Resveratrol Protects Against Nicotine-Induced Apoptosis by Enhancing Autophagy in BEAS-2B Lung Epithelial Cells

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Abstract

Background: Nicotine (Nic), the major component of tobacco products, can induce apoptosis in lung epithelial cells, and the resulting damage contributes to chronic obstructive pulmonary disease. Apoptosis is closely related to autophagy. Resveratrol (Res) can induce autophagy and inhibit apoptosis. Therefore, the present study investigated whether Nic induces apoptosis of lung epithelial cells by regulating autophagy and the effect of Res on apoptosis of Nic-exposed lung epithelial cells. Methods: The BEAS-2B lung epithelial cell line was used to study the harmful effects of Nic and the potential benefits of Res as well as the underlying mechanisms. Viability and apoptosis were examined using the Cell Counting Kit-8 and annexin V-propidium iodide staining, respectively. The expression of levels of apoptosis-related proteins, autophagy-related proteins, and members of the PI3K/Akt/mTOR pathway was measured by western blotting. Autophagic flux was detected via mRFP-GFP-LC3 double-labeled adenovirus transfection and transmission electron microscopy. Results: Nic significantly reduce the viability and increased the apoptosis of BEAS-2B cells in a concentration-dependent manner. Nic treatment also increased the numbers of autophagosomes in BEAS-2B cells and upregulated LC3II and p62 expression. Moreover, Res at concentration of 2, 10, and 50 μM protected BEAS-2B cells from Nic apoptosis, and the expression of LC3II increased further and p62 decreased in Res pretreatment group. Apart from this, Res reduced Akt and mTOR phosphorylation. Subsequently, upon inhibiting PI3K phosphorylation by PI3K inhibitors, BEAS-2B cell autophagy induced by Res was obviously abolished. Conclusions: Nic-induced BEAS-2B cell apoptosis by inhibiting the late-stage autophagic flux, but Res could protect BEAS-2B cells from the detrimental effects of nicotine by enhancing autophagy via the PI3K/Akt/mTOR pathway. These results will provide an experimental basis for the prevention and treatment of COPD.

Keywords
BEAS-2B cells, nicotine, resveratrol, autophagy, apoptosis

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Introduction

Repeated exposure to harmful environmental stimuli can generate chronic airway inflammation, and as this inflammation leads to progressive and irreversible airflow limitation and lung parenchymal damage, chronic obstructive pulmonary disease (COPD) develops. Chronic exposure to tobacco smoke is the top risk factor for COPD. Despite major efforts to educate the public on the dangers of smoking, there continue to be large numbers of smokers (including those who smoke traditional cigarettes and those who use e-cigarettes) in China, and many nonsmokers are exposed to second-hand smoke. Cessation is difficult for long-term smokers due to the addictive component of tobacco products, nicotine (Nic). Research has shown that upon cell entry through nicotinic acetylcholine receptors (nAChRs), Nic can induce apoptosis in a variety of cell types, including lung epithelial cells. Although lung epithelial cells are not classic phagocytes, they are the first line of defense against invading microorganisms and are involved in bacterial uptake and clearance. Moreover, a role for lung epithelial cell apoptosis has been demonstrated in the development and progression of COPD. Therefore, inhibiting lung...
epithelial cell apoptosis may be a potential therapeutic strategy for the prevention and treatment of COPD.

Resveratrol (Res) is a polyphenolic compound that exists naturally in a variety of plants including peanuts, cranberries, and mulberries. Multiple studies have shown that Res has a wide range of pharmacological activities, from anti-inflammatory and antioxidant activities and antitumor functions. The induction of basal autophagy is a potential mechanism for the beneficial effects of Res. In addition, a growing body of evidence suggests that enhancement of autophagy can lead to inhibition of cell apoptosis. Autophagy is an evolutionarily conserved process that is closely linked to several major classes of human disease, including neurodegeneration, cancer, and infectious diseases. Although numerous studies have shown that Res can protect a variety of cell types from oxidative stress or apoptosis as an autophagy inducer, its protective effect on lung epithelial cells as an autophagy inducer remains to be demonstrated.

To investigate whether Res can protect lung epithelial cells from the damaging effects of Nic, we studied the effects of Res in the human lung epithelial cell line BEAS-2B after treatment with Nic and explored the underlying mechanism of action, to gain novel insights into the development of future strategies for the prevention and treatment of COPD.

Materials and Methods

Materials

Nic was purchased from Desite (DY0032, ≥98%). Bafilomycin A1 (Baf A1), Res (HY-16561, 99.9%), and LY294002 (LY) were purchased from MedChemExpress. Antibodies to B-cell lymphoma 2 (Bcl-2), Bcl-2-like protein 4 (Bax), p62, LC3II, Akt, phosphorylated (p)-Akt, mammalian target of rapamycin (mTOR), and p-mTOR were obtained from Cell Signaling Technology, and antibodies to caspase-3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Signalway Antibody. All other reagents were of analytical grade and purity and were manufactured in China.

Cell Culture

The human bronchial epithelial cell line BEAS-2B was kindly provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco) supplemented with 10% fetal bovine serum (PAN, GER) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Cell Apoptosis Assay

After BEAS-2B cells were exposed to different conditions in 6-well plates, the apoptotic rate was analyzed using an Annexin V-PI Apoptosis Detection Kit (Beyotime) via flow cytometry. Briefly, BEAS-2B cells were collected and washed 3 times with phosphate-buffered saline (PBS), resuspended in 500 μL 1X binding buffer, and stained with 5 μL annexin V-fluorescein isothiocyanate and 5 μL propidium iodide (PI) for 15 min at room temperature in darkness. The samples were then analyzed by flow cytometry using a BD Influx Cell Sorter (BD Biosciences).

Cell Viability Assay

Cell viability was monitored using the Cell Counting Kit-8 (CCK-8, Beyotime) according to the manufacturer’s instructions. Briefly, 100 μL fresh medium containing 10 μL CCK-8 solution was added to each well of cells at the end of the treatment period. After incubation for 2 h at 37 °C, the absorbance of the solution at 450 nm was recorded by a microplate reader.

Adenovirus Infection

To analyze autophagic flux, BEAS-2B cells were transfected with mRFP-GFP-LC3 adenoviral vectors (HanBio, China) at a multiplicity of infection of 10 for 48 h before treatment with different experimental conditions. Autophagy was visualized under a confocal microscope (Nikon, Japan).

Transmission Electron Microscopy

After treatment with the different experimental conditions, BEAS-2B cells were washed with PBS 3 times and transferred to 1.5-mL centrifuge tubes for centrifugation. After removal of the supernatant, the pellet was fixed in 2.5% glutaraldehyde at 4 °C for 24 h. The pellets of fixed cells were then dehydrated, embedded in paraffin, sectioned into ultrathin slices, and stained with uranyl acetate and lead nitrate. Finally, the ultrathin sections of cells were observed by transmission electron microscopy (TEM) (Hi-7100; Hitachi).

Western Blot Analysis

For western blot analysis of protein expression, BEAS-2B cells were collected using a cell scraper, placed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors, and then centrifuged at 12 000 g for 20 min. The protein concentration in the obtained supernatant was determined using a BCA Kit (Beyotime). Proteins were separated on 10% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk at room temperature for 2 h, washed with Tris-buffered saline containing Tween 20 (TBST), and then incubated with primary antibodies at 4 °C overnight. After 3 additional washes with TBST, incubation with secondary antibodies (horseradish peroxidase-coupled goat antirabbit antibodies) was conducted for 2 h at room temperature.
temperature. The protein bands were detected using an enhanced chemiluminescence detection kit (Beyotime).

Statistical Analysis
The dates are expressed as mean ± standard deviation (SD). SPSS 22.0 software (SPSS Inc) was used for all statistical analyses, and significant differences among the treatment groups were detected by one-way analysis of variance, with P < .05 indicating statistical significance. Each experiment was repeated 3 times.

Results

Nic-Induced Apoptosis of BEAS-2B Cells in a Concentration-Dependent Manner

To establish suitable experimental conditions for our BEAS-2B cell model of Nic-induced apoptosis, we examined the expression of apoptosis-related proteins in BEAS-2B cells after exposure to different concentrations of Nic by western blotting. We found that the expression levels of Bax and cleaved Caspase-3 in BEAS-2B cells increased with increasing Nic concentration, while Bcl-2 expression decreased with increasing Nic concentration (Figure 1A and B). Flow cytometric analysis showed that the apoptosis rate among BEAS-2B cells increased with exposure of BEAS-2B cells to increasing Nic concentration (Figure 1C and D). In addition, CCK-8 assay results showed that BEAS-2B cell viability decreased with the use of increasing Nic concentrations (Figure 1E). Based on these results, we found that Nic can induce BEAS-2B cell apoptosis in 1, 2, and 4 mM. In addition, the cells showed better activity at 2 mM. Therefore, BEAS-2B cells treated with 2 mM Nic were employed as a Nic-induced apoptosis model in subsequent experiments.

Nic Inhibited Late-Stage Autophagy in BEAS-2B Cells

Increased autophagy is considered a protective mechanism against apoptosis. Therefore, we hypothesized that the mechanism of Nic-induced apoptosis may involve inhibition of autophagy. To test this hypothesis, we examined the expression levels of autophagy markers, including LC3II and p62, in BEAS-2B cells following treatment with Nic. BEAS-2B cells treated with Nic showed increased expression of both LC3II and p62 compared with control cells (Figure 2A and B). The increase in LC3II expression may be due to the activation of autophagy flow or the inhibition of autophagy degradation.

To further verify the effect of Nic on autophagic flux, BEAS-2B cells were pretreated with Baf A1, a well-known autophagy inhibitor, before treatment with Nic. The results showed that LC3II and p62 expression in cells treated with only Baf A1 was significantly increased compared with that in control cells. However, LC3II expression in cells pretreated with BAF A1 and then exposed to Nic did not differ significantly from that in cells treated with only Baf A1 (Figure 2A and B). We also transfected BEAS-2B cells with mRFP-GFP-LC3 double-labeled adenovirus to observe changes in autophagy flow in response to treatment with NIC.

In this experiment, the numbers of autophagosomes (yellow spots in the assay) were greater in the Nic-treated samples than in the control samples (Figure 2C and D). We also observed greater numbers of autophagosomes in Nic-treated cells versus control cells on TEM (Figure 2E). Taken together, these results indicate that Nic treatment inhibited the late-stage autophagic flux in BEAS-2B cells.

Res Attenuated Nic-Induced Apoptosis in BEAS-2B Cells

To investigate whether Res affects Nic-induced apoptosis in BEAS-2B cells, we subjected BEAS-2B cells to pretreatment with different concentrations of Res before exposure to Nic. Western blot analysis of LC3II and p62 protein expression, as markers of autophagy, showed that pretreatment with Res resulted in increased expression of LC3II and decreased expression of p62 after Nic exposure (Figure 3A and B). Western blot analysis of Bax, Bcl-2, and cleaved Caspase-3 expression levels indicated that Bax and cleaved Caspase-3 expression decreased with increasing Res concentration, whereas Bcl-2 expression increased with increasing Res concentration compared with levels in control cells (Figure 3A and B). Flow cytometric analysis revealed that the apoptosis rates among cells in the Res pretreatment groups were lower than that in the Nic-treated positive control group and decreased with increasing Res concentration (Figure 3C and D). Moreover, compared with that in the Nic-treated positive control group, the viability of BEAS-2B cells pretreated with Res increased with increasing Res concentration (Figure 3E). Together these experimental data indicate that Res protected BEAS-2B cells from the detrimental effects of Nic by enhancing autophagy.

Res-Induced Autophagy via PI3K/Akt/mTOR Pathway

Previous studies have established that the PI3K/Akt/mTOR pathway is the main regulatory pathway of autophagy. To further elucidate the potential mechanism of Res-mediated autophagy in BEAS-2B cells, the expression profiles of 2 critical molecules, mTOR and Akt, with Res and Nic treatment were examined by western blot assay. This analysis showed that Res pretreatment significantly decreased the expression levels of p-Akt, p-mTOR, and p62, while increasing the expression of LC3II in Nic-exposed cells (Figure 4). To further identify the roles of Akt and mTOR in the Res-mediated PI3K/Akt/mTOR signaling in BEAS-2B cells, we treated the cells with LY294002 (LY), an established inhibitor of PI3K. Pretreatment with Res and LY further decreased the expression levels of p-Akt, p-mTOR, and p62, while further increasing the expression of LC3II compared with the levels observed after only Res pretreatment (Figure 4). These data together imply
that the mechanism of Res-induced autophagy may involve the PI3K/Akt/mTOR pathway.

**Discussion**

COPD is currently the fourth leading cause of mortality and a major health burden worldwide. As the global population has continued to age, the incidence of COPD has also increased in recent years. Therefore, the need to identify more potential therapeutic targets related to protecting against the harmful effects of cigarette smoking is urgent, as such knowledge will provide new avenues for the prevention and treatment of COPD.

Previous studies have shown that a low concentration of Nic (100 μM) does not induce apoptosis in BEAS-2B cells, but does increase the intracellular oxidative stress index significantly. Oxidative stress is well-established as a common cause of apoptosis. In the present study, Nic at higher concentrations of 1, 2, and 4 mM did increase apoptosis and decrease cell activity among BEAS-2B cells. Therefore, Nic at both low and high concentrations can have detrimental effects on BEAS-2B cells. The use of e-cigarettes as a replacement for traditional cigarettes has been proposed as a safe and effective strategy for the...
cessation of traditional cigarette smoking, but the latest research revealed that e-cigarettes also can cause various health problems.28–30 Furthermore, the concentration of Nic found in e-liquid varies, even reaching as high as 52 mg/mL.31 Consequently, e-cigarettes are not a safe and reliable substitute for traditional cigarettes, and safer and more reliable tobacco substitutes need to be developed to help smokers quit smoking.

Autophagy is an evolutionarily conserved lysosomal degradation pathway.32 Under various physiological and pathological conditions, intracellular substances are isolated by double-membrane autophagy and then transported to lysosomes for degradation and circulation. The expression levels of LC3II and p62 proteins are often used to measure the status of autophagic flux.33 The experiments in the present study showed that LC3II and p62 expression levels were significantly increased in BEAS-2B cells exposed to Nic. The increase in LC3II expression could be due to the activation of autophagic flux or to the inhibition of autophagy degradation. Therefore, we transfected BEAS-2B cells with a double-labeled adenovirus that allowed us to observe the changes in autophagic flow with Nic treatment. The observed patterns of fluorescence demonstrated that the autophagic flux was inhibited in Nic-treated BEAS-2B cells. In addition, fewer autolysosomes were observed after Nic treatment by TEM. These results suggest that Nic can inhibit the late-stage autophagic flux in BEAS-2B cells.

A complex interplay is known to exist between autophagy and apoptosis, with research showing that autophagy is able to both promote and suppress apoptosis.34 Previous studies have shown that Nic also plays different roles in different diseases via the regulation of autophagy. In an animal model of ulcerative colitis, Nic was found to promote autophagy and

Figure 2. Nic inhibited late-stage autophagy in BEAS-2B cells. BEAS-2B cells were pretreated with Baf A1 (10 nM) for 24 h and then treated with Nic (2 mM) for 6 h. (A) Western blot analysis of the expression levels of autophagy-related proteins (LC3II and p62) in each group. (B) Quantification of relative expression of apoptosis-related proteins (LC3II and p62) in each group. (C) The fluorescence signal of mRFP-GFP-LC3 in transfected BEAS-2B cells after different treatments, as detected by confocal microscopy (scale bar, 10 μm). (D) Number of autophagosomes and autolysosomes counted in BEAS-2B cells after different treatments. (E) Representative TEM images of BEAS-2B cells; arrows indicate autophagosomes (scale bar, 1 μm). All data are mean ± SD. *P < .05, **P < .01 versus control group.
relieve the symptoms of ulcerative colitis, whereas in vitro studies in Leydig cells demonstrated that Nic can inhibit autophagy and induce apoptosis. The data obtained in the present study indicate that Nic-induced apoptosis in BEAS-2B cells by inhibiting late-stage autophagy. These discrepancies in the activities of Nic may be related to differences in treatment factors, cell types, and models. Thus, more research is needed to characterize the complex relationship between apoptosis and autophagy.

Increasing evidence supports the possibility that enhancing autophagy can alleviate symptoms or delay the progression of disease. For example, enhanced endothelial cell autophagy was shown to protect the glomerulus from oxidative stress and maintain the integrity of glomerular capillaries, thus inhibiting the severity and progression of glomerular diseases. In a model of nervous system injury, enhanced autophagy could protect neurons from injury. Notably, autophagy activation was able to alleviate excessive cytokine production and lung injury in septicemia animal models. Res is a polyphenol compound that is known to induce basal autophagy and to have great potential in the treatment of respiratory diseases. Our experiments demonstrated that Res pretreatment protected BEAS-2B cells from the detrimental effects of Nic by enhancing autophagy in a concentration-dependent manner.

Figure 3. Protective effect of Res may be mediated by autophagy upregulation. BEAS-2B cells were pretreated with Res (2, 10, or 50 μM) for 24 h before treatment with Nic (2 mM) for 6 h. (A) Western blot analysis of apoptosis-related proteins (Bax, Bel-2, and cleaved Caspase-3) and autophagy-related proteins (LC3II and p62) in each group. (B) Relative protein expression of apoptosis-related proteins (Bax, Bel-2, and cleaved Caspase-3) and autophagy-related proteins (LC3II and p62) in each group. (C) Flow cytometric analysis of specific cell populations (live, early apoptosis, and late apoptosis) among BEAS-2B cells. (D) Percentages of apoptotic cells in each group. (E) Viability of BEAS-2B cells in each group. All data are mean ± SD. *P < .05 versus control group; #P < .05 versus Nic-only group.
Subsequently, we analyzed the molecular mechanism of Res-induced autophagy. Activation of the PI3K/Akt/mTOR pathway is known to inhibit autophagy, and our experiment demonstrated the involvement of the PI3K/Akt/mTOR pathway in Res-mediated autophagy. Specifically, our results showed that Res activated the PI3K/Akt/mTOR pathway in Nic-treated BEAS-2B cells. Moreover, treatment of the cells with a PI3K inhibitor (LY) further enhanced Res-induced autophagy. Therefore, these results indicate that Res may protect lung epithelial cells from the harmful effects of Nic by inducing autophagy through the PI3K/Akt/mTOR pathway.

In conclusion, Nic-induced apoptosis of BEAS-2B cells by blocking the late-stage autophagic flux. However, Res pretreatment before Nic exposure protected BEAS-2B cells from Nic-induced apoptosis by enhancing autophagy through the PI3K/Akt/mTOR pathway. These findings provide experimental insight for the future development of novel strategies for the prevention and treatment of COPD.

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