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

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NTRODUCTION

An imbalance between oxidative stress and antioxidant activity plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD). Cigarette smoke induces cellular oxidative stress, but antioxidant levels such as heme oxygenase-1 (HO-1) are reduced in severe COPD patients. This study aimed to elucidate the molecular mechanism underlying reduced HO-1 expression in human bronchial epithelial cells.

METHODS

Cells: BEAS-2B cell line and primary human bronchial epithelial cells
Treatment: Cigarette smoke extract (CSE) and neutrophil elastase (NE)
Analytical techniques:

- Western blot for protein expression analysis
- qRT-PCR for mRNA level measurement
- DNA-binding ELISA for Nrf2 activity
- Immunofluorescence staining to confirm NE internalization
- In vitro cleavage assay for direct degradation
- **Clinical samples:** Lung tissue from non-smokers, smokers, and COPD patients

RESULTS

1. **CSE increases HO-1 expression via Nrf2 activation:** CSE increased Nrf2 DNA-binding activity and HO-1 mRNA/protein expression in a dose-dependent manner, which was completely suppressed by dominant-negative Nrf2.
2. **NE suppresses CSE-induced HO-1 expression:** NE pretreatment significantly inhibited CSE-induced HO-1 mRNA and protein expression.
3. **NE does not affect Nrf2 pathway:** NE did not suppress CSE-induced KEAP1 degradation, Nrf2 nuclear translocation, or DNA-binding activity.
4. **NE decreases SIRT1 to suppress HO-1:** NE treatment rapidly decreased SIRT1 protein levels, and SIRT1 overexpression completely blocked NE-mediated HO-1 suppression.
5. **Mechanism of SIRT1 reduction:**
   - Not due to decreased transcription (mRNA levels maintained)
   - Not via proteasomal/lysosomal degradation (not blocked by inhibitors)
   - Not due to insolubilization (not detected in insoluble fraction)
6. **Direct cleavage by NE:**
   - Immunofluorescence confirmed NE internalization into cells
   - In vitro experiments showed NE directly cleaves SIRT1
   - Elastase and serine protease inhibitors blocked cleavage
   - Multiple cleavage sites on SIRT1 identified
7. **Clinical relevance:** SIRT1 levels were significantly decreased in primary human bronchial epithelial cells and lung tissue from smokers and COPD patients.

Conclusion

NE enters bronchial epithelial cells and suppresses CSE-induced HO-1 expression by directly cleaving SIRT1. This finding demonstrates the importance of cross-talk between oxidative stress (CSE) and protease responses (NE) in COPD pathogenesis. NE weakens antioxidant defense mechanisms, perpetuating oxidative stress in a vicious cycle that may contribute to COPD development and progression.

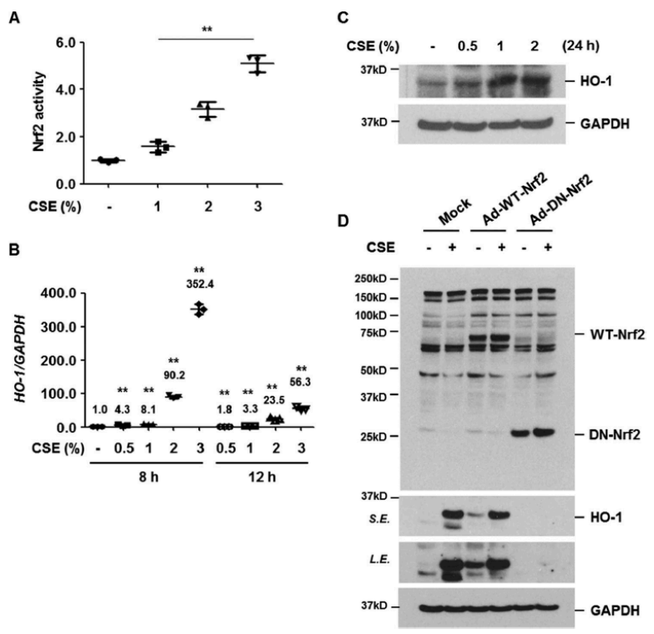

Figure 1. CSE increases the level of HO-1 expression via Nrf2 activation. *A*, BEAS-2B cells were treated with CSE (0, 1, 2, and 3%) for 4 h. Nrf2 activity using nuclear proteins was measured using a DNA-binding ELISA kit for activated Nrf2 transcription factor. Data represent the mean ± S.D. of triplicate experiments. *B*, BEAS-2B cells were stimulated with CSE (0, 0.5, 1, 2, and 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 ± S.D. of triplicates. **, $p < 0.05$. *C*, BEAS-2B cells were treated with CSE (0, 0.5, 1, and 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* and *D*). The results are representative of three independent experiments. *S.E.*, short exposure; *L.E.*, long exposure.

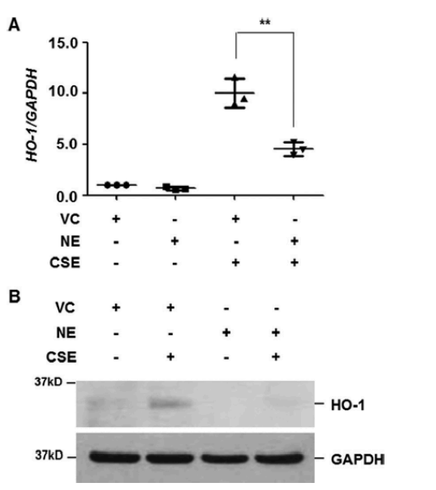

Figure 2. NE suppresses CSE-induced HO-1 expression. BEAS-2B cells were pretreated with vehicle control (*VC*) or NE (1 units/ml) for 4 h and then stimulated with CSE (1%) for 12 (*A*) or 24 h (*B*). *A*, total RNA was isolated and quantitative real-time PCR for *HO-1* and *GAPDH* was performed. Data represent the mean ± S.D. 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.

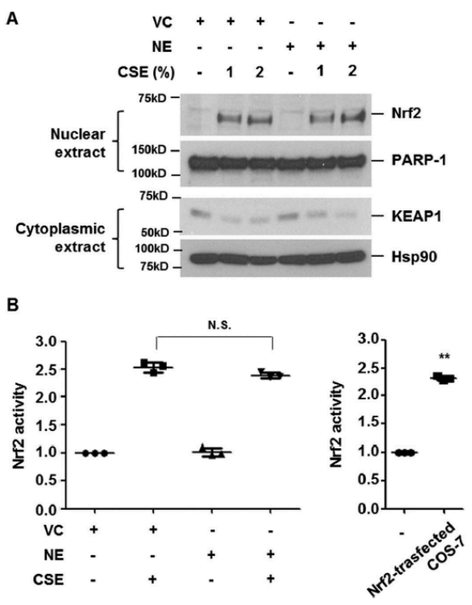

Figure 3. NE does not inhibit CSE-induced nuclear translocation and DNA-binding activity of Nrf2. *A* and *B*, BEAS-2B cells were pretreated with vehicle control (*VC*) or NE (1 units/ml) for 4 h and then stimulated with CSE (1 or 2%) for 4 h. Nuclear and cytoplasmic proteins were extracted and subjected 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 ± S.D. of triplicate experiments. **, $p < 0.05$. The results are representative of three independent experiments. *N.S.*, not

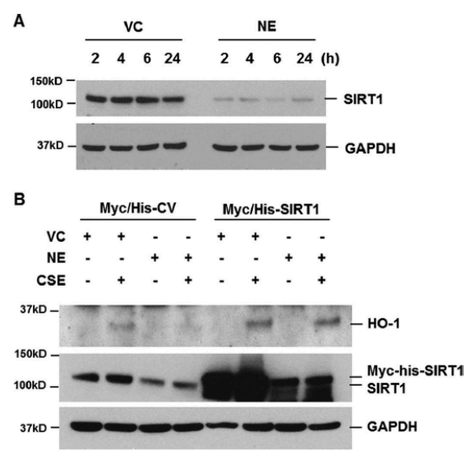

Figure 4. NE decreases SIRT1 expression, which mediates the reduction of HO-1 expression in CSE-treated cells. *A*, BEAS-2B cells were treated with vehicle control (*VC*) or NE (1 units/ml) for the indicated times. *B*, cells were transiently transfected with Myc/His-tagged SIRT1 (Myc/His-SIRT1) expression vector or control vector (Myc/His-CV). Forty-eight hours after transfection, the cells were pretreated with VC or NE (1 units/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.

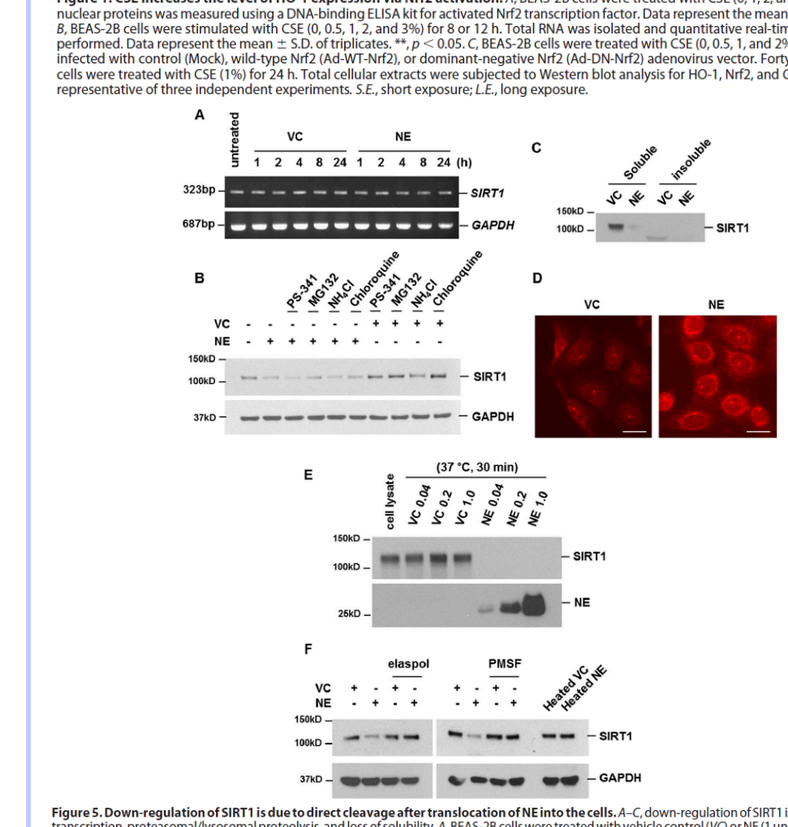

Figure 5. Down-regulation of SIRT1 is due to direct cleavage after translocation of NE into the cells. *A–C*, down-regulation of SIRT1 is not due to decreased transcription, proteasomal/lysosomal proteolysis, and loss of solubility. *A*, BEAS-2B cells were treated with vehicle control (*VC*) or NE (1 unit/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 PS-341, 10 μM MG132) or lysosomal inhibitors (50 mM ammonium chloride, 100 μM chloroquine) for 1 h and then stimulated with VC or NE (1 unit/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 units/ml) for 2 h. Immunofluorescence staining for internalized NE was performed. Cells were analyzed using an ECLIPSE TE300 (Nikon) fluorescence microscope. *Scale bar*: 20 μm. *E*, cell lysates were incubated with VC or NE (0.04, 0.2, and 1 unit/ml) in vehicle at 37 °C for 30 min. The reaction was stopped by adding 4× 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 units/ml) in the presence or absence of elastase inhibitor (elaspol, 100 μg/ml) or serine protease inhibitor (PMSF, 1 mM). Cells were stimulated with heated forms of VC or NE (1 units/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.

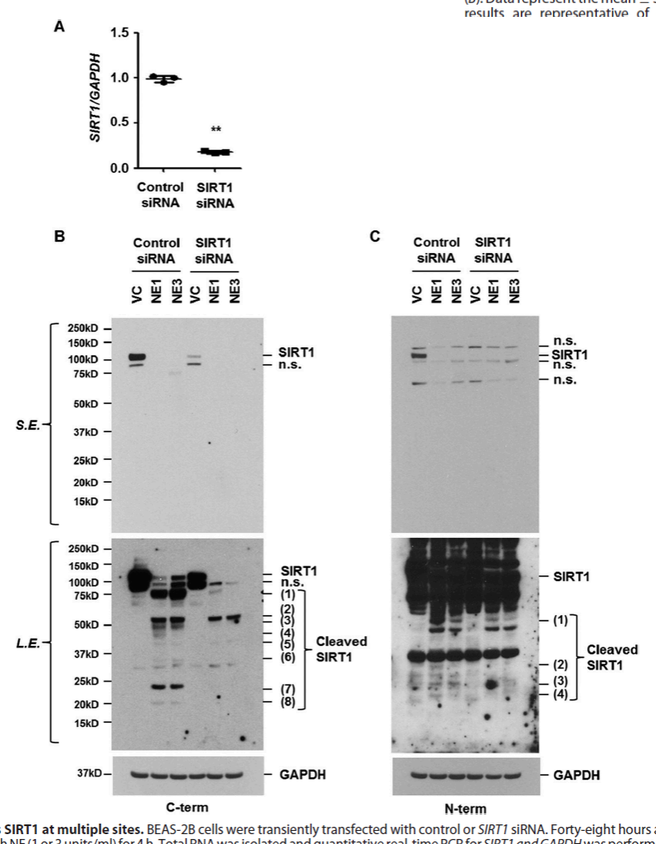

Figure 6. NE cleaves SIRT1 at multiple sites. BEAS-2B cells were transiently transfected with control or *SIRT1* siRNA. Forty-eight hours after transfection, the cells were treated with NE (1 or 3 units/ml) for 4 h. Total RNA was isolated and quantitative real-time PCR for *SIRT1 and GAPDH* was performed (*A*). Data represent the mean ± S.D. 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 carboxyl-terminal region or the amino-terminal region of SIRT1, respectively. The results are representative of three independent experiments. *n.s.*, nonspecific; *S.E.*, short exposure; *L.E.*, long exposure.

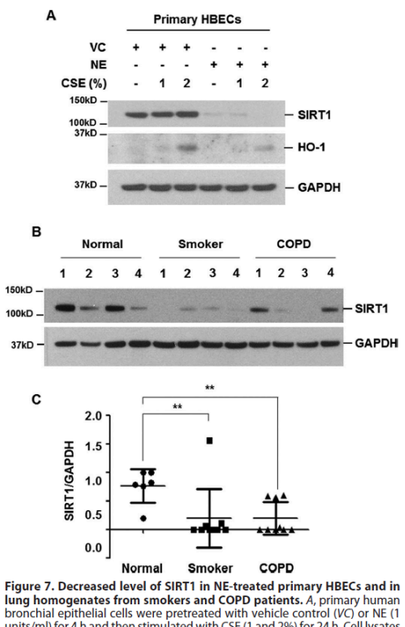

Figure 7. Decreased level of SIRT1 in NE-treated primary HBECs and in lung homogenates from smokers and COPD patients. *A*, primary human bronchial epithelial cells were pretreated with vehicle control (*VC*) or NE (1 units/ml) for 4 h and then stimulated with CSE (1 and 2%) for 24 h. Cell lysates were 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 ± S.D. normal *versus* smoker: **, $p = 0.0299$, normal *versus* COPD: **, $p = 0.0027$.

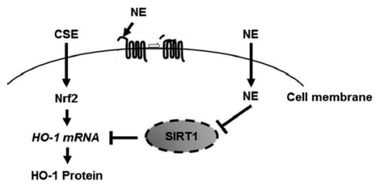

Figure 8. Schematic diagram of the effect of NE on CSE-induced HO-1 expression. CSE up-regulates HO-1 through Nrf2 activation as a defense mechanism in human bronchial epithelial cells. NE suppresses CSE-induced HO-1 by cleaving SIRT1 protein.