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

Materials

TNF-α was from R&D Systems (Minneapolis, MN, USA), prepared as a stock solution in distilled water, and stored at -70 °C until needed. Proteinase-activated receptor activating peptides (PAR-AP) and proteinase-activated receptor blocking peptides (PAR-BP) were purchased from Peptides International, Inc. (Louisville, KY, USA). PAR2 siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-IκBα, anti-caspase-1, anti-phospho-Akt (Ser473) (p-Akt), anti-Akt, anti-phospho-ERK (Thr202/Tyr204) (p-ERK), anti-ERK antibodies were from Cell Signaling (Danvers, MA, USA). Human PAR2 primers (fwd: 5'-CTGGCCATTGGGGTCTTT CTGTTC-3', rev: 5'-GGCCCTCTTCCTTTTCTTCTCTGA-3') were used.

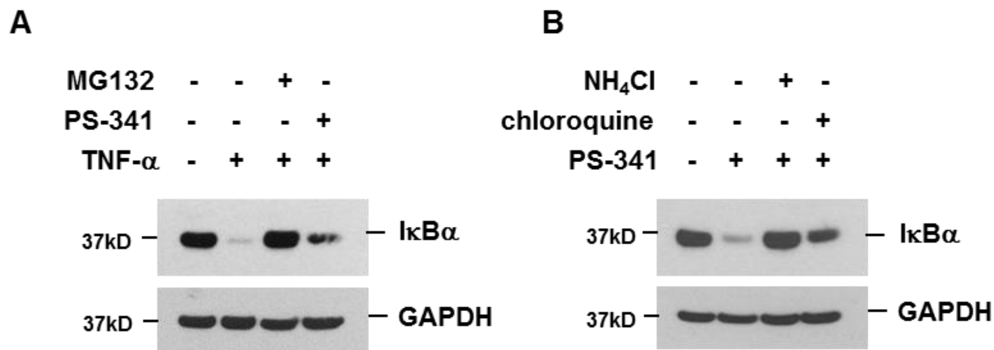

Figure S1. Proteasome or lysosome activity is effectively blocked by proteasome inhibitors (MG132, PS-341) or lysosome inhibitors (ammonium chloride, chloroquine). (A) IκBα, a cytoplasmic inhibitor of NF-κB, is well-known to be degraded via the proteasome in response to TNF-α. BEAS-2B cells were pretreated with MG132 (10μM) or PS-341 (200nM) for 1 h and then stimulated with TNF-α (10ng/ml) for 30 min. Pretreatment with PS-341 or MG132 (short-term incubation) completely blocked IκBα degradation by TNF-α. (B) Long-term incubation with proteasome inhibitors induces IκBα degradation via the lysosomal pathway (1). BEAS-2B cells were pretreated with NH_4Cl (50mM) or chloroquine (100μM) for 1 h and then stimulated with PS-341 (50nM) for 6 h. Total cellular extracts were subjected to western blot analysis for IκBα and GAPDH. PS-341-induced IκBα degradation was suppressed by lysosomal inhibitors.

A

ExPASY Proteomics Server: PeptideCutter

Human SIRT1 (555 aa)

```
  1 migtdprtil kdllpetipp pelddmtlwq ivinilsepp krkkrkdint iedavkllqe
 61 ckkiivltga gvsvscgipd frsrdgiyar lavdfpdlpd pqamfdieyf rkdprpffkf
121 akeiypgqfq pslchkfial sdkegkllrn ytqnidtleq vagiqriiqc hgsfatascl
181 ickykvdcea vrgalfsqvv prcprcpade plaimkpeiv ffgenlpeqf hramkydkde
241 vdllivigss lkvrpvalip ssiphevpqi linreplphl hfdvellgdc dviinelchr
301 lggeyaklcc npvklseite kpprtqkela ylselpptpl hvsedssspe rtsppdssvi
361 vtlldqaaks nddldvsesk gcmeekpqev qtsrnvesia eqmenpdlkn vgsstgekne
421 rtsvagtvrk cwpnrvakeq isrrldgnqy lflppnryif hgaevysdse ddvlsssscg
481 snsdsgtcqs psleepmede seieefyngl edepdvpera ggagfgtdgd dqeaineais
541 vkqevtdmny psnks
```

Enzyme : Neutrophil elastase

No. of cleavages : 63

Positions of cleavage sites : 32, 54, 55, 66, 70, 72, 74, 89, 92, 93, 103, 121, 139, 161, 162, 175, 177, 186, 190, 191, 194, 199, 200, 208, 213, 220, 233, 241, 246, 253, 256, 257, 267, 284, 292, 306, 313, 330, 342, 359, 361, 367, 368, 376, 390, 396, 400, 411, 424, 425, 428, 436, 437, 463, 465, 473, 516, 520, 523, 534, 538, 541, 545

B

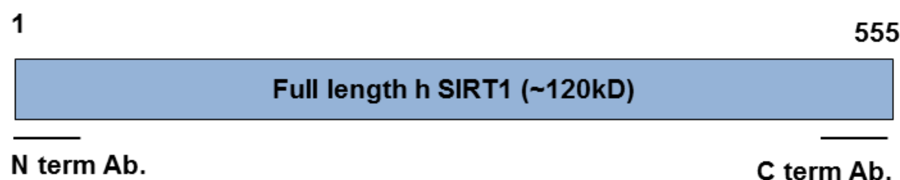

Figure S2. NE recognition sites identified in hSIRT1 protein sequence. (A) Human SIRT1 protein sequence. 63 sites predicted by PeptideCutter to be recognized by NE. (B) Schematic representation of hSIRT1 protein and the recognition region of two different SIRT1 antibodies. N term Ab. (Millipore, #07-131), C term Ab. (Cell signaling, #2496)

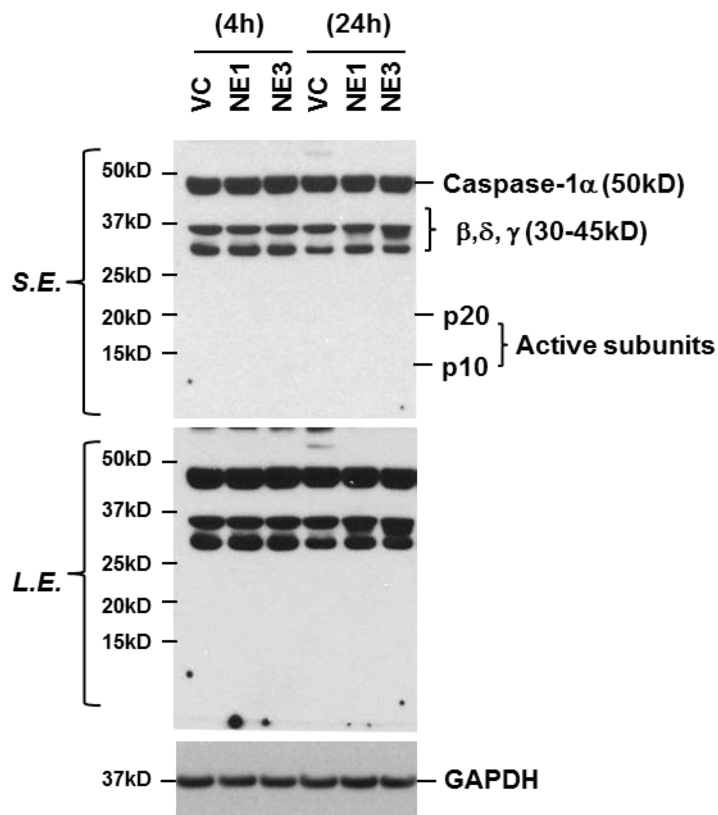

Figure S3. NE dose not activate caspase-1. BEAS-2B cells were treated with VC or NE (1 or 3U/mL) for 4 or 24 h. Total cellular extracts were subjected to western blot analysis for caspase-1 (Cell signaling, #2225) and GAPDH. *S.E.*, short exposure; *L.E.*, long exposure

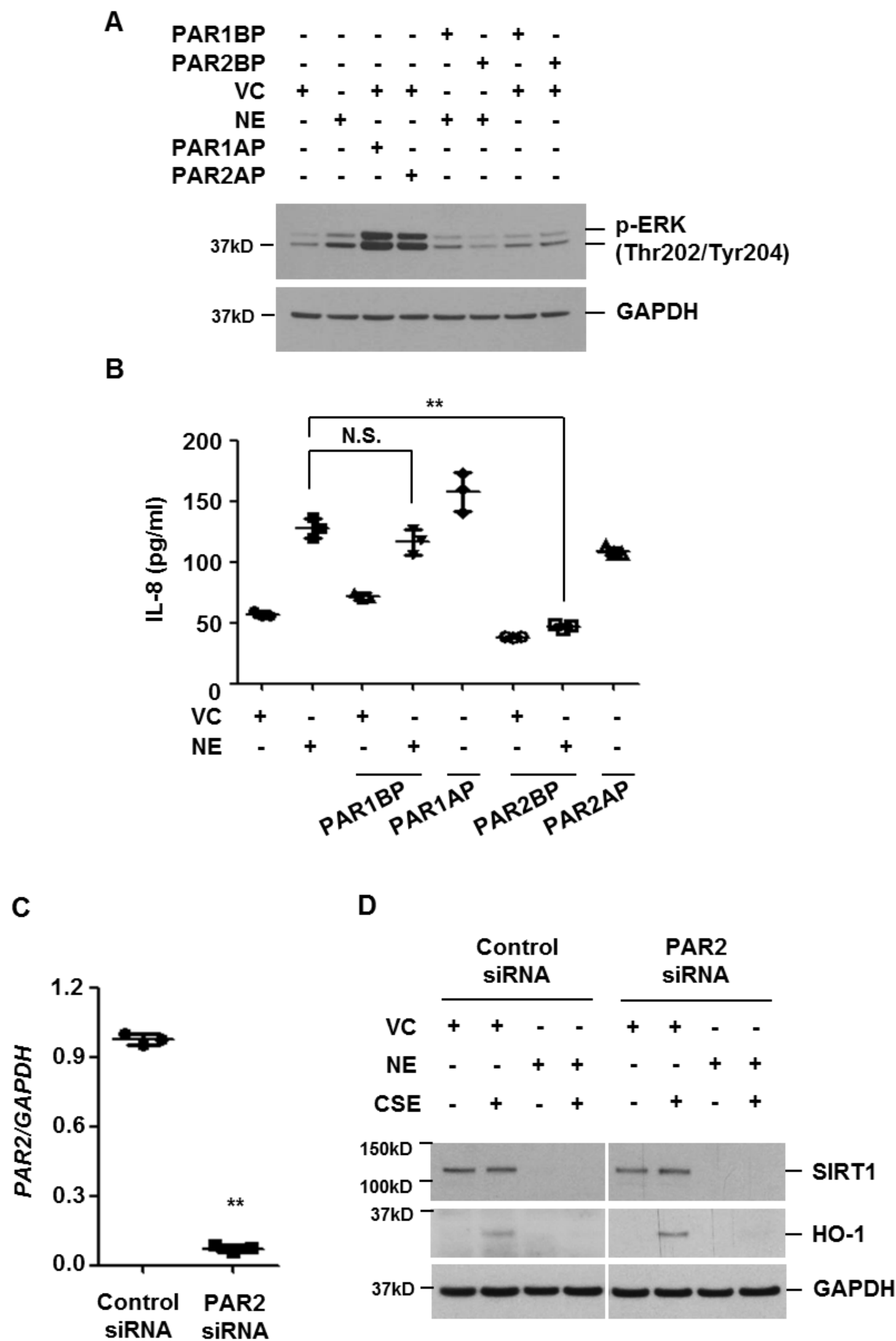

Figure S4. NE-induced cleavage of SIRT1 is not mediated through receptor. (A-B) NE activates extracellular signal-regulated kinase (ERK) and induces interleukin-8 (IL-8) production via PAR2. BEAS-2B cells were pre-incubated with 100μM of PAR1-BP or PAR2-BP for 1 h and then stimulated with VC or NE (1U/mL) for 30 min (A) or 20 h (B). Cells were treated with PAR1-AP or PAR2-AP for 30 min (A) or 20 h (B) as a positive control. Total cellular extracts were subjected to western blot analysis for p-ERK and GAPDH. IL-8 concentrations in culture media were measured by

multiplex bead assay. Data represent the mean ± SD of triplicates. ** $P < 0.05$ N.S., not significant (C-D) Knock-down of PAR2 does not affect NE-mediated down-regulation of SIRT1. BEAS-2B cells were transiently transfected with control or PAR2 siRNA. Forty eight hours after transfection, cells were pretreated with VC or NE (1U/mL) for 4 h and then stimulated with CSE (1%) for 24 h. Total RNA was isolated and quantitative real-time PCR for *PAR2 and GAPDH* was performed (C). Data represent the mean ± SD of triplicates. ** $P < 0.05$ Total cellular extracts were subjected to western blot analysis for SIRT1, HO-1, and GAPDH (D).

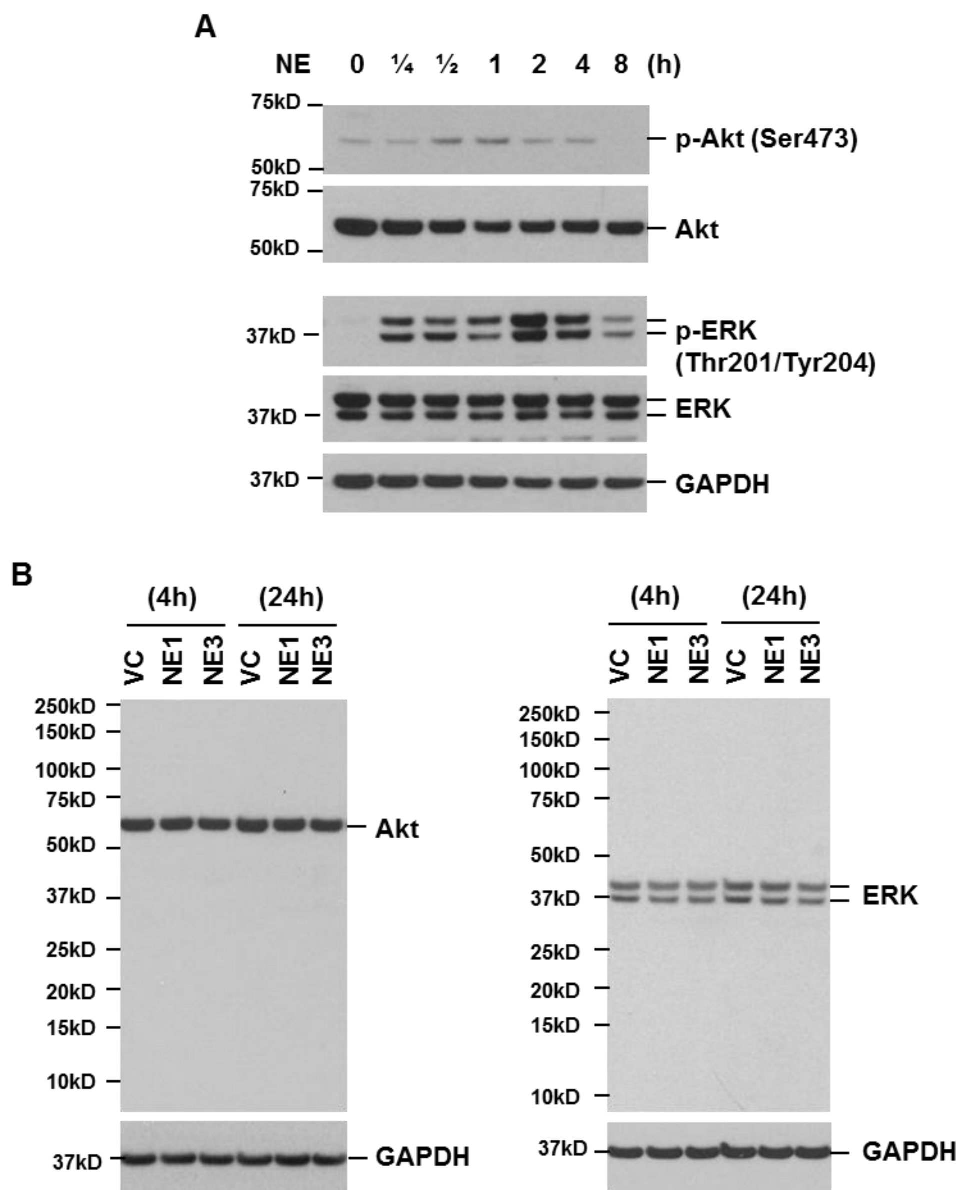

Figure S5. NE does not cleave Akt and ERK. (A) BEAS-2B cells were treated with NE (1U/mL) for the indicated times. (B) Cells were incubated with VC or NE (1 or 3U/mL) for 4 or 24 h. Total cellular extracts were subjected to western blot analysis for p-Akt, total Akt, p-ERK, total ERK, and GAPDH.

REFERENCE

1. Lee, K.H., Jeong, J., Yoo, C.G. (2013) Long-term incubation with proteasome inhibitors (PIs) induces IκBα degradation via the lysosomal pathway in an IκB kinase (IKK)-dependent and IKK-independent manner. *J. Biol. Chem.* **288**(45), 32777-32786.