*Clinical Science* (2021) **135** 2575–2588
https://doi.org/10.1042/CS20210802

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Research Article

# Paternal long-term $PM_{2.5}$ exposure causes hypertension via increased renal $AT_1R$ expression and function in male offspring

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Maternal exposure to fine particulate matter ($PM_{2.5}$) causes hypertension in offspring. However, paternal contribution of $PM_{2.5}$ exposure to hypertension in offspring remains unknown. In the present study, male Sprague-Dawley rats were treated with $PM_{2.5}$ suspension (10 mg/ml) for 12 weeks and/or fed with tap water containing an antioxidant tempol (1 mM/L) for 16 weeks. The blood pressure, 24 h-urine volume and sodium excretion were determined in male offspring. The offspring were also administrated with losartan (20 mg/kg/d) for 4 weeks. The expressions of angiotensin II type 1 receptor ($AT_1R$) and G-protein–coupled receptor kinase type 4 (GRK4) were determined by qRT-PCR and immunoblotting. We found that long-term $PM_{2.5}$ exposure to paternal rats caused hypertension and impaired urine volume and sodium excretion in male offspring. Both the mRNA and protein expression of GRK4 and its downstream target $AT_1R$ were increased in offspring of $PM_{2.5}$-exposed paternal rats, which was reflected in its function because treatment with losartan, an $AT_1R$ antagonist, decreased the blood pressure and increased urine volume and sodium excretion. In addition, the oxidative stress level was increased in $PM_{2.5}$-treated paternal rats. Administration with tempol in paternal rats restored the increased blood pressure and decreased urine volume and sodium excretion in the offspring of $PM_{2.5}$-exposed paternal rats. Treatment with tempol in paternal rats also reversed the increased expressions of $AT_1R$ and GRK4 in the kidney of their offspring. We suggest that paternal $PM_{2.5}$ exposure causes hypertension in offspring. The mechanism may be involved that paternal $PM_{2.5}$ exposure-associated oxidative stress induces the elevated renal GRK4 level, leading to the enhanced $AT_1R$ expression and its-mediated sodium retention, consequently causes hypertension in male offspring.

## Introduction

Cardiovascular disease has become an uncontrolled global epidemic and a burgeoning cause of morbidity and mortality. Hypertension is the leading risk factor for cardiovascular disease and all-cause mortality worldwide [1]. Now, it is well accepted that hypertension, not a simple genetic disease, is a complex heterogeneous disorder caused by genetic, epigenetic, behavioral, and environmental factors and their intricate interactions [2–4].

Currently, an increasing number of studies have shown that fetal programming through different environmental exposures during a critical window in the early stages of life, including pre-conceptional,

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Received: 10 August 2021
Revised: 10 November 2021
Accepted: 15 November 2021

Version of Record published: 26 November 2021

Clinical Science (2021) 135 2575–2588
https://doi.org/10.1042/CS20210802

*in utero* and early post-natal periods, affect long-term health outcomes, including hypertension, in later adulthood, which is also named developmental origins of health and disease (DOHaD) [5,6]. Our and other studies have shown that mothers can transmit the influences induced by some factors such as infection, hyperglycemia, temperature and anxiety, to their offspring [7–10].

Exposure to ambient fine particles, particulate matter with an aerodynamic diameter of ≤2.5 μm ($PM_{2.5}$), is one of the leading preventable threats to global health [11]. Evidence have shown that ambient fine particulate matter exposure increases the blood pressure in female subjects or animals [12,13]. Furthermore, our and other studies have found that maternal exposure to fine particulate matter causes elevated blood pressure in their offspring [14,15]. On the other hand, we also reported that long-term exposure of $PM_{2.5}$ enhances the level of blood pressure in Sprague-Dawley (SD) rats [16]. However, whether or not paternal long-term $PM_{2.5}$ exposure causes hypertension in their offspring remains unclear. We hypothesized that paternal exposure to environmental fine particulate matter may lead to increased blood pressure in offspring. Therefore, in the present study, we investigated paternal $PM_{2.5}$ exposure-induced regulation on the blood pressure in male offspring and determined the underlying mechanisms.

# Methods

## $PM_{2.5}$ sampling

The sampling period began on March 1, 2018 and ended on June 1, 2018. The $PM_{2.5}$ sample collection site was located at Daping Hospital, about 1 km from the center of Chongqing city. The closest main road is 100 meters northeast of the hospital. The monitoring location has a radius of about 200 meters and is almost completely surrounded by residential areas.

The method of $PM_{2.5}$ sampling has been reported in our previous studies [14,16]. In brief, a medium volume sampler (model TH-150; Tianhong Co, Wuhan, China) with a filtering system was used to collect $PM_{2.5}$ samples on the filter (diameter, 150 mm). A total of 30 filters were used to collect $PM_{2.5}$ samples. The flow of the medium volume sampler is modulated to 30 $m^3$/h. After sampling, the filter was shredded into small pieces, and then ultrasound was carried out in the double distilled water soaking the pieces for 1 h using an ultrasonic machine (KQ-250DE; Shumei, Kunshan, Jiangsu, China). The extract was frozen, freeze-dried and concentrated, and the extraction efficiency was measured by weighing. The farinose solids were stored at −80°C for the next use.

## Animal treatment

Six-week-old SD rats were purchased from the Animal Centre of The Third Military Medical University, Chongqing, China. All procedures used in this study were approved by the Third Military Medical University Animal Use and Care Committee. All experiments conformed to the guidelines for the ethical use of animals. Animals were maintained and treated in the Animal Centre of Daping Hospital.

Male SD rats with body weight of 160–190 g were divided into $PM_{2.5}$ treatment group and control group with 10 rats in each group. The models were established with $PM_{2.5}$ suspension and PBS solution by drip irrigation, respectively. After anesthesia with isoflurane inhalation, the head and neck were backward exposed to the airway, and the tongue was fixed with rubber band. Then $PM_{2.5}$ suspension (10 mg/ml) 30 μl was slowly infused into the tongue base of the rats. The control group was infused with the same amount of PBS solution as vehicle-treated. After 12 weeks (twice a week) of drip irrigation, male rats were mated with normal SD female rats at a ratio of 1:2, and two groups of offspring were obtained, respectively. To avoid the influence of estrogen on the blood pressure, we only used the male offspring in our present study. Then, at the age of 12 weeks, the male offspring of vehicle-treated paternal rats were assigned into control group (vehicle-exposed paternal offspring administrated with vehicle) and control+losartan group (vehicle-exposed paternal offspring administrated with losartan); the offspring of $PM_{2.5}$-treated paternal rats were divided into $PM_{2.5}$ group ($PM_{2.5}$- exposed paternal offspring administrated with vehicle) and $PM_{2.5}$+losartan group ($PM_{2.5}$-exposed paternal offspring administrated with losartan). Treatment with losartan in offspring means that offspring of $PM_{2.5}$-treated paternal rats were selected to receive intragastric administration with losartan (20 mg/kg/d) for 4 weeks (once a day). The diagram of the above animal experiment is shown in Flow diagram 1.

In another set of animal experiment, paternal rats were treated with both $PM_{2.5}$ and an antioxidant tempol (Flow diagram 2). In brief, six-week-old paternal rats treated with $PM_{2.5}$ or vehicle were randomly assigned into the following experimental groups: paternal rats were exposed with vehicle for 12 weeks and fed with normal tap water or tap water containing 1 mM/l tempol (Sigma, Poole, Dorset, U.K.) for 16 weeks; paternal rats were exposed with $PM_{2.5}$ for 12 weeks and fed with normal tap water or tap water containing 1 mM/L tempol for 16 weeks. After above treatments, paternal rats were then mated with normal female SD rats, and offspring were obtained. Then, at the age of 12 weeks, the blood pressure measurement and urine/blood analysis of male offspring were performed. There are four

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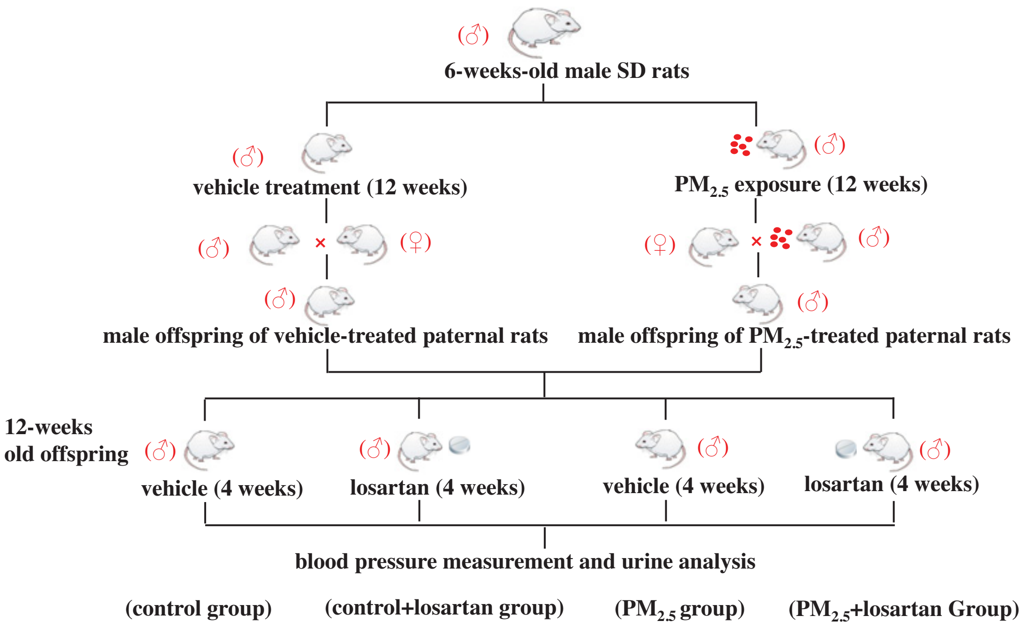
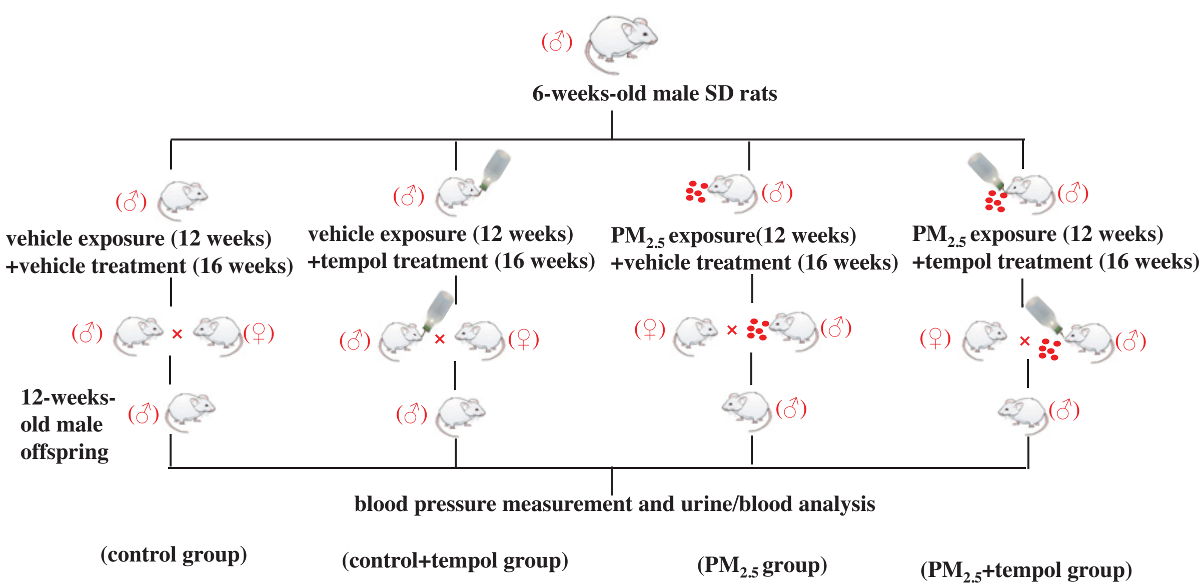



**Flow diagram 1. The diagram of the animal experiment set 1**

Control offspring: the vehicle-treated paternal offspring treated with saline; control+losartan offspring: the vehicle-treated paternal offspring treated with losartan (20 mg/kg/d, 4 weeks, once a day); $PM_{2.5}$ offspring: the $PM_{2.5}$-exposed paternal offspring treated with saline; $PM_{2.5}$+losartan offspring: the $PM_{2.5}$-exposed paternal offspring treated with losartan (20 mg/kg/d, 4 weeks, once a day).




**Flow diagram 2. The diagram of the animal experiment set 2**

Control offspring: offspring of paternal rats treated with vehicle and PBS; Control+ tempol offspring: offspring of paternal rats treated with tempol (1 mM/L, 16 weeks); $PM_{2.5}$ offspring: offspring of paternal rats treated with $PM_{2.5}$; $PM_{2.5}$+tempol offspring: offspring of paternal rats treated with both $PM_{2.5}$ and tempol (1 mM/L, 16 weeks).

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groups of offspring as shown accordingly: control group (offspring of vehicle-exposed- and vehicle-treated paternal rats), $PM_{2.5}$ group (offspring of $PM_{2.5}$-exposed- and vehicle-treated paternal rats), control+tempol group (offspring of vehicle-exposed- and tempol-treated paternal rats) and $PM_{2.5}$+tempol group (offspring of $PM_{2.5}$-exposed- and tempol-treated paternal rats).

After paternal or offspring rats were sacrificed under pentobarbital anesthesia (60 mg/kg), the kidneys were homogenized in ice-cold lysis buffer with proteinase inhibitor cocktail (Thermo Scientific, Waltham, MA, U.S.A.), sonicated, placed on ice for 1 h and centrifuged at 12,000 rpm for 30 min at 4°C. The upper layer of the pellet was re-suspended in the homogenization buffer, which was considered as the total protein of renal tissue. Finally, the supernatants were stored at −70°C until use for immunoblotting.

## Blood pressure measurement and urine/blood analysis

To ensure the reliability of the measurements, rats were trained for one week to acclimatize them to the process of measurement. Blood pressure was measured using a computerized noninvasive tail-cuff manometry system (MODEL MK-2000; Muromachi Kikai Co. Ltd, Tokyo, Japan) in conscious rats between 2 and 5 PM every day, as reported in our previous studies [17,18].

Urine was collected in metabolic cages, and the 24 h urine volumes and sodium excretions were also measured at the indicated times. The urine sodium concentration in the urine was analyzed by a flame photometer 480 (Ciba Corning Diagnostics, Norwood, MA, U.S.A.). Serum creatinine and urea nitrogen levels were measured with commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In addition, random blood glucose was also measured with a glucose analyzer (Roche, Indianapolis, IN, U.S.A.).

## Histological analysis

Kidneys of offspring rats and lungs of paternal rats were isolated, washed several times with PBS and fixed with 4% paraformaldehyde buffer for 48 h at 4°C. Then, samples were dehydrated and embedded in paraffin, cut into 5-μm-thick sagittal sections, and mounted on glass slides. Then deparaffinizing and rehydrating using xylene and different concentrations of ethanol. At last, slides were stained with hematoxylin and eosin (H&E). Slides were observed using a microscope (ECLIPSE Ti; Nikon, Tokyo, Japan).

## Biochemical markers of oxidative stress

To assess the level of systematic oxidative stress, the lipid peroxidation product malondialdehyde (MDA) in the kidney was quantified using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). To assess the level of antioxidants, renal samples from rats were used to measure superoxide dismutase (SOD) activity using a SOD assay kit (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions.

## Immunoblotting

The protein expressions of angiotensin II type 1 receptor ($AT_1R$), G-protein–coupled receptor kinase type 4 (GRK4), GAPDH and tubulin were determined by immunoblotting, as reported in our previous studies [14,16–18]. In brief, equal amounts of total extracted proteins (100 μg) were separated on SDS-PAGE and were transferred onto nitrocellulose membranes (Amersham Life Science, Arlington, TX). The blots were subjected to immunoblot analyses with the primary polyclonal antibodies for rabbit anti-$AT_1R$ (1:1000; Proteintech Group, Rosemont, IL, U.S.A.), anti-GRK4 (1:500; Abcam, Cambridge, U.K.), anti-GAPDH (1:1000; Beyotime, Shanghai, China) and for mouse anti-tubulin (1:1000; Beyotime, Shanghai, China) overnight at 4°C. The membranes were washed with phosphate buffered saline with Tween 20 (PBST, 0.05% Tween-20 in 10 mmol/L phosphate-buffered saline) and then incubated with infrared-labeled secondary antibodies for 1 h at room temperature. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences). The images were analyzed using the ImageJ Application Software (National Institutes of Health, Bethesda, MD) to obtain the integrated intensities.

## Real-time (RT) quantitative PCR

Total RNA was isolated and quantified as described previously [18,19]. cDNA was synthesized from 2 μg of total RNA using cDNA synthesis kit (High Capacity RNA to cDNA Kit; Takara, Tokyo, Japan). PCRs were carried out using the Brilliant SYBR Green QPCR Master Mix kit (High Capacity RNA to cDNA Kit; Takara, Tokyo, Japan) in a total volume of 25 μl. For $AT_1R$, the forward primer was 5′-TCCACCCAATGAAGTCTCGC-3′ and the reverse primer was 5′-ATTCTTGGTAAGGCCCAGCC-3′. For GRK4, the forward primer was 5′-ACTTCAGCAGACTGGAAGCA-3′, and the reverse primer was 5′-GGTGTCCAGGTTGACTCCTT-3′. For GAPDH served as a housekeeping/reference

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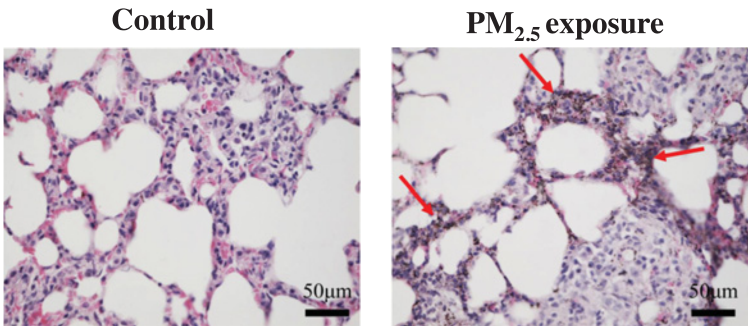


**Figure 1. Lung histology from control- and $PM_{2.5}$-exposed SD rats**

Representative light microscopy sections of lung tissues from vehicle- and $PM_{2.5}$-treated SD rats. Unabsorbed particles in the alveoli are presented in different power images. Red arrow means fine particulate matter deposition; magnification 400×.

**Table 1 Basic characteristics of the control- and $PM_{2.5}$-treated SD rats before fine particulate matter exposure**

| Characteristics | Control | $PM_{2.5}$ |
|---|---|---|
| Body Weight (g) | 168.00 ± 1.89 | 166.60 ± 1.61 |
| SBP (mm Hg) | 101.20 ± 3.92 | 102.90 ± 4.55 |
| 24 h urine volume (ml/kg weight) | 23.03 ± 1.59 | 21.52 ± 1.55 |
| 24 h sodium excretion (mmol/kg weight) | 1.69 ± 0.12 | 1.64 ± 0.11 |

These data were collected in 6-week-old vehicle (control)- and $PM_{2.5}$-treated paternal SD rats before fine particulate matter exposure. Results are mean ± SEM, $n$=10.

gene for normalization, the forward primer was 5′-GCCCAGCAAGGATACTGAGA-3′ and the reverse primer was 5′-GATGGTATTCGAGAGAAGGGAGG-3′. The amplification profile used on the BIO-RAD CFX96 (Bio-Rad Laboratories) was 95°C for 3 min followed by 40 cycles of 95°C per 10 s and 72°C per 30 s. qRT-PCR experiments were repeated for three times.

## Statistical analysis

All data are expressed as means ± SEMs. Statistical significance between experimental groups was determined using the ANOVA with Tukey's post hoc test or unpaired *t* test when only two groups were compared. Statistical analysis was carried out using a software program (GraphPad Prism version 7; GraphPad Software, San Diego, CA). A value of $P<0.05$ was considered statistically significant.

# Results

## $PM_{2.5}$ exposure increases the blood pressure and decreases sodium excretion in SD rats

Results of HE staining showed that $PM_{2.5}$ exposure model was successfully established, which demonstrated that, as compared with the control rats, there was obvious particulate matter deposition in the lung of $PM_{2.5}$-exposed SD rats (Figure 1).

No difference was found between the control and $PM_{2.5}$-treated rats, regarding to body weight, blood pressure, 24 h urine volume and sodium excretion before $PM_{2.5}$ exposure (Table 1). However, long-term (12 weeks) $PM_{2.5}$ exposure caused a remarkable elevation in both systolic blood pressure (SBP) and diastolic blood pressure (DBP) in SD rats (Figure 2A,B), accompanied with decreased sodium excretion, determined by basal levels of 24 h urine volume and sodium excretion (Figure 2C,D). Moreover, the differences in the levels of blood pressure between the control and $PM_{2.5}$-treated rats progressively increased with time (Figure 2E). It is noticed that there was no difference in the weights of the $PM_{2.5}$-treated rats and control rats (Figure 2F).

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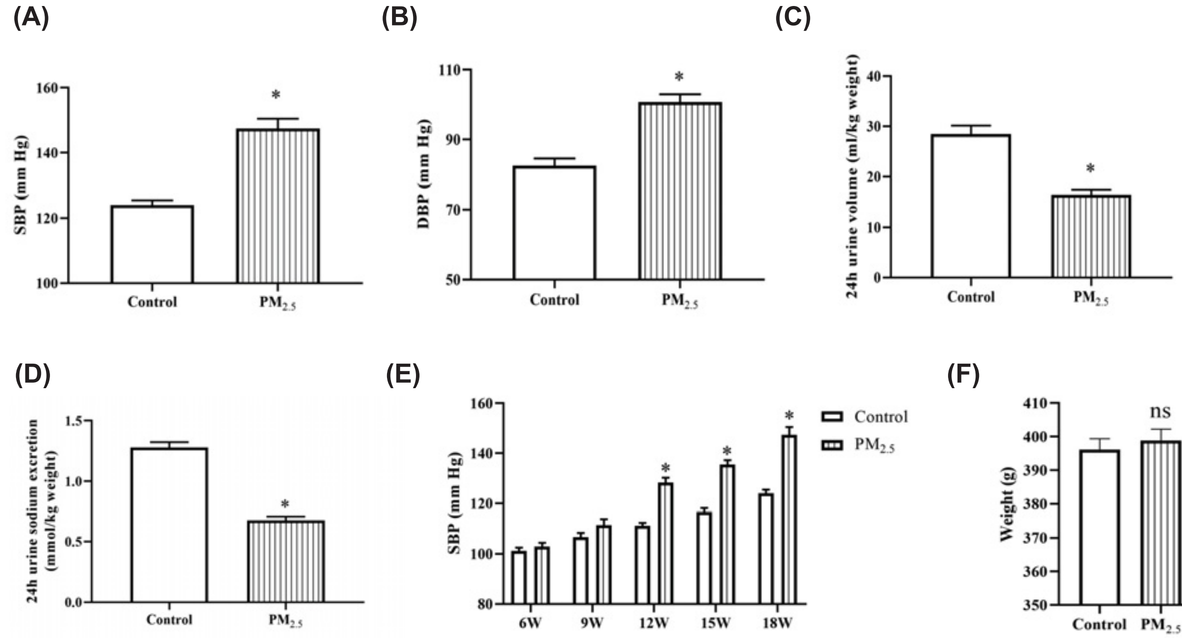


**Figure 2. Effect of $PM_{2.5}$ exposure on the regulation of blood pressure and sodium excretion in SD rats**

(**A** and **B**) Systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) were measured by the tail-cuff method after 12-weeks $PM_{2.5}$ exposure (A, two-tailed, unpaired *t* test with Welch's correction, $^{*}P < 0.05$ vs. control, $n = 10$; B, two-tailed, unpaired *t* test, $^{*}P < 0.05$ vs. control, $n = 10$). (**C** and **D**) 24 h urine volume (C) and sodium excretion (D) were determined in the control and $PM_{2.5}$-exposed SD rats after 12-week $PM_{2.5}$ exposure (C and D): two-tailed, unpaired *t* test, $^{*}P < 0.05$ vs. control, $n = 10$). (**E**) Differences of the SBP among vehicle- (control) and $PM_{2.5}$-exposed SD rats aged 6-, 9-, 12-, 15- and 18-week-old (one-way ANOVA with Tukey's post hoc test, $^{*}P < 0.05$ vs. control, $n = 10$). (**F**) Weights of vehicle- (control) and $PM_{2.5}$-exposed SD rats (two-tailed, unpaired *t* test, ns. $P > 0.05$ vs. control, $n = 10$).

## Paternal $PM_{2.5}$ exposure causes hypertension and impaired sodium excretion in male offspring rats

To show the effect of $PM_{2.5}$ exposure on the blood pressure of male offspring rats, $PM_{2.5}$ exposed-male SD rats were inbred with vehicle-treated female SD rats to get the offspring rats. Our results showed that SBP was higher in the offspring of $PM_{2.5}$-treated paternal rats than the offspring of vehicle-treated control rats, which was in age-dependent manner (Figure 3A). The elevated SBP may be, at least in part, due to the impaired natriuresis because the 12-week-old offspring rats also showed decreased sodium excretion, determined by 24 h urine volume and sodium excretion (Figure 3B,C). It is noted that the renal structure (Figure 3D) and functions, determined by plasma urea nitrogen and creatinine concentrations, were normal, and there were no differences, regarding to body wight, heart rate, random plasma glucose level and kidney/weight ratio (Table 2), between $PM_{2.5}$-offsprings and controls.

## Increased $AT_1R$ and GRK4 expression and function in the offspring of $PM_{2.5}$-treated paternal rats

Renal $AT_1R$, a G-protein–coupled receptor (GPCR), plays a vital role in the regulation of sodium balance and blood pressure [20]. To determine whether or not $AT_1R$ is involved in the paternal $PM_{2.5}$ exposure-induced hypertension in offspring, the transcriptional and translational levels of $AT_1R$ were measured. We found that both renal $AT_1R$ mRNA and protein expressions were elevated in the 12-week-old male offspring of $PM_{2.5}$-exposed paternal rats compared with the offspring of vehicle-treated paternal rats (Figure 4A,B). Then, losartan, an $AT_1R$ antagonist, was used to determine whether the aggravated expression of $AT_1R$ was reflected in its function. Results showed that treatment with losartan markedly decreased SBP in the offspring of $PM_{2.5}$-exposed paternal rats as compared with the offspring of control rats (Figure 4C). We also found that treatment with losartan normalized the impaired 24 h urine volume and sodium excretion in the offspring of $PM_{2.5}$-treated paternal rats; however, losartan had no natriuretic and diuretic effects in the offspring of control paternal rats (Figure 4D,E).

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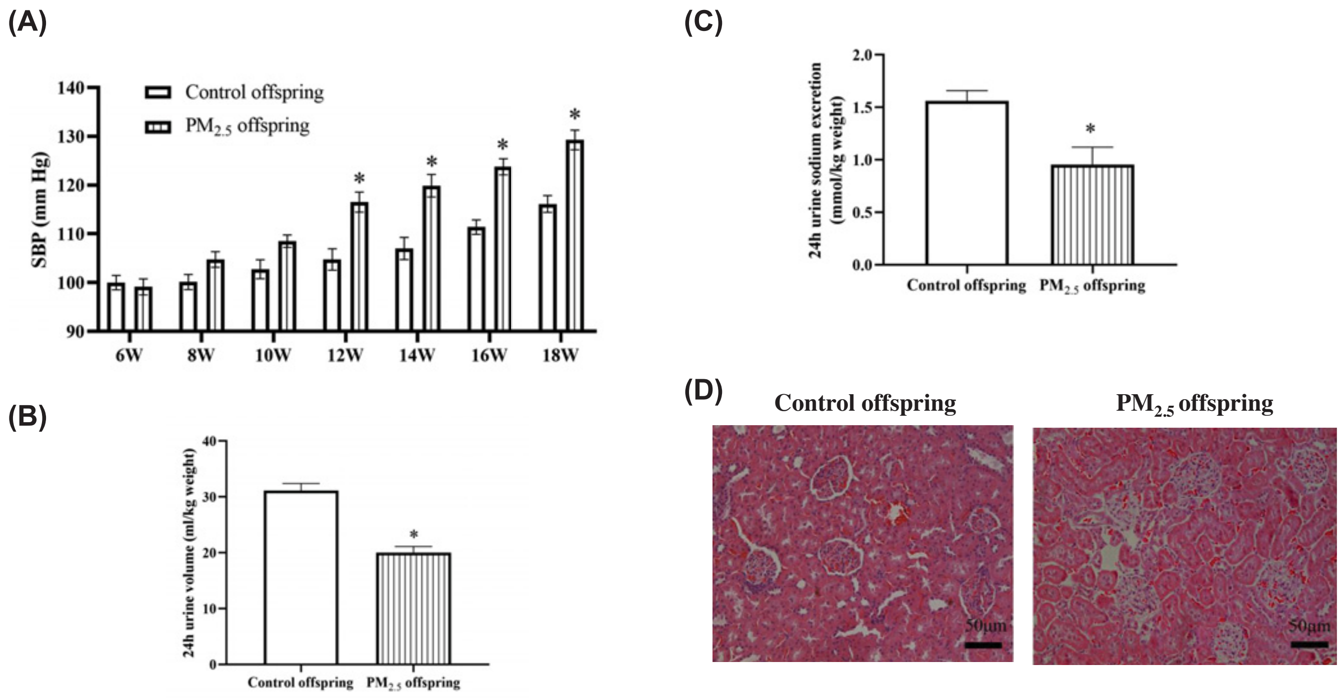


**Figure 3. Effects of paternal $PM_{2.5}$ exposure on the regulation of blood pressure and renal functions in offspring**
(**A**) Systolic blood pressure (SBP) in the different weeks old $PM_{2.5}$ offspring (one-way ANOVA with Tukey's post hoc test, *$P <$ 0.05 vs. control offspring, $n = 8$). (**B** and **C**) 24 h urine volume (B) and sodium excretion (**C**) in the 12-week-old $PM_{2.5}$ offspring (B and C, two-tailed, unpaired *t* test, *$P < 0.05$ vs. control offspring, $n = 8$). (**D**) Renal histopathology was examined by H&E staining in the 12-week-old $PM_{2.5}$ offspring; magnification 200×. Control offspring: offspring of vehicle-exposed paternal SD rats; $PM_{2.5}$ offspring: offspring of $PM_{2.5}$-exposed paternal rats.

**Table 2 Basic characteristics of the male offspring from paternal rats with different treatments**

| Characteristics | Control | Control+tempol | $PM_{2.5}$ | $PM_{2.5}$+tempol |
|---|---|---|---|---|
| Body weight (g) | 263 ± 1.73 | 267 ± 3.53 | 264 ± 4.98 | 271 ± 4.49 |
| Kidney/weight (g/kg weight) | 3.48 ± 0.07 | 3.57 ± 0.20 | 3.52 ± 0.05 | 3.37 ± 0.11 |
| Heart rate (beats/min) | 337 ± 4 | 340 ± 6 | 342 ± 8 | 343 ± 7 |
| Plasma creatinine (mmol/l) | 28.30 ± 0.87 | 28.47 ± 0.96 | 30.02 ± 1.13 | 28.68 ± 1.04 |
| Plasma urea nitrogen (mmol/l) | 5.86 ± 0.26 | 5.49 ± 0.3 | 5.70 ± 0.24 | 5.67 ± 0.22 |
| Random plasma glucose (mmol/l) | 7.14 ± 0.16 | 7.07 ± 0.12 | 6.98 ± 0.07 | 7.12 ± 0.15 |

These data were collected in 12-week-old male offspring of paternal rats with different treatments. Results are mean ± SEM, $n = 8$. Control group: offspring of vehicle-exposed- and vehicle-treated paternal rats; $PM_{2.5}$ group: offspring of $PM_{2.5}$- exposed- and vehicle-treated paternal rats; Control+tempol group: offspring of vehicle-exposed- and tempol-treated paternal rats; $PM_{2.5}$+tempol group: offspring of $PM_{2.5}$-exposed- and tempol-treated paternal rats.

Our previous studies have shown that the expression and function of $AT_1R$ are mainly regulated by GRK4 [21,22]. Thus, we determined whether the increased expression and function of renal $AT_1R$ were regulated by GRK4 in the offspring of $PM_{2.5}$-treated paternal rats. Results showed that compared with the offspring of vehicle-treated paternal rats, both renal GRK4 mRNA and protein expressions were elevated in the 12-weeks-old offspring of $PM_{2.5}$-exposed paternal rats (Figure 4F,G). These suggested that the GRK4/$AT_1R$ pathway may be involved in the paternal $PM_{2.5}$ exposure-induced hypertension in male offspring.

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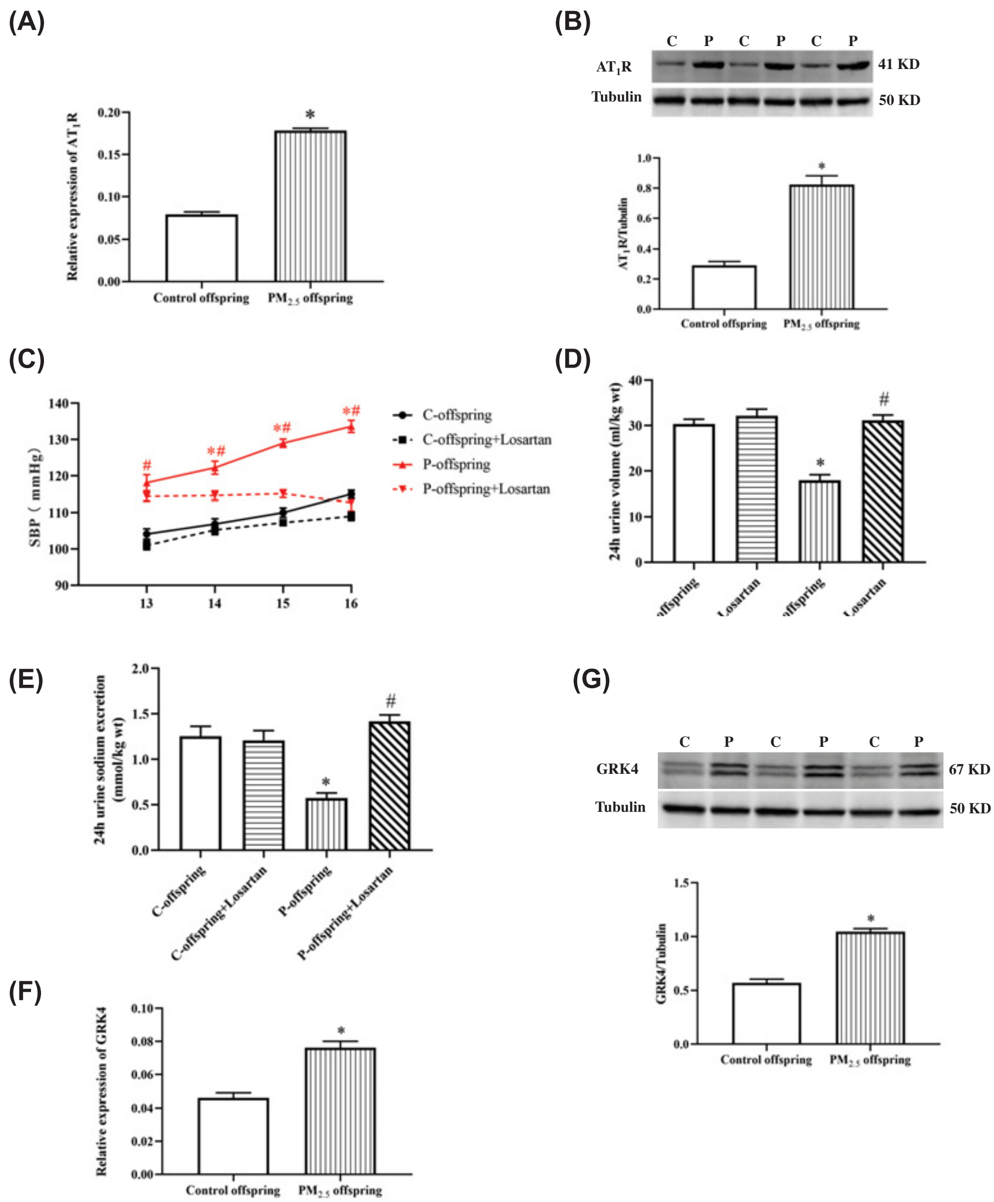


**Figure 4. $AT_1R$ and GRK4 expression and function in the offspring of $PM_{2.5}$-exposed paternal rats**

(**A** and **B**) The mRNA (A) and protein expression (B) of $AT_1R$ were determined by qt-PCR and immunoblotting in the 12-week-old $PM_{2.5}$ offspring. $AT_1R$ mRNA level was normalized using GAPDH. The protein expression of $AT_1R$ was normalized using tubulin expression (A and B, two-tailed, unpaired *t* test, $^*P < 0.05$ vs. control, $n = 6$). (**C**) Effect of losartan (20 mg/kg/d, 4 weeks) on the systolic blood pressure (SBP) in the $PM_{2.5}$ offspring (two-way ANOVA with Tukey's post hoc test, $^*P < 0.05$ vs. P- offspring +losartan, $n = 8$; $^{\#}P < 0.05$ vs. C-offspring, $n = 8$). (**D** and **E**) Effect of losartan (20 mg/kg/d, 4 weeks) on the 24 h urine volume (D) and sodium excretion (E) in the $PM_{2.5}$ offspring. (D and E, two-way ANOVA with Tukey's post hoc test, $^*P < 0.05$ vs. C-offspring, $n = 8$; $^{\#}P < 0.05$ vs. P-offspring, $n = 8$). (**F** and **G**) The mRNA (F) and protein expression (G) of GRK4 were determined by qt-PCR and immunoblotting in the 12-week-old $PM_{2.5}$ offspring. $AT_1R$ mRNA level was normalized using GAPDH. The protein expressions of GRK4 were normalized using tubulin expression (F and G, two-tailed, unpaired *t* test, $^*P < 0.05$ vs. C-offspring, $n = 6$). Control offspring (C or C-offspring): offspring of vehicle-exposed paternal SD rats; $PM_{2.5}$ offspring (P or P-offspring): offspring of $PM_{2.5}$-exposed paternal rats.

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### Role of oxidative stress in the paternal $PM_{2.5}$ exposure-induced hypertension in the offspring

Our and other studies have shown that exposure to fine particulate matter causes increased systematic and local oxidative stress levels [23,24]. However, whether paternal oxidative stress is involved in the pathogenesis of their $PM_{2.5}$ exposure-induced hypertension in the offspring is still unknown. Thus, we measured oxidative stress levels in the paternal $PM_{2.5}$- and vehicle-treated rats. Results showed that levels of MDA, a lipid peroxidation product, were higher, whereas levels of SOD, an antioxidant, were lower in the kidney of $PM_{2.5}$-treated paternal rats compared the kidney of control rats (Figure 5A,B), indicating that oxidative stress was increased in $PM_{2.5}$-treated paternal rats.

To confirm the role of oxidative stress in the paternal $PM_{2.5}$ exposure-induced hypertension in the offspring, a SOD mimetic tempol, as an antioxidant, was administered in combination with fine particulate matter to paternal rats. Results showed that administration with tempol for 16 weeks in $PM_{2.5}$-treated paternal rats restored the increased SBP in their male offspring, which was accompanied with elevated 24 h urine volume and sodium excretion (Figure 5C–E). Furthermore, treatment with tempol in $PM_{2.5}$-treated paternal rats also reversed the enhanced expressions of renal $AT_1R$ and GRK4 in their male offspring (Figure 5F,G).



## Discussion

It is well established that the maternal nutrition and lifestyle during the periconceptional period impact offspring's long-term health [25,26]. However, in recent years, studies have focused on exploring the influence that paternal exposures, including nutrition, lifestyle, drug and environment can have on the development of their offspring [27,28]. For example, paternal low protein diet attenuates vascular dysfunction, impairs glucose tolerance and elevates circulating TNF-$\alpha$ level in adult offspring [29]. Paternal healthy lifestyles such as exercise suppress the effects of paternal high-fat diet on offspring, including reversing the impairment in glucose tolerance, decreasing the percentage of fat mass, and increasing glucose uptake in skeletal muscles [30]. Streptozotocin-induced paternal hyperglycemia in SD rats exacerbates the development of obesity in offspring [31]. These suggest that paternal exposures have significant effects on offspring development and life-long health.

Among maternal environmental stimulus, air pollution has attracted more attentions on its adverse effects on the development of cardiovascular diseases such as hypertension in adult offspring [32,33]. Our and other studies have shown that *in utero* exposure to air pollution causes hypertension in offspring [14,15,34]. But there are few studies reporting the effect of paternal adverse environmental exposure in offspring. Chen et al. reported that paternal concentrated ambient $PM_{2.5}$ exposure resulted in significant hypophagia and weight loss in male offspring [35]. Another study showed that paternal and maternal collective exposure, not only paternal exposure, to concentrated ambient $PM_{2.5}$ caused a significant decrease in the body weight of adult male offspring [36]. In addition, paternal $O_3$ exposure has a direct effect on offspring hay fever [37]. All these studies did not observe the perinatal outcomes such as fetal biometrics, mortality or pre-term birth in the offspring. Thus, until now, whether or not paternal particulate matter exposure has any significant perinatal outcomes remains still unknown. Moreover, whether or not paternal $PM_{2.5}$ exposure leads to the increased blood pressure in offspring remains unknown. Our present study showed that compared with the offspring of vehicle-treated rats, the offspring of paternal rats with long-term $PM_{2.5}$ exposure had higher SBP, which was accompanied with the decreased 24 h urine volume and sodium excretion. These suggested that paternal long-term exposure to fine particulate matter causes hypertension in their offspring, which may be associated with the impaired renal functions.

The renin–angiotensin system (RAS) principal effector peptide Ang II, via its receptors, exerts its physiological functions [20]. The vast majority of actions of Ang II are transmitted via $AT_1R$, including elevation of sodium reabsorption and vasoconstriction [20,38,39]. In our present study, we found that compared with the offspring of vehicle-treated paternal rats, the expression and anti-natriuretic function of renal $AT_1R$ were aggravated in the offspring of $PM_{2.5}$-exposed paternal rats, which was accompanied by increased expression of its upstream GRK4 [22,40]. Further study showed increased oxidative stress, one of the fundamental mechanisms responsible for the development of hypertension [41,42], in paternal rats may be involved in it because administration with an antioxidant tempol for 16 weeks in paternal $PM_{2.5}$-treated rats restored the increased SBP and impaired sodium excretion, reversed the increased renal $AT_1R$ and GRK4 expression in offspring.

It is still unknown how elevated oxidative stress in paternal $PM_{2.5}$-treated rats causes increases the expressions of renal GRK4 and $AT_1R$ in offspring. In fact, the mechanisms underlying the effects of paternal exposures on offspring phenotype are only beginning to be elucidated, but many factors are hypothesized to be involved. There are at least two mechanisms involved in it: genetic impacts and epigenetic changes [43,44]. Paternal genetic information provides

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https://doi.org/10.1042/CS20210802

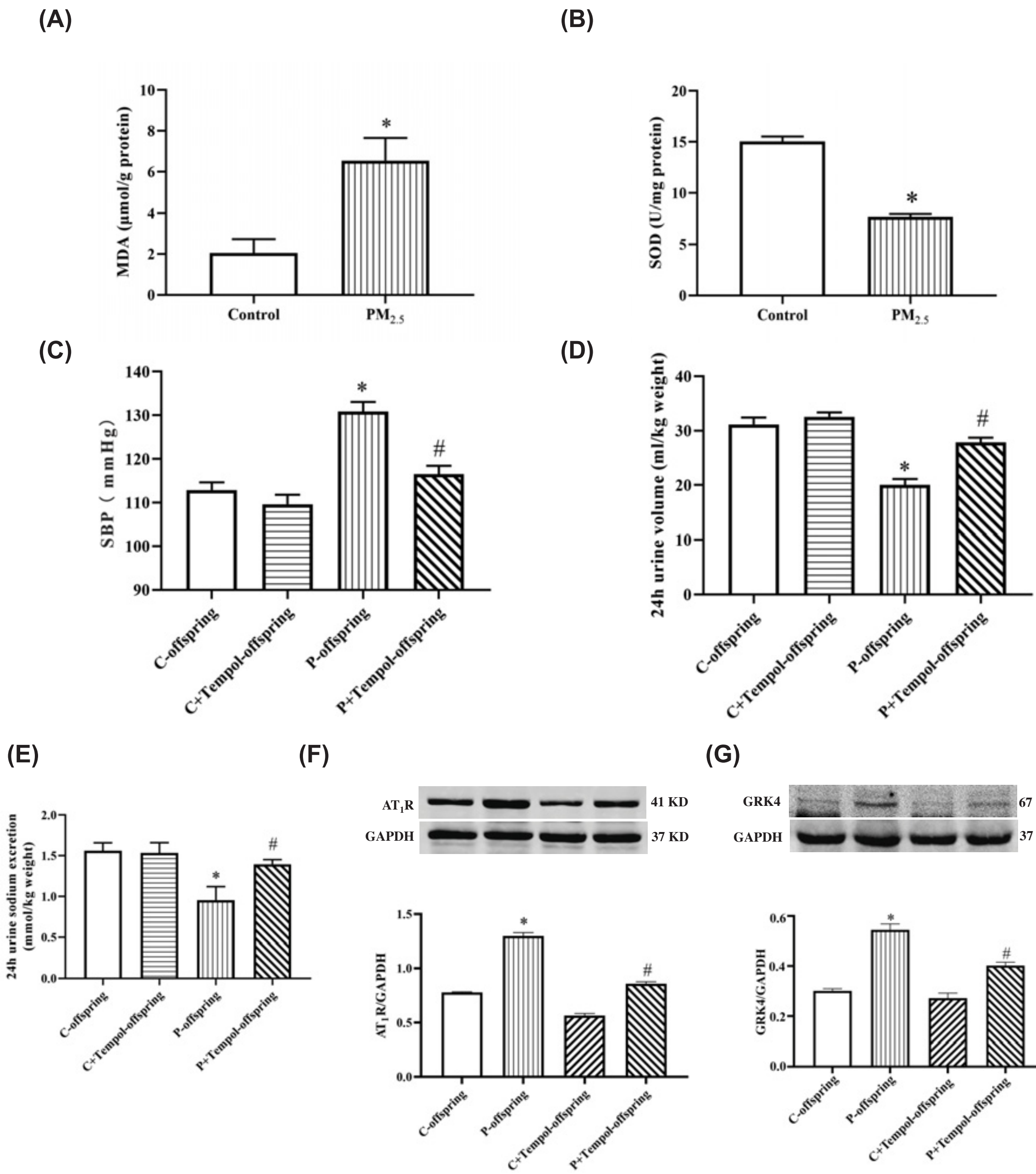


**Figure 5. Role of oxidative stress in the paternal $PM_{2.5}$ exposure-induced hypertension in offspring**

(**A** and **B**) Renal MDA (A) and SOD (B) levels were measured in vehicle (control)- and $PM_{2.5}$-treated paternal SD rats (A and B, two-tailed, unpaired *t* test, $^*P < 0.05$ vs. control, $n = 6$). (**C–E**) Systolic blood pressure (SBP) (**C**), 24 h urine volume (**D**) and sodium excretion (**E**) were determined in the 12-week-old offspring of 12 weeks $PM_{2.5}$-exposed- and 16 weeks tempol-treated paternal rats (C–E: two-way ANOVA with Tukey's post hoc test, $^*P < 0.05$ vs. control offspring, $n = 8$; $^\#P < 0.05$ vs. $PM_{2.5}$ offspring, $n = 8$). (**F** and **G**) Protein expressions of $AT_1R$ (F) and GRK4 (G) were determined by Western blot in the kidney from the 12-week-old offspring of 12 weeks $PM_{2.5}$-exposed- and 16 weeks tempol-treated paternal rats. $AT_1R$ or GRK4 protein expression was normalized using GAPDH expression (F and G, two-way ANOVA with Tukey's post hoc test, $^*P < 0.05$ vs. control, $n = 6$; $^\#P < 0.05$ vs. $PM_{2.5}$, $n = 6$). Control: vehicle-exposed paternal rats; $PM_{2.5}$: $PM_{2.5}$-exposed-paternal rats; C-offspring: offspring of vehicle-exposed and vehicle-treated paternal rats; P-offspring: offspring of $PM_{2.5}$-exposed and vehicle-treated paternal rats; C+Tempol-offspring: offspring of vehicle-exposed- and tempol-treated paternal rats; P+Tempol-offspring: offspring of $PM_{2.5}$-exposed- and tempol-treated paternal rats.

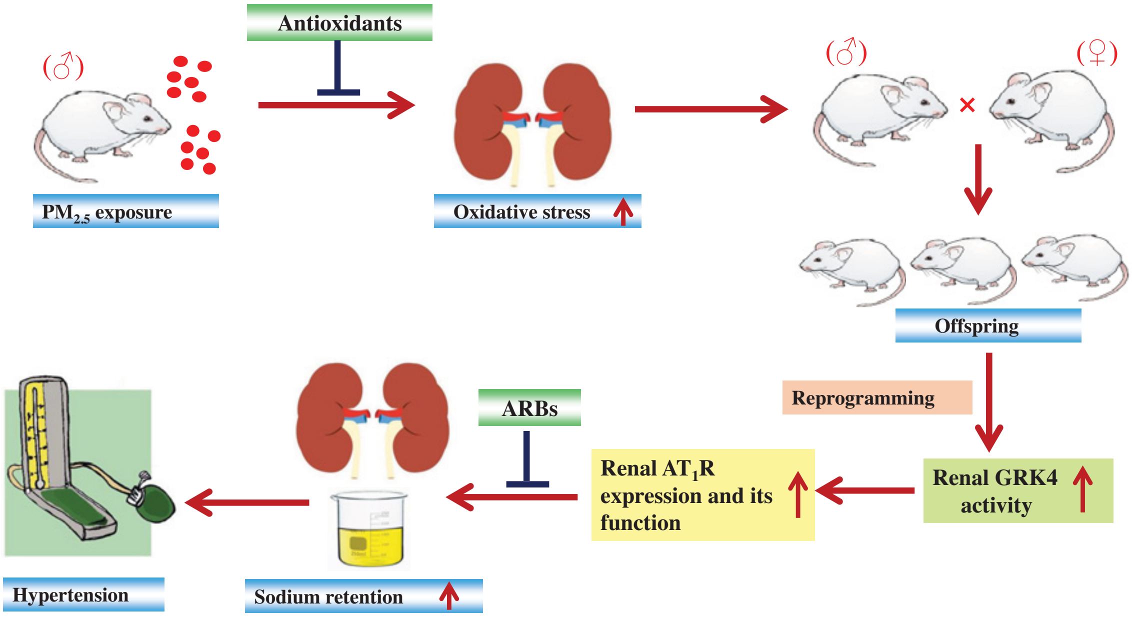


**Figure 6. Schematic diagram of the effect of paternal long-term $PM_{2.5}$ exposure on renal $AT_1R$ function and blood pressure in offspring**

Paternal $PM_{2.5}$ exposure, via increased oxidative stress, elevates renal expressions of GRK4 and its downstream target $AT_1R$ in their offspring, which leads to the enhanced $AT_1R$-mediated urinary sodium retention, and ultimately hypertension.

roughly half of their offspring's nuclear DNA. Thus, paternal environmental exposures directly impact offspring genotype and phenotype via inducing DNA damage and *de novo* genetic mutations in the male germline [45]. However, although paternal genetic changes are assumed as a mechanism for adverse effects in offspring, current reports do not often provide enough evidence for an exposure-related mutagenic effect. An increasing number of experiments have shown that paternal sperm epigenetic alterations induced by environmental exposures influence epigenetic profiles of offspring such as DNA methylation, chromatin modifications and non-coding RNAs, thereby impact their health status [43,46]. In our present study, we found increased oxidative stress level in paternal rats, which has been reported to cause epigenetic alterations [47,48]. Thus, although we cannot exclude the paternal genetic changes, we presume that oxidative stress-induced epigenetic changes may, at least in part, be involved in the increased renal GRK4 and $AT_1R$ expression and subsequently hypertension in offspring. However, it is should be noted that compared with prenatal exposure, more studies have been focused on the mechanisms of maternal and placental exposures, including impaired nephrogenesis, epigenetic reprograming, increased oxidative stress, over-activation of RAS, dysregulation of the immune system and hypothalamic–pituitary–adrenal axis [49,50].

There is a limitation in our present study. To avoid the influence of estrogen on the blood pressure [51,52], we only used male offspring. It should be noted that there may be different between male and female offspring after paternal exposures. For example, paternal high-fat diet leads to glucose intolerance due to impairment of pancreatic insulin secretion in female offspring [53] but causes a growth defect, impaired adipogenesis and decreased muscle growth in male offspring [54]. Paternal ambient $PM_{2.5}$ exposure causes hypophagia and weight loss in male, but not female, offspring [35]. Paternal bisphenol A exposure causes impaired glucose tolerance in female, not male, offspring [55]. Thus, whether or not paternal long-term $PM_{2.5}$ exposure causes hypertension as well as impaired renal functions in offspring with a sex-specific manner needs to be studied in the future.

In summary, we have demonstrated that paternal $PM_{2.5}$ exposure leads to hypertension in male offspring, which is, at least in part, due to the decreased urine volume and sodium excretion. $PM_{2.5}$ exposure-associated oxidative stress increases the level of renal GRK4, leading to the enhanced $AT_1R$ expression and its-mediated urinary sodium retention, and consequently causes hypertension in the offspring of $PM_{2.5}$-exposed paternal rats (Figure 6).

*Clinical Science* (2021) **135** 2575–2588
https://doi.org/10.1042/CS20210802

## Clinical perspectives

- Our and other studies have shown that *in utero* exposure to air pollution causes hypertension in offspring. However, whether or not paternal $PM_{2.5}$ exposure leads to the increased blood pressure in offspring still remains unknown.
- Long-term $PM_{2.5}$ exposure to paternal rats causes hypertension in male offspring. The mechanism may be involved that paternal $PM_{2.5}$ exposure-associated oxidative stress induces the increased renal GRK4 expression, causing the elevated $AT_1R$ level and its-mediated sodium retention, consequently leads to hypertension in male offspring.
- Exploring the pathogenesis of hypertension in early life development will provide new strategies for its prevention and treatment.

### Data Availability

All supporting data are included within the main article.

### Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

### Funding

These studies were supported in part by grants from the National Natural Science Foundation of China [grant numbers 31730043 and 81700375]; Postdoctoral Science Special Foundation of Chongqing; National Key R&D Program of China [grant number 2018YFC1312700]; Program of Innovative Research Team by National Natural Science Foundation [grant number 81721001].

### CRediT Author Contribution

**Cuimei Hu:** Data curation, Investigation, Methodology and Writing—original draft. **Yu Tao:** Data curation, Investigation and Methodology. **Yi Deng:** Investigation and Methodology. **Qi Cai:** Investigation and Methodology. **Hongmei Ren:** Investigation and Methodology. **Cheng Yu:** Investigation and Methodology. **Shuo Zheng:** Investigation and Methodology. **Jian Yang:** Supervision, Investigation, Methodology, Writing—review & editing. **Chunyu Zeng:** Supervision, Investigation, Methodology, Project administration and Writing—review & editing.

### Abbreviations

$AT_1R$, angiotensin II type 1 receptor; DBP, diastolic blood pressure; DOHaD, developmental origins of health and disease; GPCR, G-protein–coupled receptor; GRK4, G-protein–coupled receptor kinase type 4; H&E, hematoxylin and eosin; MDA, malondialdehyde; $PM_{2.5}$, fine particulate matter; RAS, renin–angiotensin system; SBP, systolic blood pressure; SD rat, Sprague–Dawley rat; SOD, superoxide dismutase.

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