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Research Article

Biphasic Autophagy: A Solution to Cigarette Smoke-Induced Autophagy Conundrum

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Abstract

Cigarette smoke-induced protein accumulation and aggresome formation is a major pathological hallmark of COPD disease. Aggresome formation results from a defect in autophagy. To understand the mechanism of cigarette smoke-induced autophagy defect, the current study examined autophagy kinetics and the status of mTOR and AMPK proteins of cigarette smoke extract (CSE)- treated A549 cells. The result showed that CSE-induced autophagy is biphasic. At the beginning of the first phase, phospho-AMPK level was high, and phospho-mTOR was low in CSE-treated cells compared to control indicating a nutrient-deprived condition conducive to autophagic induction. The resulting autophagosomes were LC3II positive. However, at the end of this phase, the phospho- AMPK level was reduced, and the phospho-mTOR level became high. The number of autophagosomes got reduced, but they became larger in size, indicating their fusion with lysosomes. All these suggest the reversal of nutrient-depleted situations; thus, this phase is considered beneficial. On the other hand, the second phase ended with apoptosis. Similar to the first phase, the second phase commenced with high phospho-AMPK and low phospho-mTOR. While the autophagosome population increased, most of them failed to fuse with lysosomes due to CSE-mediated microtubule disruption, which subsequently triggered apoptosis. Thus, instead of reverting nutrient deficiency, it caused cellular death, and therefore, the second phase is cytotoxic. Thus, the current study provides insight into how CSE-induced autophagy, while not initially detrimental, may lead to apoptotic cell death.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is associated with chronic inflammation of the airways [1], wherein an imbalance of proteostasis plays a crucial role [2-4]. The cell needs to maintain a healthy proteome to preserve cell function and respond to a changing environment. The healthy proteome is regulated by a proteostasis network consisting of molecular chaperones and protein degradation machinery that prevent the toxicity associated with the protein misfolding and accumulation of toxic aggregate [5]. Thus, proteostasis refers to the biological mechanism of cells that maintains the biogenesis, processing, and degradation of proteins. So, cellular degradation machinery comprising autophagy and proteasome system constitutes an important component in proteostasis [6]. In a cell, 80 to 90% of the short-lived, damaged, and misfolded proteins are degraded through proteasome, and autophagy is responsible for eliminating the protein aggregates, dysfunctional organelles (mitochondria, peroxisomes, ribosomes) and plays a crucial role in coping up with various cellular stresses including nutrient deprivation, hypoxia, oxidative stress. Ubiquitination is utilized as a degradation signal for both processes [6-8]. Cigarette smoke (CS) causes proteostasis imbalance [9], that results in the accumulation of ubiquitinated proteins in COPD patients [10]. It has also been known that many chronic inflammatory diseases are associated with autophagy impairment, as autophagy has a vital role in the induction and modulation of inflammatory reactions [11-13]. CS causes defects in autophagy, and a growing number of observations suggest that CS-induced defect in autophagy causes the formation of aggresome bodies containing polyubiquitinated protein that induces inflammation, resulting in the development of COPD [10,14,15]. However, the detailed mechanism is largely unknown [16]. Again, increased autophagy and accumulation of autophagosomes in the lung tissue of COPD patients have also been well documented. However whether this increased autophagy functions as homeostatic or plays a maladaptive role in COPD is unclear [17]. Increased autophagosomes can be formed either by increased autophagy induction or by a decrease in the

clearance of autophagosomes [18], or both. We hypothesize that CS causes impairment in the fusion between autophagosomes and lysosomes and leads to the accumulation of autophagosomes. This accumulation, in turn, contributes to cellular cytotoxicity. Towards a better understanding, the current study examined the kinetics of CS extract (CSE)-induced autophagy in alveolar epithelial A549 cells and showed that CSE-induced autophagy is biphasic. It acts in both beneficial and detrimental manner; the first phase is beneficial, and the second is harmful to the cells. Consistent with our hypothesis, this study documented that autophagosomes failed to fuse with the lysosomes in the second phase. Thus, it resulted in hindering autophagic clearance and caused the accumulation of autophagosomes and cellular death.

MATERIALS AND METHODS

Preparation of cigarette smoke extracts (CSE)

CSE was prepared using a commercially available filter-tipped cigarette with a tar content of 15 mg and nicotine content of 1 mg manufactured by the Indian Tobacco Company Limited (ITC Ltd.) as described previously [19,20]. The smoke from a cigarette was bubbled through 1 ml of 50 mM PBS, pH 7.4, till the cigarette was consumed completely. The pH of this aqueous CSE was adjusted to 7.4 using NaOH and was filter-sterilized by passing the extract through a 0.2 µm syringe filter. Different batches of CSE preparations were normalized by measuring their absorbance at 270 nm. The OD270 of 0.6 was considered as 100% CSE. This 100% stock solution was diluted to 3% as per the requirement of the experiment.

Cell culture, CSE treatment, and transfection

Alveolar epithelial A549 cells were used for in vitro experiments wherein Ham's F12-nutrient mixture was used for culturing the cells. Cells were treated with different concentrations of CSE for different periods as required. Transient transfections were performed using PolyFect reagent (Qiagen) with the pBABE-puro-mCherry-eGFP-LC3B plasmid vector (Addgene; Catalog number:22418)

Western Blotting

Western blotting was performed according to the standard procedure with a loading of 30 µg protein for each sample. Primary antibodies were against p-mTOR (Cell Signaling Technology 5536S), p- AMPK (Abcam, ab195946), LC3 B(Cell Signaling Technology, 2775s), p62 (Bio Bharati, BB- AB0130), p4EBP1 (Cell Signaling Technology, 28555s), Caspase 3 (Santa Cruz sc:222) and tubulin (Santa Cruz sc-23948).

Immunofluorescence

Cells were grown on a sterile coverslip on a 35 mm plate at 37^{0}C overnight. The next day, the media was discarded and washed with PBS two times. Fresh media containing 3% CSE was added. After CSE treatment for the desired time, cells were fixed with 4% formaldehyde for 10 mins and then permeabilized by 0.1% triton X 100 for 10 mins at 4^{0}C. Then the cells were blocked with 5% FBS and the primary antibody was added at 1:200 dilution and kept in the humidified chamber overnight. After washing with PBST the next day, the secondary antibody was added at 1:300 dilution and kept at room temperature for 2 h. This was counterstained with DAPI and the image was viewed under the confocal microscope. (Olympus, Model IX81)

FACS analysis

Following CSE treatment, cells were treated with 1µg/ml acridine orange dye for 15 mins. Then the media was discarded, and cells were washed with PBS and harvested after trypsinization. Red fluorescence was measured by FACS (BD FACSVerse, USA).

RESULTS

Treatment of A549 cells with CSE causes induction of biphasic autophagy

To understand the role of autophagy in cigarette smoke-induced cellular stress, time kinetics of CSE- induced autophagy was performed in A549 cells. To monitor autophagy, CSE-treated A549 cells were stained with acridine orange dye. Acridine orange accumulates in acidic vesicles, the formation of which is characteristic of autophagy induction [21]. In acidic vesicles, it becomes protonated and emits red fluorescence [22]. Based on this property, we have analyzed the percentage of cells undergoing autophagy in different cell populations treated with CSE for different periods by FACS (Figure 1A). The results showed a gradual increase in the percentage of autophagic cells from 0 h to 2 h post-CSE-treatment followed by a drop between 3 h and 4 h, and then again a rise at 5 h post-exposure (Figure 1A). Thus, this result showed that there is a biphasic induction of autophagy. For further analysis, immuno-fluorescence was done for LC3B, a well- known autophagosome marker, in CSE-treated

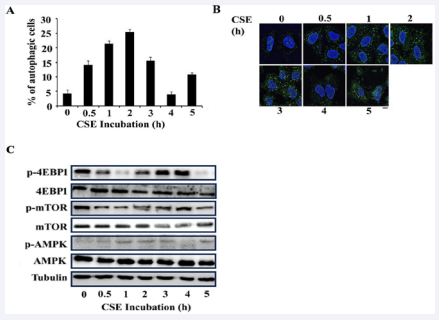


Figure 1 CSE treatment causes induction of autophagy in two phases. A. Autophagy detection by acridine orange dye. A549 cells were treated with CSE for different periods (as indicated), incubated with acridine orange dye (1µg/ml), and analyzed by FACS. B. Autophagy detection by LC3B protein. A549 cells were treated with CSE for different periods (as indicated) and fixed. Thereafter, cells were immunostained with anti-LC3B antibody and viewed under a confocal microscope. C. Coordinated activation/inhibition of AMPK and mTOR. Cell lysates were prepared from CSE-treated and -untreated cells and analyzed for different proteins (as indicated in the figure) by western blotting. Tubulin was used as a loading control. All the experiments were done at least three times. Scale bar: 5 µm

cells, as indicated in (Figure 1B). During autophagy induction, the cytosolic form of microtubule-associated protein 1A/1B light chain 3 (LC3I) conjugates to phosphatidyl-ethanolamine to form LC3-phosphatidyl-ethanolamine conjugate (LC3II), and thereafter, it is recruited to autophagosome membrane [23]. Consistent with the previous observations with acridine orange dye, the result of L3CB staining also showed a biphasic nature of CS-induced autophagy (Figure 1B). The result showed that up to 2 h, the number of autophagic puncta gradually increased, and thereafter, the number decreased gradually with time. At four h, the number of puncta was reduced maximally. However, the number of autophagic puncta again increased markedly at 5 h. Based on the congruence of the results of the above two experiments, it can be said that CSE exposure may induce autophagy in two phases.

As further confirmation of this biphasic autophagy, the activation status of two kinases, AMPK and mTOR, was monitored during the above-mentioned post-CSE treatment period as their coordinated activation/inhibition regulates the induction of autophagy [24]. Activation of mTOR, indicated by p-mTOR, inhibits autophagy, while activation of AMPK, indicated by p-AMPK, induces autophagy. Besides p-mTOR and p-AMPK, levels of p-4EBP1 were also examined in cell lysates as 4EBPI is a direct substrate of activated mTOR kinase (Figure 1C). Consistent with our previous observation, during initial autophagy induction (0.5 h to 2 h), while p-mTOR level decreased, there was an increase in the level of p-AMPK. Thereafter, between 2 h and 4 h, p-mTOR started to increase and reached the maximum at 4 h, while there was a decrease in the level of p- AMPK (Figure 1C). Change in p-4EBP1 levels was closely mirrored. p-mTOR pattern, thus confirming the mTOR activation/deactivation pattern with time. At 5 h, again, the level of p-mTOR decreased with a concomitant increase in p-AMPK level (Figure 1C). All of the above results indicate that biphasic autophagy takes place during prolonged CSE treatment, with an initial phase peaking ~2 h and a second phase commencing ~5 h.

Autophagosomes do not fuse with lysosomes during the second phase of autophagy induction

A change in the size of the autophagic puncta was observed with time (Figure 1B). Initially, their numbers were high, but the size was small. At 2 h, both large and small size puncta were observed. By 3 h, a majority of the puncta were significantly larger in size. At 5 h, again, the number increased, but the size was small. We hypothesized that the reduced number but larger size of autophagosomes during the first phase resulted from their fusion with lysosomes. This may have resulted in the clearance of damaged cellular components, whose recycling regenerated nutrients. However, at 5 hr post-CSE treatment, the observed increase in the number of smaller compartments most likely resulted from impaired fusion of autophagosomes with lysosomes, resulting in a lack of autophagic degradation. To test this hypothesis, the fusion of autophagosomes with lysosomes was assessed by monitoring the flux of autophagic markers LC3II and p62. When there is no defect in autophagosome-lysosome fusion, LC3II levels rise, and p62 decreases, but both accumulate when fusion is impaired **[25]**. Between 0 and 3 h following CSE exposure, there was a marked increase in LC3II levels and significant degradation of p62, with the maximum degradation occurring between 1 and 2 h (Figure 2A). This indicated that autophagy was induced, and there was no defect in autophagosome-lysosome fusion. But at 5h, elevated levels of both LC3II and p62 compared to 4 h were found. While increased, LC3 indicated induction of autophagosome formation, increased p62 level indicated impaired autophagosome-lysosome fusion. Thus, these results demonstrated the induction of the second autophagic event without autophagic flux.

For further confirmation, autophagosome-lysosome fusion was investigated with a chimeric probe in which LC3 was tandemly tagged with-mCherry and GFP (mCherry-GFP-LC3). When autophagosome-lysosome fusion is not impaired, delivery of this reporter results in lysosomes fluorescing red as the GFP signal is quenched in the acidic environment of the lysosome, while m- Cherry fluorescence remains unaffected. On the other hand, impaired fusion results in mCherry- GFP-LC3 positive compartments having yellow fluorescence due to the stability of both fluorescent proteins. A549 cells, transfected with mCherry-GFP-LC3, were treated with CSE for 3 h and 5 h. In comparison to 3 h, there were far more mCherry-positive compartments in 5 h, indicating an accumulation of autophagosomes at the later time point (Figure 2B). In addition, at 5 h, most of these mCherry-positive compartments were also positive for GFP as these

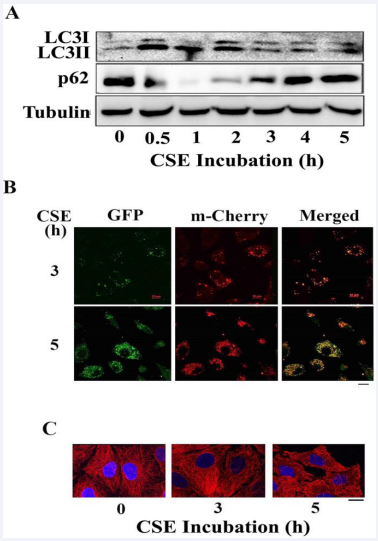


Figure 2 Autophagic flux is blocked in the second phase of autophagy. A. LC3II and p62 proteins profile. Cell lysates were prepared after CSE treatment and were analyzed for LC3II and p62 by western blotting. Tubulin was used as loading control B. Autophagosome-lysosome fusion profile. A549 cells were transfected with mCherry-GFP-LC3 construct. After 24 h of transfection, cells were treated with CSE for 3 h and 5 h. Cells were fixed and visualized by confocal microscopy. Scale bar: 10 µm. C. Microtubule structure profile. CSE-treated (as indicated) A549 cells were fixed, immunostained with anti-beta tubulin antibody, and visualized under a confocal microscope. Scale bar: 10 µm. All the experiments were done at least three times.

appeared yellow in the overlay. In contrast, at 3 h, fewer mCherry-positive compartments appeared yellow, indicating that the GFP signal is quenched. Based on the increase in the number of fluorescent compartments at 5 h, compared to 3 h, and that these compartments exhibit less quenching of the GFP signal, it may be concluded that autophagosome-lysosome fusion is inhibited at the later time point; this resulted in defective autophagic flux.

Movement along microtubules guides autophagosomes toward lysosomes [26,27]. We hypothesized that prolonged CSE treatment cause's depolymerization of microtubules, which is the underlying reason for impaired fusion between autophagosomes and lysosomes. Tos verify this, the microtubule structure was monitored at 0, 3, and 5 h after CSE treatment by performing immunofluorescence with anti-tubulin antibody. A perusal of microtubule structure indicated that while there was very little difference between 0 and 3 h, at 5 h, the microtubules appeared disrupted as their length was considerably shorter (Figure 2C). Thus, at 5 h, the inability of autophagosomes to fuse with lysosomes can be attributed to CS-mediated disruption of microtubule structure.

Apoptosis induction occurs during the second phase of autophagy induction

The existing literature shows that autophagosome accumulation is cytotoxic to cells and can serve as a platform for activating apoptotic or necrotic pathways [28]. Therefore, at 5 h, apoptosis was verified by looking into the presence of cleaved caspase 3 in western blot, and also immunofluorescence was performed for annexin V. Consistent with the expectation, Figure 4 showed the presence of cleaved caspase 3 only at 5 h and also the cells that were treated with CSE for 5 h exhibited annexin V staining. Taken together, the results showed that in the second phase of autophagy, autophagosomes do not get fused with lysosomes and thus accumulate. This, in turn, induces apoptosis and cellular death (Figure 3A,3B).

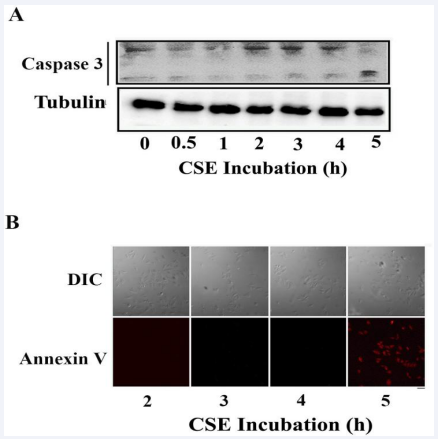
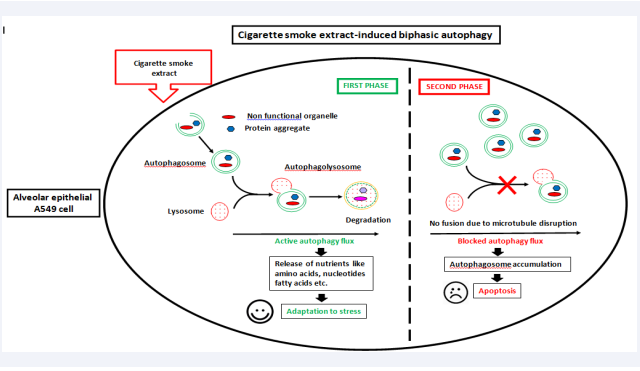


Figure 3 Apoptosis occurs during the second phase of autophagy. A. Detection of cleaved caspase 3. Cell lysates were prepared after CSE treatment for different periods as indicated and were analyzed for cleaved caspase 3 by western blotting. Tubulin was used as a loading control. Experiments were performed three times. B. Annexin V staining profile. A549 cells were treated with CSE for different periods as indicated and fixed. Thereafter, cells were stained with annexin V and visualized under a fluorescence microscope. Experiments were performed three times. Scale bar: 50 µm.



DISCUSSION

Many of the chronic inflammatory diseases are associated with autophagy dysfunction. COPD is a chronic inflammatory disease, and it has also been reported that CS-induced defects in autophagy contribute toward accelerating COPD and lung aging **[11,10].** It has also been reported that there is a defect in autophagy in the alveolar macrophages of smokers. Smoker alveolar macrophage accumulates increased autophagosomes and p62, which is a marker of autophagy flux **[29].** On the contrary, reports also showed that autophagy constitutes a stress adaptation that prevents cell death **[30,31]**. The present study documented that CS-induced autophagy is biphasic. The first phase constitutes the adaptation phase, as fusion occurs between autophagosomes and lysosomes, and mTOR becomes reactive. Nutrient availability controls the energy consumption and amount of ATP production. AMPK protein plays a crucial role in the case of nutrient starvation conditions. During the nutrient-deprived condition, it becomes active and regulates certain enzymes that control growth to reduce ATP consumption [32]. At the initial time points of CSE treatment, the results showed activation of AMPK with a concomitant inactivation of mTOR. Since it is known that nutrient deprivation causes activation of AMPK with a concomitant inactivation of mTOR, it can be said that treatment of cells with CSE causes nutrient deprivation, and thus autophagy is induced. Congruent with this, in a previous report, Das et al. showed that CSE causes downregulation of branch chain amino acid transporter (LAT1) and causes nutrient limiting conditions [33]. To maintain metabolic homeostasis and viability, cells respond to changes in nutrient availability. The upregulation of the autophagy process plays a vital role in nutrient deprivation conditions [34]. Therefore, CSE-induced autophagy initiation can be attributed to nutrient starvation. Nutrient-rich conditions generally activate mTOR protein, and its function is inhibited during starvation [35]. As mTOR was found inactive during the initial time point, it can again support the view that cigarette smoke causes nutrient deprivation that induces autophagy. Congruent with this, in a previous report, Das et al. showed that CSE causes downregulation of branch chain amino acid transporter (LAT1) and causes nutrient limiting conditions

[33]. The results of the present study showed the reactivation of mTOR and deactivation of AMPK at 3h, and this activation of mTOR and deactivation of AMPK continued till 4 h. Subsequently, the number of autophagosomes decreased at 3 h and reached the minimum at 4 h. Consistent with this degradation of p62 is reduced at 3 h compared to 2 h and reached the minimum at 4 h. The level of LC3II was also reduced markedly at 4 h. Taken together, it can be said that, during the first phase of autophagy, autophagic flux generates enough nutrients to reactivate mTOR and attenuation of autophagy At 5 hr, p-mTOR level is reduced along with the activation of AMPK. Subsequently, LC3II protein is again increased. All these results indicate that again cells are facing a nutritional deprived condition. It may be that autophagy (first phase) generated nutrients are exhausted during this period, and therefore, cells initiate the induction of autophagy again. However, at 5 h, even though autophagy was induced, cells failed to generate desired nutrients due to the failure in the fusion of autophagosomes and lysosomes. Finally, cells died through apoptosis. The failure in the fusion between autophagosomes and lysosomes can be attributed to the disrupted microtubule structure caused by prolonged exposure to CSE. Thus, it can be said that the second phase of autophagy is cytotoxic, and the increased autophagy or enhanced number of autophagosomes is harmful in this case. Therefore, chronic CS exposure may activate apoptosis by increased autophagosome formation or increased autophagy induction. Thus, the result of this study explains why enhanced autophagy in COPD is detrimental.

In conclusion, cigarette smoke-induced autophagy is biphasic. The primary phase of autophagy induction is beneficial for cellular survival, but the second phase is cytotoxic and associated with cell death. Inhibiting autophagosome formation may be beneficial when there is a defect in the fusion between autophagosome and lysosome.

AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTION

AM performed the experiments, analyzed the results, and participated in manuscript writing. AKS and SS conceived the idea, designed the experiments, analyzed the results, and wrote the manuscript.

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