Int J Mol Epidemiol Genet 2020;11(3):39-45
www.ijmeg.org /ISSN:1948-1756/IJMEG0120474

Original Article

Chrysophanol protects human bronchial epithelial cells from cigarette smoke extract (CSE)-induced apoptosis

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Received August 17, 2020; Accepted October 16, 2020; Epub December 15, 2020; Published December 30, 2020

Abstract: Objective: Chronic obstructive pulmonary disease (COPD) is a common respiratory disease characterized by the persistent airflow obstruction. Chrysophanol, an anthraquinone derivative isolated from the rhizomes of *Rheum palmatum*, has been reported to be protective for some inflammatory diseases. The present report aimed to dissect its effect on cigarette smoke extract (CSE)-induced apoptosis in 16HBECs, a human bronchial epithelial cell line. Methods: CCK8 cell viability assay was conducted to evaluate the protective effect of chrysophanol on 16HBECs after CSE induction. Western blot analysis, Annexin V/PI staining and TUNEL assay were conducted to test the effect of chrysophanol on 16HBECs apoptosis induced by CSE. Then the western blot assay measured associated molecular pathways to dissect the mechanisms underlying protective effect of chrysophanol on 16HBECs. Results: Chrysophanol protects 16HBECs against CSE-induced apoptosis in a dose dependent manner. Specifically, pre-treatment of 16HBECs with 20 mmol/l of chrysophanol, reduced CSE-induced apoptosis by almost 10%. Mechanistically, chrysophanol manifested high potency to attenuate CSE-induced expression of apoptotic markers, Bax and cleaved caspase 3. In particular, chrysophanol not only represses CSE-induced oxidative stress by inhibiting CYP1A1 expression, but also suppresses CSE-induced ER stress by inhibiting pPERK, ATF4 and ATF6 expression. Conclusion: Chrysophanol showed protective effect on CSE-induced epithelial injuries in cell line 16HBECs. And our data support that chrysophanol could be employed to reduce the toxicity of cigarette smoke in bronchial epithelial cells, which may have the potential to decrease the risk for developing COPD in smoking subjects.

Keywords: Chrysophanol, COPD, CSE, 16HBECs, apoptosis, oxidative stress, ER stress

Introduction

Epidemiological studies have demonstrated that smoking is the one of most critical pathogenic factors for chronic obstructive pulmonary disease (COPD) [1, 2], a chronic inflammatory disorder manifested by the persistent airflow limitation. Cigarette smoke (CS) contains nearly 4,500 chemical compounds abundant of reactive oxygen species (ROS) and free radical organic compounds [3]. It is believed that long-term exposure to cigarette smoke would induce inflammatory responses along with the production of a variety of cytokines, which contribute to the development of COPD. Indeed, among diagnosed COPD patients, 80-90% of whom were smokers or ex-smokers [4]. Accumulated evidence supports that programmed cell death (i.e., extrinsic and intrinsic apoptosis) in bronchial epithelial cells serves as a crucial risk for the induction of emphysema, a condition essentially for COPD pathogenesis [5-7]. Therefore, characterization of compounds with potential to protect epithelial cells from cigarette smoke-induced apoptosis could be a viable approach to prevent and treat COPD in clinical settings.

Chrysophanol (1,8-dihydroxy-3-methylanthracenedione), a 1,8-dihydroxyanthraquinone derivative, is a natural ingredient that can be eas-

ily extracted from a number of Chinese herbs such as *Rheum palmatum* L.*, Polygonum multiflorum*, and *Polygonum cuspidat*um [8]. Previous studies provided feasible evidence that chrysophanol may possess properties against inflammatory responses [9, 10], we thus in the present report assessed its possibility to protect epithelial cells against cigarette smoke extract (CSE)-induced oxidative and ER stress. Our results support that chrysophanol possesses the capability to attenuate CSE-induced epithelial injury and apoptosis, which may have great potential to reduce tobacco toxicity in subjects with smoking addiction.

Materials and methods

Cigarettes, reagents and antibodies

The cigarettes used in this study were provided by the Jinsheng Tobacco Corporate Ltd. (Nanchang, China). One cigarette contained 50 mg of nicotine, which is equivalent to 30 g of tobacco leaf [11]. Chrysophanol (National Institute for Food and Drug Control, Beijing, China) with a purity > 98% was dissolved in phosphate-buffered saline (PBS) to final concentrations of 10 mmol/l, 20 mmol/l, and 40 mmol/l for treatment of human bronchial epithelial cells (16HBECs).

Antibodies against cleaved caspase-3 (Sc-22171), BAX (Sc-6236), phospho-PERK (Sc-32577) and GAPDH (Sc-47724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies targeting ATF-4 (11815S) and ATF-6 (65880S) were obtained from Cell Signaling (Danvers, MA, USA); and antibody for CYP1A1 (13241-1-AP) was ordered from Proteintech Group (Proteintech, Wuhan, China).

Cell culture

The 16HBECs were originally purchased from ATCC, which were maintained in RPMI1640 culture medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The prepared 16HBECs were treated in the presence or absence of CSE with different concentrations of chrysophanol (10 mmol/l, 20 mmol/l, and 40 mmol/l) for 24 h.

CSE preparation

CSE was prepared as described by Su et al. [12] with modifications. Briefly, the mainstream smoke from a cigarette (containing 0.05 g nicotine) was bubbled through 10 ml of RPMI16-40 culture medium and sterilized by filtering through a 0.22 μm filter membrane (Merck Millipore, Shanghai, China). The prepared CSE stock solution was next diluted with serum-free RPMI1640 medium into different concentrations and used for experimental purpose within 30 min.

CCK8 cell viability assay

The Cell Counting Kit-8 (CCK8) method was used to measure the cell viability. The 16HBECs were seeded in 96-well plates at a density of 1×10^5 cells/ml. The cell viability was assessed using the CCK8 reagent (MCE, China) according to the manufacturer's protocol, and the absorbance was read on a microplate reader (Bio-Rad 680; Bio-Rad Laboratories, Inc. Hercules, CA, USA) at 450 nm wavelength (A450).

Annexin V and PI staining

The cells were harvested by digestion with EDTA-free trypsin (Gibco; Thermo Fisher Scientific, Inc. Shanghai, China) and washed twice with PBS. The cells ($1\text{-}5\times10^5$) were then stained with 5 μl of Annexin V-FITC (KenGen Biotech Co., Ltd., Nanjing, China) and 5 μl propidium iodide (PI) sequentially. After 5-15 min incubation, the cells were analyzed by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA) as reported [13].

TUNEL assay

The 16HBECs were seeded in a 12-well plate, and subjected to 24 h of CSE stimulation in the presence or absence of chrysophanol. After washing with PBS for 3 times, the cells were fixed with 4% PFA for 5 min. The cells were next incubated with permeabilization solution on ice for 5 min. Apoptosis was then determined using a TUNEL kit (C1088, Beyotime) as previously reported [14].

Western blot analysis

The cells were homogenized in RIPA lysis buffer. The proteins were subjected to western

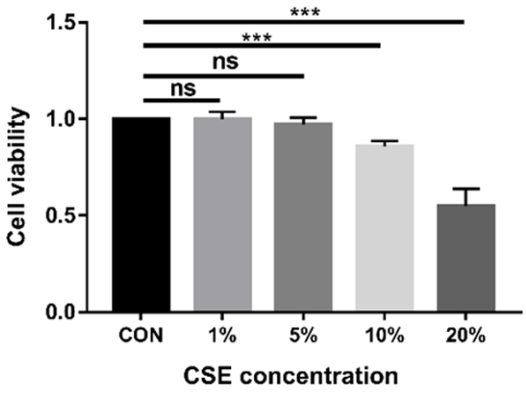

Figure 1. CCK8 assays to optimize the CSE dose in 16HBEC viability. The 16HBECs were treated with indicated dose of CSE for 24 h, and cell viability was measured by a CCK8 assays as described. **, P < 0.01, and ***, P < 0.001.

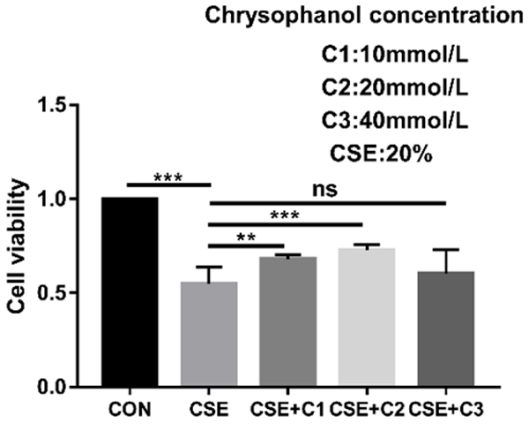

Figure 2. Chrysophanol protects 16HBECs against CSE-induced death. The HBECs were pre-treated with indicated concentrations of chrysophanol prior to CSE stimulation as described, and cell viability was determined by CCK8 assays following 24 h of CSE challenge. **, P < 0.01, and ***, P < 0.001.

blotting with the indicated primary antibodies using established techniques as reported [15]. The relative optical densities of the reactive bands were quantified using the ImageJ software. GAPDH was used for normalization.

Statistical analysis

All experiments were conducted with at least 3 independent replications and the data were presented as the mean ± standard deviation. All data were analyzed using the GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA, USA). The comparison among groups was performed using one-way analysis of variance, and pairwise comparisons between groups were conducted using Student's t test. A value of $P < 0.05$ was considered with statistical significance.

Results

The optimal CSE concentration on 16HBEC viability

Given that bronchial epithelial cells serve as the first barrier to protect airways from microorganism invasion and toxic substances, we thus employed 16HBECs, a human bronchial epithelial cell line, to assess the impact of chrysophanol on CSE-induced bronchial epithelial injury. Cell viability assays were first employed to optimize the appropriate concentration of CSE on 16HBEC viability. To this end, the cells were treated with 1%, 5%, 10%, and 20% CSE for 24 h, respectively. The CCK8 assays revealed a dose dependent reduction of 16HBEC viability (**Figure 1**). In particular, the viability of 16HBECs was only around 50% once 20% CSE was added into the culture. We thus selected 20% CSE as the optimal dose for the following experiments.

Chrysophanol protects 16HBECs against CSE-induced reduction of viability

We next sought to check the impact of chrysophanol on the protection of 16HBEC viability following CSE challenge. For this purpose, 16HBECs were pretreated with chrysophanol (10 mmol/l, 20 mmol/l, and 40 mmol/l) 1 h before 20% CSE stimulation, respectively. Interestingly, chrysophanol protected 16HBECs from CSE-induced death in a dose dependent manner (**Figure 2**). Specifically, significantly higher cell viability was noted in 10 mmol/l chrysophanol pre-treated 16HBECs, but much higher cell viability was observed in 20 mmol/l chrysophanol pre-treated 16HBECs as compared to that of 10 mmol/l chrysophanol pre-treated 16HBECs (68% vs. 72%, P < 0.001). However, chrysophanol concentration higher than 20 mmol/l, such as 40 mmol/l, did not further enhance cell viability.

Chrysophanol attenuates CSE-induced Bax expression in 16HBECs

To address the mechanisms underlying chrysophanol protection of 16HBECs from CSE-

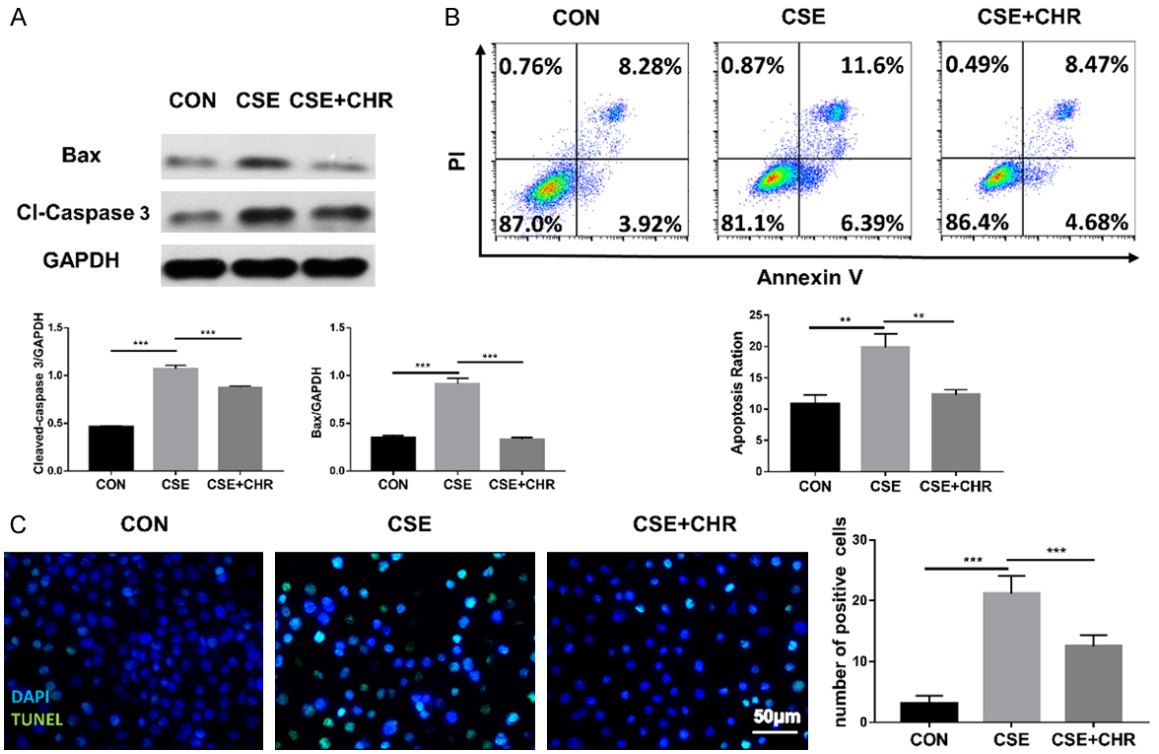

Figure 3. Chrysophanol provides protection for 16HBECs against CSE-induced apoptosis. A. Western blot results for analysis of Bax and cleaved caspase-3 expression. B. Results for Annexin V and PI staining of 16HBECs following CSE or/and chrysophanol + CSE (CHR) challenges. C. TUNEL assay results of above 16HBECs. **, $P < 0.01$, and ***, $P < 0.001$.

induced death, we examined the impact of chrysophanol on the apoptotic pathways. After screening a number of molecules, it was interestingly noted that chrysophanol possesses high potency to attenuate CSE-induced expression of Bax, a pro-apoptosis regulator, in 16HBECs (**Figure 3A**). In line with this observation, 16HBECs pre-treated with chrysophanol displayed significantly lower levels of cleaved Caspase 3 as compared to that of 16HBECs without chrysophanol pre-treatment (**Figure 3B**). Together, these results support that chrysophanol provides protection for 16HBECs against apoptosis following CSE challenge.

To confirm the above results, we first checked apoptosis by Annexin V and propidium iodide (PI) staining in 16HBECs following above stimulations. As expected, CSE induced 16HBECs to undergo extensive apoptosis as compared to that of untreated cells (10% vs. 20%, $P < 0.01$). However, pre-treatment of 16HBECs with chrysophanol significantly attenuated CSE-induced epithelial apoptosis (20% vs. 12%, $P < 0.01$) (**Figure 3B**). To further confirm this result, TUNEL assays were carried out in those treated cells. Consistently, more than 20% of 16HBECs were TUNEL positive, while chrysophanol pre-treatment reduced the TUNEl positive cells by 40% (**Figure 3C**).

Chrysophanol inhibits CSE-induced oxidative stress and ER stress

To dissect the molecular pathways by which chrysophanol attenuates CSE-induced epithelial apoptosis, we first examined the impact of chrysophanol on apoptosis. Interestingly, chrysophanol significantly repressed CSE-induced CYP1A1 expression in 16HBECs (**Figure 4A**, **4B**). Given that CYP1A1 is a critical enzyme involved in the induction of oxidative stress [16], this result suggests that chrysophanol may suppress CSE-induced oxidative stress in 16HBECs. We further checked the effect of chrysophanol on CSE-induced endoplasmic reticulum (ER) stress. Remarkably, chrysophanol also exhibited high potency to attenuate CSE-induced ER stress as evidenced by the reduced expression of p-PERK, ATF4 and ATF6 (**Figure**

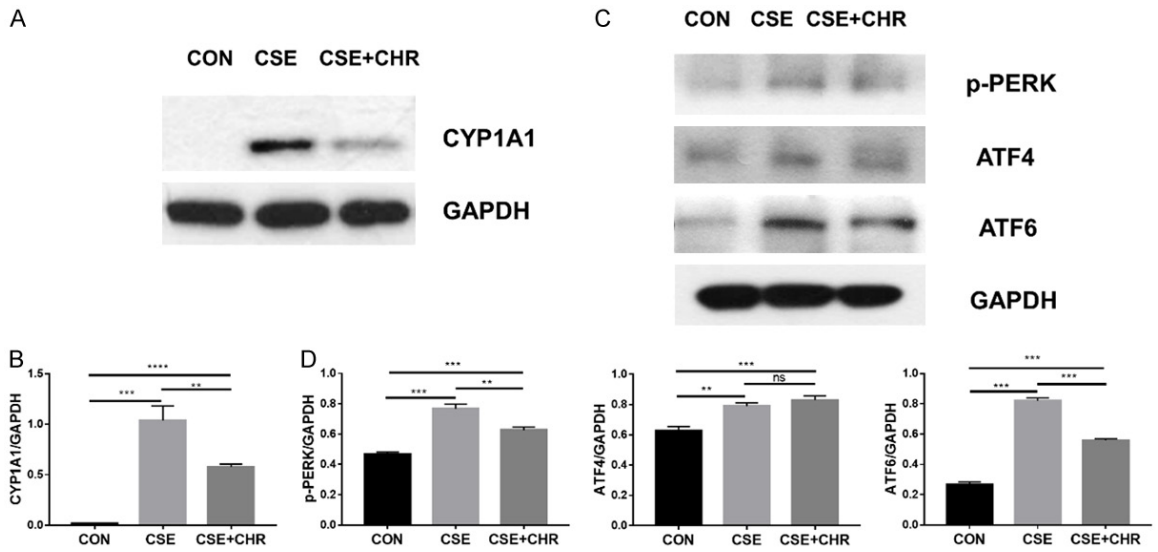

Figure 4. Chrysophanol attenuates CSE-induced oxidative stress and ER stress in 16HBECs. A. Representative Western blot results for analysis of CYP1A1 expression. B. A bar graphic figure showing the Western blot results of CYP1A1 expression derived from 3 independent replications. C. Representative Western blot results for analysis of pPERK, ATF4 and ATF6 expression. D. A bar graphic figure showing the expression levels of p-PERK, ATF4 and ATF6 obtained from 3 replications. **, $P < 0.01$, and ***, $P < 0.001$.

4C, **4D**). Collectively, those data support that chrysophanol protects 16HBECs from CSE-induced apoptosis by suppressing oxidative stress and ER stress.

Discussion

It has been well recognized that lungs derived from COPD patients display increased number of apoptotic epithelial cells and endothelial cells, supporting that apoptosis of the structural cells might be an important mechanism contributing to the development of COPD [17]. Moreover, mounting evidence from emphysema models also suggests that the balance between the apoptosis and regeneration of the structural cells in the lung is disturbed in response to cigarette smoking [18]. Furthermore, oxidative stress and ER stress are also important mechanisms underlying epithelial apoptosis in COPD patients [19].

Accumulated evidence supports that some components of Chinese herbs may manifest protective effect on the initiation and progression in a variety of diseases. Chrysophanol is one of such components extracted from the traditional Chinese medicine, rhubarb. A number of previous studies have suggested that active components extracted from rhubarb such as emodin, aloe-emodin, rhein, and chrysophanol, possess properties against some diseases [20-23]. In particular, chrysophanol has been found to attenuate cerebral ischemia/reperfusion injury in a mouse model [24], and additional studies further revealed that chrysophanol represses LPS-induced activation of RAW 264.7 cells, a macrophage cell line [25]. Given that cigarette smoke serves as an independent risk factor to induce bronchial epithelial cell injuries and dysfunction contributing to the pathogenesis of COPD, we thus in the present report examined the impact of chrysophanol on CSE-induced cell death in 16HBECs, a human bronchial epithelial cell line. We demonstrated experimental evidence that chrysophanol possesses high potency to protect 16HBECs from CSE-induced apoptosis, which involves its properties against the induction of oxidative stress and ER stress. At the same time, the protective effect of chrysophanol on bronchial epithelial cells was also reported in another human pulmonary epithelial BEAS-2B cells through nuclear factor-kappa B (NF-κB) signaling pathway in asthma [26], which is consistent with our conclusion. While in other diseases such as cancer, the chrysophanol shows different effect on carcinoma cell lines, with upregulation of ROS levels and apoptotic ratios. Our results suggest that chrysophanol could be a viable compound to protect

normal bronchial epithelial cells from cigarette smoke-induced dysfunction, thereby reducing the risk for the development of COPD in those smoking subjects.

We tested the effect of CSE on 16HBEC apoptosis in the presence or absence of chrysophanol pre-treatment prior to CSE stimulation. Remarkably, Western blotting analysis revealed that chrysophanol significantly represses CSE-induced expression of CYP1A, a crucial enzyme relevant to oxidative stress caused by excessive ROS production. Other than oxidative stress, we further found that chrysophanol attenuates CSE-induced ER stress as well. Generally, PERK is activated in response to ER stress through a mechanism involving PERK dimerization and autophosphorylation. Our studies revealed that chrysophanol also attenuates the expression of phosphorylated PERK (p-PERK), which plays a critical role in defining cellular survival in response to pathologic insults that disrupt ER proteostasis (i.e., ER stress). In line with this observation, the expression of other ER stress markers, such as ATF4 and ATF6, are also decreased following chrysophanol pre-treatment. Indeed, both flow cytometry analysis and TUNEL assay confirmed that chrysophanol pre-treatment provides protection for 16HBECs against CSE-induced apoptosis.

In conclusion, we examined the effects of chrysophanol on CSE-induced epithelial injuries using 16HBECs, a human bronchial epithelial cell line. We demonstrated evidence that chrysophanol possesses high potency to protect bronchial epithelial cells from CSE-induced apoptosis. Our data support that chrysophanol could be useful to reduce the toxicity of cigarette smoke in bronchial epithelial cells, which may have the potential to reduce the risk for developing COPD in smoking subjects.

Acknowledgements

This work was supported by the Integrated Innovative Team for Major Human Disease Programs of Tongji Medical College, Huazhong University of Science and Technology, and the Innovative Funding for Translational Research from Tongji Hospital.

Disclosure of conflict of interest

None.

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