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Original article

ROS-AKT-mTOR axis mediates autophagy of human umbilical vein endothelial cells induced by cooking oil fumes-derived fine particulate matters in vitro

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ABSTRACT

Cooking oil fumes-derived $PM_{2.5}$ (COFs-derived $PM_{2.5}$) exposure can induce oxidative stress and cytotoxic effects. Here we investigated the role of ROS-AKT-mTOR axis in COFs-derived $PM_{2.5}$-induced autophagy in human umbilical vein endothelial cells (HUVECs). HUVECs were treated with different concentrations of COFs-derived $PM_{2.5}$, together with or without N-acetyl-L-cysteine (NAC, a radical scavenger) or 3-methyladenine (3-MA, an autophagy inhibitor). Cell viability was assessed with MTT assay, and ROS level was measured with DCFH-DA assay after the treatment. Transmission electron microscopy (TEM) was used to evaluate the formation of autophagosomes, while immunofluorescent assay and western blot were used to assess the expression of LC3-I/II and beclin 1. Proteins involved in the PI3K-AKT-mTOR signaling pathway were measured with western blot. The results showed that the treatment of COFs-derived $PM_{2.5}$ dose-dependently reduced the viability of HUVECs and increased the ROS levels in the cells. Both immunofluorescent assay and western blot showed that treatment with COFs-derived $PM_{2.5}$ significantly increased LC3-II and beclin 1 levels, as well as the ratio of LC3-II/LC3-I, which could be rescued by the co-incubation with NAC or 3-MA. TEM also confirmed the increased formation of autophagosomes in the cells treated with COFs-derived $PM_{2.5}$, while co-treatment with NAC evidently decreased autophagosomes formation. In addition, western blot also showed that the phosphorylation of PI3K, AKT, and mTOR all decreased by the treatment of COFs-derived $PM_{2.5}$, which was effectively rescued by the co-treatment with NAC. These findings demonstrate ROS-AKT-mTOR axis plays a critical role in HUVECs autophagy induced by COFs-derived $PM_{2.5}$.

1. Introduction

Cooking oil fumes-derived $PM_{2.5}$ (COFs-derived $PM_{2.5}$) is an important source of indoor air pollution, especially in East Asian countries such as China. In contrast to ambient $PM_{2.5}$, COFs-derived $PM_{2.5}$ contains more noxious components, such as polycyclic aromatic hydrocarbons, and could persistently exist in the microenvironment at relatively high concentration [1]. Although the China Food-Based Dietary Guidelines (FBDGs, 2008) issued by the Ministry of Health recommended that the daily intake of fats and oils should be 25–30 g per reference man [2], the actual consumption in China is much higher [3], thus the contribution of COFs-$PM_{2.5}$ to indoor air pollution in Chinese families is substantial. In a previous survey, we found that the COFs-derived $PM_{2.5}$ level was as high as 1.679–7.023 mg/m^3 in domestic

Abbreviations: $PM_{2.5}$, Fine particulate matter of < 2.5 μm in aerodynamic diameter; COFs, Cooking oil fumes; LBW, Low birth weight; ROS, Reactive oxygen species; PI3K, Phosphatidylinositol 3-kinase; mTOR, Mechanistic target of rapamycin; HUVECs, Human umbilical vein endothelial cells; DMSO, Dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DCFH-DA, 2',7'-dichlorofluorescin diacetate; NAC, N-acetyl-L-cysteine; TEM, Transmission electron microscopy; DAPI, 4',6-diamidino-2-phenylindole; PI, Propidium iodide; 3-MA, 3-methyladenine; TBST, Tris-Buffered-Saline with Tween; PAHs, Polycyclic aromatic hydrocarbons; BaP, Benzo(α)pyrene; ANOVA, Analysis of variances

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kitchen in China (Supplementary Table S1), which was confirmed by another recent study [4]. COFs-derived $PM_{2.5}$ could especially affect the women in China, as they are generally the ones cooking food for the families. While for the pregnant women, such exposure could possibly affect the fetus. Previous studies in our group have demonstrated that maternal exposure of COFs-derived $PM_{2.5}$ during pregnancy could lead to low birth weight (LBW) in mice (Supplementary Fig. S1). However, the underlying mechanisms are still unclear.

Umbilicus is the only link between the mother and fetus during pregnancy, and is responsible for the exchange of oxygen, nutrients, and waste materials between fetus and mother [5]. Veras et al. found that ambient $PM_{2.5}$ could induce oxidative stress and alter endothelin receptor expression to affect the umbilical cord blood vessels, and therefore contribute to the decreased fetal weight [6]. Peyter et al.[7] also demonstrated that the umbilical cord diameter was significantly lower in growth-restricted newborns than appropriate for gestational age term newborns; in addition, the relaxation of the umbilical vein induced by nitric oxide was significantly attenuated in growth-restricted neonates than controls. Previous in vitro [8] and in vivo [9] studies in our group have further confirmed that COFs-derived $PM_{2.5}$ exposure could evidently affect the umbilical cord blood vessels. These findings suggest that $PM_{2.5}$ exposure could increase the risk of endothelial dysfunction of the umbilical cord blood vessels, and possibly increase the risk of decreased fetal weight.

Previous studies have shown that COFs-derived $PM_{2.5}$ can induce oxidative damage not only to type II alveolar epithelial cells in vitro [1], but also to umbilical cord blood vessels in vitro and vivo [8,9]. Oxidative stress can cause severe damages to DNA, RNA, and proteins, and thus trigger autophagy and apoptosis [10]. Autophagy enables eukaryotic cells to capture cytoplasmic components for degradation within lysosomes. Although baseline autophagy is important for the maintenance of normal cellular homeostasis, abnormal autophagy contributes to many physiological and pathological processes, including morphogenesis, cancer, neurodegenerative disorders, and infectious diseases [11,12]. Therefore, we hypothesize that the elevated ROS production induced by the exposure of COFs-derived $PM_{2.5}$ could disturb the balance of autophagy in umbilical vein endothelial cells, thus consequently impair the functions of umbilical veins.

PI3K/AKT/mTOR pathway plays an important role in the modulation of cell autophagy [13]. Mechanistic target of rapamycin (mTOR) is one of the major modulators of autophagy that can be regulated by various signaling pathways [14]. For instance, several studies have demonstrated that the PI3K/AKT/mTOR signaling pathway participates in the regulation of autophagy induced by $PM_{2.5}$ exposure [15,16]. However, the effects of COFs-derived $PM_{2.5}$ exposure on autophagy of human umbilical vein endothelial cells (HUVECs), as well as the role of the PI3K/AKT/mTOR signaling pathway in this process have not been investigated to date. Here we investigated the effects of COFs-derived $PM_{2.5}$ on autophagy of HUVECs and the role of the ROS-AKT-mTOR axis in this event. The findings of this study could provide experimental evidence of the adverse effects of COFs-derived $PM_{2.5}$ on the functions of umbilical cord.

2. Materials and methods

2.1. Collection of COFs-derived $PM_{2.5}$

To generate and collect the COFs-derived $PM_{2.5}$ in the laboratory, 200 ml peanut oil (5S pressing first-class peanut oil; Luhua, Luhua Group, Shandong, China) was poured into an iron pot, and heated to smoke point (280 ± 10 °C) by an electric heater to generate cooking oil fumes. The fumes were collected with filter paper connected to a total suspended particulates sampler (XY-2200, Qaingdao Xuyu Environmental Co., Ltd, Qingdao, China), which is designed to collect different sizes of particulate matters (including $PM_{2.5}$), at 50 cm above the oil surface (Supplementary Fig. S2). The filter paper was renewed every 2 h. Then the filter paper with fumes attached was processed separately with 50 ml acetone for 24 h in Soxhlet extractor. The extracts were dried by rotary evaporation at 40 °C, and then further evaporated to yellow viscous solution. Dimethyl sulfoxide (DMSO) was used to dilute the solution to 200 mg/ml. The solution was transferred to brown glass vials, sealed, and stored at −80 °C until use. For the experiments in this study, the stoke solution was diluted to 12.5, 25, 50, 100, and 200 μg/ml with the cell culture medium. The compositions of the COFs-derived $PM_{2.5}$ have already been measured and reported by our group [1].

2.2. Cell culture

This study was approved by the Ethics Committee of Anhui Medical University. HUVECs were kindly provided by the School of Life Sciences, University of Science and Technology of China. The cells were cultured with DMEM culture medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed every day. Cells were used in the following experiments after the culture reached 80% confluence.

2.3. Cell viability assay

Cytotoxicity of COFs-derived $PM_{2.5}$ was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, HUVECs were incubated with 0 (control, containing 1‰ DMSO), 12.5, 25, 50, 100, and 200 μg/ml of COFs-derived $PM_{2.5}$ for 12, 24, and 36 h in 96-well culture plate, respectively. To confirm the effects of ROS on cell viability, N-acetyl-L-cysteine (NAC), a ROS inhibitor, was used along with different concentrations of COFs-$PM_{2.5}$ for 24 h. Then the culture medium was carefully discarded, 200 μl MTT solution (prepared with the culture medium at a ratio of 9:1) was added to each well, and the plate was incubated for another 4 h at 37 °C. The formazan crystals were dissolved with 150ul of DMSO, and the absorbance in each well was read at 490 nm using ELx800 microplate reader (Bio-TEK, USA).

2.4. ROS measurement

To determine the ROS levels in the cells, the HUVECs were loaded with the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). The HUVECs were seeded into 6-well plates with 5.0×10^5 cells/well. Before the following treatment, the cells were synchronization for 24 h by serum starvation. According to the results of the MTT assay, the cells were treated with different concentrations (0, 25, 50, 100 μg/ml) of COF-derived $PM_{2.5}$, 100 μg/ml COF-derived $PM_{2.5}$ + 10 mmol/L NAC, and 10 mmol/L NAC for 12, 24, and 36 h, respectively. The fluorescent probe DCFH-DA was added to each well with the final concentration of 10 μM and incubated at 37 °C for 30 min in dark. The plate was washed three times with PBS, trpsinized, re-suspended, and immediately subjected to fluorospectrophotometer analysis and fluorescence microscopy, at the excitation and emission wavelength of 488 and 525 nm, respectively. To further determine the mitochondrial reactive oxygen species in HUVECs, the cells were treated with COFs-derived $PM_{2.5}$, with or without 10 mmol/L NAC, for 24 h, and then stained with MitoSOX Red dye according to manufacturer's instructions. The pictures were taken with a fluorescence microscopy and analyzed with Image-Pro Plus 6.0 software. All these experiments were performed in triplication.

2.5. Observing autophagosome with TEM

Transmission electron microscope (TEM) was adopted to observe autophagosomes in the HUVECs. In brief, HUVECs were treated with 0 and 100 μg/ml COFs-derived $PM_{2.5}$ for 24 h, respectively. Cells treated