Fine particulate matter 2.5 upregulates melanogenesis in A375 human melanoma cells

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To the Editor: Epidemiological studies have shown that fine particulate matter 2.5 (PM2.5) exposure increases the risk of skin hyperpigmentation.[1] A recent study proved that PM2.5 could induce melanogenesis by regulating tyrosinase (TYR), and tyrosinase-related protein 1 (TYRP1) and 2 (TYRP2) expression via aryl hydrocarbon receptor (AhR)/mitogen-activated protein kinase signaling activation.[2] The AhR is widespread in skin cells and its ligation controls oxidation/anti-oxidation, epidermal barrier function, photo-induced response, innate immunity, and melanogenesis. It can be activated by dioxin and polycyclic aromatic hydrocarbons (the constituents of PM2.5) and its activation induces the transcription of genes encoding both phase I and phase II xenobiotic metabolism enzymes (cytochromes P450 [CYP] 1A1, CYP1A2, and CYP1B1).[3,4] BaP and hazardous dioxins activate AhR with a robust reactive oxygen species (ROS) generation, which is at least partly mediated by CYP1A1. Moreover, CYP1A1-mediated oxidative stress is responsible for the production of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8 in environmental contaminant-treated human keratinocytes.[3,5] However, there are very few studies on the toxic effects of PM2.5 on melanogenetic cells. Herein, we treated human melanoma cells A375 with various concentrations of PM2.5 to investigate whether PM2.5 can promote melanogenesis, and its relationship with AhR signaling, oxidative stress, and inflammatory response.

The PM2.5 was collected during winter (from December to January) in Beijing. HY-1000 intelligent large-flow total suspended particle (TSP) sampler (optional PM2.5 cutter, Qingdao Hengyuan Technology Development Co., Ltd., Qingdao, China) was used for quartz filter sampling. The quartz membrane was immersed in 75% ethanol followed by ultrasonic shaking. Sterile water was used to prepare a high concentration stock solution. Human melanoma cells A375 were cultured in 5% CO_2 at 37°C in regular Dulbecco's Modified Eagle's Medium (Invitrogen Co., Ltd., Carlsbad, CA, USA) containing fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA). A375 cells were treated with different concentrations of PM2.5 (0, 100, 200, and 400 μg/mL) for 24 hours, and the cells were used for the following studies. Unpaired Student's *t* test and one-way analysis of variance were used for statistical analysis, and $P < 0.05$ was considered as statistically significant.

To investigate the influence of PM2.5 on A375 cells, the cell proliferation was detected using a CCK-8 kit (Sigma-Aldrich, St. Louis, MO, USA) and cell apoptosis was detected using Annexin V fluorescein isothiocyanate/propidium iodide (FITC/PI) apoptosis kit (Multisciences, USA), based on the manufacturer's instructions. The results indicated that PM2.5 exhibited concentration-dependent cytotoxicity to A375 cells, but did not affect the apoptosis [Supplementary Figure 1, http://links.lww.com/CM9/A917].

The NaOH method was used to determine melanin content. The PM2.5-treated A375 cells were washed with phosphate buffer saline (PBS) and lysed with water. The lysates were centrifuged at 12, 000 r/min for 5 minutes, washed with 10% trichloroacetic acid (500 μL) (Sigma, St. Louis, MO, USA) and 75% ethanol (500 μL), and then clarified again. Thereafter, 50 μL of 1 mol/L NaOH containing 10% dimethyl sulfoxide was added to the pellets for 1 hour at 80°C. The absorbance at 490 nm (OD 490) was measured using a microplate reader. As TYR is the most important melanogenic enzyme, we next detected the TYR activity using the levodopa (L-DOPA) assay. Six-well plates (Corning, USA) were seeded with A375 cells at a density of 3×10^5 cells/well. The cells were then washed with PBS and lysed with 0.5% sodium deoxycholate (Sigma). The lysate was divided into three groups, and 0.1% L-DOPA (Sigma) was added to each sample. After

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Quick Response Code:

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DOI: 10.1097/CM9.0000000000001965

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Chinese Medical Journal 2022;135(15)
Received: 25-11-2021; **Online:** 27-01-2022 **Edited by:** Lishao Guo

5 minutes, OD 490 was measured and 0.1% L-DOPA was used as the standard. We also measured the mRNA and protein expression levels of TYR. Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the ReverTra Ace qPCR RT kit (TOYOBO, Japan). Thereafter, quantitative real-time polymerase chain reaction (qRT- PCR) was performed on Bio-Rad CFX96 Touch^{TM} Realtime PCR Master Mix (TOYOBO, Japan). The primers from Invitrogen are presented in Supplementary Table 1, http://links.lww.com/CM9/A917, and the expression level of each mRNA was calculated using the $2^{-\Delta\Delta CT}$ method. The protein expression levels were analyzed by Western blotting. Cells were washed with PBS and lysed in RIPA buffer (Beyotime, China) containing phenylmethylsulfonyl fluoride (protease inhibitor mix, 20:1, Sigma-Aldrich). The protein concentrations were measured using a BCA protein assay kit (Beyotime, China). The total protein was boiled at 70°C for 10 minutes, separated in a 4%–12% gel at 200 V for 40 minutes, transferred to a membrane at 4°C using 120 mA for 75–100 minutes, and then incubated with 5% non-fat milk for 1 hour. Membranes were then probed with anti-TYR, anti-AhR, anti-CYP1A1, and anti-CYP1B1 antibodies (Abcam) overnight at 4°C. Horseradish peroxidase- conjugated anti-mouse IgG antibody (Proteintech, USA) was used as a secondary antibody. The results indicated that PM2.5 increased melanin content and TYR activity in a dosedependent manner. Both mRNA and protein expression levels of TYR were upregulated [Supplementary Figure 2 A–D, http://links.lww.com/CM9/A917].

PM2.5 can upregulate TYR activity by activating the AhR pathway. To determine whether PM2.5 affects AhR signaling, we detected the transcription levels of AhR and its classical downstream genes *CYP1A1* and *CYP1B1* by qRT-PCR and western blotting. The results showed that PM2.5 upregulated the mRNA expression levels of AhR and CYP1A1, as well as the protein expression of CYP1A1 [Supplementary Figure 2 E–G, http://links.lww.com/CM9/A917].

AhR activation causes ROS generation, which can also increase TYR activity.[5] Hence, we evaluated the ROS production using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and flow cytometry. After 24 hours treatment with PM2.5, A375 cells were incubated with DCFH-DA (5 μmol/L) for 30 minutes at 37°C in the dark. Cells were washed twice in PBS, and the fluorescence was measured by a flow cytometer. The mean fluorescence intensity was quantified using the FACS Diva 6.0 software (BD Bioscience, New York, NY, USA). As CYP1A1-induced ROS production is thought to be responsible for the inflammatory response,[5] the mRNA expression levels of pro-inflammatory cytokines (IL-1α, IL-6, IL-8, and tumor necrosis factor [TNF]-α) were measured. It was found that PM2.5 increased ROS production, as well as IL-1α, IL-6, and IL-8 mRNA levels in A375 cells [Supplementary Figure 2 H and I, http://links.lww.com/CM9/A917].

The findings of this study indicated that PM2.5 promoted melanogenesis, and increased both activity and expression of TYR in A375 cells. Moreover, PM2.5 could activate AhR signaling, induce oxidative stress and inflammatory response, which may be a melanogenic signaling cascade responsible for hyperpigmentation.

Acknowledgments

We express our sincere gratitude to the following: Prof. Dr. Masutaka Furue and Prof. Dr. Tsuji Gaku from Kyushu University Hospital who improved the manuscript and helped with the AhR-related information. We are also grateful for the English editing provided by Prof. Dr. Robert Schwartz from Rutgers New Jersey Medical School.

Funding

This study was supported by a grant from the National Natural Science Foundation of China (No. 2101000271).

Conflicts of interest

None.

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How to cite this article: Peng F, Yang X, Li W, Huang J, Chen Z, Zhang J. Fine particulate matter 2.5 upregulates melanogenesis in A375 human melanoma cells. Chin Med J 2022;135:1881–1882. doi: 10.1097/CM9.0000000000001965