






Alteration in angiotensin-converting enzyme 2 by PM₁ during the development of emphysema in rats

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ABSTRACT

Introduction: Angiotensin-converting enzyme 2 (ACE2) provides an adhesion site for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Patients with COPD could have severe outcomes after SARS-CoV-2 infection. The objective of this study was to investigate ACE2 regulation by air pollution during the development of COPD.

Methods: Sprague Dawley rats were exposed to unconcentrated traffic-related air pollution for 3 and 6 months. We examined lung injury markers, oxidative stress, inflammation, emphysema, ACE2 and angiotensin II receptor type 1 (AT1) and 2 (AT2) in the lungs after exposure.

Results: Lung injury occurred due to an increase in permeability and lactate dehydrogenase cytotoxicity was observed after 6 months of exposure to fine particulate matter of <1 µm in aerodynamic diameter (PM₁). An α₁-antitrypsin deficiency and neutrophil elastase production with emphysema development were observed after 6 months of PM₁ exposure. 8-isoprostane and interleukin-6 were increased after 3 and 6 months of PM₁ exposure. Caspase-3 was increased after exposure to PM₁ for 6 months. Upregulation of ACE2 was found after 3 months of PM₁ exposure; however, ACE2 had decreased by 6 months of PM₁ exposure. AT1 and AT2 had significantly decreased after exposure to PM₁ for 6 months. Furthermore, smooth muscle hypertrophy had occurred after 6 months of PM₁ exposure.

Conclusions: In conclusion, short-term exposure to PM₁ increased the ACE2 overexpression in lungs. Long-term exposure to PM₁ decreased the ACE2 overexpression in emphysema. Air pollution may be a risk for SARS-CoV-2 adhesion during the development of COPD.

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Short-term exposure to PM₁ increases ACE2 overexpression in lungs. Long-term exposure to PM₁ decreases the ACE2 overexpression in emphysema. Air pollution may be a risk for #SARSCoV2 adhesion during the development of COPD. <https://bit.ly/2Vfykur>

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or coronavirus disease 2019 (COVID-19) has been declared a public health emergency of international concern by the World Health Organization. The SARS-CoV-2 epidemic has resulted in 334981 confirmed cases with 14652 deaths reported (as of 24 March 2020). SARS-CoV-2 was identified as a highly infectious novel coronavirus [1], which is highly infectious in adults and older adults. A recent report indicated a lower incidence rate of SARS-CoV-2 infection in patients with chronic lung diseases [2]; however, patients with a chronic lung disease could have severe outcomes once they are infected.

A report indicated that SARS-CoV-2 uses angiotensin-converting enzyme 2 (ACE2) for entry and the serine protease TMPRSS2 for S protein priming [3]. The S1 domain contains the ACE2 receptor-binding domain, which is responsible for host cell entry in the first stage [4]. The S2 domain facilitates fusion between the cell and virus membrane, which is required for cellular infiltration [5]. Recent studies demonstrated that the modified S protein of SARS-CoV-2 has a higher affinity for ACE2 [6]. SARS-CoV-2 is likely to bind to ACE2 in human cells [6]. ACE2 is involved in the synthesis of bioactive components of the renin-angiotensin system (RAS). The RAS is involved in regulating proinflammatory pathways as well as reactive stress in inflammatory-related diseases [7]. ACE2 acts as an anti-inflammatory protein by counteracting the actions of angiotensin II (AngII) [8]. Therefore, alteration in ACE2 may not only result in regulation of SARS-CoV-2 infection ability, but also associate with inflammatory responses.

COPD is known to cause deficits in ACE2 in the lungs [9, 10]. Air pollution is a risk factor for the development of COPD, which is also suspected to be associated with the SARS-CoV-2 infection ability [11, 12]. The objective of this study was to investigate the expressions of ACE2 by air pollution during the development of COPD. The data can explain the role of air pollution in SARS-CoV-2 infections.

Materials and methods

Animals

Male 6-month-old Sprague Dawley rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan), and were housed at a constant temperature of 22±2°C and a relative humidity of 55±10% with a 12-h light-dark cycle. Animal experiments were conducted in compliance with the Animal and Ethics Review Committee of the Laboratory Animal Center at Taipei Medical University (Taipei, Taiwan).

Chronic air pollution exposure

The experimental design was previously reported [13]. Rats were randomly divided into three groups: 1) a clean-air control group housed in the Laboratory Animal Center of Taipei Medical University (Taipei, Taiwan); 2) a high-efficiency particulate air (HEPA) group (exposed to gaseous pollution only; New Taipei City, Taiwan); and 3) a PM₁ (particulate matter <1 µm in aerodynamic diameter) exposure group (exposed to particulate and gaseous pollution; New Taipei City, Taiwan). There were 16 rats in each group in this study. Rats were whole-body exposed to PM₁ using a whole-body exposure system for 3 and 6 months. The whole-body exposure system for rodents was described previously [13, 14] and was located at a traffic-dominated area near a highway and an expressway. PM₁ mass (by a tapered element oscillating microbalance) and number concentrations (by a scanning mobility particle sizer and average particle size), black carbon (by an aethalometer) and lung deposition surface area concentrations (by a nanoparticle surface area monitor) were continuously monitored during the exposure period. Gaseous pollution was referenced from the nearby Taiwan Environmental Protection Administration Yonghe air quality monitoring station (<https://taqm.epa.gov.tw/taqm/en/default.aspx>). The left lung was used for bronchoalveolar lavage fluid (BALF) and tissue collection, whereas the right lung was used for histology. An animal necropsy was performed and tissues were collected as described previously [15].

Biochemistry

Total protein (Thermo Fisher Scientific, Waltham, MA, USA) and lactate dehydrogenase (LDH) cytotoxicity (Thermo Fisher Scientific) were determined in BALF. α₁-antitrypsin (Cusabio, Wuhan, Hubei, China), neutrophil elastase (Cusabio), 8-isoprostane (Cayman, Ann Arbor, MI, USA), interleukin (IL)-6 (R&D Systems, Minneapolis, MN, USA), caspase-3 (Elabscience, Houston, TX, USA), ACE2 (Elabscience), AngII receptor type 1 (AT1) (Elabscience) and AngII receptor type 2 (AT2) (Elabscience) were determined in lung lysates using ELISA. All analyses were in accordance with the manufacturers' instructions. Data obtained from lung lysates are presented after adjusting for the β-actin level (Cell Signaling, Danvers, MA, USA).

Histology and emphysema score

Haematoxylin and eosin staining of the lungs was performed according to our previous report [16]. The mean linear intercept (MLI), an indicator of the mean alveolar diameter, was assessed in 10

nonoverlapping fields according to a previous report [17]. Histological examinations were conducted under light microscopy.

Statistical analysis

Comparisons among multiple groups were performed using an ANOVA with Tukey's *post hoc* test. Statistical analyses were performed using GraphPad version 5 for Windows. The level of significance was set to $p < 0.05$. Data are expressed as the mean \pm SD.

Results

Emphysema occurred after PM₁ exposure

Rats were whole-bodily exposed to unconcentrated traffic-related air pollution for 3 and 6 months. After exposure, we observed that the total protein and LDH in BALF had significantly increased at 6 months compared to the control and the HEPA groups ($p < 0.05$) (figure 1a). Levels of α_1 -antitrypsin had significantly decreased in both the HEPA and PM₁ groups after exposure ($p < 0.05$), especially at 6 months (figure 1a), whereas neutrophil elastase had significantly increased after 6 months of exposure ($p < 0.05$). Levels of 8-isoprostane and IL-6 were significantly increased by 3 and 6 months of PM₁ exposure ($p < 0.05$) (figure 1b). We observed that caspase-3 had significantly increased in rats after PM₁ exposure for 6 months ($p < 0.05$) (figure 1b). Next, we examined the MLI score in lungs after 3 and 6 months of exposure (figure 2). The MLI score had significantly increased due to PM₁ exposure compared to the control and HEPA groups at 6 months ($p < 0.05$).

ACE2 regulation by PM₁ exposure

Expression of ACE2 was examined in rat lungs after air pollution exposure (figure 3). Levels of ACE2 had increased at 3 months of PM₁ exposure ($p < 0.05$). However, ACE2 was significantly decreased at 6 months of PM₁ exposure ($p < 0.05$).

Smooth muscle hypertrophy occurred after PM₁ exposure

Both AT1 and AT2 had significantly decreased by PM₁ at 3 months ($p < 0.05$) (figure 3). Furthermore, we observed that smooth muscle hypertrophy occurred after 6 months of PM₁ exposure in rat lungs (figure 4).

Discussion

Air pollution has been reported to increase the risk of SARS-CoV-2 infection [11, 12]. ACE2 is considered to play an important role in regulation of SARS-CoV-2 infection ability [18]. In the present study, we observed that 3 months of exposure to fine particles (PM₁) upregulated ACE2 levels of the lungs. The 6 months of exposure to air pollution, particularly PM₁, caused an ACE2 deficiency in the lungs with the occurrence of emphysema-combined smooth muscle hypertrophy in rats. Our results suggest that: 1) short-term exposure to PM₁ increased the ACE2 overexpression in lungs, and 2) long-term exposure to PM₁ decreased the ACE2 overexpression in emphysema. The alteration in ACE2 expression of lungs may associate with SARS-CoV-2 infection during emphysema development.

Air pollution has been identified as a risk factor for COPD; however, the scientific evidence is mainly based on epidemiological evidence. Currently, there is still no natural route for air pollution exposure in a rodent model. To our best knowledge, this is the first report to study ACE2 expression in a rodent model with 6 months' inhalation of unconcentrated traffic-related ambient air using a whole-body exposure system. Our previous study indicated that the whole-body exposure system had the higher penetration rate of PM (from outdoor to exposure system) with a particle size $< 1 \mu\text{m}$; therefore PM₁ was the main size fraction for the whole-body exposure in this system [13, 14]. Rats were exposed to an average of $16.3 \mu\text{g}\cdot\text{m}^{-3}$ PM₁, a particle number concentration of $11\,257 \text{ particles}\cdot\text{m}^{-3}$, $1800 \text{ ng}\cdot\text{m}^{-3}$ of black carbon and $55.1 \mu\text{m}^2\cdot\text{cm}^{-3}$ of lung deposition surface area in the alveolar region during the exposure period [13]. The mean geometric mean diameter was 55.8 nm during the study period. The average ambient PM_{2.5} was 19.7 ± 9.8 (obtained from the Yonghe air quality monitoring station). The PM₁ to PM_{2.5} ratio 0.827, which is commonly observed from traffic-dominated urban area. The correlation (r^2) between PM₁ and PM_{2.5} was 0.829 ($p < 0.001$) (supplementary figure S1), suggested that the rats were mainly exposed to the outdoor air pollution during the experimental periods. Gaseous pollutants averaged 32.9 ppb for oxides of nitrogen, 2.5 ppb for SO₂ and 29.7 ppb for O₃. After 6 months of exposure to PM₁, the permeability (total protein) and cytotoxicity (LDH) of the lungs had increased. For the pathogenesis of COPD, we observed an α_1 -antitrypsin deficiency and neutrophil elastase elevation in the lungs due to PM₁ exposure. The data suggest that subchronic and chronic PM₁ exposure may cause extensive tissue damage and malfunctioning of the airways. We next investigated the development of emphysema, which is considered a phenotype of air pollution-induced COPD [19], after exposure in rats. First, we observed elevated levels of caspase-3 by PM₁ and gaseous pollutants. The data showed activation of apoptosis in lung cells after exposure.

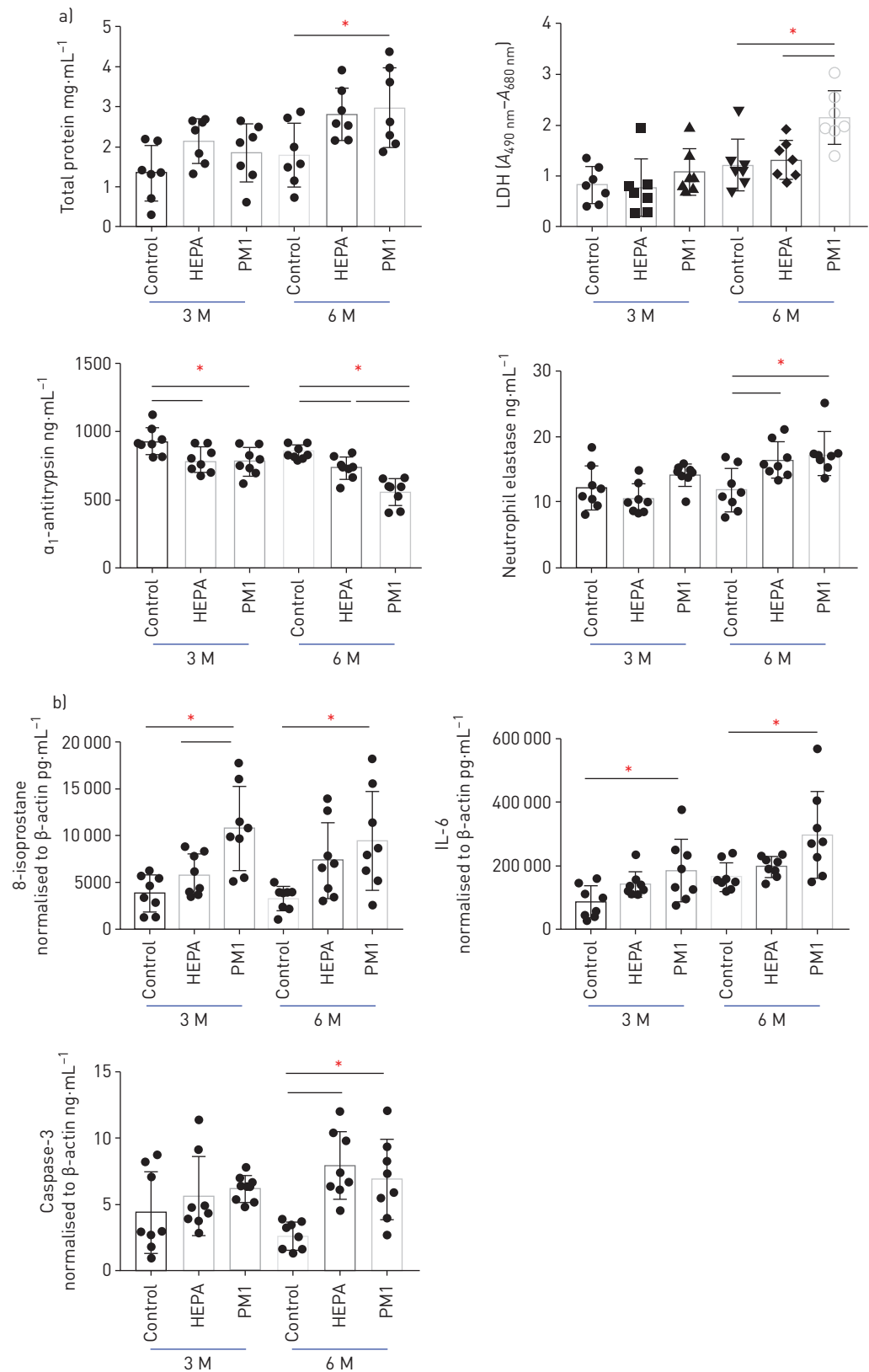


FIGURE 1 Levels of a) total protein, lactate dehydrogenase (LDH), α_1 -antitrypsin and neutrophil elastase in bronchoalveolar lavage fluid (BALF) and b) 8-isoprostane, interleukin (IL)-6 and caspase-3 in lungs after 3 and 6 months (M) of exposure to traffic-related ambient air. Rats were exposed to particulate matter with an aerodynamic diameter $<1\ \mu\text{m}$ (PM₁; PM₁ group) or high-efficiency particulate air (HEPA)-filtered air (HEPA group, gaseous pollution). Rats in the control group were housed in the animal centre. $n=8$ per group. *: $p<0.05$.

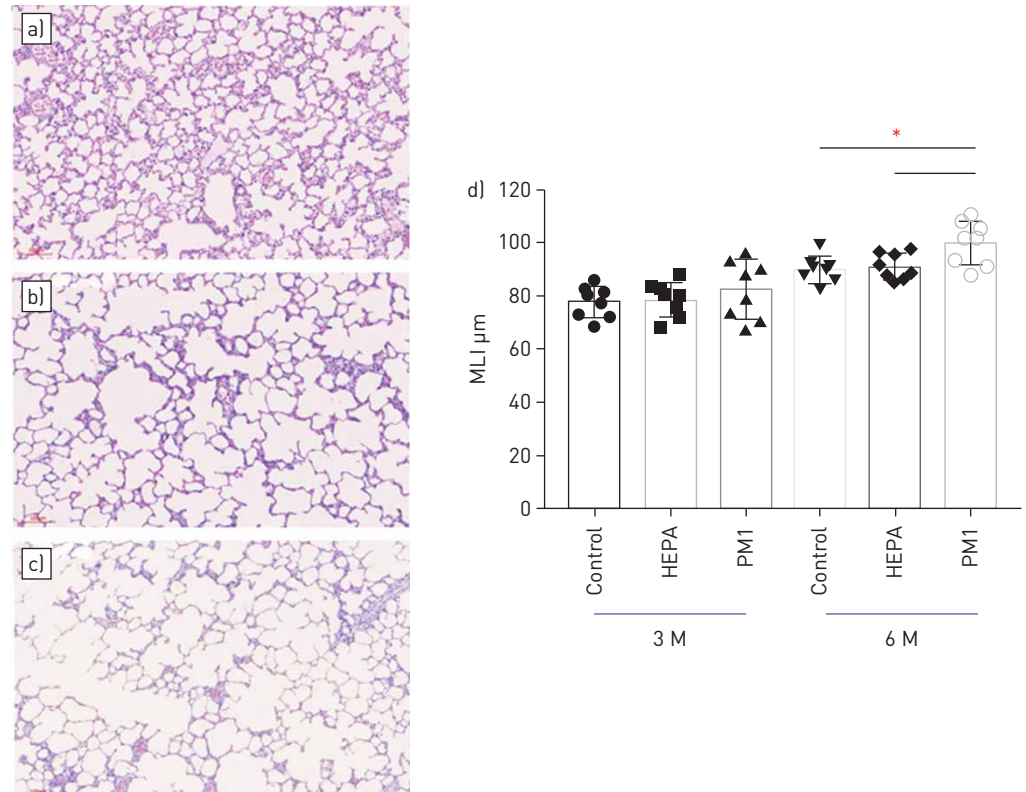


FIGURE 2 Haematoxylin and eosin staining of lungs after 6 months (M), from a) control rats, and from rats exposed to b) high-efficiency particulate air (HEPA)-filtered air or c) particulate matter with an aerodynamic diameter <1 µm (PM₁). d) Emphysema mean linear intercept (MLI) score after air pollution exposure. Significant emphysema occurred after 6 months of PM₁ exposure in rats. n=8 per group. *: p<0.05.

Consistent with caspase-3 activation, emphysema was induced by 6 months of PM₁ exposure. Our data indicated that emphysema occurred after 6 months of exposure to PM₁.

SARS-CoV-2 uses ACE2 receptors to invade cells, particularly in the lungs and heart [20]. SARS-CoV-2 shares similar mechanisms with other coronaviruses, and the outer membrane spike glycoprotein is the prime host-interacting protein with host cell targets [21]. ACE2, for example, plays important roles in cell adhesion and virulence [22]. In the present study, we first observed that 3 months of exposure to PM₁ increased ACE2 expression in rat lungs. This observation is similar to that with smoking in a previous

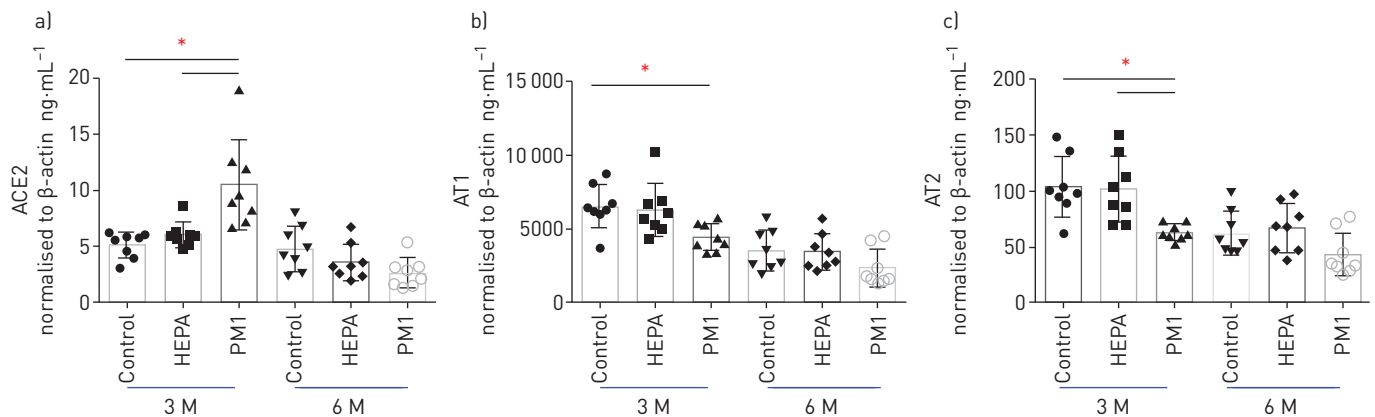


FIGURE 3 Levels of a) angiotensin-converting enzyme 2 (ACE2), b) angiotensin II receptor type 1 (AT1) and c) angiotensin II receptor type 2 (AT2) in rat lungs after 3 and 6 months (M) of exposure to traffic-related ambient air. Rats were exposed to high-efficiency particulate air (HEPA)-filtered air or particulate matter with an aerodynamic diameter <1 µm (PM₁). Rats in the control group were housed in the animal centre. n=8 per group. *: p<0.05.

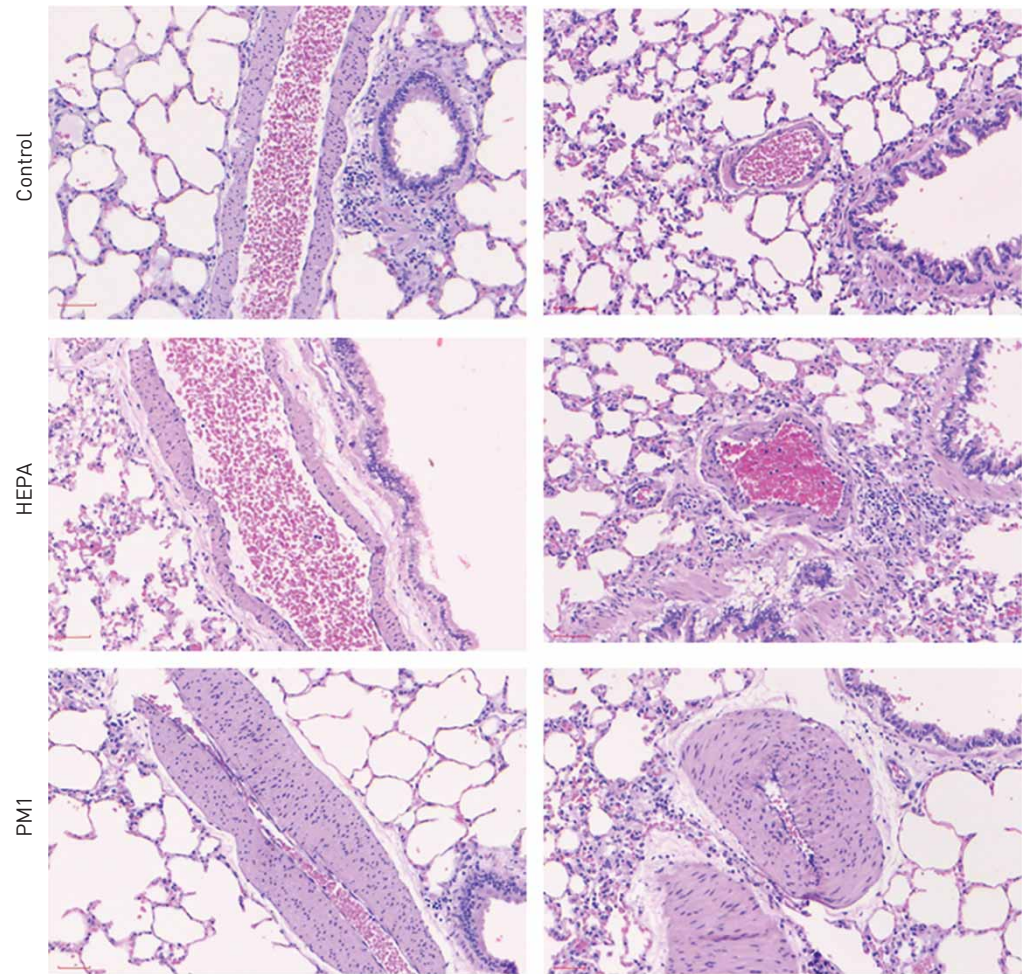


FIGURE 4 Haematoxylin and eosin staining of blood vessels after 6 months of air pollution exposure. Rats were exposed to high-efficiency particulate air (HEPA)-filtered air or particulate matter with an aerodynamic diameter $<1 \mu\text{m}$ (PM_{10}). Rats in the control group were housed in the animal centre. Two different sections of blood vessels are shown for each group. A significant increase in smooth muscle hypertrophy occurred after 6 months of PM_{10} exposure in rats.

report [20]. BRAKE *et al.* [20] indicated that smoking upregulated ACE2 expression, which provides an adhesion site for SARS-CoV-2, leading to an increased risk of SARS-CoV-2 infection. In contrast, we observed ACE2 deficiencies after 6 months of exposure to PM_{10} in the development of emphysema. A report indicated that ACE2 mRNA expression is significantly reduced in COPD rat lung [23]. ACE2 overexpression mitigated reduction of lung function and pathological manifestations of COPD [10]. The reduction of ACE2 in emphysema could decrease the risk of SARS-CoV-2 infection. However, increasing reports indicated that COPD patients had higher severity after SARS-CoV-2 infection [24, 25].

The particle effects on ACE2 were also found in a previous study, which indicated that ACE2-knockdown mice showed increased pulmonary phosphorylated signal transducer and activator of transcription 3 (STAT3) and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 levels after fine particle-induced acute lung injury [26]. It is worth noting that emphysema occurred after 6 months of exposure to PM_{10} . The ACE2 deficiency implies that RAS dysregulation may play a pivotal role in the development of COPD. Inflammation is also an adverse effect resulting from ACE2 reduction in the lungs. The ACE2 deficiency may cause uncontrolled oxidative stress and inflammatory responses in COPD. Indeed, we observed that 8-isoprostane and IL-6 persisted at higher levels in lungs even after 6 months of PM_{10} exposure. Such adverse effects by ACE2 deficiency may induce more severe outcomes in COPD patients once they have been infected by SARS-CoV-2. Therefore, although there may be low levels of ACE2 adhesion sites for SARS-CoV-2, there may be higher levels of inflammatory responses in COPD after 6 months of PM_{10} exposure.

ACE2 is elevated during episodes of lung inflammation, which is a consequence of an increase in the vascular permeability. The main active peptides of the RAS include AngII, AngIII, AngIV and Ang1-7.

AngII and Ang-1-7 play more important roles in terms of health and disease [7]. Recently, ACE2 and their peptides were reported to play key roles in inflammatory processes in conditions such as pulmonary hypertension [27]. Therefore, in the present study, we further investigated AT1 and AT2 expression. We observed reductions in AT1 and AT2 after 3 months of PM₁ exposure. Our observations were consistent with a previous study of placental structures [28], which suggested that PM₁ altered the RAS pathway. Downregulation of AT1 and AT2 by PM₁ could result in vasodilation and vasoconstriction dysfunction. Importantly, we observed smooth muscle hypertrophy of the pulmonary artery after 6 months of PM₁ exposure. An association of air pollution with hypertrophy was also evident in cardiovascular diseases [29, 30]; however, this is the first study to identify smooth muscle hypertrophy of lungs by PM₁ exposure. Together, patients with pulmonary hypertension could have low risk for SARS-CoV-2 infection.

The strength of this study is that the ACE2 expression during air pollution-induced emphysema was investigated. ACE2 has different expression between short-term and long-term PM₁ exposure. We further showed that smooth muscle hypertrophy occurred due to PM₁. The limitations of this study included that the contributions of physicochemical characteristics of PM₁ to COPD remain unclear. Expression of other proteins such as TMPRSS2 and cathepsin L was not determined in the present study. A confirmatory experiment may need to be conducted on SARS-CoV-2 adhesion sites in the future. SARS-CoV-2 might replicate more efficiently in cells in COPD; therefore, leading to more severe outcomes.

Conclusions

In conclusion, we provide biological evidence to explain how short-term exposure to PM₁ upregulated ACE2 for SARS-CoV-2 adhesion sites. Moreover, long-term exposure to PM₁ downregulated ACE2 in emphysema. Air pollution may be a risk factor for SARS-CoV-2 infection during the development of COPD.

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