The Role of Estrogen Receptor $\beta$ in PM$_{2.5}$ Organic Extract-Induced Pulmonary Inflammation in Female and Male Mice

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Research Article

Keywords: PM$_{2.5}$, organic extract, estrogen receptor $\beta$, pulmonary inflammation, sex difference, cytokine

Posted Date: January 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1192765/v1

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Abstract

Automobile exhaust is one of the main sources of PM$_{2.5}$ in urban areas, and traffic-originated PM$_{2.5}$ organic extract (Po) can promote inflammation in different organs, especially in the lung. In addition, sex differences were reported in many inflammatory diseases, but the triggering factors of sex differences in inflammatory responses are still unclear. In this study, we investigated the effects of Po exposure on pulmonary inflammatory responses and evaluated the role of sex in this process. Mice were exposed to 100 µg/m$^3$ Po for 12 weeks by an inhalation exposure system in a polycarbonate chamber. In Po-exposed mice, the lung histopathological analysis shown obvious inflammation, the cell numbers in bronchoalveolar lavage fluid (BALF) were significantly increased, and most inflammatory cytokines in BALF were upregulated. Factorial analysis of variance was carried out to investigate the interaction between sex and Po exposure in these effects. The results shown that the increase of inflammatory cell numbers and the fluctuation of cytokine levels (TNF-α, IL-5, and GRO/KC) in BALF induced by Po were significantly different between female and male mice. Notably, these differences were diminished while Po-exposed mice were injected with PHTPP, an ER$\beta$ antagonist (1 mg/kg, i.p.). We hypothesized that Po-induced inflammatory responses were different between female and male mice, and ER$\beta$ was involved in these processes. To our knowledge, this is the first investigation about the role of sex in Po-induced adverse effects. This study provided a theoretical basis for understanding the sex differences in the adverse effects of environmental pollutants.

1. Introduction

With rapid urbanization and economic growth, the use of motorized transport in China is increasing (Jiang et al. 2017). Li et al. reported that automobile exhaust is the main source of PM$_{2.5}$ in urban areas (Li et al. 2019). As PM$_{2.5}$ pollution has seriously affected human health, it has been identified as a public health problem (Atkinson et al. 2014). In our previous study, traffic-originated PM$_{2.5}$ was used to prepare water-soluble extract (Pw), organic extract (Po) and carbon core component (Pc). More importantly, the experimental results suggested that the biological effects of Po were greater than Pw and Pc (Wei et al. 2016). In addition, the chemical components of PM$_{2.5}$ and Po, including 23 types of metals and 16 types of PAHs, also have been described in the previous study (Wei et al. 2016). Existing epidemiological and experimental studies suggested that PAHs in PM$_{2.5}$ could promote inflammation in different organs, especially in lung tissue (Lag et al. 2020, Vogel et al. 2020, Zhang et al. 2016).

Several epidemiological studies have reported that there were sex differences in many inflammatory diseases. For example, there are gender biases in disease incidences for many autoimmune diseases, such as Addison's disease, scleroderma, systemic lupus erythematosus, Sjogren's syndrome, and thyroiditis (Cooper & Stroehla 2003). The incidence rates of some types of cancer were significantly different between men and women, such as kidney cancer (Lucca et al. 2015), lung cancer (Mederos et al. 2020) and colorectal cancer (Nguyen et al. 2009). The pulmonary inflammatory disease - chronic obstructive pulmonary disease (COPD) was originally recognized as a male disease in history, but now...
COPD is more and more common in women (Aryal et al. 2013). The prevalence of COPD in women was higher than that in men among the non-smoking people (Birring et al. 2002). Chronic inflammation in the lung may arise from a combination of environmental influences and genetic susceptibilities (Racanelli et al. 2018). For example, the susceptibility to tobacco smoke among patients with COPD was different between men and women (Dransfield et al. 2006).

Clougherty reviewed 209 epidemiological studies to discuss the role of sex in respiratory effects of air pollution. The author reported that more studies observed stronger adverse effects in women, although the sources of effect modification by sex remained unclear and some studies reported controversial results (Clougherty 2010). However, few experimental articles reported sex differences in the influences of environmental pollutants. And to our knowledge, there is no experimental evidence of sex differences in the Po-induced adverse effects. Considering that PAHs in PM$_{2.5}$ could promote inflammatory responses and there were sex differences in many inflammatory diseases, we supposed that there might be differences in the process of Po-induced pulmonary inflammation between men and women.

Estrogen receptors (ER) family, including estrogen receptor $\alpha$ (ER$\alpha$), estrogen receptor $\beta$ (ER$\beta$) and GPER, not only can act as signal transducers or transcription factors to regulate the expression of target genes (Flamment et al. 2012, Zhou et al. 2015), but also can regulate inflammation in different organs. For example, ER$\alpha$ is closely related to the occurrence and development of vascular inflammation and atherosclerosis (Kassi et al. 2015); The inflammatory response orchestrated by nuclear factor-κB signaling can be more serious by aberration in the ER$\beta$ (Wang et al. 2020); and GPER plays an important role in inflammation-mediated diseases because GPER can be expressed in immune cells and have an effect on the immune cells (Notas et al. 2020). Most notably, both Po and sex might be closely associated with the activation of ER signaling pathways. On the one hand, the PAHs in Po have estrogenic properties and were regarded as endocrine disruptors (Li et al. 2012, Zhang et al. 2016); on the other hand, ER might contribute to the reported sex differences in innate immune pathways (Kovats 2015). Therefore, we supposed that ER might be involved in the sex differences of Po-induced pulmonary inflammatory responses.

In this study, we performed an animal experiment in female and male mice. We investigated the effects of Po on pulmonary inflammation in mice, evaluated the differences of Po-induced inflammatory responses between female and male mice and further explored whether ERs took part in this process.

2. Materials & Methods

2.1 Collection of PM$_{2.5}$ and preparation of PM$_{2.5}$ organic extract

PM$_{2.5}$ and its organic extract were prepared according to our previous study (Wei et al. 2016). In order to obtain comparable components of the samples to the most extent, we followed strictly the same procedure, including the sampler, sampling season, sampling place, and the extraction of organic
components. Briefly, the PM\textsubscript{2.5} samples were collected in winter (November to December) by a large-volume PM\textsubscript{2.5} sampler (Intelligent 2031, Qingdao Laoying Inc., China), which was set on the top of a building (approximately 30 meters high) near Chongqing South Road (a two-way two-lane road with very dense traffic). The sampling filter membranes were taken out from the sampler after collecting PM\textsubscript{2.5} for 5 to 7 days, balanced in the dryer for 24 hours, wrapped in tin foil and stored at -20°C. The extraction method of Po has been demonstrated in our previous articles (Guo et al. 2020, Wei et al. 2016). Finally, the Po extract was dissolved in dimethyl sulfoxide in 400 µg/ml and stored at -20°C.

2.2 Animals and experimental design

6-7 weeks old C57BL/6 mice (24 female and 24 male mice) were obtained from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China) and raised in the Animal Experimental Center of the Ninth People’s Hospital of Shanghai, and their care was in accordance with institution guidelines which was approved by the Committee on Animal Use and Care of Shanghai Jiao Tong University School of Medicine, China. All mice were fed in cages of the same size with a maximum of three mice per cage and placed in the same room with fresh air in the SPF level animal experimental center. An inhalation exposure system (Shanghai Raymain Information Technology Co., Ltd.) was used for the animal experiment, which can real-time monitor dynamic parameters in the exposure chamber, including the concentration of Po aerosols, temperature, humidity, O\textsubscript{2} and CO\textsubscript{2} concentration. The experimental procedure has been described in our previous article (Luo et al. 2020), with additional male mice in the present study. Briefly, the female and male mice were respectively divided randomly into three groups (8 mice/group): a, Control groups; b, Po groups; c, Po + PHTPP groups. The mice in the control groups were left untreated; the mice in other two groups were exposed to 100 µg/m\textsuperscript{3} Po for 12 weeks (four hours a day, five days a week) by the inhalation exposure system in a polycarbonate chamber. Meantime, the mice of Po + PHTPP groups were injected with PHTPP (Sigma-Aldrich) once per week (1 mg/kg, i.p.) from the fifth week. The weight of mice was measured and recorded on the first day of each week during these experiments. The mice were sacrificed at the day after the last exposure. The lung tissues and bronchoalveolar lavage fluid (BALF) were collected.

2.3 Western blotting

The protein levels of lung tissues were quantified by western blotting. The standard protocol for western blotting has been described previously (Luo et al. 2017). Briefly, the proteins were separated and transferred onto membranes. The membranes were incubated with primary antibodies against ER\textalpha (1:1000, #8644, CST), ER\beta (1:1000, ab3576, abcam), GPER (1:1000, ab137479, abcam), overnight with agitation at 4°C. Then, the appropriate secondary antibodies were used to incubate the bands for one hour at room temperature. The protein band density was detected using Quantity One Software (Bio-Rad, USA), and quantified using Image J software.

2.4 Preparation and Cell Counts of Bronchoalveolar Lavage Fluid (BALF)
The detailed method of obtaining BALF has been described in a previous article (Luo et al. 2020). Briefly, BALF were centrifuged at 1000×g for 5 min at 4°C. The supernatant was stored at -80°C to measure the levels of inflammatory cytokines, and the cells at the bottom were resuspended with 1 ml PBS (contains 0.1% BSA). The total cell numbers for each sample were counted by the hemocytometer.

### 2.5 Hematoxylin and Eosin (HE) staining

The left lung tissues were fixed in 4% paraformaldehyde and then were embedded in paraffin after dehydration. After embedding with paraffin, these tissues were cut into 5-µm-thick sections and stained with hematoxylin and eosin. The sections were examined, photos were taken with a Pannoramic SCAN (3DHISTECH, Hungary).

### 2.6 Measurement of inflammatory cytokine levels in BALF

The inflammatory cytokine levels in BALF (including IL-1β, IL-6, GRO/KC, IL-10, IL-12p70, TNF-α and IFN-γ) were determined by the MSD proinflammatory panel 1 (mouse) according to the manufacturer's instructions (#K15048G-1, Maryland), and the data were acquired by Meso QuickPlex SQ 120 plate reader.

### 2.7 Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. The experiment data are shown as the mean ± SD. The significance level was defined as 0.05. Interaction between the factors was analyzed by factorial analysis of variance ($P<0.05$, there is an interaction between the factors; $P>0.05$, there is no interaction between the factors). In addition, when there was a significant difference in the test of homogeneity of variance for experimental data, the data were log10-transformed before statistical modeling. If there was still a significant difference in the homogeneity test of variance for log10-transformed data, the data were analyzed by Scheirer-Ray-Hare test (Hughes et al. 2020). The graphs were made by GraphPad Prism 7.0 software.

### 3. Results

#### 3.1 Changes in body weight

We evaluated the body weights of female and male mice once a week to see if the mice were healthy. As shown in Fig. 1 and our previous report (Luo et al. 2020), during the entire period of the animal experiment, there was no obvious difference in the changes of body weight between groups of female or male mice.

#### 3.2 The effects of Po on pulmonary inflammation in female and male mice

Our previous study has proved that Po can induce pulmonary inflammation in female mice (Luo et al. 2020). We further included male mice in the present experiment. Histological analysis shown that obvious inflammatory cell infiltration was observed in the lung tissue of female and male mice after Po
exposure (Fig. 2a). Po could also increase total cell numbers in the BALF of the mice (Fig. 2b; Table 1 and 2). Meanwhile, Po could upregulate the levels of several inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-5, IL-6, GRO/KC and IL-12p70) in the BALF of the mice (Fig. 2c-2i; Table 1 and 2).

We further investigated if there were significant differences in inflammatory cell numbers and cytokine levels in BALF between female and male mice. We found that the inflammatory cell numbers and IFN-γ level in the BALF of the female mice were significantly higher than those of the male mice (Fig. 2a and 2b; Table 1 and 2), and the IL-1β level in the BALF of the female mice was significantly lower than that of the male mice (Fig. 2e; Table 1 and 2). There were no significant differences in the levels of TNF-α, IL-5, IL-6, GRO/KC and IL-12p70 in BALF between female and male mice (Fig. 2d and 2f-2i; Table 1 and 2). The results indicated that there were differences in the numbers of inflammatory cell and the levels of IFN-γ and IL-1β between female and male mice.

To explore the cause of inflammatory response differences induced by Po between female and male mice, factorial analysis of variance was used to analyze the interaction of sex and Po exposure in experimental mice. The results shown that there was an interaction between sex and Po exposure in the inflammatory cell numbers and the levels of TNF-α, IL-5, and GRO/KC (Table 1 and 2). The results implied that the increase of inflammatory cell numbers and the fluctuation of TNF-α, IL-5, and GRO/KC levels induced by Po were different between female and male mice.

### 3.3 The effects of Po on the levels of estrogen receptors in lung tissues of mice

To determine whether Po exposure affect the expression of estrogen receptors, the protein levels of ERα, ERβ and GPER in the lung tissues of experimental mice were investigated. As shown in Fig. 3, while the mice were exposed to Po, the protein levels of ERβ in the lung tissues of female and male mice were upregulated, and the protein levels of ERα and GPER were no obvious change in the lung tissues of female and male mice (Fig. 3a and 3b).

### 3.4 The effects of ERβ on Po-induced pulmonary inflammation in mice

We further explored whether ERβ could play an important role in Po-induced pulmonary inflammation in mice by using the ERβ antagonist, PHTPP. As shown in Fig. 4, PHTPP could significantly reduce the infiltration degree of inflammatory cell in lung tissues as well as the inflammatory cell numbers in BALF induced by Po (Fig. 4a and 4b; Table 1 and 2). Meanwhile, the Po-induced increases of IFN-γ, TNF-α IL-5, IL-6, GRO/KC and IL-12p70 levels in BALF were diminished by PHTPP (Fig. 4d and 4h; Table 1 and 2). The results suggested that ERβ contributed to Po-induced inflammatory cell infiltration in lung tissues as well as Po-induced increases of some proinflammatory cytokines levels in the BALF of the mice. Therefore, we summarized that ERβ was one of the contributors for Po-induced pulmonary inflammation.
As shown in Table 1 and 2, we speculated that the Po-induced increase of inflammatory cell numbers and the fluctuation of TNF-α, IL-5, and GRO/KC levels in BALF were different between female and male mice by factorial analysis of variance. Then, we further evaluated whether ERβ participated in the Po-induced inflammatory response differences. We investigated the interaction between sex and Po+PHTPP treatment in experimental mice. The results indicated that there was no interaction in inflammatory cell numbers and the levels of TNF-α, IL-5, and GRO/KC between sex and Po+PHTPP treatment (Table 1 and 2). It means that PHTPP might diminish the interaction between sex and Po exposure which have been reported in 3.2. The results implied that PHTPP could eliminate the differences in the Po-induced increase of inflammatory cell numbers and the fluctuation of TNF-α, IL-5 and GRO/KC levels in BALF between female and male mice.

4. Discussion

In this study, we investigated the effects of Po exposure on pulmonary inflammatory responses and evaluated the role of sex in this process in female and male mice. Most importantly, based on the above findings, we elucidated the role of ERβ in this process. To our knowledge, this is the first investigation about the role of sex in Po-induced adverse effects.

Existing epidemiological and experimental studies have shown that PAHs in PM$_{2.5}$ can promote the release of inflammatory cytokines, such as IL-1β, IL-8, TNF-α, and IL-6 (Falcon-Rodriguez et al. 2016, Kuang et al. 2020, Manzano-Leon et al. 2016). Our previous study has proved that Po contained more than half (53.75%) of the total polycyclic aromatic hydrocarbons (PAHs) in PM$_{2.5}$ (Wei et al. 2016). Therefore, we further evaluated whether Po could induce pulmonary inflammation in mice. We found that Po could induced inflammatory cell infiltration in lung tissues of mice, increased the inflammatory cell numbers and upregulated the levels of several inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-5, IL-6, GRO/KC and IL-12p70) in the BALF of the mice (Fig. 2; Table 1 and 2). Thus, Po could cause pulmonary inflammation in mice, and might have an influence on the immune microenvironment through regulating the inflammatory cytokines, which might lead to lung injury. Park et al. reported that Po can induce inflammatory responses of lung cells, which is consistent with this study (Park et al. 2021).

Existing evidences have suggested that pulmonary inflammation may arise from a combination of genetic susceptibilities and environmental influences (Racanelli et al. 2018), and there are differences in the susceptibility to tobacco between women and men (Dransfield et al. 2006). In addition, there are sex differences in some inflammatory diseases (Carey et al. 2007, Klein &Flanagan 2016, Laws et al. 2018, Ngo et al. 2014, Rainville et al. 2018, Samet et al. 2009, Zore et al. 2018). Among nonsmoking patients with COPD, there were more women than men (Birring et al. 2002). These studies indicated that sex might plays an important role in the pulmonary inflammation. In this study, we found that the increase of inflammatory cell numbers, and the fluctuation of TNF-α, IL-5, and GRO/KC levels in BALF induced by Po were different between female and male mice (Table 2). TNFα, GRO/KCC and IL-5 are pro-inflammatory factors that have been reported to participate in inflammatory and immune responses. The function of GRO/KC is similar to IL-8 in rat (Dong et al. 2012). IL-5 is important to eosinophils which is related to the
fibrosis and long-term tissue injury. Some asthma patients will suffer from diseases characterized by eosinophilic airway inflammation (Dougan et al. 2019). Studies reported that inflammatory cytokines may contribute to some inflammatory diseases, such as COPD, pulmonary fibrosis, asthma (Hou et al. 2018, Narendra & Hanania 2019, Russell & Brightling 2016, Zhang et al. 2019). Therefore, Po might cause differences in these diseases between men and women through regulating the levels of inflammatory cytokines.

As we all know, hormonal difference is an important reason why sex differences appear in many diseases. Our previous study revealed that Po contained more than half (53.75%) of the total PAHs with DBA, PHE, BPE, IPY, BaP, BbF, BkF and CHR as the main compositions (Wei et al. 2016), and these substances, as environmental endocrine disruptors, have been confirmed to have estrogenic activity (Zhang et al. 2016). An existing study shown that PAHs could selectively induce ERβ transcriptional activity, while did not activating ERα (Sievers et al. 2013); Additionally, PAHs were able to competitively bind ERβ, induce ERβ homodimers, and regulate ERβ target genes (Sievers et al. 2013). ERα, ERβ and GPER belong to the estrogen receptor family, and they are major mediators of estrogenic signals (Barzi et al. 2013, Prossnitz & Barton 2011). The present study was the first to explore the association of Po with ERs (ERα, ERβ and GPER). The results shown that Po exposure could regulate the protein levels of ERβ, but not ERα and GPER, in lung tissues of both female and male mice, which indicating that Po exposure could activate ERβ signaling in lung tissues of mice.

ERβ not only can acts on the breast and uterus whose function were closely related to estrogen, but also can acts on the lung (Chen et al. 2011, Niikawa et al. 2008). Abnormal ERβ signaling can lead to inflammation in different organs, such as the lung (Jia et al. 2015, Watanabe et al. 2019), cardiovascular (Novella et al. 2019) and uterus (Spence & Voskuhl 2012). Considering that Po exposure might activate ERβ signaling in the process of Po-induced pulmonary inflammation in mice, we further evaluated whether ERβ played a key role in this process by using PHTPP, an ERβ antagonist. The results shown that PHTPP treatment can interfere with Po-induced inflammatory cell infiltration in lung tissues and Po-induced increases of the inflammatory cell numbers and the levels of IFN-γ, TNF-α IL-5, IL-6, GRO/KC and IL-12p70 in the BALF of the mice (Fig. 4; Table 1 and 2). Therefore, we speculated that ERβ was one of the contributors for Po-induced pulmonary inflammation in mice. Moreover, ERs have been reported to contribute to sex differences in pulmonary fibrosis, asthma and allergic inflammation (Elliot et al. 2019, Keselman & Heller 2015). According to the above results, we focused on the ERβ signaling pathway and explore whether ERβ participate in the process of Po-induced inflammatory response differences by sex. Intriguingly, we found that ERβ did play a role in the Po-induced inflammatory response differences (including the increase of inflammatory cell numbers and the fluctuation of the TNF-α, IL-5 and GRO/KC levels in BALF) between female and male mice and the differences were diminished by PHTPP (Table 2).

In summary, we explore the effects of sex in Po-induced inflammatory responses and evaluate the role of ERβ in this process, which provided a theoretical basis for understanding the sex difference in the adverse effects of environmental pollutants.
5. Conclusion

Po could induce pulmonary inflammation in both female and male mice. While exposed to Po, the increase of inflammatory cell numbers induced by Po was different between female and male mice. And the fluctuation of several inflammatory cytokine levels in BALF shown different pattern between female and male mice. Further analysis suggested that ERβ was involved in these processes.

Declarations

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Authors contributions

Huaqi Guo: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. Hengyi Yu: Methodology, Formal analysis, Investigation, Writing - Original Draft. Yan Feng: Investigation, Resources. Wei Cheng: Resources. Yan Li: Resources. Yan Wang: Supervision, Funding acquisition, Writing - review & editing.

Funding

This work was supported by the Natural Science Foundations of China (21777100). We are sincerely thankful for the constructive suggestions of anonymous reviewers.

Ethical approval

This work was approved by the Committee on Animal Use and Care of Shanghai Jiao Tong University School of Medicine, China.

Competing interests

All authors declared they had no competing interests.

Consent to participate

All authors known and agreed to participate in this manuscript.

Consent to publish

All authors agreed to the publication of the article if the article is accepted.

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Tables

Table 1, 2 is available in the Supplemental Files section.

Figures
Figure 1

*The body weight changes of experimental mice.* C57BL/6 mice (24 female and 24 male mice) divided randomly into three groups (8 mice/group), and the mice were weighed once a week. Control groups: untreated, Po groups: Po exposure, Po+PHTPP groups: Po exposure and PHTPP treatment.
The effects of Po on pulmonary inflammation in female and male mice. C57BL/6 female mice and male mice with or without Po exposure (8 mice/group). (a) Representative images of the lung histopathological section by HE staining (200×). (b) the number of inflammatory cells in BALF. (c-i) The IFN-γ, TNF-α, IL-1β, IL-5, IL-6, GRO/KC, IL-12p70 levels in BALF. All experiment data are shown as the mean ± SD.
Figure 3

The effects of Po on estrogen receptor levels in lung tissues. C57BL/6 female mice and male mice with or without Po exposure (8 mice/group). (a) and (b) The protein levels of ERα, ERβ and GPER in lung tissue of female and male mice were quantified by western blotting.
Figure 4

The effects of ERβ on pulmonary inflammation induced by Po in female and male mice. C57BL/6 female mice and male mice exposed to Po with or without PHTPP treatment (8 mice/group). (a) Representative images of the lung histopathological section by HE staining (200x). (b) the number of inflammatory cells in BALF. (c-i) The IFN-γ, TNF-α, IL-1β, IL-5, IL-6, GRO/KC, IL-12p70 levels in BALF. All experiment data are shown as the mean ± SD.

Supplementary Files

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