Cigarette smoke-induced autophagy is regulated by SIRT1-PARP-1-dependent mechanism: implication in pathogenesis of COPD

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Cigarette smoke-induced autophagy is regulated by SIRT1-PARP-1-dependent mechanism: Implication in pathogenesis of COPD

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Abstract

Autophagy is a fundamental cellular process that eliminates long-lived proteins and damaged organelles through lysosomal degradation pathway. Cigarette smoke (CS)-mediated oxidative stress induces cytotoxic responses in lung cells. However, the role of autophagy and its mechanism in CS-mediated cytotoxic responses is not known. We hypothesized that NAD^+-dependent deacetylase, sirtuin 1 (SIRT1) plays an important role in regulating autophagy in response to CS. CS exposure resulted in induction of autophagy in lung epithelial cells, fibroblasts and macrophages. Pretreatment of cells with SIRT1 activator resveratrol attenuated CS-induced autophagy whereas the SIRT1 inhibitor, sirtinol, augmented CS-induced autophagy. Elevated levels of autophagy were induced by CS in the lungs of SIRT1 deficient mice. Inhibition of poly(ADP-ribose)-polymerase-1 (PARP-1) attenuated CS-induced autophagy via SIRT1 activation. These data suggest that the SIRT1-PARP-1 axis plays a critical role in the regulation of CS-induced autophagy and have important implications in understanding the mechanisms of CS-induced cell death and senescence.

Keywords

SIRT1; PARP-1; resveratrol; cigarette smoke; autophagy

INTRODUCTION

Cigarette smoke (CS) contains numerous oxidants/free radicals as well as chemical compounds that induce oxidative stress and are involved in the pathogenesis of lung and heart diseases. CS accelerates cell death and senescence both through direct mechanisms mediated by oxidants/free radicals as well as via generation of oxidants from inflammatory cells in chronic inflammatory lung diseases including chronic obstructive pulmonary disease (COPD) [1–2]. Although there is emerging evidence that CS-mediated cell death and

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senescence increase the susceptibility to diseases, the exact mechanism by which cigarette smoking accelerates cell death and senescence remains unclear.

Autophagy is a fundamental cellular process that eliminates long-lived proteins and damaged organelles through a lysosomal degradation pathway, and has been suggested to have an essential function in maintaining cellular homeostasis [3]. The autophagic process is initiated by sequestering redundant cytoplasmic contents within double-membrane structures termed autophagosomes. The autophagosome fuses with a lysosome, and its contents are degraded and recycled [4]. Although autophagy occurs at basal levels in all cells to maintain cellular homeostasis, recent reports show that autophagy is also induced in response to environmental stresses, such as pathogen infections, starvation and oxidative stress [4–7]. Although, autophagy plays a protective role in overcoming the exogenous stress, prolonged and excessive autophagy can lead to cell death [8–9]. Failure to regulate autophagy has been implicated in pathogenesis of cancer, cardiovascular failure, immune disease, skeletal muscle atrophy and neurodegenerative disorders [10–17]. Recent studies have showed that increased autophagy occurs in lungs of patients with COPD and in lung cells of mouse exposed to CS [18–19]. However, the underlying mechanism for CS-induced autophagy was not studied.

Sirtuin 1 (SIRT1), the mammalian ortholog of yeast silent information regulator 2 (Sir2), is an NAD^+-dependent deacetylase which is shown to be an anti-inflammatory and anti-aging protein [20]. SIRT1 is involved in diverse physiological functions, including gene silencing, stress resistance, apoptosis, inflammation, senescence and aging [2,21–26]. These physiological functions of SIRT1 are mediated by deacetylation of histones and several important transcription factors such as forkhead box O3 (FoxO3), p53 and nuclear factor-κB (NF-κB) [20,22–23,27–29]. SIRT1 activity is also regulated by NAD^+ depletion induced by oxidative stress or activation of the NAD^+-dependent enzyme poly(ADP-ribose)-polymerase-1 (PARP-1) [30–31]. It has recently been shown that SIRT1 regulates autophagy under calorie restriction/starvation [32]. Moreover, we have recently shown that SIRT1 levels/activity is decreased in response to CS exposure *in vitro* in macrophages and epithelial cells as well as in lungs of smokers and patients with COPD [20,25,30]. However, the role of SIRT1 and PARP-1 on CS-mediated autophagy is not known. Therefore, we hypothesized that SIRT1 plays an important role in regulating CS-mediated autophagy in lung cells. We studied the effect of CS on induction of autophagy in different lung cell types *in vitro* and in mouse lung *in vivo*, and determined that the SIRT1-PARP1 axis regulates autophagy.

MATERIALS AND METHODS

Reagents

Penicillin-Streptomycin, L-glutamine and RPMI-1640 were obtained from Gibco BRL (Grand Island, NY). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT). Dulbecco's modified Eagle's medium-Ham's F12 50:50 mixture (DMEM/F-12) was purchased from Mediatech (Manassas, VA). Amphotericin B was purchased from Lonza (Walkersville, MD). Resveratrol was purchased from Biomol (Plymouth Meeting, PA). Sirtinol was procured from Sigma (St. Louis, MO). 3-aminobenzamide (3-AB) was purchased from Calbiochem (La Jolla, CA).

Cell culture and treatments

Human bronchial epithelial cells (H292) and human fetal lung fibroblasts (HFL1) were obtained from American Type Culture Collection (Manassas, VA). H292 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin and

100 U/ml streptomycin. HFL1 cells were cultured in DMEM-F12 supplemented with 10% FBS, 100 μg/ml penicillin, 100 U/ml streptomycin, and 1 μg/ml amphotericin B. Human bronchial epithelial cells (Beas-2B) were grown in DMEM-F12 supplemented with 5% FBS, 15 mM HEPES, 100 μg/ml penicillin, and 100 U/ml streptomycin. Human monocyte-marcophage cell line (MonoMac6), which was established from peripheral blood of patient with monoblastic leukemia, were grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin, 1% nonessential amino acid, 1 mM sodium pyruvate, 1 μg/ml human holo-transferrin, and 1 mM oxaloacetic acid. The cells were incubated at 37 °C in a humidified atmosphere containing 7.5% CO_2 and 92.5% air. The cells were pretreated with resveratrol (10 μM), sirtinol (10 μM) or 3-aminobenzidine (3-AB, 1mM) for 2 hrs before treated with cigarette smoke extract (CSE, 0.5% – 5%) for 24 hrs. To avoid induction of autophagy through the serum starvation pathway, all treatments were done in complete culture medium.

Preparation of cigarette smoke extract

Research grade cigarettes 2R4F were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). These cigarettes contain 11.7 mg of total particulate matter (TPM), 9.7 mg of tar, and 0.76 mg of nicotine per cigarette. Cigarette smoke extract (CSE) was prepared by bubbling smoke from one cigarette into 10 ml serum-free media at a rate of one cigarette/min as described previously [33–34]. The pH of the CSE was adjusted to 7.4, and was sterile-filtered through a 0.45 μm filter (25 mm Acrodisc; Pall Corporation, Ann Arbor, MI). CSE preparation was standardized by measuring the absorbance (OD: 1.00 ± 0.05) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at 320 nm showed very little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 10% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml serum-free media, adjusting pH to 7.4, and sterile-filtered as described above.

Transfection

For the autophagy assays, H292 cells were plated on chamber slides and transfected with 1 μg of GFP-LC3 expression construct, a kind gift of Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan), using lipofectamine^{TM} 2000 (Invitrogen, CA) according to the manufacturer's protocol. Images were captured using a fluorescent microscope (BX51, Olympus Optical, Tokyo, Japan).

Immunoblotting

Whole cell extracts were separated on a 6.5% to 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis. Separated proteins were transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL), and blocked for 1 hr at room temperature with 5% bovine serum albumin (BSA) (Sigma-Aldrich). The membranes were then probed with specific primary antibodies of LC3, β-actin (Sigma-Aldrich), SIRT1, acetylated p53 on lysine 382, GAPDH (Cell Signaling Technology, Beverly, MA) or p53, poly(ADP-ribose) (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for overnight. After three washing steps, the levels of protein were detected by probing with secondary anti-rabbit or anti-mouse antibody linked to horseradish peroxidase for 1 hr, and bound complexes were detected using the enhanced chemiluminescence method (Perkin Elmer, Waltham, MA). Equivalent loading of the gel was determined by quantification of protein as well as by reprobing membranes for β-actin or GAPDH. ImageJ software (Version 1.41, National Institutes of Health, Bethesda, MD) was used for gel band quantitative densitometric analysis.

Animals and cigarette smoke exposure

SIRT1 heterozygous knockout ($Sirt1^{+/-}$) mice [35] and wild-type mice of genetic background 129/SvJ were bred and maintained under specific pathogen-free condition in the vivarium facility of the University of Rochester. These SIRT1 deficient mice and WT littermates were housed in the vivarium facility of the University of Rochester with a 12 hrs light/dark cycle (light on at 6:00 am). All animal procedures were approved by the Committee on Animal Research at the University of Rochester. In brief, mice were exposed to CS using research grade cigarettes (2R4F, University of Kentucky, Lexington, KY) according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35-ml volume) with a Baumgartner-Jaeger CSM2072i automatic CS generating machine (CH Technologies, Westwood, NJ). Mainstream CS was diluted with filtered air and directed into the exposure chamber. The smoke exposure (TPM in per cubic meter of air) was monitored in real-time with a MicroDust Pro-aerosol monitor (Casella CEL, Bedford, UK) and verified daily by gravimetric sampling. The smoke concentration was set at a value of ~300 mg/m^3 TPM by adjusting the flow rate of the diluted medical air, and the level of carbon monoxide in the chamber was 350 ppm [36]. Mice (n=4 per group) received two 1 hr exposures (1 hr apart) daily for 3 consecutive days and were sacrificed at 24 hrs post-last exposure. Control mice were exposed to filtered air in an identical chamber according to the same protocol described for CS exposure. Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium (100 mg/kg; Abbott Laboratories, Abbott Park, IL) and then sacrificed by exsanguination 24 hrs after last exposure. The lungs were removed *en bloc* and frozen for immunoblot analysis.

Statistical analysis

Data were presented as mean±SEM for three independent repeats of each experiment. Statistical analysis of significance was calculated using one-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test for multigroup comparisons using StatView software. $P < 0.05$ considered as significant whereas $P > 0.05$ considered as non-significant.

RESULTS

Cigarette smoke extract (CSE) induces autophagy in different lung cell types

We investigated whether CSE could affect the induction of autophagy in different lung cell types (epithelial cells and fibroblasts), and in macrophages. Treatment of human bronchial epithelial cells (H292) with CSE caused a dose-and time-dependent increase in the conversion of LC3-I to LC3-II, a hallmark of autophagic activity (Fig. 1A) [37]. At the concentration of 1% CSE, approximately 5-fold increase in the amount of LC3-II/LC3-I was found as compared to controls. CSE (1%) time-dependently increased the LC3-II/LC3-I for up to 36 hrs following CSE treatment. The formation of GFP-LC3 punctae, a characteristic during the formation of autophagosomes [37], was also significantly increased in response to CSE (Fig. 1B), and was correlated with the conversion of LC3-I to LC3-II by immunoblot analysis. The number of GFP-LC3 dots per cell in CSE-treated H292 cells was also significantly increased in a dose-dependent manner. Another human bronchial epithelial cell line Beas-2B also showed the similar results to dose-dependent increase in the conversion of LC3-I to LC3-II in response to CSE (Fig. 1C). Furthermore, CSE treatment of human fetal lung fibroblasts (HFL1) and human monocyte-macrophage cell line (MonoMac6) also caused a dose-dependent increase in the conversion of LC3-I to LC3-II (Fig. 1D). These data clearly suggest that CSE induces autophagy in different lung cell types.

SIRT1 activator attenuates CSE-induced autophagy

We recently reported that the levels and activity of SIRT1 are decreased in response to CS exposure in lungs of smokers and patients with COPD as well as in MonoMac6 and lung epithelial cells [20,25,30]. Based on this, we hypothesized a decrease in SIRT1 levels/activity is involved in induction of CS-induced autophagic response. To investigate the role of SIRT1 in CSE-induced autophagy, H292 cells were pretreated with a non-specific activator of SIRT1, resveratrol (10 μM) for 2 hrs, followed by treatment with CSE (0.5% and 1%) for 24 hrs or H_2O_2 (100 μM) for 1 hr. The levels of SIRT1 were significantly reduced in response to CSE, whereas resveratrol pretreatment prevented the decrease in SIRT1 levels in response to CSE (Fig. 2). SIRT1 deacetylase activity was also assessed by measuring levels of acetylated p53 on lysine 382. CSE significantly increased acetylation of p53, which was partially attenuated by resveratrol pretreatment. Resveratrol treatment alone without CSE challenge showed increased levels and activity of SIRT1 but did not affect induction of autophagy, as assessed by immunoblot analysis of LC3 levels. As shown in Fig. 2, however, pretreatment of H292 cells with resveratrol showed attenuation in levels of LC3-II/LC3-I in response to CSE and H_2O_2 as compared to H292 cells that were not pretreated with resveratrol. These data suggest that resveratrol attenuates CSE-induced autophagy, implying that decreased levels/activity of SIRT1 under stress condition is involved in induction of autophagy.

Inhibition of SIRT1 leads to augmentation in CS-induced autophagy

To determine whether the decreased level of SIRT1 was associated with CSE-induced autophagy, H292 cells were pretreated with pharmacological inhibitor of SIRT1, sirtinol (10 μM). After pretreatment for 2 hrs, cells were treated with CSE (0.5% and 1%) for 24 hrs or H_2O_2 (100 μM) for 1 hr and subjected to immunoblot analysis. The levels of SIRT1 were significantly decreased in response to CSE, which was augmented by pretreatment with sirtinol (Fig. 3). CSE significantly increased acetylation of p53 on lysine 382 indicating reduction in SIRT1 activity, which was further enhanced in sirtinol pretreated cells. As expected, CSE increased induction of autophagy and sirtinol pretreatment further increased autophagic activity. Interestingly, sirtinol treatment alone without CSE challenge showed decreased SIRT1 levels and activity but this did not induceLC3-II suggesting that SIRT1 reduction *per se* is not sufficient to induce autophagy.

To further demonstrate the involvement of SIRT1 in regulation of CS-induced autophagy, SIRT1 deficient heterozygous (*SIRT1*$^{+/-}$) and *wild-type* (WT) littermate mice were exposed to CS for 3 days and the levels of autophagy estimated from induction of LC3-II. As shown in Fig. 4, an increase in conversion of LC3-I to LC3-II was observed *in vivo* in CS-exposed SIRT1 deficient and WT mice lung. However, no significant different was seen between air-exposed *SIRT1* deficient and WT mice. These data suggest that SIRT1 has a role in the induction of autophagy in response to CS but reduction of SIRT1 alone without any stress was not sufficient to induce autophagy in the lung.

PARP-1 inhibition attenuates CSE-induced autophagy

PARP-1 is a NAD^+-dependent nuclear enzyme that generates poly(ADP-ribose) (PAR) polymer from NAD^+. Hence, activation of PARP-1 depletes the nuclear NAD^+ pool which will result in reduction of NAD^+-dependent deacetylase SIRT1 activity [30,38,39]. To determine whether PARP-1 activity contributed to the CSE-induced autophagy via downregulation of SIRT1 activity, HFL1 fibroblasts were treated with CSE (1%) for 24 hrs or H_2O_2 (100 μM) for 1 hr in the presence or absence of PARP-1 inhibitor (3-AB, 1 mM) for 2 hrs. The formation of PAR polymer was detected by immunoblot assay. As shown in Fig. 5, PAR polymer formation was induced by CSE treatment accompanied with reduction in SIRT1 activity. Pretreatment with 3-AB significantly inhibited CSE-induced PAR

formation and the reduction in SIRT1 activity specifically in HFL1 fibroblasts. Interestingly, 3-AB pretreatment attenuated CSE-induced autophagy, which was similar to the inhibitory effect of resveratrol on LC3-I to LC3-II conversion. These observations suggest that SIRT1-PARP-1 axis has a role in induction of autophagy in response to CSE.

DISCUSSION

Recent studies have reported that downregulation of histone deacetylase (HDAC) activity can induce autophagy. HDAC inhibitors, such as sodium butyrate and suberoylanilide hydroxamic acid (SAHA) can induce autophagy [40–41]. In addition, Chen *et al.* demonstrated that decreased HDAC activity in response to CS triggers autophagy [19]. Despite increasing reports of the association between decreased HDAC activity and induction of autophagy, little is known about the relationship between decreased SIRT1 deacetylase activity and induction of autophagy especially under oxidative stress conditions. We tested the hypothesis that SIRT1 plays an important role in regulating CS-mediated autophagy which is mediated by SIRT1-PARP1 axis in lung cells. We found that reduction in SIRT1 activity by CS induced autophagy in different lung cell types. SIRT1 activator resveratrol attenuated CSE-induced autophagy through prevention of SIRT1 reduction, whereas SIRT1 inhibitor sirtinol enhanced CSE-induced autophagy through SIRT1 reduction.

Recently, Lee *et al.* demonstrated that SIRT1 upregulates starvation-induced autophagy, which resulted from deacetylation of the autophagy machinery [32]. SIRT1 is NAD^+-dependent and its activity is regulated by intracellular NAD^+ level. Calorie restriction/starvation increases the NAD^+ levels through upregulation of the NAD^+ salvage pathway, thus increasing SIRT1 activity [42]. Unlike calorie restriction, oxidative stress imposed by CS and H_2O_2 leads to a decrease in SIRT1 activity possibly via depletion of intracellular NAD^+ pool. Moreover, we and others have shown that SIRT1 activity was decreased in lungs of smokers and patients with COPD as well as in lung cells exposed to CSE [20,25,30,43]. Our results are in discordance with the findings of Lee *et al* for the role of SIRT1 in upregulating autophagy during starvation stress and suggest that CS or oxidants-induced autophagy is regulated by another mechanism which is linked with SIRT1.

Huang *et al.* reported that NAD^+-dependent enzyme PARP-1 promotes autophagy under oxidative stress [44]. Under oxidative stress, PARP-1 is activated and causes rapid depletion of NAD^+, leading to reduction of SIRT1. We found that PARP-1 was activated in response to CS, as shown by increased formation of PAR polymer, which results in depletion of NAD^+ and subsequent reduction of SIRT1 activity. PARP-1 inhibitor (3-AB) attenuated CSE-induced autophagy with partial increase in SIRT1 activity specifically in fibroblasts. These findings suggest that SIRT1-PARP-1 axis is required for regulation of autophagy in response to CS. Resveratrol is shown to enhance SIRT1-dependent cellular processes, including life-span extension, cell cycle regulation and apoptosis from yeast to mammals [45–48]. Hence pharmacological activation of SIRT1 may be beneficial in attenuating cigarette smoke/oxidants-induced autophagy.

Interestingly, we showed that decrease in SIRT1 activity by pharmacological SIRT1 inhibitor sirtinol could not induce autophagy without stimuli/stresses. This phenomenon was also confirmed in lung tissues from SIRT1 deficient mice where autophagy was not seen in lung cells of SIRT1 overexpressing mice or induced by CS in SIRT1 overexpressing mice (Hwang *et al.* unpublished data). However, autophagy was induced in lungs of SIRT1 deficient mice when exposed to CS compared to WT mice exposed to CS or SIRT1 deficient mice alone. We speculated that SIRT1 reduction *per se* is not sufficient to cause autophagy

and possibly requires PARP-1 activation and/or other molecules linked with SIRT1 to trigger autophagy in response to CS.

The mammalian target of rapamycin (mTOR) plays a critical role in maintaining nutrient and energy status through a pathway that regulates many essential biological processes, including autophagy. AMP-activated protein kinase (AMPK) is one of the major upstream regulators of mTOR and its activation stimulates autophagy induction [44,49]. Accumulating evidence suggests the relevance of SIRT1, mTOR and AMPK to a defect in biological processes, including energy expenditure, muscle loss and senescence [50–52]. Whether AMPK has any role in CS-induced reduction of SIRT1 activity and subsequent induction of autophagy in lung cells remains to be determined. As AMPK has been well established as key regulators of autophagy in response to alteration of SIRT1 activity, it is reasonable to postulate that AMPK may have a direct role in CS-induced reduction of SIRT1 activity and subsequent induction of autophagy in lung cells.

Intriguingly, SIRT1 and autophagy have been implicated in cellular senescence and aging [59]. SIRT1 has been shown to regulate aging and longevity in mammals [21], and CS also induces aging-like alterations in tissue and organ structure [60]. The failure in clearance of proteins due to decline of autophagy is associated with age-related pathogenesis such as neurodegenerative disease [61]. CS-induced autophagy is involved in pathogenesis of CS-mediated lung age-related diseases, such as emphysema and COPD [62]. Emphysema and COPD are associated with loss of regenerative capacity in lungs and cellular senescence aggravates adequate cell replacement by autophagy. Based on our data showing CS-mediated induction of autophagy via SIRT1, it is tempting to speculate that SIRT1 is not only a key player in regulation of autophagy but also involved in aging and cellular senescence in smokers.

COPD and lung cancer are CS-associated chronic diseases but a relationship between both of these conditions with respect to regulation of autophagy is not fully understood [63]. Although we have reported reduction of SIRT1 abundance and activity in lungs of smokers and patients with COPD [20], it is highly debatable whether SIRT1 functions as tumor suppressor or tumor promoter [64]. SIRT1 acts as a tumor promoter which deacetylates and inactivates tumor suppressor genes p53 and p73, leading to down-regulation of p53-and p73-mediated transcriptional activity [27–28,65]. On the other hand, overexpression of SIRT1 suppressed the age-related transcriptional change and tumor formation [66], which showed that SIRT1 serves as tumor suppressor. Recent reports showed that resveratrol (an indirect activator of SIRT1) and its analogs have anti-tumor effects through inhibition of cancer cell growth and induction of apoptosis in lung cancer cells [67–68]. Although resveratrol showed promising efficiency as anti-tumor agent, further investigation on the function of SIRT1 in various lung cancer types and its relevance with COPD is required for the clinical applications.

In summary, our data show that CS induces autophagy in lung epithelial cells, fibroblasts and macrophages through the reduction in level and activity of SIRT1. We further showed that the SIRT1-PARP-1 axis plays a pivotal role in regulation of CS-induced autophagy, as evidenced by the studies using the pharmacological SIRT1 activator and inhibitor, SIRT1 deficient mice and PARP-1 inhibitor in response to CS. These findings have implications in understanding the underlying mechanism that CS cause cell death and senescence in chronic inflammatory lung diseases. Pharmacological activation of SIRT1 may be a novel therapeutic approach in conditions where oxidative stress plays a critical role in autophagy-mediated cell death.

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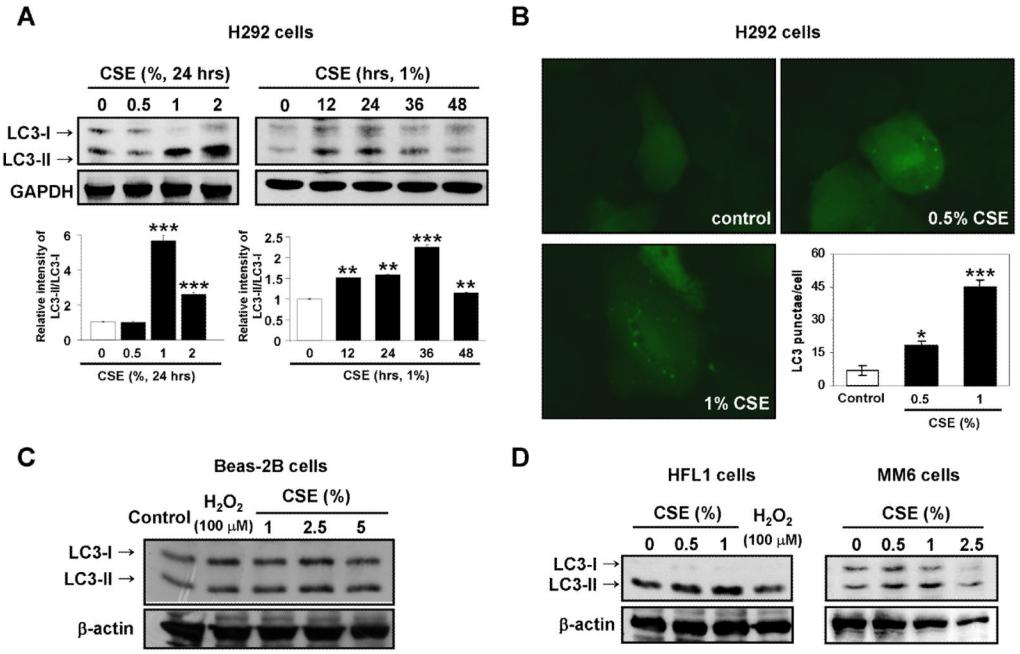

Fig. 1. CSE increases autophagy in different lung cell types

(A) Human bronchial epithelial cells (H292) were treated with indicated concentrations of CSE for 24 hrs or with 1% CSE for indicated time periods. Cell lysates were subjected to immunoblot analysis for detection of LC3 levels. GAPDH was used as loading control. Quantification of results is presented as the amount of LC3-II normalized against LC3-I. **(B)** H292 cells were transfected with GFP-LC3 expression plasmid, and treated with CSE (0.5% and 1%) for 24 hrs. The cells were examined by fluorescent microscopy, and representative cells were selected and photographed. Autophagic activities were measured by the number of GFP-LC3 dots per cell at the designed concentration of CSE treatment. **(C)** Human bronchial epithelial cell line Beas-2B were treated with indicated concentration of CSE for 24 hrs, and subjected to immunoblot analysis for detection of LC3 levels. **(D)** Human fetal lung fibroblasts (HFL1) and human monocyte-macrophage MonoMac6 cells (MM6) were treated with indicated concentrations of CSE for 24 hrs, and subjected to immunoblot analysis for detection of LC3 levels. β-actin was used as loading control. The immunoblot results and microscopic data are representative of three independent repeats of each experiment. Data are shown as mean ± SEM (n=3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, significant compared to controls.

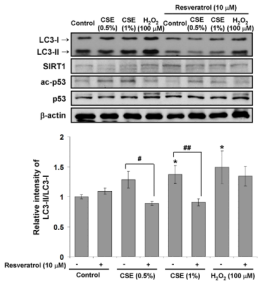

Fig. 2. SIRT1 activation attenuates CSE-induced autophagy

H292 cells were treated with CSE (0.5% and 1%) for 24 hrs or H_2O_2 (100 μM) alone for 1 hr, pretreatment with or without SIRT1 activator resveratrol (10 μM) for 2 hrs. Whole cell extracts were used for immunoblot analysis of LC3, SIRT1, acetylated p53 on lysine 382 and p53. β-actin was used as loading control. Quantification of results is presented as the amount of LC3-II normalized against LC3-I. The immunoblot results are representative of three independent repeats of each experiment. Data are shown as the mean±SEM (n=3). $^{*}P < 0.05$, significant compared to controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant compared to absence of resveratrol. ac-p53, acetylated p53 on lysine 382.

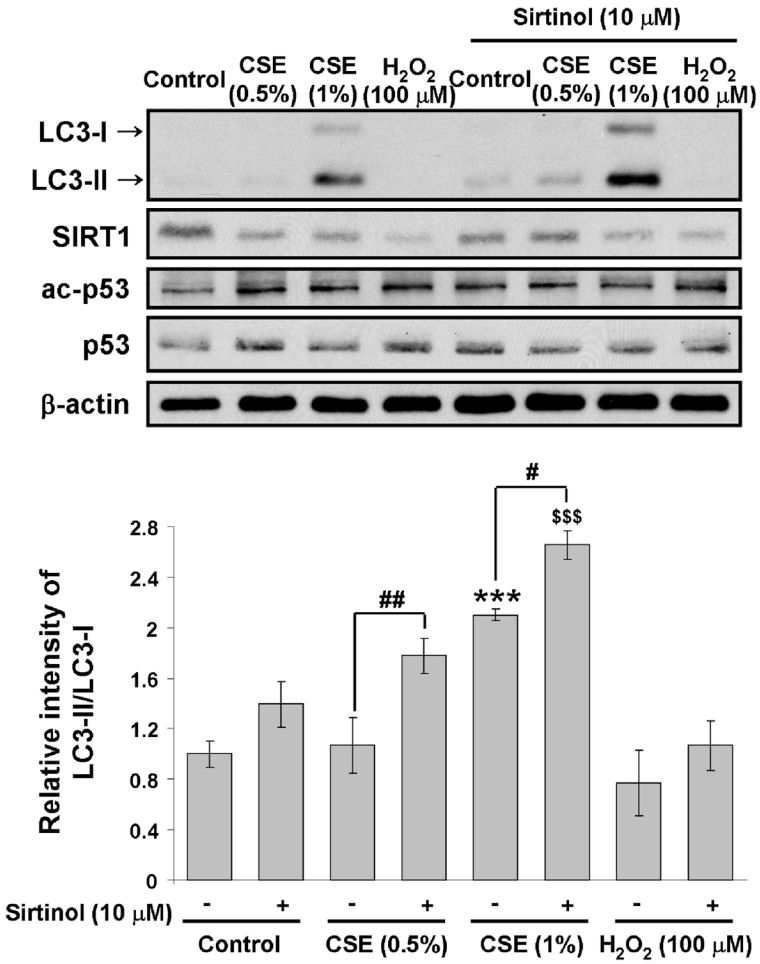

Fig. 3. SIRT1 inhibition enhances CSE-induced autophagy

H292 cells were treated with CSE (0.5% and 1%) for 24 hrs or H_2O_2 (100 μM) for 1 hr, pretreatment with or without SIRT1 inhibitor sirtinol (10 μM) for 2 hrs. Whole cell extracts were used for immunoblot analysis of LC3, SIRT1, acetylated p53 on lysine 382 and p53. β-actin was used as loading control. Quantification of results is presented as the amount of LC3-II normalized against LC3-I. The results are representative of three independent repeats of each experiment. Data are shown as mean±SEM (n=3). ***$P < 0.001$, significant compared to controls; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significant compared to absence of sirtinol; $^{\$\$\$}P < 0.001$, significant compared to sirtinol treatment without challenge. ac-p53, acetylated p53 on lysine 382.

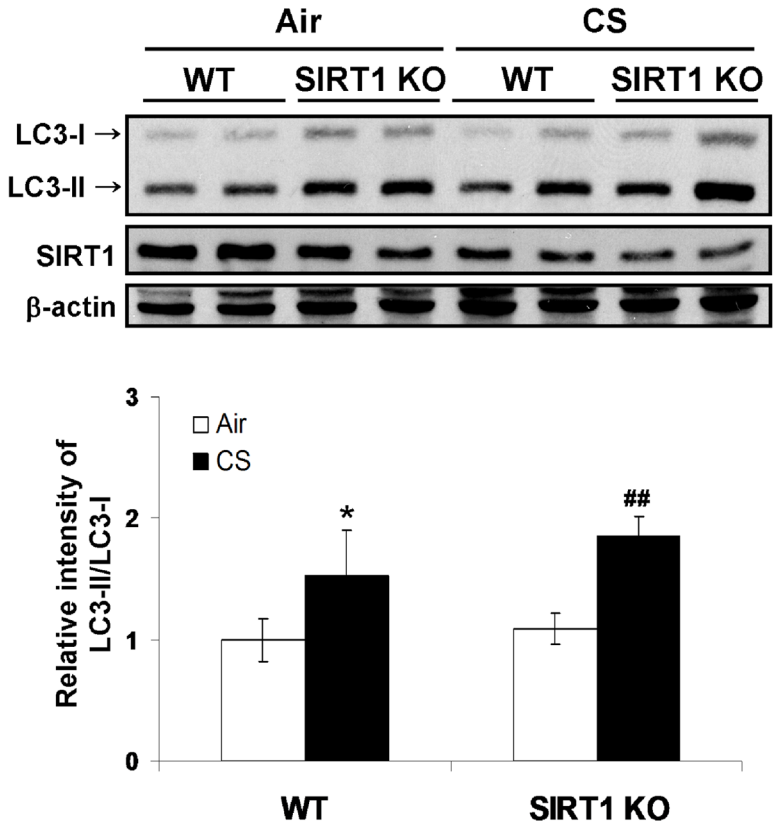

Fig. 4. SIRT1 deficiency augments CS-induced autophagy in mice lung

Whole lung homogenates from SIRT1 deficient and WT mice exposed to CS for 3 days were used for immunoblot analysis of LC3 and SIRT1. β-actin was used as loading control. Quantification of results is presented as the amount of LC3-II normalized against LC3-I. Data are shown as mean ± SEM (n=4 per group). $^{*}P < 0.05$ significant compared to air-exposed WT mice; $^{\#\#}P < 0.01$, significant compared to air-exposed SIRT1 deficient mice.

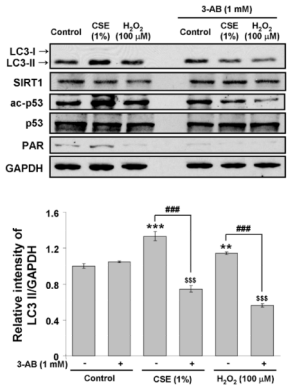

Fig. 5. PARP-1 inhibition attenuates CSE-induced autophagy

HFL1 cells were treated with CSE (1%) for 24 hrs or H_2O_2 (100 μM) for 1 hr, pretreatment with or without PARP-1 inhibitor 3-AB (1 mM) for 2 hrs. Whole cell extracts were used for immunoblot analysis of LC3, SIRT1, acetylated p53 on lysine 382 and p53. GAPDH was used as loading control. Quantification of results is presented as the amount of LC3-II normalized against GAPDH. The immunoblot results are representative of three independent repeats of each experiment. Data are shown as mean±SEM (n=3). $^{**}P < 0.01$, $^{***}P < 0.001$, significant compared to controls; $^{\#\#\#}P < 0.001$, significant compared to absence of 3-AB; $^{\$\$\$}P < 0.001$, significant compared to 3-AB treatment without challenge. ac-p53, acetylated p53 on lysine 382.