves the hemodynamics during acute pulmonary embolism [18]. Whether OVA-sensitized and -challenged pulmonary vascular inflammation and remodeling is suppressed by activation of eNOS/cGMP pathway and inhibition of MMP-9 expression remains to be investigated. The objective of this study was to determine if inhaled KMUP-1 can prevent asthmatic peri-bronchial vascular inflammatory obstruction in lung tissues.

2. EXPERIMENTAL PROCEDURES

2.1. Animals

Care and use of experimental animals (BALB/c mice) followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, 1996). The experimental protocol was approved by the animal center of Kaohsiung Medical University in Taiwan (Fig. 1B).

2.2. Short Term Nebulization

Short-term KMUP-1 (5 mM, 30 min) nebulization and pretreatment with L-NAME (12 mM, 15 mins) were accomplished using a previously described method [14]. Aerosols were generated by ultrasonic nebulizer from day 21

to 27. Mice were sacrificed on day 28 [19]. Three different experiments were performed on cohorts of 7~14 mice per experimental condition.

2.3. Allergen-Sensitization, Challenge and Treatment

In this long-term experiment, 6–8 week old BALB/c female mice were sensitized on days 1 and 8 by intraperitoneal injection (i.p.) of OVA (10 μ g) (Sigma-Aldrich, Schnelldorf, Germany) adsorbed to aluminum hydroxide (2 mg) (AlumInject; Perbio, Erembodegem, Belgium). Mice were divided into three groups. In Group 1, a control group was only exposed to saline nebulization by aerosol. The other 2 groups were subjected to OVA (1%) nebulization. Before each challenge with OVA, mice were either exposed to saline nebulization (Group 2) or KMUP-1 nebulization (5 mM) by aerosol (Group 3).

2.4. Bronchoalveolar Lavage and Cell Counts

After the sacrifice of mice, a cannula was placed in the trachea for bronchoalveolar lavage by gentle manual aspiration, using cold PBS-EDTA (0.05 mM, 1 ml) (Calbiochem, Darmstadt, Germany) as previously described [19]. The bronchoalveolar lavage fluid (BALF) was centrifuged at 4 °C for 10 min (1200 rpm). The supernatant was frozen at 80 °C for protein assessment and the cell pellet was resuspended in PBS-EDTA (0.05 mM, 1ml). Total cell counts were performed manually using a thoma chamber. The differential cell counts were performed using morphologic criteria on cytocentrifuged preparations (Cytospin) after staining with Diff-Quick (Dade, Belgium). Differential cell counts were performed by one blinded observer unaware of the experimental treatment.

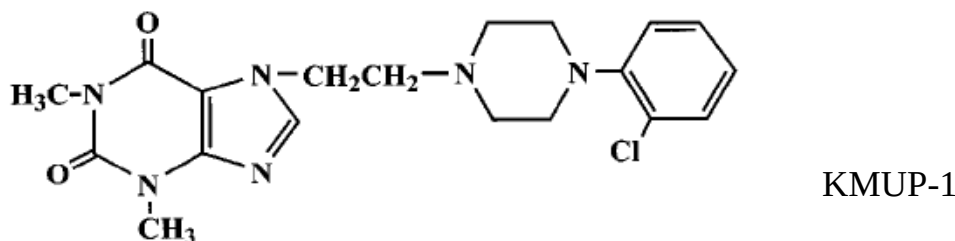
2.5. Zymography

The gelatinolytic activity of MMP-9 was measured by gelatin zymography as described previously [19]. Briefly, BALF (10 μ l) was subjected to electrophoresis in 10% polyacrylamide gel containing gelatin (1 mg/ml). The gel was washed in 2.5% Triton X-100 to permit re-naturation of gelatinases and stained with Coomassie blue after overnight incubation. Destaining visualized clear zones of lysis against a blue background indicated the gelatinase activity [20].

2.6. Western Blotting Analysis

The lung tissues were homogenized, washed with PBS and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. A Western blot analysis was performed as described previously [16]. The samples were loaded to 10% SDS-PAGE gels and were separated at 120 V for 90 min. The blots were incubated with anti-eNOS antibody diluted at a ratio of 1:500, anti-sGC $\alpha 1$ antibody diluted at a ratio of 1:2000, anti-PKG antibody diluted at a ratio of 1:500, anti-VCAM-1 antibody diluted at a ratio of 1:500, anti-ICAM-1 antibody diluted at a ratio of 1:500, and anti-MMP-9 antibody diluted at a ratio of 1:600 overnight at 4 °C. The membranes were stripped and re-blotted with β anti-actin antibody (Sigma, St. Louis, MO) to verify the equal loading of protein in each lane.

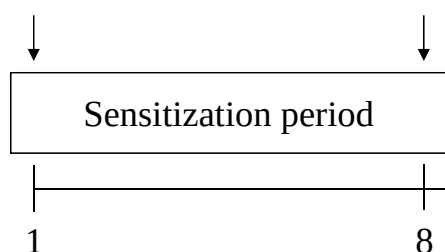
(A)



(B)

Intra-peritoneal injection

Control: Saline

Sensitization: OVA + Al (OH)₃**Nebulization**

Control : Saline

Challenge : OVA

Treatment: KMUP-1 + OVA

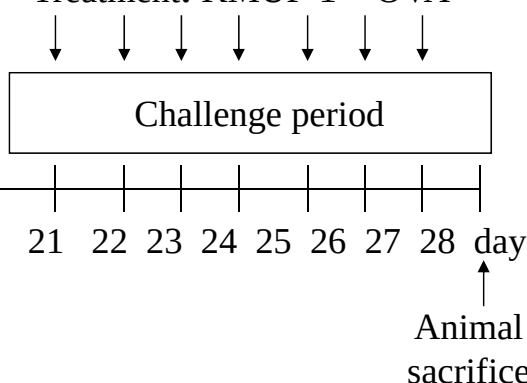


Fig. (1). Chemical structure of KMUP-1 (A) and experimental protocol (B). OVA was intraperitoneally injected on days 1 and 8. OVA (1%) or OVA (1%)+KMUP-1 was nebulized into mice airway on days 21~27 and mice were sacrificed on day 28.

2.7. NO Metabolites (NO_x)

The remaining BALF was centrifuged (900 g, 1200 rpm, 10 min) and the supernatant frozen (-80 °C) until determination of nitrite and nitrate by the Griess reaction [21]. BALF (100 μL) was incubated (37 °C) for 30 min with N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid buffer (HEPES) (50 mM, pH 7.4), flavin adenine dinucleotide (5 μM), nicotinamide adenine dinucleotide phosphate (0.1 mM), distilled water (290 μL), and nitrate reductase (0.2 U/ml) for the conversion of nitrate into nitrite. The nitrate reductase was absent for nitrite determination. Unreacted nicotinamide adenine dinucleotide phosphate was oxidized by incubating (25 °C, 10 min) with potassium ferricyanide (1 mM). The samples were then incubated (25 °C, 10 min) with Griess reagent [N-(1-naphthyl)-ethylenediamine: 0.2% (w/v), sulphanilamide: 2% (w/v), solubilized in double-distilled water: 95% and phosphoric acid: 5% (v/v)] and the absorbance measured at 540 nm. The levels of nitrite in the BALF were calibrated and compared to a sodium nitrite (0~150 μL) standard curve.

2.8. Histologic and Morphologic Analyses

Sections of fixed embedded tissues were cut at 4 μm, placed on glass slides, deparaffinized and stained sequentially with hematoxylin 2 and eosin-Y (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) [22]. To confirm the relationship between vascular inflammation/remodeling and expression of proteins, lung tissue sections from OVA experiments were prepared and stained with hematoxylin-eosin as previously described [23]. Microphotography analysis of blood vessels and bronchioles, focusing on peri-bronchial and peri-vascular region in the lung sections, was performed using a color digital camera mounted on a computer-interfaced light microscope (Eclipse TE2000-S microscope, Nikon, Tokyo, Japan).

2.9. Vascular and Bronchial Wall Thickening

The thickness of vascular and bronchial walls was measured as the distance between the external and internal

elastic laminae of each artery or bronchiole and external diameter was measured as the diameter of the external lamina using a calibrated eyepiece micrometer. The percent wall thickness (%) of each artery or bronchiole was calculated using the following formula: Wall thickness (%) = $(2 \times \text{medial wall thickness}) / (\text{external diameter}) \times 100$. The average of six measurements of pulmonary artery or bronchiole in each animal was used in analysis.

2.10. eNOS- and MMP-9-Immunohistochemical Staining

Immunohistochemical analysis of eNOS enhancement by KMUP-1 was performed as following. The lungs were perfused with saline followed by 10% formalin and then placed in 10% formalin for paraffin embedding. The lung tissues were cut into 3~5 μm sections onto slides, dewaxed in 100% xylene, and then re-hydrated in graded alcohol solutions. Throughout the procedures, slides were washed as appropriate in PBS. Antigen retrieval was performed by microwave treatment for eNOS immunostaining. Sections were treated with 3% H_2O_2 (10 min) to inhibit endogenous peroxidases and incubated with normal serum (60 min) to reduce nonspecific binding of secondary antibodies. Sections were then incubated at 4°C overnight with eNOS antibodies. After washing off unbound primary antibodies, sections were incubated with secondary biotinylated antibodies against mouse or rabbit (DAKO Co., Japan) (60 min), followed by incubation in avidin/biotin/horseradish peroxidase complex (DAKO Co., Japan) (30 min). Subsequently, peroxidase activity was visualized by incubation (3 min) with 0.05% 3,3'-diaminobenzidine (DAKO Co., Japan), which gives a brown reaction product. The reaction was stopped by washing with water. The slides were counterstained with hematoxylin. For immunostaining of MMP-9 in lung tissue, the de-paraffinized 3~5 μm sections of lung tissue was performed as above eNOS, but using MMP-9 antibody.

2.11. Measurement of Cytokines

To confirm whether allergen-sensitized and challenged mice is involved in the response to immunologic activation, we measured the plasma cytokines IL-5 and IL-13 of mice and treatment with KMUP-1 by ELISAS techniques, according to the manufacturer's guidance.

2.12. Compounds

Anti-eNOS antibody was obtained from BD Biotechnology (New York, USA); anti-PKG; anti-VCAM and anti-ICAM-1 antibodies from Santa Cruz (CA, USA); anti-sGC α 1, anti-MMP-9, ROCKII (a representative of Rho kinase), iNOS, IL-5, IL-13, and β -actin antibodies from Sigma Chemical Co. Goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody was purchased from Santa Cruz (CA, USA). KMUP-1 (7-[2-[4-(2-chlorobenzene) piperazinyl] ethyl]-1, 3- dimethyl xanthine) was synthesized in our laboratory and whose salt form KMUP-1 $\cdot$ HCl was dissolved in vehicle and the serial dilutions was made in distilled water for the experiments of inhalation. All other reagents we used were of analytical grade or higher and were obtained from commercial sources.

2.13. Statistical Evaluation of Data

The experimental results of inhaled KMUP-1 were expressed as mean $\pm$ SE. Statistical differences were determined by independent and paired Student's *t*-test in unpaired and paired samples, respectively. Whenever a control group was compared with more than one treated group, one way ANOVA or two way repeated measures ANOVA was used. When the ANOVA showed a statistical difference, the Dunnett's or Student-Newman-Keuls test was applied. A *P* value less than 0.05 was considered significant in all experiments. Analysis of the data and plotting of the figures were done with the aid of SigmaPlot software (Version 8.0, Chicago, IL, USA) and SigmaStat (Version 2.03, Chicago, IL, USA) run on an IBM compatible computer.

3. RESULTS

3.1. Pulmonary e-NOS Enhancement and MMP-9 Suppression

In the short-term experiment without OVA sensitization, KMUP-1 (1.0~2.5 mM, 30 mins) dose-dependently increased eNOS and decreased MMP-9 expression in mice lung tissues; the changes of eNOS and MMP-9 expression by KMUP-1 (2.5 mM, 30 mins) were reversed by L-NAME-pretreatment (12 mM, 30 mins) (Fig. 2A-D; **P*<0.05 versus control; ***P*<0.01 versus control, ****P*<0.01 versus KMUP-1).

3.2. Pulmonary Vascular Wall Thickening Features

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells including eosinophils were infiltrated around the peri-vascular and peri-bronchial regions (Fig. 3B, E), compared to control mice (Fig. 3A, D). Mice treated with KMUP-1 showed marked reductions of inflammatory cells in the peri-vascular (Fig. 3C) and peri-bronchiolar regions (Fig. 3F). After OVA challenge, marked increases in PA wall thickness % were found, compared to controls (Fig. 3G, ****P*<0.01 versus control; ***P*<0.01 versus OVA).

3.3. Pulmonary eNOS, iNOS, sGC α 1, PKG, ICAM-1, VCAM-1 and MMP-9 Expression

In the 28-day experiment, Western blotting analysis showed that the levels of basal eNOS (A) expression were not affected by OVA, but iNOS (B) was increased and sGC α 1 (C), PKG (D), MMP-9 (E, F), ICAM-1 (G) and VCAM-1 (H) were decreased significantly at 24 hrs after the last OVA-challenge. The increased iNOS and MMP-9 and decreased sGC α 1 and PKG expressions by OVA sensitization were significantly reduced by nebulization of KMUP-1 (Fig. 4A-H; ****P*<0.01 versus control; ***P*<0.01 versus OVA). Both expression and its zymography of MMP-9 were decreased (Fig. 4F; ***P*<0.01 versus OVA).

3.4. Immunostaining

OVA decreased vascular eNOS-immunostaining in lung sections, but KMUP-1 prevented this decrease quantitatively

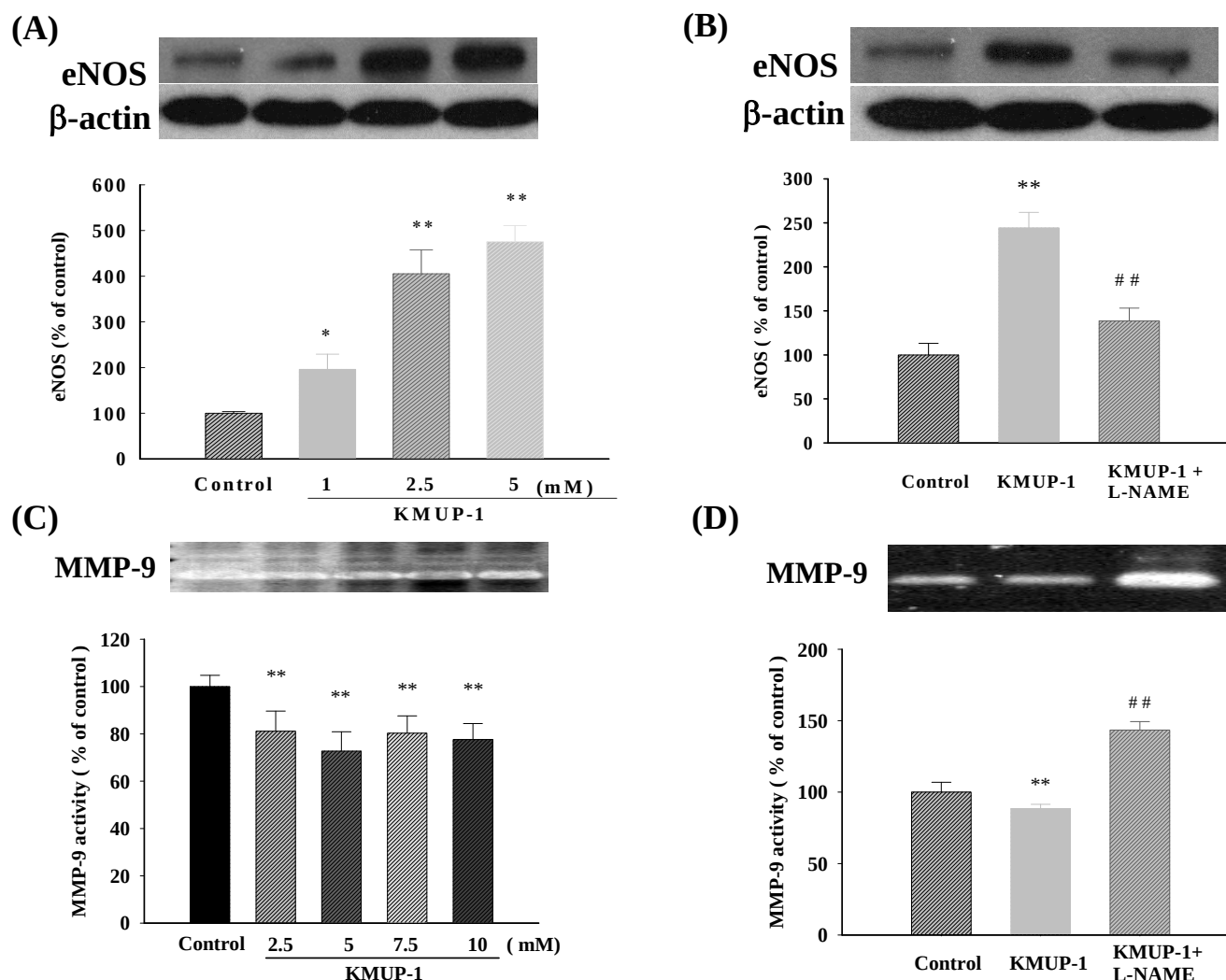


Fig. (2). Effects of short-term inhalation of KMUP-1 and L-NAME-pretreatment on eNOS and MMP-9 expression in lung tissues. Western blotting showed the expression of eNOS (A) was increased and zymography of MMP-9 (C) was decreased by KMUP-1 (1-5 mM, 30 min) after OVA challenge. L-NAME-pretreatment reversed eNOS (B) and MMP-9 (D) expression by KMUP-1 (2.5 mM, 30 min). Densitometric analyses presented the relative ratio of these proteins. The relative ratio of β -actin in the lung tissue of control is arbitrarily presented as 100%. Data represent the mean $\pm$ SE from 6 independent experiments. * P <0.05, ** P <0.01 versus control; ## P <0.01 versus KMUP-1.

(Fig. 5A-E). Immunostaining also indicated that MMP-9 was found on inflammatory cells and debris, filling the airway lumen (Fig. 5G). In control and OVA-sensitized and -challenged mice treated with KMUP-1, MMP-9 positive cells were not found and hardly detected, respectively (Fig. 5G, H).

3.5. NOx Levels and Inflammatory Cell Components

Griess reagent analysis showed that the basal levels of NOx ($65.4 \pm 8.2 \mu\text{M}$) in BALF were significantly increased to $162.8 \pm 15.7 \mu\text{M}$ by OVA at 24 hrs after the last challenge, compared with levels after saline nebulization. The increased levels of NOx (nitrate+nitrite) in BALF were significantly reduced by KMUP-1 to $86.3 \pm 17.3 \mu\text{M}$ (Fig. 6A; ** P <0.01 versus control; ** P <0.01 versus OVA).

The numbers of total cells, lymphocytes and eosinophils in BALF were significantly increased at 24 hrs after OVA

challenge. KMUP-1 nebulization significantly reduced the increase in total cells, lymphocytes and eosinophils elicited in the airway lumen 24 hrs after the last OVA challenge (Fig. 6B; ## P <0.01, versus control; ** P <0.01 versus OVA).

3.6. Plasma Cytokines

Fig. (7) showed that OVA increased IL-5 (A) and IL-13 (B), respectively and KMUP-1 inhibited IL-5 and IL-13 levels in the plasma of mice sensitized and challenged by OVA (** P <0.01 versus control; # P <0.05 versus OVA).

4. DISCUSSION

Pulmonary vascular inflammation and remodeling has been shown in the peri-bronchial blood vessels after OVA sensitization and challenge. KMUP-1 has displayed the expression of eNOS enhancement and suppression of MMP-

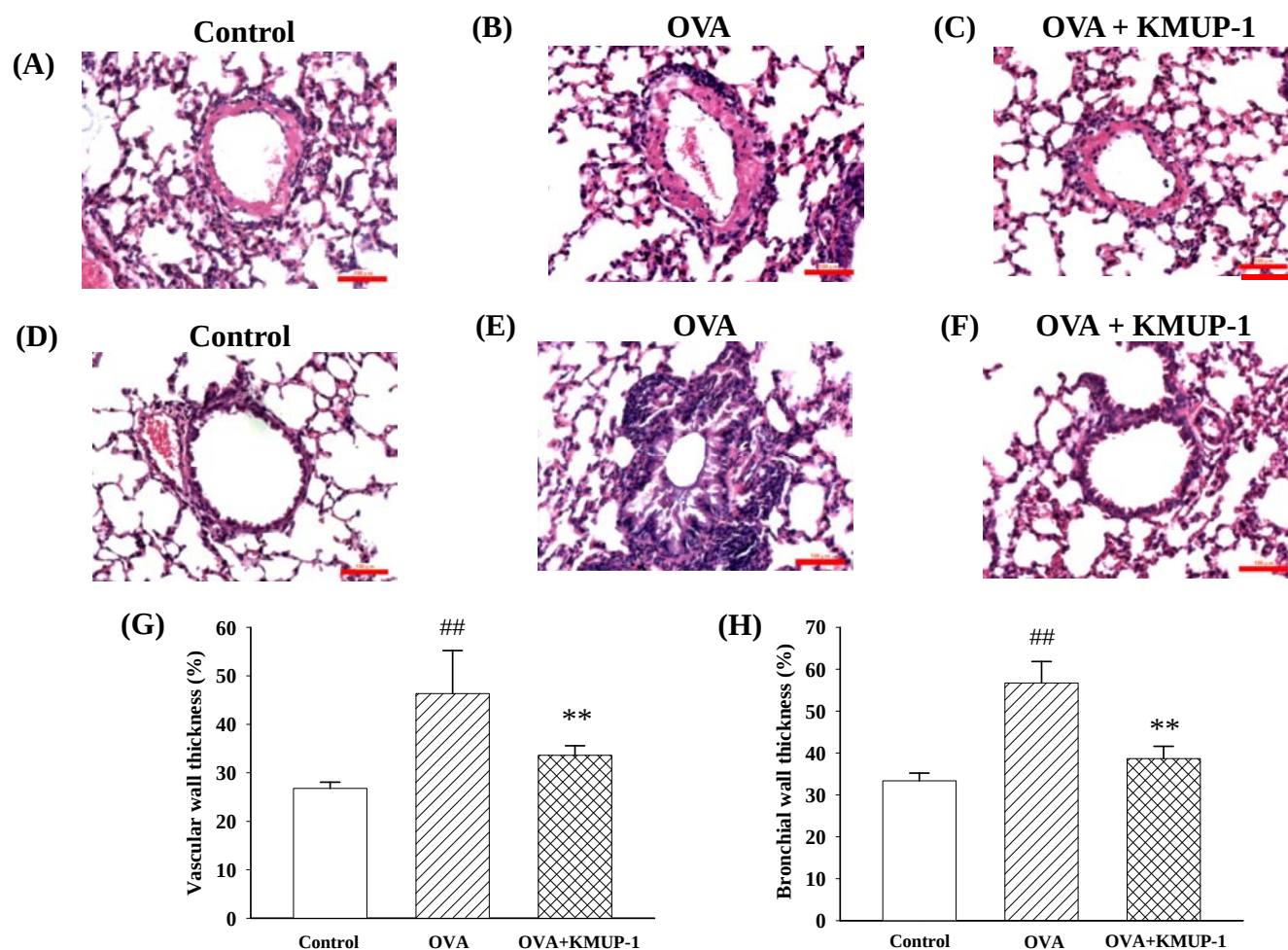


Fig. (3). Effects of KMUP-1 on OVA-sensitized and -challenged perivascular and peribronchial lung tissue inflammation. Hematoxylin and eosin staining of the peri-vascular and peri-bronchia regions showed numerous inflammatory cells, including eosinophils, around the vascular and bronchioles (**B, E**) compared to control mice (**A, D**). Histologically, mice treated with KMUP-1 (**C, F**) showed marked reductions in the infiltration of inflammatory cells in the peri-vascular (**A, B**) and peri-bronchiolar (**D, E**) regions. OVA-induced peri-vascular (**G**) and peri-bronchial (**H**) wall thickness were inhibited by KMUP-1. Grey bars indicate scale of 100 μ m. Data represent the mean $\pm$ SE from 6 independent experiments, involving control, exposure to OVA and treatment with OVA+KMUP-1. ## P <0.01 versus control; ** P <0.01 versus OVA.

9, iNOS and adhesion molecule in inhibiting OVA sensitized vascular and bronchial inflammation and remodeling. To characterize the pathophysiologic features of OVA-derived events in lung of peri-vascular and peri-bronchial area, we measured various protein expression, vascular and bronchial wall thickness and immunostaining, NO_x production, plasma cytokine levels and infiltrated cells count in BALF.

4.1. Vascular and Bronchial Wall Thickening and Immunostaining

Morphologically, cell accumulation by OVA sensitization and challenge in peri-vascular and peri-bronchial regions has created an inflammatory wall-thickening phenomenon. The muscularization or thickness of vascular and bronchial wall was measurable by micrographic analysis. OVA-sensitization increased the vascular and bronchial wall thickness, however, treatment with long-term

KMUP-1 nebulization prevented these changes. Enhanced eNOS-immunostaining on vascular endothelium indicates that eNOS is the target of KMUP-1 effect. MMP-9-immunostaining in peri-bronchial region and the reduced MMP-9-positive cells in bronchiole co-indicated the reduction of migration of infiltrated inflammatory cells.

4.2. Infiltration of Blood-Born Inflammatory Cells

Inflammation and remodeling of the bronchioles is associated with infiltration of blood-born lymphocytes, eosinophils and neutrophils, migrating into bronchial lumen. During the course of allergic disease, structural changes occur in the airways, referred to as remodeling. These changes include sub-epithelial fibrosis, increase of airway smooth muscle mass and excessive mucus secretion from hyperplastic goblet cells [24]. Allergic airway inflammation can evoke similar long-term structural alterations as those

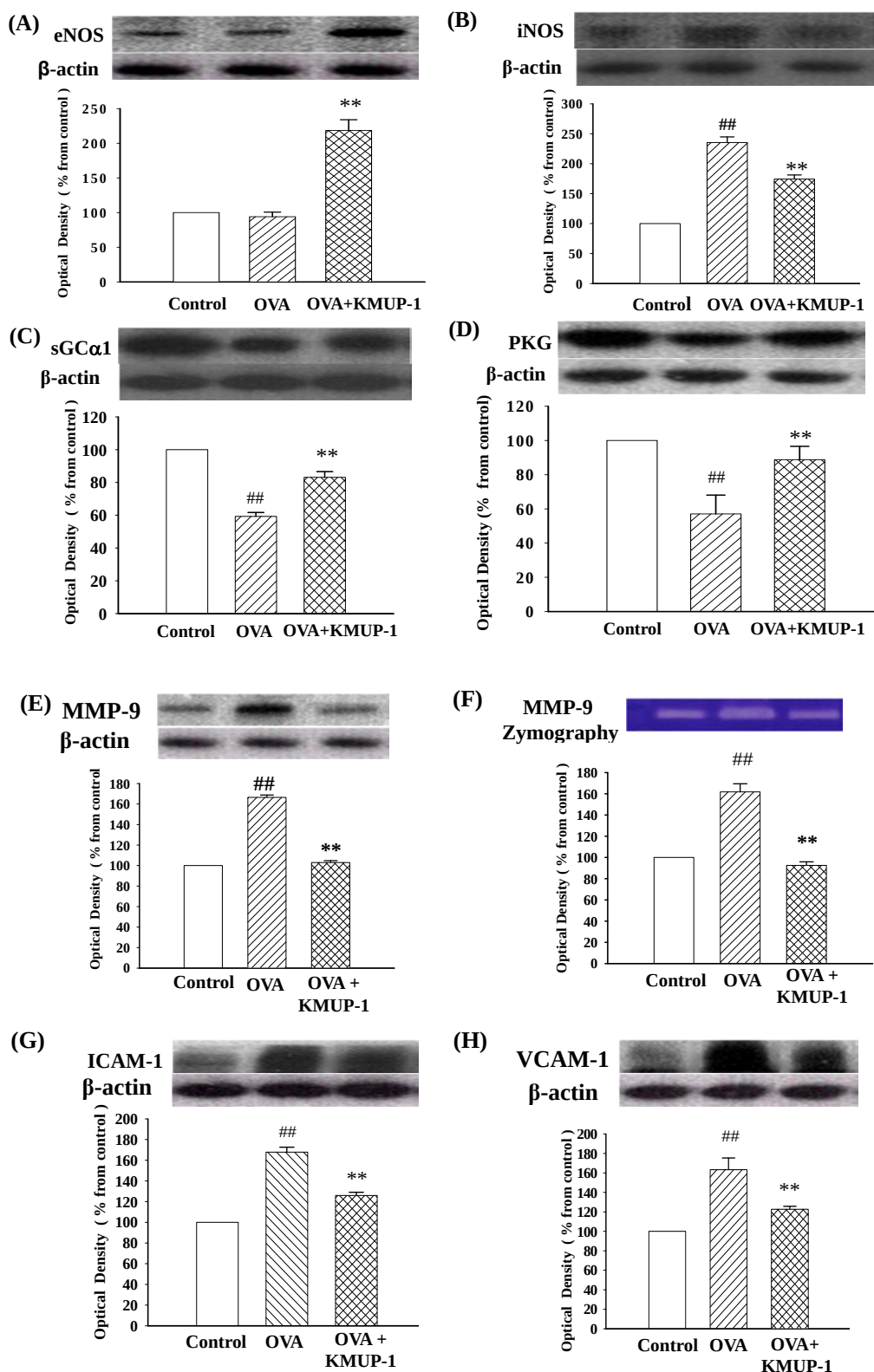


Fig. (4). Effects of KMUP-1 on OVA sensitized and challenged protein expression. Western blotting showed that the expression of eNOS (A) was unaffected, expression of iNOS (B), MMP-9 (E), ICAM-1 (G) and VCAM-1 (H) were increased, and expression of sGCβ1 (C) and PKG (D) in lung tissues were decreased significantly after OVA challenge, compared to saline nebulization control. Treatment with KMUP-1 (OVA+KMUP-1) reversed these changes caused by OVA. Zymography of MMP-9 expression (F) in BALF was also inhibited. Densitometric analyses presented the relative ratio of these proteins. The relative ratio of β-actin in the lung tissue of control mice is arbitrarily presented as 100%. Data represent the mean±SE from 6 independent experiments, involving control, exposure to OVA and treatment with OVA+KMUP-1. ##*P*<0.01 versus control; ***P*<0.01 versus OVA.

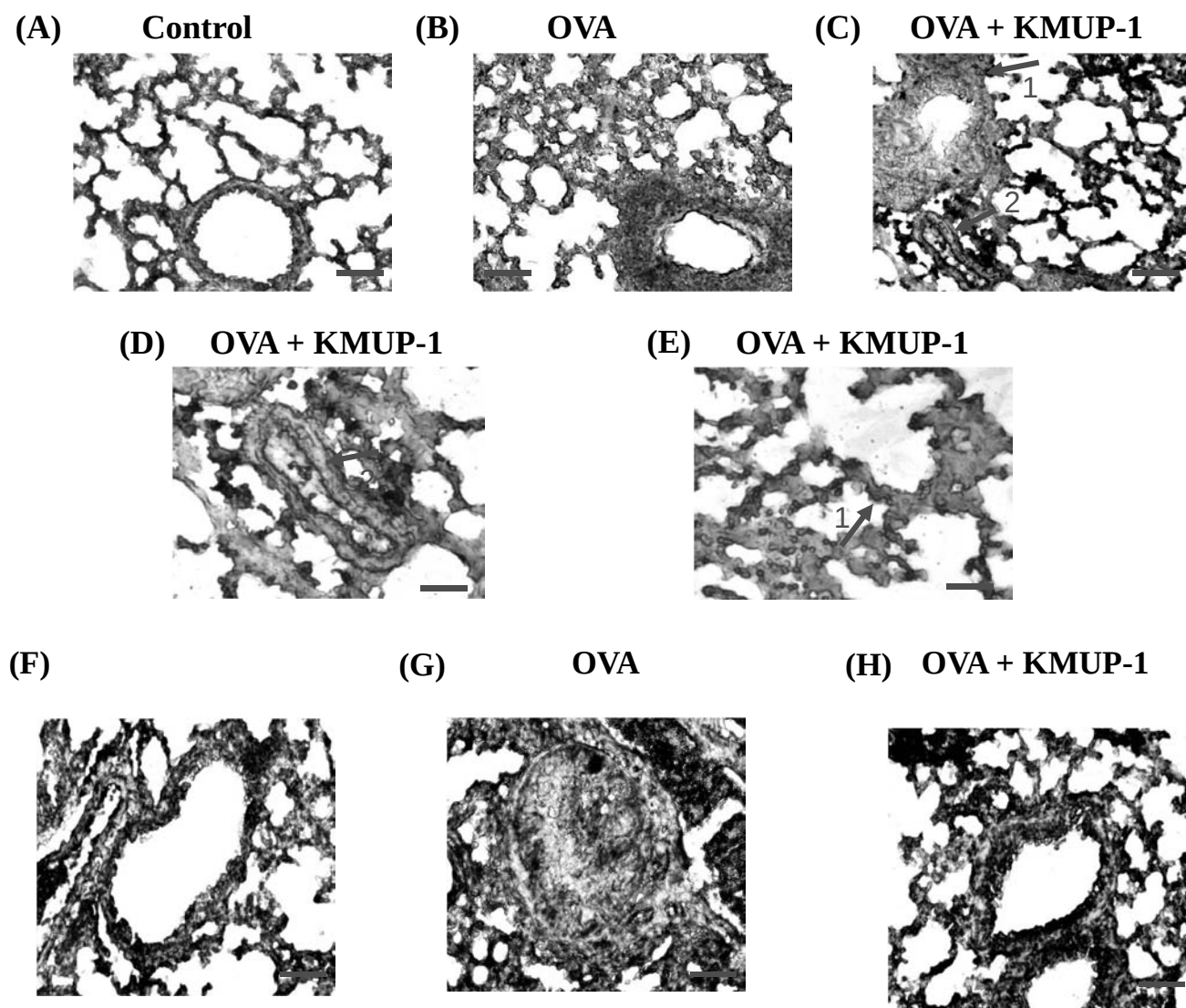


Fig. (5). Microphotographic demonstration of KMUP-1's effects on OVA-induced eNOS- and MMP-9-immunostaining in lung sections. (A) Lung section microphotography of mice inhaled with saline; Faint brown eNOS-immunostaining of the peri-bronchial region was found with hematoxylin and eosin; (B) bronchial remodeling and faint brown eNOS-immunostaining in peri-bronchial region; (C) eNOS-immunostaining is localized in the alveolar septa (grey arrow 1) and small blood vessel (grey arrow 2) after treatment with OVA+KMUP-1; (D) No eNOS-immunostaining signal was seen in cells from the bronchiole, while the endothelial cells from the small vessel, accompanying the bronchiole, shows the deep brown eNOS-immunostaining on vascular endothelium (grey arrow 2); (E): eNOS-immunostaining is present within the septa (grey arrow 1) after treatment with OVA+KMUP-1; (F): MMP-9-immunostaining was increased significantly 24 hrs after the last OVA challenge, compared to saline nebulization control. The increased MMP-9 expression by OVA sensitization (G) was significantly reduced by OVA+KMUP-1 (H). Original magnifications: 200 x (A, B, C) and 400 x (D, E). Experiments were performed at least three times and similar results were obtained. Grey bar indicates scale of 100 μ m.

seen in pulmonary blood vessels [25]. Airway remodeling is also due, at least in part, to an excess of extracellular matrix deposition in the airway wall, which leads to sub-epithelial collagen deposition [26]. However, treatment with KMUP-1 prevented the migration of blood born cells into bronchiole.

4.3. NOS/sGC/PKG Pathway

NOS was localized in a variety of cells of the respiratory tract, including endothelial, epithelial and inflammatory cells, providing the sources of NO production. In healthy situations, NO derived from eNOS activation seems to be

predominant, since low levels of NO produced by eNOS controls the airway smooth muscle tone [27].

In disease-like situations, treatment of cultured cells with inflammatory stimuli, including cytokine mixtures, leads to reduction in sGC mRNA levels. The above-mentioned cytokines have been shown to be increased in asthma and might contribute to the reduction of sGC. An additional pathway contributing to sGC down-regulation in asthmatic airway could be iNOS-derived NO; uncontrolled production of NO has been shown to decrease the mRNA stability of both the α 1 and the β 1 subunits of sGC [27]. sGC α 1 and PKG expressions were significantly reduced by OVA

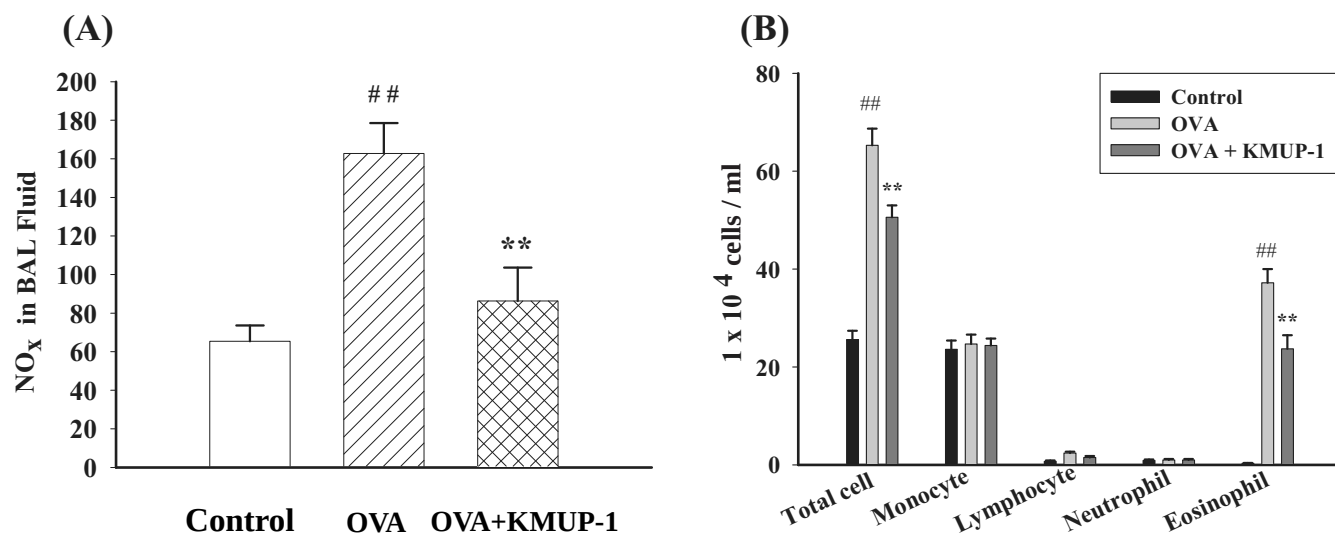


Fig. (6). Effects of KMUP-1 on NO_x levels and cellular components in BALF. Samples were taken 24 hrs after OVA challenge in mice treated with saline and KMUP-1. NO_x indicated the concentration of combined NO metabolites (mM) per 100 ml of BALF. The number of cellular components of BALF from control, OVA-sensitization or OVA+KMUP-1, including total cell, monocyte, lymphocyte, neutrophil and eosinophil, were counted at 24 hr after OVA challenge. Data represent the mean±SE from 6 independent experiments, involving control, exposure to OVA and treatment with OVA+KMUP-1; ^{##} *P*<0.01 versus control; ^{*} *P*<0.05, versus OVA.

sensitization but prevented by KMUP-1, creating an enhanced NO/sGC/PKG-dependent pathway [16, 17].

4.4. Leukocyte Migration and ICAM-1/VCAM-1

Cytokine-inducible leukocyte-endothelial adhesion molecules are important in the recruitment and migration of leukocytes to the sites of inflammation [28]. Cell adhesion molecules ICAM-1 and VCAM-1 are expressed on endothelial cells and are markedly up-regulated on subjects with asthma [28]. Previous studies have demonstrated that the production of matrix-degrading enzymes, such as MMP-9, is essential for leukocyte extravasation and recruitment to the affected sites [28].

MMP inhibitor regulates inflammatory cell migration by reducing expression of ICAM-1 and VCAM-1, leading to a reduction of cell migration through the endothelial basement membranes [29]. In the present study, KMUP-1 with MMP-9 suppressing activity was suggested to prevent the migration of leukocytes and eosinophils, entering at peri-bronchial region or even entering into BALF by reducing vascular ICAM-1 and VCAM-1.

Inhaled KMUP-1 can be absorbed into airway and blood flow to increase eNOS and down-regulate Rho-kinase expression by a cGMP-dependent pathway, protecting OVA-sensitized and challenged ICAM-1/VCAM-1 expression-associated cellular adhesion and migration activity of neutrophils [30-32].

4.5. MMP-9 vs eNOS

MMP-9 plays a role in chronic airway inflammation, including the induction of migration of eosinophils, lymphocytes and neutrophils across basement membranes, the infiltration of airways inflammatory cells and the

induction of airway hyper-responsiveness and remodeling in asthma [8, 33]. Inhibition of MMP-9 has been looked to as a good therapeutic strategy for asthma [34]. MMP-9-deficient mice in an allergen-challenged model demonstrated heightened inflammation and airway responsiveness, suggesting the role of MMP-9 in the resolution of lung injury after antigen challenge and a link to inflammation-induced tissue remodeling [35].

How endothelial eNOS regulates MMP-9 expression is less clear. However, NO and MMPs activation plays a role in flow-induced microvascular remodeling [36]. eNOS can come from the vascular endothelium and bronchial epithelium. Inhaled KMUP-1 rapidly enters at the airway epithelium and vascular endothelium and thus increases the expression of eNOS, which releases NO and causes a local accumulation of peroxynitrate, reducing OVA-induced increase of MMP-9 [16]. This is a reasonable explanation of why KMUP-1 can protect against OVA-sensitized MMP-9 expression. L-NAME-pretreatment can block KMUP-1-induced eNOS expression and MMP-9 suppression, indicating that eNOS is activated earlier than MMP-9 reduction or inactivation [37]. Thus, increased eNOS and restored sGC/PKG by KMUP-1 was suggested to prevent OVA-sensitized MMP-9 expression-associated infiltration of inflammatory cells and NO_x production.

4.6. Anti-Inflammation

MMP-9 and iNOS expression are induced by OVA sensitization. KMUP-1's anti-inflammatory activity is characterized by their inhibition. Theoretically, OVA sensitization-derived iNOS expression and KMUP-1's eNOS-enhancing activity can co-prevent MMP-9 expression by producing peroxynitrate [38-40]. However, early eNOS expression by KMUP-1 prevents cytokine-induced iNOS

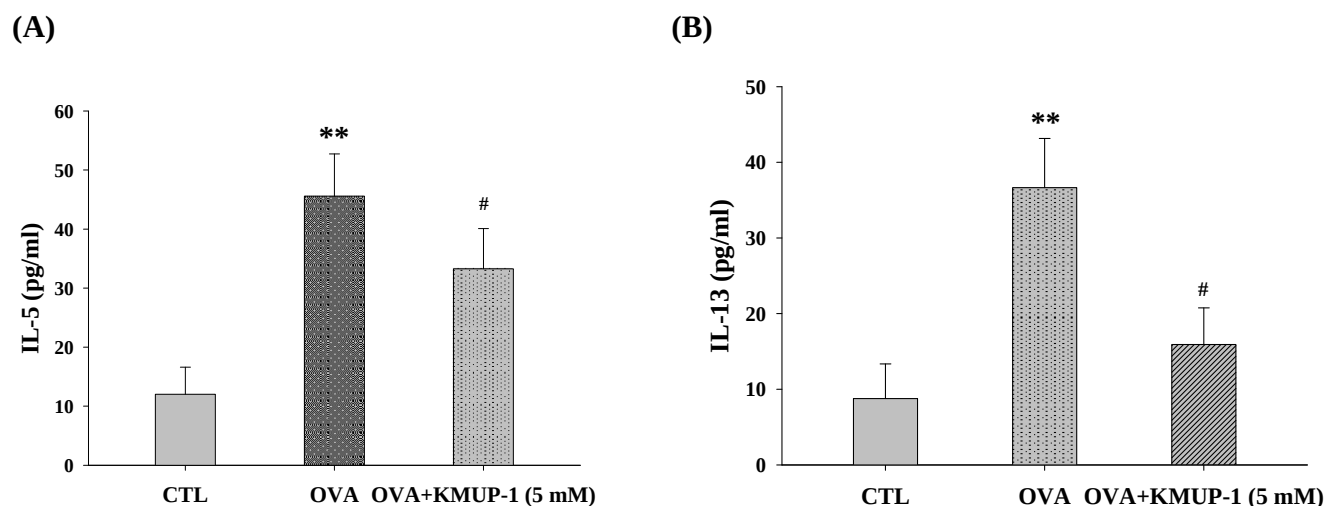


Fig. (7). Effects of KMUP-1 on OVA-sensitized and challenged mice plasma cytokines. IL-5 (A) and IL-13 (B) levels were measured by ELISA techniques. Data represents the mean $\pm$ SE from 3 independent experiments, involving control, exposure to OVA and treatment with OVA+KMUP-1. ** P <0.01 versus control; # P <0.05 versus OVA.

expression [16]. KMUP-1 decreased OVA-induced NOx in BALF, potentially reducing cytokine-induced iNOS expression in infiltrated inflammatory cells, but not in endothelial and epithelial cells [41]. This result is adaptable to the effect of overexpression of eNOS on eosinophilic lung inflammation in eNOS transgenic mice [42]. We presume that KMUP-1 enhances eNOS expression and stabilizes the aberrant sequestration of eNOS in endothelial and epithelial cells [43]. This is why KMUP-1 is favorable to prevent the late worsening of vascular and bronchial inflammation.

CONCLUSION

Inhaled KMUP-1 reduces allergic pulmonary vascular inflammation and remodeling by released NO and suppressed ICAM-1/VCAM-1 and MMP-9/iNOS. KMUP-1 inhalation displays a double anti-inflammation activity on vascular endothelium and bronchial epithelium via enhanced eNOS. KMUP-1 shows considerable promise for treating pulmonary vascular inflammation and remodeling to alleviate asthma, COPD and hypoxic PAH.

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ABBREVIATIONS

BALF	=	Bronchoalveolar lavage fluid
cGMP	=	Guanosine 3',5'-cyclic monophosphate
COPD	=	Chronic obstructive pulmonary disease
eNOS	=	Endothelial nitric oxide synthase
ICAM-1	=	Intercellular-associated molecule
iNOS	=	Inducible nitric oxide synthase
IL-5	=	Interleukin-5

IL-13	=	Interleukin 13
LFA-1	=	Lymphocyte function associated antigen
NOx	=	NO metabolite
PAH	=	Pulmonary artery hypertension
PKG	=	Protein kinase G
ROCKII	=	Rho kinase II
sGC α 1	=	Soluble guanylyl cyclase α 1
VCAM-1	=	Vascular cell adhesion molecule 1
VLA-4	=	Very late antigen-4

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