



Metformin reduces airway inflammation and remodeling via activation of AMP-activated protein kinase

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ABSTRACT

Recent reports have suggested that metformin has anti-inflammatory and anti-tissue remodeling properties. We investigated the potential effect of metformin on airway inflammation and remodeling in asthma. The effect of metformin treatment on airway inflammation and pivotal characteristics of airway remodeling were examined in a murine model of chronic asthma generated by repetitive challenges with ovalbumin and fungal-associated allergenic protease. To investigate the underlying mechanism of metformin, oxidative stress levels and AMP-activated protein kinase (AMPK) activation were assessed. To further elucidate the role of AMPK, we examined the effect of 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) as a specific activator of AMPK and employed AMPKα1-deficient mice as an asthma model. The role of metformin and AMPK in tissue fibrosis was evaluated using a bleomycin-induced acute lung injury model and *in vitro* experiments with cultured fibroblasts. Metformin suppressed eosinophilic inflammation and significantly reduced peribronchial fibrosis, smooth muscle layer thickness, and mucin secretion. Enhanced AMPK activation and decreased oxidative stress in lungs was found in metformin-treated asthmatic mice. Similar results were observed in the AICAR-treated group. In addition, the enhanced airway inflammation and fibrosis in heterozygous AMPKα1-deficient mice were induced by both allergen and bleomycin challenges. Fibronectin and collagen expression was diminished by metformin through AMPKα1 activation in cultured fibroblasts. Therefore metformin reduced both airway inflammation and remodeling at least partially through the induction of AMPK activation and decreased oxidative stress. These data provide insight into the beneficial role of metformin as a novel therapeutic drug for chronic asthma.

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1. Introduction

Asthma has unique characteristics, such as reversible airway obstruction and airway hyperresponsiveness (AHR) that are typically associated with chronic inflammation. Although airway obstruction is fully reversible with treatment in many cases, persistent airflow limitation and progressive decline of lung function are also observed in some patients. Structural changes, widely referred to airway remodeling, are critical for the development of irreversible airway obstruction and are linked to the morbidities of severe asthma. Although chronic airway

inflammation is a key contributing factor for airway remodeling, the presence of airway inflammation does not always translate into airway remodeling. Despite appropriate inhaled corticosteroid treatments, the most effective current anti-inflammatory therapy [1,2], many studies have demonstrated that corticosteroids have little or no effect on airway remodeling [3,4]. Thus, elucidation of the precise mechanisms and development of novel therapeutic approaches targeting airway remodeling and chronic inflammation in asthma are urgently needed.

Recent reports have shown that metformin, a medication commonly used for treating type 2 diabetes, has various biological functions other than its anti-diabetic effects. Metformin suppressed tumor necrosis factor-α (TNFα) production in a variety of cells, including human monocytes, umbilical vein endothelial cells, and primary bronchial epithelial cells [5–7]. Two *in vivo* studies demonstrated that metformin administration reduced experimental autoimmune encephalomyelitis (EAE) induction and improved survival rates during endotoxemia [8,9]. The cellular mechanism of

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metformin may involve adenosine mono-phosphate activated protein kinase (AMPK), a sensor of cellular energy status and oxidative stress. AMPK may function in cellular proliferation and protein synthesis, and it suppresses NADPH oxidases that generate oxidative stress [10]. Furthermore, roles of AMPK in tissue remodeling, especially during vascular remodeling and cardiac hypertrophy, have been reported [11,12].

Based on previous studies, metformin may have both anti-inflammatory and anti-airway remodeling effects during chronic asthma through AMPK activation. Currently, few studies have been conducted to elucidate the roles of metformin in the pathogenesis of airway inflammation and remodeling in asthma. We evaluated the effect of metformin on airway inflammation and remodeling and its functional mechanism in a murine model of chronic asthma.

2. Methods

2.1. Mice

Female, 6–8-weeks-old BALB/C mice were purchased from OrientBio (Kapyong, Korea). Heterozygous AMPK α 1-deficient (AMPK α 1 HT) C57BL/6 mice were generously provided by Professor Benoit Violet (INSERM U567, Paris, France) and bred in specific pathogen-free conditions. Animal experiments were approved by the Institutional Animal Care and Use Committee of Asan Medical Center, Seoul, Korea.

2.2. Generation of a murine asthma model

To generate a chronic asthma model, mice were immunized with ovalbumin (OVA) and fungal-associated allergenic protease (FAP) as previously reported [13]. BALB/C mice were anaesthetized and intranasally immunized with 25 μ g OVA (grade V, Sigma, St. Louis, MO, USA) and 50 μ g FAP (*Aspergillus oryzae*, Sigma) twice a week for 8 weeks. Endotoxin-removed metformin (250 mg/kg body weight; Sigma) or AICAR (100 mg/kg body weight; Toronto Research Chemicals, North York, ON, Canada) were intraperitoneally administered 30 min before each immunization (Fig. 1). AMPK α 1-HT and littermate wild-type (WT) C57BL/6 mice were intranasally immunized with OVA and FAP twice a week for 8 weeks as same protocol. Mice were sacrificed 48 h after the last immunization.

2.3. Generation of a murine bleomycin-induced lung injury model

AMPK α 1 HT and littermate WT C57BL/6 mice were intratracheally injected with bleomycin (1.5 mg/kg body weight; Nippon Kayaku Co., Ltd, Tokyo, Japan) on day 0 and sacrificed on day 7.

2.4. Analysis of bronchoalveolar lavage fluid cells and lung tissues and serology

Bronchoalveolar lavage fluid (BALF) cells were analyzed and Western blot and real-time PCR analysis were conducted for the

evaluation of various chemical mediators in lungs and cells. To obtain bronchoalveolar lavage fluid (BALF), 2 ml PBS was instilled and withdrawn following tracheostomy. BALF was centrifuged at 2200 g for 10 min at 4 °C, and pellets were resuspended in PBS to count total cells. For differential cell counts, BALF cells were centrifuged at 500 g for 5 min at room temperature (RT) using cyto-centrifugation (Cytofuge 12, Iris, Westwood, MA, USA) followed by staining with Diff-Quik (Sysmex Co., Japan). At least 300 cells were counted from each preparation to determine macrophage, eosinophil, neutrophil, and lymphocyte counts. Total serum IgE levels were measured using OptEIA ELISA Kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. OVA-specific IgE and IgG1 levels in serum were measured using a modified method described previously [14]. Briefly, ELISA plates were coated with 1 mg/ml OVA overnight at RT. Diluted serum (1:50 for IgE and 1:50,000 for IgG1) was added to the plate. Pooled sera from sensitized mice were used as standards. Anti-mouse IgE (BD Pharmingen, San Jose, CA, USA), HRP-conjugated anti-rat IgG (Chemicon, Billerica, MA, USA), and HRP-conjugated anti-mouse IgG1 (Serotec, Oxford, United Kingdom) were used with TMB (R&D Biosystems, Minneapolis, MN, USA) as the substrate.

2.5. Western blot and real-time PCR analysis of various chemical mediators in lungs and cells

To obtain lung protein extracts for Western blot analysis, lung tissues were homogenized in RIPA buffer containing protease inhibitors. Homogenates were incubated on ice and centrifuged at 13,000 g for 20 min at 4 °C. Supernatants were removed and protein concentrations were quantified by Bradford assays (Bio-Rad, Richmond, CA, USA). Samples were separated on SDS-PAGE gels and transferred to PVDF membranes (Amersham, Buckinghamshire, England). Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) and incubated with anti-VEGF (Abcam, Cambridge, MA, USA), anti-TGF- β 1 (R&D Systems, Minneapolis, MN, USA), anti-heparin-binding epidermal-like growth factor (HB-EGF), anti-fibronectin (Abcam, Cambridge, MA, USA), and anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies overnight at 4 °C. HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Bethyl Laboratories, Montgomery, TX, USA) were used to detect primary antibodies. Blots were visualized with ECL reagents (Amersham, Buckinghamshire, England). Total RNA was extracted from frozen lung tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and cleaned using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Purified RNA (1 μ g) was reverse-transcribed to cDNA using oligo (dT) primers and reverse transcriptase (Roche Applied Science, Mannheim, Germany). Target amplification was performed on a Lightcycler 480 real-time PCR machine using a Lightcycler 480 SYBR Green I Master System (Roche Applied Science, Mannheim, Germany). The primer sequences used were: mouse fibronectin, sense: 5'-ACCGTGTCTCAGGCTTCCGGT-3' and anti-sense: 5'-ACG-GAAGTGGCCGTGCTTGG-3'; mouse collagen-1, sense: 5'-CCTCTGACGCATGGCCAAGA-3' and anti-sense: 5'-TGCACGT-

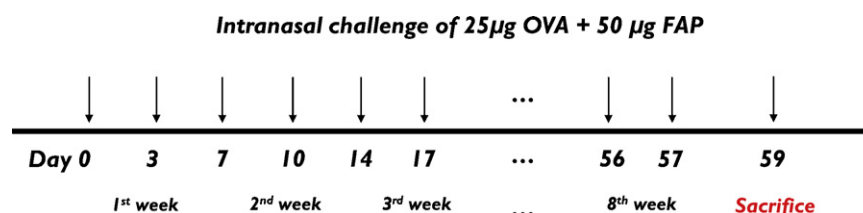


Fig. 1. Experimental protocols for inducing chronic murine models of asthma. Female BALB/C mice were intranasally challenged with 25 μ g OVA and 50 μ g FAP twice a week for 8 weeks. Mice were sacrificed 2 days after the last challenge. OVA, ovalbumin; FAP, fungal-associated allergenic protease.

CATCGCACACAGCC-3'); mouse GAPDH, sense: 5'-TGCCAG-CCTCGTCCCGTAGA-3' and anti-sense: 5'-AGGCGCCCAATACGGC-CAAA-3'. The relative quantity (RQ) of each transcript was calculated by subtracting the $\Delta\Delta C_t$ values of the control groups from the $\Delta\Delta C_t$ values of the experimental groups.

2.6. Lung histopathological examination

Slides of lung tissues were stained using hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome (MT) methods to examine the magnitude of airway inflammation, mucus production, and peribronchial collagen deposition, respectively [15].

2.7. Measurement of lung oxidative stress burden

The degree of reactive oxidative stress was assessed by measuring the ratio of reduced to oxidized glutathione (GSH: GSSG) in lung tissue. Mouse lungs were removed, washed with cold saline, weighed, and stored at -80°C . Lungs were homogenized in 10 ml ice-cold buffer (50 mmol/l phosphate buffer containing 1 mmol/l EDTA) per gram tissue. After centrifugation at 10,000 g for 15 min at 4°C , supernatants were removed and deproteinized. Total GSH and GSSG levels were determined using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. We also evaluated nitrotyrosine levels in lung tissues for detecting nitrosative stress burden using immunohistochemistry. Paraffin-embedded lung tissue sections were deparaffinized and boiled in citrate buffer to retrieve antigens. Slides were blocked with 3% bovine serum albumin (BSA, Sigma) for 1 h at RT and incubated with anti-nitrotyrosine antibodies (Millipore) for 1 h at RT. HRP-conjugated anti-rabbit IgG antibodies (Bethyl Laboratories, Montgomery, TX, USA) were used to detect primary antibodies.

2.8. AMPK activity in lung tissue

AMPK activation was assessed as the ratio of phospho-AMPK to total AMPK following correction for local background intensity and normalization to β -actin. Lung tissue was homogenized in lysis buffer [50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM sodium

pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 50 $\mu\text{g/ml}$ leupeptin, and 50 $\mu\text{g/ml}$ soybean trypsin inhibitor]. Lung tissue proteins were analyzed by 10% SDS-PAGE and Western blotting with mouse monoclonal anti-total AMPK- α antibodies (Cell Signaling technology Inc., Denver, MA, USA) and rabbit monoclonal anti-phospho-AMPK- α at Thr172 (Cell Signaling technology Inc., Denver, MA, USA) antibodies.

2.9. Lung collagen measurements

Collagen levels in lung tissues were determined using a modified Sircol Collagen Assay (Biocolor Ltd., UK) according to the manufacturer's instructions [16]. Briefly, left lung lobes were homogenized and collagen was solubilized in 0.5 M acetic acid. Extracts were incubated with Sirius red dye and absorbencies were determined at 540 nm on an Infinite M200 spectrophotometer (Tecan, Austria). Collagen levels were expressed as g/g of wet tissue.

2.10. In vitro experiments with cultured fibroblasts

The fibroblast cell lines IMR-90 and MRC5 were purchased from ATCC (Manassas, USA) and cultured in Eagle's minimum essential medium (EMEM, Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2 . IMR-90 cells were cultured for 16 h in serum-free media for serum starvation and stimulated with TGF- β 1 (2 ng/ml, R&D Biosystems) for 24 h. Cells were treated with 1 mM metformin and/or 1 μM compound C (Sigma) as an inhibitor of AMPK 30 min before TGF- β 1 stimulation. To knockdown AMPK α 1 expression, human AMPK α 1-specific small interfering RNA (siRNA) duplexes were obtained from Bioneer (Daejeon, Korea). MRC-5 cells were transfected with 10 nM AMPK α 1 siRNA or scrambled siRNA using Lipofectamine RNAiMAX (Invitrogen). After transfection, cells were treated with TGF- β 1 (2 ng/ml) to induce myofibroblast transformation for 24 h. To evaluate the regulation of extracellular matrix expression in fibroblasts, IMR-90 cells cultured on slides were fixed with methanol and blocked with 3% BSA in PBS. Slides were incubated with anti-fibronectin (Abcam, Cambridge, MA, USA) overnight at 4°C and DyLight549-conjugated anti-rabbit

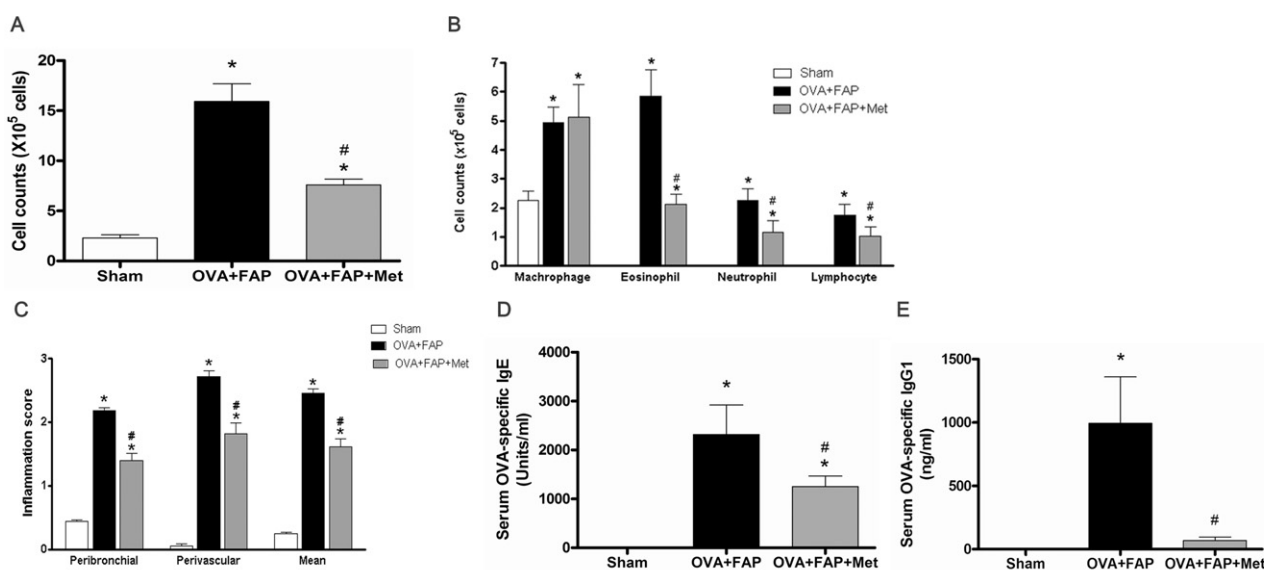


Fig. 2. The effect of metformin on airway inflammation. (A and B) The number of total and differential inflammatory cells in BAL fluid, respectively. (C) Inflammatory scores in lung tissue are shown as the mean of peribronchial and perivascular inflammation scores summed together. (D and E) Serum OVA-specific IgE level and IgG1 level by ELISA. OVA, ovalbumin; FAP, fungal-associated allergenic protease; Met, metformin. Data represent means $\pm$ SEM. $n = 6$. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

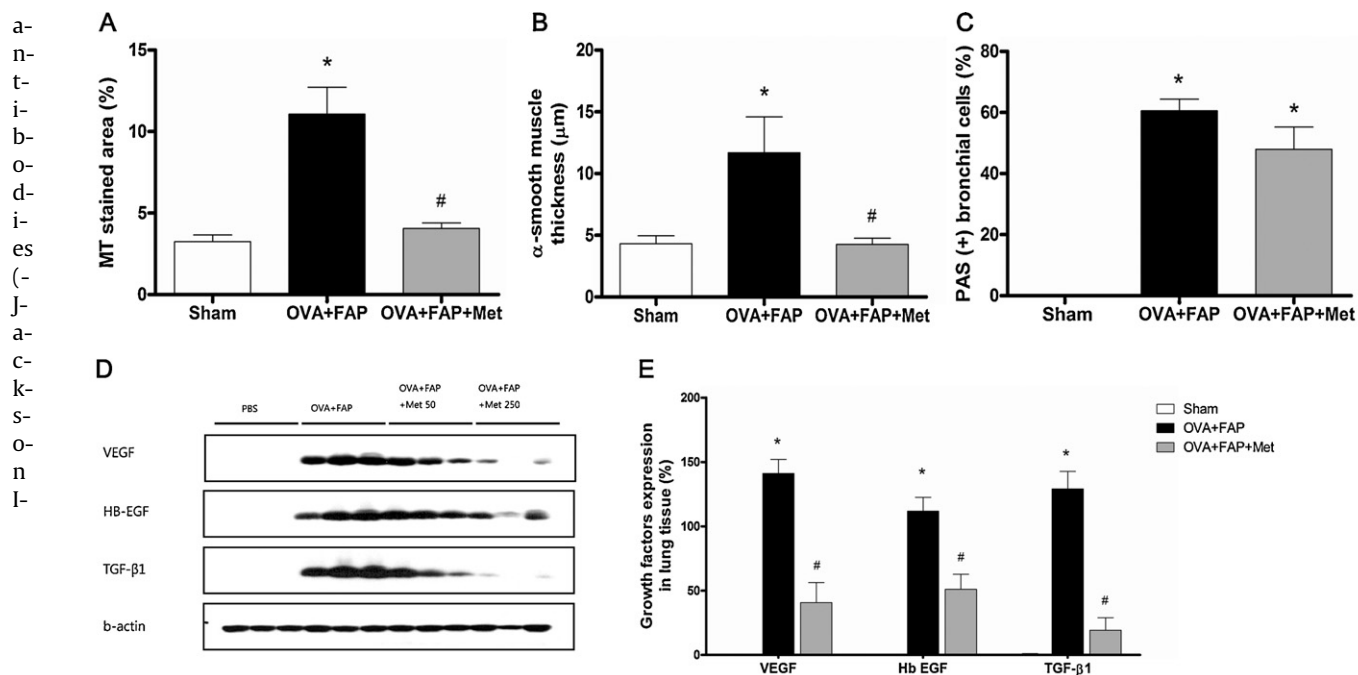


Fig. 3. The effect of metformin on airway remodeling. (A) Masson's trichrome stain. (B) α -smooth muscle actin stain. (C) periodic acid-Schiff (PAS) stain were performed and analyzed by quantitative morphometry using a computerized imaging analysis software system. (D and E) Western blot analysis of growth factors expressions. OVA, ovalbumin; FAP, fungal-associated allergenic protease; Met, metformin. Data represent means $\pm$ SEM. $n = 6$. Each data point is based on data generated from three independent Western blots. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

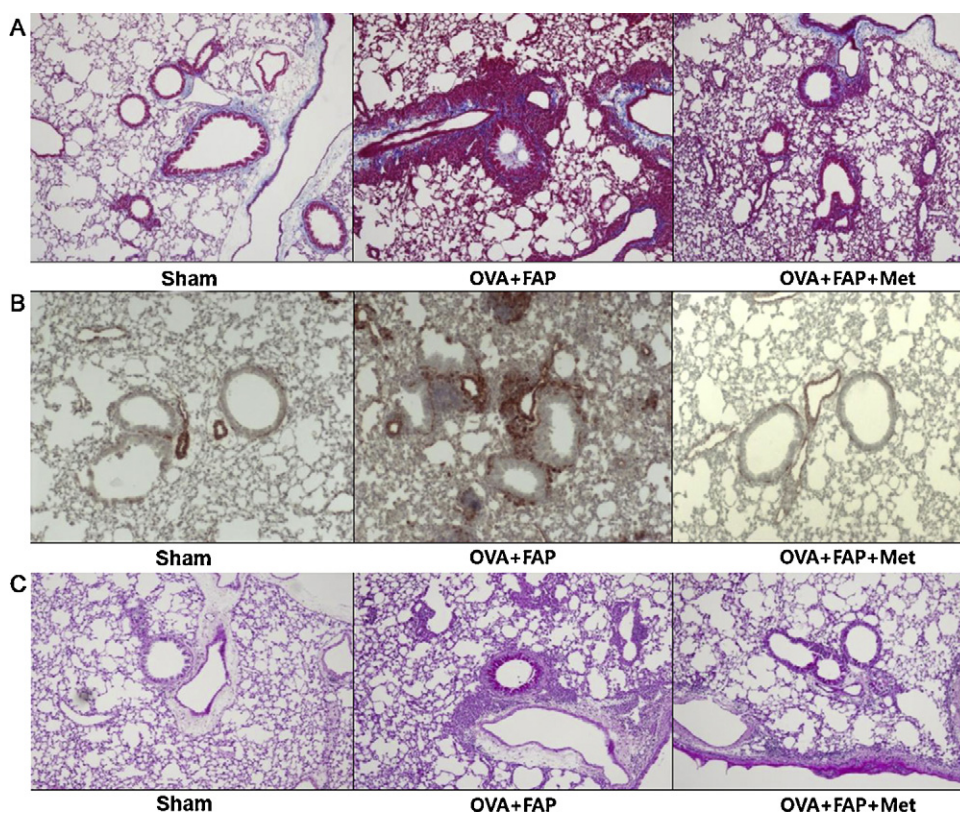


Fig. 4. The effect of metformin on airway remodeling. (A) Masson's trichrome stain, (B) α -smooth muscle actin stain, (C) periodic acid-Schiff (PAS) stains in lung tissue. Representative photomicrographs (10 $\times$). $n = 6$. OVA, ovalbumin; FAP, fungal-associated allergenic protease; met, metformine.

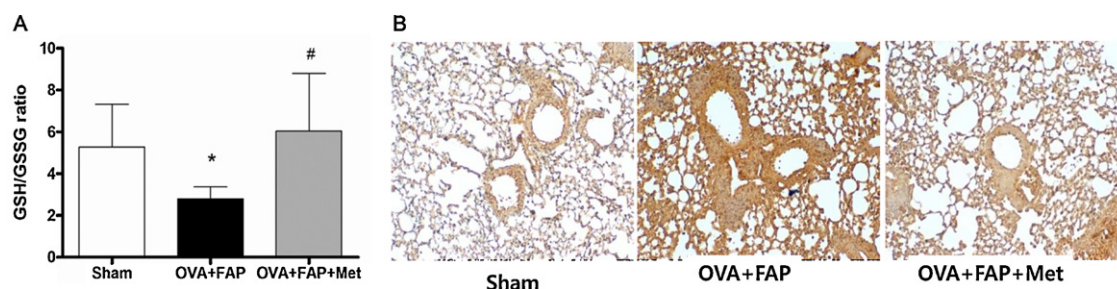


Fig. 5. The effect of metformin on oxidative stress. (A) ELISA analysis of the ratio of reduced to oxidized glutathione (GSH:GSSG) in lung tissue. (B) Immunohistochemical analysis of nitrosative stress in lung tissue. Brown indicates nitrotyrosine. $n = 6$. OVA, ovalbumin; FAP, fungal-associated allergenic protease; Met, metformin. Representative photographs (10 $\times$). * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

immunoresearch laboratories, West Grove, PA, USA) for 1 h at RT in the dark. After nuclei staining with DAPI (Invitrogen, Carlsbad, CA, USA), slides were mounted with a mounting solution (Vector laboratories, Burlingame, CA, USA) and examined by confocal microscopy (Leica, Wetzlar, Germany).

2.11. Statistical analysis

Data are expressed as means $\pm$ SEM. Analysis of variance (ANOVA) was used to determine the levels of difference between groups. For comparison between groups, Mann-Whitney and Kruskal-Wallis tests were used. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Metformin attenuates eosinophilic airway inflammation and remodeling in OVA-and FAP-induced chronic asthma models

We examined whether metformin could reduce allergic airway inflammation in murine models of chronic asthma. Mice challenged with repetitive OVA or FAP had enhanced lung tissue

inflammation and elevated levels of inflammatory cells. With metformin treatment, the number of inflammatory cells in BALF, especially eosinophils, was decreased in metformin-treated mice than that in sham-treated mice (Fig. 2A and B). Consistent with bronchial inflammatory cells, histopathological examination inflammation around the bronchial and vascular areas was significantly diminished (Fig. 2C). Metformin treatments also reduced levels of OVA-specific IgE and IgG1 (Fig. 2D and E). Thus, metformin has anti-inflammatory effects, especially on eosinophilic inflammation, in a chronic asthma model.

To evaluate the effect of metformin on airway remodeling, peribronchial collagen deposition and smooth muscle hypertrophy were assessed and both were significantly reduced in metformin-treated chronic asthmatic mice. Mucus production also tended to decrease (Figs. 3A–C and 4A–C). Additionally, metformin treatment significantly reduced the levels of various growth factors associated with tissue remodeling, including HB-EGF, TGF- β 1, and VEGF, which were increased following repetitive intranasal allergen challenges (Fig. 3D and E). Thus, metformin treatment showed a protective effect on airway remodeling induced by chronic allergen challenges by modulating growth factors expression.

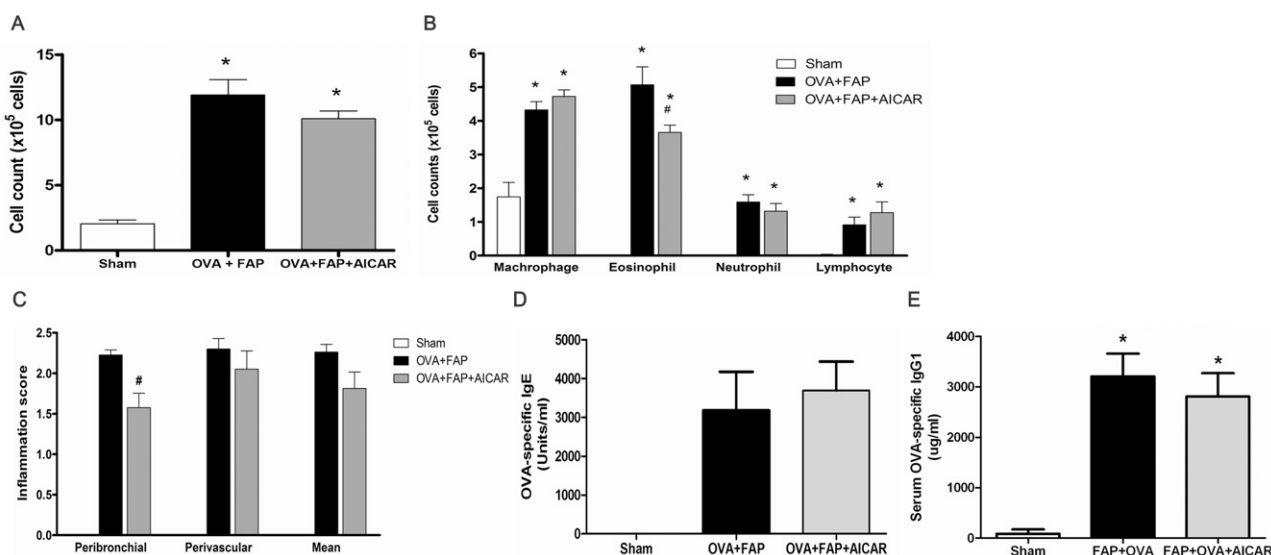


Fig. 6. The effect of AICAR on airway inflammation. (A and B) The number of total and differential inflammatory cells in BAL fluid, respectively. (C) Inflammation scores in lung tissue are shown as the mean of peribronchial and perivascular inflammation scores summed together. (D and E) Serum OVA-specific IgE and IgG1 level by ELISA. OVA, ovalbumin; FAP, fungal-associated allergenic protease; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside. Data represent means $\pm$ SEM. $n = 4$. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

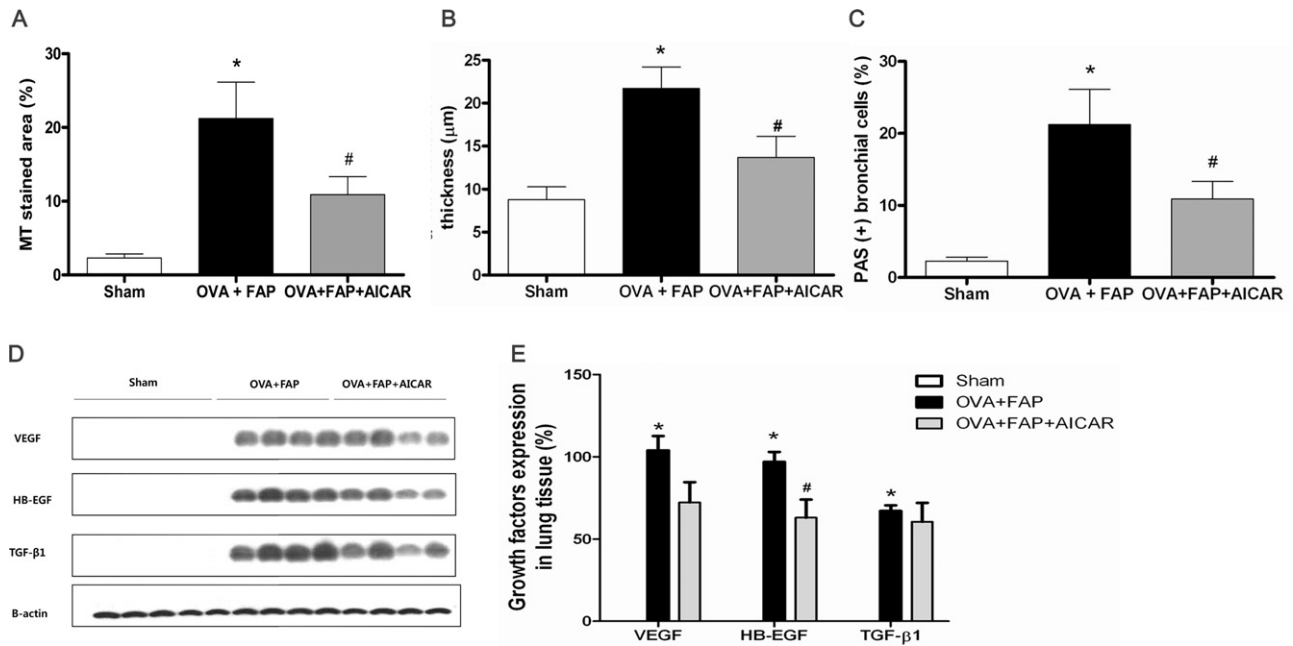


Fig. 7. The effect of AICAR on airway remodeling (A) Masson's trichrome stain. (B) α -smooth muscle actin stain. (C) periodic acid-Schiff (PAS) stain were performed and analyzed by quantitative morphometry using a computerized imaging analysis software system. (D and E) Western blot analysis of growth factors expressions. OVA, ovalbumin; FAP, fungal-associated allergenic protease; AICAR, 5-aminoimidazole-4-carboxamide-1- β -4-ribofuranoside. Data represent means $\pm$ SEM. $n = 4$. Each data point is based on data generated from three independent Western blots. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

Given that oxidative stress is critical in asthma pathogenesis, the effect of metformin on oxidative stress levels was evaluated. Repetitive allergen challenges led to decreased GSH/GSSG ratios in lung tissues and metformin treatments returned the ratios to normal

levels in asthmatic mice. This suggests that metformin plays a role in reducing oxidative stress (Fig. 5A). Immunohistochemical staining for nitrotyrosine in lung tissue showed that nitrosative stress was also mitigated by metformin treatment (Fig. 5B).

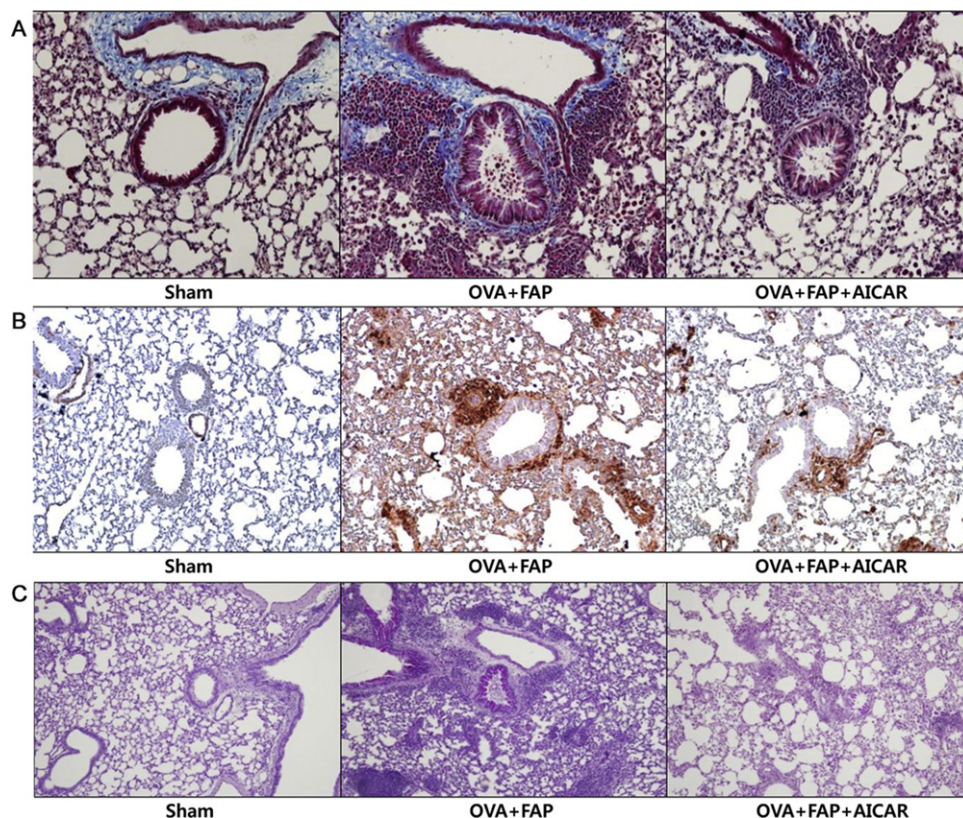


Fig. 8. The effect of AICAR on airway remodeling (A) Masson's trichrome stain. (B) α -smooth muscle actin. (C) Periodic acid-Schiff stains. Representative photomicrographs (10 $\times$). $n = 4$. OVA, ovalbumin; FAP, fungal-associated allergenic protease; AICAR, 5-aminoimidazole-4-carboxamide-1- β -4-ribofuranoside.

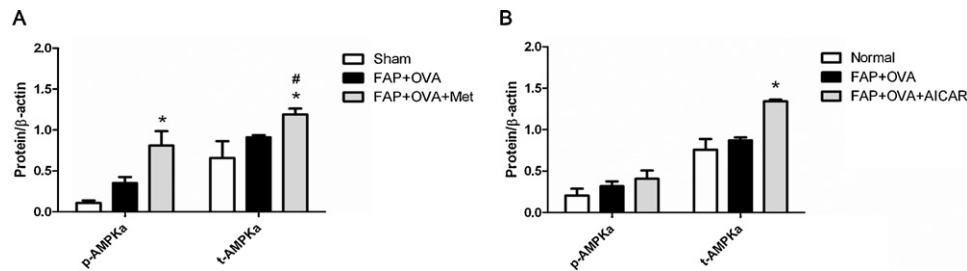


Fig. 9. The effect of AMPK activators on the activation of AMPK. Western blot analysis of phosphorylated (p)-AMPK α 1 and total (t)-AMPK α 1 in lung tissue. (A) Metformin treated chronic mouse model. (B) AICAR treated chronic mouse model. OVA, ovalbumin; FAP, fungal-associated allergenic protease; Met, metformin; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside. Data represent the means $\pm$ SEM. $n = 4-6$. Each data point is based on data generated from three independent Western blots. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

3.2. AMPK is involved in reduced eosinophilic airway inflammation and remodeling in a chronic asthma model

To evaluate whether AMPK activation is crucial for reducing airway inflammation and remodeling, mice were treated with AICAR, an AMPK activator. The number of eosinophils in particular was significantly decreased in BALF following AICAR treatment and

histopathological examination confirmed a reduction in inflammatory cells surrounding the airways (Fig. 6A–C). However, AICAR treatments did not reduce the production of OVA-specific IgE and IgG1 (Fig. 6D and E). AICAR treatment also reduced airway remodeling as the percentage of PAS-positive bronchial cells, the thickness of smooth muscles, and the areas of peribronchial collagen deposition were decreased (Fig. 7A–C and see Fig. 8A–C).

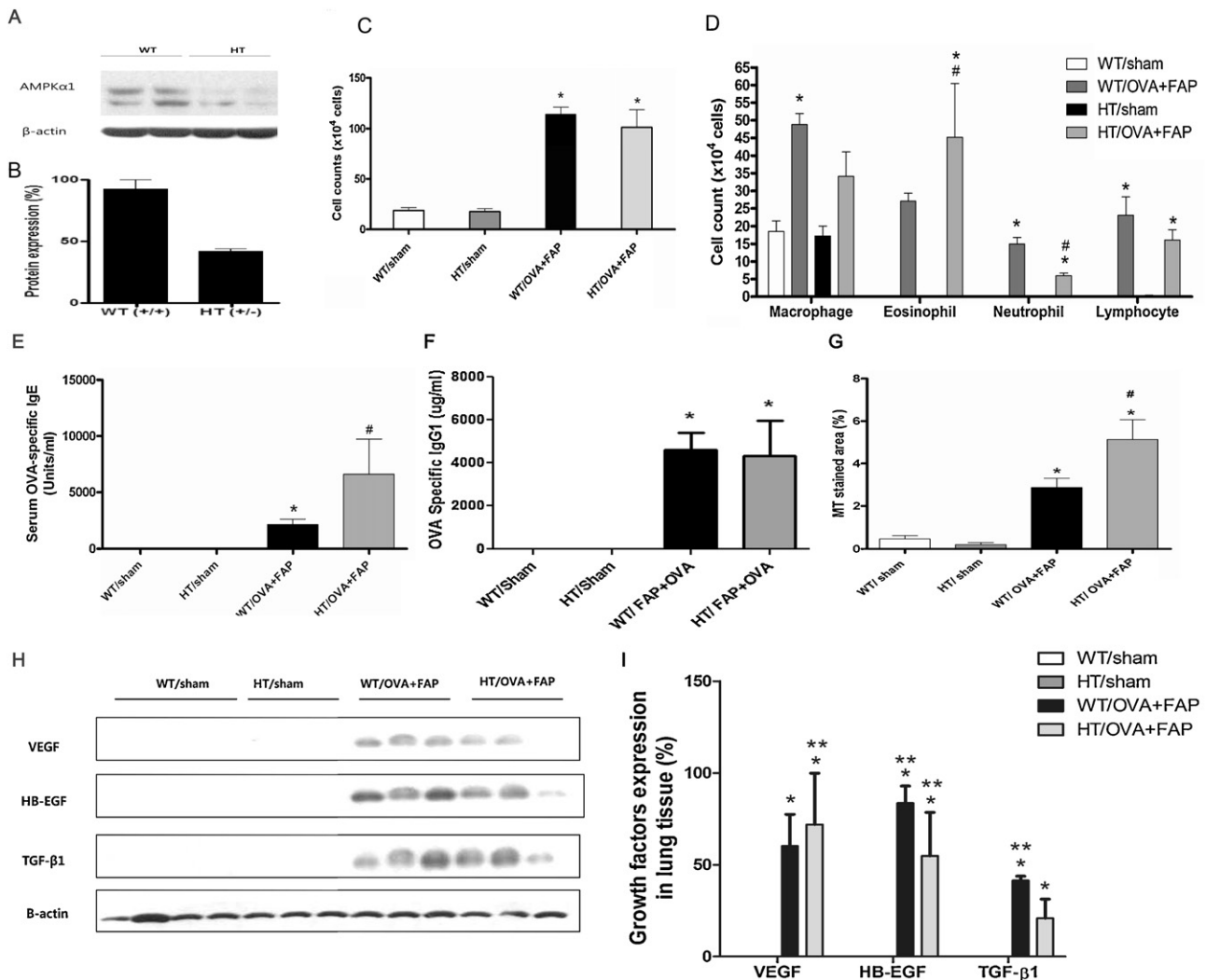


Fig. 10. The effect of AMPK deficiency on allergic inflammation and fibrosis. (A and B) Western blot analysis of the AMPK α 1 activated form in lung tissue. (C and D) The number of total and differential inflammatory cells in BAL fluid, respectively. (E and F) Serum OVA-specific IgE and IgG1 levels by ELISA. (G) Masson's trichrome stains were performed and analyzed by quantitative morphometry using a computerized imaging analysis software system. (H and I) Western blot analysis of growth factors expressions. OVA, ovalbumin; FAP, fungal-associated allergenic protease; HT, heterozygous AMPK α 1-deficient mice; WT, littermate wild-type mice. Data represent means $\pm$ SEM. $n = 3$. Each data point is based on data generated from three independent Western blots. * $p < 0.05$ vs. Sham mice. ** $p < 0.05$ vs. WT mice. # $p < 0.05$ vs. untreated mice.

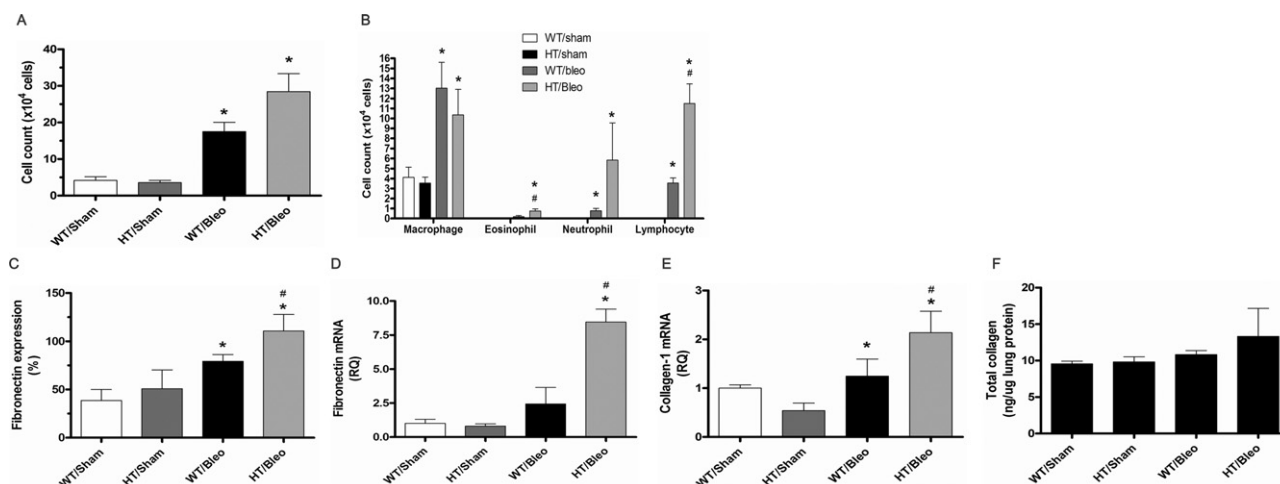


Fig. 11. The effect of AMPK deficiency on bleomycin-induced lung injury. (A and B) The number of total and differential inflammatory cells in BAL fluid, respectively. (C and D) Western blot and real-time PCR analysis of fibronectin expression, respectively. (E and F) Real-time PCR and Sircol assay analysis of collagen expression. HT, heterozygous AMPK α 1-deficient mice; WT, littermate wild-type mice. Data represent means $\pm$ SEM. $n = 4$. Three independent experiments for Western blot, RT-PCR, and Sircol assay. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. untreated mice.

Additionally, AICAR treatment significantly reduced the level of HB-EGF, with signs of decreased levels of VEGF and TGF- β 1 (Fig. 7D and E).

In addition, metformin and AICAR plays a role in AMPK activation, we examined the activation status of AMPK in lung tissues in a chronic asthma model. Total AMPK level was significantly increased in both the metformin- and AICAR-treated group compared to that of sham-treated mice. Metformin treatment also enhanced and phosphorylation of AMPK (Fig. 9A and B).

To elucidate a more precise role of AMPK in the pathogenesis of airway inflammation and remodeling in chronic asthma, AMPK α 1-deficient mice were challenged with OVA and FAP for 8 weeks and their pathologic features were evaluated. The AMPK α 1 protein levels in heterozygous AMPK α 1-deficient (AMPK α 1-HT) mice were approximately 50% of that of WT mice

(Fig. 10A and B). Allergen-challenged AMPK α 1-HT mice showed significantly exaggerated eosinophilic airway inflammation and higher serum IgE levels than did WT mice (Fig. 10C–E). However, there was no difference in the serum IgG1 level between allergen-challenged AMPK α 1-HT mice and WT mice (Fig. 10F). Furthermore, peribronchial fibrosis was more prominent in allergen-challenged AMPK α 1-HT mice than in WT mice (Fig. 10G). A tendency toward enhanced mucin secretion and smooth muscle hypertrophy was observed in allergen-challenged AMPK α 1-HT mice (data not shown). However, the levels of growth factors were no different between WT and AMPK α 1-HT asthmatic mice (Fig. 10H and I). Accordingly, the effect of metformin on asthmatic mice may be at least partially dependent on the AMPK pathway and AMPK could be an important regulator of eosinophilic inflammation and airway remodeling, including airway fibrosis.

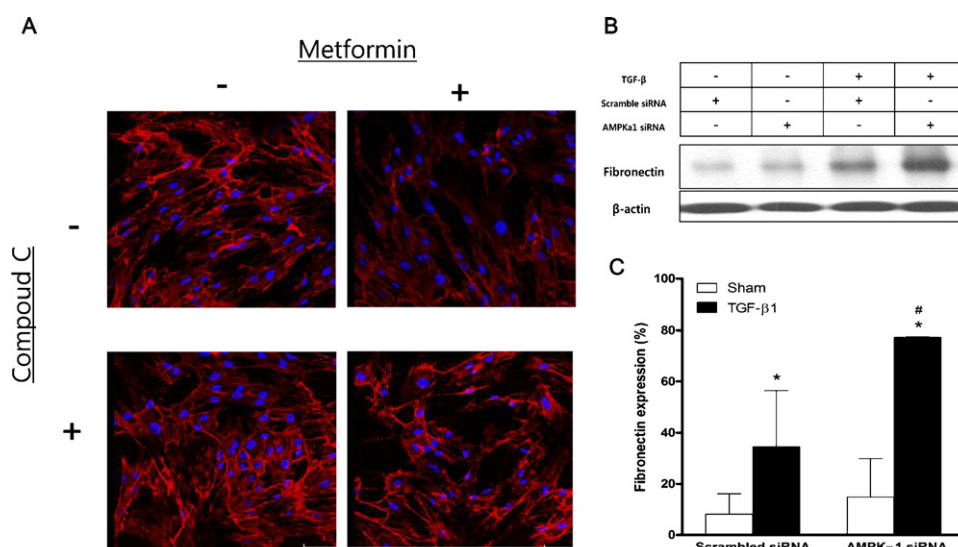


Fig. 12. The role of AMPK in *in vitro* TGF- β 1-induced fibrosis. (A) Human bronchial fibroblasts (IMR-90) were treated with 1 mM metformin and/or 1 μ M compound C 30 min before TGF- β 1 stimulation. Fibronectin expression was assessed using confocal microscopy. Red indicates fibronectin. (B and C) siRNAs knocked down AMPK α 1 expression in human bronchial fibroblasts (MRC-5). Real-time PCR analysis of collagen expression. Representative photomicrographs (10 $\times$) from three independent experiments. Data represent means $\pm$ SEMs. * $p < 0.05$ vs. sham mice. # $p < 0.05$ vs. scrambled siRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

3.3. AMPK negatively regulates eosinophilic inflammation and lung fibrosis in a bleomycin-induced lung injury model

To further elucidate the role of AMPK in reducing lung inflammation and tissue remodeling, such as lung fibrosis induced by stimuli other than allergens, a murine model of bleomycin-induced lung injury was employed. Bleomycin-treated AMPK α 1-HT mice had higher levels of total and BALF inflammatory cells than did bleomycin-treated WT mice (Fig. 11A and B). We further evaluated the effect of AMPK on tissue fibrosis by measuring the expression of molecules critically linked to fibrosis. Fibronectin1 (Fn1) expression in lung tissue were significantly enhanced in bleomycin-treated AMPK α 1-HT mice compared to WT mice (Fig. 11C and D). Total collagen levels in lung tissues as determined by real-time PCR and Sircol assays tended to increase in bleomycin-treated AMPK α 1-HT mice (Fig. 11E and F). Thus, AMPK negatively regulates airway inflammation and fibrosis in lungs treated with other stimuli such as bleomycin as well as typical repeated allergen challenges.

3.4. Metformin decreases TGF- β 1-induced fibronectin expression in cultured fibroblasts through AMPK activation

We evaluated whether metformin reduces TGF- β 1-induced fibrosis by using the human bronchial fibroblast cell lines IMR-90 and MRC-5. To elucidate the precise role of AMPK, the AMPK inhibitor compound C and AMPK α 1-specific siRNA were used. Recombinant human TGF- β 1 (rhTGF- β 1)-induced fibronectin expression was significantly decreased in metformin pre-treated IMR-90 cells compared to sham-treated cells. Furthermore, compound C treatment restored the inhibited fibronectin expression caused by metformin treatment (Fig. 12A). In siRNA AMPK α 1-knockdown MRC-5 cells, TGF- β 1-induced fibronectin expression was significantly enhanced (Fig. 12B and C). Based on these results, it is assumed that the anti-fibrotic effect of metformin at least partially relies on AMPK α 1 activation.

4. Discussion

We have demonstrated that metformin significantly reduces airway inflammation and airway remodeling in a murine model of chronic asthma at least partially through AMPK activation. Thus, metformin may function as a novel anti-asthmatic agent and AMPK may be a target molecule for the development of new drugs targeting both airway inflammation and remodeling in bronchial asthma.

Metformin possesses various properties other than its anti-diabetic effects. Several *in vitro* and *in vivo* studies indicate that metformin has anti-inflammatory effects, functions as an antioxidant, and attenuates cellular proliferation and tissue remodeling in various diseases [7,17–20]. Metformin also decreases the secretion of pro-inflammatory mediators, such as IL-6, TNF- α , fibrinogen, and soluble vascular cell adhesion molecule-1, in serum from patients with dyslipidemia and/or diabetes [21,22]. Additionally, metformin attenuates vascular remodeling and prevents cardiac hypertrophy [12,23,24] as well as improves cardiac fibrosis *in vivo* by inhibiting collagen synthesis [11]. Despite the growing evidence of the effects of metformin on tissue remodeling, there have been few studies that demonstrate the role of metformin in the pathogenesis of airway remodeling [24,25].

In the current study, metformin significantly suppressed airway inflammation in a murine chronic asthma model. Eosinophilic infiltration, which is characteristic of asthma and is critically linked to airway remodeling, was greatly reduced following metformin treatment. Furthermore, metformin treatment induced a significant reduction in pivotal features of airway remodeling, including

peribronchial fibrosis areas and smooth muscle layer thickness. Consistent with our results, metformin has been reported to reduce pro-inflammatory cytokine levels *in vitro* in human bronchial epithelial cells [7]. Taken together, it is presumed that metformin affects both airway inflammation and airway remodeling in asthma.

Although further studies are needed to determine how metformin may function as an anti-asthmatic drug, our study demonstrated the association between the effect of metformin and AICAR on modulating airway pathology of asthmatic mice and reduced expression of various growth factors such as VEGF, HB-EGF, and TGF- β 1. Consistent with our result, recent report demonstrated that AICAR ameliorated airway inflammation in toluene diisocyanate (TDI) induced murine model of asthma by regulating hypoxia-inducible factor (HIF)/VEGF pathway [26]. Although the levels of these three growth factors were no different between WT and AMPK α 1-HT asthmatic mice, one explanation may be that the mice used in this study were only partially deficient in AMPK. Further experiments that will elucidate the relationship between AMPK deficiency and the expression of HB-EGF, VEGF, and TGF- β 1 have been planned using AMPK knockout mice. Metformin also showed anti-oxidative function as oxidative and nitrosative stress was reduced in lung tissues from metformin-treated mice. Despite debates about whether oxidative stress is a simple consequence of inflammation or an important contributor, numerous studies suggest that oxidative stress is critically involved in the pathogenesis of asthma including severe refractory asthma [27–30].

To further investigate the functional mechanism of metformin, we hypothesized that AMPK plays a role in reducing airway inflammation and remodeling because metformin is known to be a pharmacological AMPK activator. Here, we showed that metformin enhanced AMPK activation in lung tissue. Metformin activates AMPK and ameliorates pro-inflammatory activities in several cell types, including hepatocytes, adipocytes, smooth muscle cells, and various inflammatory cells [7,20,31]. Metformin increases AMP levels by inhibiting AMP deaminase [32]. With regard to inflammation modulation, AMPK directly suppresses pro-inflammatory responses and promotes macrophage polarization to an anti-inflammatory phenotype [33]. Furthermore, additional evidence indicates that AMPK is also a negative regulator of protein synthesis, resulting in the inhibition of cellular proliferation and protein and DNA synthesis. Moreover, AMPK is a suppressor of NADPH oxidases that generate oxidative stress [10,34]. Here, metformin suppressed oxidative stress levels in lung tissue as assessed by GSH/GSSG ratios and nitrosative protein expression, which may be associated with AMPK activation. In summary, AMPK is a critical molecule linked to the various anti-asthmatic effects of metformin, such as its anti-inflammatory, anti-oxidative, and anti-tissue remodeling properties.

To further investigate the role of AMPK in alleviating various features of chronic asthma, the effect of AICAR, another AMPK activator, on airway inflammation and remodeling was evaluated. We previously reported that the administration of AICAR significantly attenuated AHR and airway inflammation in a poly(I:C)-treated acute asthma murine model [14]. Another study reported that AICAR administration resulted in dose-dependent inhibition of human aortic smooth muscle cell proliferation and neointimal hyperplasia [35]. Recent studies have also demonstrated that AMPK activators control the balance between CD4⁺ T helper cells and inducible regulatory T (T_{reg}) cells in different asthma models [26,36]. In the current study, AICAR also reduced airway inflammation and key features of airway remodeling, which was similar to the results of metformin-treated asthmatic mice. Additional experiments were also performed using AMPK α 1-deficient mice. Repeated airway challenge with antigens caused

significantly increased eosinophilic inflammation and more prominent peribronchial fibrosis in AMPK α 1-deficient mice compared with WT mice. Collectively, these data indicate a potentially pivotal role of AMPK in the pathogenesis of airway inflammation and remodeling in chronic asthma, although our current data do not provide a clear mechanistic explanation for the effect of AMPK activators on allergic airway inflammation.

AMPK has been known to inhibit myofibroblast transdifferentiation (MET), a fundamental cellular program associated with embryonic development, tumor metastasis, wound repair, and fibrosis in various tissues [37–39]. Therefore, we conducted experiments using a bleomycin-induced acute lung injury model, the best available experimental tool for studying pulmonary fibrosis [40], to elucidate the effect of AMPK on fibrosis, a typical structural characteristic of airway remodeling. Lung inflammation was increased and fibronectin and collagen-1 expression was enhanced in bleomycin-treated AMPK α 1-deficient mice compared to WT mice. With these results, it is suggested that AMPK play a critical protective role in airway fibrosis induced by other stimuli as well as allergens. Furthermore, an AMPK α 1 inhibitor reversed the inhibitory effect of metformin on TGF- β 1-induced fibronectin production and fibronectin expression in AMPK α 1-knockdown fibroblast cell lines in this study. These results are consistent with a previous study that demonstrated that AMPK activation suppresses TGF- β 1-induced production of collagen IV, fibronectin, and α SMA in human primary mesangial cells [38]. Therefore, AMPK activation may modulate the tissue fibrosis observed in various chronic inflammatory respiratory diseases. However, the precise intracellular mechanism of AMPK regulation of tissue fibrosis will be the focus of further studies.

In the present study, we found some discrepancies between metformin- and AICAR-treated asthma models regarding the extent of anti-inflammatory effects and effect on airway remodeling. Metformin had a more potent anti-inflammatory effect than AICAR. In addition, there was no significant reduction found in the serum OVA-specific IgE and IgG1 levels from the AICAR-treated group, whereas AICAR seems to have more pronounced effects on goblet cell hyperplasia than those in the metformin-treated group. One possible explanation is that the anti-inflammatory effect induced by different AMPK activators, metformin and AICAR, may result, at least in part, from the AMPK-dependent pathway, with other AMPK-independent pathways, including modulation of oxidative stress or other undisclosed mechanisms, still contributing to the anti-asthmatic efficacy observed in the current study. Additional experiments that investigate the precise mechanism that underlies this phenomenon could provide more decisive insight into the differences between metformin and AICAR.

Meanwhile, in regard to the clinical application of metformin in asthma treatment, one potential side effect is bronchial edema associated with the inhibition of epithelial Na⁺ channel (ENaC). In lung, transepithelial Na⁺ transport via the ENaC control the volume of the fluid layer that lines the airway. Recent studies reported that AMPK agonists, including metformin and AICAR, decrease the activity of ENaC [7,41]. The chronic inhibition of ENaC might aggravate bronchial edema therefore may be harmful by asthma. However, the results from both studies are based on *in vitro* experiments. In addition, one study showed that metformin was much less potent than AICAR in reducing transepithelial Na⁺ transport. Further studies will be needed to establish the clinical effect of ENaC inhibition following metformin treatment.

In conclusion, this study demonstrated that metformin can improve airway inflammation and remodeling, thus indicating the potential of metformin to be a novel therapeutic drug for asthmatic patients. Furthermore, the underlying mechanism of metformin may be closely related to AMPK α 1 activation. Therefore, AMPK

may be an attractive target molecule for novel drugs for regulating chronic inflammation and related tissue fibrosis.

Author contributions

Conception and design, Chan Sun Park, Ki-Young Lee, Hee-Bom Moon, and You Sook Cho.; analysis and interpretation, Chan Sun Park, Bo-Ram Bang, Hyouk-Soo Kwon, and Keun-Ai Moon.; drafting the manuscript for important intellectual content, Chan Sun Park, Bo-Ram Bang, and You Sook Cho.; revising the manuscript for important intellectual content, Chan Sun Park, Hyouk-Soo Kwon, Tae-Bum Kim, Ki-Young Lee and You Sook Cho.; and final approval of the manuscript, Chan Sun Park, Bo-Ram Bang, Hyouk-Soo Kwon, Tae-Bum Kim, Ki-Young Lee, Hee-Bom Moon, and You Sook Cho.

Conflict of interest

There is no other relationships/conditions/circumstances that present a potential conflict of interest.

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References

- [1] Kelly M, O'Connor T, Leigh R, Otis J, Gwozd C, Gauvreau G, et al. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J Allergy Clin Immunol* 2010;125:349–56.
- [2] Benayoun L, Druilhe A, Dombret M-C, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360–8.
- [3] Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β 1, IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293–8.
- [4] Molet SM, Hamid QA, Hamilos DL. IL-11 and IL-17 expression in nasal polyps: relationship to collagen deposition and suppression by intranasal fluticasone propionate. *Laryngoscope* 2003;113:1803–12.
- [5] Arai M, Uchida M, Komura H, Mizuuchi Y, Harada N, Okajima K. Metformin, an antidiabetic agent, suppresses the production of tumor necrosis factor and tissue factor by inhibiting early growth response factor-1 expression in human monocytes *in vitro*. *J Pharmacol Exp Ther* 2010;334:206–13.
- [6] Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* 2006;47:1183–8.
- [7] Myerburg MM, King JD, Oyster NM, Fitch AC, Magill A, Baty CJ, et al. AMPK agonists ameliorate sodium and fluid transport and inflammation in cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol* 2010;42:676–84.
- [8] Tsoyi K, Jang HJ, Nizamutdinova IT, Kim YM, Lee YS, Kim HJ, et al. Metformin inhibits HMGB1 release in LPS-treated RAW 264.7 cells and increases survival rate of endotoxaemic mice. *Br J Pharmacol* 2011;162:1498–508.
- [9] Nath N, Khan M, Paintlia MK, Hoda MN, Giri S. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. *J Immunol* 2009;182:8005–14.
- [10] Song P, Zou M-H. Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free Radic Biol Med* 2012;52:1607–19.
- [11] Xiao H, Ma X, Feng W, Fu Y, Lu Z, Xu M, et al. Metformin attenuates cardiac fibrosis by inhibiting the TGF β 1–Smad3 signalling pathway. *Cardiovasc Res* 2010;87:504–13.
- [12] Sasaki H, Asanuma H, Fujita M, Takahama H, Wakeno M, Ito S, et al. Metformin prevents progression of heart failure in dogs: role of AMP-activated protein kinase. *Circulation* 2009;119:2568–77.
- [13] Kheradmand F, Kiss A, Xu J, Lee SH, Kolattukudy PE, Corry DB. A protease-activated pathway underlying Th cell type 2 activation and allergic lung disease. *J Immunol* 2002;169:5904–11.
- [14] Kim TB, Kim SY, Moon KA, Park CS, Jang MK, Yun ES, et al. Five-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside attenuates poly (I:C)-induced airway inflammation in a murine model of asthma. *Clin Exp Allergy* 2007;37:1709–19.
- [15] Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Lee HK, et al. Modulation of airway remodeling and airway inflammation by peroxisome proliferator-activated receptor γ in a murine model of toluene diisocyanate-induced asthma. *J Immunol* 2006;177:5248–57.

- [16] Udalov S, Dumitrascu R, Pullamsetti SS, Al-tamari HM, Weissmann N, Ghofrani HA, et al. Effects of phosphodiesterase 4 inhibition on bleomycin-induced pulmonary fibrosis in mice. *BMC Pulm Med* 2010;10:26.
- [17] Bai A, Ma AG, Yong M, Weiss CR, Ma Y, Guan Q, et al. AMPK agonist down-regulates innate and adaptive immune responses in TNBS-induced murine acute and relapsing colitis. *Biochem Pharmacol* 2010;80:1708–17.
- [18] Bai A, Yong M, Ma AG, Ma Y, Weiss CR, Guan Q, et al. Novel anti-inflammatory action of 5-aminoimidazole-4-carboxamide ribonucleoside with protective effect in dextran sulfate sodium-induced acute and chronic colitis. *J Pharmacol Exp Ther* 2010;333:717–25.
- [19] Bergheim I, Luyendyk JP, Steele C, Russell GK, Guo L, Roth RA, et al. Metformin prevents endotoxin-induced liver injury after partial hepatectomy. *J Pharmacol Exp Ther* 2006;316:1053–61.
- [20] Zhao X, Zmijewski JW, Lorne E, Liu G, Park YJ, Tsuruta Y, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L497–504.
- [21] Gomez-Garcia A, Martinez Torres G, Ortega-Pierres LE, Rodriguez-Ayala E, Alvarez-Aguilar C. Rosuvastatin and metformin decrease inflammation and oxidative stress in patients with hypertension and dyslipidemia. *Rev Esp Cardiol* 2007;60:1242–9.
- [22] Lund SS, Tarnow L, Stehouwer CDA, Schalkwijk CG, Teerlink T, Gram J, et al. Impact of metformin versus repaglinide on non-glycaemic cardiovascular risk markers related to inflammation and endothelial dysfunction in non-obese patients with type 2 diabetes. *Eur J Endocrinol* 2008;158:631–41.
- [23] El Messaoudi S, Rongen GA, de Boer RA, Riksen NP. The cardioprotective effects of metformin. *Curr Opin Lipidol* 2011;22:445–53.
- [24] Ratnovsky A, Mellema M, An SS, Fredberg JJ, Shore SA. Airway smooth muscle proliferation and mechanics: effects of AMP kinase agonists. *Mol Cell Biomech* 2007;4:143–57.
- [25] Shore SA, Williams ES, Zhu M. No effect of metformin on the innate airway hyperresponsiveness and increased responses to ozone observed in obese mice. *J Appl Physiol* 2008;105:1127–33.
- [26] Park SJ, Lee KS, Kim SR, Chae HJ, Yoo WH, Kim DI, et al. AMPK activation reduces vascular permeability and airway inflammation by regulating HIF/VEGFA pathway in a murine model of toluene diisocyanate-induced asthma. *Inflamm Res* 2012;61:1069–83.
- [27] Cho YS, Moon H-B. The role of oxidative stress in the pathogenesis of asthma. *Allergy Asthma Immunol Res* 2010;2:183–7.
- [28] Cieniewicz J, Trivedi S, Kleeberger SR. Oxidants and the pathogenesis of lung diseases. *J Allergy Clin Immunol* 2008;122:456–68.
- [29] Kaminsky DA, van der Vliet A, Janssen-Heininger Y. Reactive nitrogen species in refractory asthma: markers or players. *J Allergy Clin Immunol* 2008;121:338–40.
- [30] Wenzel SE, Busse WW. Severe asthma: lessons from the severe asthma research program. *J Allergy Clin Immunol* 2007;119:14–21.
- [31] Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* 2005;310:1642–6.
- [32] Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. *J Biol Chem* 2011;286:1–11.
- [33] Sag D, Carling D, Stout RD, Suttles J. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J Immunol* 2008;181:8633–41.
- [34] Eid AA, Ford BM, Block K, Kasinath BS, Gorin Y, Ghosh-Choudhury G, et al. AMP-activated protein kinase (AMPK) negatively regulates Nox4-dependent activation of p53 and epithelial cell apoptosis in diabetes. *J Biol Chem* 2010;285:37503–12.
- [35] Igata M, Motoshima H, Tsuruzoe K, Kojima K, Matsumura T, Kondo T, et al. Adenosine monophosphate-activated protein kinase suppresses vascular smooth muscle cell proliferation through the inhibition of cell cycle progression. *Circ Res* 2005;97:837–44.
- [36] Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol* 2011;186:3299–303.
- [37] Caligiuri A, Bertolani C, Guerra CT, Aleffi S, Galastri S, Trappoliere M, et al. Adenosine monophosphate-activated protein kinase modulates the activated phenotype of hepatic stellate cells. *Hepatology* 2008;47:668–76.
- [38] Mishra R, Cool BL, Laderoute KR, Foretz M, Viollet B, Simonson MS. AMP-activated protein kinase inhibits transforming growth factor-beta-induced Smad3-dependent transcription and myofibroblast transdifferentiation. *J Biol Chem* 2008;283:10461–69.
- [39] Subramaniam N, Sherman M, Rao R, Wilson C, Coulter S, Atkins AR, et al. Metformin-mediated Bambi expression in hepatic stellate cells induces pro-survival Wnt/beta-catenin signaling. *Cancer Prev Res* 2012;5:553–61.
- [40] Mouratis MA, Aidinis V. Modeling pulmonary fibrosis with bleomycin. *Curr Opin Pulm Med* 2011;17:355–61.
- [41] Albert AP, Woollhead AM, Mace OJ, Baines DL. AICAR decreases the activity of two distinct amiloride-sensitive Na⁺-permeable channels in H441 human lung epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L837–L848.