Roflumilast Ameliorates Obesity-induced Airway Hyper-responsiveness and Pulmonary Fibrosis in a Murine Model

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Directed by Professor Jung-Won Park

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This certifies that the Doctoral Dissertation of Hye Jung Park is approved.

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ABSTRACT

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Background: Obese asthma patients respond poorly to conventional asthma medications, resulting in severe symptoms and poor prognosis. Roflumilast, a phosphodiesterase-4 inhibitor that lowers the levels of various cytokines and reactive oxygen species (ROS) which are implicated in obese asthmatics, may be effective to treat obese asthmatics.

Objectives: We evaluated the potential of roflumilast as a novel therapeutic agent for obese asthmatics.

Methods: We designed three models (diet-induced obesity [DIO], DIO with ovalbumin [OVA], and OVA). We fed C57BL/6J mice a high-fat (60%) diet for 3 months with or without OVA sensitization and challenge. Roflumilast or dexamethasone was administered orally thrice at 2-day intervals at the last experimental week. Airway hyper-responsiveness (AHR), bronchoalveolar fluid (BALF), lung pathology, mRNA and protein levels of cytokines and adipokines, ROS levels, and T cell activation were evaluated.

Results: AHR resulting from DIO significantly improved in the roflumilast-treated group, compared with dexamethasone-treated groups. Although DIO did not affect the cell proliferation in BALF, increased fibrosis was seen in the DIO group, which significantly improved by the treatment of roflumilast. DIO-induced changes in adiponectin and leptin levels were improved by roflumilast, while dexamethasone aggravated them. Messenger RNA levels and proteins of tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, interleukin (IL)-1β, and interferon (IFN)- γ increased in the DIO group, and decreased by roflumilast. The ROS levels was also increased in the DIO group, and decreased by roflumilast. In the DIO-with-OVA and OVA models, roflumilast improved Th1 and Th2 cell activation to a greater extent than dexamethasone.

Conclusions: Roflumilast is significantly more effective than dexamethasone against AHR and fibrosis caused by DIO in the murine model, as roflumilast treatment led to improved levels of adiponectin, leptin, TNF-α, TGF-β, IL-1β, IFN- γ and ROS. Roflumilast may represent a promising therapeutic agent for the treatment of obese asthma patients.

Key words: Airway hyper-responsiveness, Asthma, Obesity, Phosphodiesterase-4 inhibitor, Roflumilast

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I. INTRODUCTION

Asthma is a common and potentially serious chronic airway disease, with manifestating cough, chest tightness, and dyspnea. Atopic asthma, or typical asthma, is always associated with "atopy," which means a genetic predisposition to the production of immunoglobulin E (IgE) antibodies in response to allergens. Atopic asthma is usually accompanied by eosinophilia in the blood and sputum. This type of asthma can be effectively treated using anti-inflammatory drugs including corticosteroids.[1]

Overweight and obesity are related to various chronic diseases, and also obesity is generally considered a risk factor for asthma. Several mechanisms explaining the association between obesity and asthma have been proposed, including the effects of obesity on the upregulation of immunologic and inflammatory conditions. Obese itself involve in development of asthma, and also incidentally combined with atopic asthma. The prevalence of obesity in both childhood and adulthood has increased sharply in many countries.[2] The rise of obesity has paralleled the rise of asthma.[3] The

obesity related asthma patients has recently come into focus, because of the increasing prevalence.

Asthma is a heterogeneous disorder of the airways with multiple phenotypes. Many studies have revealed that the obesity-related asthma phenotype is different from the other phenotypes. Obese asthmatics usually exhibit more severe symptoms, especially dyspnea, and have poorer prognosis, as obese asthmatics respond poorly to conventional asthma medications such as inhaled corticosteroids and long-acting beta-agonists.[4-7] This difference of responses to therapy is may be due to the difference of the mechanisms of those diseases.

Obesity, independent of ovalbumin (OVA) sensitization, induces airway hyper-responsiveness (AHR) and pulmonary fibrosis and aggravates pre-existing asthma,[8-10] and the mechanisms of that are different from those underlying typical atopic asthma, which is associated with T helper type 2 (Th2) immunity including interleukin (IL)-5, IL-13, and eosinophil. In obese individuals, the excess adipose tissue surrounding the lungs has been proposed to contribute to lung stiffness. Moreover, increased levels of adipokines, tumor necrosis factor (TNF)-α, IL-1β, various cytokines and molecules and reactive oxygen species (ROS) have been considered to associate with obese asthmatics.[11-14] IL-17-producing innate lymphoid cells (ILC) are proposed to play a key role in obesity-induced AHR.[8,15] A recent study has suggested that fibrotic changes mediated by transforming growth factor (TGF)-β represent a key mechanism of AHR in diet-induced obesity (DIO).[10]

Roflumilast, a phosphodiesterase-4 (PDE4) inhibitor, is an anti-inflammatory drug. This has been approved for the treatment of chronic obstructive pulmonary disease (COPD), but not yet for the treatment of asthma. Roflumilast mediates the inflammatory signal pathway in inflammatory cells by increasing cyclic adenosine monophosphate (cAMP) levels.[16] Roflumilast reduces the plasma and sputum levels of TNF-α, TGF-β and IL-1β, which may play key roles in obesity-induced asthma. Roflumilast also reduces oxidative stress resulting from an imbalance between ROS

generation and the antioxidative machinery.[17] Moreover, myofibroblast transition, epithelial-mesenchymal transition, and TGF-β induction, which in turn induce fibrosis, can be controlled by roflumilast administration.[18-20] Together, these findings indicate that roflumilast may be an effective drug for the treatment of obese asthma. Additionally, roflumilast treatment induces weight loss and decreases adipose tissue volume;[21] these side effects may have beneficial effects in the treatment of obese asthma.

In this study, we aimed to evaluate the effects of roflumilast on obese, and obese asthma in a murine model. We designed three models (DIO; DIO with OVA; OVA), and compared the effects of roflumilast and dexamethasone (a conventional asthma medicine) in these models.

II. MATERIALS AND METHODS

1. Animals

Female C57BL/6 mice (4-weeks-old) were purchased from Japan-SLC (Hamamatsu, Japan). All mice were housed under specific pathogen-free conditions in strict accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission by the Life Sciences National Research Council, USA. The study protocol was approved by the Institutional Animal Care and Use Committee (2010-0223) of the Yonsei University College of Medicine (Seoul, Korea), which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2. DIO model

To generate DIO mice, 4-weeks-old C57BL/6 mice were fed high-fat diet (HFD) for 12 weeks. Fat accounts for 60% of the calories in the HFD (D12492; Research Diets, Inc., New Brunswick, USA). Lean mice were fed a normal chow diet (D12450B; Research Diets, Inc., New Brunswick, USA) with fat accounting for 10% of the calories. The body weights of the mice were measured weekly. We defined DIO mice as high fat diet-induced obese mice with weights >150% of the average weight in the chow diet group.

3. OVA-induced asthma model

To induce systemic sensitization to OVA in the lean or DIO mice, a mixture

of OVA (20 μg per mouse; Sigma-Aldrich, St Louis, USA) and Imject® Alum (100 μL per mouse; Thermo Scientific, Rockford, IL, USA) was administered intraperitoneally to the mice at experimental week 9 and 11. One week after the second sensitization, OVA (30 μg per mouse) was administered intranasally thrice consecutively in the 12th week. Two days after the last OVA challenge, the OVA-asthma mice were sacrificed for analysis (Figure 1).

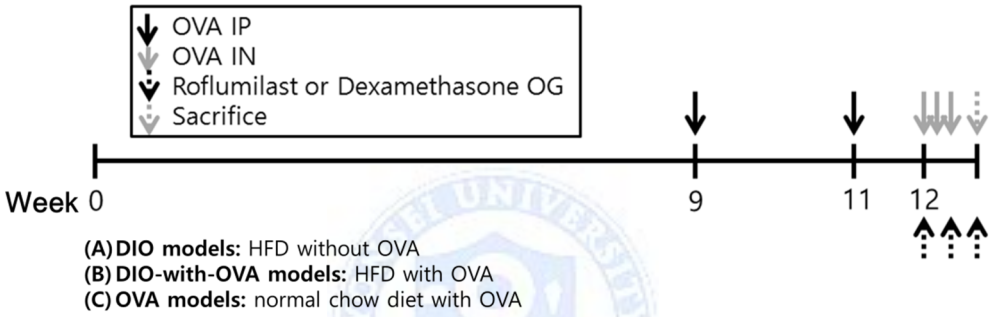


Figure 1. This figure shows the Scheme of the experiments. We designed three models (diet-induced obesity [DIO]; DIO with OVA; OVA). In DIO models (n = 24), mice were fed high-fat diet (HFD) for 12 weeks. In OVA models (n = 24), ovalbumin (OVA) was injected intraperitoneally (IP) at experimental weeks 9 and 11, followed by intranasal (IN) inoculations at 3 consecutive days in the 12th week. In DIO with OVA models (n = 24), OVA was treated in DIO models. To compare the effects of medicines, roflumilast (5 mg/kg) or dexamethasone (3 mg/kg) was administered orally (OG) thrice at 2-day intervals in the 12th week. All mice were sacrificed 4 h after the last administration of roflumilast or dexamethasone (2 days after the final OVA challenge).

4. Administration of roflumilast and dexamethasone

Roflumilast (ALTANA Pharma, Konstanz, Germany) was placed in a glass beaker, and 250 μL of PEG400 (polyethylene glycol wt. 400; Sigma-Aldrich, St Louis, USA) was added, followed by stirring and heating. After the compound dissolved completely, 4% Methocel™ (Methocel™ E15, ALTANA Pharma, Konstanz, Germany) was added, and the mixture was stirred again. The suspension was prepared daily and stirred before administration. The mice were administered roflumilast (5 mg/kg), dexamethasone (9α-fluoro-16α-methylprednisolone; Sigma-Aldrich, St Louis, USA; 3 mg/kg), or vehicle (mixture of 4% Methocel™ and PEG400) by gavage 3 times per week in the 12th week. The mice were sacrificed at 4 hours after the administration of the final dose of oral medications (Figure 1).

5. Measurement of AHR

The mice were anesthetized by peritoneal injection of pentobarbital sodium (50 mg/kg). In each mouse, a tracheostomy was performed, and an 18-gauge tube was inserted for ventilation. An animal ventilator was used for ventilation (FlexiVent® 5.1: SCIREQ, Montreal, QC, Canada), and the mice were challenge with aerosolized-saline followed by increasing doses (6.3, 12.5, 25.0, 50.0 and 100.0 mg/mL) of methacholine (MCh; Sigma-Aldrich, St Louis, USA). The aerosol was generated using an ultrasonic nebulizer (Omron Healthcare, Kyoto, Japan) and was delivered to the inspiratory line of the FlexiVent® apparatus via bias flow of medical air (respiration rate: 60 breaths/min, tidal volume: 30 mL/kg) for 12 seconds. Lung resistance to methacholine aerosols was measured using the FlexiVent ® 5.1 program.

6. BALF analysis

To collect BALF, the lungs were lavaged with 1 mL of Hank's balanced salt solution (HBSS, Gibco BRL, Massachusetts, USA) via the tracheostomy tube. The total number of inflammatory cells was counted with a hemocytometer. The BALF was centrifuged at 2,000 rpm for 3 min to collect the pellet. After removing the supernatant, cell pellets were re-suspended in HBSS. A BAL cell was smeared by cytocentrifugation (Cytospin™ 3, Thermo, Billerica, MA, USA) at 1,000 rpm for 5 min and then stained using a Hemacolor® Staining Kit (Merck, Darmstadt, Germany). BALF cells (at least 200 cells were analyzed) were classified as macrophages, lymphocytes, neutrophils, and eosinophils.

7. Pathological preparation and analysis

The lungs were filled with 10% formalin solution, embedded in paraffin, and cut into 3-μm-thick sections. We utilized hematoxylin and eosin (H&E) staining for general examination, periodic acid-Schiff staining (PAS) to measure goblet cell hyperplasia, and Masson's trichrome (MT) staining to measure fibrosis. Tissue sections were examined with an Olympus BX40 microscope in conjunction with an Olympus U-TV0.63XC digital camera (Olympus BX53F, Center Valley, PA, USA). Images were acquired using the cellSens Standard 1.6 image software.

Quantification analysis was conducted using Metamorph® (Molecular Devices). The number of goblet cells in selected bronchi along the basement membrane was counted on PAS-stained slides at 200× magnification; goblet cell numbers per micrometer of basement membrane were estimated. Fibrosis area was measured by estimating the color-pixel count over the pre-set threshold color for the entire field containing several bronchial tubes on MT-stained slides at 200× magnification.

8. RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from lung tissues prepared in TRIzol® reagent (Ambion, Life technologies, Carlsbad, CA, USA) and homogenized in a tissue homogenizer (T10 basic ULTRA-TURRAX®, IKA, Staufen, Germany) according to the manufacturer's instructions. Reverse transcription was performed with reverse transcriptase (Invitrogen, Carlsbad, CA, USA) primed with oligo (dT) primer. The synthesized cDNAs were amplified using the SYBR® green PCR master mix (BioRad, California, USA) and forward and reverse primers (Bioneer, Daejeon, Korea) with a real-time PCR system (StepOnePlus, Applied Biosystems, USA). Primer sequences were as follows: TNF-α (forward) 5′-CCCTCACACTCAGATCATCTTCT-3′ and (reverse) 5′-GCTACGACGTGGGCTACAG-3′; TGF-β (forward) 5′- TGACGTCACTG GAGTTGTACGG-3′ and (reverse) 5′-GGTTCATGTCATGGATGGTGC-3′; IL-1β (forward) 5′- TGTAATGAAAGACGGCACACC-3′ and (reverse) 5′-TCTTCTTTGGGTATTGCTTGG-3′; IFN-γ (forward) 5′-ATGAACGCTACACACTGCATC-3′ and (reverse) 5′-CCATCCTTTTGCCAGTTCCTC-3′; IL-4 (forward) 5′-GGTCTCAACCCCCAGCTAGT-3′ and (reverse) 5′-GCCGATGATCTCTCTCAAGTGAT-3′; IL-5 (forward) 5′-CTCTGTTGACAAGCAATGAGACG-3′ and (reverse) 5′-TCTTCAGTATGTCTAGCCCCTG-3'; IL-13 (forward) 5'-CCTGGCTCTTGCTTGCCTT-3' and (reverse) 5′-GGTCTTGTGTGATGTTGCTCA-3′; IL-17 (forward) 5′-AAGGCAGCAGCGATCATCC-3′ and (reverse) 5′-GGAACGGTTGAGGTAGTCTGAG-3′; and GAPDH (forward) 5′-TGCCCCCATGTTTGTGATG-3′ and (reverse) 5′-TGTGGTCATGAGCCCTTCC-3′. All the PCR experiments were performed under the following conditions: 95 °C for 5 min, 95 °C for 15 s, and 60 °C for 45 s for up to 40 cycles.

9. Enzyme-linked immunosorbent assay (ELISA)

Adiponectin and leptin levels were measured in serum. To assess cytokine levels, lung tissues were homogenized in an extraction reagent (ThermoFisher Scientific Inc., Rockford, IL, USA). The homogenates were incubated at 4 °C for 30 min and then centrifuged at 12,000 rpm for 10 min. Supernatants were collected and stored at −70 °C until further analysis. The levels of adiponectin and leptin in serum, and the levels of TNF-α, TGF-β, IL-1β, IFN-γ, IL-4, IL-5, IL-13, and IL-17A in lung homogenates were estimated by ELISA with commercially available materials (R&D Systems, Inc., Minneapolis, MN; detection range: adiponectin, 31.2–2,000 pg/mL; leptin, 125–8,000 pg/mL; TNF-α, 31.2–2,000 pg/mL; TGF-β, 31.2–2,000 pg/mL; IL-1β, 15.6–1,000 pg/mL; IFN-γ, 31.2–2,000 pg/mL; IL-4, 15.6–1,000 pg/mL; IL-5, 31.2–2,000 pg/mL; IL-13, 62.5–4,000 pg/mL; and IL-17A, 15.6–1,000 pg/mL).

10. Flow cytometry

Lung tissues were minced into small pieces, and 3 mL of phosphate-buffered saline (PBS) followed by 60 μL of collagenase (collagenase type 4, Worthington biochemical Corporation, Lakewood, NJ) were added to the homogenates. After incubation for 30 min at 37 °C, 7.5 μL of DNase (DNase I; New England Biolabs Inc., MA, USA) was added and mixed. Digested tissues were filtered through a 100-μm filter, followed by a 40-μm filter; excess cold PBS was used to wash the cells through the filter. After centrifugation for 5 min at 12,000 rpm, cells were washed with FACS buffer (PBS containing 2% fetal bovine serum). After one more round of centrifugation, cells were incubated with the blocking monoclonal antibody (2.4G2) and washed with FACS buffer. Cells were stained with fluorescent dye-

conjugated CD3 (T-cell marker), CD4 (T helper cell marker), CXCR3 (Th1 cell marker), CCR4 (Th2 cell marker), CCR6 (Th17 cell marker), and CD8 (T cytotoxic cell marker) antibodies (eBiosciences, San Diego, CA, USA) at 4 °C for 30 min. Stained cells were measured on a BD FACSVerse™ Flow Cytometer and were analyzed using the FlowJo 8.3.3 Software (BD Bioscience, San Jose, CA, USA).

11. Statistical analysis

Statistical analyses were performed using the SPSS software (version 18.0; Chicago, IL). The differences between two groups were evaluated by using the two-tailed unpaired Student's t-test or paired t-test. The results of the methacholine challenge tests for AHR and weight changes were analyzed by using two-way analysis of variance (ANOVA) with Bonferroni's post hoc analysis, while the other tests involving more than three groups were evaluated by using one-way ANOVA with Bonferroni's post hoc analysis. Differences were considered significant at $P < 0.05$.

III. RESULTS

1. HFD induced significant obesity (DIO) in the murine models

Compared to the chow diet, HFD induced significant weight gain in the mice. The mean weight in the HFD group on the day of sacrifice was 43.3 g, whereas that in the chow-diet group was 26.3 g: the weight in the HFD group was 166.5% the average weight in the chow-diet group. After administration of medications, decrease in weight was observed only in the roflumilast-treated group (final weight = 39.5 g). However, the differences in the final weight changes of the HFD groups (HFD, vehicle-treated HFD; HFD/D, dexamethasone-treated HFD; HFD/R, roflumilast-treated HFD) were not statistically significant (Figure 2A). The gross appearance and volume of organs (including heart, lung, liver, and adipose tissue) were significantly different between the HFD and chow-diet groups (Figure 2B, 2C). The HFD group developed hepatic steatosis with ballooning degeneration of hepatocytes (Figure 2D, 2E).

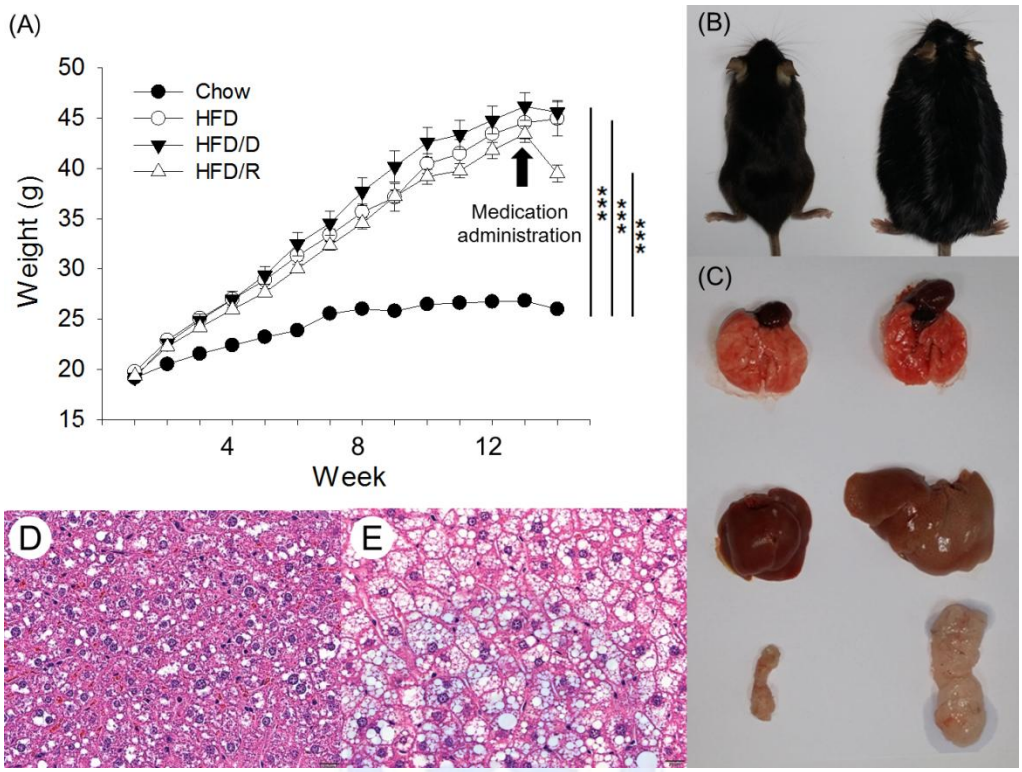


Figure 2. The high-fat diet (HFD) induced significant obesity (DIO) in the murine model. (A) The average weight in the HFD group was 166.5% of the average weight in the chow-diet group. After administration of medications decrease in weight was observed only in the roflumilast-treated group. However, the differences in the final weight changes of the HFD groups (HFD, vehicle-treated HFD; HFD/D, dexamethasone-treated HFD; HFD/R, roflumilast-treated HFD) were not statistically significant. (B, C) The gross appearance and volume of organs were significantly different between the HFD and chow-diet groups. (D, E) Hematoxylin and eosin (H&E ×40)-stained pathological differences in the livers of the chow-diet groups (D) and HFD groups (E) were observed. The HFD group developed hepatic steatosis with ballooning degeneration of hepatocytes. ***$P < 0.001$.

2. DIO-induced AHR was significantly ameliorated by roflumilast, independent of cellular proliferation

The DIO models developed significant AHR compared to the control group. Roflumilast significantly ameliorated AHR caused by DIO to the levels observed in the control group. However, dexamethasone had no effects on AHR caused by DIO (Figure 3A). BALF analysis revealed that DIO did not induce cell proliferation of macrophages, eosinophils, and neutrophils. Therefore, both roflumilast and dexamethasone had no effects on cell proliferation (Figure 3B).

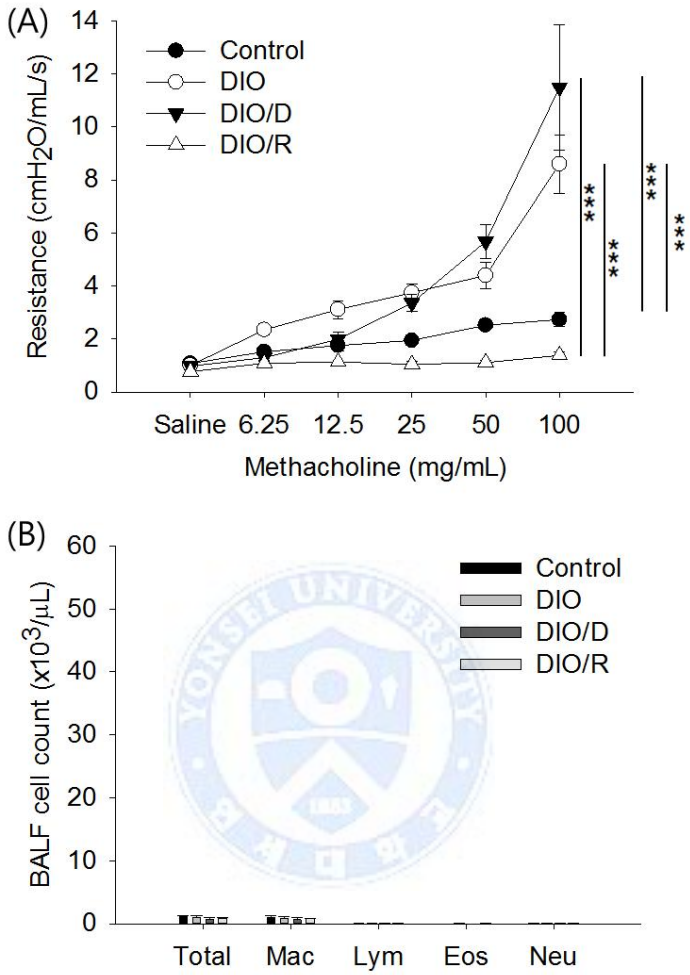
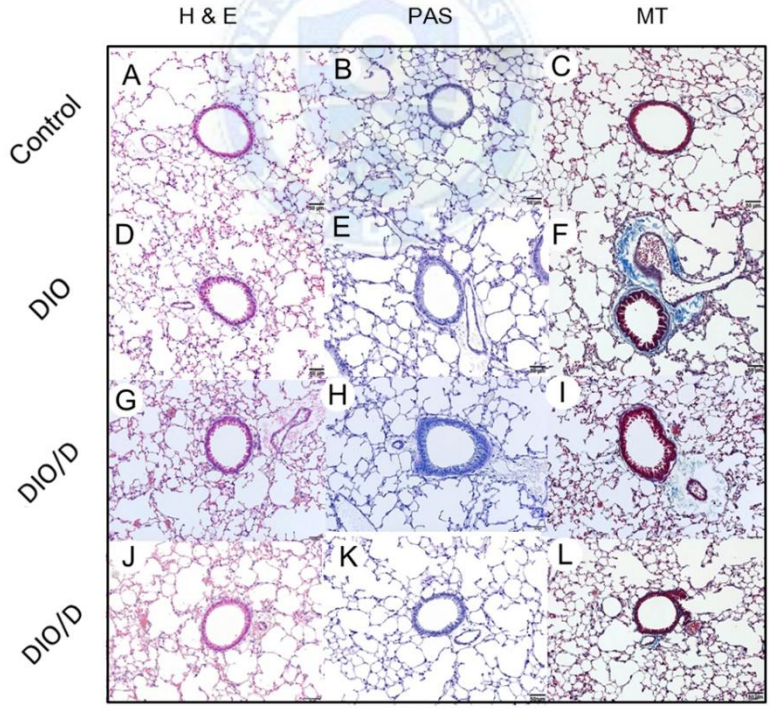


Figure 3. Diet-induced obesity (DIO)-induced airway hyper-responsiveness (AHR) was significantly ameliorated by roflumilast, independent of cellular proliferation. Dexamethasone had no effects on AHR caused by DIO. AHR (A) and bronchoalveolar fluid (BALF) analysis (B); ***$P < 0.001$; DIO/D, dexamethasone-treated DIO; DIO/R, roflumilast-treated DIO; Mac, macrophage; Lym, lymphocyte; Eos, eosinophil; Neu, neutrophil.

3. DIO-induced fibrosis was significantly ameliorated by roflumilast

Pathological analysis of lung tissue revealed that DIO did not induce cellular proliferation and infiltration, in comparison with the control group (Figure 4A-L). Moreover, DIO did not induce goblet cell proliferation, as assessed by PAS staining. Therefore, roflumilast and dexamethasone had no effects on goblet cell proliferation (Figure 4M). However, DIO induced significant fibrosis in the areas surrounding the bronchi and arteries, as assessed by MT staining (Figure 4F). This fibrosis was significantly ameliorated by roflumilast (Figure 4L), whereas dexamethasone reduced DIO-induced lung fibrosis to a lesser extent (Figure 4I). As evidenced by the results of the fibrosis-area analysis (pixels/slide) with the MetaMorph program (Figure 4N), not dexamethasone but roflumilast can significantly ameliorate DIO-induced lung fibrosis.



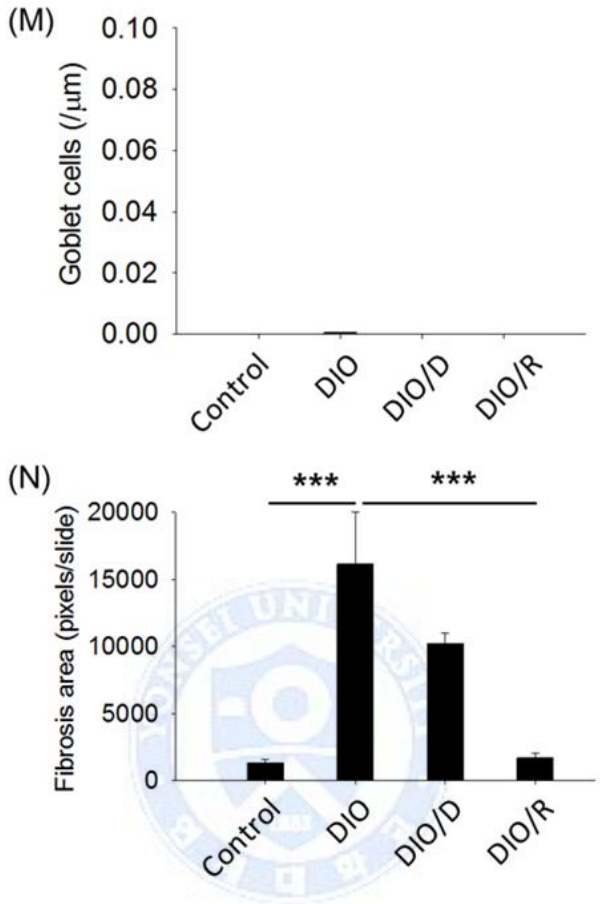


Figure 4. Diet-induced obesity (DIO) did not induce cellular proliferation and goblet cell infiltration. DIO-induced fibrosis was ameliorated not by dexamethasone but by roflumilast. Lung pathology in the control group: (A) Hematoxylin and eosin (H&E) ×40, (B) periodic acid-Schiff staining (PAS) ×40, (C) Masson's trichrome (MT) ×40; in the DIO group: (D) H&E ×40, (E) PAS ×40, (F) MT ×40; in the dexamethasone-treated DIO (DIO/D) group: (G) H&E ×40, (H) PAS ×40, (I) MT ×40; and in the roflumilast-treated DIO (DIO/R) group: (J) H&E ×40, (K) PAS ×40, (L) MT ×40. (M) Change in the goblet cell count (/μm), and (N) change in the fibrosis area (pixel/slide); ***$P < 0.001$.

4. DIO-induced changes in adiponectin and leptin levels in serum, and cytokine levels in lung homogenates were significantly improved by roflumilast

DIO induced a slight decrease in the levels of adiponectin in serum. These decreased adiponectin levels were not affected by roflumilast. However, these decreased adiponectin levels were significantly aggravated by dexamethasone (Figure 5A). DIO induced a significant increase in the levels of leptin in serum compared to the control group. These increased leptin levels were reduced by roflumilast. However, these increased leptin levels were aggravated by dexamethasone. The levels of leptin in roflumilast treated DIO group were significantly lower than those in dexamethasone treated DIO group (Figure 5B).

DIO induced a significant increase in the mRNA levels of TNF-α, TGF-β, IL-1β, and IFN-γ in the lung homogenates compared with the control group. These increased mRNA levels of TNF-α, TGF-β, IL-1β, and IFN-γ were significantly reduced by roflumilast. The mRNA levels of IL-1β and IFN-γ were also significantly ameliorated by dexamethasone. Although dexamethasone reduced the mRNA levels of TNF-α and TGF-β, the effects were not statistically significant (Figure 5C). However, the mRNA levels of IL-4, IL-5, IL-13, and IL-17A did not change in the DIO model with or without roflumilast treatment (data not shown).

DIO also induced an increase in the protein levels of TNF-α, TGF-β, IL-1β, and IFN-γ in the lung homogenates, when compared with the levels in the control group. However, only the increase in TNF-α level was statistically significant. Roflumilast tended to reduce the levels of these cytokines to a greater extent than dexamethasone. The only cytokine showing significant reduction upon roflumilast treatment was TGF-β. However, the differences between the effects of roflumilast and dexamethasone were not statistically significant (Figure 5D). Other markers, including IL-4, IL-5, IL-13, and IL-17A, showed no significant changes in the DIO models, with or without roflumilast treatment (data not shown).

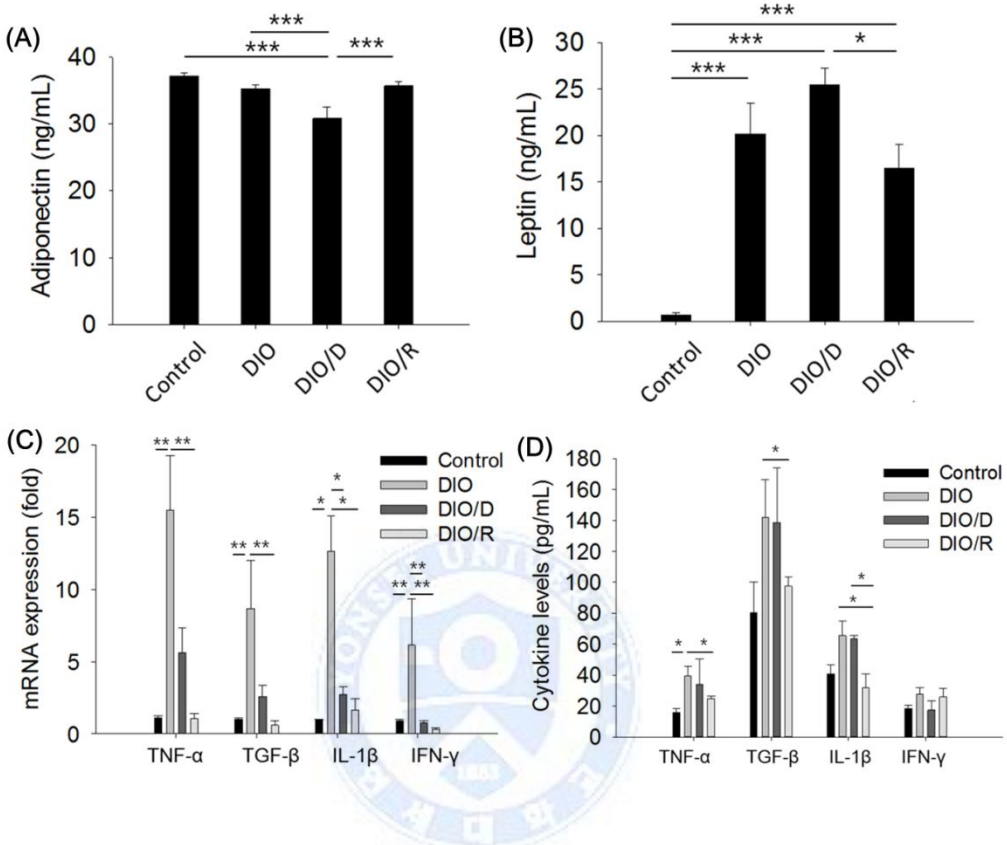
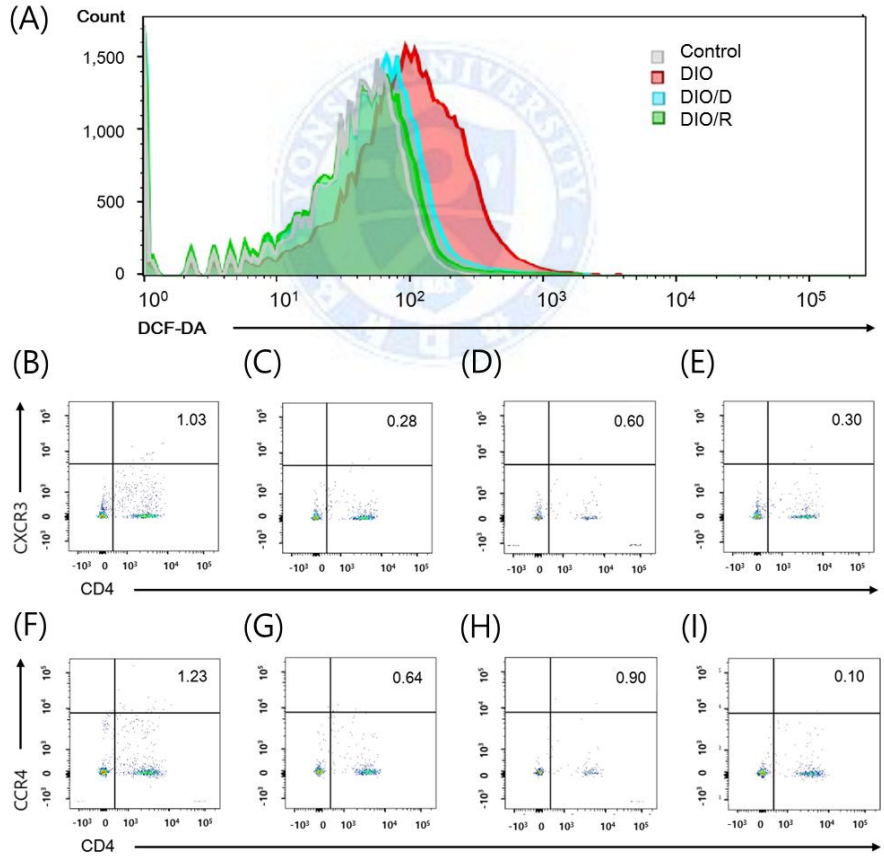


Figure 5. Diet-induced obesity (DIO)-induced changes in adiponectin (A) and leptin (B) was improved by roflumilast, not by dexamethasone. (C) DIO-induced increase in mRNA levels of TNF-α and TGF-β was significantly ameliorated not by dexamethasone (DIO/D) but by roflumilast (DIO/R). DIO-induced increase in mRNA levels of IL-1β and IFN-γ was significantly ameliorated by not only roflumilast but also dexamethasone. (D) Protein levels of cytokines showed similar pattern to mRNA levels with lesser statistical significance. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

5. DIO-induced increase in ROS level was significantly ameliorated by roflumilast

The ROS level in the DIO groups increased in comparison with the levels in the control group; this increase was markedly ameliorated by roflumilast or dexamethasone to the levels in the control group (Figure 6A). DIO did not induce an increase in the number of Th1, Th2, and Th17 cells. So, the T cell differentiation showed no changes, with or without roflumilast or dexamethasone treatment, in the DIO models (Figure 6B-P). The number of cytotoxic T cells did not change in the DIO models with or without medication (data not shown).



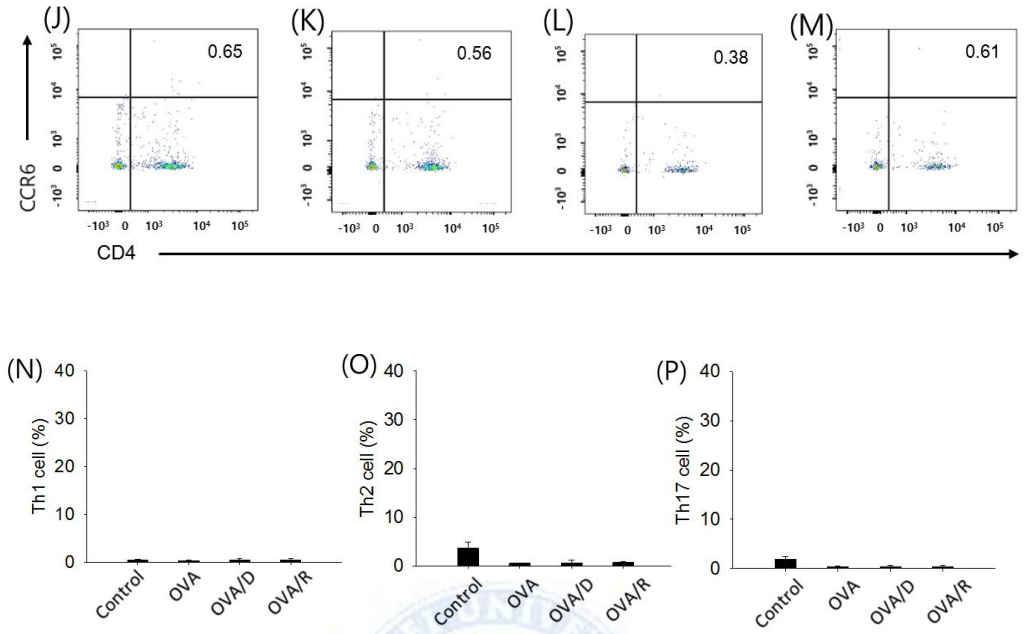


Figure 6. Diet-induced obesity (DIO)-induced increase in reactive oxygen species (ROS) level was ameliorated by roflumilast or dexamethasone. DIO did not induce an increase in the number of T helper type 1 (Th1) and Th2 cells. So, the T cell differentiation showed no changes, with or without roflumilast or dexamethasone treatment. (A) ROS, Th1 cell activation in the (B) control, (C) DIO, (D) dexamethasone-treated DIO (DIO/D), (E) roflumilast-treated DIO (DIO/R) groups. Th2 cell activation in the (F) control, (G) DIO, (H) DIO/D, (I) DIO/R groups. Th17 cell activation in the (J) control, (K) DIO, (L) DIO/D, (M) DIO/R groups. (N) Changes in the Th1 cell, (O) change in the Th2 cell, (P) change in the Th17 cell.

6. DIO with OVA-induced AHR was significantly ameliorated by roflumilast

The DIO-with-OVA models developed significant AHR, whereas the control group showed normal airway reactivity. Roflumilast significantly ameliorated AHR caused by DIO with OVA to the level observed in the control group. Dexamethasone did not diminish AHR (Figure 7A). BALF analysis revealed that the DIO-with-OVA models exhibited significant total cell and eosinophil proliferation, which is significantly ameliorated by roflumilast or dexamethasone. The difference between the effects of the two agents was not statistically significant (Figure 7B).

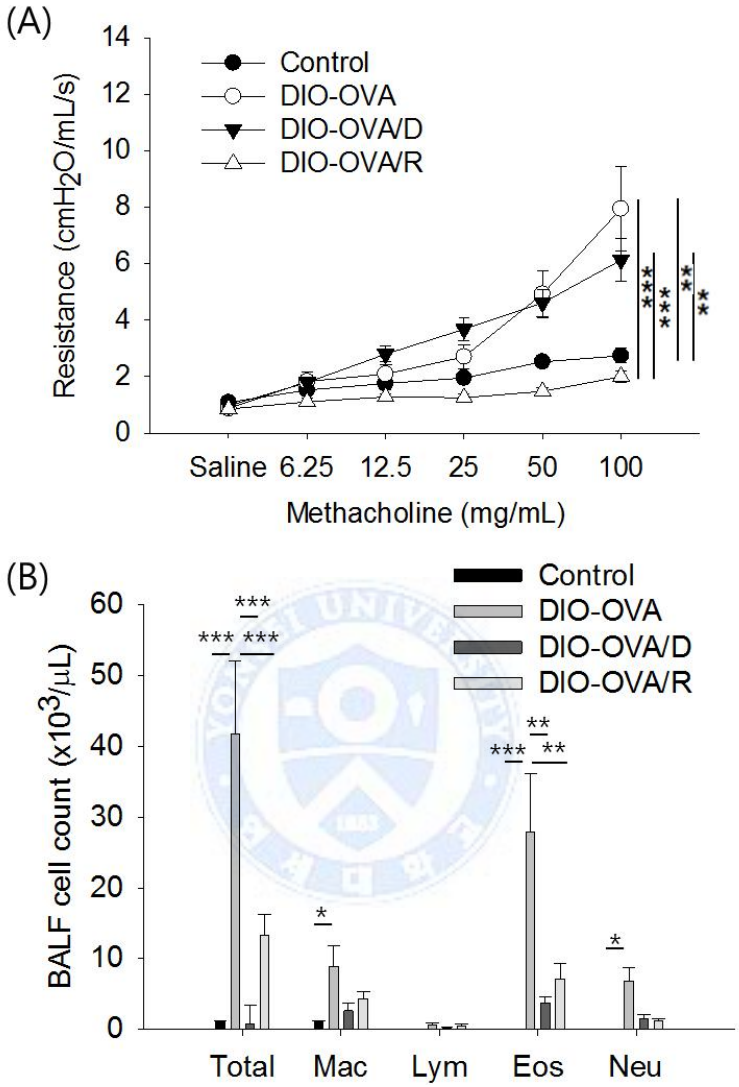
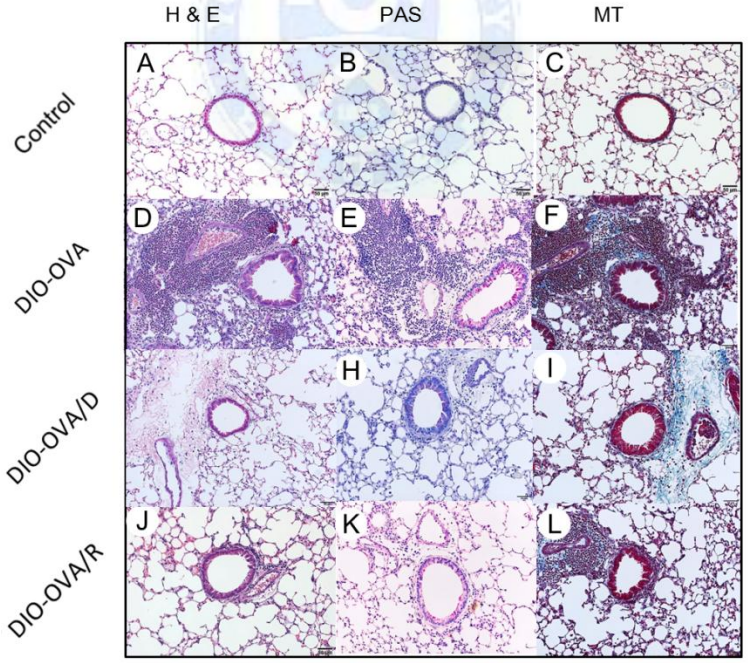


Figure 7. Diet-induced obesity (DIO)-with-ovalbumin (OVA)-induced airway hyper-responsiveness (AHR) was significantly ameliorated by roflumilast. The DIO-with OVA (DIO-OVA)-induced cellular proliferation in the bronchoalveolar lavage fluid (BALF) was markedly reduced by roflumilast (DIO-OVA/R) or dexamethasone (DIO-OVA/D). (A) AHR, (B) BALF analysis; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

7. DIO with OVA-induced fibrosis was ameliorated by roflumilast

Pathological analysis of the lung tissue revealed that DIO with OVA induced peribronchial and perivascular cellular proliferation and infiltration, in comparison with the control group. The cellular proliferation and infiltration was significantly ameliorated by roflumilast and dexamethasone (Figure 8A-L). The DIO-with-OVA models exhibited goblet cell proliferation, as assessed by PAS staining. Both roflumilast and dexamethasone had no effects on goblet cell proliferation (Figure 8M). The DIO-with-OVA models exhibited significant fibrosis around the bronchi and vessels, as assessed by MT staining (Figure 8F). This fibrosis was slightly aggravated by dexamethasone (Fig 8I), whereas roflumilast ameliorate lung fibrosis (Figure 8L). Fibrosis-area analysis revealed that lung fibrosis induced by DIO with OVA significantly improved upon roflumilast treatment, but not by dexamethasone (Figure 8N).



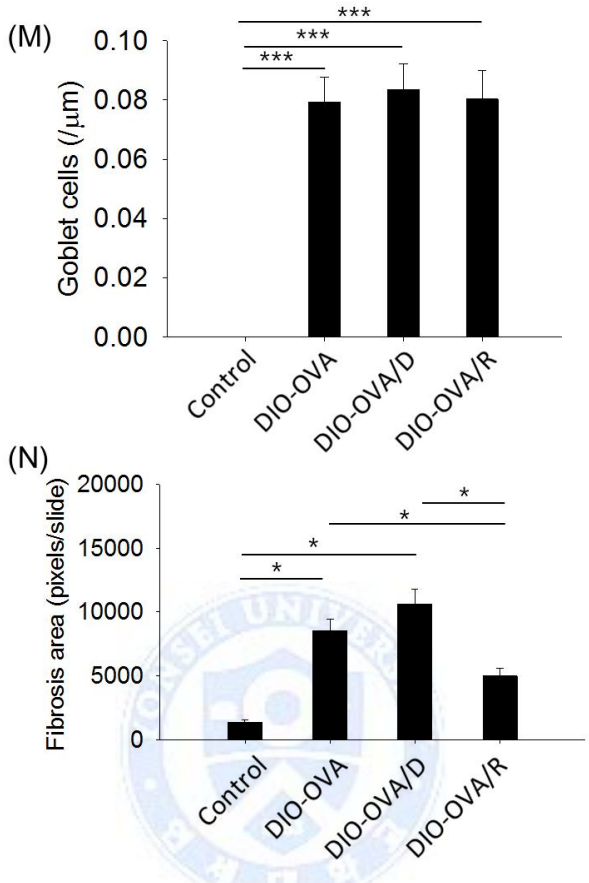


Figure 8. Diet-induced obesity (DIO)-with-ovalbumin (OVA)-induced lung fibrosis was significantly ameliorated not by dexamethasone but by roflumilast. Roflumilast ameliorated cellular infiltration but not goblet cell proliferation. Lung pathology in the control group: (A) Hematoxylin and eosin (H&E) ×40, (B) periodic acid-Schiff staining (PAS) ×40, (C) Masson's trichrome (MT) ×40; in the DIO-with-OVA (DIO-OVA) group: (D) H&E ×40, (E) PAS ×40, (F) MT ×40; in the dexamethasone-treated DIO-with-OVA (DIO-OVA/D) group: (G) H&E ×40, (H) PAS ×40, (I) MT ×40; and in the roflumilast-treated DIO-with-OVA (DOI-OVA/R) group: (J) H&E ×40, (K) PAS ×40, (L) MT ×40; (M) change in the goblet cell count (/μm), (N) changes in the fibrosis area (pixel/slide); *$P < 0.05$ and ***$P < 0.001$.

8. DIO with OVA-induced increases in ROS and T cell levels were ameliorated by roflumilast

DIO with OVA-induced changes in adiponectin and leptin in serum with similar pattern to DIO model. These changes were improved by roflumilast, while dexamethasone aggravated them. The mRNA levels of TNF-α, IL-1β, TGF-β, IL-4, IL-5, and IL-13 in the DIO-with-OVA models decreased slightly upon treatment with roflumilast or dexamethasone; however, the difference was not statistically significant (data not shown). The protein levels of cytokine mRNA showed a similar trend. The ROS level in the DIO-with-OVA models increased in comparison with the control group; this increase in the ROS level was dramatically ameliorated by roflumilast or dexamethasone (Figure 9A).

In the DIO-with-OVA models, the percentages of Th1 and Th2 cells increased significantly compared with those in the control group. Both roflumilast and dexamethasone significantly reduced the percentages of Th1 and Th2 cells to the levels in the control group. The difference between the effects of the two agents was not statistically significant (Figure 9B-K).

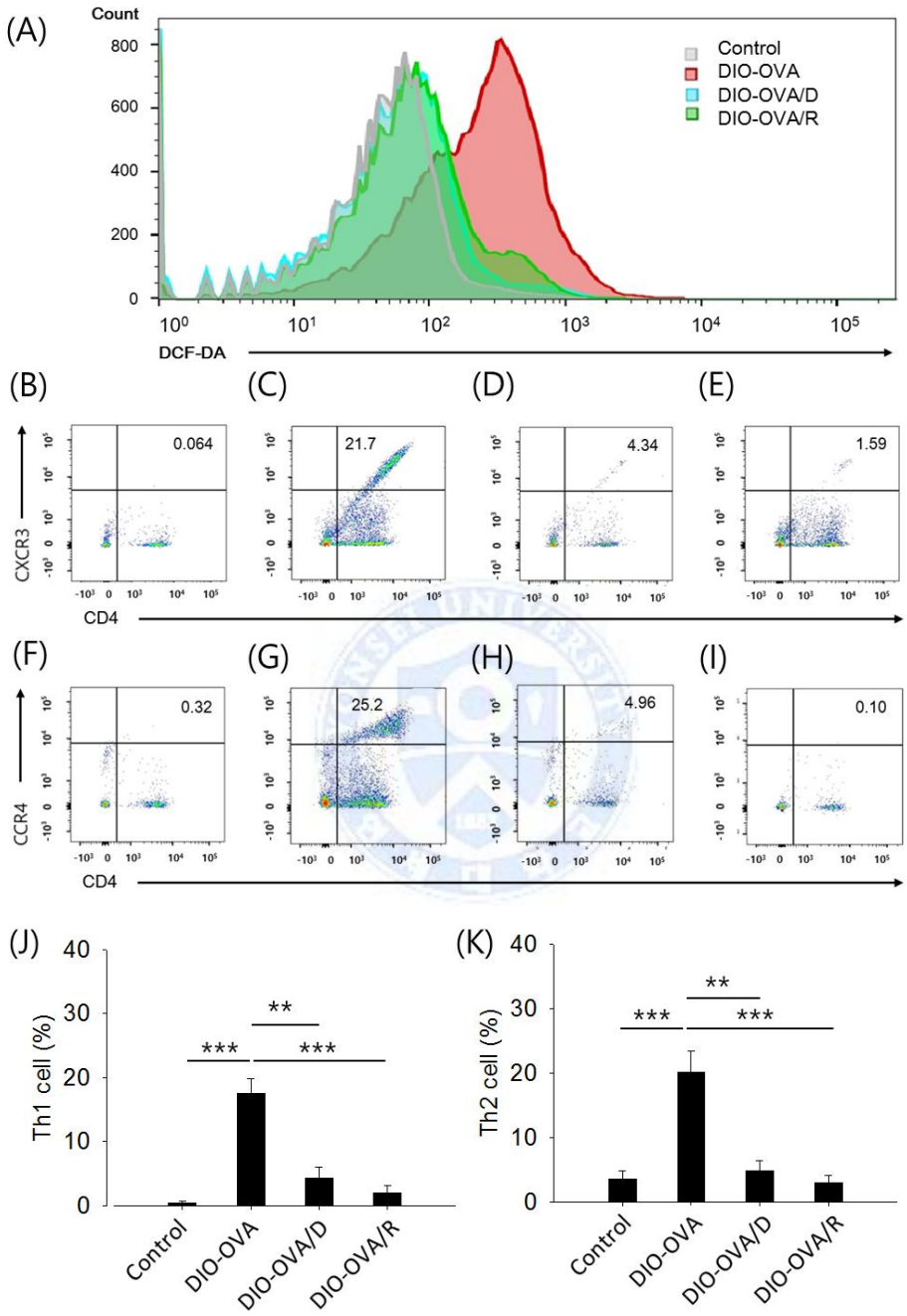

(A)
Count
800
600
400
200
0
Control
DIO-OVA
DIO-OVA/D
DIO-OVA/R
10^0
10^1
10^2
10^3
10^4
10^5
DCF-DA
(B)
(C)
(D)
(E)
0.064
21.7
4.34
1.59
CXCR3
CD4
(F)
(G)
(H)
(I)
0.32
25.2
4.96
0.10
CCR4
CD4
(J)
Th1 cell (%)
40
30
20
10
0
**
***
***
Control
DIO-OVA
DIO-OVA/D
DIO-OVA/R
(K)
Th2 cell (%)
40
30
20
10
0
**
***
***
Control
DIO-OVA
DIO-OVA/D
DIO-OVA/R

Figure 9. Diet-induced obesity (DIO) with ovalbumin (OVA)-induced increases in reactive oxygen species (ROS) and T cell levels were ameliorated by roflumilast. (A) ROS, T helper type 1 (Th1) cell activation in the (B) control, (C) DIO-with-OVA (DIO-OVA), (D) dexamethasone-treated DIO-with-OVA (DIO-OVA/D), and (E) roflumilast-treated DIO-with-OVA (DIO-OVA/R) groups. Th2 cell activation in the (F) control, (G) DIO-OVA, (H) DIO-OVA/D, (I) DIO-OVA/R groups, (J) changes in Th1 cell, (K) changes in Th2 cell activation. $^{**}P < 0.01$ and $^{***}P < 0.001$.

9. OVA-induced AHR was significantly ameliorated by roflumilast

OVA-induced AHR was lower than that induced by DIO or DIO with OVA. Moreover, OVA-induced AHR was significantly ameliorated by roflumilast. Although dexamethasone diminished AHR, the effect was not statistically significant (Fig 10A). Roflumilast and dexamethasone significantly attenuated the total cell and eosinophil proliferation induced by OVA in the BALF (Fig 10B).

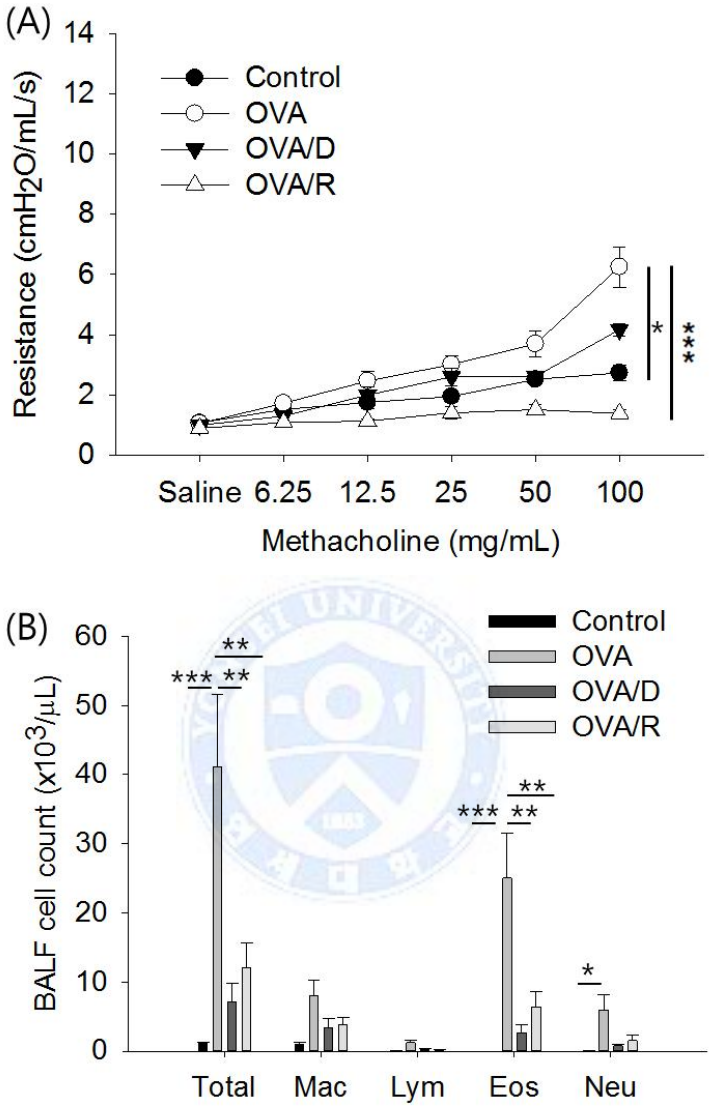
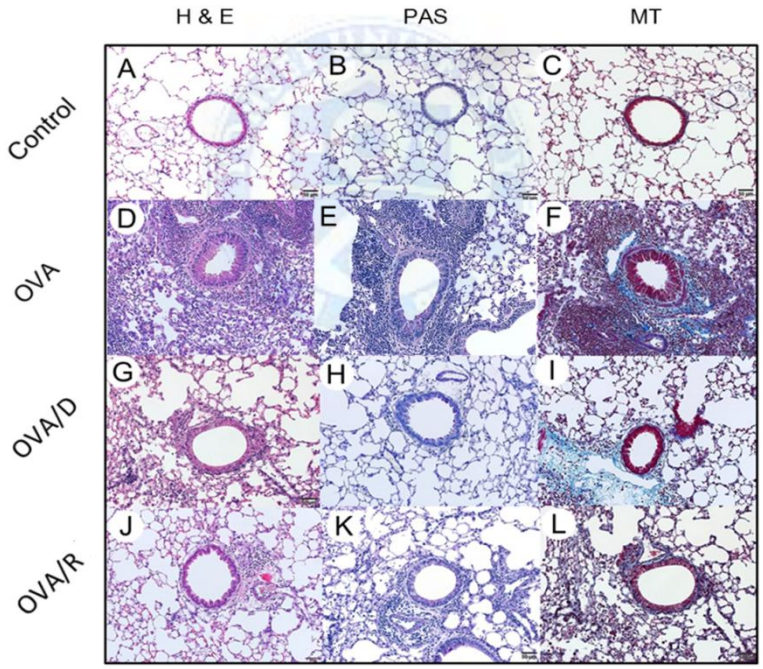


Figure 10. Ovalbumin (OVA)-induced airway hyper-responsiveness (AHR) was significantly ameliorated by roflumilast. The OVA-induced cellular proliferation in the bronchoalveolar lavage fluid (BALF) was markedly reduced by roflumilast or dexamethasone. (A) AHR, (B) BALF analysis; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

10. OVA -induced fibrosis was significantly ameliorated by roflumilast

The OVA models exhibited cellular proliferation and infiltration in the peribronchial and perivascular areas comparison with the control group, as evidenced by pathological findings in lung tissue. The cellular proliferation and infiltration was attenuated by roflumilast or dexamethasone (Figure 11A-L). The OVA models exhibited goblet cell proliferation, as assessed by PAS staining. Both roflumilast and dexamethasone had no effects on goblet cell proliferation (Figure 11M). OVA models exhibited significant fibrosis around the bronchi and arteries, as assessed by MT staining (Figure 11F). Fibrosis-area analysis revealed that fibrosis significantly improved after treatment roflumilast, but not dexamethasone (Figure 11N).



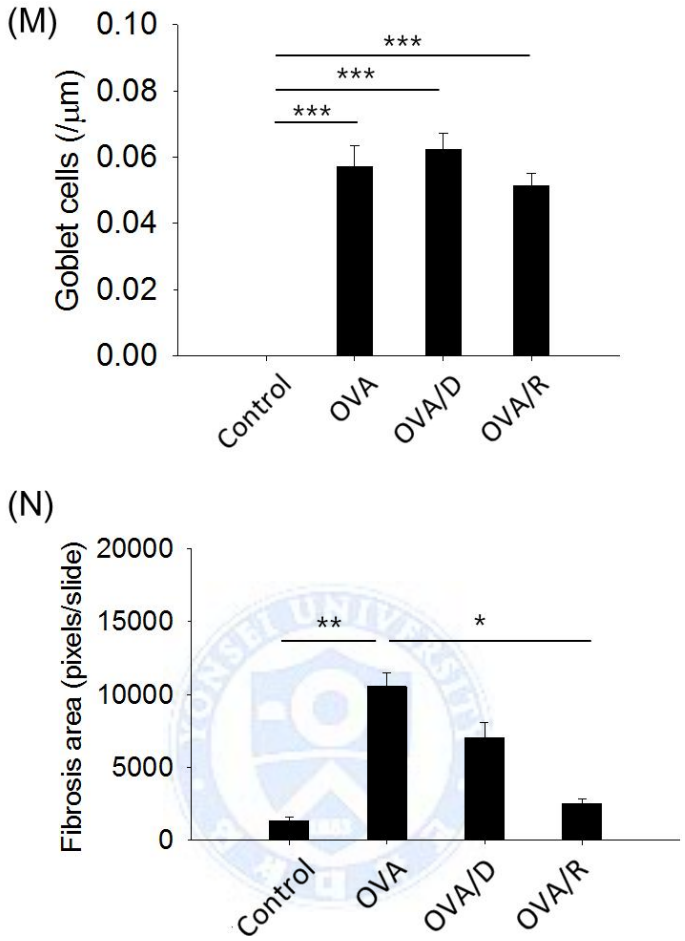


Figure 11. OVA-induced fibrosis was significantly ameliorated not by dexamethasone but by roflumilast. Lung pathology in the control group: (A) Hematoxylin and eosin (H&E) ×40, (B) periodic acid-Schiff staining (PAS) ×40, (C) Masson's trichrome (MT) ×40; in the OVA group: (D) H&E ×40, (E) PAS ×40, (F) MT ×40; in the dexamethasone-treated OVA (OVA/D) group: (G) H&E ×40, (H) PAS ×40, (I) MT ×40; and in the roflumilast-treated OVA (OVA/R) group: (J) H&E ×40, (K) PAS ×40, (L) MT ×40. (M) Change in the goblet cell count (/μm), (N) change in the fibrosis area (pixel/slide); *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

11. OVA-induced increases in ROS and T cell level were ameliorated by roflumilast

The OVA alone did not affect to the levels of adiponectin and leptin. The OVA models exhibited increased mRNA levels of IL-4, IL-5, and IL-13 in their lung homogenates (data not shown). Roflumilast reduced the mRNA levels of these cytokines; however, the differences were not statistically significant. The cytokine levels measured by ELISA showed a similar trend. The increased ROS level in the OVA models reduced upon treatment with roflumilast or dexamethasone to the level observed in the control group (Figure 12A).

OVA induced a significant increase in the percentages of Th1 and Th2 cells. Increase of Th1 cell was significantly ameliorated by roflumilast and dexamethasone, but the differences between two agents were not statistically significant. Increase of Th2 cell was significantly ameliorated by only roflumilast not by dexamethasone (Figure 12B-K).

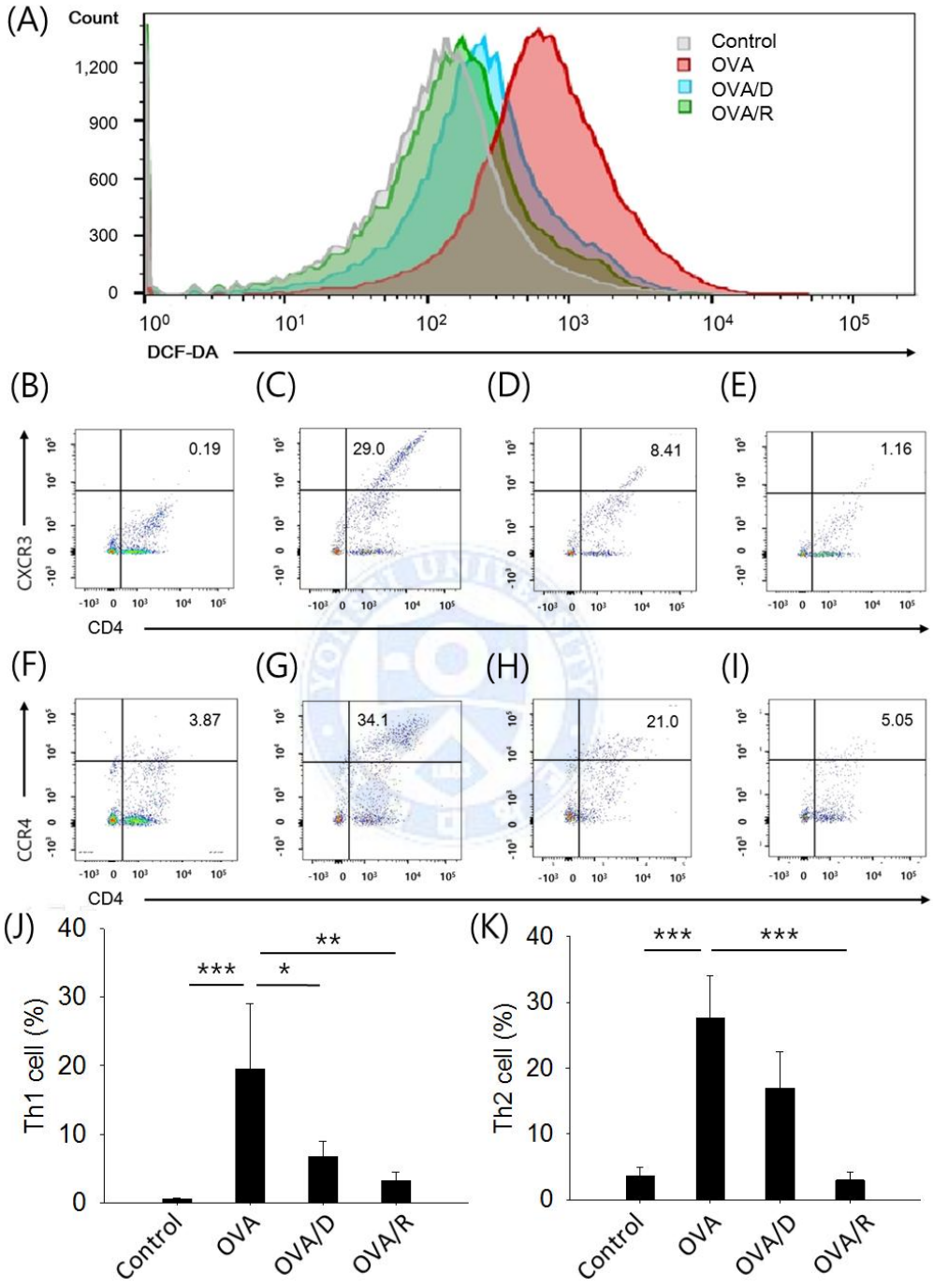

(A)
Count
1,200
900
600
300
0
Control
OVA
OVA/D
OVA/R
DCF-DA
(B)
(C)
(D)
(E)
0.19
29.0
8.41
1.16
CXCR3
CD4
(F)
(G)
(H)
(I)
3.87
34.1
21.0
5.05
CCR4
CD4
(J)
Th1 cell (%)
(K)
Th2 cell (%)
Control
OVA
OVA/D
OVA/R

Figure 12. OVA-induced increase in ROS and T cell level was ameliorated by roflumilast or dexamethasone. Dexamethasone did not reduce Th2 cell activation. (A) ROS, Th1 cell activation in the (B) control, (C) OVA, (D) dexamethasone-treated OVA (OVA/D), and (E) roflumilast-treated OVA groups (OVA/R). Th2 cell activation in the (F) control, (G) OVA, (H) OVA/D, and (I) OVA/R groups; $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

IV. DISCUSSION

Our results revealed that DIO itself induced significant AHR and lung fibrosis. Previous studies have confirmed that sufficient weight increase (more than 45%) is inevitable for significant AHR induction.[8,9,22-26] Therefore, we selected only those mice that exhibited adequate weight gain (>150% increase compared with the control group) and successfully induced AHR in all the DIO models. The various mechanisms how DIO without OVA induces AHR have been investigated. Many studies have revealed that DIO does not need to induce overt inflammation to induce AHR.[23,24] Our study also confirmed that DIO alone does not induce cellular proliferation and infiltration in the lungs and BALF. Moreover, DIO did not affect Th1, Th2, and Th17 cell proliferation, indicating that DIO has no effects at the cell and tissue levels. Many pro-inflammatory mediators produced by adipose tissue are thought to be associated with the development of AHR. We confirmed that DIO models are associated with increased mRNA and protein levels of TNF-α, TGF-β, IL-1β, and IFN-γ in lung, in concordance with previous studies.[8,9,22,27-30] Together, these findings suggest that, even in the absence of overt inflammation, DIO induces AHR by increasing the levels of multiple cytokines. The term “low-grade systemic inflammation” has been used to describe this phenomenon. Moreover, our results confirmed that DIO models are associated with increased ROS and TGF-β levels and lung fibrosis; this finding corroborated previous studies.[10,31] We can guess that lung fibrosis is induced by increased ROS and TGF-β levels. Overall, this study revealed that various mediators and mechanisms are simultaneously involved in DIO-induced AHR and fibrosis in murine models.

Our results revealed that DIO-induced AHR and fibrosis is not improved by conventional steroid treatment (dexamethasone) in murine models. Although, several clinical and *in vitro* studies have demonstrated that obesity-associated asthma patients respond poorly to conventional steroid therapy,[27,32,33] to the best of our knowledge,

this hypothesis has not been corroborated by animal studies. Although dexamethasone treatment reduces the levels of cytokines involved in the inflammatory cascade, it does not improve ROS levels. Therefore, it is necessary to investigate alternative agents as candidate drugs for the treatment of obesity-associated asthma.

Roflumilast is an anti-inflammatory drug which is known to be involved in various inflammatory cascades, where it lowers the levels of TNF-α, IL-1β, IL-2, IL-13, IFN-γ, and ROS.[34-37] In addition, roflumilast has protective effects against fibrosis and contraction in fibroblasts and smooth muscle cells.[38,39] In this study, DIO-induced increased mRNA and protein levels of TNF-α, TGF-β, IL-1β, and IFN-γ markedly reduced after roflumilast treatment. Roflumilast dramatically reduced ROS levels and fibrosis, consistent with the results of previous studies.[37,40] Although dexamethasone also reduced cytokines and ROS levels, the degree of reduction was lower than that induced by roflumilast. Moreover, dexamethasone could not improve fibrosis. These differences may explain the differential effects of dexamethasone and roflumilast on AHR and fibrosis.

In the DIO-with-OVA and OVA-alone models, both Th2 and Th1 cell proliferation was observed. The amount of OVA (20 μg) used in this experiment was very low. This dose of OVA leads to both Th2 and Th1-related immunity in C57BL/6 mice.[41] We also found that both Th2- and Th1-related cytokine levels increased in the DIO-with-OVA and OVA-alone models (data not shown). In addition, roflumilast ameliorated Th1 and Th2 cell proliferation upon OVA sensitization. The effects in DIO-with-OVA and OVA-alone model were contributed to the Th1 and Th2 cell deactivation by roflumilast.

Several studies have revealed that weight loss improves asthma in obese individuals.[42-45] The most common side effect of roflumilast is gastrointestinal effects, including diarrhea, nausea, and vomiting, all of which are associated with weight loss.

"Weight loss" accounts for 7.4% of all adverse effects induced by roflumilast in human study.[46] Some studies have suggested that the effect of roflumilast on weight loss may be attributed to loss of body fat mass.[21] Because DIO-induced AHR and fibrosis were associated with adipose tissue-derived mediators (TNF-α, TGF-β and IL-1β), loss of body fat mass accompanied with weight loss will improve AHR.[14,47] In this study, roflumilast treatment led to weight loss (8.8% reduction compared with the obesity group). This additional side effect may also explain the significant improvement of DIO-induced AHR and fibrosis upon roflumilast treatment observed in this study.

Adipokines are hormone, cytokines, chemokines, and other factors secreted by adipose tissues. Adiponectin and leptin are adipocyte-derived hormone and key regulators in metabolic process and energy homeostasis. Adiponectin is known to have protective anti-inflammatory effects, and be decreased in obese subjects. Many previous studies revealed obesity-related adiponectin deficiency induce AHR.[48] Leptin, pro-inflammatory adipokine, is known to be increased in obese subjects, and increased leptin levels induce AHR.[49] In addition, direct effects of leptin on release of VEGF from airway smooth muscle cells might be affect AHR.[50] Dexamethasone is a potent inhibitor of adiponectin release, and a potent secretagogues of leptin in obese subjects (R3, R4).[51,52] The aggravated AHR by dexamethasone in this study may be due to these effects of dexamethasone, lowering of adiponectin levels and raising of leptin levels. In contrast, roflumilast showed positive effects on the adipokines in this study. Roflumilast restored the decreased adiponectin levels, and increased leptin levels. These effects of roflumilast may be due to the loss of adipose tissues, which directly affects the cytokines. The decreased levels of TNF-α and IFN-γ which are known as a adipokines may be due to these effects of roflumilast. In contrast, the decreased levels of TNF-α and IFN-γ in dexamethasone treated DIO group may be due to the anti-inflammatory and immunosuppressive effects of dexamethasone. The

direct effects of roflumilast on airway smooth muscle through adipokines should be further studied.

In summary, we can organize the results of roflumilast effects in table below (Table 1) and outline the mechanisms how roflumilast ameliorated AHR induced by DIO (Figure 13). Roflumilast, phosphodiesterase-4 inhibitor will induce increase of active cAMP according to the basic mechanisms. These increased cAMP levels directly reduced ROS levels and blocked inflammatory cascade with reducing various cytokine levels. The decreased TGF-β levels and direct effects of roflumilast ameliorated fibrosis. The weight loss effects of roflumilast improved levels of adipokines including adiponectin, leptin, TNF-α and IFN-γ. These improved adipokines levels in serum had systemic anti-inflammatory effects with reducing various cytokine levels. Last, the weight loss and improved adipokines levels can directly affect the AHR. These various mechanisms of roflumilast simultaneously affected on the AHR induced by DIO.

Table 1. The summary of roflumilast effects in three models.

	DIO	DIO with OVA	OVA
AHR	+++	+++	+++
BALF cell proliferation	Not changed	+++	++
Lung fibrosis	+++	+	+
Adipokines	+	+	Not changed
Cytokines	++	+	+
ROS level	+	+	+
Th1/Th2 cell activation	Not changed	+++	++

+++ $P < 0.001$; ++$P < 0.01$; +$P < 0.05$ improvement in roflumilast treated group, compared to control or dexamethasone treated group

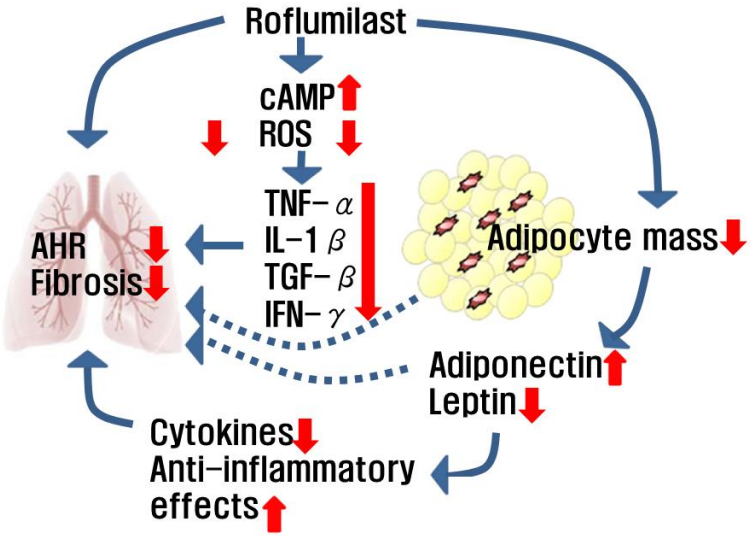


Figure 13. Mechanisms how roflumilast ameliorated AHR induced by DIO.

Our results suggest that roflumilast is a promising candidate for the treatment of obesity-associated asthma. By improving micro-inflammation, roflumilast significantly ameliorates AHR. Moreover, roflumilast induces weight loss. In clinics, roflumilast may be prescribed to obese asthma patients as an additional to conventional treatment. In particular, patients suffering from newly developed dyspnea with weight gain may benefit from roflumilast treatment. Moreover, obese patients with atopic asthma, who respond poorly to conventional treatment, may be considered for roflumilast treatment. Roflumilast may help reduce unnecessary medical cost and effort.

Obesity is often accompanied by various comorbid diseases. Several cytokines, ROS, and fibrosis, which are indicated in this study, may have effects on various organs, including the liver, pancreas, kidney, vessel, and skin. In addition to obesity-associated asthma, roflumilast may serve as a potential therapeutic agent against other diseases associated with obesity. Some studies have already suggested

that roflumilast is a potential therapeutic agent for myocardial ischemia and type II diabetes mellitus.[53,54] This study indicates that roflumilast may be useful in the treatment of other obesity related disease.

This study has some limitations. The detailed mechanisms how DIO induce AHR and fibrosis and underlying the effects of roflumilast were not well investigated. Although we observed improvements in TNF-α, TGF- β, IL-1β, and IFN-γ levels and AHR, we do not know if reduced levels of these cytokines contributed to improved AHR. Additional effects of roflumilast on airway smooth muscle cells should be further evaluated. Further studies using various cytokine agonists in roflumilast-treated DIO models and knockout mouse are required to investigate the detailed. Moreover, *in vitro* and human clinical studies need to be conducted to evaluate clinical usefulness of roflumilast treatment in obesity-associated asthma.

This is the first study to examine the effects of roflumilast on AHR and fibrosis in a DIO murine model. We suggested that adipose tissue-derived cells and adipokines including adiponectin, leptin, and cytokines in DIO models express genes associated with the inflammatory cascade, and produce abundant levels of cytokines (TNF-α, TGF-β, IL-1β, and IFN-γ) and ROS, which can lead to fibrotic changes, and finally induce significant AHR. Improvement of these all parameters by roflumilast may significantly ameliorate AHR. Our results suggest that roflumilast may be used as a novel therapeutic agent against obesity-associated asthma.

V. CONCLUSION

In conclusion, roflumilast is significantly more effective than dexamethasone against AHR caused by DIO in the murine model. Roflumilast treatment led to improved mRNA and protein levels of TNF-α, TGF-β and IL-1β. Roflumilast reduced increased ROS levels and ameliorated fibrosis. In the presence of OVA, roflumilast also ameliorated Th1 and Th2 cell proliferation. Roflumilast may represent a promising therapeutic agent for the treatment of obese asthma patients.

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ABSTRACT (IN KOREAN)

식이요법으로 유도된 비만 쥐 모델에서의 기도과민성 및 폐섬유화에 대한 Roflumilast의 효과

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박 혜 정

연구 배경 및 목적: 비만한 천식 환자는 전형적인 천식 치료제에 잘 반응하지 않아 치료 성적이 낮고 중증도가 높다. 비만의 유병률이 증가함에 따라 비만한 천식 환자에게 특화된 새로운 약물의 필요성이 대두되고 있다. Roflumilast는 Phosphodiesterase-4 억제제로서 비만 천식 환자에서 중요하다고 알려진 다양한 싸이토카인 및 활성 산소를 낮춰준다고 알려져 있어, 비만한 천식환자에서의 Roflumilast의 효과를 연구하였다.

연구 재료 및 방법: 식이요법 유도 비만 모델, OVA와 함께 처리한 비만 모델, OVA 처리 모델을 구축하고 각 모델에서 Roflumilast와 Dexamethasone의 효과를 비교하였다. C57/BL/6J 수컷 쥐를 대상으로 3개월 동안 60% 고지방 식이를 섭취하게 하였다. Roflumilast 또는 Dexamethasone는 격일로 총 3회 마지막 주에 투여하였다. 기도과민성,

기관지폐포 세척액, 폐조직, 싸이토카인, mRNA, 활성산소, T세포 활성도를 분석하였다.

연구 결과: 비만 모델에서 유도된 기도과민성은 Dexamethasone 처리군과 비교하여 Roflumilast 처리군에서 유의하게 호전되었다. 비만 모델은 기관지폐포 세척액 및 조직 소견에서 세포 증식을 유발하지 않았지만, 섬유화를 증가시켰고, Roflumilast 처리군에서 유의하게 호전되었다. 비만 모델에서 증가한 TNF-α, TGF-β, IL-1β mRNA는 Roflumilast 처리군에서 유의하게 호전되었으며, 해당하는 싸이토카인도 비슷한 경향을 보였다. 비만 모델에서 증가한 활성산소는 Roflumilast 처리군에서 호전을 보였다. OVA와 함께 처리한 비만 모델에서도 Roflumilast 는 Dexamethasone에 비해 기도과민성, 폐섬유화, 활성산소발생에 더 큰 호전을 보였으며, Th1 및 Th2 세포 증식에도 호전을 보였다. OVA 처리한 모델에서 Roflumilast는 Dexamethasone과 기도과민성에서 비슷한 효과를 보였다.

결론: Roflumilast는 비만으로 유도된 기도과민성 및 폐섬유화에 Dexamethasone에 비해 큰 효과를 보였으며, TNF-α, TGF-β, IL-1β 및 활성 산소 수준을 감소시켰다. 비만 천식 환자에서 Roflumilast는 각광받는 치료제가 될 수 있을 것이다.

핵심되는 말: 기도과민성, 천식, 비만, Phosphodiesterase-4 억제제, Roflumilast