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Fine particulate matter induces vascular endothelial activation *via* IL-6 dependent JAK1/STAT3 signaling pathway

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Exposure to PM2.5 has been strongly linked to endothelial dysfunction. However, the underlying mechanism of PM2.5 on the vascular endothelial function is poorly understood. This study examined the toxic effect and underlying mechanism of PM2.5 on human umbilical vein endothelial cells (HUVECs). Decreased cell viability and increased LDH activity were observed in the PM2.5-treated HUVECs in a dose-dependent manner. The production of ROS, MDA, and the inhibition of SOD activity were also triggered by PM2.5 in HUVECs. In addition, PM2.5 increased the intracellular levels of proinflammatory cytokines (IL-6, TNF-a, IL-1β, IL-8 and CRP), cell adhesion molecules (ICAM-1, VCAM-1) and tissue factor (TF), resulted in endothelial activation. For an in-depth study, the protein levels of IL-6, JAK1 and STAT3 were up-regulated significantly, while the expression of JAK2 and SOCS1 were down-regulated gradually in PM2.5-treated HUVECs in a dose-dependent manner. These results show that PM2.5 triggered endothelial activation *via* upregulation of the IL-6 dependent JAK1/STAT3 signaling pathway. This will provide new insights into the toxic effects and mechanisms of cardiovascular diseases triggered by ambient air pollution.

Introduction

Ambient air pollution is an environmental hazardous factor that affects the public health globally. According to the World Health Organization (WHO), air pollution leads to the premature deaths of about 2.4 million people each year worldwide.[1] Substantial epidemiological evidence confirms that the likelihood of ambient particulate matter (PM) and an increased morbidity and mortality of cardiovascular diseases.[2] Time-series studies report that an increase in the fine particulate matter (PM2.5; diameter <2.5 μm) level to 10 μg m^{-3} leads to approximately 0.4% to 1.0% of the relative risk (RR) for daily cardiovascular mortality.[3] However, the underlying mechanism of PM2.5 on the cardiovascular system is not well understood and its related research conclusions lack consistency.

Epidemiological studies suggested that PM2.5 exposure is associated with systemic inflammation, endothelial activation, impairment in the vascular reactivity and hemodynamics.[4–7] Laboratory studies further revealed that PM2.5 could increase the levels of oxidative stress and adhesion molecule expression, impair the endothelial function and vasorelaxation, as well as accelerate atherosclerosis and vascular inflammation.[8–11] In addition, the pulmonary inflammation is mediated by adhesion molecules expressed on the leukocytes and the pulmonary vasculature endothelial cells.[12] The evidence suggests that the vascular system is the major target organ induced by PM2.5. Endothelial activation may play an important role in cardiovascular diseases. Therefore, it is necessary to explore the toxic effects and underlying mechanism of PM2.5 on vascular endothelial cells.

Endothelial cells that lay between circulating blood and the vessel wall play a critical role in maintaining the vascular function and homeostasis.[13] Endothelial activation, defined as a specific and complex change in the endothelial phenotype, is pivotal to the inflammatory responses *via* endothelium–leukocyte interactions.[14] The process of endothelial activation involves the up-regulated expression of chemotactic factors, cell surface adhesion molecules, the release of proinflammatory cytokines and procoagulant factors.[15] Endothelial activation is implicated in the pathophysiology of several

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cardiovascular diseases, including atherosclerosis, hypertension, diabetic vasculopathy and coronary artery disease.[16,17]

This study examined the toxicity mechanism of PM2.5 on endothelial activation in the human umbilical vein endothelial cells (HUVECs). The PM2.5 was collected from Beijing, China, which is one of the most severe cities of ambient air pollution in the world.[18,19] It is well known that the biological effect and toxicity of PM2.5 can vary greatly from different areas. However, researchers have paid more attention to epidemiological studies rather than mechanistic studies regarding PM2.5 pollution in Beijing.[20,21] To investigate the toxic effects of PM2.5 on endothelial activation, we conducted a series of evaluations, including the cell viability, membrane integrity, intracellular ROS generation, oxidative damage, as well as the expression of adhesion molecules, the production of pro-inflammatory cytokines and procoagulant factors. We also measured whether the IL-6 dependent JAK/STAT signaling pathway plays an important role in PM2.5-triggered toxicity in HUVECs.

Materials and methods

Cell culture and exposure to PM2.5

The HUVECs line was purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China). The HUVECs were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U per mL penicillin and 100 μg per mL streptomycin and cultured at 37 °C in a 5% CO_2 humidified environment. For the experiments, the HUVECs were seeded in culture plates at a density of 1×10^5 cells per mL. The PM2.5 used in this study was provided from the School of Environment in Tsinghua University, and collected from Haidian District of Beijing in winter. The sampling sites were located in Tsinghua University, which is near the commercial area without industrial sources of pollution. The distance from the sampling sites to the main road is about 200 m. Secondary species, including organics, sulfate, nitrate, and ammonium, were the major constituents of PM2.5 during this period.[22] The stock suspensions of PM2.5 were sonicated for 5 min before HUVECs exposure. Cultured HUVECs were treated with various concentrations (0, 25, 50, and 100 μg mL^{-1}) of PM2.5 for 24 h. The controls were supplied with an equivalent volume of DMEM without PM2.5.

Assessment of cytotoxicity

The cell morphology was observed by optical microscopy (Olympus IX81, Japan). The effect of PM2.5 on the cell viability was determined using a CCK-8 kit (KeyGen, China) according to the manufacture's instruction. The optical density at 450 nm was detected by a microplate reader (Themo Multiscan MK3, USA). Lactate dehydrogenase leakage (LDH) was detected using a LDH Kit (Jiangcheng, China) according to the manufacturer's protocols. After HUVECs-treated with PM2.5 for 24 h, the supernatants were collected for the LDH measurement and measured by a microplate reader at 450 nm absorbance (Themo Multiscan MK3, USA).

Intracellular ROS measurement

The production of intracellular ROS was measured by an oxidation-sensitive probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma, USA). In brief, after exposing the HUVECs to PM2.5 for 24 h, the cells were incubated with 10 μM DCFH-DA at 37 °C in the dark for 30 min. The cells were then washed twice with cold PBS and re-suspended in PBS for analysis. The fluorescent intensity and percentage of positive cells were measured by flow cytometry (Becton-Dickison, USA).

Assessment of oxidative damage

In brief, after HUVECs exposed to PM2.5 for 24 h, the cells were lysed in ice-cold PBS buffer by an ultrasonic cell disruptor (JY 92-II, SCIENTZ, China) for 3 min. After the cell lysates were centrifuged at 12 000 rpm for 5 min, the supernatants were collected for measurements of the production of malondialdehyde (MDA) and the activity of superoxide dismutase (SOD). All examinations were carried out using commercially available kits (KeyGen, China) according to the manufacturer's instructions.

Proinflammatory cytokines measurement

The supernatants were collected after HUVEC exposed to PM2.5 for 24 h. The supernatants were centrifuged and stored at −80 °C until used. The levels of human C-reactive protein (CRP), human tumor necrosis factor-α (TNF-α), human interleukin-1β (IL-1β), human interleukin-6 (IL-6), and human interleukin-8 (IL-8) were measured using Enzyme Linked Immunosorbent Assay (ELISA) kits (Raybiotech, Inc. USA) according to the manufacturers' protocols. The supernatants were detected immediately with the absorbance of 450 nm using a microplate reader (Themo Multiscan MK3, USA).

Western blot analysis

Equal amounts of 20 μg lysate proteins were loaded onto 12% SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After blocking with nonfat milk (5%) in Tris-buffered saline (TBS) for 1 h, the PVDF membrane was incubated sequentially with intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), TF, NF-κB p65, p-NF-κB p65, c-JUN, p-c-JUN, IL-6, Janus family of tyrosine kinase 1 (JAK1), JAK2, Signal Transducers and Activators of Transcription 3 (STAT3), Suppressor of Cytokine Signaling 1 (SOCS1) (CST, USA) (1 : 1000, rabbit antibodies) at 4 °C overnight. The PVDF membrane was then rinsed with TBST and incubated with anti-rabbit Ig G secondary antibody (CST, USA) for 1 h. After rinsing with TBST three times, the proteins-bound were measured by the Enhanced Chemiluminescence (ECL) (Pierce, USA). Using the ImageJ Software, the densitometric analysis for western blot was detected.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software. Data are expressed as the mean ± standard deviation (S.D.). Three or more treatment groups were compared by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) method for multiple comparisons. All significance differences were considered at the level of $p < 0.05$.

Results

Cytotoxicity of PM2.5 in HUVECs

To assess the cytotoxicity of PM2.5 on vascular endothelial cells, the cell viability and LDH activity were measured after exposing the HUVECs to PM2.5 (0, 25, 50, and 100 μg mL^{-1}) for 24 h (Fig. 1). The cell viability of the PM2.5-treated HUVECs decreased significantly in 50 μg mL^{-1} and 100 μg mL^{-1} treated groups compared to the control group (decrease of 82.39% and 72.75%, respectively), while the activity of LDH increased remarkably in the 50 μg mL^{-1} and 100 μg mL^{-1} treated groups compared to that of control. These results indicate that PM2.5 induced the cytotoxicity of HUVECs in a dose-dependent manner.

ROS production and oxidative damage induced by PM2.5

As shown in Fig. 2A, the ROS levels of all treated groups were increased gradually in the PM2.5-treated HUVECs. At the highest concentration (100 μg mL^{-1} of PM2.5), the fluorescence intensity of ROS was elevated significantly compared to the control group (1.9-fold). In addition, the production of MDA and the activity of SOD were also measured after exposing the HUVECs to PM2.5 for 24 h (Fig. 2B and C). The intracellular level of MDA was increased significantly, while the activity of SOD was decreased markedly compared to that of the control. The results demonstrated that PM2.5 could cause ROS production and oxidative damage in HUVECs in a dose-dependent manner.

Proinflammatory cytokines triggered by PM2.5

As shown in Fig. 3, the production of IL-6 was increased significantly in all PM2.5-treated groups compared to the control group. Moreover, the levels of TNF-α and IL-1β were elevated significantly at 50 μg mL^{-1} and 100 μg mL^{-1} in the PM2.5-treated groups, respectively; while the release of IL-8 and CRP were increased slightly compared to that of the control. The data indicate that PM2.5 triggers the inflammatory response in

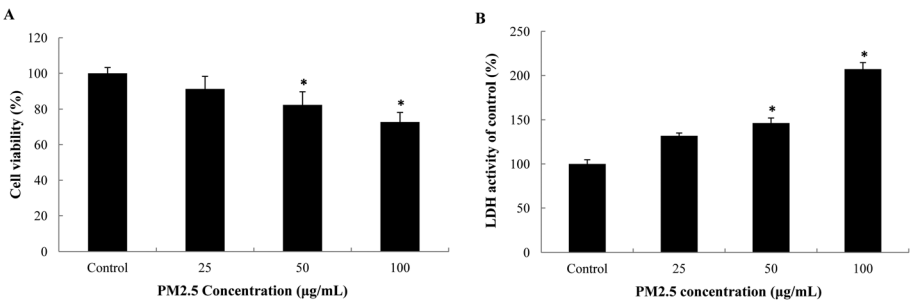

Fig. 1 Cytotoxicity induced by PM2.5 in HUVECs. (A) Cell viability of HUVECs treated with PM2.5 was measured by a CCK-8 assay after 24 h exposure. (B) LDH activity of HUVECs after exposed to PM2.5 for 24 h. Data are expressed as the means ± S.D. from three independent experiments (*$p < 0.05$).

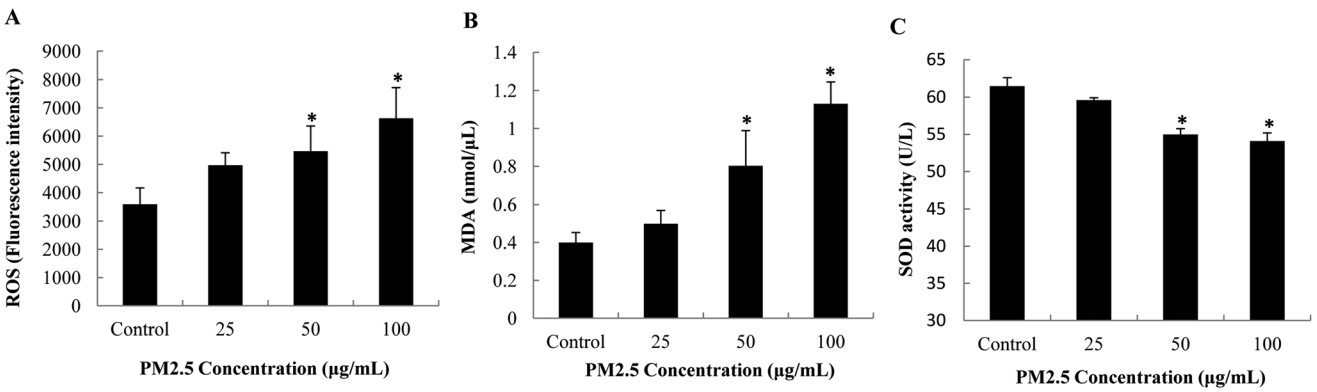

Fig. 2 Oxidative stress and oxidative damage induced by PM2.5 in HUVECs. The intracellular levels of ROS (A) and MDA (B) were significantly increased. (C) The activity of SOD was decreased markedly in a dose-dependent manner. Data are expressed as means ± S.D. from three independent experiments (*$p < 0.05$).

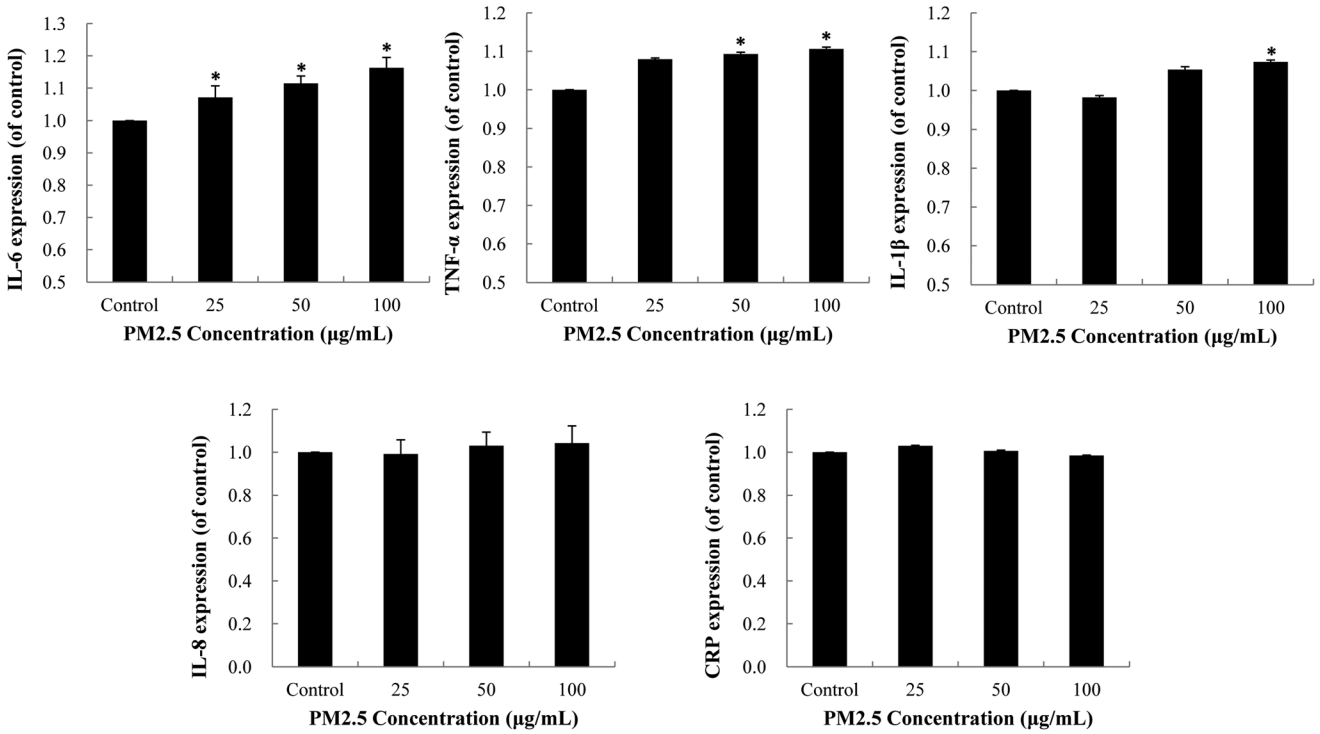

Fig. 3 Proinflammatory cytokines triggered by PM2.5 in HUVECs. The productions of IL-6, TNF-a and IL-1β were increased significantly, while the secretions of IL-8 and CRP were increased slightly compared to that of the control. Data are expressed as means ± S.D. from three independent experiments (*$p < 0.05$).

HUVECs. In addition, the secretion of IL-6 might play an important role in PM2.5-induced endothelial activation.

Effect of PM2.5 on cellular adhesion molecule expression

The expression of ICAM-1 and VCAM-1 were measured to investigate the impact of PM2.5 on cellular adhesion (Fig. 4). At the 25 μg mL^{-1} and 50 μg mL^{-1} PM2.5-treated groups, there were no marked changes in the protein expression of ICAM-1 and VCAM-1. While up to the highest concentration (100 μg mL^{-1} of PM2.5), the protein levels of ICAM-1 and VCAM-1 were increased significantly compared to that of the control group.

Procoagulant factors triggered by PM2.5

Given that TF is the primary activator of the coagulation cascade and is regulated by the transcription factors, NF-κB and AP-1/c-Jun, we measured the protein levels of TF, NF-κB p65 and c-Jun by western blot analysis (Fig. 5). The protein expression of TF, NF-κB p65 and c-Jun were up-regulated significantly in all PM2.5-treated groups compared to that of the control. At the highest concentration (100 μg mL^{-1} of PM2.5), the protein levels of TF, NF-κB p65 and c-Jun were elevated to 1.92-fold, 1.85-fold and 1.77-fold of the control group, respectively.

IL-6 dependent JAK/STAT pathways activated by PM2.5

As shown in Fig. 6, the protein expression of IL-6, JAK1 and STAT3 were increased significantly compared to that of the

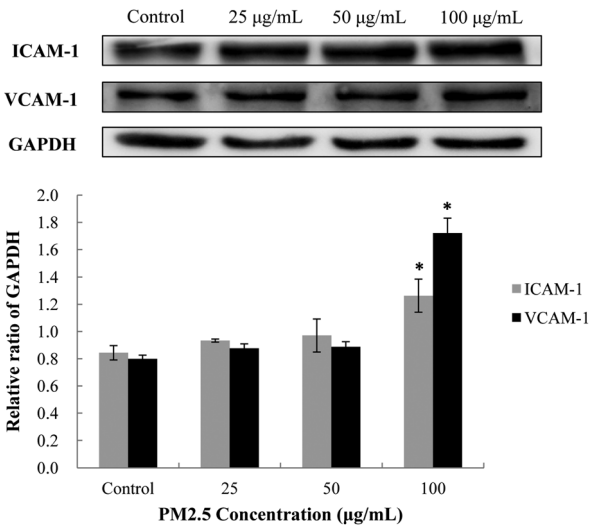

Fig. 4 Effects of PM2.5 on cellular adhesion molecule expression in HUVECs. The protein levels of VACM-1 and ICAM-1 were up-regulated significantly in the PM2.5-treated groups compared to the control. Data are expressed as the means ± S.D. from three independent experiments (*$p < 0.05$).

control. The protein levels of IL-6, JAK1 and STAT3 in the 100 μg mL^{-1} of PM2.5-treated group were up-regulated to 3.24-fold, 1.76-fold and 2.32-fold of the control group, respectively. In contrast, the protein levels of JAK2 and SOCS1 decreased

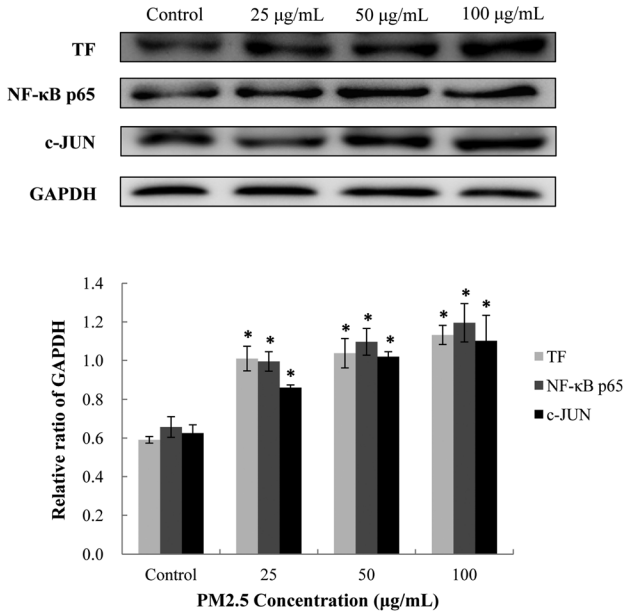

Fig. 5 Procoagulant factors triggered by PM2.5 in HUVECs. The protein levels of TF and the transcription factors NF-κB 65, AP-1/c-Jun were up-regulated markedly compared to the control group. Data are expressed as the means ± S.D. from three independent experiments (*$p < 0.05$).

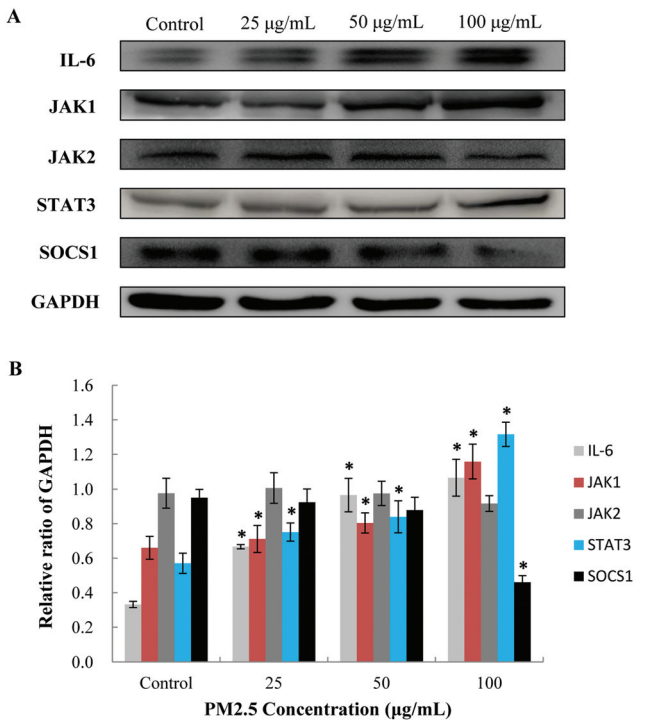

Fig. 6 Effects of PM2.5 on IL-6 dependent JAK/STAT signaling pathway. (A) Effects of PM2.5 on the expressions of IL-6, JAK1, JAK2, STAT3, and SOCS1. (B) Densitometric analysis of the proteins bands was performed and presented. Data are expressed as the means ± S.D. from three independent experiments (*$p < 0.05$).

gradually in a dose-dependent manner. In addition, the SOCS1 was significantly down-regulated in the 100 μg mL^{-1} PM2.5-treated group. Our data demonstrates that the IL-6 dependent JAK1/STAT3 signaling pathway was activated by PM2.5 in HUVECs.

Discussion

A growing body of evidence confirms that PM2.5 is associated with cardiovascular diseases such as atherosclerosis, hypertension, stroke and heart failure.[23–25] Because PM2.5 can translocate from the lungs to extrapulmonary organs *via* the systemic circulation, endothelial cells are unavoidable directly exposed to the airborne fine particles.[26] Exposure to PM2.5 has been strongly linked to the disturbance of endothelial function either in human beings or in animal models.[27,28] However, the underlying mechanism of PM2.5 on vascular endothelial cells is poorly understood. This study for the first time examined the toxicity mechanism of PM2.5 on endothelial activation *via* the IL-6 dependent JAK1/STAT3 signaling pathway in HUVECs. The PM2.5 samples used in our study were collected from Beijing, China, one of the top 15 cities with severe air pollution in the world.[18,19] This will provide new insights into the toxic effects and mechanisms of cardiovascular diseases triggered by PM2.5.

In this study, the results of the cell viability and LDH activity showed that PM2.5 induced cytotoxicity in HUVECs in a dose-dependent manner (Fig. 1). The release of LDH is a common indicator that reflects cell damage. To investigate the possible mechanism of cytotoxicity induced by PM2.5, the intracellular levels of ROS, MDA and the activity of SOD were measured. These results showed that PM2.5 caused the oxidative stress and oxidative damage in HUVECs (Fig. 2). Induction of oxidative stress by PM2.5 had been observed in various cell types, including endothelial cells.[29,30] Oxidative stress is the result of an imbalance in the pro-oxidant/antioxidant homeostasis. The antioxidant activities act as scavenging superoxide anion and H_2O_2 scavengers to prevent ROS-induced damage.[31] It was reported that the ROS acted as signal transduction agents in strained endothelium, disturb the endothelial function and are involved in the impairment of cardiovascular events.[32,33] Our data showed that the oxidative stress was responsible for the cytotoxicity caused by PM2.5 in HUVECs.

Endothelial activation, involved in the release of proinflammatory cytokines, procoagulant factors, and the upregulation of cell surface adhesion molecules, is a specific and complex change in the endothelial phenotype. During the activation process, the inflammatory response is an initial event by the activated endothelium.[34] In the present study, the data demonstrates that PM2.5 could induce the generation of proinflammatory cytokines (IL-6, TNF-a, IL-1β, IL-8 and CRP) (Fig. 3). Among them, the production of IL-6 is mainly responsible for the PM2.5-induced inflammation in HUVECs. Elevated systemic levels of IL-6 have been demonstrated to be an

independent and important predictor of cardiovascular disease.[35] IL-6 also stimulates the production of acute-phase proteins, which can promote the progress of atherogenesis by amplifying the inflammatory responses.[36] When the primary barrier of the endothelium is disturbed by the inflammatory progress, PM2.5 can impair the endothelial and vascular functions, lead to unexpected effects on the human cardiovascular system and even cause cardiovascular disease.

It is well documented that the proinflammatory cytokines (*e.g.*, IL-6, TNF-a, IL-1β) can stimulate the expression of cellular adhesion molecules.[11] Cell adhesion is crucial for regulating a series of cellular functions such as cell growth, migration, and differentiation.[37] This study showed that the ICAM-1 and VCAM-1 were increased gradually after HUVECs were exposed to PM2.5 (Fig. 4). In cardiovascular system, the upregulation of cell adhesion molecules are another characteristic of endothelial activation. ICAM-1 and VCAM-1, as members of the immunoglobulin superfamily of proteins, are important for regulating the adhesion of leukocytes to the vascular endothelium.[38] A recent study found that PM2.5 induced the cell surface expression of ICAM-1 and VCAM-1 *via* upregulation of the ERK/AKT/NF-κB signaling pathway.[11] NF-κB is the molecular target of ROS and is involved in vascular inflammation.[39] In addition, the NF-κB is one site of the human TF promoter in the distal region and the other one is AP-1/c-JUN.[40]

TF, a 47 kDa protein, regulated by the transcription factors, NF-κB and AP-1/c-JUN, is the primary activator of the coagulation cascade. To explore the in-depth mechanism of TF expression, the protein levels of TF, NF-κB p65, and c-JUN were measured by western blot analysis. Our results demonstrated that the protein levels of TF, NF-κB p65, and c-JUN were elevated significantly in the PM2.5-treated groups compared to the control group (Fig. 5). Under physiological conditions, TF is not expressed by circulating blood cells; while under pathologic conditions, TF can be expressed on the membrane surface of the activated vascular endothelium. In that way, it may lead to disseminated intravascular coagulation (DIC) and thrombosis.[41,42] Moreover, it was reported that the TF could be up-regulated by IL-6 dependent JAK/STAT signalling pathway.[43]

Finally, the IL-6 dependent JAK/STAT pathway was examined by a western blot assay. The results demonstrated that the IL-6 dependent JAK1/STAT3 signaling pathway was activated by PM2.5 in HUVECs (Fig. 6). The IL-6 family plays an important role in the pathological process of cardiovascular disease, the IL-6 mediated JAK signaling pathway mediated endothelial cells growth, survival and apoptosis, involved mechanism in the inflammatory/immune response, angiogenesis regulation.[44] JAK1 and JAK2 are the key members of the Janus family of tyrosine kinases, which are activated by ligands binding to a number of associated cytokine receptors.[45] Activated IL-6 receptor signal transduction components, glycoprotein 130, lead to the phosphorylation of the downstream protein JAK and phosphorylate their associated receptors to provide multiple binding sites for the signaling proteins. One of the main downstream signaling proteins, STAT, is targeted to increase the procoagulant factors such as TF.[46] The proinflammatory, procoagulant state of activated endothelium has been implicated in several cardiovascular diseases such as the early stages of atherosclerosis, hypertension, and ischaemic heart disease (IHD).[47]

Conclusions

In this study, we explored the toxic effects and underlying mechanism of PM2.5 on vascular endothelial cells (HUVECs), and found that PM2.5 induced cytotoxicity, followed by oxidative stress and oxidative damage in HUVECs. In addition, PM2.5 triggered the intracellular levels of proinflammatory cytokines, cell adhesion molecules and procoagulant factors, which led to endothelial activation *via* upregulation of the IL-6 dependent JAK1/STAT3 signaling pathway. This will provide experimental evidence for the pathological process of cardiovascular diseases induced by ambient air pollution.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledgements

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