의학박사 학위논문

만성폐쇄성 폐질환에서의 cereblon의 역할 및 조절 기전 규명

The functional role and mechanisms of cereblon in chronic obstructive lung disease

2021년 2월

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2021년 2월

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Abstract

The functional role and mechanisms of cereblon in chronic obstructive lung disease

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Background

Chronic obstructive pulmonary disease (COPD) is associated with chronic inflammation manifesting as emphysema and chronic airway obstruction. Although the nature of this inflammation has been heavily studied, there is no proven treatment to prevent emphysema progression due to the complexity of inflammatory mechanisms. Cereblon (CRBN) is a substrate receptor for the CRL4A E3 ubiquitin ligase complex. CRBN has been identified to play an essential role in regulating inflammatory response, oxidative stress, and endoplasmic reticulum(ER) stress, mediating the development of various diseases. However, little is known about the actions of CRBN in the lung.

Objective

The role of CRBN in modulating elastase-induced emphysema and its regulatory mechanisms were evaluated.

Methods

The correlation between CRBN expression and clinical parameters were evaluated in human lung tissues. Mouse lungs were analyzed for inflammation and emphysema after intratracheal administration of porcine pancreatic elastase (PPE). Bronchial epithelial cells and bone marrow derived macrophages (BMDM) were used after relevant siRNA transfection. With neutrophil elastase (NE), cigarette smoke extract (CSE) or lipopolysaccharide (LPS) treatment, protein expressions related to inflammation, oxidative stress and protease activity were evaluated by immunoblotting and immunohistochemistry. LPS-induced scavenger receptor expression was determined by flow cytometry.

Results

The level of CRBN expression was decreased in the lung tissue of COPD patients which correlated with the FEV1/FVC ratio. CRBN KO aggravated PPE-induced emphysema, and neutrophilic inflammation was predominantly observed preceding emphysema development in mice lungs. CSE or LPS-induced inflammatory cytokines such as KC, MIP-2, TNF-α, and IL-6 were increased in CRBN KO macrophages. In these cells, NE/CSE-induced mitogen-activated protein kinase (MAPK) activation was not affected, while CSE-induced nucelar

factor-kappa B (NF-κB) activation was increased. CRBN KO also increased oxidative stress and protease expression with NE or LPS stimulation. Cell aging, autophagy activation, and apoptosis were not affected with CRBN KO. LPS-induced scavenger receptor-A and macrophage receptor with collagenous structure upregulation were also not affected by CRBN.

Conclusion

CRBN deficit may be involved in the pathogenesis of COPD by increase in neutrophilic inflammation and augmentation of elastase-induced emphysema through NF-κB activation in mice.

Key words: CRBN, COPD, emphysema, elastase, NF-κB

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LIST of ABBREVIATIONS

COPD : Chronic obstructive pulmonary disease

CRBN : Cereblon

NF-*k*B : Nuclear factor-kappa B

LPS : Lipopolysaccharide

AMPK : Adenosine monophosphate-activated protein

KO : Knockout

WT : Wild type

8-OHdG : 8-hydroxy-2'-deoxyguanosine

4HNE : 4-hydroxynonenal

MMP9 : Matrix metallopeptidase 9

NE : Neutrophil elastase

PPE : Porcine pancreatic elastase

CSE : Cigarette smoke extract

LPS : Lipopolysaccharides

SRA : Scavenger receptor A

MARCO : Macrophage receptor with collagenous structure

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major health-care problem in the world. The Global Burden of Disease Study 2015 estimated the global prevalence of COPD at about 174 million cases, and COPD ranked the third with about 3.2 million patients dying of the disease.(1) Emphysema is a main phenotype of COPD and is defined as the irreversible destruction of alveolar structures and enlargement of the airspaces.(2) Oxidant/antioxidant or protease/antiprotease imbalances are pivotal to emphysema's pathogenesis.(3) Considerable efforts have been made over the past decade to find a novel therapeutic to slow the inflammation and progression of emphysema.(4) However, there is no effective treatment to change emphysema's natural history due to the complexity of inflammatory signaling or mechanisms.(5)

Cereblon (CRBN) was initially discovered as a candidate gene for a mild form of autosomal recessive non-syndromic mental retardation and has been reported as a direct molecular target for the teratogenicity of thalidomide and cytotoxicity of immunomodulatory drugs.(6,7) CRBN forms a substrate receptor of E3 ubiquitin ligase complex, which consists of damage-specific DNA binding protein 1(DDB1), cullin-4A/B (Cul4A or Cul4B), and regulator of Cullines1 (RoC1) and mediates the ubiquitination and degradation of endogenous substrates.(7-10) CRBN has been extensively studied and found to

regulates the large conductance calcium- and voltage-activated potassium (BK_{Ca}) channels,(11,12) chloride channel protein1 (CLC-1),(13) to bind to the immunomodulatory drug (IMiDs) and induce the death of hematologic cancer cells.(14-16) Furthermore, CRBN has lately attracted the considerable attention to the effect of regulating inflammation. It has been reported that CRBN binds to the ubiquitination domain of TRAF 6 (TNF receptor associated factor 6) and suppress nuclear factor-κ B (NF- κ B) activation by negatively regulating the Toll-like receptor 4 (TLR4) signaling pathway.(17) Besides, CRBN is known to suppress the lipopolysaccharide (LPS)-induced inflammatory response by promoting the ubiquitination and degradation of c-jun,(10) or binds to adenosine monophosphate-activated protein kinase (AMPK) and inhibits the activation of AMPK, which plays an important role in regulating metabolic process, inflammatory response, oxidative stress and endoplasmic reticulum (ER) stress, mediating the development of various diseases.(6,18,19) However, it is unknown how CRBN acts in the lung.

In this study, we investigated the role of CRBN in the development of elastase-induced emphysema and its molecular mechanisms.

MATERIALS and METHODS

Mice

Female wild-type (WT) BALB/c mice were purchased from Koatech Laboratory Animal Company (Pyeongtaek, Korea) and CRBN knockout (KO) mice in a BALB/c genetic background were kindly donated by Dr. Kyung Jin Lee (Asan Medical Center, Seoul, Korea). All mice used were sex matched at 6-8 weeks of age (20-22g) and housed in the animal facility of Seoul National University Hospital under specific pathogen-free barrier conditions. Animal experiments were approved by the Institutional Animal Care and Use Committee (number 19-0220-S1A1(1)) of Seoul National University Hospital.

In vivo porcine pancreatic elastase exposure

Mice (n=5) were anesthetized and given 0.5 units porcine pancreas elastase (PPE; Sigma-Aldrich, St. Louis, MO, USA) in 100ml of saline or saline alone via intratracheal injection. Mice were sacrificed on day 2 and day 14 after PPE instillation.

Analysis of bronchoalveolar lavage fluid (BALF)

On day 2 and 14, after PPE administration, the lungs of terminally anesthetized mice were lavaged with 1ml of cold phosphate-buffered saline (PBS). BALF was centrifuged at 1500 rpm at 4℃ for 10min, and then the supernatants were collected to measure the levels of lactate dehydrogenase (LDH) and protein. Total cell count was determined with a hemocytometer, and BALF cell distribution was quantified in cytospin preparations after staining with Diff-Quik dye (Sysmex, Kobe, Japan).

Emphysema measurement and immunohistochemistry (IHC)

The right lungs were fixed in 4% paraformaldehyde solution (USB products, USA) for 24h, then embedded in paraffin and sectioned in 4μm of thickness. The slides were stained with hematoxylin and eosin (H&E). Four randomly selected x100 fields per specimen were photographed in a blinded manner. Emphysema was quantified by measuring the mean linear intercept (MLI). The MLI was measured by placing four 1000mm lines over each field. The total length of each line divided by the number of alveolar intercepts gives the average distance. The non-parenchymal area was not included. Lung tissues were placed on slides using the Discovery XT automated IHC

stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Tissue sections were deparaffinized and rehydrated. Cell conditioning 1 (CC1) standard (pH 8.4 buffer containing Tris/Borate/EDTA) was used for antigen retrieval. The sections were incubated with anti-8-hydroxy-2'-deoxyguanosine (8-OHdG), anti-4 hydroxynonenal (4HNE), or anti-matrix metallopeptidase 9 (MMP9) antibody for 32 min at 37℃, washed and incubated with a secondary antibody for 20 min at 37℃. After successive washes, slides were incubated with 3,3-diaminobenzidine (DAB) H_2O_2 substrate for 8 min at 37℃, followed by hematoxylin and bluing reagent counter stain. Stained cells were observed under a microscope (EVOS XL Core Cell Imaging System, Thermo Fisher Scientific).

Isolation of RNA and quantitative real-time PCR

Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA). Power SYBR Green PCRMaster Mix (Applied Biosystems, Carlsbad, CA, USA) was used for amplification. The primers used in the study are as follows: mouse CRBN (fwd:5'-AGCATGGTGCGGAACTTA-3', rev:5'-ATCTCTGCT GTTGTCCCA-3'), mouse KC(fwd:5'-TGTCAGTGCCTGCAGACCAT-3' and rev:5'-CCTGAGGGCAACACCTTCA-3'), mouse MIP2 (fwd: 5'-CCAACCACCAGGCTACAGG-3' and rev:5'-GCGTCACACTCAAGC TCTG-3'), mouse GAPDH (fwd:5'-ACGGCAAATTCAACGGCA

CAG-3' and rev:5'-TGGGGGCATCGGCAGAAGG-3')

Cells and reagents

Normal human bronchial epithelial cells (BEAS-2B) were maintained in a defined keratinocyte serum-free medium (GIBCO by Life Technologies, Grand Island, NY, USA) at 37℃ under 5% CO_2. Bone marrow-derived macrophages (BMDM) were obtained from bone marrow by flushing the tibia and femur of a BALB/c mouse. Bone marrow cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 30% L929 cell supernatant for 5-7 days. BMDM were resuspended in DMEM supplemented with 10% FBS and used in experiments.

Human sputum neutrophil elastase (NE) was purchased from Elastin Products Co. (Owensville, MO, USA). NE was dissolved in a solution of 50% glycerol and 50% 0.02M NaOAc (pH 5) (vehicle control, V.C.). Antibodies used for protein detection were anti-phospho-SAPK/JNK (p-SAPK/JNK) (Thr183/Tyr185), anti-p-p38 (Thr180/Tyr182), anti-total p38, anti-p-ERK (Thr202/Tyr204), anti-total ERK, anti-Iκ Ba, anti-LC3B, anti-caspase-3, and anti-poly (ADP-ribose) polymerase (PARP) antibodies (Cell Signaling Technology, Danvers, MA, USA); anti-CRBN antibody (Novus Biologicals, Centennial, CO, USA); anti-8-OHdG (Biossantibodies Inc., Woburn, MA, USA); anti-p21,

anti-p27, anti-MMP2, anti-total JNK, and anti-GAPDH antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-4HNE and anti-MMP9 antibodies (Abcam, Cambridge, MA, USA). Control siRNA and CRBN siRNA (Assay ID: s27634) were purchased from Thermo Fisher Scientific.

Preparation of cigarette smoke extract (CSE)

Commercial cigarettes (THIS; KT&G Corp., Daejeon, South Korea) were smoked continuously by a bottle system connected to a vacuum machine (GastManufacturing Inc., MI, USA). The smoke from 20 cigarettes was bubbled in 60ml of PBS. The large insoluble particles were removed by filtering the solution through a 0.22μ m filter.

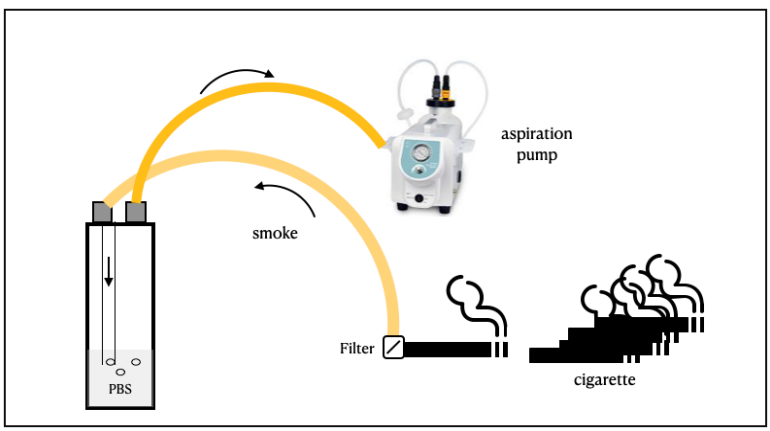

Figure 1. Diagram of preparing CSE

Enzyme-linked immunosorbent assay (ELISA) for inflammatory cytokine secretion

The levels of IL-8, KC, MIP2, TNF-a, IL-6 in cell culture supernatants were measured using a commercially available DuoSet ELISA Kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

Protein extraction and Western blot analysis

Total cellular extracts were prepared using 1X Cell Lysis Buffer (Cell Signaling Technology) supplemented with 1mM PMSF. Frozen lung tissues (SNUH IRBNumber:H-1309-073-521) were homogenized in tissue extraction buffer (Life Technologies) containing protease inhibitor mixture (Sigma) and phosphatase inhibitor mixture (Sigma). Protein concentration was determined using the Bradford assay (BIO-RAD, Hercules, CA, USA). Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were transferred to Hybond ECL nitrocellulose membranes (Thermo Fisher Scientific) for 100min at 90V. The membranes were blocked with 5% skim milk in 1X tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1h at room temperature. After successful washes, the membranes were incubated with horse

radish peroxidase (HRP)-conjugated secondary antibodies for 1h. Blots were developed using West Pico Western blot detection kit (Thermo Fisher Scientific).

Luciferase Assay

According to the manufacturer's specifications, cells were transfected with the NF-kB reporter plasmid or control plasmid using a Neon Transfection System (Thermo Fisher Scientific). Luciferase activity was determined using a luciferase assay kit (Promega, Madison, WI).

Flow cytometry

Cells were incubated with anti-macrophage receptor with collagenous structure (MARCO)-fluorescein isothiocyanate (FITC), anti-scavenger receptor A (SRA)-phycoerythrin (PE), anti-IgG FITC, or anti-IgG PE in 200ml incubation buffer for 45min. Unreacted antibodies were removed. Cell-associated FITC or PE-conjugated antibodies were analyzed by flow cytometry using a FACSCalibur or a FACSCanto™ flow cytometer (BDBiosciences, San Jose, CA).

Statistical analysis

Data were analyzed using Prism (version 5; GraphPad Software, San Diego, CA, USA) by Student's t-test and one-way analysis of variance (ANOVA). A P-value of <0.05 was considered statistically significant.

Results

Expression of CRBN is decreased in the lungs of COPD patients

To investigate the functional role of CRBN in emphysema development in a human lung. We first measured CRBN expression in surgically resected human lung tissue by immunoblotting. The expression of CRBN protein was decreased in COPD lung compared with non-COPD lung (Figure 2A). Between never smoker and smoker, CRBN expression was not significantly different (Figure 2B). Cigarette smoking is the main risk factor for COPD, yet only 20-30% of smokers develop COPD.(20) These findings suggested that CRBN plays a role in the development of COPD regardless of smoking.

The ratio of FEV1/FVC correlates with the expression level of CRBN in human lung tissue.

We further investigate the association between the clinical parameters related to COPD and the expression level of CRBN in human lung tissue. Clinical parameters included age, body mass index

(BMI), pack-year of smoking, FEV1 (forced expiratory volume in 1second), the ratio of FEV1 to FVC (forced vital capacity), DLco (diffusion capacity), questionnaires for the quality of life with COPD, and mMRC (modified Medical Research Council) scale to measure the degree of dyspnea. Among these various clinical parameters, only the ratio of FEV1/FVC had a significant correlation with the expression level of CRBN (Figure 3). The ratio of FEV1/FVC less than 70% indicates the airflow limitation and the possibility of COPD. The correlation between the ratio of FEV1/FVC and the expression of CRBN shows that a decreased level of CRBN is associated with COPD development consistently.

Emphysematous change is exaggerated in PPE-treated CRBN KO BALB/c mice.

Rodent models of elastase-induced emphysema have been established. To evaluate whether the expression of CRBN affected the development of porcine pancreatic elastase (PPE)-induced emphysema in mice, we treated WT and CRBN KO BALB/c mice (Figure 4A) with intratracheal PPE administration. On day 14 after intratracheal PPE administration, more prominent emphysematous change was observed in CRBN KO mice lung tissue than WT mice (Figure 4B). The mean linear intercept (MLI) was measured to quantify emphysema in mice lung tissue. The MLI was increased in PPE-treated mice lung

compared to saline-treated mice lung (Figure 4C). In CRBN KO mice lung, PPE-induced emphysema was further increased compared to WT mice.

PPE-induced neutrophilic inflammation is augmented in CRBN KO mice preceding the development of emphysema.

PPE-treated mice lung showed marked inflammation with cellular infiltration, especially in CRBN KO mice on day 2 (Figure 5A). In addition to significant inflammatory response on day 2, emphysematous change was more prominent on day 14 in CRBN KO mice. To investigate whether PPE-induced inflammation mimicked those of COPD patients, we analyzed BALF from BALB/c mice. The total number of BALF cells was significantly higher in CRBN KO mice on day 2 after PPE administration (Figure 5B). Along with the total cell count, neutrophils were markedly increased in BALF of CRBN KO mice treated with PPE (Figure 5C). However, on 14 days after PPE administration, most of the inflammatory cells in the BALF disappeared. Neutrophils are the most abundant inflammatory cells in the bronchus of patients with COPD, and increased neutrophilic inflammation is characteristic of acute exacerbation of COPD.(21) These findings suggested PPE-induced inflammation was similar to

patients with COPD, and CRBN KO could augment neutrophilic inflammatory response induced by PPE.

NE/CSE-induced IL-8production was increased by CRBN KO

IL-8 is a major neutrophil chemoattractant and activating factor, and levels of IL-8 in sputum samples of COPD patients are increased.(22) To investigate the effect of CRBN on IL-8 production, we transfected BEAS-2B cells with CRBN siRNA. After 48hrs, the cells were treated with neutrophil elastase (NE) 1u/ml or cigarette smoke extract (CSE) 1% for 24hours. The concentrations of IL-8 were higher in NE/CSE-treated BEAS-2B cells. In CRBN KO cells, NE/CSE-induced IL-8 production was further increased compared to WT BEAS-2B cells (Figure 6).

CSE/LPS-induced inflammatory cytokine release from BMDM cells were increased by CRBN KO

Alveolar macrophage plays a central role in modulating inflammation by producing many inflammatory proteins in patients with COPD. We next investigate the effect of CRBN KO not only on bronchial

epithelial cells but also on macrophages. Bone marrow-derived macrophages (BMDM) were treated with NE 1U/ml, CSE 1% for 24hours, or with LPS 100ng/ml for 6 and 24 hours. We measured the amount of KC, macrophage inflammatory protein 2 (MIP2), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in the supernatant of BMDM stimulated by NE, CSE or LPS. In NE-treated BMDM, concentrations of KC, MIP2, TNF-α , and IL-6 were not significantly different by CRBN KO (Figure 7A-7D). In CSE-treated BMDM, KC and MIP 2, known as neutrophilic chemokines, significantly increased in CRBN KO cells compared with WT cells (Figure 7E and 7F). In LPS-treated BMDM, the production of KC, MIP2, TNF-α , and IL-6 were significantly higher in CRBN KO cells compared with WT cells (Figure 7I - 7L).

NE/CSE-induced MAPK activation is not influenced by CRBN KO in bronchial epithelial cells.

Given increased neutrophilic inflammation in CRBN KO mice and increased IL-8 release in CRBN KO BEAS-2B cells by NE or CSE, we investigated the effect of CRBN on mitogen-activated protein kinases (MAPKs) that includes p38, extracellular signal-regulated kinases (ERK), and c-*jun*NH_2-terminal kinase (JNK). In BEAS-2B cells treated with NE 1u/ml or CSE 1%, expressions of phosphorylated

and total forms of p38, ERK and JNK were analyzed by western blotting. NE and CSE significantly increased phosphorylated forms of MAP kinases in BEAS-2B cells. However, the density of electrophoretic bands of CRBN KO cells did not differ compared with the control cells (Figure 8). These findings suggested that CRBN KO enhanced NE/CSE-induced neutrophilic inflammation and cytokine production independently of the MAPK pathway.

Effect of CRBN KO on NE, CSE or LPS induced I*k*B α degradation and NF-*k*B activation in bronchial epithelial cells

NF-κB is an important transcription factor that participates in a broad spectrum of inflammatory signaling pathways regulating cytokine activity in COPD.(23) NF-κB regulated the expression of KC, MIP2, TNF-α, and IL-6 genes. We evaluated whether CSE/LPS -induced inflammatory cytokine release in CRBN KO cells was dependent on NF-κB activation. Phosphorylation and degradation of cytoplasmic IκBα is required for NF-κB pathway activation.(24) IκBα degradation induced by NE, CSE, or LPS was observed, yet CRBN KO did not affect the degree of IκBα degradation in BEAS-2B cells (Figure 9A - 9E). However, when we measured the NF-κB activity by Luciferase assay, CSE induced NF-κB activity was significantly

increased in CRBN KO cells compared with WT cells (Figure 9G). These results suggest that exaggerated neutrophilic inflammation induced by CSE or LPS in CRBN KO cells is dependent on the NF-κB activation pathway.

PPE/NE-induced oxidative stress is increased by CRBN KO

Reactive oxygen species (ROS) have been shown to increase gene expressions of inflammatory cytokines and partially through NF-κB activation.(25) To confirm whether CRBN KO affects the inflammatory response mediated by oxidative damage, we performed IHC for 8OHdG and 4HNE on day 2 after PPE administration in BALB/c mice lung. The intensity of 8OHdG and 4HNE staining after PPE-treatment was greater in CRBN KO mice than WT mice (Figure 10A & 10B). 4HNE expression also significantly increased in NE-treated CRBN KO cells compared to WT cells (Figure 10C).

Elastase/LPS-induced protease expression is increased by CRBN KO

Emphysema is characterized by increases in proinflammatory cytokines and MMP activity in the lung.(26) MMP-9 is the predominant elastolytic enzyme in patients with COPD. We investigate the effect of CRBN on PPE-induced protease expression by immunohistochemical staining with MMP-9 on day 2 after PPE administration (Figure 11A). We found an increased intensity of MMP-9 staining in CRBN KO mice's lungs compared with WT mice lungs. Next, we performed *in vitro* study using BEAS-2B and BMDM cells. NE (Figure 11B) or LPS (Figure 11C) stimulation increased MMP-9 protein expressions in both bronchial epithelial cells and macrophages. These results indicate that CRBN KO also affects expressions of protease induced by elastase or LPS.

CRBN KO contributes to cellular injury yet does not affect cellular senescence, autophagy activation, and apoptosis

We additionally investigated the role of CRBN on the cellular injury, aging, autophagy activation, and apoptosis. In cell cytotoxicity assay (Figure 12A& 12B), the concentration of LDH and protein in BALF

of CRBN KO mice significantly increased on day 2 after PPE administration compared with WT mice. On day 14 after PPE administration, markers of cellular injury were reduced to baseline. p21 is a member of a family of cell cycle inhibitors. p21 potentiates inflammatory response and inhibits apoptosis and proliferation, leading to cellular senescence.(27) With higher CSE treatment concentration, p21 expression increased in BEAS-2B cells (Figure 12C). However, the density of electrophoretic bands of CRBN KO cells did not differ compared with control cells. Neither CSE-induced autophagy activation nor apoptosis markers were significantly different between CRBN KO and control BEAS-2B cells (Figure 12C). These findings suggest that CRBN KO augmented PPE-induced cellular injury. However, CSE-induced cell aging, autophagy activation, or apoptosis were independent with CRBN.

CRBN KO does not affect the expression of LPS-induced scavenger receptors

Acute exacerbations are associated with disease progression and two-thirds of exacerbations are related to a bacteria infection in COPD patients.(28,29) Alveolar macrophages express various cell surface receptors and play a critical role in the clearance of the pathogens. MARCO and SRA are class A scavenger receptors, which are highly

expressed in the lung.(30,31) Cell surface expression of MARCO or SRA was determined by flow cytometry. LPS treatment upregulated MARCO and SRA protein on the plasma membrane was independent with CRBN KO (Figure 13).

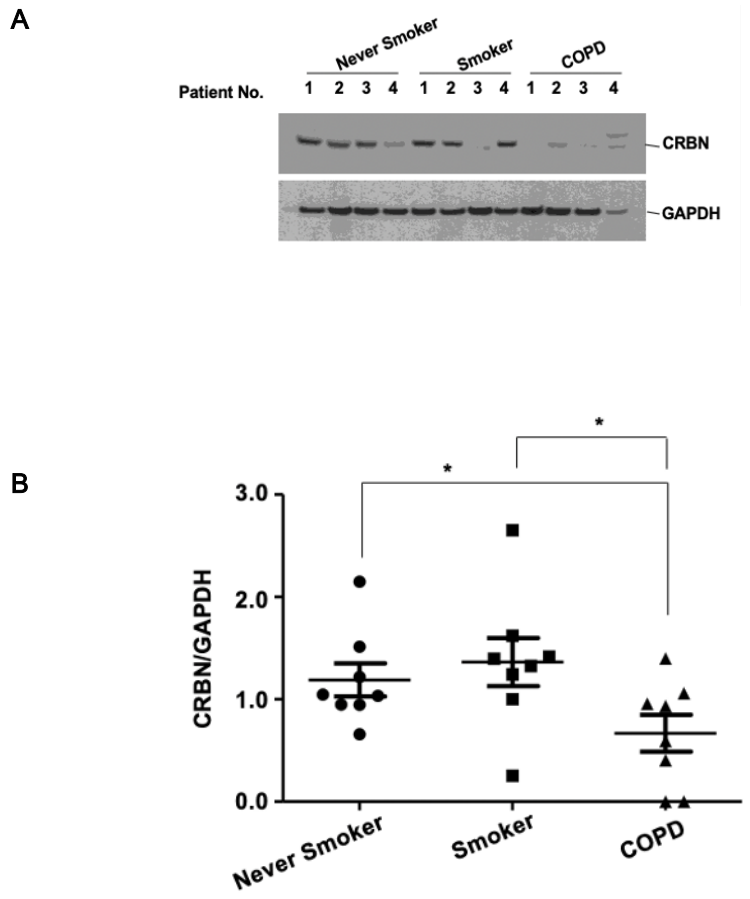

Figure 2. Decreased expression of CRBN protein in lung tissue of COPD patients. A: Expression of CRBN protein was measured by Western blot analysis in lung tissue of never smokers, smokers, and patients with COPD. **B**: Results are expressed as a ratio of the band intensity of CRBN protein versus the band intensity of GAPDH protein. **Abbreviations**: CRBN, cereblon; COPD, chronic obstructive lung disease; **P*<0.05 versus neversmoker/smoker group.

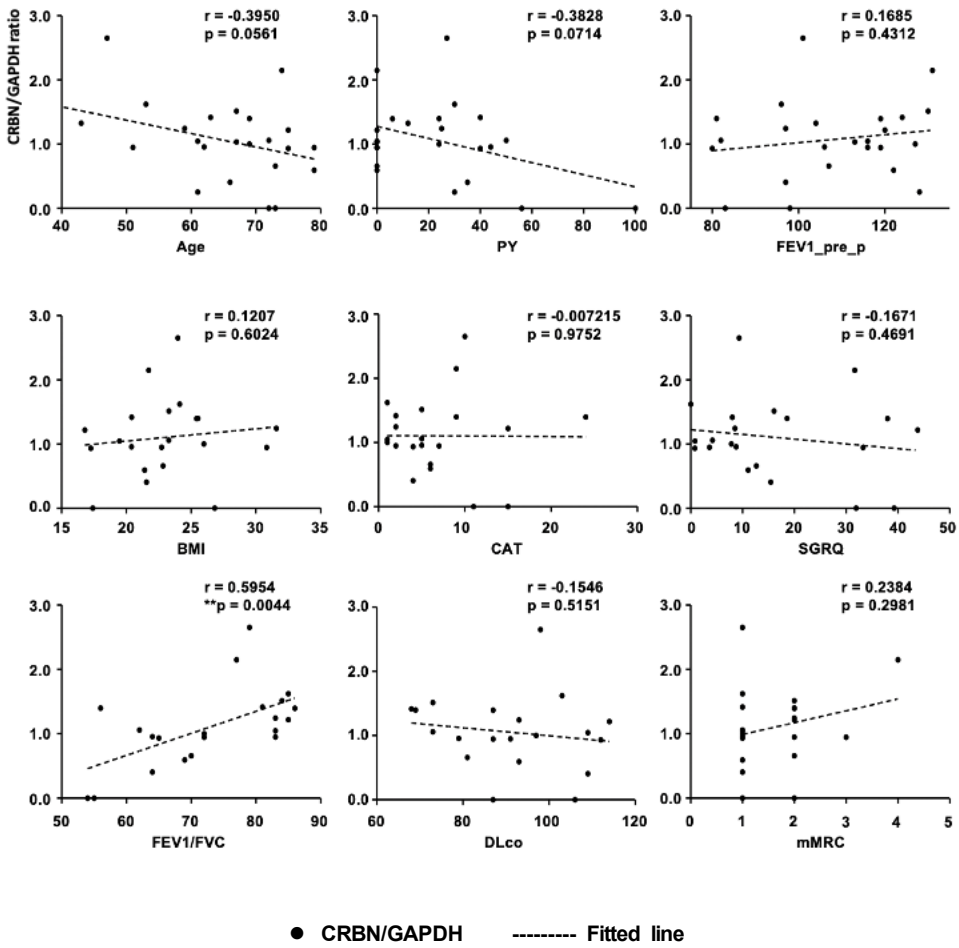

Figure 3. The correlation between the expression levels of CRBN protein in human lung tissue and clinical parameters of 24 subjects. Clinical parameters including age, pack-year, FEV1/FVC ratio, SGRQ, etc. were compared according to the expression level of CRBN protein. Results are expressed as a ratio of the band intensity of CRBN protein versus the band intensity of GAPDH protein. **Abbreviations:** PY, pack-year; FEV1, forced expiratory volume in 1 second; BMI, body mass index; CAT, COPD assessment test; SGRQ, St. George's Respiratory Questionnaire; FVC, forced vital capacity; DLco, diffusion capacity; mMRC, modified Medical Research Council; other abbreviations as in Fig.1. $^{**}P<0.01$

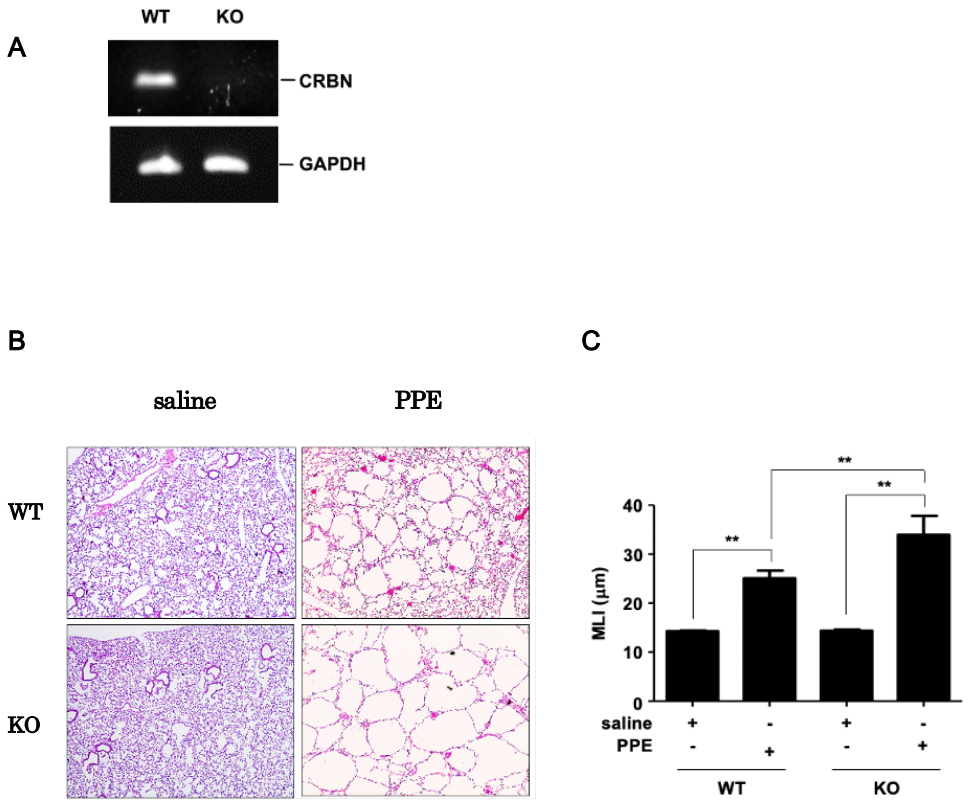

Figure 4. PPE-induced emphysema is exaggerated in CRBN KO BALB/c mice. Wild type (WT) and CRBN KO mice were given 0.5 units PPE via intratracheal injection. A: RT-PCR of CRBN from total lung tissues of BALB/c WT and CRBN KO mice. B: Representative images by H&E staining in lungs from WT and CRBN KO mice 14 days after intratracheal instillation of PPE (x40). C: The MLI was measured with resected lung tissue on day 14 after intratracheal instillation of PPE. Histogram bars represent means ± S.D. (WT: 5 mice per group, KO:5mice per group). **Abbreviations**: RT-PCR, reverse transcription polymerase chain reaction; PPE, porcine pancreatic elastase; H&E, hematoxyline & eosin; MLI, mean linear

intercept; WT, wild type; KO, knock out; S.D., standard deviation; other abbreviations as in Fig.1. *P<0.05, ***P<0.001 versus saline/WT BALB/c mouse group

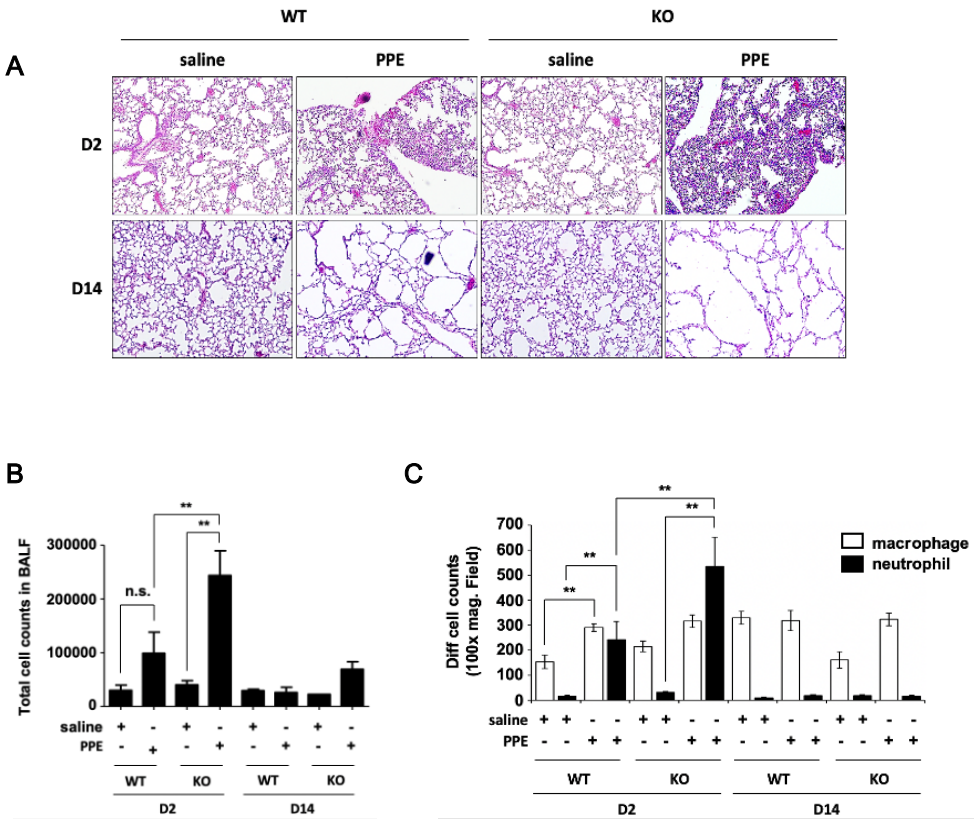

Figure 5. CRBN KO increases PPE-induced inflammation on day 2, which is returned to baseline on day 14. A: Representative images by H&E staining in lungs from WT and CRBN KO mice on day 2 & day 14 after intratracheal instillation of PPE. B, C: BALF was collected on day 2 and 14 after intratracheal instillation of PPE. Total cells (B) and differential cells (C) including macrophages and neutrophils were counted in BALF. Histogram bars represent means ± S.D. (WT: 5 mice per group, KO: 5 mice per group). Abbreviations: BALF, bronchoalveolar lavage fluid; n.s., not significant; other abbreviations as in Fig. 3. ** $P<0.01$ versus saline/ WT BALB/c mouse group.

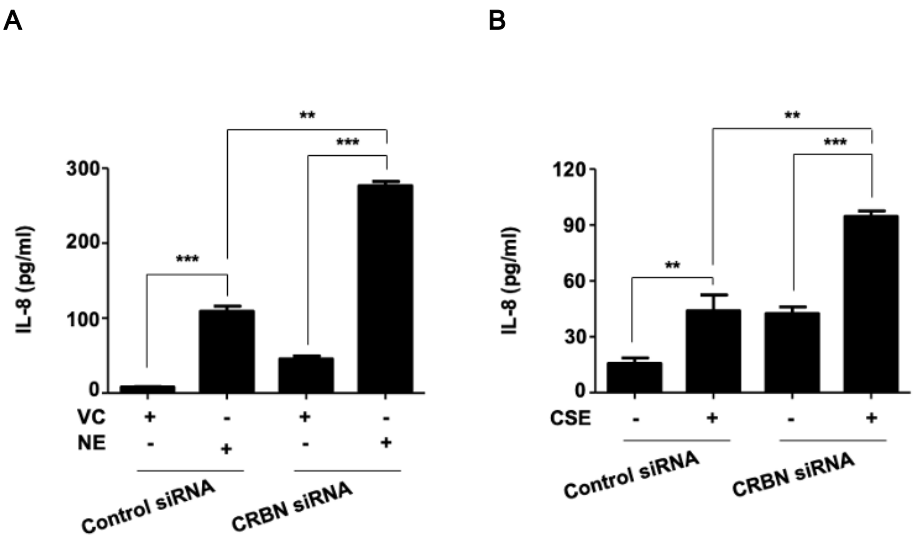

Figure 6. CRBN KO augments IL-8 release by NE or CSE stimulation in bronchial epithelial cells. A, B: BEAS-2B cells were transfected with control siRNA or CRBN siRNA. After 48 hours, BEAS-2B cells were treated with NE 1u/ml (A) and CSE 1% (B) for 24h, and supernatants were collected for measuring IL-8 level by ELISA. Histogram bars represent means ± S.D. Abbreviations: VC, vehicle control; NE, neutrophil elastase; CSE, cigarette smoke extract; siRNA, small interfering RNA. **P*<0.05, ***P*<0.01, *** *P*<0.001 versus VC/Control siRNA

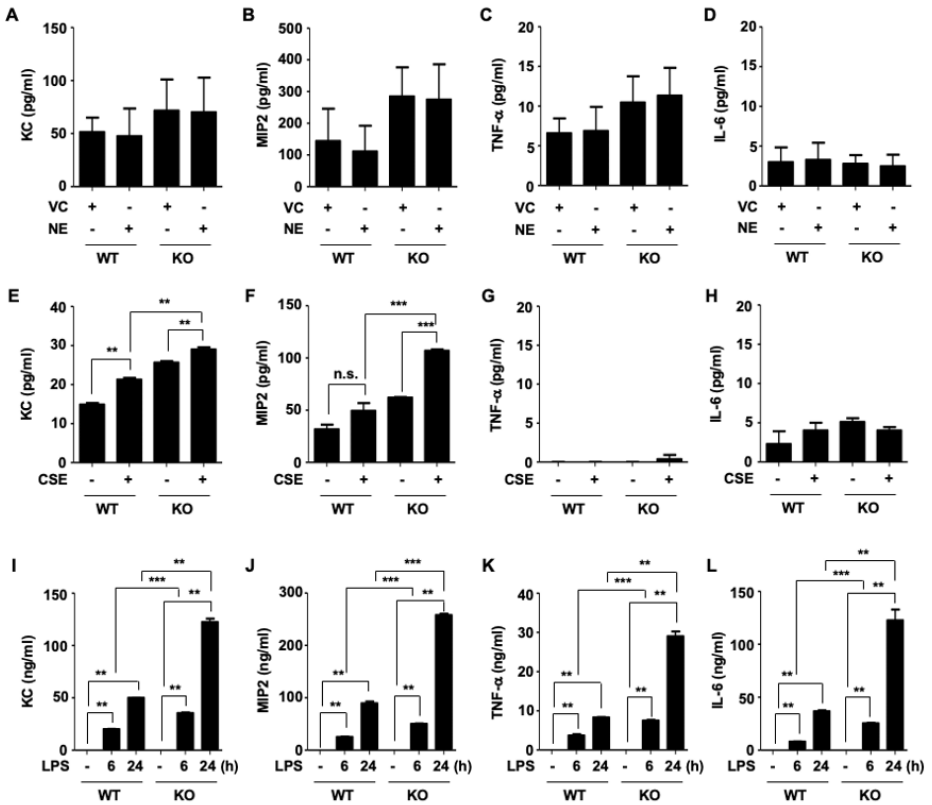

Figure 7. CRBN KO increases the release of inflammatory cytokines induced by LPS in BMDM cells. BMDM cells were treated with NE 1U/ml (A-D), CSE 1% (E-H) for 24hours or with LPS 100ng/ml (I-L) for 6, 24 hours. The concentration of inflammatory cytokines in cell culture media was analyzed by ELISA. Data represents the mean ± S.D. Abbreviations: MIP2, macrophage-inflammatory protein-2; TNF-α , tumor necrosis factor-α ; IL-6, interleukin-6; LPS, Lipopolysaccharides; BMDM, bone marrow derived macrophage; other abbreviationsas Fig. 3, 4 &5.

P<0.01,*P<0.001 versus untreated control/WT

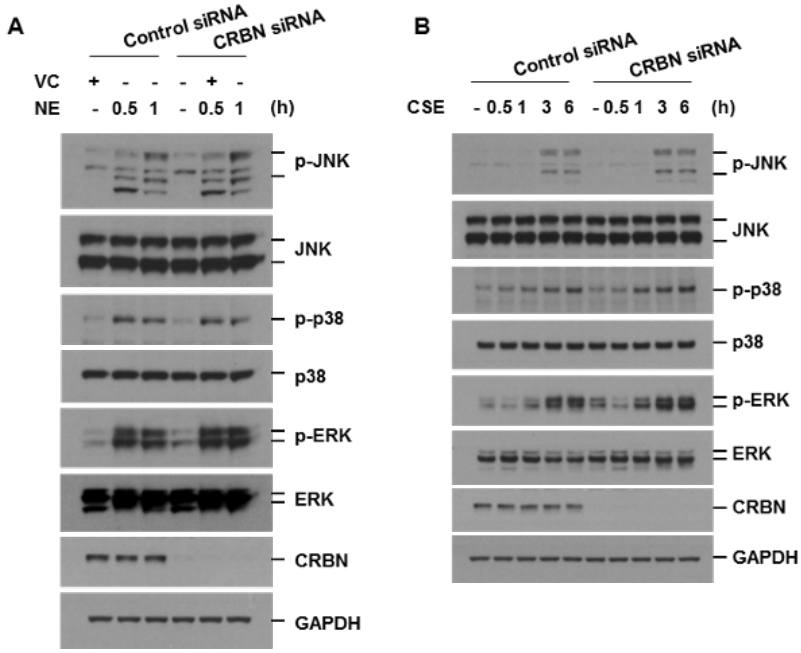

Figure 8. NE/CSE-induced MAPK activation is not influenced by CRBN KO in bronchial epithelial cells. In BEAS-2B cells treated with NE 1u/ml (A) or CSE 1% (B), expression of phosphorylated and total forms of p38, ERK and JNK were analyzed by western blotting. GAPDH was used as an indicator for equal protein loading. Abbreviations: JNK, c-*jun* NH2-terminal kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; other abbreviations as Fig.1&5.

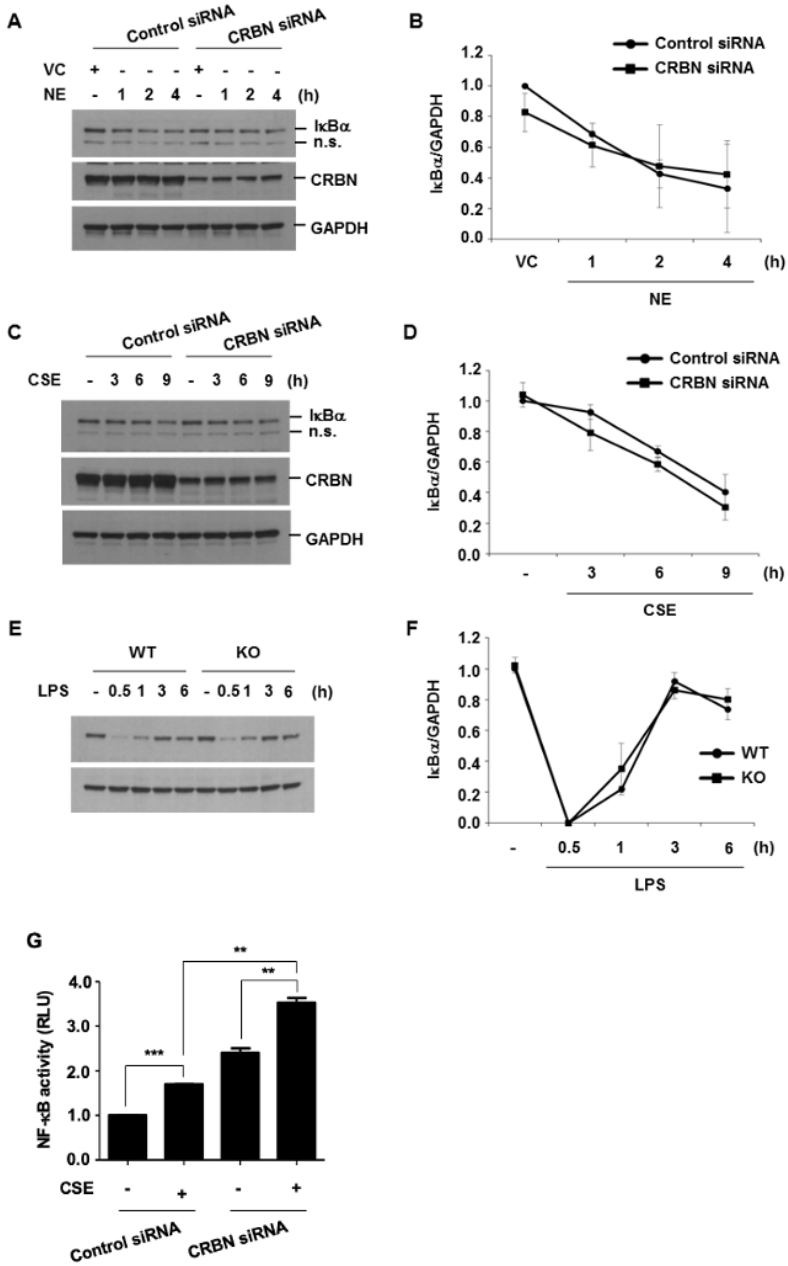

Figure 9. CRBN KO does not affect NE, CSE or LPS-induced I*k*B*α* degradation but increases CSE-induced NF-*k*B activity in bronchial epithelial

cells. A, C, E: Western blots of soluble proteins extracted from BEAS-2B cells treated with NE 1U/ml (A), CSE 1% (C), or LPS 100ng/ml (E). B, D, F: Relative density of I*k*B*α* against GAPDH in BEAS-2B cells treated with NE (B),CSE (D), or LPS (F). The relative density ratio was determined by the average intensity of the bands. G: BEAS-2B cells were transfected with the NF-*k*B reporter plasmid or control plasmid. Luciferase assay was done to measure the NF-*k*B activity with CSE treatment. Histogram bars represent means ± S.D. Abbreviations: NF-*k*B, nuclear factor kappa B; other abbreviations as Fig. 1, 5, & 6. ***P*<0.01,****P*<0001 versus untreated control/control siRNA

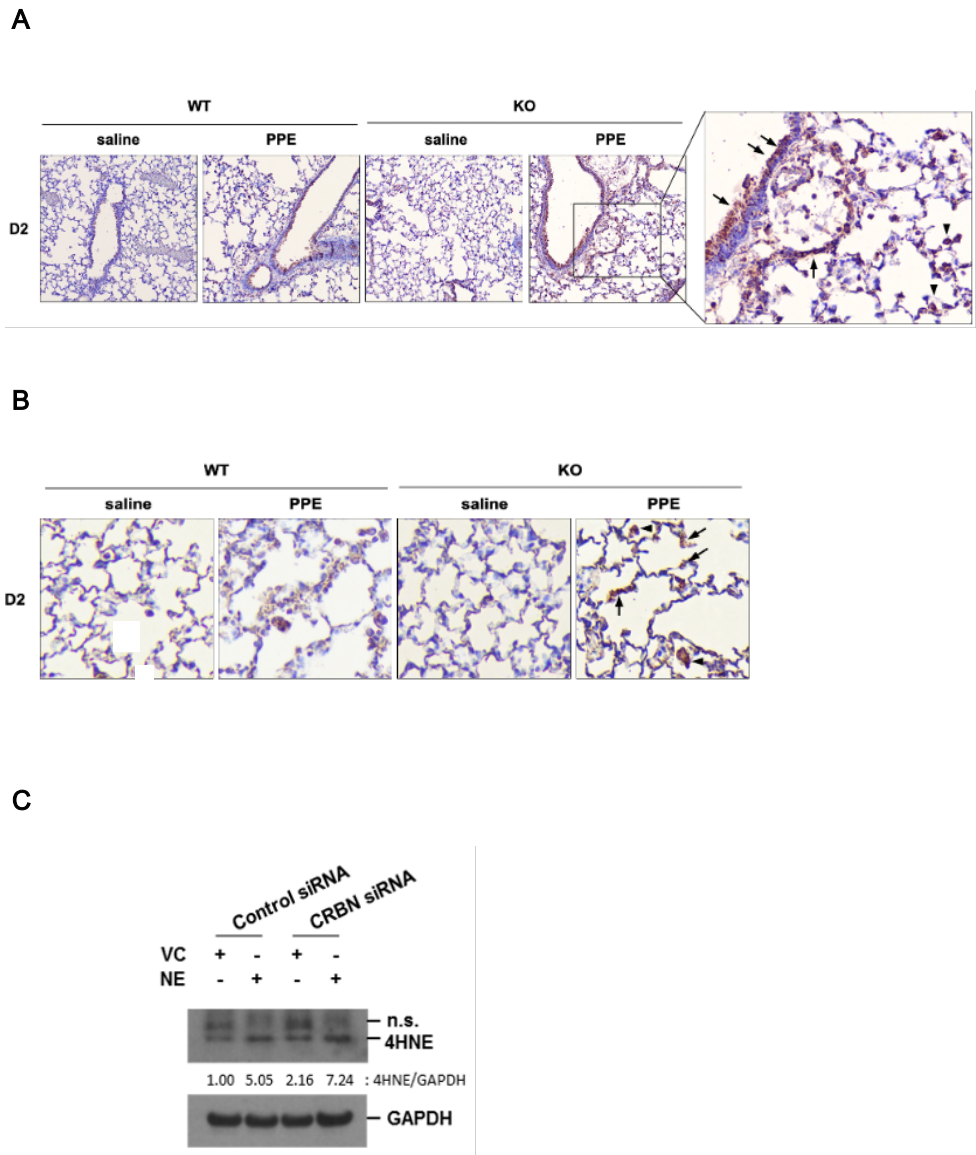

Figure 10. PPE/NE-induced oxidative stress was increased by CRBN KO in mice lung and bronchial epithelial cells A, B; Representative oxidative stress images by 8OHdG (A) and 4HNE (B) immunohistochemical staining in the lungs of BALB/c mice on day 2 after intratracheal administration of PPE

(WT: 5 mice per group, KO: 5 mice per group). Arrow indicates positive staining for 8OHdG (A) and4NHE(B). C: Western blots of 4HNE protein expression in BEAS-2B cells treated with NE 1u/ml for 24 hours. Signals corresponding to 4HNE were expressed as a ratio of the band intensity of the protein versus the band intensity of GAPDH. Abbreviations:8OHdG, 8-hydroxy-2'-deoxyguanosine; 4HNE, 4-hydroxynoneal; other abbreviationsas Fig.1, 3 & 5.

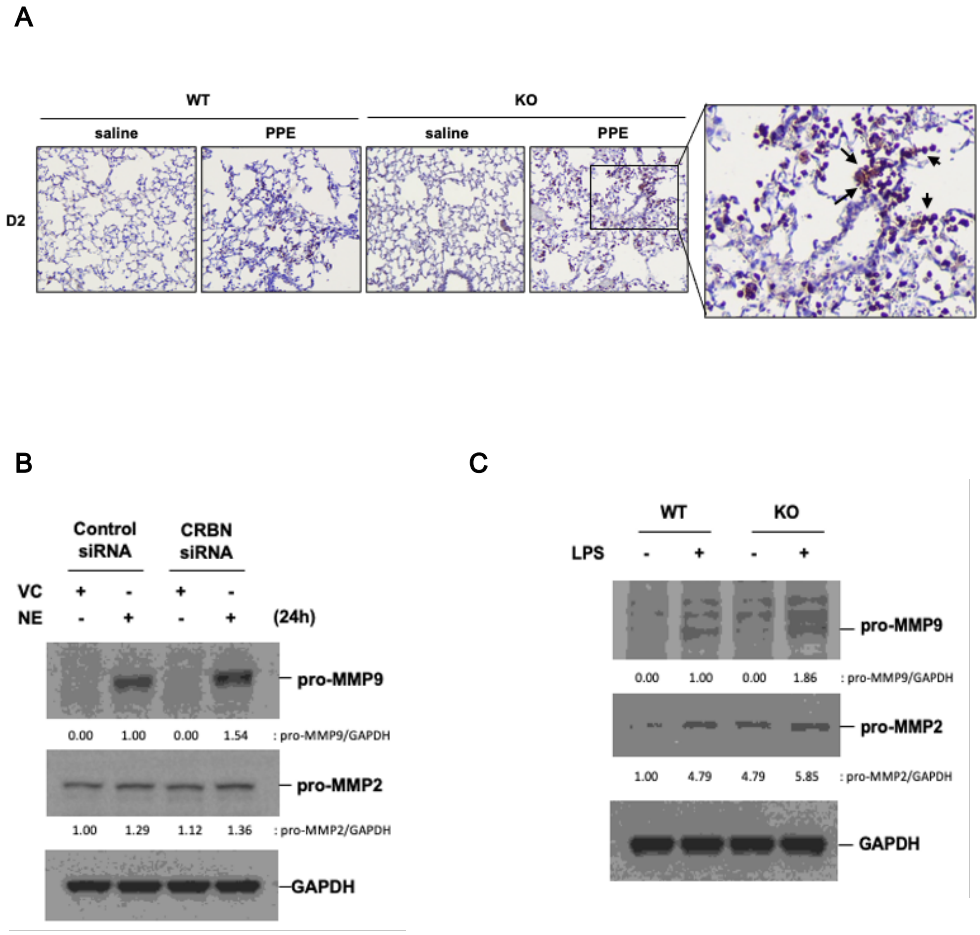

Figure 11. Elastase or LPS induced protease expression was increased by CRBN KO in mice lung and in bronchial epithelial cells. A: Representative images of MMP9 immunohistochemical staining in the lungs of BALB/c mice on day 2 after intratracheal administration of PPE (WT: 5 mice per group, KO: 5 mice per group). Arrow indicates positive staining for MMP9. B: Western blots of pro-MMP9, and pro-MMP2 protein expression in BEAS-2B cells treated with NE 1u/ml for 24 hours. C: Western blots of pro-MMP9,

and pro-MMP2 protein expression in CRBN WT and KO BMDM cells treated with LPS (100ng/ml) for 24 hours. Signals corresponding to pro-MMP9, and pro-MMP2 were expressed as a ratio of the band intensity of the protein versus the band intensity of GAPDH. Abbreviations: MMP9, matrixmetallopeptidase 9; MMP2, matrix metallopeptidase 2; other abbreviations as Fig. 3, 5 & 6.

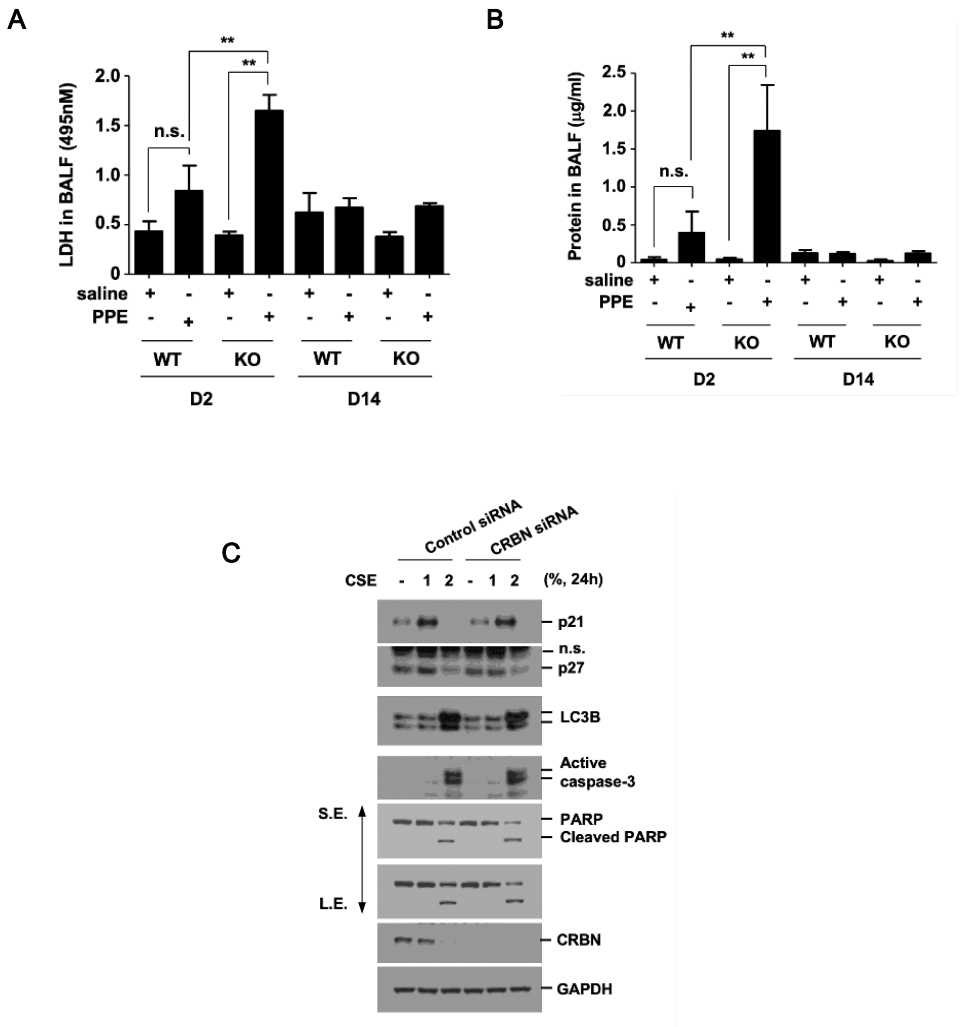

Figure 12. CRBN KO contributes to cellular injury but not to cell aging, autophagy activation or apoptosis. A, B: Levels of LDH (A) and protein (B) of BAL fluid were measured on day 2 & 14 after intratracheal administration of PPE. Histogram bars represent means ± S.D. (WT: 5 mice per group, KO: 5 mice per group). C: Western blots of cellular senescence markers (p21, p27), autophagy marker(LC3B) and apoptosis markers

(active caspase-3, PARP) in BEAS-2B cells treated with CSE for 24hours. Abbreviations: LDH, lactate dehydrogenase; LC3B, light chain 3B; PARP, poly (ADP-ribose)polymerase; S.E., shortexposure; L.E., long exposure; other abbreviations as Fig. 1, 3, 4, &5. **P<0.01versus saline/WT

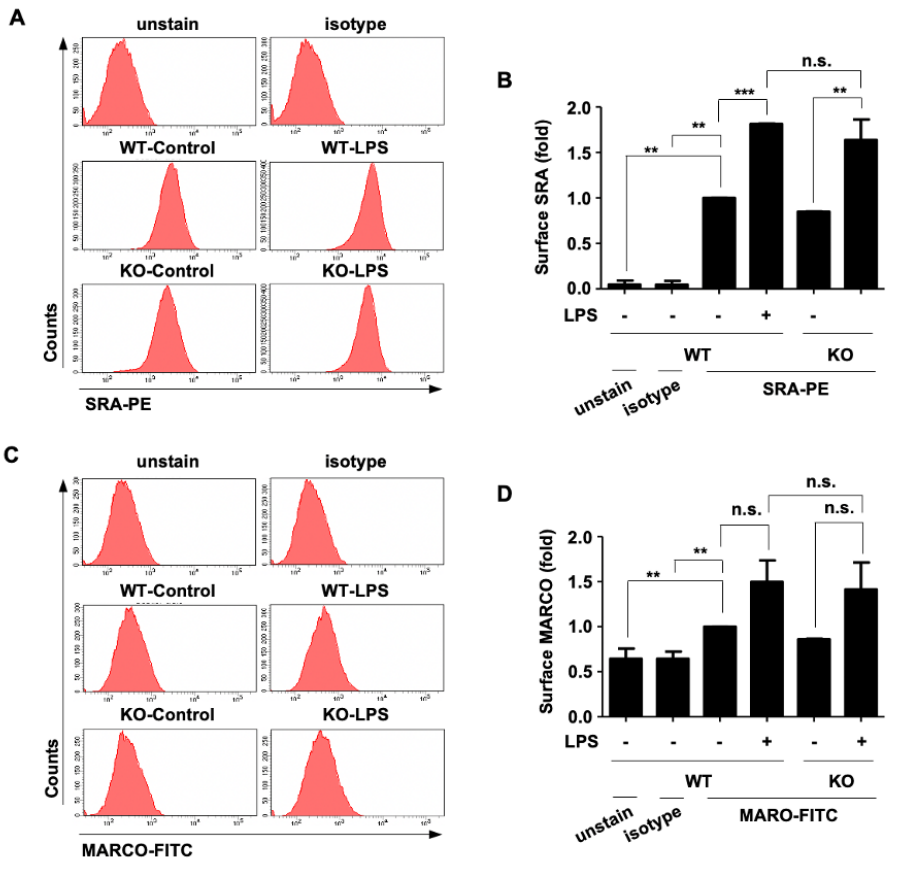

Figure 13. Effect of CRBN KO on the expression level of SRA or MARCO upon LPS stimulation. BMDM cells were stimulated with LPS 100ng/ml (A~D) for 24 hours. Cell surface expressions of SRA and MARCO were determined by flow cytometry. Results are shown as mean fluorescence intensity (MFI) histograms (A, C). Data represent the mean ± SD (B, D). Abbreviations: SRA, scavenger receptor A; MARCO, macrophagereceptor with collagenous structure; other abbreviations as Fig 1.3, 4 & 6. $^{**}P<0.01$, $^{***}P<0.001$ versus the corresponding control/WT

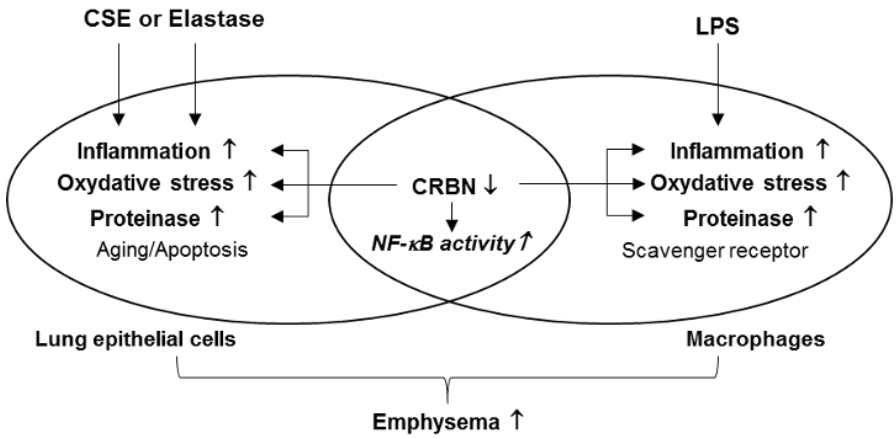

Figure 14. Proposed mechanism for the role of CRBN KO on the development of emphysema. CRBN KO increases NF-*k*B activity, leading to the enhanced inflammatory response, oxidative stress, and proteinas eexpression in lung epithelial cells and in alveolar macrophages by elastase, CSE or LPS stimulation. Thus, CRBN KO augments the development of emphysema. Abbreviations as Fig 1, 5, 6 & 8.

Discussion

Emphysema is a main phenotype of chronic obstructive pulmonary disease (COPD) and is defined as the irreversible destruction of alveolar structures and enlargement of the airspaces.(2) Oxidant/antioxidant or protease/antiprotease imbalances, which are pivotal to the pathogenesis of emphysema.(3) However, the precise molecular mechanisms for emphysema remain still unclear. Our present findings suggest that CRBN deficiency aggravates elastase-induced emphysema by augmenting neutrophilic inflammation through NF-κ B activation.

Our study aimed to elucidate the role of CRBN in emphysema development. We first evaluated the degree of PPE-induced emphysema in CRBN KO mice. PPE-induced emphysema was exaggerated in CRBN KO mice compared to WT mice. On 2 days after PPE intratracheal administration, a more prominent neutrophilic inflammatory response was observed, and PPE-induced emphysema was exaggerated in CRBN KO mice compared to WT mice. This finding is consistent with that neutrophils predominate in the sputum and BALF of patients with COPD and correlate with disease severity.(32) Neutrophils recruit macrophages and lymphocytes and might play a key role in emphysema progression by the release of elastase.(33)

We observed that IL-8 release was significantly increased in BEAS-2B cells transfected with CRBN siRNA after NE or CSE

treatment. IL-8 is a well-known neutrophilic specific chemotactic factor, and IL-8 expression in humans mediated through MAPK signaling.(34) However, we found that MAPK activation by NE or CSE was independent with CRBN KO in bronchial epithelial cells.

The alveolar macrophage is a primary defense cell along with airway epithelial cells and a key regulator of inflammation of COPD.(26, 35) However, bronchial epithelial cell and alveolar macrophage exhibit different inflammatory responses to various microbes or cigarette smoking.(35) According to the study using paired airway epithelial cells and alveolar macrophages, alveolar epithelial cells exhibited a greater pro-inflammatory response to the toll-like receptor (TLR) 3 agonist, alveolar macrophages were a stronger responder to TLR 4 agonists.(35) In our experiments, BMDMs were not effectively stimulated by elastase or CSE compared to bronchial epithelial cells. Therefore, we used LPS along with NE or CSE to stimulate macrophages in our experiments using BMDMs. LPS is a toxic component of gram-negative bacteria that provokes profound inflammation.(35) COPD patients with exacerbations showed a significantly increased number of inflammatory cells in sputum and accelerated emphysema progression compared with those without exacerbations.(36,37) Most exacerbations are precipitated by viral of bacterial infections, and the Gram-negative bacteria is the most commonly isolated pathogens.(38) Even single LPS intratracheal administration to mice with elastase-induced emphysema can provoke inflammation, increase protease production and finally lead to more

severe emphysematous change.(39) Therefore, we stimulated macrophages with LPS to mimic the exacerbation status of humans and elucidate the role of CRBN more clearly on the development of elastase/CSE-induced emphysema. CRBN KO significantly increased productions of KC, MIP-2, TNF-a and IL-6 in macrophages stimulated by CSE or LPS. NF-κB regulates gene expression of these inflammatory cytokines. NF-κB is a ubiquitous transcription factor that regulates most of the inflammatory proteins in patients with COPD.(40) NF-κB activation involves IκBα phosphorylation and dissociation from IκB. Liberation from IκB allows NF-κB to translocate into the nucleus, where it induces gene transcription by binding to the promoter of NF-κB responsive genes.(41) Our study observed that CRBN KO did not affect NE, CSE, or LPS-induced degradation of IκBα , yet CSE-induced NF-κB activation increased by CRBN KO. In this regard, CRBN KO might increase neutrophilic inflammation and leads to profound emphysema through NF-κB activation by affecting the following step after IκBα degradation.

Increased oxidative stress and elastase activity are also involved in the pathogenesis of COPD. 4-HNE, is a major product of the lipid peroxidation, is a key mediator of oxidant-induced cell signaling and apoptosis.(42) Alveolar septal apoptosis is also increased in COPD patients compared with the lungs of normal individuals and non-COPD smokers. The patients with emphysema had significantly higher percentages of type II cells positive for senescence-associated cyclin-dependent kinase inhibitors, such as p16 and p21 than the

asymptomatic smokers and nonsmokers.(43) Although 4HNE expression was increased in CRBN KO mice compared with CRBN WT mice, CRBN KO did not affect the expression of apoptosis or cellular senescence markers with elastase or CSE treatment. Given the increased expression of 4HNE in CRBN KO mice and BEAS-2B cells transfected with CRBN siRNA, CRBN may have a protective effect against oxidative stress induced by elastase. We also evaluated the effect of CRBN on the phagocytic function of macrophages. The upregulated surface SRA with LPS stimulation in BMDM was not influenced in CRBN KO mice.

When we measure the CRBN expression in human lung tissue, CRBN expression was decreased in COPD patients compared with non-smokers or smokers without COPD. And the ratio of FEV1/FVC, which is an essential parameter for COPD diagnosis, was significantly correlated with CRBN expression levels. This suggests CRBN is involved in the pathogenesis of COPD in the human lung.

Taken together, these findings indicate that CRBN KO aggravates elastase-induced emphysema by increased neutrophilic inflammation, oxidative stress and proteinase expression through NF-κB activation, and LPS-induced inflammation mimicking exacerbations of human lung is also influenced by CRBN (Figure 14).

Conclusion

In conclusion, our study suggests that CRBN deficit may contribute to the susceptibility to emphysema via NF-κB activation and CRBN can be a new therapeutic candidate in treatment of COPD/emphysema.

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국문 초록

연구의 배경 : 만성 폐쇄성 폐질환은 폐기종과 만성 기도 폐쇄를 특징으로 하는 만성 염증성 질환이다. 이러한 염증 반응에 대한 연구는 오랫동안 계속되어 왔지만, 여전히 질병의 진행을 멈추고 경과를 바꿀 수 있는 치료법은 아직 없다. 세레브론 (CRBN) 은 CRL4A E3 유비퀴틴 연결효소의 기질 수용체로 다양한 질환에서 염증 반응, 산화 스트레스 등을 조절하는데 그 역할을 하는 것으로 알려져 있다. 그러나 아직 세레브론의 폐 내 역할에 대한 것은 알려진 바가 없다.

연구의 목적: 엘라스타제 투여로 유도된 폐기종 발생에서의 CRBN의 역할 및 그 조절 기전을 확인하는 것이다.

연구방법: 환자들의 폐조직을 이용하여 임상적 지표와 CRBN 발현과의 연관성을 평가하였다. 이후 각 그룹당 5마리의 쥐에 엘라스타제 (PPE)를 기도내로 투여하여 CRBN 유전자 제거된 쥐 그룹과 야생주 쥐 그룹 간의 염증 반응 및 폐기종 발생 정도를 분석하였다. 기전 확인을 위한 세포 실험을 위해서는 기관지 상피세포 및 쥐 대퇴골에서 추출된 대식세포에 CRBN 짧은 간섭 RNA 형질주입을 시행하였다. 형질 주입 48시간 이후 각 세포군에 엘라스타제, 담배 연기 추출물 및 지질다당류(LPS) 처리하여 세포를 자극시킨 후 염증성 사이토카인, 산화 스트레스 및 프로테아제 활성 정도를 웨스턴 블랏으로 평가하였으며, 쥐 조직에서도 면역 형광 염색을 통하여 같은 지표들을 확인하였다. 또한 대식세포 기능에 대한 CRBN의 역할 확인을 위해서 대식세포에 LPS 처리를 한 후 청소제 (scavenger) 수용체 발현 정도를 유세포 분석으로 평가하였다.

결과: 만성 폐쇄성 폐질환 환자들의 폐조직에서 CRBN의 발현이 감소되어 있었으며 임상 지표 중에서는 FEV1/FVC 값이 CRBN의 발현 정도

와 유의한 상관관계를 보였다. CRBN 유전자 제거 쥐는 대조군에 비해 엘라스타제 처리 후 2일 째 폐 조직내 호중구 침윤이 증가하였으며 14일 째는 폐기종이 더욱 진행하였다. KC, MIP-2, TNF-α 및 IL-6 는 담배 연기 추출물 또는 LPS 처리를 하였을 때 CRBN 결핍 세포주에서 분비가 더욱 증가하였다. 염증성 사이토카인 증가의 기전을 알아보기 위해 진행한 실험에서 MAPK 시그널은 CRBN 유무에 영향을 받지 않았으나 NF-κB는 담배 연기 추출물 자극을 주었을 때 CRBN 결핍 세포주에서 유의하게 그 활성도가 증가하는 것을 확인하였다. 또한 엘라스타제 및 LPS 자극을 주었을 때 유도되는 산화 스트레스 및 프로테아제 발현 정도도 CRBN 결핍인 경우 더욱 증가되어 나타나는 결과를 관찰하였다. 그러나 세포 노화, 오토파지 활성 및 세포사멸 과정은 CRBN 유무와 관련이 없었으며 LPS 자극에 따른 대식 세포의 청소제 수용체 발현의 증가도 CRBN 의 영향을 받지 않음을 확인하였다.

결론: CRBN 결핍은 NF-κB 활성화를 통한 호중구성 염증 촉진 및 폐기종 발생 증가를 통해 COPD 의 병인에 관여할 것이다.

주요어: CRBN, COPD, emphysema, elastase, NF-κB

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