Probucol protects circulating endothelial progenitor cells from ambient PM$_{2.5}$ damage via inhibition of reactive oxygen species and inflammatory cytokine production in vivo

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Abstract. Bone marrow-derived circulating endothelial progenitor cells (EPCs) contribute to angiogenesis and vascular repair. The number and function of EPCs are significantly decreased following exposure to ambient fine particulate matter of ≤2.5 µm in diameter (PM$_{2.5}$) through reactive oxygen species (ROS) generation and inflammatory cytokine secretion. The anti-oxidant drug probucol reduces ROS and inflammatory cytokine production. The present study was designed to determine the protective effects of probucol on EPCs from PM$_{2.5}$-associated impairment in vivo and to explore the potential underlying mechanisms. Male C57BL/6 mice were exposed to ambient air containing PM$_{2.5}$ for one month with or without probucol treatment. Mice that breathed filtered air were used as a control group. Serum and blood cells were collected for analysis. The results indicated that PM$_{2.5}$ exposure induced increases in blood intracellular ROS, serum inflammatory cytokine levels and the blood cell apoptotic rate, while it decreased the number and proliferation rate of circulating EPCs in the mice with PM$_{2.5}$ exposure. These effects were significantly reduced/abrogated by probucol treatment. The present in vivo study suggested that probucol protects EPCs from damage through PM$_{2.5}$ exposure by inhibiting ROS generation and inflammatory cytokine production.

Introduction

Deficiency or injury of the endothelium is closely associated with the initiation of the atherosclerotic process (1). Endothelial progenitor cells (EPCs) are mainly involved in vascular regeneration, angiogenesis and re-endothelialization following vascular damage (2). Thus, it is important to retain the number and function of EPC at normal levels in patients with cardiovascular disease.

Ambient particulate matter (PM) has become a major health threat. Increases in mortality and morbidity due to PM exposure have been reported (3). PM is a mixture of different types of debris, the major components of which are crustal material, metals and bio-aerosols (4). Small PM with a median aerodynamic diameter of ≤2.5 µm (PM$_{2.5}$) is the most harmful type and numerous cardiovascular diseases are associated with exposure to PM$_{2.5}$ (5). PM$_{2.5}$ exposure causes a reduction in heart rate variability, as well as vascular dysfunction, vascular inflammation, an increased coagulation-thrombosis risk and the acceleration of atherosclerosis (5). More importantly, in humans and mice, a decreased number and function of EPC are also associated with PM$_{2.5}$ exposure (6,7). A previous study by our group indicated that exposure of mice to PM of ≤4 µm in size (including PM$_{2.5}$) through intranasal instillation for one month significantly suppressed the number and function of EPCs via increasing blood intracellular reactive oxygen species (ROS) production and inflammatory cytokine generation (8). However, the detailed mechanisms by which the number and function of EPCs decrease after inhalation of atmospheric PM$_{2.5}$ have remained to be elucidated.

A notable reduction in atherosclerosis and restenosis in the coronary artery were observed in subjects treated with probucol (9,10). This potent drug has anti-oxidant and anti-inflammatory effects and preserves endothelial function by
reducing the amount of endogenous nitric oxide (NO) synthase inhibitor, increasing prostacyclin generation, inhibiting the expression of various adhesion molecules and promoting the proliferation of endothelial cells, while preventing the apoptosis of endothelial cells due to oxidative injury (9,10). Of note, cigarette smoking and oxidized high-density lipoprotein (Ox-HDL) induced EPC dysfunction may also be reversed by probucol treatment (11,12). However, the effects of probucol on EPCs exposed to PM$_{2.5}$ remain elusive.

The aim of the present study was to determine whether probucol has any protective effects on EPCs in mice exposed to PM$_{2.5}$. It was observed that the diminished EPC levels in mice under PM$_{2.5}$ exposure were indeed restored to normal levels with probucol treatment.

**Materials and methods**

**Animal model of PM$_{2.5}$ exposure.** All animal procedures were performed in accordance with the Guidelines of the Animal Care Committee of the Shandong Provincial Hospital affiliated with Shandong University (Jinan, China). The Animal Care Committee of Shandong University (Jinan, China) approved the experimental protocols. A total of 40 male wild-type C57 BL/6 mice (age, 6-8 weeks; weight, 20-25 g) were purchased from Better Biotechnology Co., Ltd. (Nanjing, China). All mice were housed at the animal facility for 1 week prior to exposure. The center of Jinan city (China), a highly polluted area, was selected for the experiment. The exposure period lasted one month from December 12, 2016 to January 12, 2017. Ambient PM with a diameter equal to 2.5 µm was collected using a high flow rate with an aerosol-into-liquid collector (HRH-PM186; Beijing Huironghe Technology Co., Ltd., Beijing, China). The mean concentration of PM in Jinan measured during the experiment was 135.23±42.12 µg/m³. This data was in accordance with previously published data (13). The concentration of PM for mice with exposure and for mice with filtered air (FA; the control mice) was adjusted to 130±65.51 and 2.4±1.1 µg/m³, respectively. A total of 10 mice in each group were subjected to PM exposure or used as controls who inhaled FA. All mice were exposed in a chamber system for the experiment as described (14). The high-efficiency particulate air filter ( Pall Life Sciences; Pall Corporation, Port Washington, NY, USA) was used for mice with FA exposure. Probucol was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). To evaluate the protective effect of probucol on EPC from PM$_{2.5}$ mice, 5 mg probucol was first dissolved in 100 µl 99% ethanol, then diluted in 99.9 ml PBS to give a final concentration of 50 µg/ml. The final concentration of ethanol was adjusted to 1% in PBS. Then, the probucol solution was used to treat mice at a dose of 500 mg/kg/day (10). In a preliminary experiment, probucol at 100, 250, 500 and 600 mg/kg/day was used to treat the mice exposed to PM$_{2.5}$. The maximum anti-oxidant and anti-inflammatory effects of probucol were observed at the dosage of 500 mg/kg/day, while no further increases in the protective effects of probucol were achieved when the dose was 600 mg/kg/day. Probucol was administered by oral gavage for three days prior to PM$_{2.5}$ exposure and for one month during PM$_{2.5}$ exposure. A total of 40 mice were equally divided into four groups: The FA group, 10 mice with FA exposure; the PM$_{2.5}$ group, 10 mice with PM$_{2.5}$ exposure; the Prob+FA group, 10 mice with probucol treatment and FA exposure; the Prob+PM$_{2.5}$ group, 10 mice with probucol treatment and PM$_{2.5}$ exposure.

**Measurement of pro-inflammatory factors.** Mouse serum was collected after one month of PM$_{2.5}$ exposure. The pro-inflammatory cytokines tumor necrosis factor (TNF)-α (cat. no. 430904), interleukin (IL)-1β (cat. no. 432604) and IL-6 (cat. no. 431304) were measured with an ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocols.

**Assessment of EPC proliferation, apoptosis and intracellular ROS formation.** After PM$_{2.5}$ or FA exposure, murine blood was collected, followed by the elimination of red blood cells (RBCs) with an RBC lysis buffer (cat. no. 420301; BioLegend). The in vivo EPC number and proliferation rate were measured at 12 h after i.p. injection of 1 mg bromodeoxyuridine (BrdU). CD34-Alexa Fluor® 700 (cat. no. 560518; BD Biosciences, Franklin Lakes, NJ, USA) and CD133-phycocerythrin (cat. no. 141204; BioLegend) antibodies were used to mark the EPC population. Specifically, 1 µl CD34 and 1 µl CD133 in 100 µl cell staining buffer (420201; BioLegend) were added to 1x10⁶ cells. Then the mixture solution was incubated in ice in a dark room for 30 min. In order to quantify the EPC population, the CD34⁺/CD133⁺ cells were detected in a sample containing at least 50,000 cells. Anti-BrdU fluorescein isothiocyanate (FITC) contained in the BrdU Flow Kit (cat. no. 559619; BD Biosciences) was used to measure the cell proliferation. Blood EPC apoptosis was determined by using the FITC Annexin V Apoptosis Detection Kit (cat. no. 556547; BD Biosciences). Early (Annexin V FITC-positive and propidium iodide (PI)-negative cells) and late (Annexin V FITC and PI double-positive cells) apoptotic cells were measured. Total ROS Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which contained dichlorofluorescein (DCF)-FITC, was used to measure the intracellular ROS production. Specifically, following the staining of cells with CD34⁺ and CD133⁺ antibodies, cells were washed twice with PBS. Then, DCF-FITC was added in the mixture solution for 10 min at 37°C. The DCF-FITC-labelled cells were washed twice with PBS and then suspended in warm PBS (37°C) for analysis using flow cytometry. The CD34⁺/CD133⁺ cells with DCF-FITC fluorescence-positive cells were quantitatively evaluated using a BD™ LSR II flow cytometer (BD Biosciences) at the wavelength of 525 nm, as previously described (8).

**Statistical analysis.** Values are expressed as the mean ± the standard deviation. PRISM version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analyses. The unpaired Student's t-test (two-sided) was used for comparison between two groups. One-way analysis of variance followed by a post-hoc conservative Tukey's test were used for comparison between three or more groups to minimize type-I errors as appropriate. A two-tailed P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PM$_{2.5}$ treatment reduces circulating EPCs in association with increased apoptosis and decreased proliferation.** After exposure to PM$_{2.5}$ for one month, murine blood cells were collected
for EPC analysis. The results indicated that PM$_{2.5}$ significantly decreased the CD34$^+$/CD133$^+$ cell population (0.017±0.007%) compared with that in the control (0.037±0.012%; Fig. 1A). To identify the possible reasons for the decrease in the EPC population, the EPC proliferation and apoptotic rate were assessed. As presented in Fig. 1B, the EPC proliferation rate was significantly decreased compared with that in the control group (0.012±0.004% vs. 0.026±0.005%). Furthermore, the early apoptotic rate (6.74±0.67%) and the late apoptotic rate (14.04±4.38%) of EPCs were substantially elevated compared with those in the control group (3.48±0.51 and 6.58±1.77%, respectively; Fig. 1C).

PM$_{2.5}$ increases serum inflammatory factors. The serum levels of inflammatory factors, including TNF-α, IL-1β and IL-6, are known to be closely associated with an increased blood cell apoptosis and decreased circulating EPC proliferation (8,15). In the present study, the serum levels of TNF-α, IL-1β and IL-6 were measured after PM$_{2.5}$ exposure. As presented in Fig. 2, the TNF-α and IL-1β levels were increased up to 2-fold of those in the control group, while IL-6 was 6-fold of that in the control group.

PM$_{2.5}$ increases intracellular ROS levels in blood EPCs. It has been previously reported that intracellular ROS may cause an elevation of blood cell apoptosis and a decline of EPC proliferation (8,15). After exposure to PM$_{2.5}$, for one
As presented in Fig. 3, the blood intracellular ROS levels were significantly increased in the mice with PM$_{2.5}$ exposure compared to those in the control group (29.14±2% vs. 18.96±3.14%). Probucol treatment attenuates the detrimental effects of PM$_{2.5}$ on EPCs. To evaluate the protective effect of probucol against the reduction of circulating EPCs due to PM$_{2.5}$ exposure, mice were pre-treated with probucol prior to PM$_{2.5}$ exposure for 3 days and treatment was continued for 1 month with PM$_{2.5}$ exposure. The intracellular ROS production was completely blocked by probucol treatment (Fig. 3). In the probucol treatment group, the PM$_{2.5}$-associated elevated serum inflammatory factors (TNF-α, IL-1β and IL-6) were also reduced to the normal level of the control group (Fig. 2).

To determine whether probucol treatment of mice exposed to PM$_{2.5}$ is able to restore the circulating EPC proliferation and blood EPCs apoptotic rate to the normal level, the EPC proliferation and blood cell apoptotic rate were measured after probucol treatment. As presented in Fig. 4B, the EPC proliferation rate was recovered with probucol treatment, while the early and late apoptotic rate of blood cells were also restored to the normal level in the mice with probucol treatment (Fig. 4C).

Finally, it was assessed whether probucol was able to prevent the reduction in EPCs after PM$_{2.5}$ exposure. As presented in Fig. 4A, the decrease in CD34+/CD133+ cells (0.017±0.007%) associated with PM$_{2.5}$ exposure was inhibited by probucol treatment, resulting in near normal levels (0.029±0.002%). These data indicated that probucol effectively inhibited the effects of PM$_{2.5}$ on murine circulating EPCs via the inhibition of blood cell apoptosis and increase in EPCs proliferation through the blocking of intracellular ROS production and inflammatory cytokine secretion (Fig. 5).

**Discussion**

In the present study, it was demonstrated that exposure to PM$_{2.5}$ induced apoptosis of blood cells and suppressed EPC proliferation to reduce the number of EPCs via increasing the blood intracellular ROS levels and serum inflammatory cytokines and EPC apoptosis. The proliferation rate and the percentage of circulating EPCs were also recovered with probucol treatment.

It has been widely reported that PM$_{2.5}$ exposure is associated with various systemic diseases, including cardiovascular (5), neuronal (16) and hepatic disease (17), as well as diabetes (18). The mechanisms mainly involve PM$_{2.5}$ triggering systemic oxidative stress and inflammation (19). Of note, the increases in ROS production and the levels of inflammatory factors following PM exposure are associated with endothelial injury as well as a decreased number of EPCs (20,21). The results of the present study also suggested that intracellular ROS production as well as serum inflammatory factors were increased after PM$_{2.5}$ exposure. These contributed to the decrease in the number of circulating EPCs, which is associated with an increased risk of cardiovascular disease.

It is well documented that after endothelial injury, EPCs contribute to angiogenesis and vascular regeneration, as well as maintaining a normal endothelial function (2). Of note, increases in cardiovascular disease are associated
with a decreased number and function of EPCs after PM exposure (5). It has been reported that after PM$_{2.5}$ or nickel exposure, the number of murine bone marrow and circulating EPCs ($CD34^+$/$CD31^+$/CD45$^-$/$CD133^+$) was decreased (21), and the function of EPCs ($CD34^+$/vascular endothelial growth factor receptor-2$^+$/$CD11b^-$), including tube formation and chemotaxis, were also significantly suppressed (6).

A study on a Chinese cohort also reported that circulating EPCs ($CD34^+$/kinase insert domain receptor (KDR)$^+$, $CD34^+$/$KDR^+$/$CD45^-$ or $CD34^+$/KDR$^+$/$CD133^+$) were notably reduced following PM$_{2.5}$ exposure (22). Furthermore, in accordance with a previously published study (8), the present results indicated that PM exposure significantly suppressed the circulating EPC population in mice through promoting apoptosis of EPCs ($CD34^+$/CD133$^+$) in association with an elevated ROS generation as well as serum TNF-α and IL-1β levels in vivo. Furthermore, the present study confirmed that inhalation of ambient PM$_{2.5}$ suppressed the proliferation of EPCs and promoted their apoptosis via increasing the levels of ROS in blood cells and of TNF-α, IL-1β and IL-6 in the serum. However, certain studies reported that circulating EPCs increased following short-term PM$_{2.5}$ exposure (7,23). The mechanisms were described to mainly involve sympathetic nervous system activation and decreased mobilization of bone marrow EPCs into the circulation through a systemic reaction to an acute ‘endothelial injury’ following PM exposure.

Reagents and methods for the protection of EPCs have been reported in numerous studies and included the use of microRNA, triterine, granulocyte-macrophage colony-stimulating factor, urinary trypsin and inhibition of
CD40 (24-27). Of note, probucol, as a cholesterol modulator, significantly inhibits the initiation and progression of atherosclerosis. The mechanisms mainly include the suppression of ROS formation (10), promotion of endothelial recovery, inhibition of monocyte activation and adhesion (28), attenuation of vascular smooth muscle cell (VSMC) growth and migration (29), an influence on VSMC and macrophage proliferation and apoptosis, as well as a decrease of cytokine secretion by macrophages (30,31). Of note, the cigarette smoke-induced impairment and ischemia-triggered neovascularization was rescued by probucol through its protective effects on EPCs (11). The deleterious effects of Ox-HDL on EPCs were also reversed by probucol (12). In addition, probucol prevents ROS-induced inactivation of endothelium-derived NO, decreases endogenous NO synthase inhibitor formation and increases the level and function of NO to further benefit EPCs (9,10,32).

In the present study, after the treatment of mice with probucol at 500 mg/kg/day for one month, it was observed that the population and the proliferation of circulating EPCs were effectively restored, EPC apoptosis was inhibited, ROS formation blocked and serum inflammatory cytokines were reduced in the mice with PM2.5 exposure.

Although the benefits of probucol on EPCs have been reported in numerous studies, the detailed mechanisms of its protective effects on EPCs following PM2.5 exposure have remained elusive. Further questions, including what specific type of ROS is generated following PM2.5 exposure, whether any other mechanisms are involved in the protection of EPCs by probucol, and whether ROS and inflammatory cytokines that are induced by PM2.5 exposure are the two major factors that impair EPCs remain to be addressed in further studies. Future projects, including an experiment to discriminate between different types of ROS generated in EPCs and the application of vitamin C after PM2.5 exposure, are currently in planning.

In conclusion, the present study indicated that probucol effectively prevented the effects of PM2.5 on murine circulating EPCs via inhibition of blood cell apoptosis and recovery of EPC proliferation through blocking of blood intracellular ROS generation and inflammatory cytokine secretion. Thus, probucol may be an effective medicine for the prevention and treatment of PM2.5-induced cardiovascular diseases.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

YC, LC, QS and ZL designed the experiments, and YC, KH, LY, HX, PZ and HS performed them. ZS, HL, LC and HB collected and analyzed the data. YC wrote the manuscript.

Ethical approval and consent to participate

The Animal Care Committee of Shandong University (Jinan, China) approved the experimental protocols.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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