이학석사 학위논문

담배연기에 노출된 폐 상피세포에서 progranulin 의 조절역할

Regulatory Role of Progranulin in Cigarette Smoke Extract–Exposed Lung Epithelial Cells

울산대학교대학원

의 학 과

이경영

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이 논문을 이학석사 학위 논문으로 제출함

2016년 2월

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Abstract

Background

Emphysema is characterized by irreversible destruction of alveolar wall with enlargement of distal air spaces. Cigarette smoke (CS) is considered as a main causative factor for the development of emphysematous change in chronic obstructive airway disease. Progranulin (PGRN) has been reported to be induced in response to various stimuli including CS. Recently, PGRN is reported to participate in apoptosis process that is a well known cause of alveolar wall destruction. However, the role of PGRN in emphysema is currently unknown.

Aims

The aim of this study is to evaluate if there is a regulatory role of PGRN in human alveolar epithelial cells exposed to cigarette smoke extract (CSE).

Methods

The PGRN expression levels were measured in A549 cells after exposure to CSE. The effect of PGRN on CSE-mediated apoptosis was determined by using Western blot with anti-PARP and anti-caspase3, and flow cytometry analysis with annexin V and PI staining in PGRN-silenced or over-expressed A549 cells. In addition, to investigate the underlying mechanism of PGRN, ER stress markers such as molecules of PERK, IRE1, and AFT6 pathways were also evaluated in CSE exposed A549 cells.

Results

Intracellular PGRN expression was increased in A549 cells treated with CSE. A significant increase in active caspase-3 and active PARP expression was detected in PGRN-silenced A549 cells after CSE exposure. Apoptotic cell death was also significantly increased in

PGRN knock-down A549 cells and decreased in PGRN over-expressed cells. CSE-mediated apoptosis was associated with the molecules in PERK.

Conclusion

PGRN may play a role in the prevention of the development of emphysema by inhibiting apoptosis of alveolar epithelial cells.

Keyword : PGRN , CSE , ER stress, apoptosis , Emphysema ,

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Abbreviations

COPD, chronic obstructive pulmonary disease

PGRN, progranulin

GRN, granulin

ER, endoplasmic reticulum

CSE, cigarette smoke extract

PERK, pancreatic ER kinase

IRE-1, inositol-requiring protein

ATF6, activating transcription factor 6

GRP78, glucose-regulated protein 78

IL-8, interleukin-8

PARP, poly (ADP-ribose) polymerase

CHOP, C/EBP homologous protein

Introduction

Chronic obstructive pulmonary disease (COPD) including chronic bronchitis and emphysema is an important chronic inflammatory airway disease with big burden. Cigarette smoking is a well known major risk factor for the development of COPD[1]. Cigarette smoke (CS) contains numerous harmful compounds and various oxidants including free radicals, reactive oxygen species, and reactive nitrogen species which cause oxidative stress and severe damage to the lungs [2]. As time progresses, damaged cellular components tend to aggregate and accumulate in cells, potentially disturbing normal cellular functions and initiating cell death[3]. In fact, emphysematous lung pathology accompanied by alveolar wall destruction was typically found in COPD patients. However, the precise pathogenesis of emphysema has not been clearly defined.

Progranulin (PGRN), also known as several different names such as granulin epithelin precursor (GEP), PC-cell-derived growth factor (PCDGF), proepithelin, or acrogranin [4], is a 593 amino acid glycoprotein that functions as a growth factor [5] and is secreted from cells mediated by its signal peptide. PGRN is secreted in an intact form (88 kDa) and undergoes proteolysis, leading to release of its constituent granulin (GRN) peptides [6-8]. PGRN contains 7.5 repeats of a cysteine-rich motif (CX5–6CX5CCX8CCX6CCXDX2HCC PX4CX5–6C) in the order P-G-F-B-A-C-D-E, where A-G are full repeats and P is the half motif [5] **(Fig. 1)**. PGRN is also known to have multiple functions[9], and plays a crucial role in wound healing [10], inflammation [11], and host defense [5]. So far, PGRN has been reported to be combined with more than 20 proteins **(Table.1)**. Interestingly, PGRN is expected to have an anti-inflammatory characteristic, whereas the additional pro-inflammatory function of released 6-kDa GRN peptides was reported[12, 13]. **(Fig. 2)** In addition, PGRN is reported to be digested by several enzymes including neutrophil elastase (NE), proteinase 3 (PR3), and MMP-12, all of which are well known to be critically important in the COPD inflammation.

Meanwhile, in eukaryotic cell, the endoplasmic reticulum (ER) is an organelle responsible for protein synthesis, folding, post-translational modification, and transport of proteins to be used in cells. However, enhanced oxidative stress can result in the accumulation of misfolded or unfolded proteins in the ER lumen, known as ER stress [14]. For maintenance of ER homeostasis, ER stress response activates typical 3 signaling pathways including ER membrane including pancreatic ER kinase (PERK), inositol-requiring protein (IRE-1), and activating transcription factor 6 (ATF6) **(Fig. 3)**. ER chaperone GRP78 dissociates from ER stress sensors and then PERK, IRE1 and ATF6 activates a transcriptional response which can be adaptive or apoptotic processes for cell survival during ER stress. In cases where ER stress is severe or prolonged and the unfolded protein response (UPR) cannot restore homeostasis, apoptosis is activated to remove the damaged cells[15]. Recently, it has been reported that cigarette smoke extract (CSE) induced ER stress-mediated apoptosis for lung epithelial cells [3]. Interestingly enough, PGRN is known to be regulated by a network of ER molecular chaperones as a novel substrate of ERp72 [16]. However, whether PGRN directly regulates ER stress response pathway and ER stress-mediated apoptosis has not yet been elucidated.

In this study, we hypothesized that the intracellular expression of PGRN plays a critical role in alveoli epithelial cell apoptosis found in CS associated airway inflammation and COPD. The results of the current study show that CS induces PGRN expression and triggers the ER stress response including PERK and IRE1 signaling in human alveolar epithelial cells. In addition, it is suggested that the enhanced expression of PGRN caused by CS exposure is critically linked to regulating ER stress response and protection of ER stress-mediated cellular apoptosis.

PGRN-associated proteins	Interaction sites	Interaction domain PGRN	Function
ADAMTS7	Matrix	GRN A–G	Inhibition of ADAMTS 7 activity
ADAMTS12	Matrix	GRN A–G	Inhibition of ASAMTS 12 activity
COMP	Matrix	GRN A	COMP enhances PGRN-induced proliferation
Perlecan domain V	Matrix	GRN F + B	Promotion of tumor growth
HDL/apo A–I	Circulation	NA	HDL inhibits PGRN convertion into GRN
SLPI	Circulation	NA	Convertion of PGRN into GRN
Tat (of HIV-1 and -2 and CAEV)	Circulation	GRN B + A	Unknown
TNFR1 and -2	Membrane	GRN F + A + C	Inhibition of TNF-[α] signaling
TLR9	Membrane	GRN ACDE	Assisting the binding of CpG to TLR9
Sortilin	Membrane	GRN E	Delivery of PGRN to lysosome for degradation
Hexokinase	Cytoplasm	NA	Unknown
TPM3	Cytoplasm	NA	Promotion of hepatocarcinogenesis
BiP, calreticulin, GRP94, ERp57, ERp5, HSP70	ER	NA	Chaperones networking
Cyclin T1	Nuclear	GRN PFGBA DE	Inhibition of transcription
Tat of HIV	Nuclear	GRN PFGBA DE	Inhibition of transcription

NA, unknown.

Table 1. PGRN interacting proteins (from ref.[5])

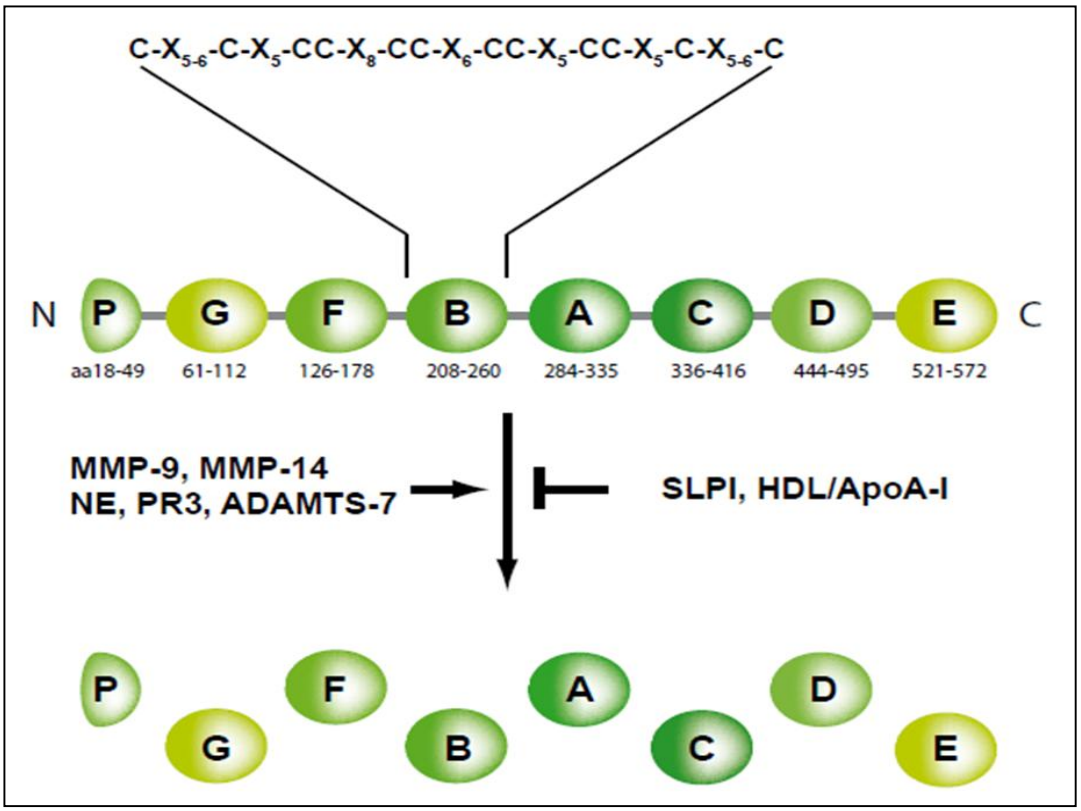

Figure 1. Stucture and cleavage of PGRN. (from ref.[17]). Each circle represents granulin domain containing the consensus motif of 12 cysteine residues. PGRN can be cleaved in single granulin domains by MMP-9, MMP-14, NE, PR3 and ADAMTS-7. Both SLPI and high density lipoprotein(HDL)/apoliporotein A-l(Apo A-l) can bind PGRN and inhibit PGRN cleavage.

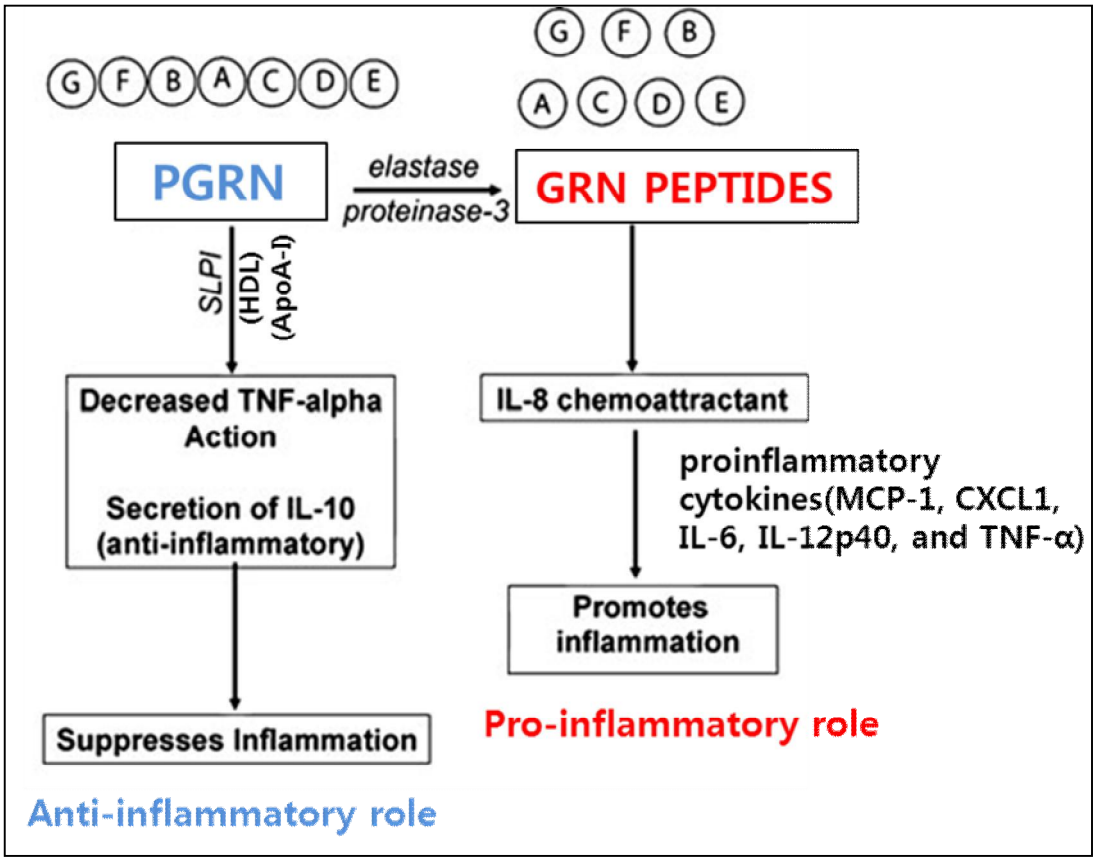

Figure 2. PGRN and GRNs have opposing inflammatory effects. (from ref.[13]). Intact PGRN, which is represented schematically as a chain of granulin domains (linked circles) supresses inflammtion by, among other processes, blocking TNF-α receptors and signaling. SLPI(secretory leukocyte protease inhibitor) prevents the proteolysis of PGRN at the site of injury or inflammation. Proteloytiec enzymes degrade PGRN to granulin domain peptides (unlinked circles) which may enhance inflammation by stimulating the secretion of the chemokines interleukin-8 (IL-8).

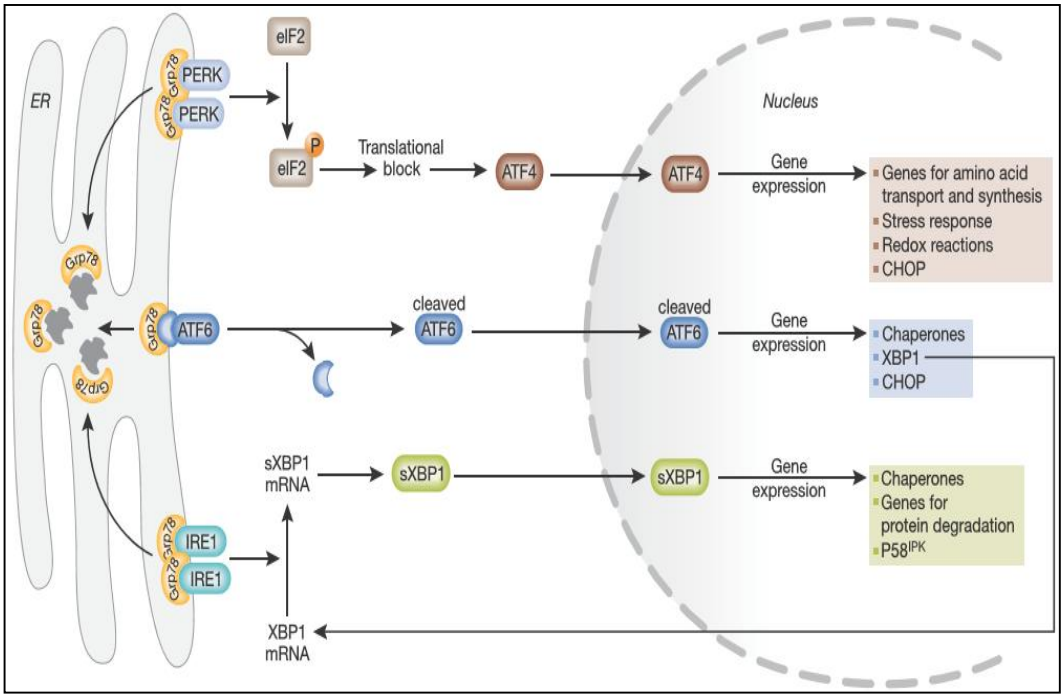

Figure 3. Signaling of unfolded protein response. (from ref.[18]).GRP78 dissociates from the three endoplasmic reticulum(ER) stress receptors, pancreatic ER Kinase (PKR)-like ER kinase **(PERK)**, activating transcription factor 6 **(ATF6)** and inositol-requiring enzyme 1 **(IRE1)**, allowing their activation. The activation of the receptors occurs sequentially, with PERK being the first, rapidly followed by ATF6, whereas IRE1 is activated last. Activated PERK blocks general protein synthesis by phosphorylating eukaryotic initiation factor 2α (eIF2α). This phosphorylation enables translation of ATF4, which occurs through an alternative, eIF2α-independent translation pathway. ATF4, being a transcription factor, translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis. ATF6 is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus. Active ATF6 is also a transcription factor and it regulates the expression of ER chaperones and X box-binding protein 1 (XBP1), another transcription factor. To achieve its active form, XBP1 must undergo mRNA splicing, which is carried out by IRE1. Spliced XBP1 protein (sXBP1) translocates to the nucleus and controls the transcription of

chaperones, the IPK co-chaperone and PERK-inhibitor P58 , as well as genes involved in protein degradation. This concerted action aims to restore ER function by blocking further build-up of client proteins, enhancing the folding capacity and initiating degradation of protein aggregates. CHOP(C/EBP homologous protein).

Materials & methods

Cell cultures and experimental design

The A549 cells (ATCC, Manassas, VA, USA), established from human lung epithelial alveolar cell carcinoma, were cultured in RPMI 1640 (WELGENE Inc. Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 1% penicillin-streptomycin (WELGENE Inc.) in a humidified incubator with 5% CO_2 at 37°C.

Firstly, to evaluate the effect of CSE on A549 cells, the cells were seeded at an optimal density of 5×10^5 cells per well into 6-well plates and cultured till the cultures are reached 90% confluence. The following day, cultured cells were washed in PBS, and then treated with different concentrations of CSE (1, 3, 5, 10, 15 and 20%) for various times (3, 6, 12, and 24 hours). At the end of treatment, the cells were washed with cold PBS and lysed before being used for real time PCR and western blot analysis.

Next, to investigate the effect of PGRN on CSE-exposed cells, PGRN knock down or PGRN overexpressed A549 cells, generated by transfection of PGRN specific siRNA or plasmids respectively, were used for further experiments in this study. The transfection was performed and the cultured medium was replaced with fresh CSE-exposed medium. Followed by incubation for 24 hours, adhered and floating cells were then collected together and analyzed with western blot and FACSCanto for assessing the status of cellular apoptosis. In addition, cultured cells and media were collected and centrifuged at 14,000 x g for 30 seconds to pellet the cells. The supernatant was then collected and stored at -80°C until further analysis.

Mouse model of cigarette smoke induced emphysema

To generate a murine model of CS induced emphysema, mice (C547BL/6J, female) were divided into air-exposed control group (5/group) and CS-exposed groups (5/group) at

8weeks of age. CS-exposed animals were subjected for 5 day a week during 6months to the smoke of research cigarettes (3R4F) generated by cigarette smoke exposure system (Cigarette Laboratory at the Tobacco and Health Research Institute, University of Kentucky, Lexington, KY). All mice were killed 2hr after the last CS exposure. Then, to assess the expression of PGRN in the lung of mice with CS induced emphysema, we performed immunohistochemical staining of lung sections.

Preparation of cigarette smoke extract

To prepare CSE, we used research cigarettes (British American Tobacco, Republic of Korea) including 11.0 mg TPM /cigarette, tar (8.0 mg/cigarette) and nicotine (0.7 mg/cigarette) or fresh air as a control for in vitro cultures. CSE was prepared by methods developed in our laboratory. Briefly, CSE was prepared from one cigarette by blowing smoke, generated using a vacuum syringe system on a smoking machine, through 5 ml of serum-free medium. Large particles and bacteria were eliminated from the solution through a 0.22 μm filter. The solution was standardized by measuring the absorbance at 320 nm (optical density of 0.74±0.05). This solution was regarded as 100% CSE and was freshly generated for each experiment, and subsequently serially diluted with medium to obtain a final 10% working concentration.

Generation of PGRN knock down or overexpression cells

To deplete human PGRN in A549 cells, a siRNA targeted against a portion of the human PGRN mRNA open reading frame (5´- GGGAAGGACACUUCUGCCAUGAUAA -3´) as well as control RNA duplex of random sequence were purchased from Bioneer Corporation (Daejeon, Republic of Korea). Cells were seeded into 6-well plates at a density of 3×10^5 cells/well. The next day, the cells were transfected with 10 nM PGRN siRNA or control siRNA using lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) and were

incubated for 48 hours.

For overexpression of human PGRN in A549 cells, a pCMV3-SP-N-flag-hPGRN and pCMV-SP-N-flag as negative control vector were purchased from Sino Biological Inc. (Beijing, China) and were induced into these cells using ViaFect Transfection Reagent (Promega, Madison, WI, USA) with 1μg of the indicated plasmids at a density of 3×10^5 cells/well for 48 hours. Downregulation (by siRNAs) and upregulation (by plasmids) of PGRN were determined by western immunoblotting.

Cell viability assay

To determine a suitable concentration and duration of the CSE intervention, Cell Counting Kit-8 (Enzo Life Science, Farmingdale, NY, USA) assay was used to monitor cellular viability. Cells were subsequently seeded in a 96-well plate with 100 μl of culture medium at a density of 2×10^4 cells/well and incubated overnight in a humidified incubator with 5% CO_2 at 37°C. After washing with PBS, cells were treated medium containing CSE in 1, 3, 5, 10, 15, 20% respectively for 24 hours. Then the cultures were added 10 μl CCK-8 solution to each well and incubated for 15 min at 37°C. Absorbance intensity was measured at 450 nm using a SpectraMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA)

Western blot analysis

For western blot analysis, each well of cells was lysed into lysis buffer containing 50mM Tris-HCl (pH7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, protease inhibitor (1mM ABSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and phosphotase inhibitor (1mM Na_3VO_4 and 50 mM NaF). Cell lysates were centrifuged at 14,000 x g for 10 min at 4°C, and the samples were boiled in 5 x loading buffer for 5 min. 40 μg of proteins per sample was loaded into each well. Proteins were separated by 10-14% SDS-PAGE and separated proteins were transferred electrophoretically to polyvinylidene fluoride membranes

(GE Healthcare, Pittsburgh, PA, USA). After blocking with 5 % nonfat dry milk (BD Biosciences, San Jose, CA, USA) in Tris-buffered saline-0.1% Tween (TBS-T) for 1 hour at room temperature, each membrane was incubated overnight at 4°C with primary antibodies against PGRN (1:1000; Invitrogen), GRP78 (1:1000; BD Biosciences), cleaved caspase-3 (1:1000; Cell Signaling Technology), PARP (1:1000; Cell Signaling Technology), p-eIF2α (1:1000; Cell Signaling Technology), total eIF2α (1:1000; Cell Signaling Technology), ATF6 (1:1000; Novus Biologicals), p-ERK1/2 (1:1000; cell signaling Technology), total ERK1/2 (1:1000; cell signaling Technology), p-JNK (1:1000; cell signaling Technology), total JNK (1:1000; cell signaling Technology), p-Akt (1:1000; cell signaling Technology) and total Akt (1:1000; cell signaling Technology). After incubation with HRP-conjugated secondary antibodies (Enzo Life Science), membranes were developed using West-Q Chemiluminescence Substrate Kit (GenDEPOT, Barker, TX, USA). Membranes were reblotted with anti- β-actin (1:5000; GeneTex Inc.) for normalization and densitometry analysis was performed using the ImageJ software (NIH). The ratio of remaining indicators to β-actin bands was taken as the relative expression levels of proteins.

RT-PCR and quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen) and the concentration of RNA was determined by optical density measurement at 260 nm. First-stand cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Standard cDNA synthesis kit (Roche, Diagnostics, GmbH, Mannheim, Germany) with random primers according to the manufacturer's protocol.

Gene transcript levels of PGRN and GAPDH were quantified by real-time PCR with use of an SYBR Green 1 Master (Roche). cDNA was amplified for PGRN and GAPDH as an internal control. The specific primers used were as follows: for PGRN, forward primer 5'-GAG GAC TAA CAG GGC AGT GG -3' and reverse primer 5'- GCC TCT GGG ATT GGA

CAG -3'; for GAPDH, forward primer 5'- TGC ACC ACC AAC TGC TTA -3' and reverse primer 5'- GGC ATG GAC TGT GGT CAT -3' . The reaction was performed in 20 µl SYBR Green mixture containing 1µl of each 10 µM forward and reverse primer and in a LightCycler 480 system (Roche Applied Science, Penzberg, Germany) using the following PCR conditions: initial incubation step of 2 min at 50 °C, reverse transcription of 60 min at 60 °C and 95 °C for 2 min, followed by 40 cycles of 15 sec at 95 °C for denaturation and 1 min at 60°C for annealing and extension. The presence of a single specific PCR product was verified by melting curve analysis and confirmed on a 1.5% agarose gel.

RT-PCR analysis of total RNA was performed to simultaneously detect both unspliced (XBP1u) and spliced (XBP1s) XBP1 mRNA and GAPDH was used for the normalization of target gene expression data. In brief, 1 µg of total RNA was reversed transcribed with cDNA synthesis kit (Roche) as described above and amplified with AccuPower PCR Premix (Bioneer) using the following pair of primers: 5'-TCTGCTTGATGTGTGTCCTCTT-3' and 5'-GTCGTTCACCTTCGTAGACCT-3' for XBP1, PCR product size is 289 bp amplicon was generated from unspliced XBP1, a 263 bp amplicon was generated from spliced XBP1. The reaction was performed in 20 µl premixture containing 1ul of each 10 µM forward and reverse primer and in a S1000 Thermal Cycler (Bio-Rad Laboratories Inc.). The PCR cycling conditions were followed by 35 cycles of 5 min at 95 °C, 1min at 95 °C, 30 sec at 58 °C, 30 sec at 72 °C and 5 min at 72 °C. The PCR products were separated by electrophoresis using 1.5 % agarose gel.

Immunohistochemistry for PGRN

Formalin-fixed, paraffin-embedded lung tissues from mice exposed to cigarette smoke (CS) for 6 months were obtained for immunohistochemical (IHC) analysis. 4 µm sections were deparaffinized, rehydrated, subjected to antigen retrieval by boiling in 10 mM sodium citrate buffer, pH 6.0, for 15 min, and blocked in 3% H_2O_2 (DaKo Peroxidase Blocking Solution)

for 10 min at room temperature in humidity chamber. The slides were washed three times in PBS, blocked with 0.25% casein in PBS (DaKo Protein Block Serum-Free) for 15 minutes at room temperature in humidity chamber, and then incubated with rabbit polyclonal anti-PGRN antibody (Santa Cruz Biotechnology; 1:100) overnight in dark at 4°C. After washing three times in PBS, slides were incubated polyclonal HRP-conjugated anti-rabbit immunoglobulin G (DakoCytomation) for 1 hour at room temperature in dark and detection was by the DaKo EnVision HRP/DAB system (Dako, Carpinteria, CA, USA). Slides were counterstained for 5~15 sec in hematoxylin (Sigma) and then dehydrated and mounted using Permount (Thermo Fisher Scientific Inc. Rockford, IL, USA) with a cover-slide. The positive DAB-stained areas produced brown pigmentations.

Enzyme-linked immunosorbent (ELISA) assay

The levels of IL-8 release in the cultured media were determined using the respective duo set kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer instructions. Each well of a 96-well plate (SPL) was pre-coated overnight at room temperature in 100 μl of capture antibody, diluted to 1μg/ml in PBS. Wells were then aspirated and washed 3 times with 300 μl of wash buffer before 100 μl of blocking solution was added to each well and incubated at room temperature for 1 hour. Wells were again washed 3 times with 300 μl of wash buffer, before 100 μl of sample or standard was added to each well and incubated for 2 hours at room temperature. hIL-8 standards were made up at: 0, 31.25, 62.5, 125, 250, 500, 1000 and 2000: pg/ml in blocking solution. Following another 3 washes, 100 μl of detection antibody was added, also diluted to 1μg/ml and incubated at room temperature for 2 hours. The plate was again washed 3 times and 100 μl of streptavadin solution (R&D Systems) was added to each well and the plate incubated again for 20 minutes at room temperature. The plate was given 3 final washes before 100 μl of substrate solution (R&D Systems) was added to each well and the plate incubated for 20 minutes at room temperature. Finally, 50 μl of

stop solution was added to each well, Plate was measured absorbance 450 nm using a microplate reader.

Flow cytometry

A FACSCanto (BD Biosciences) was used to analyze the membrane and nuclear events during apoptosis. The excitation wavelength was 488 nm (FITC and PI), and the observation wavelength was 530 nm for green fluorescence (FITC) and 585 nm for red fluorescence (PI). Cells were harvested by trypsinization and then resuspended in 100 µl of binding buffer (10 mM HEPES-NaOH pH 7.4, 140 mM NaCl, 2.5mM $CaCl_2$) at concentration of 1 x 10^5 cells/ml. A total of 5 µl of Annexin V-FITC (BD Pharmingen) and propidium iodide (BD Pharmingen; 50 µg/ml) were added to these cells and then was incubated for 15 min at 25 °C in the dark. After adding 400 µl binding buffer in each sample, the debris of labeled samples were eliminated through a 0.22 µm filter. The samples were analyzed with a FACSCanto flow cytometer within 1 hour. All experiments were at least repeated three times.

Results

CSE induces PGRN expression in airway epithelial cells

To investigate if the expression of PGRN is regulated by CSE exposure in airway epithelial cells, both cell culture model and in vivo animal model were used. We performed western blot, RT-PCR, and immunohistochemistry for detection of PGRN. Firstly, to determine a suitable concentration and duration of the CSE treatment, cell viability assays were performed under the treatment with various concentrations of CSE (0, 1, 3, 5, 10, 15 and 20 %) for 24 hour. The survival rate of A549 cells was ≥ 50% at 10% CSE exposure, which was considered suitable for further experiment **(Fig. 4A)**.

Protein levels of cellular PGRN were increased in CSE-exposed A549 cells in a dose-and time-dependent manner and confirmed by western blot **(Fig.4B)**. The results from RT-PCR experiments demonstrated that the PGRN mRNA levels were significantly increased in the cells treated by 10%, 6 hours after exposure to CSE **(Fig. 4C)**. Therefore, we decided to use 10% CSE as stimulating concentration and 24 hours as exposure time.

Next, lung tissues from mice exposed to CS for 6 months were obtained and immunohistochemistry was conducted. The results showed that PGRN positive staining was limited to epithelium and macrophage in mouse lung tissues. In addition, the levels of PGRN expression were increased in CS-exposed lung tissues compared to those from the mice exposed to fresh air for 6 months **(Fig. 4D)**.

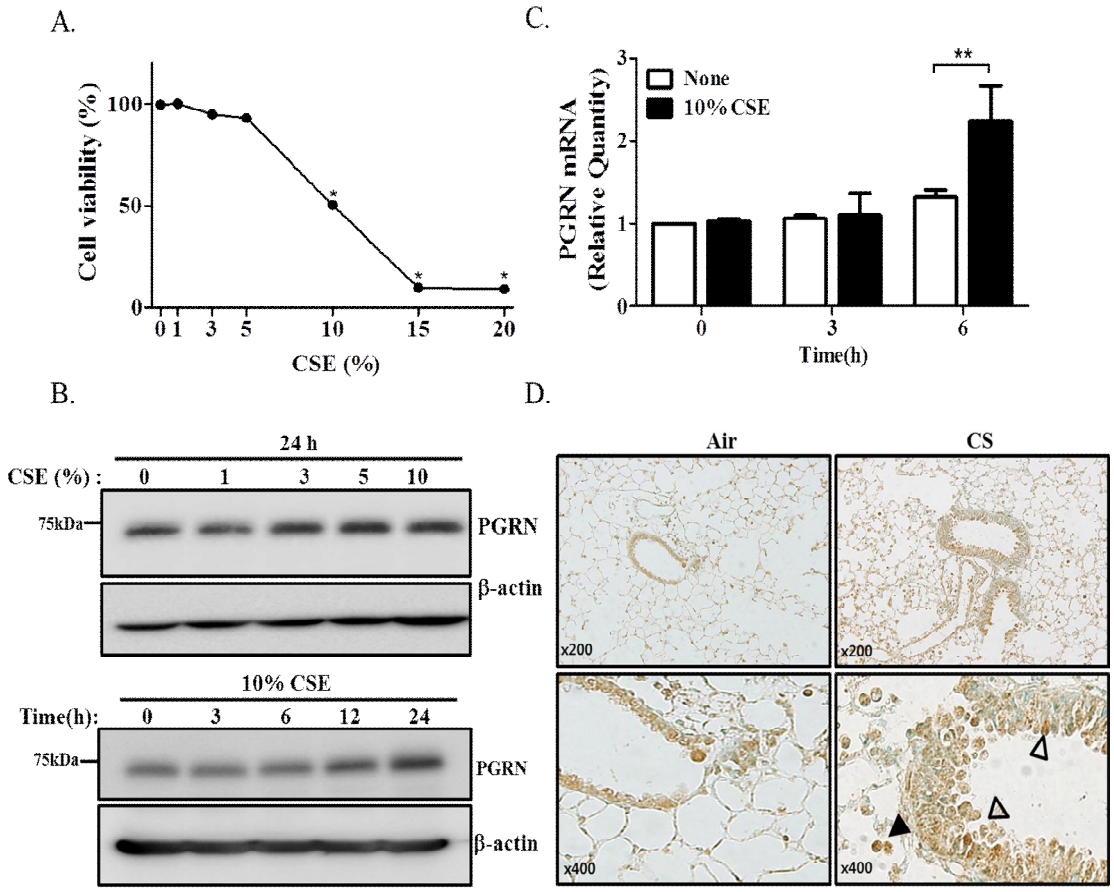

Figure 4. Induction of PGRN in airway epithelial cells by cigarette smoke exposure (CSE). (**A**) A549 cells were exposed to various concentrations of CSE for 24hr and the cell viability of A549 reduced at high concentrations of CSE. (**B**) A549 cells were treated with various concentrations of CSE for 24 hours or for different time with 10% CSE, as indicated. Protein levels of PGRN were analyzed by western blots. H, hours. (C) PGRN mRNA transcription was confirmed by qRT-PCR in A549 cells harvested after 3 and 6hrs of 10% CSE exposure. PGRN mRNA was normalized by GAPDH. Asterisks indicate statistical significance, $^{**}P<0.01$; NS, not significant. Error bars are SEM. (D) Immunohistochemical staining of PGRN expression in mouse lung tissue obtained from C57BL/6J mice exposed to CS for 6 months, and were increased in macrophages (solid arrowheads), type II alveolar, and airway epithelial cell (open arrowheads). Brown: positive PGRN.

Increased levels of PGRN expression were associated with CSE-induced ER stress via PERK or IRE1 signaling in airway epithelial cells

Exposure to CSE is well known to induce cellular oxidative stress which lead to up-regulation of multiple heat shock proteins and ER-associated chaperones, called ER stress[3]. To investigate if CSE induced PGRN expression is associated with ER stress, we examined 3 distinct signaling pathways including PERK, inositol requiring enzyme1 (IRE1), and activating transcription factor6 (ATF6) which are well known molecules to sense accumulation of misfolded proteins in the ER[15].

A549 cells or PGRN deficient cells were stimulated with various concentration of CSE and then the expression levels of each ER stress related molecule were evaluated. High levels of CSE (10%) had been applied to induce an ER stress response. Firstly, to evaluate PERK pathway, expression of phosphorylated eIF2α (p- eIF2α) and GRP78, both of which are directly related to activation of PERK, was assessed.

The expression of chaperone protein GRP78 was slowly increased in a time dependent manner in both PGRN intact and knock-down epithelial cells. The GRP78 expression was a little bit enhanced in PGRN knock-down cells than that in PGRN intact cells. The levels of p-eIF2α was significantly increased after 6hr of incubation with 10% CSE and further increased upon prolonged exposure to CSE. In PGRN knock down cells, p-eIF2α expression was further increased at earlier time point (**Fig. 5A and 5B)** Interestingly, the expression of PGRN was increased and well correlated with the expression patterns of p-eIF2α (**Fig. 5A**).

To evaluate ATF6 pathway, ATF6 proteolysis analysis was carried out since ATF6 undergoes proteolytic cleavage by S1P and S2P proteases followed by migration into the nucleus to activate further transcription of UPR responsive genes. We analyzed CSE-exposed lysates by Western blot. No significant change in cleaved ATF6 level (50kDa) was found in PGRN intact cells while significantly enhanced expression of that was observed in PGRN deficient cells **(Fig. 5A and 5B)**.

Next, for the evaluation of IRE1 pathway, XBP1splicing analysis was conducted since XBP1 is a known molecule to be spliced by IRE-1 and lead to its translation into protein and further up-regulation of chaperones in an effort to prevent overloading the misfolded proteins of the ER. RNA samples from CSE-exposed cells were collected at multiple time points and were analyzed a semi-quantitative RT-PCR with primers that it possible to detect both the splice and unspliced form of XBP1. Intensity of spliced XBP1 (XBP1s) mRNA represented by ER stress in CSE-exposed PGRN intact cells was slightly increased as exposure time progresses, whereas spliced XBP1 in PGRN knock down cells was significantly enhanced at even earlier time points **(Fig. 5C and D)**.

Collectively, PGRN expression was upregulated under the condition of CSE exposures and this response was closely associated with CSE-induced ER stress responses mainly medicated by PERK and IRE1 pathways in PGRN intact alveolar epithelial cells. Meantime, all 3 ER stress pathways seem to be involved with exaggerated ER stress response under PGRN deficient alveolar epithelial cells.

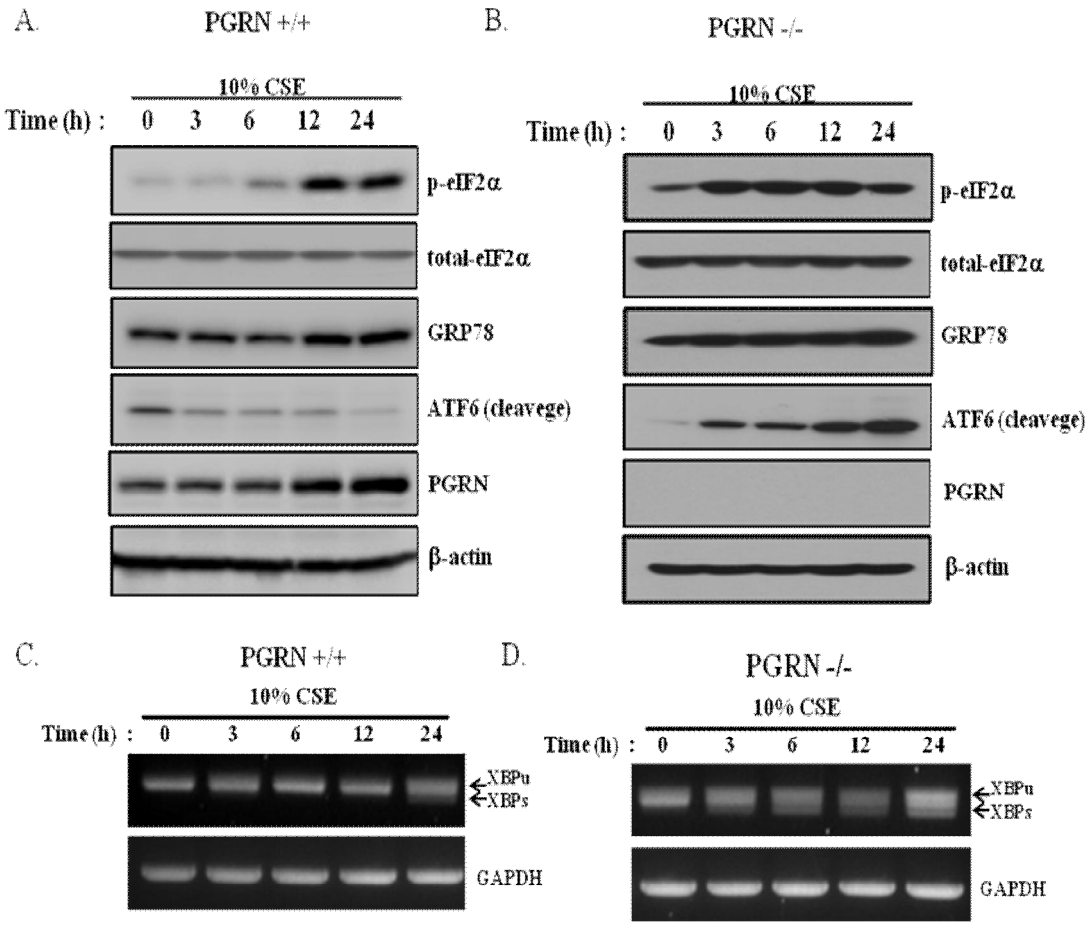

Figure 5. CSE-induced ER stress responses in PGRN intact and deficient cells.

PGRN intact A549 cells (A and C) or PGRN-deficient A549 cells (B and D) were exposed to 10% CSE for various time periods as indicated, and then p-eIF2α, GRP78, and Cleaved ATF6 levels (50kDa) were determined by Western blot (A and B). β-actin was used as a loading control. RNA was extracted from both PGRN intact and PGRN-deficient A549 cells exposed to CSE over multi-time points and XBP1 splicing was assessed with RT-PCR (C and D). Unspliced (XBP1u) and spiced(XBP1s) XBP1 mRNAs was separated on 1.5% agarose gel using RT-PCR analysis with primer set flanking the spliced-out region in XBPs mRNA. GAPDH was used as a normalization control.

Airway alveolar cells exposed to CSE augmented PGRN secretion and proteolytic cleavage by extracellular proteases

Since PGRN and GRNs have been suggested to have an important function in the inflammatory diseases and GRNs in particular are well known molecules to induced neutrophilic inflammation, we evaluated whether CSE affects secretion and cleavage of PGRN and GRN production. A549 cells were treated with various concentrations of CSE for 24 hours in addition to being treated with 10% CSE for various durations till 24 hours. The expression of PGRN and GRNs was analyzed in both cell lysates **(Fig. 4B)** and cultured media (**Fig. 6A)**. Secretion of PGRN (88kDa) was detected after 12hr CSE treatment and increased. In the culture supernatant media, cleavage of full length PGRN to GRN fragments was observed in the region molecular mass <80kDda on SDS-PAGE. Variant GRNs (cleaved PGRN) were detected with apparent band (55kDa) after prolonged 24hours exposure and were increased CSE dose-dependently at 24hr time period **(Fig. 6A)**.

The relative density of each band was measured by using Image J software and The GRNs/PGRN ratio increased significantly at high concentration CSE (10%) for 24hr **(Fig. 6B)**. The results showed that PGRN was constantly secreted and underwent proteolytic cleavage to GRNs after CSE exposure.

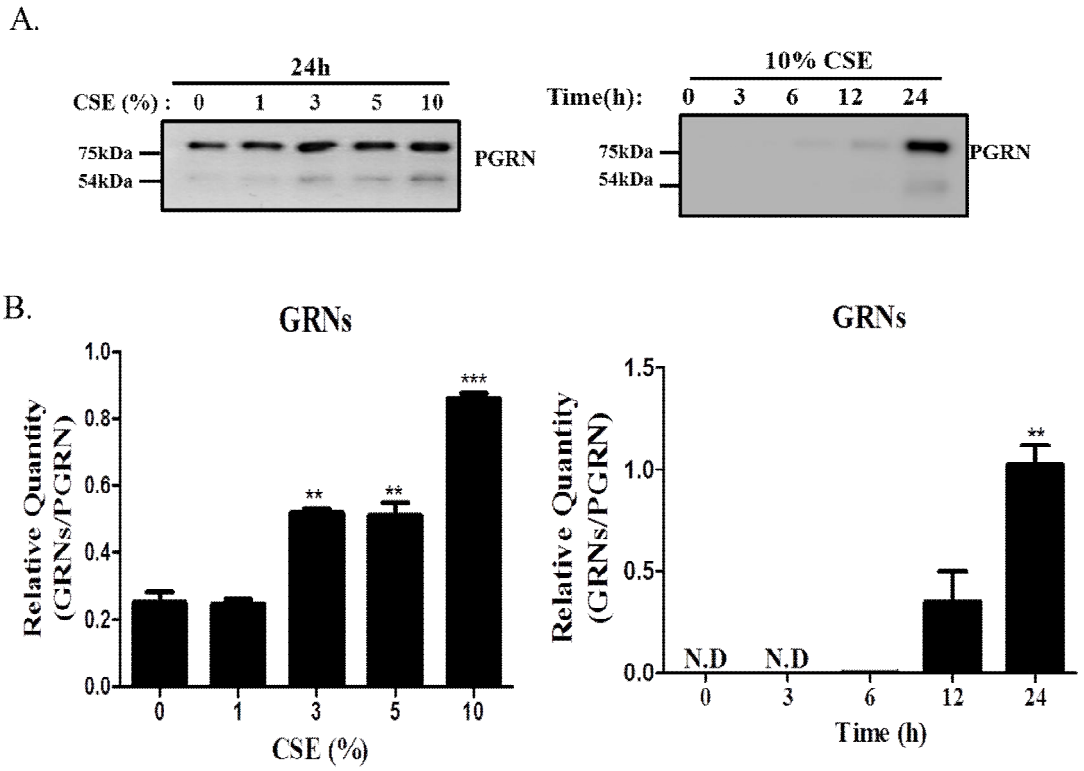

Figure 6. Increased proteolytic cleavage of the secreted PGRN to GRN in culture supernatants from CSE exposed A549 cells. (A) A549 cells were treated as described above for Fig.4B. The levels of secreted PGRN in the cell culture supernatant were detected by Western blot. GRNs occurs as variants, reflecting probably intact PGRN and cleaved GRNs. (B) The levels of GRNs/PGRN conversion were confirmed subsequent quantitative analysis of band density. The bar diagram represents mean values ± SEM. Asterisks indicate statistical significance, ***P*<0.01, *** *P*<0.001compared with the group without treatment; ND, not detected.

CSE-induced PGRN modulates release of IL-8 from epithelial cells

GRNs originated from proteolysis of secreted PGRN are closely related with induction of neutrophilic inflammation probably through strong induction of IL-8, the most critical neutrophil chemoattractant, which was observed in A549 cells under the influence of CSE in many previous reports[19]. In the current study, to elucidate the role of PGRN in the secretion of IL-8 in airway epithelial cells, A549 were cultured with treatment of various increasing concentration of CSE and for various durations. The levels of secreted IL-8 were measured for each experimental condition. The results showed that the levels of IL-8 release in CSE exposed A549 cells were increased in a time and dose dependent manner **(Fig. 7A and 7B)**.

To investigate whether the level of IL-8 secretion is regulated by PGRN, we used siRNA to knock down the expression of PGRN **(Fig. 7C)**. Interestingly, IL-8 secretion showed a remarkable increase in the PGRN knock down cells compared with in the cells treated with the random siRNA **(Fig. 7D)**.

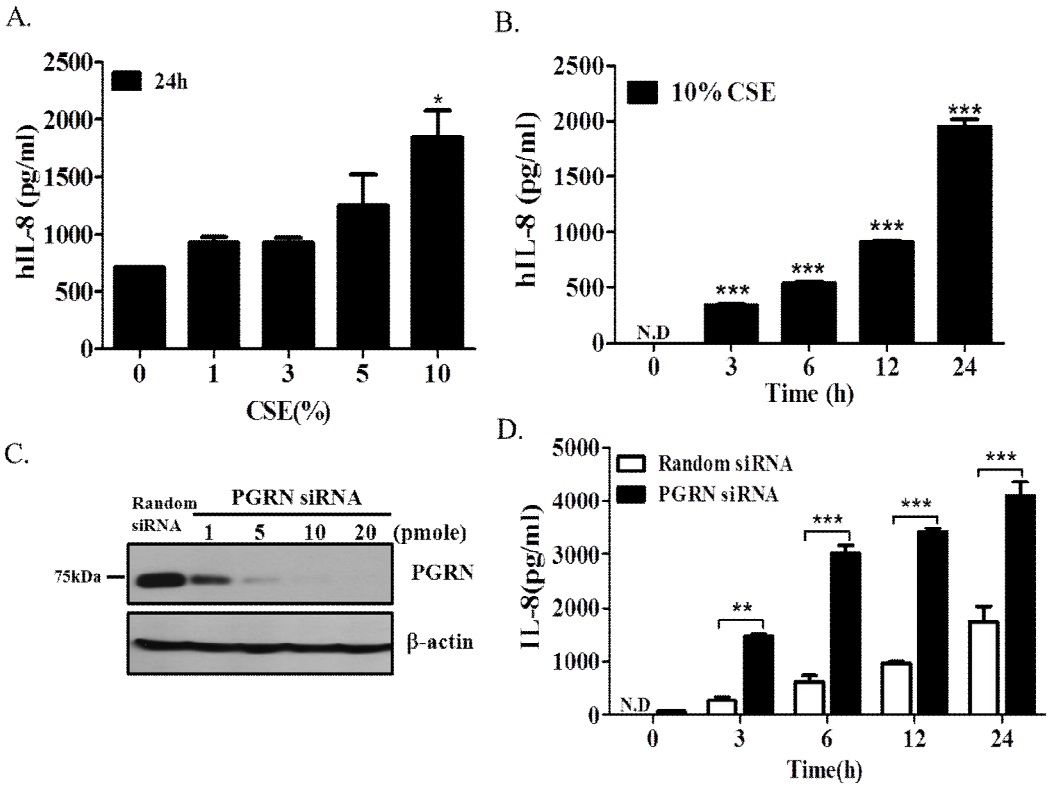

Figure 7. Elevation of the CSE-induced IL-8 secretion in PGRN deficient epithelial cells. (A) After treatment with various concentration of CSE for 24h or (B) for different time with 10% CSE, cell-free supernatants were collected from culture medium of A549 cells and measured for IL-8 release by ELISA. Data are based on three experiments performed in triplicates, and expressed as mean values ± SEM. Asterisks indicate statistical significance, $^{**}P<0.01$, $^{***}P<0.001$ compared with the group without treatment; ND, not detected. (C and D) A549 cells were transiently transfected with random siRNA or specific PGRN siRNA. After 24h, cells were exposed to CSE at a concentration of 10% and cell-free supernatants were harvested at 0, 3, 6, 12 and 24 h. The efficiency of siRNA knockdown of PGRN in cells and measurement of IL-8 release in medium were determined using Western blot and ELISA analysis. Asterisks indicate statistical significant difference from the random siRNA control, as determined by Student's *t*-test, $^{**}P<0.001$ and $^{***}P<0.001$.

Intracellular PGRN prevents ER stress-induced cellular apoptosis under CSE treated conditions

It has been reported that ER stress caused by CS contributes to apoptosis of lung epithelial cells. In the current study, we determined whether PGRN plays a role in ER stress-induced cellular apoptosis, and if so, whether overexpressed PGRN could restore the ER stress responses. High-concentration CSE (10%) was used to lead to cellular apoptosis in both PGRN depleted or overexpressed A549 cells.

The results from flowcytometry analysis showed remarkably enhanced cellular apoptosis after exposure to 10% CSE in PGRN deleted A549 cells**,** while cellular apoptosis was greatly declined in the cells with PGRN overexpression **(Fig. 8A and 8B)**.

In addition, we investigated the anti-apoptotic effects of PGRN and observed apoptosis markers such as cleaved caspase-3 and PARP protein expression after exposure 10% CSE by using Western blot analysis. The expression level of cleaved caspase-3 and PARRP was enhanced in 10% CSE treated PGRN depleted cells **(Fig. 9A)**, while those were not detected in PGRN overexpressed cells **(Fig. 9B)**. These results demonstrated that intracellular PGRN expression is directly linked to a regulatory role in CSE-induced apoptosis of epithelial cells.

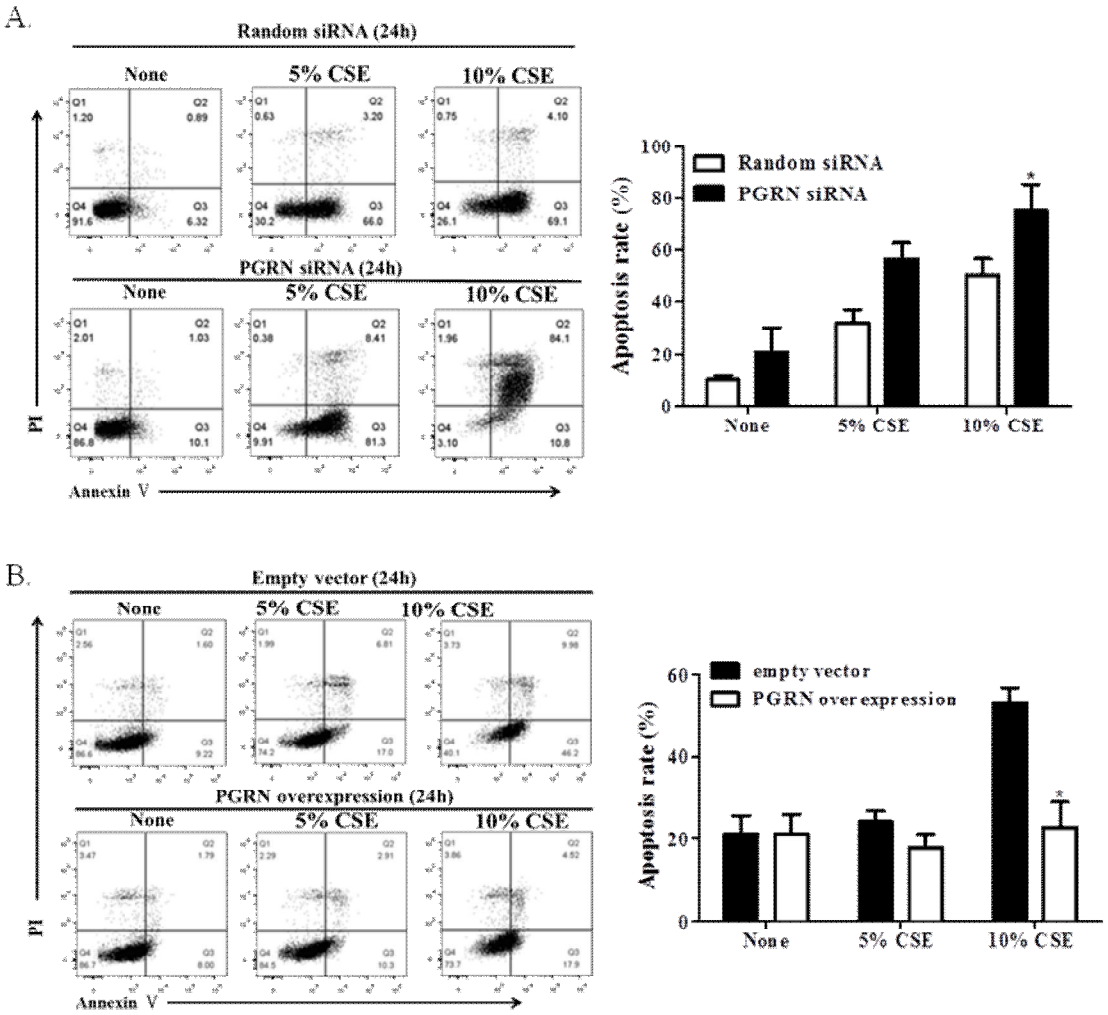

Figure 8. The effect of PGRN on CSE-mediated apoptosis in epithelial cell by using flowcytometry analysis.

PGRN-depleted (A) and PGRN-overexpressed (B) A549 cells were exposed to 5% or 10% CSE for 24hr, representative images of cells obtained using flow cytometry analysis following Annexin V/propidium iodide (PI) staining. The percentages of apoptotic cells are shown as mean values ±S.D. of three independent experiments. Asterisks indicate statistical significance, as determined by Student's *t*-test, $^{**}P<0.001$ versus Random siRNA and $^{*}P<0.05$ versus Empty vector (bottom left panel).

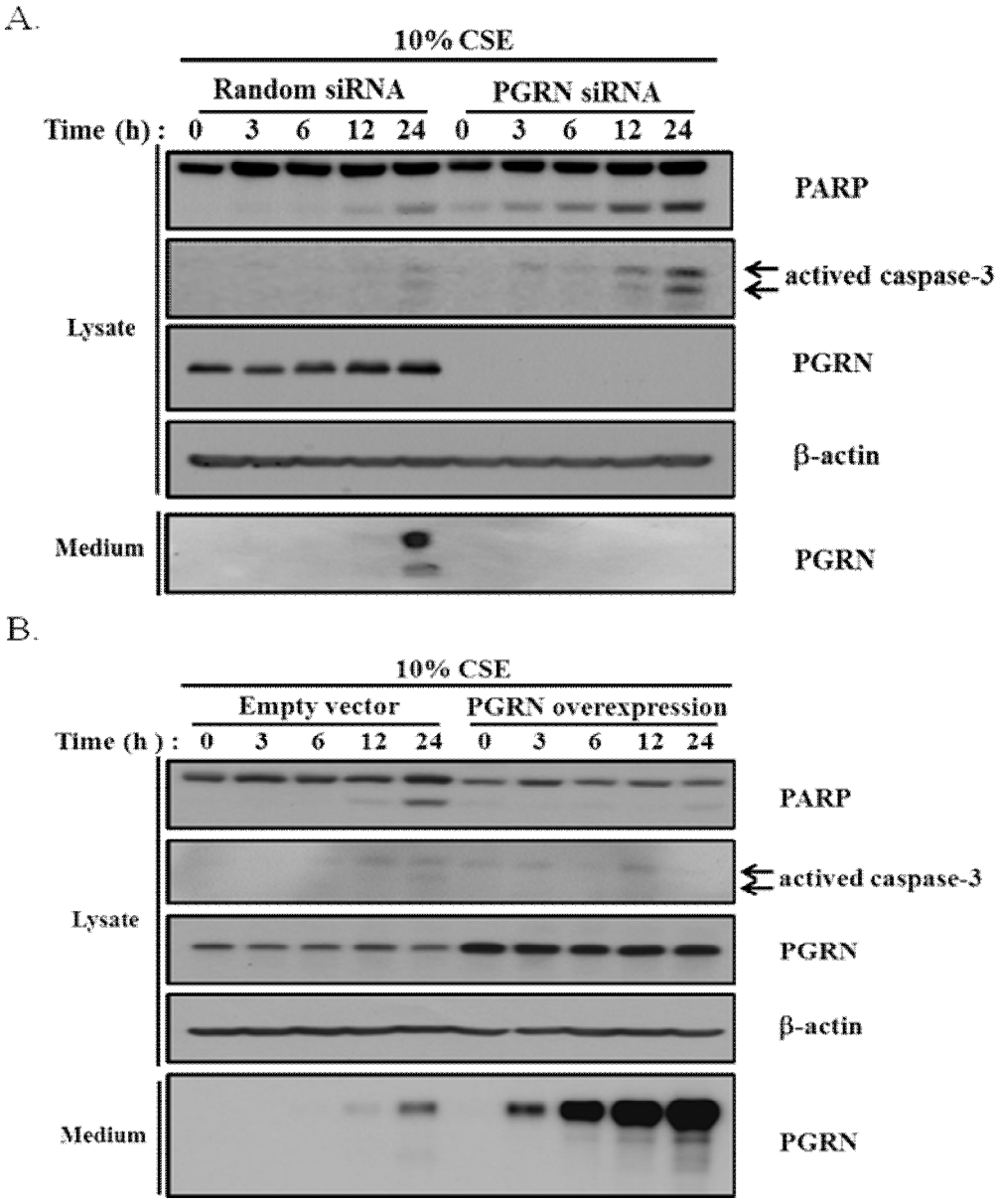

Figure 9. PGRN prevented CSE-mediated apoptosis in epithelial cell. PGRN-depleted (A) and PGRN-overexpressed (B) A549 cells were exposed to 10% CSE for various time periods as indicated. Apoptotic cells were determined by Western blot with either anti-cleaved caspase 3 or anti-PARP antibody. β-actin was used as a loading control.

The regulatory role of PGRN in MAPK pathway and Akt signaling

IRE pathway is known to lead to apoptosis through the JNK activation.[20] In addition, it is also reported that activation of some kinases is important in the development of inflammation and cellular apoptosis as well. Phosphorylated ERK1/2 is a critical factor in the production of pro-inflammatory cytokines such as IL-8 and phosphorylated AKT is also a pivotal pro-survival.[21] In this study, to further investigate the relations between PGRN and activation of these kinases, the expression of those phosphorylated molecules were investigated under the conditions with or without PGRN expression.

The results herein clearly revealed that the levels of phosphorylated AKT were decreased in PGRN deficient cells **(Figure 10A)**. On the other hand, PGRN-deficient cells increased the levels of both phosphorylated JNK and phosphorylated ERK1/2 **(Figure 10B)**. These results suggest that PGRN can modulate CSE associated cellular apoptosis and pro-inflammatory cytokine production through regulation of Akt signaling and ERK1/2 and JNK MAPK pathways as well.

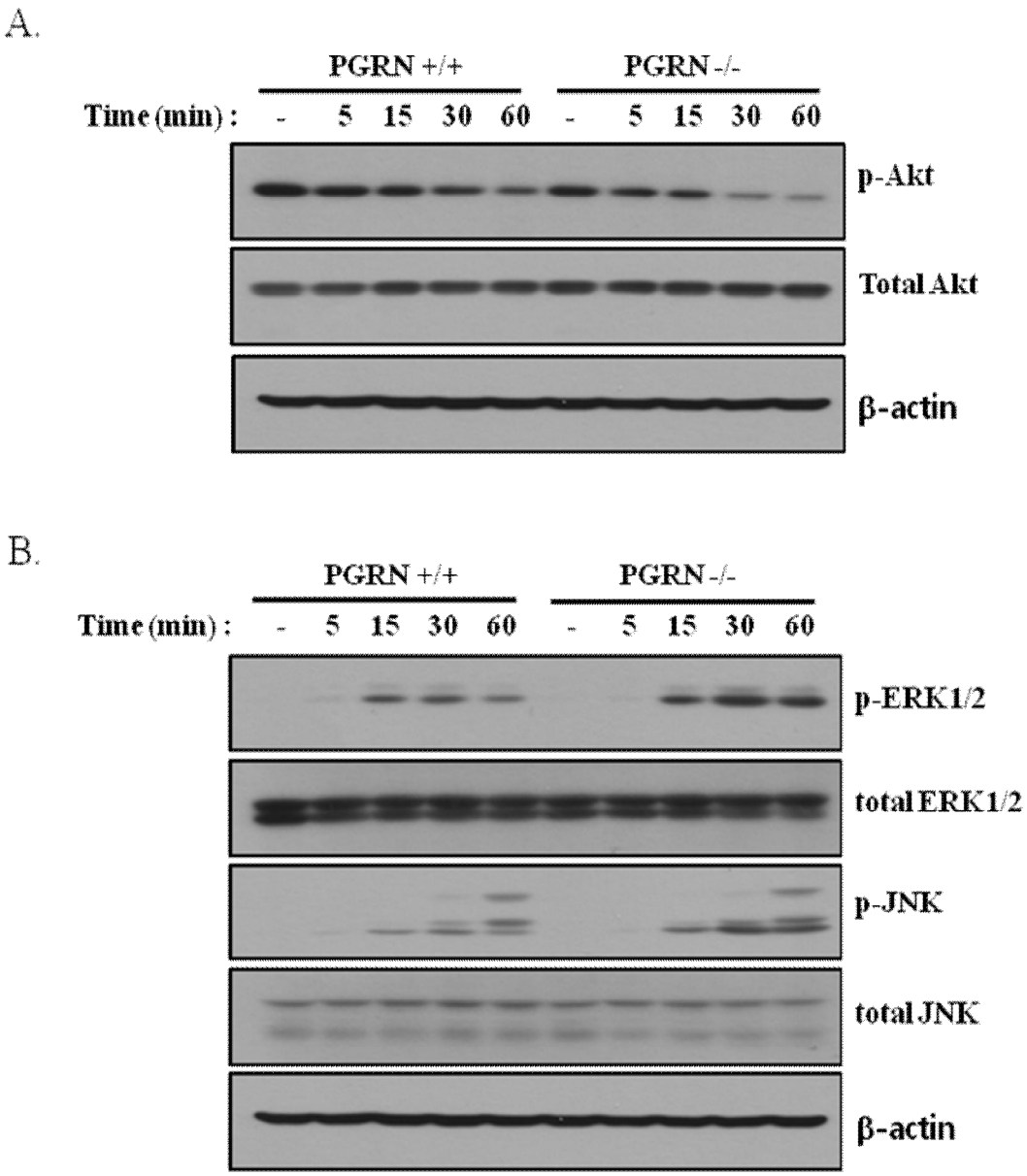

Figure 10. CSE stimulation induced apoptosis through MAPK signaling pathway. Both PGRN intact A549 cells and PGRN-deficient cells were exposed to 10% CSE for the indicated various short time periods. (A) The expressions of total and phosphorylated Akt were determined by Western blotting. β-actin was used as a loading control. (B) Cell lysates were detected with specific antibodies against total and phosphorylated Erk1/2 and JNK. β-actin was used as a loading control.

Discussion

In this study, it is demonstrated that exposure to CS induced PGRN expression as well as triggering ER stress indicated by activation of the ER stress response markers in alveolar epithelial cells. PGRN expression is associated with attenuation of both IL-8 production and cellular apoptosis, which is well known to be critical in COPD airway inflammation and development of emphysema respectively. These results of the current study suggest that intracellular PGRN may have an anti-inflammatory and anti-apoptotic effect on CSE exposed airway epithelial cells probably through modulation of ER stress responses and involvement of apoptosis pathway.

Numerous studies have demonstrated that CS directly linked to induction of airway inflammation [22] and alveoli epithelial cell apoptosis as well [23, 24] although the precise functioning mechanism of CS underlying COPD development has been poorly understood. PGRN is the well known molecule which is abundantly expressed in epithelial cells, macrophages and certain types of neuron[25]. Recently, many studies reported that PGRN has some anti-inflammatory efficacy in various diseases. At the same time, however, it is also well known that GRN, cleaved products from PGRN, has a strong pro-inflammatory molecule recruiting neutrophils into certain organs and tissues. To dates, this is the first study to evaluate the precise role of PGRN in COPD development.

In this study, it is clearly demonstrated that CSE induced intracellular PGRN production in alveolar epithelial cells. In addition, we also demonstrated CSE induces intracellular ER stress responses. CSE-exposed cells undergo a prolonged phase of severe ER stress and both PERK-mediated phosphorylation of elF2a and IRE-1-mediated splicing of XBP1 were closely related to induction of PGRN. PGRN is also regulated by a network of ER molecular chaperones including Bip/GRP78, calreticulin, GRP94, ERp57, and ERp5, as well as HSP70 [26]. Especially, PGRN binds with GRP78, a major ER chaperone, which is protects from

estrogen starvation-induced apoptosis[27, 28]. In the current study, GRP78 was enhanced in CSE-exposed PGRN deficient cells, comparing with control cells (data not shown). Taken together, it can be assumed that upregulation, secretion, folding, and degradation of intracellular PGRN are tightly regulated under the cellular stress. Thus, to understand posttranslational regulation of PGRN, we may need to research others binding partners of PGRN. Furthermore, the study on the relationship between GRP78 and PGRN in ER stress should be needed. Probably, CSE is a cellular stimulus strong enough to elicit ER stress response. Given that various cellular responses should be followed by ER stress, it is assumed that the increased production of intracellular PGRN can be closely linked to the ER stress response in order to induce cellular homeostasis and protection against stimuli from outside.

In COPD, there is definite chronic inflammation of the small airways and the lung parenchyma, which could lead to fixed narrowing of small airways and alveolar wall destruction (emphysema)[23, 29]. Despite the reports that PGRN had anti-inflammatory effects on various diseases, we firstly observed that intracellular PGRN could control the production of a major pro-inflammatory cytokine, IL-8. The results herein clearly revealed that the levels of IL-8, critically important in the recruitment of neutrophils, were increased in PGRN deficient cells. Although the precise relationship between IL-8 secretion and PGRN has not been clarified in detail, several evidences were reported that ER stress-induced IL-8 production was enhanced by NF-kB activation [19, 30]. There has been other report that CSE enhanced CCN1 expression and secretion via induction of ER stress and the secreted CCN1 regulated augmented IL-8 release through the activation of Wnt pathway [31]. The results of this study suggest that intracellular PGRN can attenuate IL-8 expression in airway epithelial cells exposed to CSE. The precise mechanism for these factors in inducing ER stress remains to be further investigated.

This anti-inflammatory function might be affected by PGRN binding to the receptor for

TNF-α. The binding may inhibit the signaling of TNF-α, therefore, which prevents the inflammation process[13, 32]. Although PGRN has also presented with features of binding with over 20 proteins at extracellular and intracellular levels including cytoplasm and nuclear the intracellularly, precise mechanism and biological significance of this interaction are still not fully understood. In a pilot study, we observed that levels of PGRN were significantly increased in serum obtained from COPD patients, comparing with those from non-smokers (data not shown). In addition, in a murine model of COPD, elevated PGRN expression was confirmed using IHC staining on lung tissue of mice exposed to CS. It has been also reported that PGRN was involved in inflammatory response with its recruitment into sites of inflammation and competition between inflammatory mediators in acute skin injury. [12, 32] Taken together, it can be presumed that PGRN may be associated with inflammatory process of the COPD airway. At this moment, however, the precise mechanism of how to regulate IL-8 production by PGRN is not clearly defined. Further investigated should be warranted.

Interestingly enough, while inflammation occurs, infiltration neutrophils and macrophages release neutrophil elastase, proteinase 3, and MMP-12, which are suggested to digest PGRN into individual 6 kDa GRNs [33]. In the current study, increased GRN was also detected in CSE exposed alveolar epithelial cells. PGRN has been known to be a potent anti-inflammatory molecule[5] based on the fact that PGRN can binds with the receptor for tumor necrosis factor-α (TNF- α), sortilin [34] as well as Toll-like receptor 9 (TLR9) [5, 25] and lead to inhibition of those mediators. On the contrary, GRN peptides are well known pro-inflammatory molecule[5]. GRN peptides have been shown to increase the expression of pro-inflammatory cytokines[32]. It might be possible that the increased PGRN secretion caused by CSE under the condition of airway inflammation with neutrophils and monocytes like COPD can induce enhanced production of GRNs, which can further aggravate airway inflammation. However, this vicious cycle would not be occurred since secretory leukocyte proteinase inhibitor (SLPI), reported to play a role in preventing the release of enzymes which can

cleave the PGRN, has been known to be increased when cells are exposed to the CSE [34].

With regard to emphysema, it has been suggested that exposure of alveoli epithelial cells to CSE elicited a oxidative damage of the respiratory epithelial cells followed by DNA damage and cellular apoptotic death[35]. The number of studies on COPD patients with a smoking history is increasing, however, the mechanism underlying the development of emphysema has not been clarified yet [36, 37]. More attention has been paid to the mechanism of alveolar epithelial injury in addition to alveolar matrix destruction. The presence of apoptosis has recently being investigated in animal models of emphysema and in several studies in human disease[38-40]. In addition, the curative treatment for emphysema has not been known. Thus, COPD is a disease that the new therapeutic agents are urgently needed. For the development of new drugs for COPD, our understanding of exact pathogenic mechanisms between inflammation and apoptosis should be improved.

Recently, PGRN was discovered as a novel ligand TNFR1 and TNFR2 receptors, and blocks TNF-α–mediated signaling pathway by competing with TNF-α [5]. TNF-α activates the caspase pathway and NF-κB pathway to induce apoptosis and expression of proinflammatory cytokines through TNFR1 and activates the AP-1 and NF-κB pathway to promote proliferation through TNFR2[41]. It is demonstrated in this study that PGRN deficient cells became extremely susceptible to ER stress-induced apoptosis. In addition, overexpressed PGRN restored ER stress responses and inhibited ER stress-mediated apoptosis. It has been reported that secreted PGRN is bind to the receptor for TNF-α, which induces caspase-3 activation, In results of the current study showed that caspase-3 was activated in PGRN deleted epithelial cell at high dose of CSE and PGRN may have protective effect against CSE-induced apoptosis.

Meanwhile, BiP is known to adjust the Akt / PI3K pathway in the plasma membrane[42]. In addition, it was reported that PGRN can induce phosphorylation Akt[43]. It is suggested that PGRN efficiently inhibited CSE-mediated pro-inflammatory signaling or ER stress-induced

apoptosis in A549 cells through activation of the Akt pathway, indicating its protective roles in lung epithelial cells against ER stress-induced apoptosis. Taken together, these findings indicate that CSE-induced PGRN plays a role in ER stress response of the COPD airway that is reasonably associated with protection against epithelial apoptosis.

Conclusion

In conclusion, the results of the current study suggest that intracellular PGRN may have an anti-inflammatory and anti-apoptotic effect on CSE exposed airway epithelial cells probably through modulation of ER stress responses and involvement of apoptosis pathway. Thus, PGRN might be an useful target for the development of a novel therapeutic drug of emphysema.

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국문요약

만성폐쇄성폐질환(COPD)은 장기간 유해한 가스나 먼지의 흡입으로 인한 기도 내 만성 염증 및 기관지 폐쇄가 비가역적으로 발생하는 질환으로, 흡연이 핵심 요인으로 제시되고 있다. 흡연은 폐포상피세포의 세포자연사(apoptosis)를 일으켜 폐기종을 유발시킴으로써 폐의 가장 중요한 기능인 가스 교환이 정상적으로 이루어지지 않게 만든다. 이는 현존하는 어떠한 치료법으로도 회복이 불가능하며, 증상의 진행을 늦추는데 급급한 시점이기 때문에 심각한 문제를 초래하고 있다. 따라서 COPD 에 대한 새로운 치료제의 개발이 시급하며, 이를 위해서는 COPD 의 발병기전을 보다 정확히 이해하고 제어하는 방법을 찾는 노력이 필요한 시점이다.

Progranulin(PGRN)은 다양한 자극에 의해 발현이 유도되는 물질로써 다양한 질환에서 염증을 억제하는 효과 및 세포자연사로부터 세포를 보호하는 기능이 보고 되어 있다. 흡연 시 폐포상피세포에서 생성되는 과도한 소포체 스트레스(ER stress)가 apoptosis 를 활성화 시킴으로써 폐기종을 발생시키므로, 본 연구에서는 PGRN 의 항염증 및 세포보호 역할이 폐포상피세포의 apoptosis 를 억제할 것이라는 가설 하에 연구를 수행하였다.

본 연구에서는 폐상피세포종인 A549 를 대상으로 흡연 상태를 재현하고자 담배추출물(CSE)을 처리하였다. 예상했던 바와 같이, CSE 의 처리는 ER stress 를 생성함과 동시에 apoptosis 를 유발하였다. PGRN 가 apoptosis 에 미치는 영향을 확인하기 위해 PGRN 발현을 억제 혹은 촉진시킨 A549 에 CSE 를 처리하여 apoptosis 를 확인한 결과 PGRN 이 ER stress 및 apoptosis 를 억제함을 확인하였다. 추가적으로 PGRN 의 항염증성 기능을 A549 가 CSE 의 자극에 의해 분비하는 대표적인 염증성 사이토카인인 IL-8 을 통해 확인해 본 결과, PGRN 발현을 억제시킨 A549 에서 정상 A549 에 비해 IL-8 발현이 증가되어 있는 것을 관찰하였다.

본 연구를 통하여 PGRN 이 CSE 로부터 폐포상피세포를 보호하여 apoptosis 및 IL-8 의 분비를 효과적으로 감소시킴을 확인하였다. 이는 PGRN 이 폐기종의 주요 발병기전을 억제 할 수 있는 효과적인 조절기능을 수행할 수 있음을 의미한다. 현존하는 치료법으로 완치가 불가능한 COPD 의 치료에 있어서, 병인 기전을 효과적으로 억제시킬 새로운 치료 타겟 후보물질로서의 PGRN 의 가능성을 제시한다.