Repositioning of Ezetimibe to treatment of idiopathic pulmonary fibrosis via mTORC1-autophagy axis

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ABSTRACT

Repositioning of Ezetimibe to treatment of idiopathic pulmonary fibrosis via mTORC1-autophagy axis

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Although idiopathic pulmonary fibrosis (IPF) has an overall poor prognosis, there have been no proven effective therapeutic strategies. Here, we investigated the potential capacity of ezetimibe, a FDA-approved lipid-lowering agent, for IPF treatment. Ezetimibe decreased COL1A1 expression proportional to the treatment dose and time without inducing cytotoxicity in lung fibroblasts. Ezetimibe increased the LC3B-II to LC3B-I ratio and quantities of GFP-LC3B puncta in mouse lung fibroblast; this finding suggests that ezetimibe may promote autophagy flux. Additionally, autophagic inhibition by chloroquine accumulated COL1A1 in lung fibroblasts and averted the activity of ezetimibe. Treatment of ezetimibe in lung fibroblasts ameliorated TGF-β1-induced phosphorylation of p70S6k and RPS6, indicating that ezetimibe inhibited mTORC1 activation. Moreover, ezetimibe prevented fibrosis progression via autophagy activation in a bleomycin induced lung fibrosis mouse model in vivo. We demonstrated that ezetimibe ameliorated lung fibrosis

via induction of autophagy flux by down-regulating the mTORC1 signaling pathway. We suggest that ezetimibe can be potentially repurposed for the treatment of IPF.

Key words : IPF, autophagy, mTORC1, ezetimib

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I. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrotic interstitial lung disease, characterized by excessive production and deposition of extracellular matrix and remodeling of abnormal lung tissue structure[1-3]. IPF has been considered a rare disease; however, its prevalence and incidence have increased in recent years[4-6]. Despite recent advance, IPF has a poor prognosis, with a median survival of 3–4 years[7,8]. Two antifibrotic drugs, pirfenidone and nintedanib, have been shown to slow IPF progression[9-11], and increase survival[12-14]. However, the only curative option is lung transplant[5,15], and treatment options are still limited. Accordingly, there is an urgent need to explore the pathophysiology of IPF and identify strategies for its prevention and treatment.

The profibrotic role of transforming growth factor- β1 (TGF-β1) in IPF has been demonstrated in previous studies[16-18]. TGF-β1 induces macrophage and fibroblast recruitment as well as fibroblasts proliferation[16]. TGF-β1 also stimulates the expression of proinflammatory and fibrogenic cytokines and excessive extracellular matrix production,

leading to the destruction of the lung architecture[16,19,20]. Although the exact mechanisms of TGF-β1 in IPF are not fully understood, recent studies have demonstrated that pulmonary fibrosis is related to insufficient autophagy inhibition by TGF-β1 signaling[21]. Autophagy is a cellular process that degrades damaged organelles, which plays an improtant role in the homeostatic process[22-24]. An increasing number of studies have shown that TGF-β1/non-Smad pathways, incluiding the mTOR signaling pathway, inhibits autophagy[25-29]. Therefore, inibition of the mTOR signaling pathway may activate autophagy, leading to anti-fibrotic effects.

Ezetimibe is a FDA-approved lipid-lowering agent that is prescribed to patients with hyperlipidemia[30]. A recent study suggested that ezetimibe treatment might lead to an improvement in hepatic steatosis[31]. Another study demonstrated that ezetimibe exhibited pharmacological activities in hepatic steatosis via autophagy activation[32]. According to this study, ezetimibe is thought to ameliorate hepatic steatosis, inflammation, and fibrosis by inducing autophagic activation[32].

However, the anti-fibrotic effects of ezetimibe in lung tissue have not yet been investigated. Therefore, this study aimed to assess the potential therapeutic role of ezetimibe on IPF *in vivo* and *in vitro*, and evaluate whether the mTOR-autophagy axis is involved in the pathogenesis.

II. MATERIALS AND METHODS

1. Bleomycin induced mouse lung fibrosis model

Male mice aged 7~9 weeks were used for the experiments. Briefly, 2U/kg of bleomycin (Sigma-Aldrich #B5507; resolved in 50uL sterile normal saline) or injection-quality normal saline was administered to each mouse by oropharyngeal instillation under 4% isoflurane-induced anesthesia. The mice were randomly divided into three groups: control (N=23), bleomycin (N=25), and ezetimibe-treated (N=25) groups. Ezetimibe was administered to each mouse three times a week from day 7 to day 21. Body weight was measured three times a week, including the day of the intervention. For bleomycin-aspirated mice, samples and data were excluded from further analysis if the body weight change was ≤ 5% from baseline until day seven. Twenty-one days after the instillation, the mice were anesthetized and euthanized for tissue harvest. The lungs were perfused with 6 mL of ice-cold sterile phosphate buffered saline, and processed as described below.

2. Animal subjects

Six-to seven-week-old C57BL/6J male mice were purchased from Japan SLC Inc. (SLC-M-0133). Green fluorescent protein-light chain 3B (GFP-LC3B) transgenic mice were kindly provided by Dr. Y.H. Lee and J.W. Ryu, and RFP-GFP-LC3B transgenic mice were provided by Dr. Y.W. Chung and J.W. Ryu. All experimental procedures in this study were approved by Yonsei University Health System Institutional Animal Care and Use Committee (IACUC number: 2020-0099).

3. Primary lung fibroblast isolation and culture

Human and mouse primary lung fibroblast were isolated and cultured as described previously[33,34]. Aseptically harvested lungs were minced into 1mm pieces, and digested with Liberase™ TM for 40 minutes. After a few rinses with culture media, the tissue pieces were cultured under a 3% hypoxic environment, and the resulting fibroblasts were subcultured until passage three. At least 95% of lung fibroblasts were stained positive of vimentin and negative of pan-cytokeratin on immunofluorescence (data not shown).

Human lung specimens were obtained from patients who underwent lobectomy or pneumonectomy for clinical necessities. Only remnant non-neoplastic sections were used in this study. All patients who participated in this study provided informed consent. All procedures and protocol were approved by the Institutional Review Board at Severance Hospital (IRB number: 4-2019-0447). Human lung fibroblasts were used for experiments before passage eight. For mouse fibroblasts, the right and left lungs of 6 to 8 weeks old male mice were used. Mouse fibroblasts were used for experiments before passage four.

4. Cytotoxicity assay

Lung fibroblasts were seeded at a density of 2~4 x 10^4 cells/well in a final volume of 100uL in 96-well plates, and incubated overnight. The culture media was aspirated, and changed into media containing variable doses of ezetimibe. After 24 hours of incubation, cell viability was measured using a CellTiter-Glo Luminescent cell viability assay kit (G7570, Promega Corporation) according to the manufacturer's protocol.

5. Immunoblot analysis

Samples were lysed in ice-cold lysis buffer containing 20 mM Hepes-KOH (pH 7.9), 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 0.3% Triton X-100, 10% glycerol, 5mM NaF, 1mM Na3VO4, 1mM EDTA, aprotinin, leupeptin, and PMSF. The right lobes of the mouse lungs were homogenized with TissueLyser II. The resulting lysates were centrifuged for 15

minutes at 13,500 rpm and the supernatants were analyzed with Bradford assay to determine the sample concentrations. The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to polyvinylidene fluoride membranes (PVDF; IPVH00010, MERK Millipore). After blocking with 5% skim milk in Tris-buffered saline and 0.1% Tween 20 (TBS-T), the membranes were then incubated with specific primary antibodies at 4 ℃ overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Proteins were visualized using an enhanced chemiluminescence solution (34580, Thermo Scientific).

6. Quantitative RT-PCR analysis

Total RNA from cultured cells or lung tissue was prepared using TRIzol™ Reagent (15596018, Invitrogen) according to the manufacturer's protocol. After quantitation with a NanoDrop 2000, 1μg of RNA was used for cDNA synthesis using the PrimeScript™ RT Master Mix (RR036A, Takara). The resulting cDNA was subjected to quantitative RT-PCR analysis using SYBR™ Green PCR Master Mix (4301955, Applied Biosystems™) and specific paired primers for each genes.

7. Collagen assay

The amount of soluble collagen in the right lungs of each mouse was quantified using the Sircol Soluble Collagen Assay (S1000, Biocolor Ltd.) according to the manufacturer's protocol.

8. Histological analysis

The lungs were intra-tracheally infused with low-melting agarose, immersed in 10%

formalin for 24hrs, and then embedded in paraffin. Lung architecture was evaluated using right-field microscopy. Masson's trichrome staining was used to histologically assess fibrosis, and the severity of lung fibrosis was quantified by using a collagen assay.

9. RFP-GFP-LC3 autophagic flux assay

To detect autophagosomes and autolysosomes, mouse lung fibroblasts isolated from RFP-GFP-LC3 transgenic mice were cultured on coverslips, and treated with the indicated drug and protein for 24 hours. The cells were fixed and stained with ProLong™ Glass Antifade Mountant with NucBlue™ Stain. Fluorescent proteins were imaged using an LSM 780 confocal microscope at 63x objective magnification. The number of vesicular structures was counted semi-automatically using Imaris 9.4.1

10. Statistical analysis

Analysis of variance (ANOVA) and Tukey's multiple comparisons test were performed using the build-in analysis package in Prism 9.4.0 (GraphPad LLC, San Diego, CA).

III. RESULTS

1. Ezetimibe inhibits TGF-β1 induced myofibroblast differentiation without cytotoxicity in lung fibroblasts.

The distribution of Niemann-Pick C1 like intracellular cholesterol transporter 1 (NPC1L1) mRNA in mouse tissues was evaluated (Figure 1). NPC1L1 was predominantly expressed in the small intestine, and was also detectable in the liver, lung, and lung fibroblasts. As NPC1L1 is a cholesterol absorption transporter that appears to be the target of ezetimibe[35,36], these results suggest that ezetimibe may play a role in lung tissue.

To determine whether ezetimibe exerts an inhibitory effect on TGF-β1-induced differentiation of fibroblasts into myofibroblasts, the expression of COL1A1 was examined in both primary human and mouse lung fibroblasts. Western blot analysis demonstrated that ezetimibe decreased COL1A1 expression in proportion to the treatment dose and time (Figure 2A and 2C). Consistent with the results of the western blot analysis, RT-qPCR analysis also demonstrated that administration of ezetimibe resulted in a decline in COL1A1 mRNA level in both a dose and time dependent manner, respectively (Figure 2B and 2D). The MTT assay was performed to evaluate the effect of ezetimibe on the proliferation viability. The cells were exposed to the indicated doses of ezetimibe (0-100uM) for 24h. The results showed that ezetimibe exhibited no evident cytotoxicity (Figure 2E).

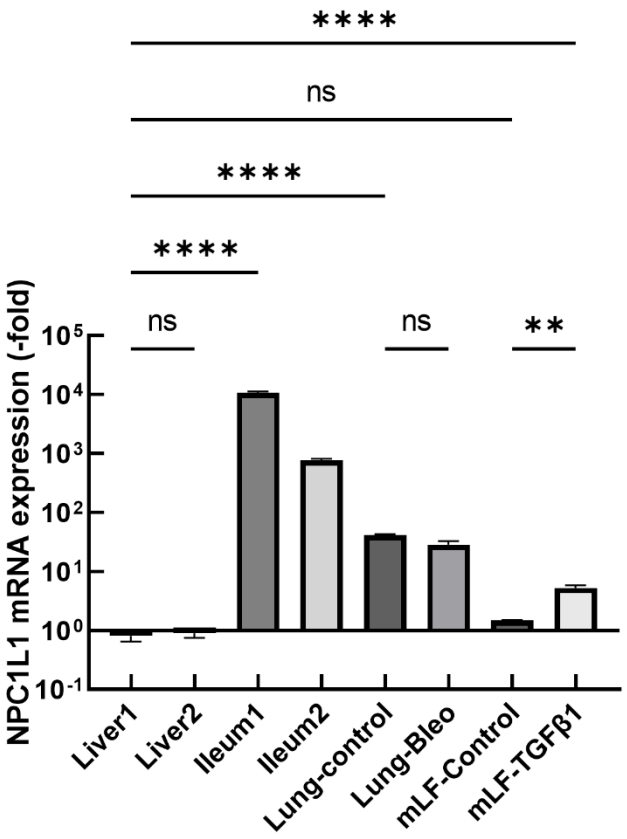

Fig 1. NPC1L1, the target protein of Ezetimibe, is expressed in lung and lung fibroblasts.

mRNA expression levels of NPC1L1 in murine liver, ileum, lung, and lung fibroblast were quantified using RT-qPCR and normalized to 18S rRNA using delta-delta CT method. Log-scaled data are presented as the mean ± SEM, and statistically analyzed with one-way ANOVA and Tukey's multiple comparisons test.

**p<0.01, ****p<0.0001, and ns, not significant.

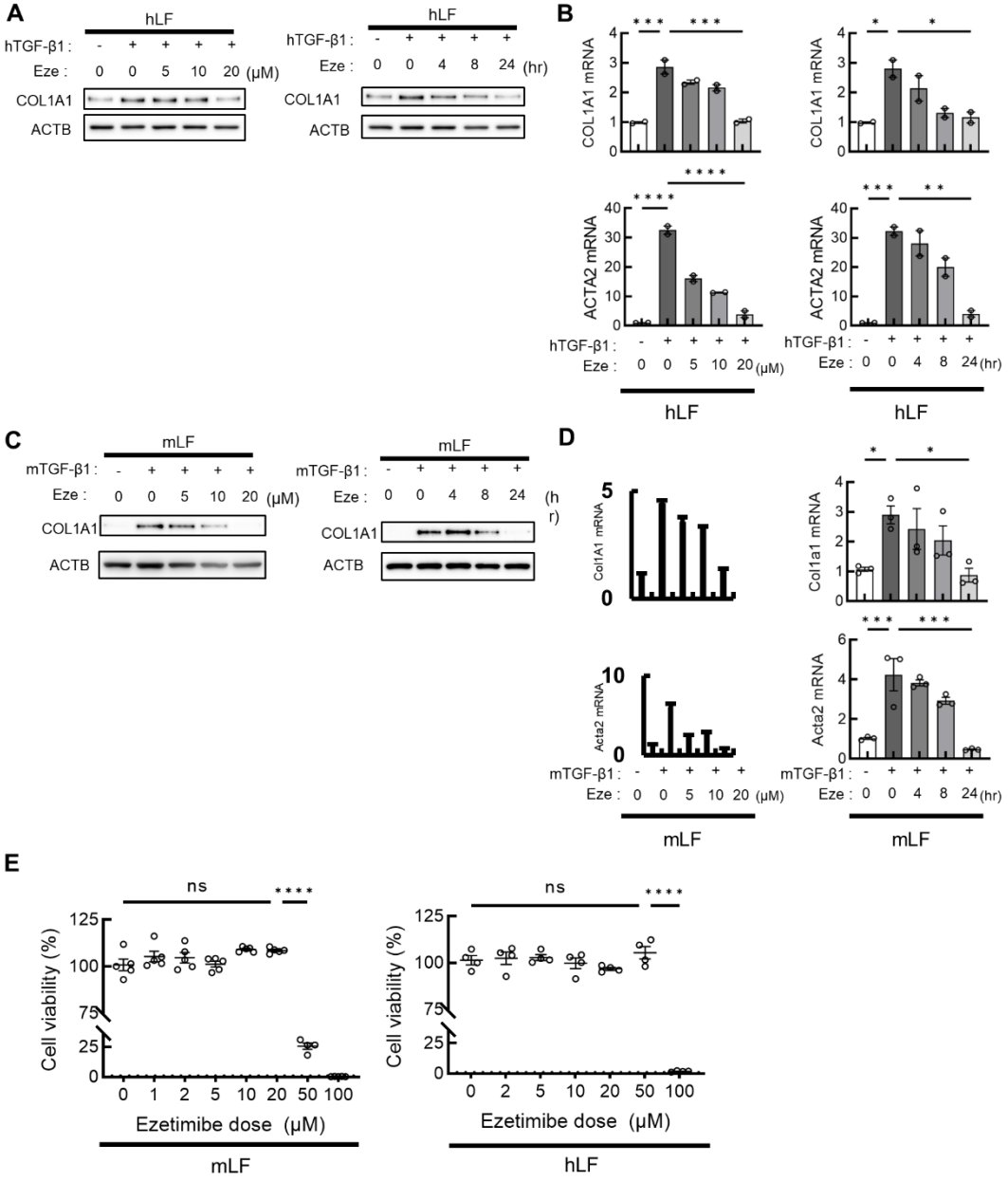

Fig 2. Ezetimibe inhibits TGF-β1 induced myofibroblast differentiation without cytotoxicity in lung fibroblasts.

(A-B) Treatment of TGF-β1-activated primary human lung fibroblasts (hLFs) isolated from normal lung tissue with ezetimibe led to decrease in (A) protein and (B) mRNA levels of COL1A1 proportional to the treatment dose and time. hLFs were activated with 2ng/ml of human recombinant TGF- β1 and treated with ezetimibe as indicated.
(A) Representative immunoblots are shown.
(B) mRNA levels analyzed by RT-qPCR were relatively normalized to the control sample.
(C-D) Ezetimibe inhibits myofibroblast differentiation in primary mouse lung fibroblasts (mLFs) in a treatment dose- and time-dependent manner. mLFs isolated from lungs of a 6 to 8 week-old C57BL/6J mouse were activated with 2ng/ml of mouse recombinant TGF-β1 and treated with ezetimibe as increasing dose or time as indicated.
(C) Representative immunoblots are shown.
(D) mRNA levels analyzed by RT-qPCR were relatively normalized to the control sample.
(E) Cell viability of hLFs and mLFs treated with ezetimibe as indicated doses for 24h was estimated using a Cell titer-Glo assay kit. Live cell numbers are expressed as absorbance at luminescence, and normalized to control.

Data are presented as the mean ± SEM.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, and ns, not significant.

2. Canonical TGFβ-Smad3 pathway is not significantly affected by ezetimibe treatment.

It was established that ezetimibe inhibits TGF-β1-induced myofibroblast differentiation; therefore, we evaluated whether ezetimibe is involved in the canonical TGFβ-Smad3 pathway. Western blot analysis revealed that phosphorylation of smad3 was not affected by ezetimibe treatment (Figure 3A). Additionally, the expression of Serpine1 and Ctgf mRNA, which are downstream target genes of the canonical TGFβ-Smad3 pathway, was

also not suppressed by ezetimibe treatment (Figure 3B), suggesting that ezetimibe was independent of the canonical TGFβ-Smad3 pathway.

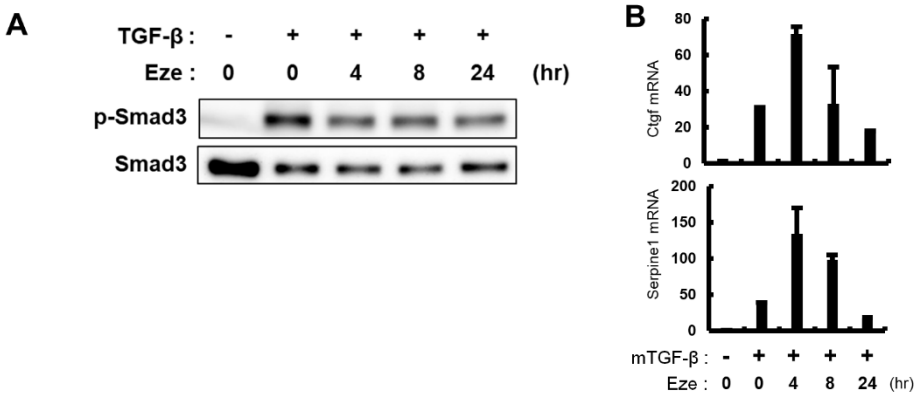

Fig 3. Canonical TGFβ-Smad3 pathway is not significantly affected by Ezetimibe treatment.

Ezetimibe treatment to TGF-β1 activated primary mouse lung fibroblasts (mLFs) isolated from normal lung tissue did not significantly attenuate Smad3 phosphorylation and canonical TGFβ-Smad3 pathway target gene Serpine1 and Ctgf expression. mLFs were activated with 2ng/ml of mouse recombinant TGF-β1 and treated with ezetimibe for different durations.

(A) Western blotting for phosphorylated, total Smad3 indicated that ezetimibe treatment did not induce a significant change in the ratio of p-Smad3 over total Smad3.

(B) mRNA levels analyzed by RT-qPCR are shown. Ezetimibe treatment did not suppress Serpine1 and Ctgf mRNA expression.

3. Ezetimibe stimulates autophagic flux in lung fibroblasts.

We investigated whether the autophagy machinery was triggered by ezetimibe treatment. Experimental data showed that ezetimibe increased LC3B-II to LC3B-I, representing LC3 net flux, proportional to the time and dose of the treatment administered (Figure 4A and 4B). Next, the RFP-GFP-LC3 probe was used to monitor autophagy flux. The numbers of both yellow (mRFP and GFP) and red (mRFP only) puncta were increased after ezetimibe treatment (Figure 4C and Figure 4D), indicating that ezetimibe could increase the accumulation of autophagosomes/autolysosomes. The amount of free GFP fragments resulting from the degradation of GFP-LC3 within autolysosomes increased with ezetimibe treatment (Figure 4E and Figure 4F). Western blot analysis demonstrated that prolonged chemical autophagic inhibition by chloroquine resulted in the accumulation of COL1A1 in lung fibroblasts and averted ezetimibe activity (Figure 4G). Taken together, it is hypothesized that ezetimibe stimulates autophagic flux in lung fibroblasts.

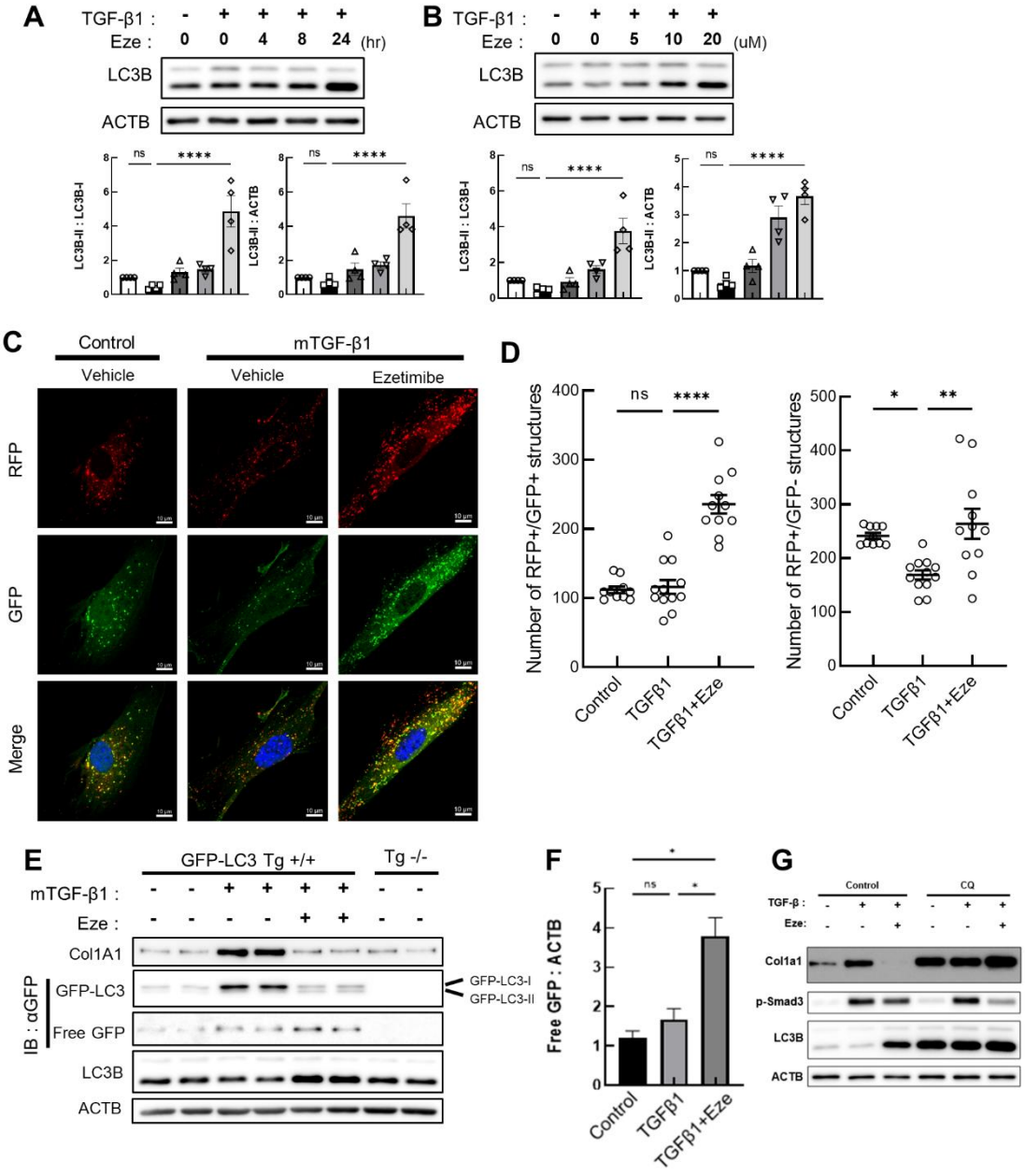

Fig 4. Ezetimibe stimulates autophagic flux in lung fibroblasts.

(A-B) Increase in the LC3B-II to LC3B-I ratio and protein level of LC3B-II is proportional to the time (A) and dose (B) of ezetimibe treatment (N=4).

(C-D) The number of RFP-GFP-LC3 puncta reveals increased autophagic activity with ezetimibe treatment.

(C) Mouse lung fibroblasts (mLFs) isolated from RFP-GFP-LC3 transgenic mice were cultured on a coverslip, and treated with indicated drug and protein for 24 h. The cells were fixed and stained with the mountant ProLong™ Glass Antifade Mountant with NucBlue™ Stain. Fluorescent proteins were imaged using confocal microscope. The representative optical section and merged image are shown. Scale bar, 10 μm. (D) Autophagy-related structures visualized with RFP-GFP-LC3 in (C) were semi-automatically counted. n=9-12.

(E-F) GFP-LC3 cleavage reveals increased autolysosomal activity with ezetimibe treatment.

(E) Western blot analysis results are shown. Free GFP fragments resulting from the degradation of GFP-LC3 within the autolysosome were observed by immunoblotting with GFP protein. mLFs isolated from GFP-LC3 transgenic mice or non-transgenic mice were treated with the indicated drug.

(F) GFP-LC3 cleavage observed by immunoblotting with GFP protein in (E) was semi-automatically counted.

(G) Prolonged chemical autophagic inhibition by chloroquine (CQ) accumulated COL1A1 in lung fibroblasts, and averted the activity of ezetimibe.

Data are shown as mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001.

4. Ezetimibe inhibits mTORC1 activation.

The mTORC1 pathway is a major regulator of autophagy; therefore we performed western blotting analysis of the mTORC1 substrates. Activation was reflected by increased phosphorylation of the well-known mTORC1 substrates, p70S6k and RPS6 (Figure 5). Lung fibroblasts were treated with ezetimibe for 24h, resulting in the amelioration of TGF-β1-induced phosphorylation of p70S6k and RPS6 in a time-and dose-dependent manner.

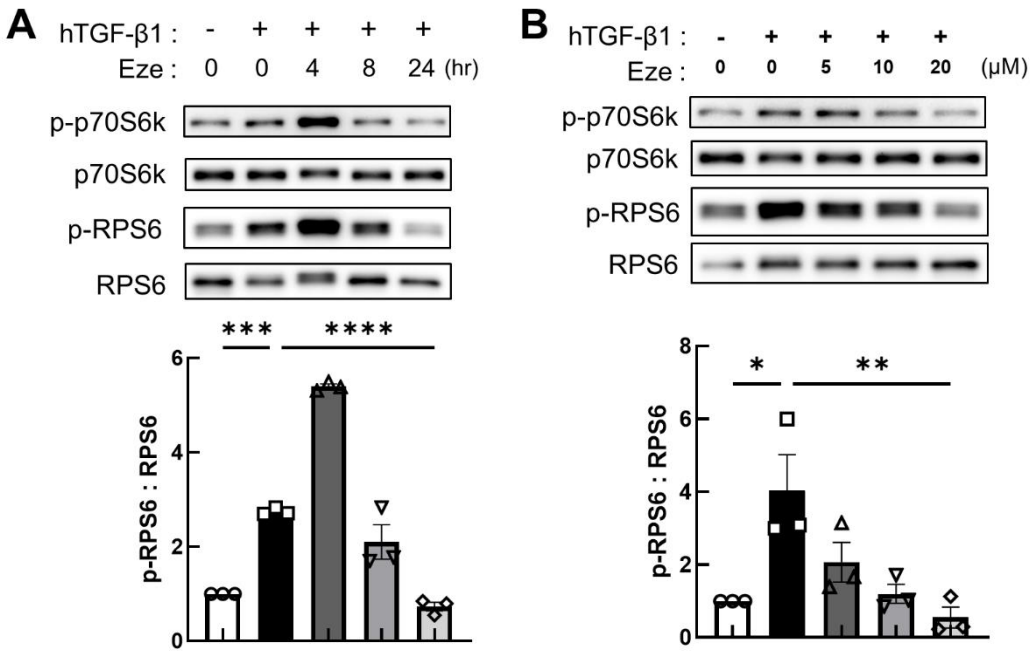

Fig 5. Ezetimibe inhibits mTORC1 activation.

(A-B) Treatment of lung fibroblasts with ezetimibe ameliorated TGF-β1-induced phosphorylation of the mTORC1 substrates, p70S6k and RPS6, proportional to the treatment (A) time and (B) dose.

5. Ezetimibe prevents fibrosis progression in bleomycin induced lung fibrosis mouse model.

The ameliorative effects of ezetimibe on lung fibrosis were assessed in vivo (Figure 6). After bleomycin-induced lung injury, ezetimibe-treated mice recovered their body weights faster than vehicle-treated mice (Figure 6B). Masson's trichrome staining showed that bleomycin increased the density of the collagen matrix, and ezetimibe significantly decreased the fibrotic lesions (Figure 6C). To further verify that ezetimibe promoted autophagy in the bleomycin-induced lung fibrosis mouse model, expression of the autophagy-related marker LC3 was analyzed using immunofluorescence staining (Figure 6D). An increased number of LC3B puncta-like structures were observed in myofibroblasts from ezetimibe-treated mice. Mice treated with ezetimibe showed a significant reduction lung collagen content (Figure 6E). The mRNA levels of fibrosis-related genes, Col1a1, Acta2, and Eda-Fn, were down-regulated in the ezetimibe-treated group compared to the control group (Figure 6F). Western blot analysis demonstrated that ezetimibe treatment increased autophagosome conversion and decreased mTORC1 activity and fibrosis (Figure 6G and Figure 6H).

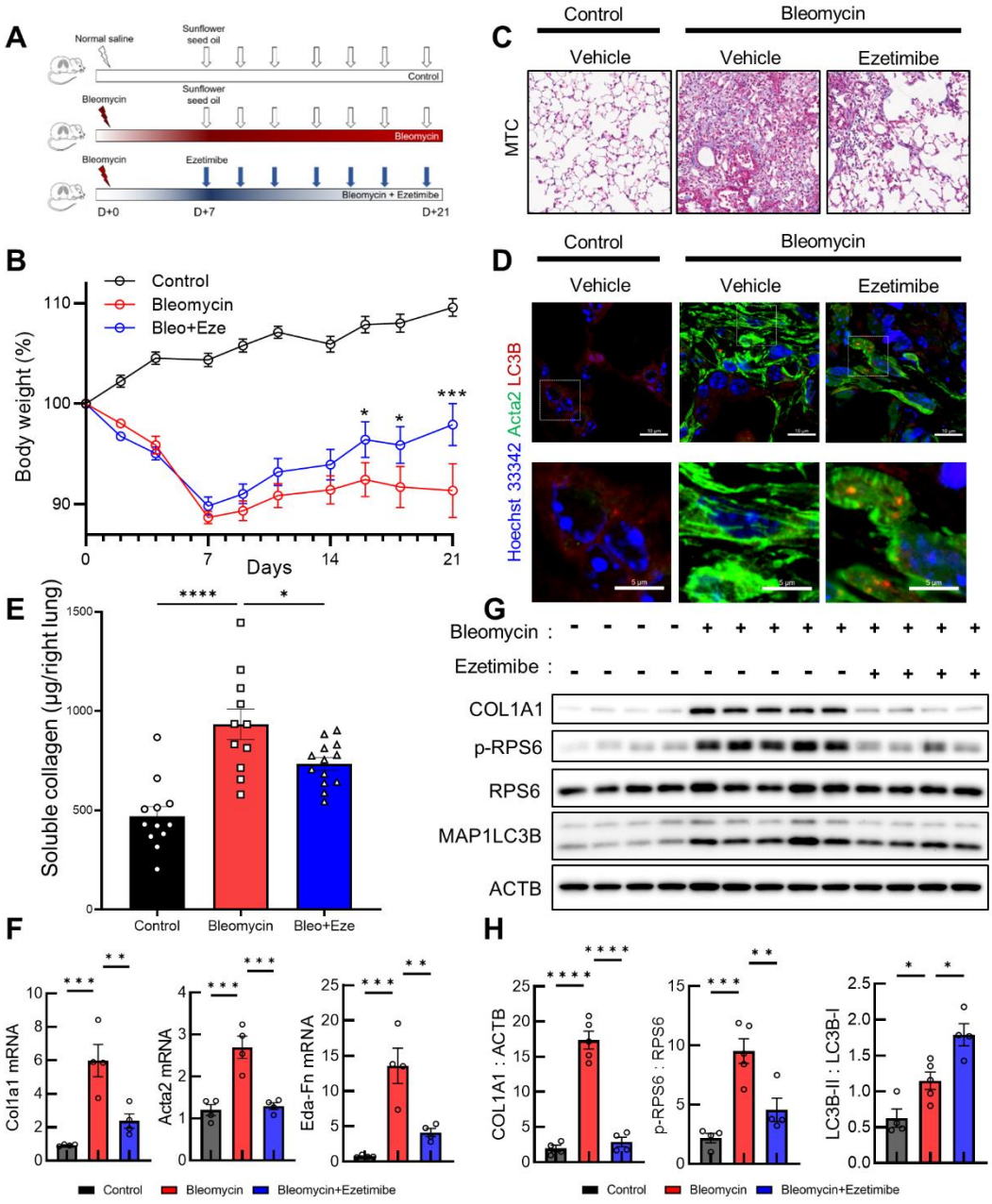

Fig 6. Ezetimibe prevents fibrosis progression in bleomycin-induced lung fibrosis mouse model.

(A) Age-matched mice were randomly assigned to three groups. Bleomycin (2U/kg) or normal saline was instilled by oropharyngeal aspiration. After 7 days from exposure, 2mg/kg of ezetimibe or sunflower seed oil was gavaged orally to the mice 3 times a week. The mice were sacrificed 21 days after initial bleomycin instillation.

(B) After bleomycin-induced lung injury, ezetimibe-treated mice recovered their body weights faster than vehicle-treated mice. (N=18-23/group)

(C) Fibrotic lesion was smaller in lungs of ezetimibe-treated mice. Representative Masson's trichrome staining image of paraffin-embedded slide section of left lung are presented.

(D) Immunofluorescence staining of Acta2 and LC3B revealed LC3B-stained autophagosome-like structures in myofibroblasts. Representative merged images are shown. (upper) Scale bar, 10 μm. (lower) Scale bar, 5 μm.

(E) Ezetimibe alleviated collagen accumulation in murine lung (N=11-13/group).

(F) mRNA levels of fibrosis-related genes, Col1a1, Acta2, and Eda-Fn, were down-regulated in ezetimibe-treated group compared to the positive control (N=4-5/group).

(G-H) Oral ezetimibe treatment resulted in increase in autophagosome conversion and decrease in mTORC1 activity and fibrosis.

(G) Representative immunoblots of single lysates from right lungs of each group of mice (N=4-5/group) are shown.

(H) Densitometry quantification of (G) is shown.

*p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Data are shown as mean ± SEM. Each dot represents single individual mouse.

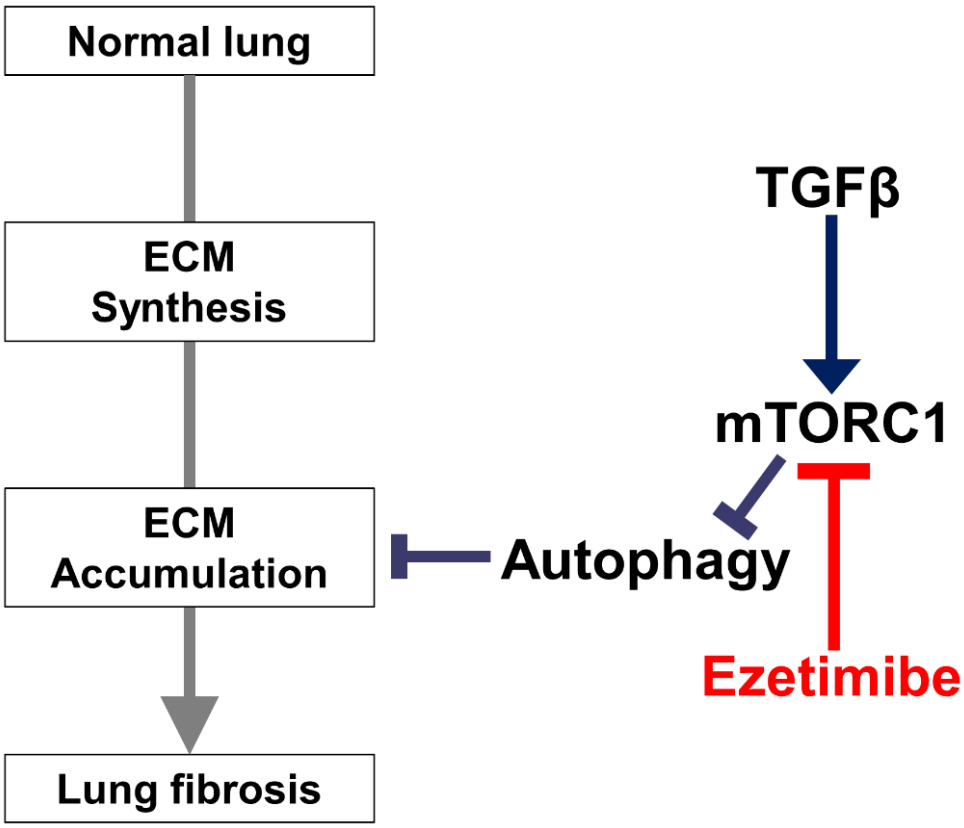

Fig 7. Potential scheme of the anti-fibrotic effects of ezetimibe mediated by mTORC1-autophagy axis

IV. DISCUSSION

Although IPF has an overall poor prognosis, there have been no proven effective therapeutic strategies. Therefore, there is an urgent need for in-depth research into effective treatment strategies for IPF. In the current study, we evaluated the potential of ezetimibe in the treatment of IPF for IPF.

Ezetimibe was originally developed as lipid-lowerering agent that inhibits cholesterol absorption by blocking the cholesterol transporter, NPC1L1[30,37,38]. Previous studies have suggested that NPC1L1 inhibition by ezetimibe ameliorates hepatic steatosis[39-41]. Nozaki *et al*.[42] demonstrated that long-term treatment with ezetimibe improved the histological signs of nonalcoholic fatty liver disease. Lee *et al*.[43] identified the therapeutic role of ezetimibe, particularly its anti-oxidant function. They identified the role of ezetimibe as a p62-dependent nuclear factor erythroid 2-related factor 2 activator in hepatic steatohepatitis. Kim *et al*.[32] have suggested that ezetimibe prevents hepatic steatosis by promoting AMP-activated protein kinase-mediated autophagic activation. Although human NPC1L1 mRNA is expressed predominantly in the small intestine and liver, it is also expressed in other tissues, such as the stomach, ovaries, muscles, and lungs, suggesting its possible role in other tissues[44]. Besides, accumulating evidence shows that drugs for fibroproliferative diseases are useful tools across different organs and are not limited to specific organs[45]. In the current study, we speculated that ezetimibe may be a candidate drug for elucidating the pathophysiology of fibrosis in the lungs. As a result, we demonstrated that ezetimibe ameliorated lung fibrosis by modulating the mTORC1-augophagy axis (Figure 7). We explored whether ezetimibe inhibits TGF-β1-induced myofibroblast differentiation in lung fibroblasts. Then we specifically demonstrated that ezetimibe could inhibit mTORC1 activation and stimulate autophagic flux in lung fibroblasts. Furthermore, we investigated the potential mechanism of the anti-fibrotic effects of ezetimibe in a bleomycin-mediated lung fibrosis model in vivo.

Autophagy is a self-eating regulated process that helps maintain cellular homeostasis[22-24]. Growing evidence suggesets that autophagy plays an essential role in fibrogenesis[24]. Dysregulated autophagy has been demonstrated in fibrotic diseases such as cardiac, liver, and kidney fibroses[46-48]. Similar results were also obtained for IPF[49]. Patel *et al*.[50] found that autophagy was reduced in the lung tissues of patients with IPF. Kesireddy *et al*.[51] reported that LC3B -/- mice displayed an increased susceptibility to bleomycin-induced lung injury and fibrosis. Sosulski *et al*.[52] revealed that autophagy flux was reduced in bleomycin- and TGF-β1-induced IPF mouse models. Additionally, overexpression of miR-449a, an autophagy-regulated miRNA, promotes autophagy in fibroblasts and significantly reduces both the distribution and severity of fibrotic lesions induced by silica[53]. The current study showed that ezetimibe increased the LC3B-II to LC3B-I ratio and the number of autophagosomes and autolysosomes in mouse lung fibroblasts, suggesting that ezetimibe may promote autophagy flux. Additionally, we revealed that autophagic inhibition by chloroquine resulted in the accumulation of COL1A1 in lung fibroblasts and averted ezetimibe activity. Additionally, an increased number of LC3B puncta-like structures were observed in myofibroblasts from ezetimibe-treated mice.

However, the exact mechanism of autophagy in pulmonary fibrosis remains unclear. The mTOR signaling pathway is considered an essential sensor for autophagy regulation[54,55]. Lipopolysaccharides were reported to promote lung fibroblast proliferation through autophagy inhibition by activating the PI3K-Akt-mTOR pathway, which was reversed by rapamycin, a specific mTOR inhibitor[56]. Moreover, several studies have investigated the potential protective effects against lung fibrosis via the Akt/mTOR-regulated autophagy axis. Mohamed *et al*.[57] showed that coenzyme Q 10 improved the histological features of liver and lung fibrosis by increasing the expression of markers of autophagy and downregulating mTOR expression. Divya *et al*.[58] documented that celastrol, a quinine methide triterpenoid, reduced collagen deposition in a bleomycin-induced fibrosis model via autophagy activation by inhibiting PI3K/Akt-mediated mTOR expression. Ligustrazin was also found to attenuate paraquat-induced lung fbirosis by blocking PI3K/Akt/mTOR

signaling[59]. These findings are consistent with those of the prestent study. We revealed that ezetimibe treatment in lung fibroblasts ameliorated TGF-β1-induced phosphorylation of p70S6k and RPS6, which are well-known mTORC1 substrates.

V. CONCLUSION

In conclusion, the potential mechanisms by which ezetimibe may affect the development of IPF were identified. The present study demonstrated that ezetimibe inhibits TGF-β1-induced myofibroblast differentiation in lung fibroblasts without cytotoxicity. Ezetimibe ameliorates lung fibrosis by inducing autophagy flux. Moreover, ezetimibe ameliorates lung fibrosis by modulating the mTORC1-augophagy axis, independent of the canonical TGFβ-Smad3 pathway. Based on these results, we suggest that ezetimibe could potentially be repurposed for the treatment of IPF.

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ABSTRACT (IN KOREAN)

특발성 폐섬유화증에서 mTORC1-autophagy 기전을 기반으로 한 Ezetimibe의 치료 효능 규명

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곽 세 현

특발성 폐섬유화증은 진단 이후 평균 수명이 약 3년에 불과하며 예후가 불량한 것으로 알려져 있다. 그러나, 아직 발병 기전이 명확히 밝혀지지 않았으며, 여전히 폐 이식만이 유일한 완치법으로 알려져 있는 실정이다. 이에 본 연구에서는 mTOR-autophagy axis에 주목하고, 이를 바탕으로 미국 식품의약국의 사용 승인을 받은 항지질제인 ezetimibe의 특발성 폐섬유화증에서의 잠재적 치료제로서의 가능성을 탐구하였다.

Ezetimibe의 투여 용량과 시간에 비례하여 COL1A1의 발현이 감소함을 human lung fibroblasts와 mouse lung fibroblast에서 확인하였으며, MTT assay를 통하여 ezetimibe이 20mM 이하의 농도에서 세포 독성이 없음을 검증하였다. LC3I이 LC3II로 전환되는 비율, 즉 LC3 net flux를 ezetimibe이 증가시킴을 확인하였으며, RFP-GFP-LC3 transgenic mice에서 분리한 mouse lung fibroblast에서 GFP-LC3B puncta가 ezetimibe에 의해 증가함을 확인하였다. Chloroquine을 통해 autophagy를 억제시킨 결과, COL1A1이 축적된 것을 확인하였다. 또한, ezetimibe의 투여에 의해 mTORC1의 기질인 p70S6k와

RPS6의 인산화가 저하됨을 확인함으로써 ezetimibe이 TGF-β1-mTORC1을 억제하는 방향으로 작용함을 확인하였다. 마지막으로, C57BL/6J male mice를 사용한 bleomycin 유도 폐섬유화 모델을 통해 ezetimibe이 autophagy 활성화를 통해 폐 섬유화의 진행을 억제함을 in vivo에서 검증하였다.

본 연구에서는 ezetimibe이 mTORC1 기전 억제를 통해 autophagy를 활성화하여 폐 섬유화를 억제하는 방향으로 작용함을 확인하였다. 본 연구 결과를 근거로 ezetimibe의 특발성 폐섬유화증 치료제로서의 잠재적 유용성을 제시하고자 한다.

핵심 되는 말: 특발성 폐섬유화증, 자가 포식 작용, mTORC1, ezetimibe