

Exogenous neutrophil elastase enters bronchial epithelial cells and suppresses cigarette smoke extract-induced heme oxygenase-1 by cleaving sirtuin 1

Kyoung-Hee Lee¹, Jiyeong Jeong¹, Yoon-Jung Koo¹, An-Hee Jang¹, Chang-Hoon Lee^{1,2}, and Chul-Gyu Yoo^{1,2}

From the ¹Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Seoul National University Hospital, Seoul 110-744, Korea and ²Department of Internal Medicine, Seoul National University College of Medicine, Seoul 110-799, Korea

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To whom correspondence should be addressed : Chul-Gyu Yoo, Tel.: +82-2-2072-3760, Fax: 82-2-762-9662, E-mail: cgyoo@snu.ac.kr

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ABSTRACT

An imbalance between oxidative stress and antioxidant activity plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD). Cigarette smoke, a major risk factor of COPD, induces cellular oxidative stress, but levels of antioxidants such as heme oxygenase-1 (HO-1) are reduced in individuals with severe COPD. In this study, we evaluated the molecular mechanism of reduced HO-1 expression in human bronchial epithelial cells. We found that cigarette smoke extract (CSE) increases HO-1 levels via activation of NFE2-related factor 2 (Nrf2). However, pretreating cells with the protease neutrophil elastase (NE) suppressed the CSE-induced expression of HO-1 mRNA and protein. NE also decreased sirtuin 1 (SIRT1) level, but did not inhibit CSE-induced nuclear translocation and DNA-binding activity of Nrf2. Transfection of cells with a Myc/His-tagged SIRT1 expression vector completely blocked the NE-mediated suppression of HO-1 expression. We further noted that the NE-induced downregulation of SIRT1 was not due to decreased transcription or proteasomal/lysosomal degradation or loss of

solubility. Immunofluorescence staining revealed that NE enters the cell cytoplasm, and we observed that NE directly cleaved SIRT1 in vitro, indicating that SIRT1 levels are decreased via direct degradation by internalized NE. Of note, we observed decreased SIRT1 levels in NE-treated primary human bronchial epithelial cells and in lung homogenates from both smokers and patients with COPD. In conclusion, NE suppresses CSE-induced HO-1 expression by cleaving SIRT1. This finding indicates the importance of cross-talk between oxidative stress and protease responses in the pathogenesis of COPD.

Chronic obstructive pulmonary disease (COPD) is a type of obstructive lung disease characterized by chronic bronchitis and remodeling of the small airways and destruction of the lung parenchyma (1). Although the mechanism of COPD development and progression is not fully understood, various hypotheses have been proposed, including oxidative stress, inflammation, protease-antiprotease imbalance, autophagy, apoptosis, and aging (2). Among these, oxidative stress is a major factor in the pathogenesis of

COPD (3). Oxidative stress arises from environmental exposure such as cigarette smoking and inflammatory responses to viral and bacterial infections in the lungs. Oxidative stress causes protein oxidation and lipid peroxidation, injuring the lung tissue (4). Therefore, targeting oxidative stress with antioxidants or increasing the endogenous levels of antioxidants is thought to be beneficial for COPD treatment.

Heme oxygenase (HO)-1 is a strongly inducible and highly regulated anti-oxidant (5). *HO-1* deleted cells were more sensitive to oxidative stress such as hydrogen peroxide and paraquat (6), and adenoviral overexpression of HO-1 in the lungs protected mice from elastase-induced inflammation, pro-inflammatory cytokine production, and emphysema (7). In addition, polymorphisms in the HO-1 promoter that reduced HO-1 expression were reported to be associated with an increased susceptibility to emphysema in smokers (8). Pharmacological inhibition of HO-1 or knock-down of the HO-1 gene increased mucosal permeability and enhanced cigarette smoke (CS)-induced secretion of mucus (9, 10). Moreover, in lung epithelial cells, cigarette smoke extract (CSE)-induced apoptosis was suppressed by HO-1 induction (11). Additionally, HO-1 plays protective roles against destruction, mucus hypersecretion, and structural cell death in the lungs. These findings support the protective role of HO-1 in the development of COPD. When lung cells are exposed to oxidants, anti-oxidants such as HO-1 are typically up-regulated as a defense mechanism. However, patients with severe COPD show reduced levels of HO-1 (12); the mechanism for this has not been elucidated.

Nuclear factor (erythroid-derived 2) like-2 (Nrf2) is a major transcription factor that regulates the expression of antioxidant HO-1 (13). Under normal conditions, Nrf2 is maintained in the cytoplasm by Kelch like-ECH-associated protein 1 (KEAP1) and cullin3. Cullin3 ubiquitinates Nrf2 and mediates proteasomal degradation of Nrf2 (13). Oxidative stress such as CS and H₂O₂ increases the stability of Nrf2 by down-regulating the adaptor protein Keap1, leading to increased

Nrf2 nuclear accumulation/DNA binding activity and the induction of antioxidant HO-1 (13, 14). Genetic ablation of *Nrf2* as well as *HO-1* deficiency enhanced the susceptibility to CS-induced emphysema. In Nrf2-deficient mice, greater lung cell apoptosis and inflammation were observed compared to in control mice (15). In addition to Nrf2, several factors have been reported to regulate *HO-1* gene expression. Signal transducer and activator of transcription 3 is associated with *HO-1* gene activation (16, 17) and SIRT1 mediates CS-induced HO-1 expression via an Nrf2-independent and Foxo3-dependent mechanism (18).

In our previous study, we found that CSE induces the production of the neutrophilic chemokine interleukin-8 (IL-8) in lung epithelial cells and co-exposure to CSE and elastase (released from recruited neutrophils) enhances IL-8 secretion by accelerating extracellular receptor kinase (ERK) activation (19). CSE up-regulates anti-oxidative and anti-inflammatory HO-1 protein as a defense mechanism. In this study, we evaluated the effect of neutrophil elastase (NE) on CSE-induced HO-1 expression and its regulatory mechanism in human bronchial epithelial cells. We found that NE suppresses the CSE-induced expression of HO-1 by cleaving SIRT1. This indicates the importance of cross-talk between oxidative stress (CSE) and protease (NE) in the pathogenesis of COPD.

RESULTS

CSE increases HO-1 expression via Nrf2 activation in human bronchial epithelial cells - Oxidative stress is well-known to activate Nrf2, which in turn binds cis-acting ARE enhancers and induces the expression of protective antioxidant genes (13). We first determined whether CSE activates Nrf2 and induces antioxidant in human bronchial epithelial cells (BEAS-2B) as a defense mechanism. BEAS-2B cells were treated with CSE (0, 1, 2, 3%) for 4 h and DNA-binding activity of Nrf2 was measured using nuclear

extracts. CSE increased the DNA binding activity of Nrf2 in a dose-dependent manner (Fig. 1A). The levels of antioxidant HO-1 mRNA and protein were assessed by quantitative real-time PCR and western blot analysis after treatment with CSE. CSE significantly up-regulated HO-1 mRNA and protein expression (Fig. 1B and 1C). To evaluate whether Nrf2 activation mediates CSE-induced up-regulation of HO-1 protein, cells were infected with control (Mock), wild-type Nrf2 (Ad-WT-Nrf2), or dominant negative Nrf2 (Ad-DN-Nrf2) adenovirus vector. Overexpression of DN-Nrf2, which lacks an N-terminal trans-activating domain and appears as a 29-kDa immunoreactive band, completely suppressed CSE-induced HO-1 expression (Fig. 1D). These results indicate that CSE up-regulates HO-1 via Nrf2 activation in human bronchial epithelial cells.

NE suppresses CSE-induced HO-1 expression - We next evaluated the effect of NE treatment on CSE-induced HO-1 expression. Cells were pretreated with NE (1 U/mL) and then stimulated with CSE. NE pretreatment alone slightly decreased the basal level of HO-1 mRNA and protein expression (Fig. 2A and 2B). Moreover, up-regulation of HO-1 by CSE was significantly suppressed in NE-pretreated cells (Fig. 2A and 2B).

NE does not inhibit CSE-induced nuclear translocation and DNA-binding activity of Nrf2 - Under non-stress conditions, Nrf2 is anchored in the cytoplasm by binding to KEAP1, which, in turn, facilitates the ubiquitination and subsequent proteolysis of Nrf2 (13). CSE exposure leads to KEAP1 degradation, resulting in the stabilization and nuclear translocation of Nrf2 (20). To determine the molecular mechanism of NE-mediated suppression of HO-1, we first investigated whether NE suppresses CSE-induced KEAP1 degradation and nuclear translocation of Nrf2. BEAS-2B cells were pretreated with NE and then stimulated with the indicated concentrations of CSE for 4 h. Cytoplasmic and nuclear proteins were used to detect KEAP1 and Nrf2 expression, respectively. CSE decreased cytoplasmic KEAP1 expression and mediated nuclear translocation of Nrf2, which was not suppressed by NE

pretreatment (Fig. 3A). We next evaluated the effect of NE on CSE-induced Nrf2 binding to ARE. Nuclear extracts from Nrf2-transfected COS-7 cells were used as a positive control (Fig. 3B). NE pretreatment did not inhibit the DNA-binding activity of Nrf2 induced by CSE. These results suggest that NE-induced suppression of HO-1 is Nrf2-independent.

NE decreases SIRT1 expression, which mediates the reduction of HO-1 expression in CSE-treated cells - SIRT1 deficiency has been reported to reduce the mRNA level of HO-1 in mouse lungs exposed to CS. In contrast, SIRT1 transgenic overexpression or treatment with SIRT1 activator increased HO-1 mRNA in both WT and Nrf2-deficient mouse lungs in response to CS exposure. This suggests that the protective effect of SIRT1 against CS-induced oxidative stress is caused by HO-1 up-regulation independently of Nrf2 (18). Therefore, we evaluated the role of NE on SIRT1 expression. Interestingly, NE treatment significantly decreased SIRT1 expression. SIRT1 began decreasing at 2 h after NE treatment and its decrease was prolonged for up to 24 h (Fig. 4A). Moreover, when the cells were transiently transfected with Myc/His-tagged SIRT1 expression plasmids, CSE-induced HO-1 expression was enhanced and NE-mediated HO-1 suppression was completely blocked (Fig. 4B). These results demonstrate that NE negatively regulates HO-1 expression by down-regulating SIRT1.

NE-mediated down-regulation of SIRT1 is not due to decreased transcription, proteasomal/lysosomal proteolysis, and loss of solubility - We next evaluated the mechanism of SIRT1 down-regulation by NE. To evaluate whether down-regulation of SIRT1 by NE is regulated at the transcriptional level, the mRNA levels of SIRT1 were assessed by RT-PCR after treatment with NE. NE treatment did not suppress the transcript levels of SIRT1 (Fig. 5A). We next examined whether NE-induced down-regulation of SIRT1 protein expression is due to intracellular degradation of SIRT1 protein. As the main sites of intracellular protein degradation are the proteasome and lysosome, we tested whether the

down-regulation of SIRT1 protein by NE was caused by degradation via these sites. Proteasome or lysosome activity was blocked by proteasome inhibitors (PS-341, MG132) or lysosome inhibitors (ammonium chloride, chloroquine), respectively (Supplementary Fig. S1). When both proteolysis sites were blocked, NE-induced down-regulation of SIRT1 was not affected (Fig. 5 B). Because decreased solubility of protein contributes to reduced protein activity or expression (21), we tested this possibility for NE-induced down-regulation of SIRT1. To evaluate whether the decreased level of SIRT1 is mediated by the loss of solubility, SIRT1 expression level in the soluble extracts and insoluble pellet was examined. Although SIRT1 expression in the soluble fraction decreased after NE treatment, SIRT1 in the insoluble pellet was not detected, indicating that NE-mediated SIRT1 down-regulation was not related to SIRT1 insolubilization (Fig. 5C).

SIRT1 is down-regulated because of direct cleavage after translocation of NE into cells - We previously reported that NE can enter into normal lung epithelial cells (19). We next investigated whether decreased expression of SIRT1 occurs by direct degradation by NE in the cytoplasm. Immunofluorescence staining for cytoplasmic/internalized NE was performed and analysis of NE-treated cells showed that NE was introduced into cells (Fig. 5D). To test whether the reduction of SIRT1 expression at the post-translational level occurs because of direct cleavage of SIRT1 by internalized NE, we assessed whether NE is capable of degrading SIRT1 *in vitro*. SIRT1 was highly sensitive to NE cleavage. Most SIRT1 was degraded after 30 min of incubation with NE (Fig. 5E). When cells were treated with NE in the presence of elastase inhibitor (elastol) or serine protease inhibitor (phenylmethylsulfonyl fluoride, PMSF), NE-mediated SIRT1 cleavage was completely blocked. Heated NE also failed to cleave SIRT1 (Fig. 5F). Taken together, these data suggest that NE enters into the cell and directly cleaves SIRT1 and activity of NE is required for

SIRT1 cleavage.

To investigate cleavage sites of SIRT1 by NE, we used the program PeptideCutter to search for consensus cleavage sites. We identified 63 sites in the human SIRT1 amino acid sequence predicted to be recognition sites for the NE (Supplementary Fig. S2). To confirm that NE cleaves SIRT1 to small fragments and the fragments are cleaved forms of SIRT1, cells were transiently transfected with control or SIRT1 siRNAs and then stimulated with NE. The expression of SIRT1 mRNA and protein was significantly reduced in SIRT1 siRNA transfected cells (Fig. 6A-6C). Western blot analysis, using antibodies that recognize the carboxy- or amino-terminal peptide of SIRT1, revealed the presence of faster migrating fragments in control siRNA/NE-treated cells. In SIRT1 knock-down cells, the expression level of migrating fragments was decreased or not detected, verifying that the fragments are cleaved SIRT1 (Fig. 6B and 6C). These results demonstrate that NE cleaves SIRT1 at multiple sites.

Decreased level of SIRT1 in NE-treated primary human bronchial epithelial cells (HBECs) and in lung homogenates from smokers and patients with COPD - We confirmed the effect of NE on SIRT1 expression in primary HBECs. Normal HBECs were collected using a bronchial brush. Airway brushings were obtained from the right lower lobe anterobasal segmental bronchus wall. The epithelial lineage was verified by immunocytochemical staining. Cultured HBECs stained intensely and exclusively for the epithelial specific markers (cytokeratin and E-cadherin). No expression was observed for macrophage and endothelial lineage markers (CD11b and CD31). Verified HBECs (passage No. 2) were used in the experiment. NE pretreatment significantly fragmented SIRT1 and CSE-induced HO-1 expression was suppressed in NE-pretreated primary HBECs (Fig. 7A). We compared the expression level of SIRT1 in protein extracts of lung tissues from normal (non-smoker), smoker, and COPD patients. SIRT1 expression was significantly lower in lung extracts of smokers and COPD patients (Fig. 7B, 7C).

DISCUSSION

COPD is a complex disease process. Oxidative stress, inflammation, and protease-antiprotease imbalance have been suggested to act in a pathogenic triad in COPD and many studies have demonstrated that these processes closely interact with each other. We recently reported that CSE induces IL-8, a well-known neutrophil chemotactic factor. CSE-induced IL-8 mediates neutrophil recruitment, and recruited neutrophils release several proteinases including elastase. When lung epithelial cells were co-stimulated with CSE and NE, IL-8 production was enhanced. Consequently, the interactive action of oxidative stress (CSE) and protease (NE) lead to continued neutrophil accumulation and inflammation (19). Both CS and CSE exert strong oxidative stress and this action is limited by the simultaneous activation of anti-oxidative pathways. Although NE enhanced CSE-induced inflammation synergistically, the effect of NE on CSE-induced activation of the anti-oxidative pathway has not been elucidated. Therefore, in this study, we evaluated the interactive action of CSE and NE on the anti-oxidative pathway and its molecular mechanism in bronchial epithelial cells.

In this study, we demonstrated that CSE-induced up-regulation of HO-1 depends on the activation of its transcriptional factor Nrf2, which agrees with the results of previous studies (22). In contrast, NE markedly suppressed the level of HO-1 mRNA and protein expression induced by CSE. Considering the well-known protective effects of HO-1 in lung diseases (23), these results suggest that NE plays a role in maintaining oxidative stress by CS or CSE. We next examined the molecular mechanism of down-regulation of HO-1 by NE.

Since Nrf2 is the representative transcriptional factor regulating the expression of antioxidant genes (13), we first evaluated the role of Nrf2 in the down-regulation of HO-1 by NE. NE not only did not affect the basal level of Nrf2 mRNA and

protein (data not shown), but also did not suppress CSE-induced KEAP1 down-regulation in the cytoplasm and nuclear translocation/DNA-binding activity of Nrf2. These results indicate that NE-mediated suppression of HO-1 is independent of Nrf2 activity.

SIRT1 is a member of the sirtuin family (class III histone/protein deacetylases), which play important roles in a variety of cellular processes including aging, inflammation, and oxidative stress resistance (24-26). A recent report showed that SIRT1 protected mice against CS-induced lung oxidative stress via a FOXO3-dependent mechanism (18). While SIRT1 deficiency reduced the level of CS-induced *HO-1* mRNA, transgenic overexpression of SIRT1 or treatment with SIRT1 activator augmented *HO-1* in response to CS exposure (18). This study is the first to show that NE treatment rapidly and effectively decreased the SIRT1 protein level, which was not due to decreased transcription. NE-mediated reduction of HO-1 was restored by SIRT1 overexpression, suggesting that abrogation of CSE-induced HO-1 up-regulation by NE occurred because of down-regulation of SIRT1. In accordance with this result, the level of SIRT1 was reported to be significantly lower in the nuclear extracts of peripheral lung tissue of smokers and patients with COPD than in the lungs of nonsmokers (27). We also observed decreased levels of SIRT1 protein expression in lung tissues from smokers and COPD patients.

We next evaluated how SIRT1 is down-regulated by NE treatment. SIRT1 was reported to be cleaved in adipose tissue by high-fat diet. SIRT1 cleavage was mediated by inflammation-activated caspase-1 (28). However, NE did not activate caspase-1 (Supplementary Fig. S3). The concentration of CSE or NE used in this study did not change the cell viability and CSE treatment up to 24 h did not decrease the level of SIRT1 protein expression (data not shown). CSE or H₂O₂ decreased SIRT1 protein levels in both a human monocyte-macrophage cell line (MonoMac6) and primary and transformed epithelial cell lines (27, 29) because of proteasomal degradation (29). In

this study, however, NE-mediated SIRT1 degradation was not blocked by proteasome inhibition. Although NE has been shown to activate intracellular signaling mediators through cell membrane receptors including protease-activated receptors (PARs) (30, 31), we did not find an association between NE-mediated down-regulation of SIRT1 and cellular reactive oxygen species generation or Akt/ERK activation. Moreover, we found that NE activated intracellular signaling mediators via receptor, PAR2, in human bronchial epithelial cells and knock-down of PAR2 did not affect NE-mediated down-regulation of SIRT1 (Supplementary Fig. S4). Finally, as previous studies, including ours, reported that NE was internalized into cells (19, 32), we assessed the direct cleavage of SIRT1. We found that NE can fragment SIRT1 *in vitro*. On the other hand, NE did not cleave several cellular proteins including Akt, ERK, PARP, Hsp90, KEAP1 (Supplementary Fig. S5, Fig. 3) and IL-8 (19), which indicates that NE selectively fragments SIRT1 protein. NE pre-mixed with elaspol (NE inhibitor) or PMSF (serine protease inhibitor) failed to degrade SIRT1 and heated NE had no effect on SIRT1 expression, suggesting that NE activity is necessary for SIRT1 degradation.

NE activity in patients with COPD is known to be increased (33), which may be associated with a decreased level of SIRT1. This indicates the importance of NE-induced SIRT1 down-regulation in the pathogenesis of COPD. Although many studies have demonstrated the anti-inflammatory and anti-oxidative effects of SIRT1 activator (18, 25, 34), pretreatment with resveratrol, a well-known SIRT1 activator, did not suppress NE-mediated reduction of SIRT1 (data not shown).

In conclusion, our study demonstrates the importance of the cross-talk between oxidative stress (CSE) and protease (NE) in the pathogenesis of COPD. NE suppresses CSE-induced activation of the anti-oxidant pathway by downregulating SIRT1.

EXPERIMENTAL PROCEDURES

Cells-Normal human bronchial epithelial cells, BEAS-2B, were maintained in defined keratinocyte-SFM (GIBCO by Life Technologies, Grand Island, NY, USA) at 37°C under 5% CO₂. Normal primary HBECs were obtained after review and approval by the Seoul National University Hospital Institutional Review Board (SNUH IRB number: H-1602-108-742). Primary HBECs were isolated from bronchial brushing samples during bronchoscopy. The bronchial brushing samples were obtained from the right lower lobe anterobasal segmental bronchus wall. The brush was immediately immersed in a tube containing 10 mL of ice-cold RPMI containing 20% fetal bovine serum. Within a few minutes, the cells were centrifuged and resuspended in defined keratinocyte-SFM. Submerged cells were grown as monolayers to 80–100% confluence and then used for experiments.

Cigarette smoke extract-CSE was prepared as previously described (35). Commercial cigarettes (THIS; 84 mm long, diameter of 8 mm; purchased from KT&G, Republic of Korea) were smoked continuously by a bottle system connected to a vacuum machine. The smoke from 20 cigarettes was bubbled in 60 mL of phosphate-buffered saline (GIBCO). The large, insoluble particles in the resulting suspension were removed by filtering the solution through a 0.22-μm filter (35).

Reagents-Rabbit monoclonal anti-SIRT1 (C-term), and anti-KEAP1 antibodies were from Cell Signaling (Danvers, MA, USA). Rabbit polyclonal anti-SIRT1 (N-term) antibody was from Millipore (Darmstadt, Germany). Rabbit polyclonal anti-Nrf2 (to detect C-terminus of Nrf2), anti-poly (ADP-ribose) polymerase-1 (PARP-1), goat polyclonal anti-heme oxygenase 1 (HO-1), anti-GAPDH, mouse monoclonal anti-heat shock protein 90 (Hsp90) antibodies, and secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human

sputum leucocyte elastase (NE) was purchased from Elastin Product Company (Owensville, MO, USA). Elastase was dissolved in 50% glycerol and 50% 0.02 M NaOAc (pH 5). PS-341 was obtained from Selleckchem (Houston, TX, USA), and MG132 was from Calbiochem (Darmstadt, Germany). Chloroquine, ammonium chloride, and PMSF were from Sigma-Aldrich (St. Louis, MO, USA). Elaspol was from Ono Pharmaceutical Co., Ltd. (Osaka, Japan).

Nrf2 activity assay-Nrf2 activity in nuclear extracts was determined using the TransAM[®] Nrf2 activity assay kit (Active Motif, Carlsbad, CA, USA). Briefly, nuclear extracts were incubated in a 96-well plate, to which oligonucleotide containing the antioxidant response element (ARE) consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3') had been immobilized. The primary antibody recognized an epitope on Nrf2 protein upon DNA binding. A horseradish peroxidase-conjugated secondary antibody was added to each well and a colorimetric reaction was performed. Absorbance was read at 450 nm with a reference wavelength of 655 nm.

Real-time PCR and reverse transcription-PCR (RT-PCR)-Total RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 µg of total RNA, using the Reverse Transcription system (Promega, Madison, WI, USA). PCR amplification was performed with 2X TaqMan gene expression master mix (Applied Biosystems, Foster City, CA, USA) or SYBR Green (Applied Biosystems). HO-1 probe (Hs00157965_m1) and GAPDH probe (Hs99999905_m1) were obtained from Applied Biosystems. Human SIRT1 primers (fwd 5'-TCGCAACTATACCCAGAACATAGACA-3', rev 5'-CTGTTGCAAAGGAACCATGACA-3') and GAPDH (fwd 5'-GAAGGTGAAGGTCGGAGTC-3', rev 5'-GAAGATGGTGATGGGATTTC-3') were used. RT-PCR was performed using the following primers: human SIRT1 (fwd 5'-

TCAGTGGCTGGAACAGTGAG-3', rev 5'-TCTCCATCAGTCCCAAATCC-3') and GAPDH (fwd 5'-CTTTTGCGTCGCCAGCCGAG-3', rev 5'-CAGCCTTGGCAGCGCCAGTA-3').

Protein extraction-Cells were allowed to equilibrate in ice-cold cytoplasmic extraction buffer (CEB) consisting of 10 mM Tris-HCl (pH 7.8), 10 mM KCl, 1.5 mM EDTA, and 0.5 mM DTT for 5 min. The cells were lysed on ice for 10 min in 0.4% NP-40/CEB/protease inhibitor cocktail (Roche Diagnostics Corporation, Basel, Switzerland). Following centrifugation at 3500 rpm for 5 min, the supernatants (cytoplasmic extracts) were collected. The nuclear pellets were washed with CEB and then suspended in nuclear extraction buffer consisting of 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 50 mM KCl, 1.5 mM EDTA, 5 mM DTT, and 0.4% NP-40/protease inhibitor cocktail. Following centrifugation at 13,000 rpm for 15 min, the supernatant (nuclear extract) was collected. Total cellular extracts were prepared in 1X cell lysis buffer (Cell Signaling). Triton X-100-insoluble proteins remaining in the pellets were dissolved in SDS sample buffer consisting of 10 mM Tris-Cl (pH 7.4), 1 mM sodium orthovanadate, and 2% SDS. Frozen lung tissues (SNUH IRB Number: H-1309-073-521) were homogenized in tissue extraction buffer (Life Technologies) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

Western blot analysis-Equal amounts of protein were resolved by gradient SDS-polyacrylamide gel electrophoresis (Invitrogen) and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The membranes were blocked with 5% skim milk blocking buffer for 1 h before being incubated overnight at 4°C with primary antibodies. The membranes were then washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies in

blocking buffer for 1 h. After successive washes, the membranes were developed using SuperSignal West Pico Chemiluminescent kit (Thermo Scientific, Waltham, MA, USA).

Transfection of plasmid vectors or siRNA-Transfection of control vector, Myc/His-tagged SIRT1 expression vector, control siRNA, or SIRT1 siRNA was carried out using the Neon Transfection System (Thermo Fisher Scientific) according to the manufacturer's specifications. After 48 h, the cells were used in experiments.

Transduction of adenoviruses vectors-Cells were plated in a 60-mm tissue culture plate. After overnight incubation, the cells were transduced at multiplicities of infection of 50 by adenovirus vectors expressing wild-type Nrf2 (WT-Nrf2) or dominant-negative Nrf2 (DN-Nrf2), which lacks an N-terminal transactivating domain, in media for 2 h with gentle shaking and then incubated at 37°C and 5% CO₂. After 48 h, the cells were used in the experiments as indicated.

Immunofluorescent staining for NE-BEAS-2B cells grown in 35-mm dishes in the presence or absence of NE for 2 h were fixed in methanol and incubated with rabbit polyclonal anti-NE antibody diluted 1:100 in 3% bovine serum albumin for 24 h. The cells were subsequently incubated with Alexa Fluor 555 goat anti-rabbit antibody diluted 1:100 in 3% bovine serum albumin for 30 min. After successive washes, the cells were analyzed with a fluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan).

Fragment generation of SIRT1-Cell lysates were incubated in vehicle control (50% glycerol 50% 0.02 M NaOAc, pH 5) or NE (0.04, 0.2, 1 U/mL) at 37°C for 30 min. The reaction was stopped by adding western blotting sample buffer and samples were subjected to western blotting analysis for SIRT1 and NE.

Statistical analysis-Data were subjected to Student's *t* test to analyze statistical significance, and a P value of <0.05 was considered significant.

Conflict of interest : The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions : KHL and CGY designed the study; KHL, JJ, YJK, AHJ and CHL conducted the experiments; KHL, CHL and CGY contributed to the data analysis; KHL and CGY wrote the manuscript.

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FOOTNOTES

The abbreviations used are: NE, neutrophil elastase; HO-1, heme-oxygenase 1; CS, cigarette smoke; CSE, cigarette smoke extract; COPD, chronic obstructive pulmonary disease; Nrf2, nuclear factor (erythroid-derived 2) like-2; KEAP1, kelch like-ECH-associated protein1; ERK, extracellular receptor kinase; HBEC, human bronchial epithelial cell; PARP-1, poly (ADP-ribose) polymerase-1; Hsp90, heat shock protein 90

Figure legends

Figure 1. CSE increases the level of HO-1 expression via Nrf2 activation in HBECs. A, BEAS-2B cells were treated with CSE (0, 1, 2, 3%) for 4 h. Nrf2 activity using nuclear proteins was measured using a DNA-binding ELISA kit for activated Nrf2 transcription factor. Data represent the mean \pm SD of triplicate experiments. B, BEAS-2B cells were stimulated with CSE (0, 0.5, 1, 2, 3%) for 8 or 12 h. Total RNA was isolated and quantitative real-time PCR for *HO-1* and *GAPDH* was performed. Data represent the mean \pm SD of triplicates. ** $P < 0.05$ C, BEAS-2B cells were treated with CSE (0, 0.5, 1, 2%) for 24 h. D, BEAS-2B cells were infected with control (Mock), wild-type Nrf2 (Ad-WT-Nrf2), or dominant negative Nrf2 (Ad-DN-Nrf2) adenovirus vector. Forty-eight hours after infection, the cells were treated with CSE (1%) for 24 h. Total cellular extracts were subjected to western blot analysis for HO-1, Nrf2, and GAPDH (C, D). The results are representative of three independent experiments. *S.E.*, short exposure; *L.E.*, long exposure

Figure 2. NE suppresses CSE-induced HO-1 expression. BEAS-2B cells were pretreated with vehicle control (VC) or NE (1 U/mL) for 4 h and then stimulated with CSE (1%) for 12 h (A) or 24 h (B). A, Total RNA was isolated and quantitative real-time PCR for *HO-1* and *GAPDH* was performed. Data represent the mean \pm SD of triplicate experiments. ** $P < 0.05$ B, Total cellular extracts were subjected to western blot analysis for HO-1 and GAPDH. The results are representative of three independent experiments.

Figure 3. NE does not inhibit CSE-induced nuclear translocation and DNA binding activity of Nrf2. A and B, BEAS-2B cells were pretreated with VC or NE (1 U/mL) for 4 h and then stimulated with CSE (1 or 2%) for 4 h. Nuclear and cytoplasmic proteins were extracted and subjected to western blot analysis for Nrf2, PARP-1, Keap1, and Hsp90 (A). Nrf2 activity using nuclear extracts was determined. Nuclear proteins from Cos-7 cells transfected with Nrf2 expressing vector were used as a positive control (B). Data represent the mean \pm SD of triplicate experiments. ** $P < 0.05$. The results are representative of three independent experiments. N.S., not significant

Figure 4. NE decreases SIRT1 expression, which mediates the reduction of HO-1 expression in CSE-treated cells. A, BEAS-2B cells were treated with VC or NE (1 U/mL) for the indicated times. B, Cells were transiently transfected with Myc/His-tagged SIRT1 (Myc/His-SIRT1) expression vector or control vector (Myc/His-CV). Forty-eight hours after transfection, the cells were pretreated with VC or NE (1 U/mL) for 4 h and then incubated with CSE (1%) for 24 h. Total cellular extracts were subjected to western blot analysis for SIRT1, HO-1, and GAPDH. The results are representative of three independent experiments.

Figure 5. Down-regulation of SIRT1 is due to direct cleavage after translocation of NE into the cells.

A–C, Down-regulation of SIRT1 is not due to decreased transcription, proteasomal/lysosomal proteolysis and loss of solubility. A, BEAS-2B cells were treated with VC or NE (1 U/mL) for the indicated times. Total RNA was isolated and reverse transcription PCR (RT-PCR) for the SIRT1 and GAPDH genes was performed. B, Cells were pretreated with proteasome inhibitors (200 nM of PS-341, 10 μ M of MG132) or lysosomal inhibitors (50 mM of ammonium chloride, 100 μ M of chloroquine) for 1 h and then stimulated with VC or NE (1 U/mL) for 2 h. Total cellular extracts were subjected to western blot analysis for SIRT1 and GAPDH. C, BEAS-2B cells were stimulated with VC or NE for 2 h. SIRT1 expression in soluble extracts or insoluble pellets were detected by western blot analysis. D, Cells were treated with VC or NE (20 U/mL) for 2 h. Immunofluorescence staining for internalized NE was performed. Cells were analyzed using an ECLIPSE TE300 (Nikon) fluorescence microscope. Scale bar : 20 μ m. E, Cell lysates were incubated with VC or NE (0.04, 0.2, 1 U/mL) in vehicle (50% glycerol, 50% 0.02 M NaOAc, pH 5) at 37°C for 30 min. The reaction was stopped by adding 4X sample buffer. Samples were subjected to western blot analysis for SIRT1 and NE under reducing conditions. F, BEAS-2B cells were treated with VC or NE (1 U/mL) in the presence or absence of elastase inhibitor (elaspol, 100 μ g/mL) or serine protease inhibitor (PMSF, 1 mM). Cells were stimulated with heated forms of VC or NE (1 U/mL) for 4 h. Total cellular extracts were subjected to western blot analysis for SIRT1 and GAPDH. The results are representative of three independent experiments.

Figure 6. NE cleaves SIRT1 at multiple sites. BEAS-2B cells were transiently transfected with control or SIRT1 siRNA. Forty-eight hours after transfection, the cells were treated with NE (1 or 3 U/mL) for 4 h. Total RNA was isolated and quantitative real-time PCR for *SIRT1* and *GAPDH* was performed (A). Data represent the mean \pm SD of triplicates. ** $P < 0.05$ B and C, Total cellular extracts were subjected to western blot analysis for SIRT1 using two different SIRT1 antibodies that recognize the carboxy-terminal region or the amino-terminal region of SIRT1, respectively. The results are representative of three independent experiments. n.s., non-specific; *S.E.*, short exposure; *L.E.*, long exposure

Figure 7. Decreased level of SIRT1 in NE-treated primary HBECs and in lung homogenates from smokers and COPD patients. A, Primary human bronchial epithelial cells were pretreated with VC or NE (1 U/mL) for 4 h and then stimulated with CSE (1, 2%) for 24 h. Cell lysates were subjected to western blot analysis for SIRT1, HO-1, and GAPDH. B, Lung homogenates from normal (non-smoker) (n=6), smoker (n=9), and COPD (n=9) patients were subjected to western blot analysis for SIRT1 and GAPDH. C, Gel data were quantified using Scion image densitometry. Data represent the mean \pm SD. normal versus smoker : ** $p=0.0299$, normal versus COPD : ** $p=0.0027$.

Figure 8. Schematic diagram of the effect of NE on CSE-induced HO-1 expression. CSE up-regulates HO-1 through Nrf2 activation as a defense mechanism in human bronchial epithelial cells. NE suppresses CSE-induced HO-1 by cleaving SIRT1 protein.

Figure 1

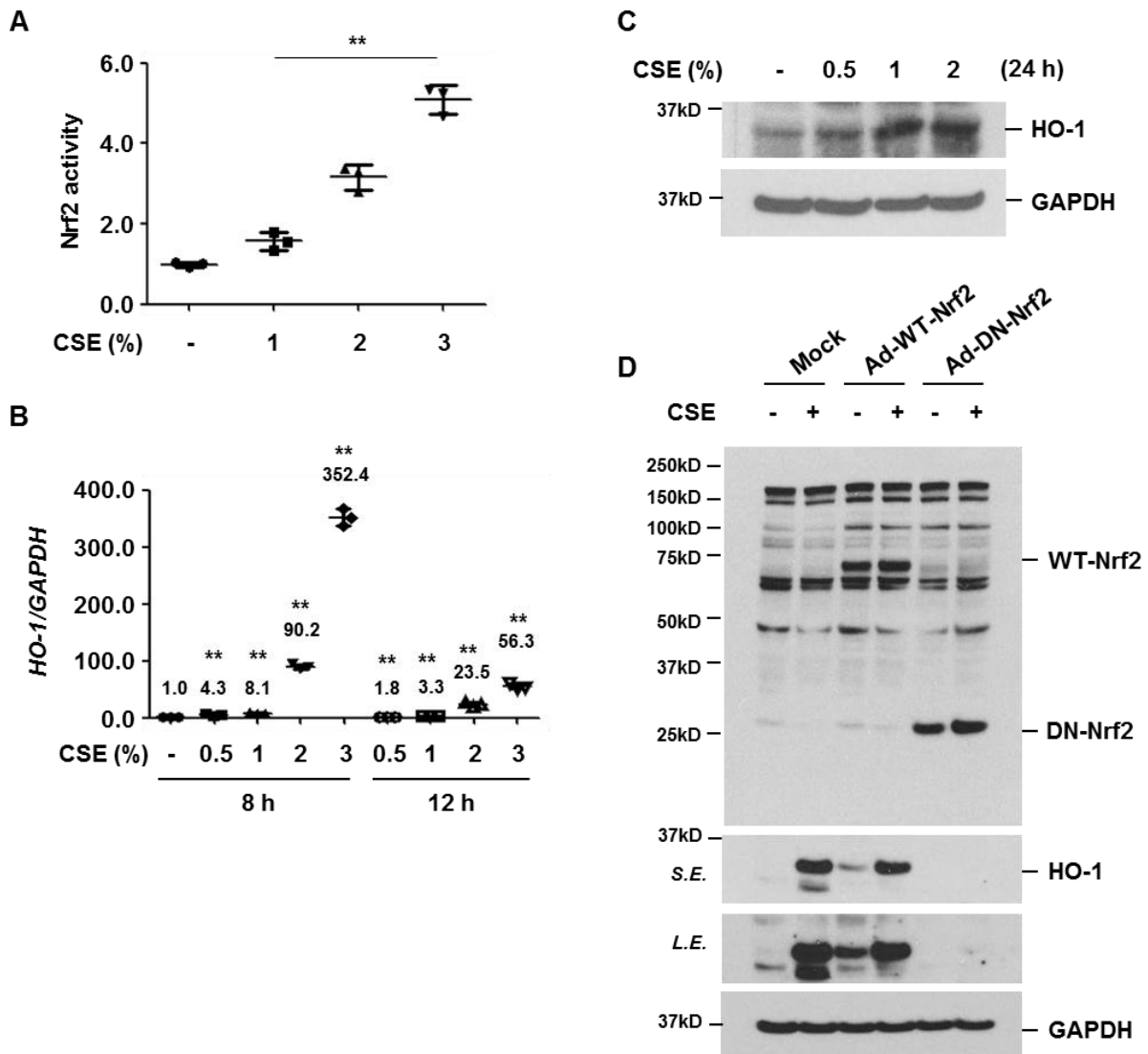
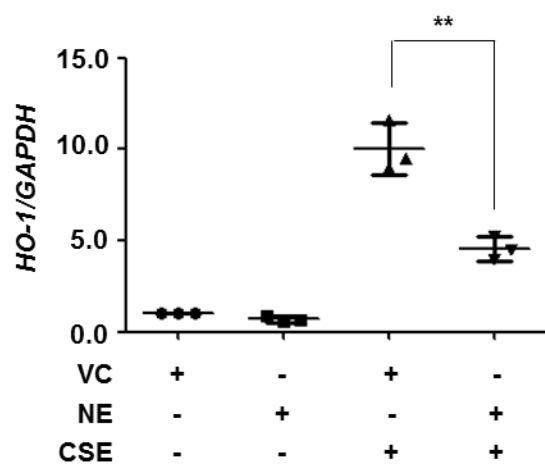


Figure 2

A



B

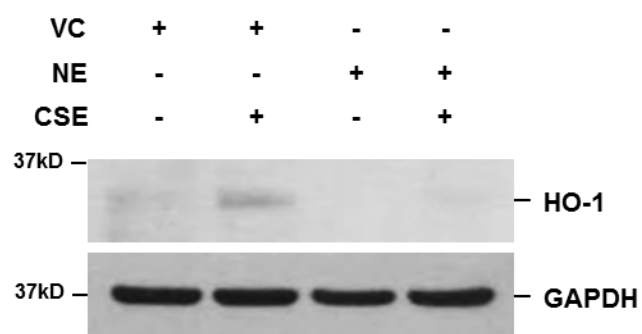


Figure 3

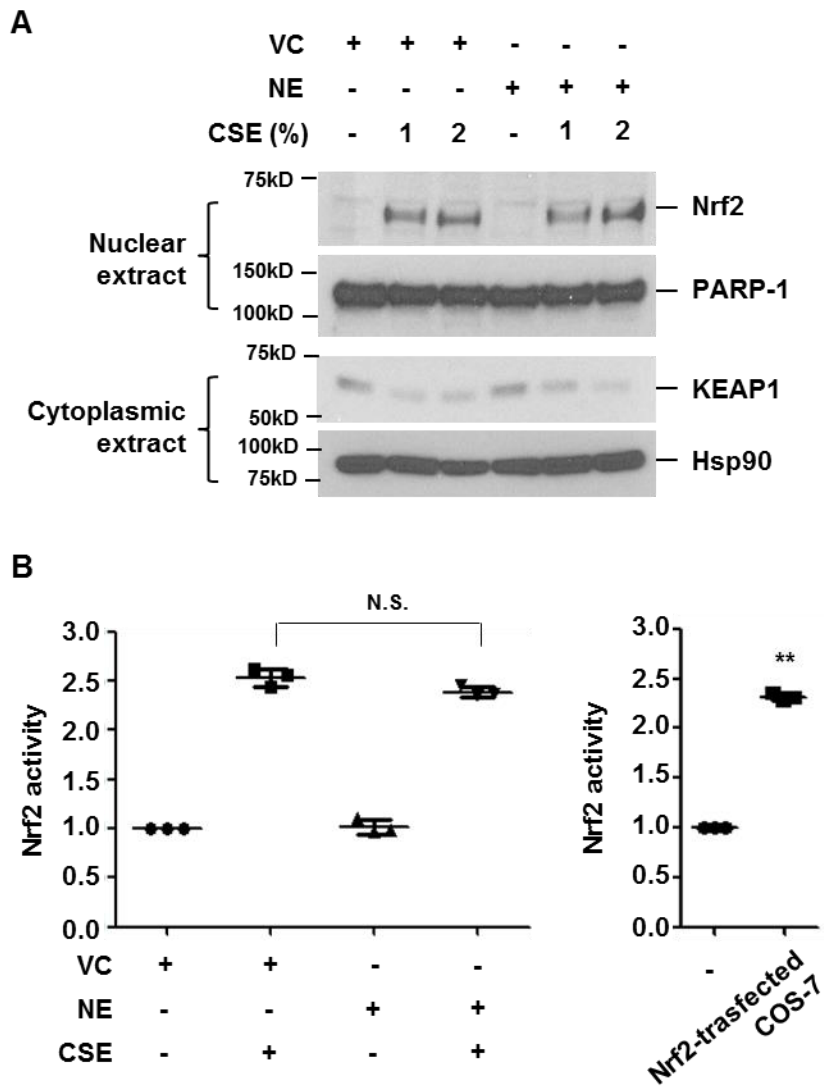


Figure 4

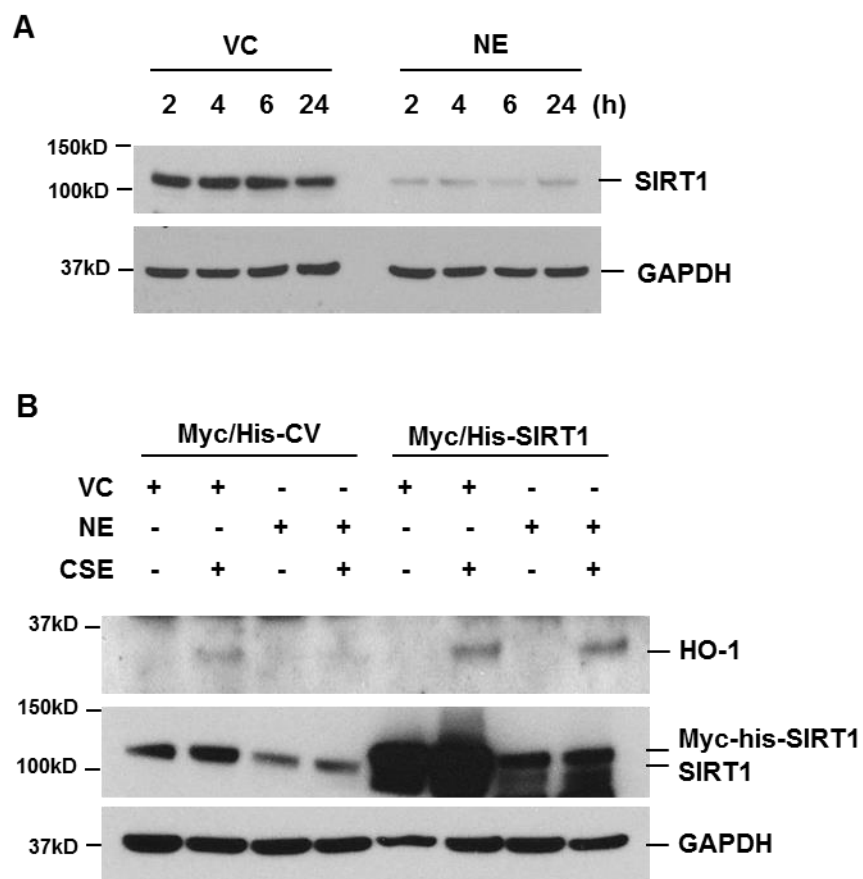


Figure 5

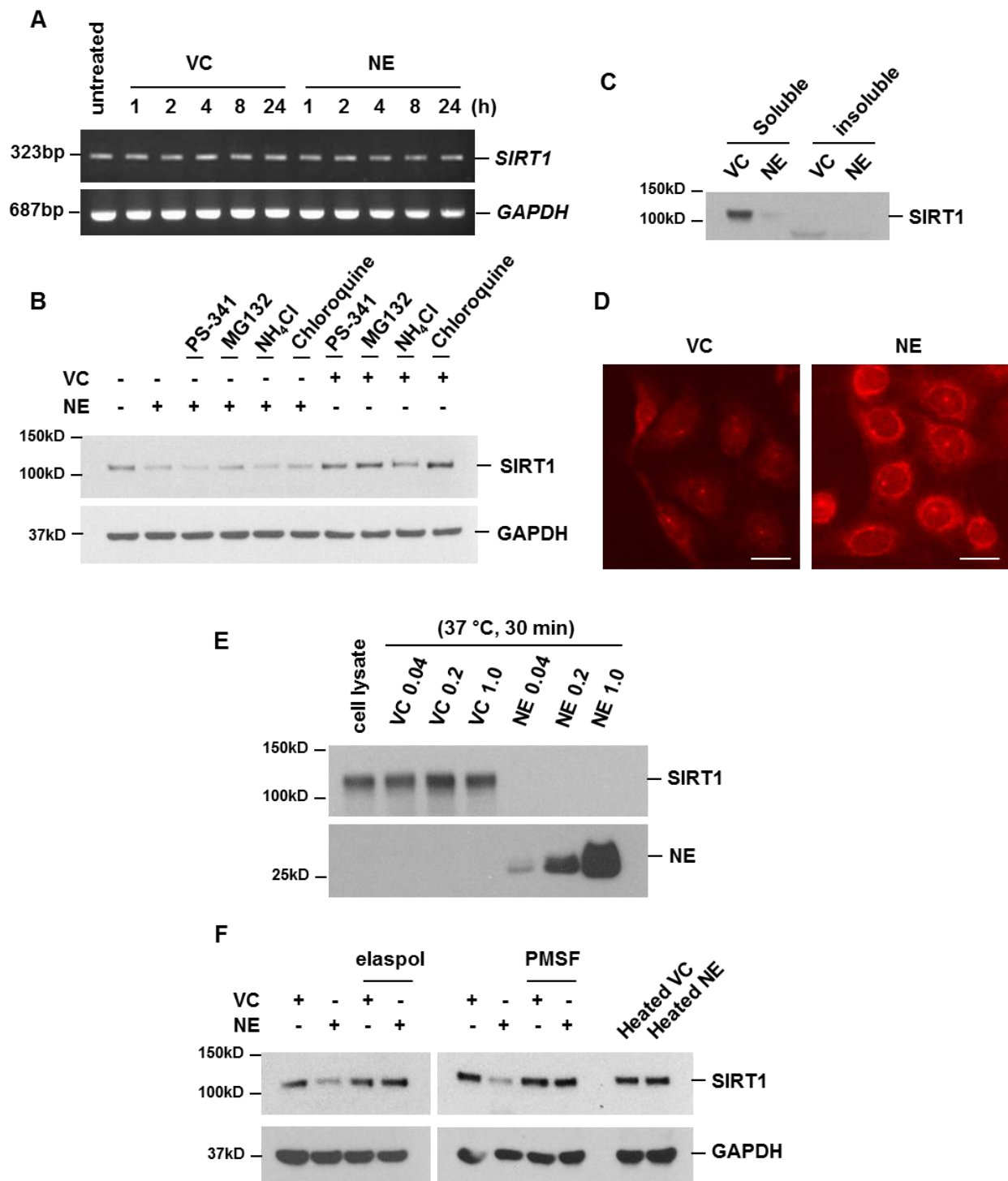


Figure 6

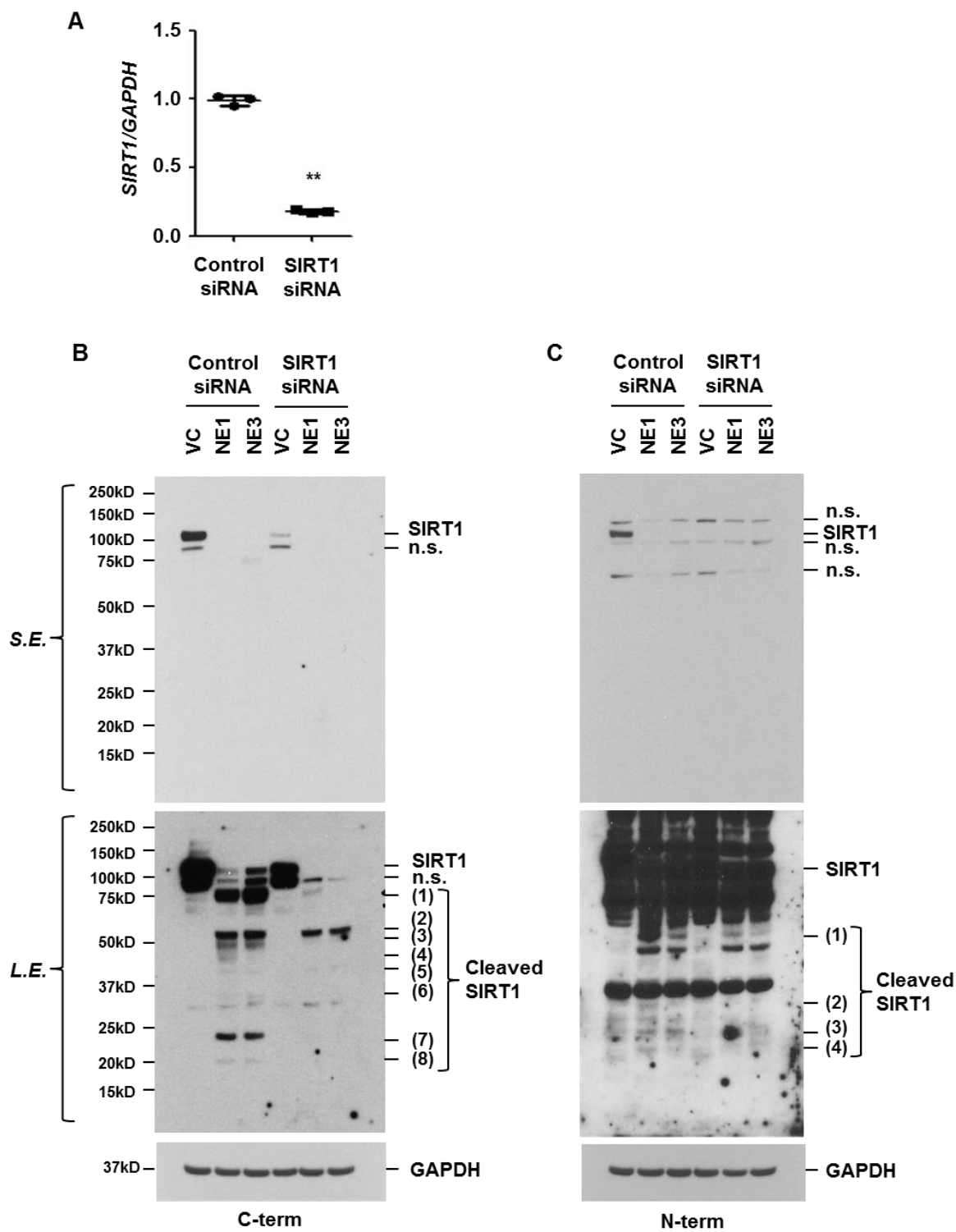


Figure 7

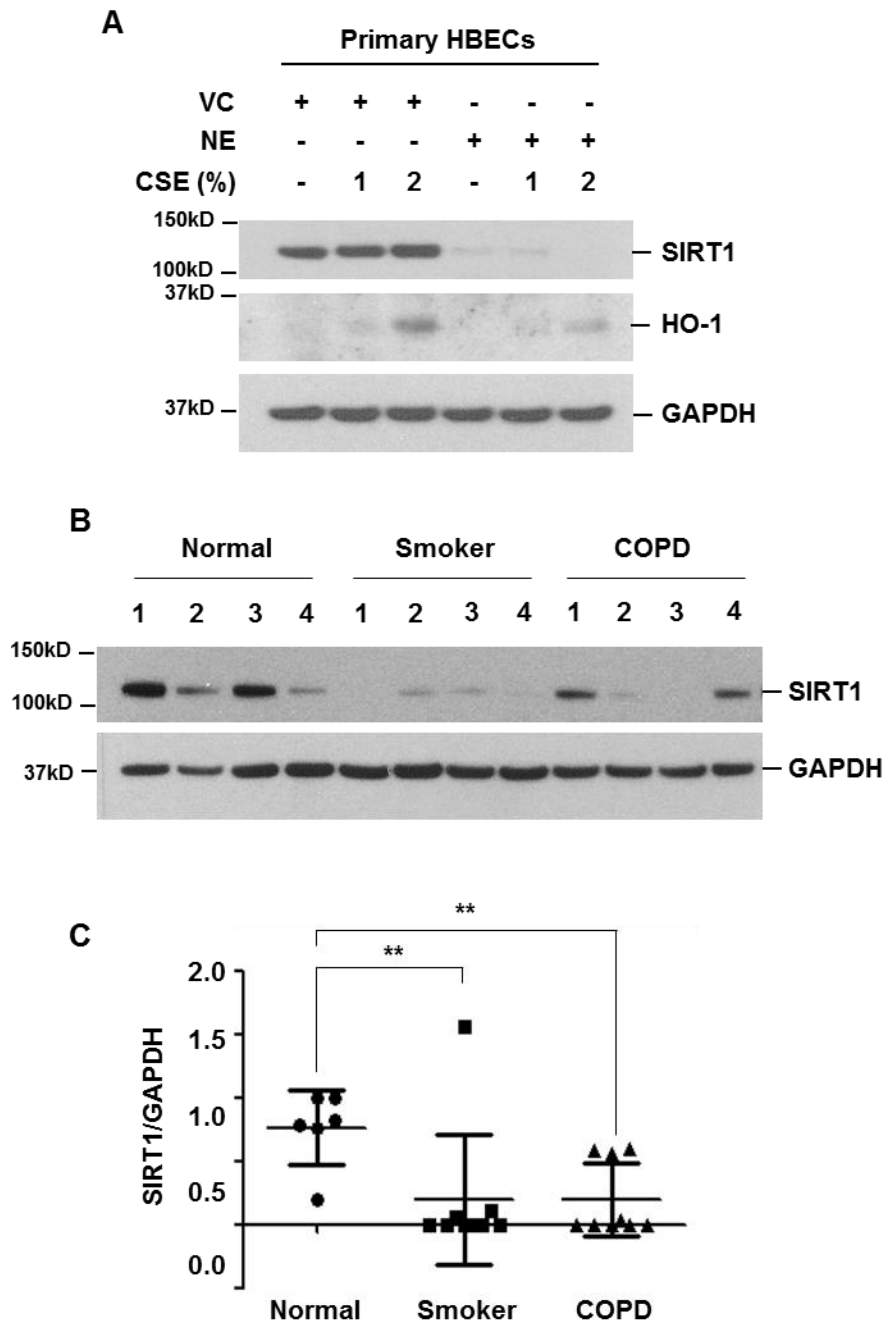
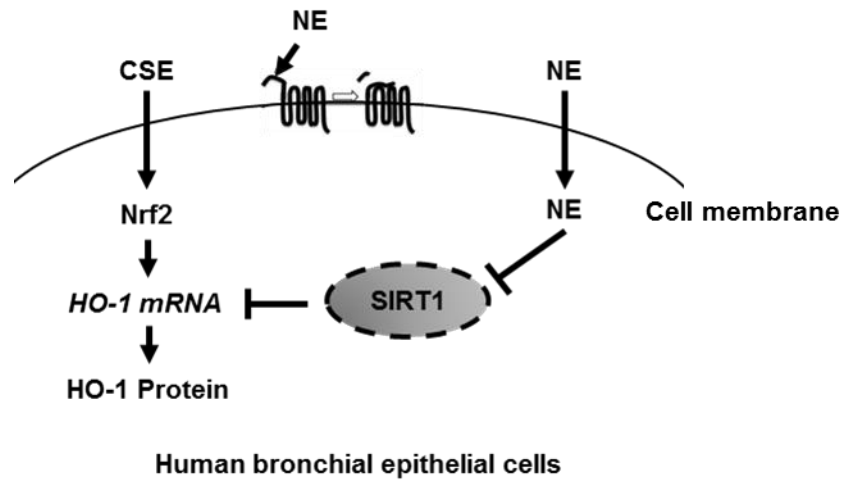


Figure 8



Exogenous neutrophil elastase enters bronchial epithelial cells and suppresses cigarette smoke extract-induced heme oxygenase-1 by cleaving sirtuin 1
Kyoung-Hee Lee, Jiyeong Jeong, Yoon-Jung Koo, An-Hee Jang, Chang-Hoon Lee and Chul-Gyu Yoo

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