ORIGINAL RESEARCH

Long-Term Exposure of Fine Particulate Matter Causes Hypertension by Impaired Renal D_1 Receptor–Mediated Sodium Excretion via Upregulation of G-Protein–Coupled Receptor Kinase Type 4 Expression in Sprague-Dawley Rats

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***Background*—**Epidemiological evidence supports an important association between air pollution exposure and hypertension. However, the mechanisms are not clear.

***Methods and Results*—**Our present study found that long-term exposure to fine particulate matter ($PM_{2.5}$) causes hypertension and impairs renal sodium excretion, which might be ascribed to lower D_1 receptor expression and higher D_1 receptor phosphorylation, accompanied with a higher G-protein–coupled receptor kinase type 4 (GRK4) expression. The in vivo results were confirmed in in vitro studies (ie, $PM_{2.5}$ increased basal and decreased D_1 receptor mediated inhibitory effect on Na^+-K^+ ATPase activity, decreased D_1 receptor expression, and increased D_1 receptor phosphorylation in renal proximal tubule cells). The downregulation of D_1 receptor expression and function might be attributable to a higher GRK4 expression after the exposure of renal proximal tubule cells to $PM_{2.5}$, because downregulation of GRK4 by small-interfering RNA reversed the D_1 receptor expression and function. Because of the role of reactive oxygen species on D_1 receptor dysfunction and its relationship with air pollution exposure, we determined plasma reactive oxygen species and found the levels higher in $PM_{2.5}$-treated Sprague-Dawley rats. Inhibition of reactive oxygen species by tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) reduced blood pressure and increased sodium excretion in $PM_{2.5}$-treated Sprague-Dawley rats, accompanied by an increase in the low D_1 receptor expression, and decreased the hyperphosphorylated D_1 receptor and GRK4 expression.

***Conclusions*—**Our present study indicated that long-term exposure of $PM_{2.5}$ increases blood pressure by decreasing D_1 receptor expression and function; reactive oxygen species, via regulation of GRK4 expression, plays an important role in the pathogenesis of $PM_{2.5}$-induced hypertension. **(*J Am Heart Assoc*. 2018;7:e007185. DOI: 10.1161/JAHA.117.007185.)**

Key Words: dopamine receptor • GRK4 • hypertension • kidney • $PM_{2.5}$ • sodium excretion

Epidemiological evidence supports an important association between air pollution exposure and cardiovascular risks.[1] The World Health Organization reported that >800 000 premature deaths worldwide per year can be directly attributed to particulate matter air pollution.[2] The ESCAPE (European Study of Cohorts for Air Pollution Effects) showed that a 5 $\mu g/m^3$ increase in estimated annual mean fine particulate matter ($PM_{2.5}$) was associated with a 13% increased risk of coronary events.[3] Recently, the relationship between particulate matter and hypertension is getting more and more attention.[4] Dai-Hua Tsai *et al* found that inhalable particulate matter short time exposure, a 10 $\mu g/m^3$ increase

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Accompanying Figures S1 through S4 are available at http://jaha.ahajournals.org/content/7/1/e007185/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- Fine particulate matter exposure causes hypertension because of the dysfunction of the renal dopamine D_1 receptor.
- Increased oxidative stress, associated with increased G-protein–coupled receptor kinase type 4 levels, impairs renal D_1 receptor function and leads to hypertension.

What Are the Clinical Implications?

- Particulate matter air pollution might play an important role in the pathogenesis of hypertension.
- Restoration of dopamine D_1 receptor function by antioxidants may be a therapeutic target for fine particulate matter–caused hypertension.

in inhalable particulate matter level, is associated with higher nighttime blood pressure and is preceded by associations with the reduced ability of the kidney to excrete sodium during the daytime.[5] Numerous investigations have shown that exposure to $PM_{2.5}$ elucidated potential biological consequences, such as modulation of blood pressure, including systemic proinflammatory oxidative responses, DNA damage, and autonomic nervous system imbalance.[5–12] However, the underlying pathophysiological mechanisms of airborne $PM_{2.5}$-mediated hypertension are complex and remain to a large extent unexplored. Studies have shown that hypertension is caused by prohypertensive factors, which include the increased activity of the sympathetic nervous system and the decreased activity of antihypertensive mechanisms, such as sodium excretion.[13] The kidney is endowed with local hormonal systems that play major roles in the regulation of sodium transport across the renal proximal tubule (RPT).[14] Among those systems, dopamine is taken as an important one, which is synthesized in and secreted from RPT cells and decreases RPT sodium transport,[13–17] via the D_1 and D_3 receptors, by inhibition of NHE3 at the brush border membrane and Na^+-K^+-ATPase at the basolateral membrane.[13] In hypertensive states, the renal D_1 receptor is hyperphosphorylated, which leads to its dysfunction, and is ascribed to the higher G-protein–coupled receptor kinase type 4 (GRK4) expression[13,18] and activity in spontaneously hypertensive rats or hypertensive patients.[19–21] This can be linked to increased oxidative stress.[22–25] Many studies have shown that the impaired intrarenal D_1 receptor signaling in hypertension can cause or be caused by oxidative stress.[24–27] Antioxidant supplementation with tempol (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), a superoxide dismutase (SOD) mimetic, decreases oxidative stress, restores D_1 receptor signaling, and lowers blood pressure.[24,25]

There are reports that $PM_{2.5}$ induces hypertension through impaired sodium excretion and increased reactive oxygen species (ROS) and oxidative stress[28–31]; ROS has been reported to decrease dopamine receptors in animals.[24–27] Therefore, we hypothesize that $PM_{2.5}$, via increased ROS levels, increases GRK4 expression and consequently impairs renal D_1 receptor function, and leads to hypertension. To test this hypothesis, we exposed Sprague-Dawley (SD) rats to $PM_{2.5}$ in vivo. Concurrently, we also exposed immortalized RPT cells in vitro, because they behave similarly to freshly obtained RPT cells, at least with regard to dopamine receptors and its responses to G-protein stimulation, in this present study.

Methods

The data, analytic methods, and study materials will be made available on request to other researchers for purposes of reproducing the results or replicating the procedure.

$PM_{2.5}$ Sampling

The sample site was the Daping Hospital, which is ≈1 km from the city center. The nearest main road is in the northeast at a distance of 100 m. Within an ≈200 m radius of the monitoring site is almost completely surrounded by residential areas.

We used a medium volume sampler (model TH-150; Tianhong Co, Wuhan, China) equipped with a $PM_{2.5}$ filtration system to collect $PM_{2.5}$ samples on filters (diameter, 150 mm), according to the reported method.[32–34] The flow rate of medium volume sampler was adjusted to 30 m^3/h. After sampling, filters were shredded into small pieces and sonicated using an ultrasonator (KQ-250DE; Shumei, Kunshan, Jiangsu, China) for 3× 40 minutes in double-distilled water. The extracted material was frozen, concentrated using lyophilization, and weighed to determine the extraction efficiency. The farinose solid was stored at −80°C until further use. The sampling period started on April 8, 2013, and ended on July 28, 2013. A total of 25 filters were used to collect the $PM_{2.5}$ samples. To make sure the $PM_{2.5}$ sample diameter was correct, we performed a scanning electron microscope analysis of the samples to prove the purity of the samples. The analysis confirmed that the particulate matter collected had a diameter of = or <2.5 μm (Figure S1).

Animal Treatment

Male SD rats, 8 to 10 weeks old and initially weighing 198±6 g, were given a standard laboratory chow and ad libitum access to clean drinking water. They were randomly divided into 3 groups (n=56 for control, n=32 for lower

dosage $PM_{2.5}$ treatment, and n=56 for higher dosage $PM_{2.5}$ treatment). The details of each group are shown in Figure S2. The rats were individually housed in metabolic cages for urine collection and maintained at a temperature of 25±2°C, a relative humidity of 50% to 60%, and a 12-hour dark-light cycle. An acclimatization period of 2 days was allowed for the rats before the start of the experiments. The rats were weighed at the beginning of the experiment and just before urine collection and euthanasia. This study was approved by the Third Military Medical University Animal Use and Care Committee. All experiments conformed to the guidelines of the ethical use of animals, and all efforts were made to minimize animal suffering.

$PM_{2.5}$ was suspended in PBS. To minimize aggregation, particle suspensions were always sonicated for 15 minutes, 3 times, and vortexed before their dilution and before intratracheal administration. Control animals received PBS treatments. The animals were anesthetized with isoflurane and placed supine with extended necks on an angled board.[33,35,36] An oropharynx instillation of the $PM_{2.5}$ suspension (3 or 30 μg $PM_{2.5}$ in 25 μL) or PBS only was instilled (25 μL) via a sterile syringe. SD rats were treated with the $PM_{2.5}$ solution every 3 days, for 8 weeks. In the present study, 2 dosages of $PM_{2.5}$ treatment of either lower or higher dose were used. The lower dosage given (3 μg $PM_{2.5}$) represented 35 $\mu g/m^3$ per 24 hours $PM_{2.5}$ in the air and was marked as $PM_{2.5}$-L. The higher dosage given (30 μg $PM_{2.5}$) represented 350 $\mu g/m^3$ per 24 hours $PM_{2.5}$ in the air and marked as $PM_{2.5}$-H. To confirm the success of the exposure model, we performed a hematoxylin-eosin stain of the lung after the rats were euthanized (Figure S3). Light microscope detection found the $PM_{2.5}$ in the lung tissue.

The blood pressure and 24-hour urine volume and sodium excretion were measured in conscious rats on the indicated exposure time points. After 8 weeks of being exposed to $PM_{2.5}$-L, $PM_{2.5}$-H, and PBS, treated rats were randomly assigned into 2 groups: one group that drank tap water served as a control, and the other group drank tap water with 1.0 mmol/L tempol that was changed 2 times per day, for 3 weeks (n=12 in each group).

Blood Pressure and Urine Collection

Systolic blood pressure, diastolic blood pressure, and mean arterial blood pressure were measured every week using a computerized, noninvasive, tail-cuff plethysmography system in conscious rats (BP-98A; Softron, Tokyo, Japan). The animals were individually restrained in a clear acrylic restrainer at an ambient temperature of 37°C to 38°C for 15 minutes. Five blood pressure values were recorded per rat, and the average was taken. To ensure the reliability of the measurements, a training period of 1 week was established before the start of the experiment for the rats to adapt to the method. Blood pressure measurements were performed between 3 and 5 PM.

SD rats were acclimated in metabolic cages for at least 2 days before urine was collected for 24 hours every week. After urine collection, sodium and potassium concentrations in urine samples were measured using a flame photometer 480 (Ciba Corning Diagnostics, Norwood, MA).

Cell Culture and Sample Preparation

Immortalized RPT cells from microdissected S_I segments of the RPTs from 4- to 8-week-old Wistar-Kyoto rats were cultured at 37°C in 95% air/5% CO_2 atmosphere in DMEM/F-12 with transferrin (5 μg/mL), insulin (5 μg/mL), epidermal growth factor (10 ng/mL), dexamethasone (4 μg/mL), and 5% fetal bovine serum on a 100-mm Petri dish. The final concentration of $PM_{2.5}$ (1–200 μg/mL) was mixed evenly into the culture medium, 24 hours prior for the $PM_{2.5}$ exposure group, and an equal volume of PBS was mixed into the culture medium for the control group. Cells were made quiescent by incubating for 2 hours in the media without fetal bovine serum before adding the drugs. The cells (80% confluence) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1 hour, and centrifuged at 16 000*g* for 30 minutes. All samples were stored at −80°C until use.

Immunoblotting

In the in vivo study, immediately after euthanasia (or after thawing), the renal cortex (upper pole, left kidney) was homogenized in buffer (10 mmol/L Tris-HCl, 250 mmol/L sucrose, 2 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail; pH 7.4) and centrifuged at 24 000*g* for 25 minutes at 4°C. The upper fluffy layer of the pellet, which is considered as the membrane fraction, was resuspended in the homogenization buffer.[37,38] The samples were quickly frozen and stored at −80°C until use. For the in vitro study, the RPT cells were lysed in a lysis buffer, sonicated, placed on ice for 1 hour, and centrifuged at 16 000*g* for 30 minutes. The supernatant was stored at −80°C until use.

After boiling the homogenates in sample buffer (35 mmol/L Tris-HCl, pH 6.8, 4% SDS, 9.3% dithiothreitol, 0.01% bromophenol blue, and 30% glycerol) at 95°C for 5 minutes, 50 μg of protein was separated by SDS-PAGE (10% polyacrylamide) and then electroblotted onto nitrocellulose membranes (Amersham Life Science, Arlington, TX). The blots representing the nonspecific antibodies were blocked overnight with 5% nonfat dry milk in PBS with Tween 20 (0.05% Tween 20 in 10 mmol/L PBS [isotonic]) at 4°C under constant shaking, then incubated with polyclonal rabbit anti-rat GRK4 receptor antibodies (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), overnight at 4°C. The other antibodies

were anti-D_1 dopamine receptor antibody (1:400; Millipore, Billerica, MA), anti–c-Myc (phosphorylated T58+S62) antibody (1:400; Abcam, Cambridge, UK), and rabbit polyclonal GAPDH (1:500; Santa Cruz Biotechnology). The membranes were then further incubated using infrared-labeled secondary antibodies (donkey anti-rabbit IRDye 800; Li-Cor Biosciences, Lincoln, NE) to bind to the primary antibody at room temperature for 1 hour. The membranes were washed 3 times in PBS with Tween 20. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences). The images were analyzed using the Odyssey Application Software to obtain the integrated intensities.

Determination of D_1 Receptor Phosphorylation by Coimmunoprecipitation

Equal amounts of lysates (1.0 μg protein/mL supernatant from RPT cells or renal cortex membranes) were incubated with dopamine D_1 receptor (D_1R) antibody (2 μg; Millipore) for 2 hours, followed by protein A/G agarose overnight, with constant rocking at 4°C for 12 hours. The immunoprecipitates were pelleted and washed 3 times with PBS. Then, the pellets were suspended in sample buffer, boiled for 10 minutes, and subjected to immunoblotting with phosphoserine antibody (1:400; Immunechem, Burnaby, BC, Canada). To determine the specificity of the bands, normal rabbit IgG (negative control) and D_1 receptor antibody (positive control) were used as the immunoprecipitants. The bound complexes were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Band density of the serine-phosphorylated D_1R was normalized by band density of D_1R. All the bands were quantified by densitometry.

Small-Interfering RNA

Small-interfering RNA (siRNA) against GRK4 mRNA and its control, scrambled RNA, was synthesized and purified with reverse-phased high-performance liquid chromatography as 25-mer phosphorothioate-modified oligodeoxynucleotides (GRK4 siRNA sequence, 5′-AUCUAAAGAGGUGCAUUGAAUUCUUdTdT-3′; scrambled RNA sequence, 5′-TGACGATAAGAACAATAACdTdT-3′), from nucleotides 412 to 436 and 1752 to 1776, respectively, of the rat GRK4 cDNA. The effects of 50 nmol/L siRNA was compared with scrambled RNA (control). Briefly, cells were grown in 6-well plates until 60% confluence, and 50 nmol/L siRNA or control RNA was mixed with 6 μL of Oligofectamine in Optimem medium (Invitrogen Life Technologies) and incubated for 24 hours, then switched to growth medium and incubated for another 24 hours. The cells were collected and processed for reverse transcription–polymerase chain reaction for GRK4 to determine the efficiency of siRNA-induced GRK4 gene silencing.

Cell Counting Kit-8 Assay

As previously described,[39] the number of viable cells was assayed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), per the manufacturer's protocol. Briefly, RPT cells were plated into a 96-well plate and incubated with Cell Counting Kit-8 for 2 hours at 37°C. The optical density (OD) values were subsequently measured on a spectrophotometer (PerkinElmer) at 490 nm. Three independent experiments were performed.

Real-Time Quantitative Polymerase Chain Reaction Detecting System

A total of 2 to 3 μg of total RNA extracted from RPT cells or renal cortex membranes was used to synthesize cDNA, which served as template for the amplification of receptor and GAPDH (as the reference housekeeping gene). For GAPDH, the forward primer was 5′-GACATGCCGCCTGGAGAAAC-3′ and the reverse primer was 5′-AGCCCAGGATGCCCTTTAGT-3′ (GeneBank Accession No. NM_022928.1). For GRK4, the forward primer was 5′-ACTTCAGCAGACTGGAAGCA-3′ and the reverse primer was 5′-GGTGTCCAGGTTGACTCCTT-3′ (GeneBank Accession No. NM_017008.4). For c-Myc, the forward primer was 5′- ACTTCAGCAGACTGGAAGCA-3′ and the reverse primer was 5′-GGTGTCCAGGTTGACTCCTT-3′ (GeneBank Accession No. AY679730.1). The amplification was performed with the following conditions: denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 10 seconds and 60°C for 30 seconds. At the end of each run, a melting curve analysis was performed from 65°C to 95°C to monitor primer dimers or nonspecific product formation. The reactions were performed in triplicate. The GRK4 and c-Myc mRNA expression was normalized for GAPDH mRNA.

Na^+-K^+-ATPase Activity Assay

Na^+-K^+-ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain.[40,41] Rat RPT cells were treated with vehicle (distilled water) and D_1 receptor agonist,fenoldopam, at the indicated concentrations and durations of incubation. To prepare membranes for Na^+-K^+-ATPase activity assay, RPT cells cultured in 21-cm^2 plastic culture dishes were collected and centrifuged at 3000*g* for 10 minutes. The cells were then placed on ice and lysed in 2 mL of lysis buffer (1 mmol/L $NaHCO_3$, 2 mmol/L $CaCl_2$, and 5 mmol/L $MgCl_2$). Cellular lysates were centrifuged at 3000*g* for 2 minutes to remove intact cells, debris, and nuclei. The resulting supernatant was suspended in an equal volume of 1 mol/L sodium iodide, and the mixture was centrifuged at 48 000*g* for 25 minutes. The pellet (membrane fraction) was washed 2 times and suspended in 10 mmol/L Tris containing 1 mmol/L EDTA (pH

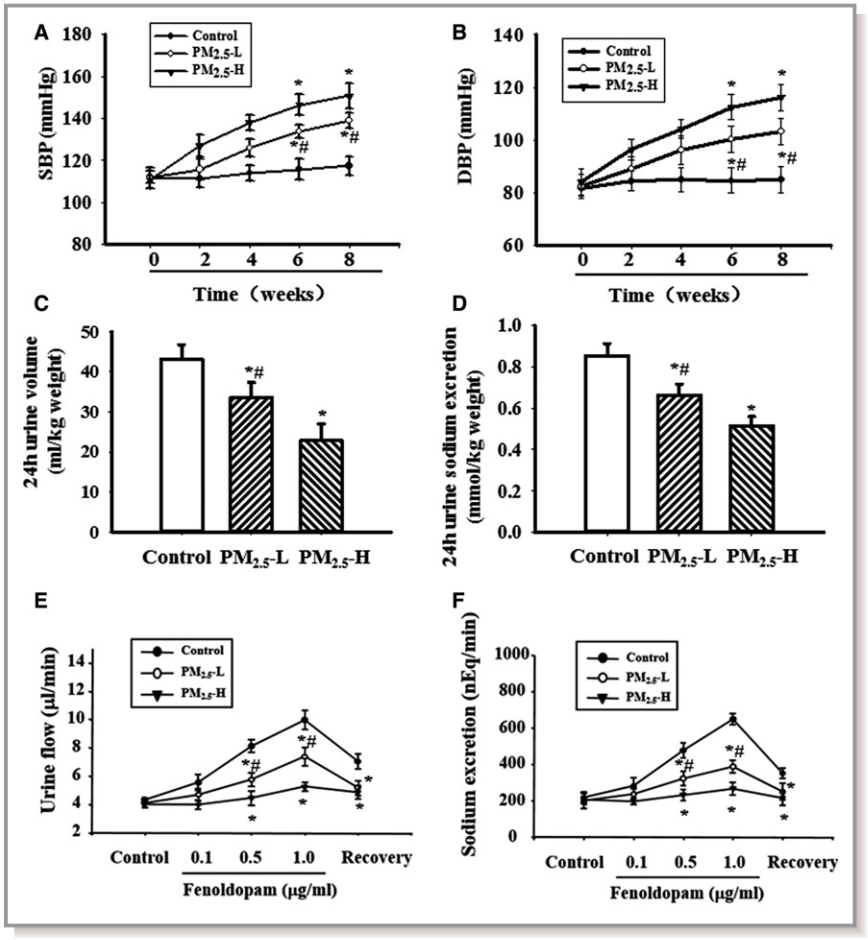

Figure 1. Effect of fine particulate matter ($PM_{2.5}$) exposure on blood pressure and sodium excretion in Sprague-Dawley (SD) rats. A and B, Effect of $PM_{2.5}$ exposure on blood pressure. SD rats received lower (3 μg $PM_{2.5}$ per time [$PM_{2.5}$-L]) or higher (30 μg $PM_{2.5}$ per time [$PM_{2.5}$-H]) dosage of $PM_{2.5}$. Systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) were recorded by the tail-cuff method (n=12 in each group). C and D, Effect of $PM_{2.5}$ exposure on urine volume (C) and urine sodium excretion (D) in SD rats. Urine was collected in SD rats after exposure with $PM_{2.5}$ or vehicle for 8 weeks (n=20 in each group). E and F, Effect of $PM_{2.5}$ exposure on renal dopamine D_1 receptor (D_1R) function in SD rats. Varying dosages of the D_1-like receptor agonist, fenoldopam (0.1, 0.5, and 1.0 μg/kg per minute), were infused into the right suprarenal artery in SD rats after 8-week exposure with $PM_{2.5}$ or vehicle. The fenoldopam-associated urine volume (E) and urine sodium excretion (F) were measured (n=12 in each group). G and H, Effect of $PM_{2.5}$ exposure on renal D_1R expression (G) and phosphorylation (H) in SD rats. Renal cortical membranes were prepared in SD rats exposed to $PM_{2.5}$ or vehicle for 8 weeks. D_1R phosphorylation was normalized by total D_1R expression (n=10 in each group). I and J, Effect of $PM_{2.5}$ exposure on renal G-protein–coupled receptor kinase 4 (GRK4) mRNA (I) and protein (J) expressions in SD rats (n=10 in each group). *P<0.0001 vs control rats, $^{\#}P$<0.0001 vs $PM_{2.5}$-H group.

7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA) and adjusted to 1 mg/mL. The membranes were stored at −70°C until use.

To measure Na^+-K^+-ATPase activity, 100-μL aliquots of membrane fraction were added to an 800-μL reaction mixture (75 mmol/L NaCl, 5 mmol/L KCl, 5 mmol/L $MgCl_2$, 6 mmol/L sodium azide, 1 mmol/L Na_4EGTA, 37.5 mmol/L imidazole, 75 mmol/L Tris-HCl, and 30 mmol/L histidine; pH 7.4), with or without 1 mmol/L ouabain (final volume=1 mL), and preincubated for 5 minutes in a water bath at 37°C. Reactions were initiated by adding Tris-ATP (4 mmol/L) and terminated after 15 minutes of incubation at 37°C by adding 50 μL of 50% trichloroacetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 mL of coloring reagent

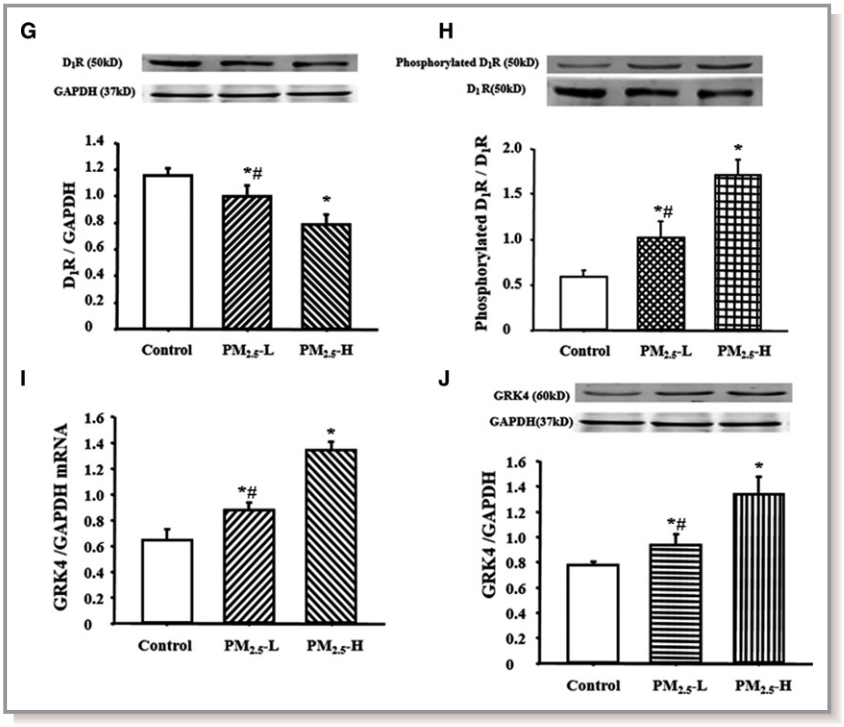

Figure 1. Continued.

(10% ammonium molybdate in 10N sulfuric acid and 10N ferrous sulfate mix buffer) was added to the reaction mixture. The mixture was then mixed thoroughly and centrifuged at 3000*g* for 10 minutes. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm, against a standard curve prepared from K_2HPO_4. Na^+-K^+-ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as percentage change of control.

To eliminate the effect of proteases and phosphatases, protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 10 μg/mL each leupeptin and aprotinin) and a phosphatase inhibitor (50 μmol/L sodium orthovanadate) were added in all solutions used after drug/vehicle incubations.

Fenoldopam-Induced Diuresis and Natriuresis

Twelve rats were selected from each group randomly and used for fenoldopam infusion study. A total of 36 rats from control, $PM_{2.5}$-L, and $PM_{2.5}$-H groups were anesthetized with pentobarbital (50 mg/kg IP), placed on a heated board to maintain body temperature at 37°C, and tracheotomized (PE-100). Catheters (PE-50) were placed into the external jugular and femoral veins and left carotid artery for fluid administration and blood pressure monitoring. Systemic blood pressure was monitored electronically using Cardiomax II (Columbus Instruments, Columbus, OH). Laparotomy was performed to expose the left and right ureters, which were then separately catheterized for urine collection. The right suprarenal artery (which originates from the right renal artery) was located and catheterized (PE-10; heat stretched to 180 μm). After a 60-minute stabilization period, a normal saline load equivalent to 5% of body weight was infused intravenously for 30 minutes. After the short-term saline load, a 60-minute stabilization period was allowed before starting the urine collection.[37]

Five consecutive 40-minute urine samples were collected: Vehicle was given during the first collection period (control). In the next 3 periods, the mice received fenoldopam, a D_1-like receptor agonist, at 3 different dose rates of 0.1, 0.5, and1 μg/kg per minute, each dose that has been shown not to affect blood pressure in previous studies.[30,31] During the last collection period (recovery), only the vehicle, normal saline, was infused. This was considered as the recovery phase of the experiment. The change in all infusates (vehicle and reagents) was commenced 10 minute before each period to account for the dead space in the delivery catheter. All infusions (vehicle and reagents) were given at a rate of 40 μL/h. Fluid losses throughout the experiment were replaced intravenously with 5% albumin at 1% body weight over 30 minutes. The rats were euthanized with an overdose of pentobarbital (100 mg/kg) at the end of the experiment right after the last sample was collected. Urine samples were stored at −80°C until use.

Biochemical Markers of Oxidative Stress

To assess total oxidative stress, plasma samples from rats were used to measure SOD activity using an SOD Assay Kit (Dojindo Laboratories), following the manufacturer's

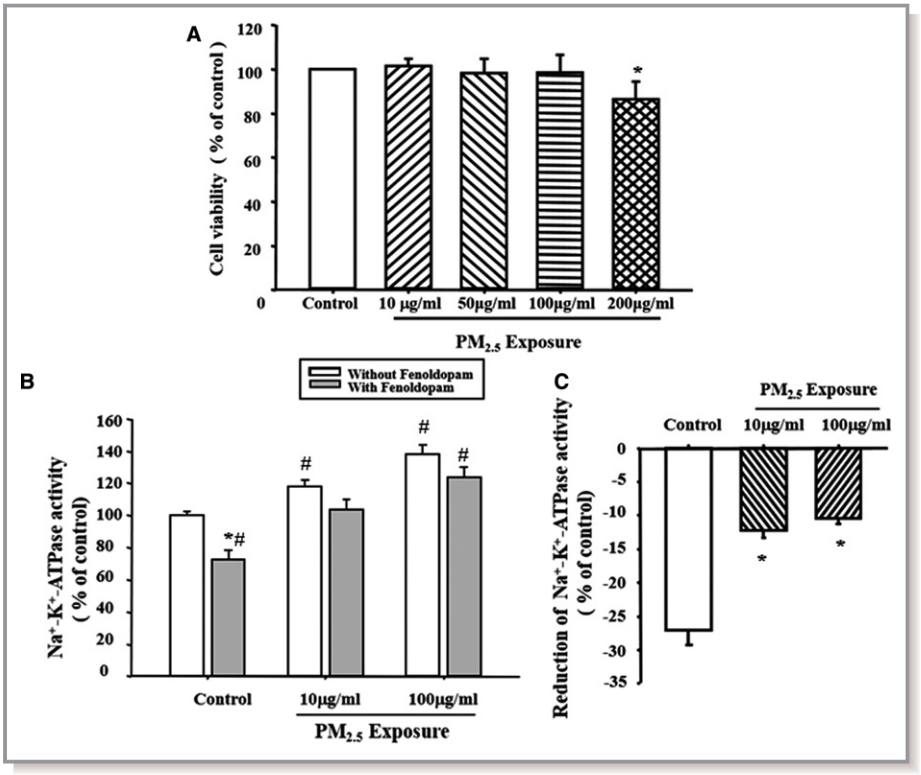

Figure 2. Effect of fine particulate matter ($PM_{2.5}$) exposure in in vitro study in renal proximal tubule (RPT) cells. A, Effect of $PM_{2.5}$ exposure on cell viability in RPT cells. Cell viability was assayed using Cell Counting Kit-8 after exposure to vehicle (distilled water [dH_2O]) or $PM_{2.5}$ for 24 hours. **P*<0.0001 vs control (n=9 in each group). B and C, Effect of $PM_{2.5}$ exposure on Na^+-K^+-ATPase activity in RPT cells. The cells were treated with $PM_{2.5}$ or vehicle (dH_2O) for 24 hours. Results are expressed as percentage change of control (B). **P*<0.0001 vs nonfenoldopam treatment in each group, $^{\#}$*P*<0.0001 vs control without fenoldopam and $PM_{2.5}$ treatment (n=10 in each group). C showed the fenoldopam-induced reduction of Na^+-K^+-ATPase activity in each group. **P*<0.0001 vs control in C (n=10 in each group). D and E, Effect of $PM_{2.5}$ exposure on dopamine D_1 receptor (D_1R) protein expression (D) and serine phosphorylation (E) in RPT cells. RPT cells were exposed with 10 or 100 μg/mL concentration of $PM_{2.5}$ for 24 hours. Results were expressed as ratio of D_1R phosphorylation/total D_1R expression. **P*<0.05 vs control, $^{\#}$*P*<0.05 vs $PM_{2.5}$-H group (n=9 in each group). F and G, Effect of $PM_{2.5}$ exposure on G-protein–coupled receptor kinase 4 (GRK4) mRNA (F) and protein (G) expressions in RPT cells. RPT cells were exposed with 10 or 100 μg/mL concentration of $PM_{2.5}$ for 24 hours. **P*<0.01 vs control, $^{\#}$*P*<0.05 vs $PM_{2.5}$-H group (n=9 in each group).

instructions. Malondialdehyde and glutathione peroxidase in the plasma were quantified using a commercially available kit (Nanjing Jianchen Bioengineering Institute, Nanjing, China). For in vitro study, the RPT cell culture medium supernatants were collected to determine SOD, glutathione peroxidase, and malondialdehyde levels.

Statistical Analysis

The data are expressed as mean±SEM. A repeated-measures ANOVA (dosages of $PM_{2.5}$×time points) was used to determine the differences of blood pressure, among 3 groups (control, $PM_{2.5}$-L, and $PM_{2.5}$-H) at 0, 2, 4, 6, and 8 weeks. To analyze the effect of fenoldopam infusion on urine flow and urinary sodium excretion in different dosages of $PM_{2.5}$-treated groups (control, $PM_{2.5}$-L, and $PM_{2.5}$-H), another repeated-measures (dosages of $PM_{2.5}$×infusion periods) ANOVA was used. Dosages of $PM_{2.5}$ were the predictor in these analyses. The Mauchly test of sphericity was performed using Greenhouse-Geisser adjustment. Moreover, to determine the difference between groups that have been split on 2 dependent variables (whether fenoldopam treatment in cells and the dosage of $PM_{2.5}$ [0, 10, and 100 μg/mL]) and their interaction, the 2-way ANOVA was also performed. Besides, other comparisons among groups (or *t* test when only 2 groups were compared) was made by 1-way ANOVA with Holm-Sidak test. Post hoc multiple comparisons were performed by least significant difference method. *P*<0.05 was considered significant.

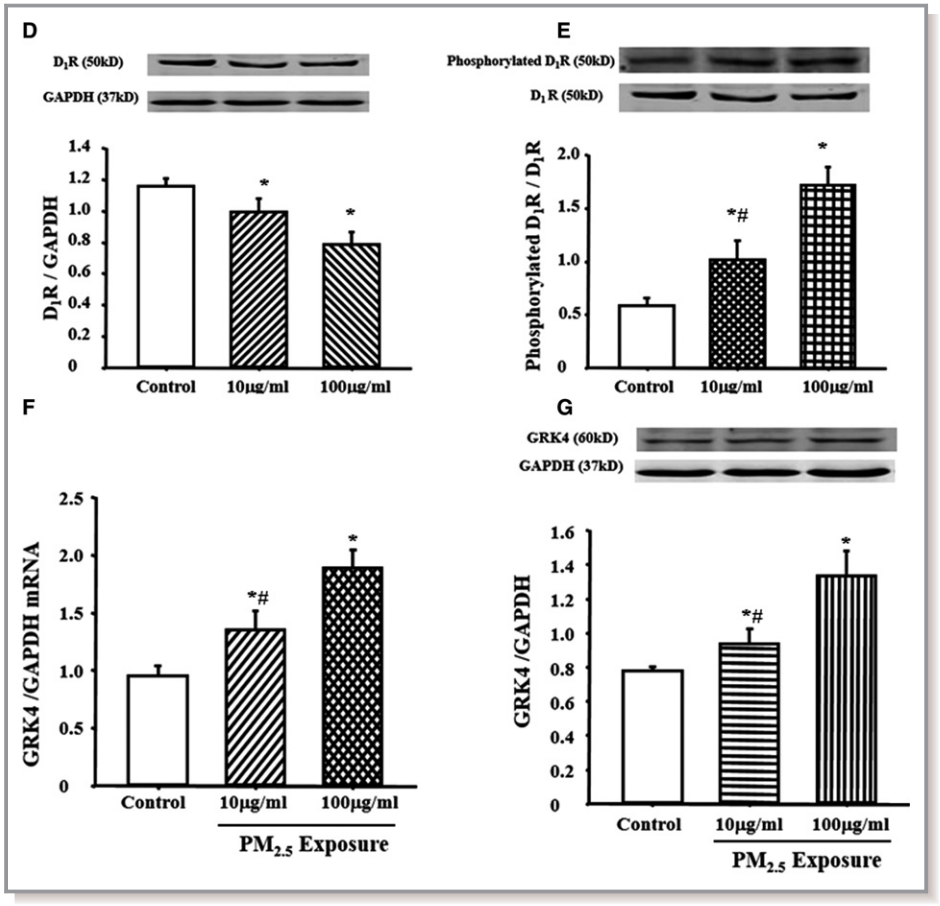

Figure 2. Continued.

Results

Effects of $PM_{2.5}$ Exposure on Blood Pressure and D_1 Receptor–Mediated Sodium Excretion in SD Rats

Long-term exposure to $PM_{2.5}$ caused a significant increase in both systolic and diastolic blood pressures, which were measured by the tail-cuff method, whether by the high dose ($PM_{2.5}$-H) or lower dose ($PM_{2.5}$-L) of $PM_{2.5}$ (Figure 1A and 1B). The differences in blood pressure between the control and $PM_{2.5}$-treated rats progressively increased with time (Figure 1A and 1B). The high blood pressure might be ascribed to impaired sodium excretion, because the basal levels of sodium excretion were lower in $PM_{2.5}$-treated SD rats. Moreover, the higher dose of $PM_{2.5}$ decreased the sodium excretion to a greater degree compared with the lower dose (Figure 1C and 1D).

Because of the important role of the dopamine receptor on sodium excretion, we also determined the effect of fenoldopam, a D_1-like receptor agonist, on sodium excretion. Consistent with previous reports,[41,42] fenoldopam induced natriuresis (Figure 1E) and diuresis (Figure 1F) in SD rats. However, after the treatment of SD rats by $PM_{2.5}$ for 8 weeks, the fenoldopam-induced natriuresis and diuresis were significantly decreased (Figure 1E and 1F). Moreover, the higher dose of $PM_{2.5}$ worsened the D_1 receptor–mediated sodium excretion to a higher degree compared with the lower dose of $PM_{2.5}$ (Figure 1E and 1F). Our previous studies indicated that hyperphosphorylation of D_1 receptor leads to the dysfunction of the renal D_1 receptor, which is ascribed to the higher GRK4 expression and activity in spontaneously hypertensive rats or hypertensive patients.[17–20] Our additional study found that D_1 receptor expression was lower and GRK4 expression and D_1 receptor phosphorylation were higher in $PM_{2.5}$-treated SD rats. The degrees of change were greater with the higher dose of $PM_{2.5}$ than the lower dose (Figure 1G through 1J), indicating GRK4 might be involved in the dysfunction of D_1 receptor in $PM_{2.5}$-induced hypertension.

Role of GRK4 on $PM_{2.5}$-Impaired D_1 Receptor Expression and Function in RPT Cells

We used RPT cells to uncover the mechanism of $PM_{2.5}$-induced hypertension. We first determined the optimal concentration of $PM_{2.5}$ required, and it showed that

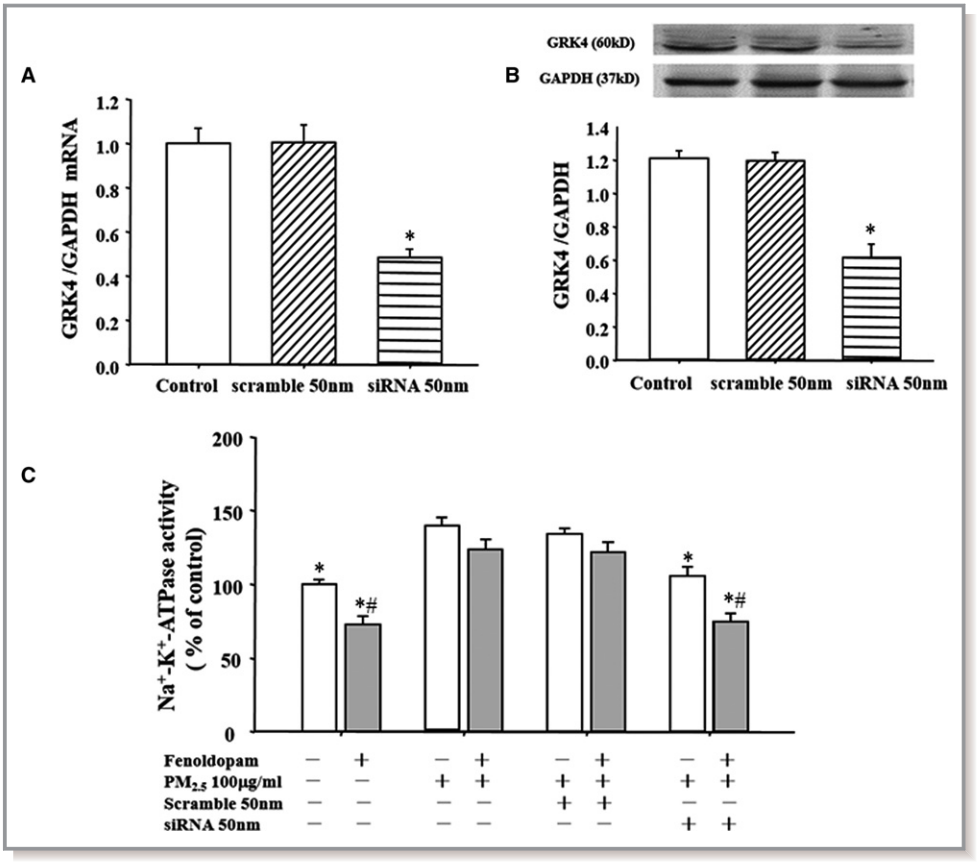

Figure 3. Role of G-protein–coupled receptor kinase 4 (GRK4) on fine particulate matter ($PM_{2.5}$)–mediated impairment in renal proximal tubule (RPT) cells. A and B, Effect of GRK4 small-interfering RNA (siRNA) on GRK4 expression. RPT cells were transfected with siRNA for 24 hours. GRK4 mRNA expression (A) and protein expression (B) were determined by quantitative polymerase chain reaction or immunoblotting. **P*<0.0001 vs control (n=10 in each group). C and D, Effect of GRK4 siRNA on Na^+-K^+-ATPase activity in RPT cells. After transfection with GRK4 siRNA for 24 hours, RPT cells were treated with $PM_{2.5}$ (100 μg/mL) or vehicle (distilled water) for another 24 hours. Na^+-K^+-ATPase activity was checked with or without the presence of fenoldopam. C, Results are expressed as percentage change of control. **P*<0.0001 vs $PM_{2.5}$ only (100 μg/mL), $^{\#}P$<0.0001 vs nonfenoldopam treatment in each group (n=10 in each group). D, Fenoldopam-induced reduction of Na^+-K^+-ATPase activity in each group. **P*<0001 vs fenoldopam only, $^{\#}P$<0001 vs siRNA treatment (n=10 in each group). E and F, Effect of GRK4 siRNA on dopamine D_1 receptor (D_1R) phosphorylation and GRK4 protein expressions in RPT cells exposed to $PM_{2.5}$ (100 μg/mL). After transfection with GRK4 siRNA for 24 hours, D_1R phosphorylation (E) and GRK4 expression (F) were checked in $PM_{2.5}$-exposed RPT cells. **P*<0001 vs control, $^{\#}P$<0001 vs $PM_{2.5}$ only (100 μg/mL) (n=10 in each group).

200 μg/mL $PM_{2.5}$ decreased RPT cell viability; $PM_{2.5}$ concentrations from 10 to 100 μg/mL had no effect (Figure 2A). Therefore, we used 10 and 100 μg/mL as lower and higher concentrations, respectively, in the in vitro study.

Consistent with previous research findings,[43,44] the selective D_1 receptor agonist fenoldopam caused an inhibition of the Na^+-K^+-ATPase activity, achieving a maximum of ≈30% in the control rats. With $PM_{2.5}$ exposure, the basal Na^+-K^+-ATPase activity was increased and the fenoldopam-mediated inhibitory effects on Na^+-K^+-ATPase activity were reduced (Figure 2B and 2C). Moreover, we also found that, compared with control, $PM_{2.5}$-treated cells had lower D_1 receptor expression and higher D_1 receptor phosphorylation (Figure 2D and 2E) and GRK4 expression (Figure 2F and 2G).

To further clarify the role of GRK4 on the Na^+-K^+-ATPase activity after $PM_{2.5}$ exposure, we used a specific GRK4 siRNA to decrease the expression of GRK4 (Figure 3A and 3B). Downregulation of GRK4 by the siRNA reduced the increase in the basal Na^+-K^+-ATPase activity and reversed the inhibitory effect of fenoldopam on Na^+-K^+-ATPase activity in $PM_{2.5}$-treated cells (Figure 3C and 3D). Moreover, the decreased D_1 receptor expression (Figure 3E) and increased D_1 receptor phosphorylation (Figure 3F) by $PM_{2.5}$ were also reversed after GRK4 siRNA treatment in RPT cells.

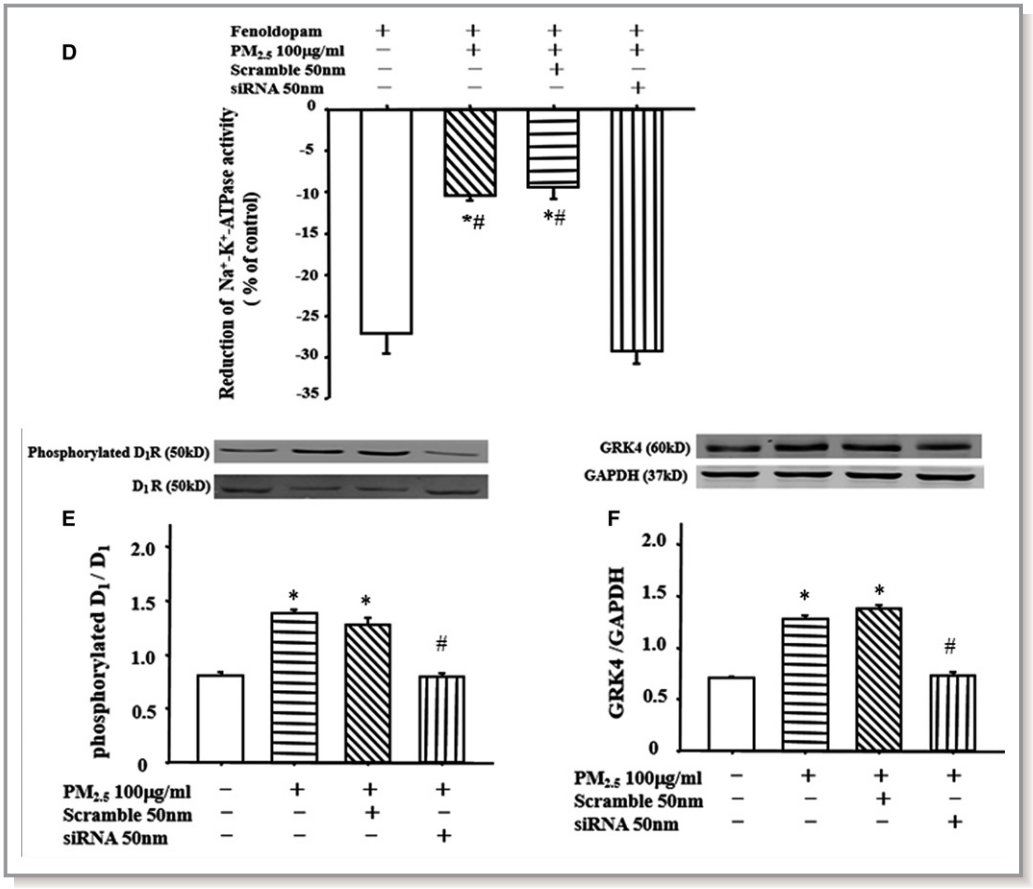

Figure 3. Continued.

Role of ROS in $PM_{2.5}$-Induced Hypertension and Impaired Renal Function

ROS has been reported to play a role in the dysfunction of D_1 receptor in obese Zucker rats,[23,26,27] and there is evidence showing that $PM_{2.5}$ increases inflammation and ROS production.[28,31] Therefore, we wondered whether ROS is involved in the pathogenesis of $PM_{2.5}$-induced hypertension. We first measured ROS production in the $PM_{2.5}$- and vehicle-treated (control) rats. We found that plasma malondialdehyde levels were higher, whereas SOD and glutathione peroxidase levels were lower, in $PM_{2.5}$-treated rats versus the vehicle-treated rats (Figure 4A through 4C). The dichloro-dihydro-fluorescein diacetate staining showed ROS level was significantly increased after $PM_{2.5}$ treatment in both RPT alone and RPT/THP-1 cocultured cells, whereas the extent of ROS elevation was much higher in RPT/THP-1 cocultured cells than those in RPT cells alone with $PM_{2.5}$ treatment (Figure S4).

It is known that c-Myc is a transcription factor for GRK4, and there is evidence that ROS increases c-Myc expression.[45–47] We, therefore, tried to determine whether $PM_{2.5}$ increased GRK4 via the c-Myc pathway. Results showed that c-Myc expression was higher in $PM_{2.5}$-treated than control cells (Figure 4D and 4E), indicating that $PM_{2.5}$ might increase GRK4 expression via an increased c-Myc expression.

To determine directly the role of ROS in $PM_{2.5}$-induced hypertension, tempol, an SOD mimetic, was used to treat $PM_{2.5}$ rats, which were exposed to $PM_{2.5}$ for 8 weeks. After tempol (1.0 mmol/L in drinking water[24,25]) treatment for 3 weeks, the increased malondialdehyde and the decreased SOD/ glutathione peroxidase levels were recovered in $PM_{2.5}$ rats (Figure 5A through 5C). Furthermore, the sodium excretion was increased, and blood pressure was lowered, in $PM_{2.5}$ rats (Figure 5D through 5G), and the fenoldopam-induced natriuresis and diuresis were also recovered in $PM_{2.5}$-exposed rats (Figure 5H and 5I), accompanied with a recovered renal D_1 receptor expression and phosphorylation and GRK4 expression (Figure 5J and 5K). It is interesting to find that the renal c-Myc expression is also reduced after treatment with tempol in $PM_{2.5}$-exposed rats (Figure 5L and 5M), which further indicated the role of c-Myc in the $PM_{2.5}$-induced hypertension.

Consistent with the in vivo study, $PM_{2.5}$ increased malondialdehyde and ROS concentrations in the supernatants from RPT cells, which were normalized by tempol (200 μmol/L) treatment (Figure 6A and 6B), whereas the high expression of

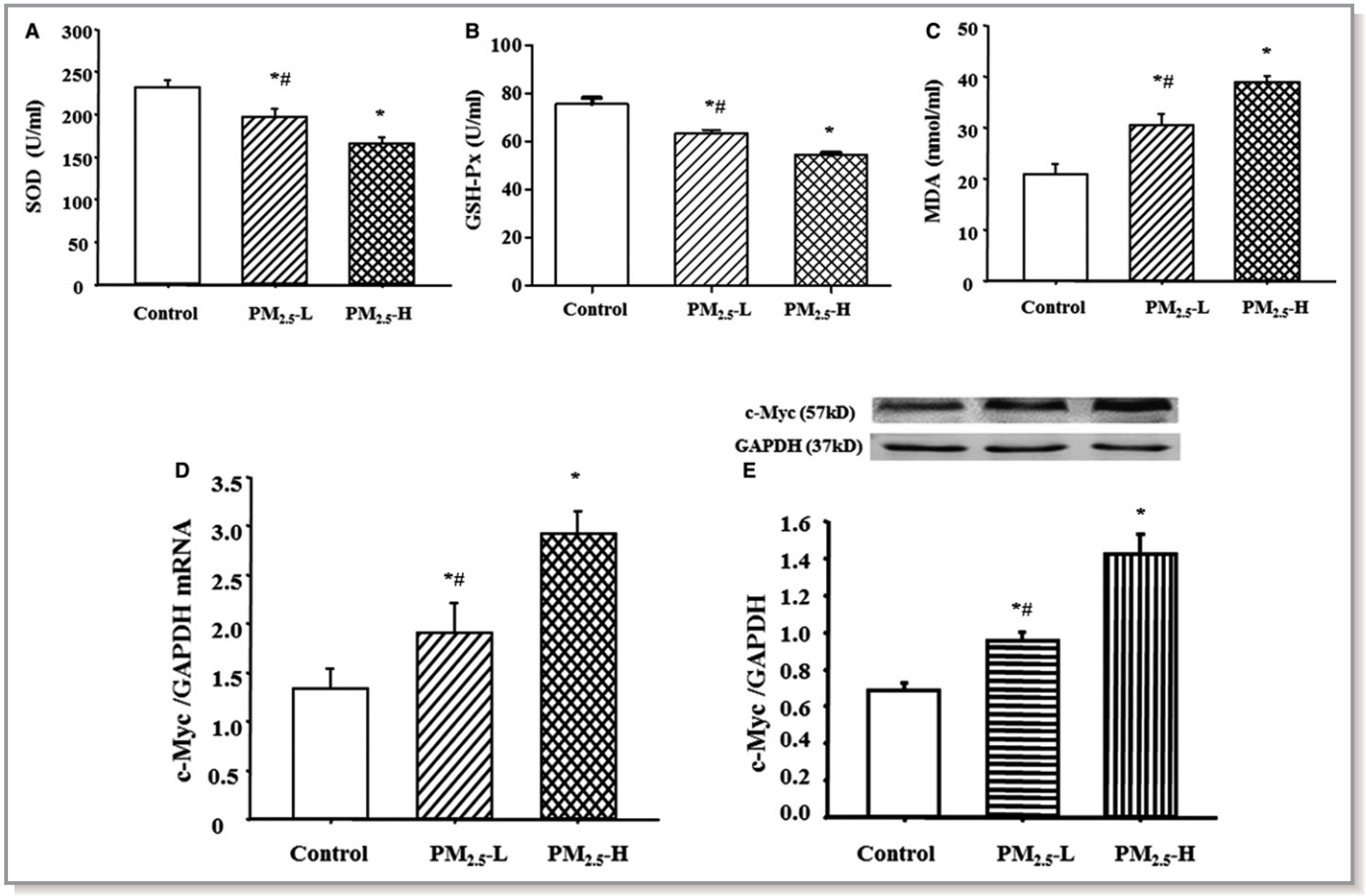

Figure 4. Effect of fine particulate matter ($PM_{2.5}$) exposure on oxidative stress and c-Myc expression in Sprague-Dawley (SD) rats. SD rats were exposed with lower (3 μg $PM_{2.5}$ per time [$PM_{2.5}$-L]) or higher (30 μg $PM_{2.5}$ per time [$PM_{2.5}$-H]) dosage of $PM_{2.5}$ for 8 weeks. Plasma superoxide dismutase (SOD) (A), glutathione peroxidase (GSH-Px) (B), and malondialdehyde (MDA) (C) levels, and c-Myc mRNA (D) and protein (E) expressions were determined in those rats. *P<0.005 vs control rats, #P<0.001 vs $PM_{2.5}$-H group (n=12 in each group).

c-Myc was decreased (Figure 6C and 6D). Moreover, tempol reversed the fenoldopam-mediated inhibitory effects on Na^+-K^+-ATPase activity in $PM_{2.5}$-treated RPT cells (Figure 6E and 6F). Meanwhile, the lowered renal D_1 receptor expression and the upregulated renal D_1 receptor phosphorylation and GRK4 expression were normalized by tempol in $PM_{2.5}$-treated RPT cells (Figure 6G and 6H).

Discussion

Exposure to ambient particulate matter has been associated with increased hospital admission and mortality for cardiovascular diseases.[4,6,7,29] However, the mechanisms are not completely understood. Hypertension is an established risk factor for cardiovascular disease and may be implicated in the association between ambient particulate matter and cardiovascular morbidity and mortality.[11] Epidemiological studies have shown that traffic[4,48] or household[49–51] $PM_{2.5}$ exposure can increase blood pressure in both the short- and long-term. Many animal experimental studies have validated the view of the above-mentioned evidence-based-studies.[8,31,52–54] In this study, $PM_{2.5}$ was confirmed to be associated with increased blood pressure, and the blood pressure was positively correlated with the exposure dose within a certain range. Even a lower concentration of $PM_{2.5}$ exposure can cause an increased blood pressure.

Although the mechanisms of $PM_{2.5}$-induced hypertension are not clear, some mechanisms, including oxidative stress, inflammatory reaction, changes in sympathetic nerve activity, DNA damage, and apparent genetic changes, have been reported to be involved in the pathogenesis of hypertension.[6,8,10–12] Our present study and others show the role of impaired sodium excretion in $PM_{2.5}$-induced hypertension. It is known that sodium excretion is regulated by numerous hormones and humoral factors, including dopamine. Renal dopamine, independent of renal nerves, plays an important role in maintaining sodium homeostasis and blood pressure regulation, especially during moderately increased sodium intake. Under conditions of moderate sodium excess, the renal dopaminergic system, mainly the D_1R, is responsible for >50% of renal sodium excretion.[13] Abnormalities in renal dopamine and D_1R response to an increased sodium load

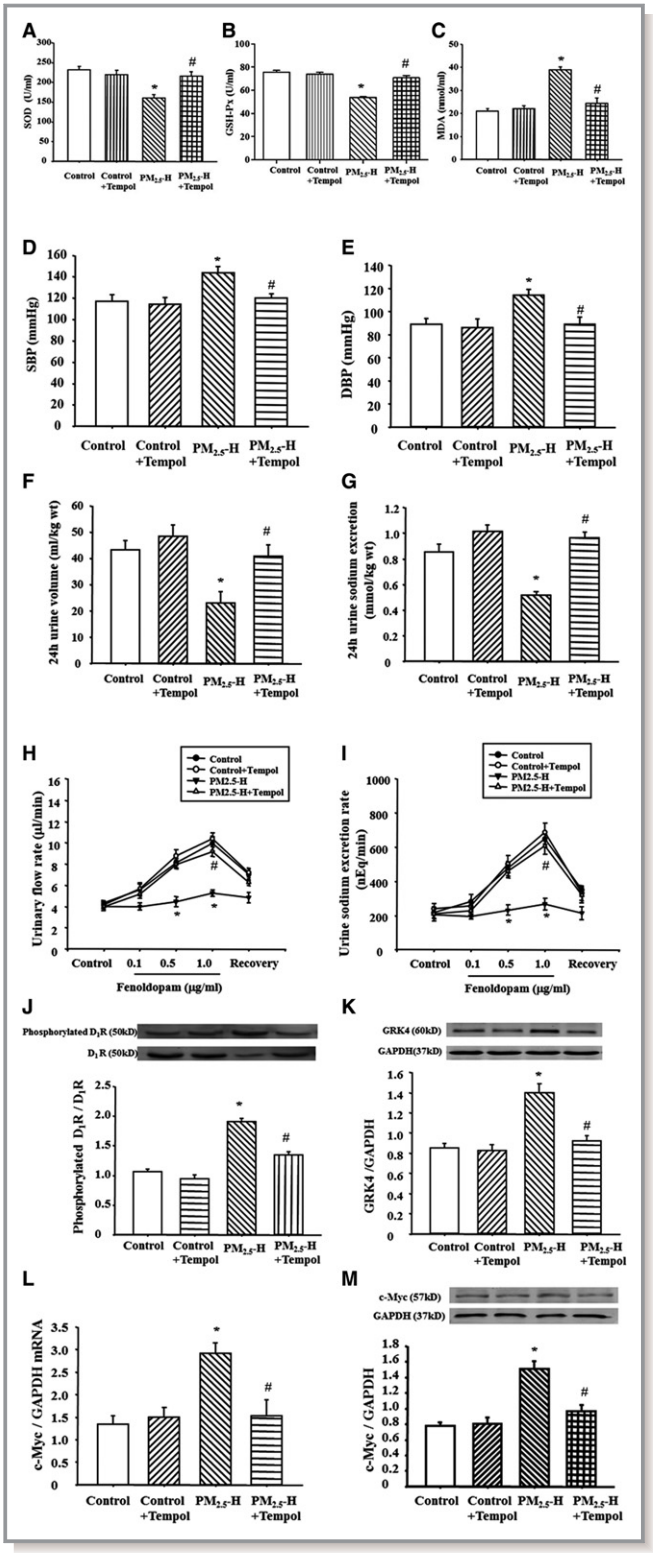

Figure 5. Effect of tempol on blood pressure and sodium excretion in Sprague-Dawley (SD) rats exposed to fine particulate matter ($PM_{2.5}$). SD rats exposed to $PM_{2.5}$ were treated with tempol for 3 weeks. Plasma superoxide dismutase (SOD) (A), glutathione peroxidase (GSH-Px) (B), and malondialdehyde (MDA) (C) levels were checked in those rats (n=12 in each group). The blood pressure (D and E) and sodium excretion (F and G) were recorded (n=12 in each group). Moreover, the fenoldopam-mediated urinary flow rate (H) and urine sodium excretion rate (I), G-protein–coupled receptor kinase 4 (GRK4) expression (J), and dopamine D_1 receptor (D_1R) phosphorylations (K), as well as c-Myc mRNA (L) and protein expressions (M) were checked (n=10 in each group). DBP indicates diastolic blood pressure; $PM_{2.5}$-H, 30 μg $PM_{2.5}$ per time; and SBP, systolic blood pressure. *P<0.0001 vs control rats, $^{\#}P$<0.0001 vs $PM_{2.5}$-H rats.

have been implicated in the diminished natriuretic response and increase in blood pressure in hypertensive patients and rodents.[13–16,23] The impaired D_1R function is ascribed to the hyperphosphorylation, which leads to the uncoupling of D_1R with G protein.[13–16] Our previous study found that the hyperphosphorylated D_1R is caused by a constitutively increased GRK4 activity and expression in hypertension.[13,18–21] Consistent with hypertension, our present study found that D_1R-mediated diuresis and natriuresis are impaired in $PM_{2.5}$-exposed rats, accompanied with D_1R hyperphosphorylation. The increased phosphorylation of D_1R is ascribed to GRK4, because in both in vivo and in vitro studies, GRK4 expression is increased because of $PM_{2.5}$ exposure; after downregulation of GRK4 with siRNA, the impaired inhibitory effect of D_1R on Na^+-K^+-ATPase activity was reversed in RPT cells exposed to $PM_{2.5}$. Thus, the current studies support the notion that GRK4 is involved in the impaired function of renal D_1R in $PM_{2.5}$-exposed hypertension.

Many studies have indicated that both hypertensive patients and animals have decreased antioxidant capacity and produce excessive amounts of ROS.[27,55–57] Antioxidant treatment could mitigate the production of ROS and further increase antioxidant capacity, such as SOD and glutathione in plasma and tissue, consequently decreasing the blood pressure in hypertensive animal models. $PM_{2.5}$ is known to increase serum tumor necrosis factor-α level and stimulate macrophages to generate ROS and enhance oxidative stress.[28–31,33,58–60] Multiple studies have shown that an impaired intrarenal D_1R signaling in hypertension can cause or be caused by oxidative stress.[24–27,61] Our present study found that tempol treatment would reduce ROS, lower blood pressure, and reverse the impaired D_1R-mediated natriuresis and diuresis in $PM_{2.5}$-exposed rats.

As stated previously and in other reports, ROS is related to the regulation of GRK4 on D_1R expression and function and tempol has been shown to reduce GRK4 level, restore D_1R expression and function, and normalize blood pressure by decreasing oxidative stress.[22,24,25] However, the molecular mechanisms involved are not well understood. c-Myc, a human homolog of the avian myelocytomatosis viral oncogene v-Myc, is involved in cancer progression, which has been implicated in hypertrophy and fibrosis of the heart, atherosclerosis, and hypertension.[45–47] c-Myc was shown to

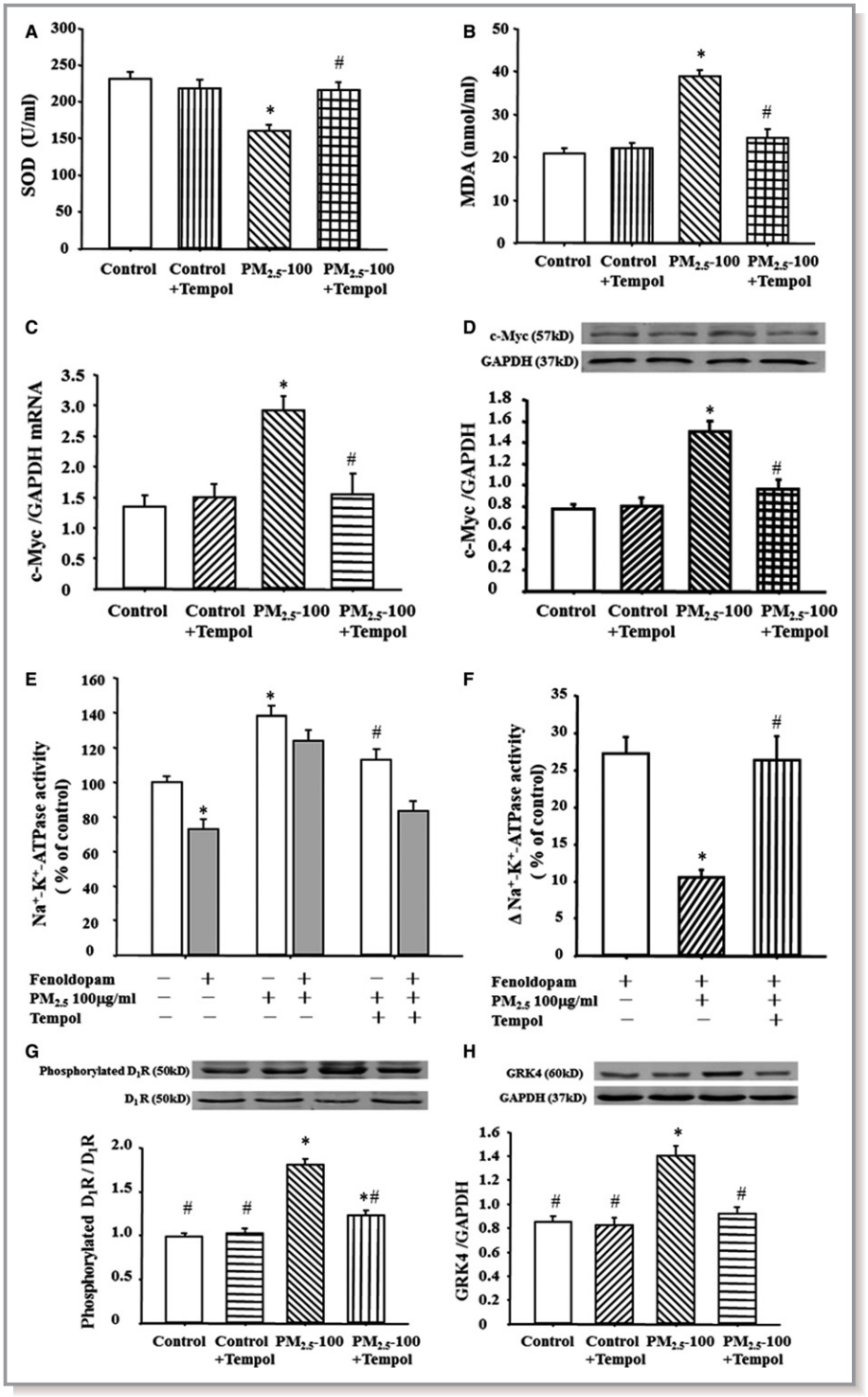

Figure 6. Effect of tempol on Na^+-K^+-ATPase activity and G-protein–coupled receptor kinase 4 (GRK4) expression in renal proximal tubule (RPT) cells exposed to fine particulate matter ($PM_{2.5}$). RPT cells exposed to vehicle (control) or $PM_{2.5}$ were treated with tempol (200 μmol/L) for 24 hours. Superoxide dismutase (SOD) (A) and malondialdehyde (MDA) levels (B) were quantified in the cell culture medium supernatant. $^*P<0.0001$ vs control, $^{\#}P<0.0001$ vs $PM_{2.5}$ only (100 μg/mL) (n=12 in each group). c-Myc mRNA (C) and protein (D) expressions were checked in those cells. $^*P<0.0001$ vs control, $^{\#}P<0.0001$ vs $PM_{2.5}$ only (100 μg/mL) (n=10 in each group). Moreover, the Na^+-K^+-ATPase activity (E and F) and GRK4 expression (G) and dopamine D_1 receptor (D_1R) phosphorylations (H) were tested in those cells. $^*P<0.05$ vs control, $^{\#}P<0.001$ vs $PM_{2.5}$ treated (100 μg/mL) (n=10 in each group).

bind to the promoter region of GRK4 in Burkitt lymphoma cells. Gildea et al found c-Myc may be involved in the increase in blood pressure in hypertension that is mediated by increased activity of the renin-angiotensin system and decreased activity of the renal dopaminergic system.[45] Many other studies also report that ROS increased c-Myc expression.[46,47] Our present study found that ROS associated with increased c-Myc levels and increased GRK4 expression in $PM_{2.5}$-exposed rats and cells.

In the present study, the particulate sampling method is universally accepted,[32–34] and we performed a scanning electron microscope analysis of the samples to determine the reliability of the samples. To ensure the success of the exposure model, we also performed a hematoxylin-eosin stain of the lung after the rats were euthanized, and a light microscope detection confirmed the presence of $PM_{2.5}$ in the lung tissue. According to the World Health Organization's Air Quality Guideline, an annual mean $PM_{2.5}$ concentration of 35 $\mu g/m^3$ was selected as the level 1 interim target, which corresponds to the highest mean concentrations reported in studies of long-term health effects. This level has been shown to be associated with significant mortality in the developed world.[62] For this reason, in this study, the rats received 2 dosages of $PM_{2.5}$. A lower dosage represented 35 $\mu g/m^3$ per 24 hours $PM_{2.5}$, whereas the higher dosage represented 350 $\mu g/m^3$ per 24 hours $PM_{2.5}$ in the air.

In summary, our study shows direct evidence supporting the hypothesis that $PM_{2.5}$ causes hypertension and, for first time, links the dysfunction of the renal D_1R to the hypertension attributable to $PM_{2.5}$ exposure. Increased oxidative stress, associated with increased GRK4 levels, impairs renal D_1R function and leads to hypertension. Restoration of D_1R function by antioxidants may be a therapeutic target for $PM_{2.5}$-caused hypertension.

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Disclosures

None.

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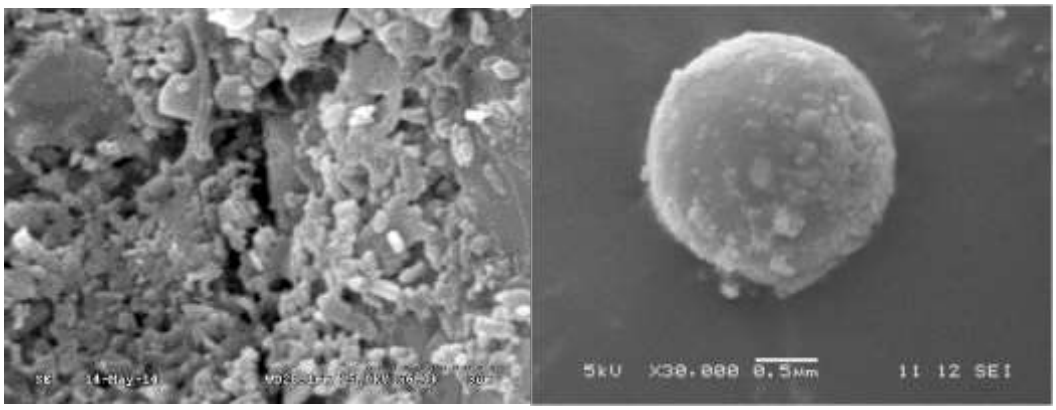
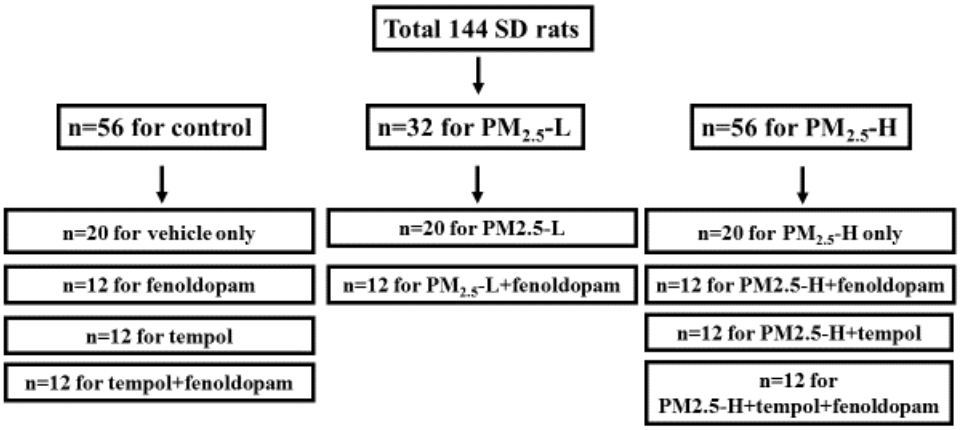
SUPPLEMENTAL MATERIAL

Figure S1. Scanning electron microscopy analysis of $PM_{2.5}$ samples.



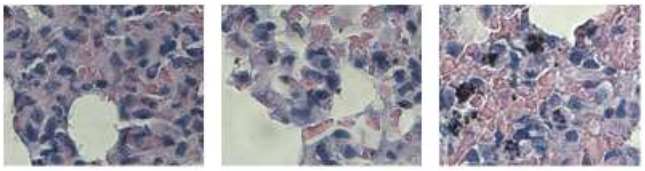
The scanning electron microscopy analysis of $PM_{2.5}$ samples showed that the collected samples were less than 2.5 μm in diameter.

Figure S2. The flowchart of animal studies.



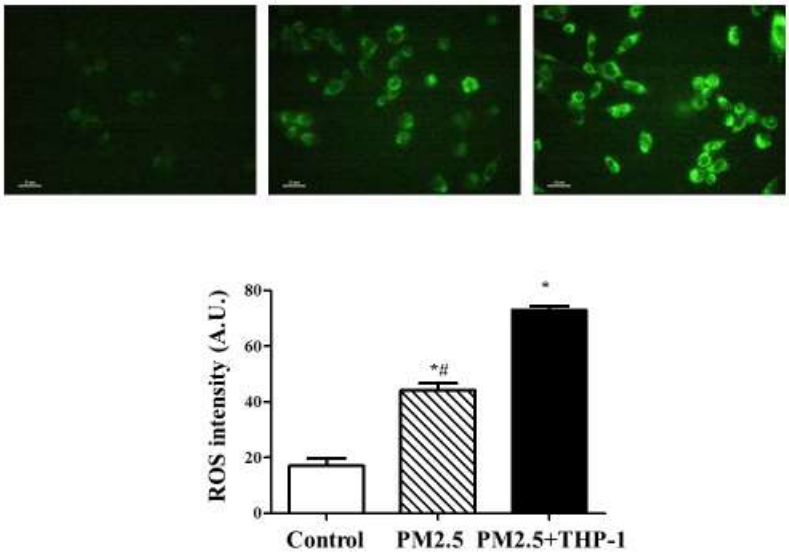
A total 144 SD rats were randomly divided into control, lower dosage $PM_{2.5}$ treatment, and higher dosage $PM_{2.5}$ treatment group. And subgroups were further divided into vehicle (or $PM_{2.5}$), +fenoldopam, +tempol, and fenoldopam+tempol.

Figure S3. Lung histology from control and exposed rats.



Representative light microscopy sections of lung tissue from control and $PM_{2.5}$-treated rats, showing particulate matter deposition and without obvious alveolar inflammatory response.

Figure S4. The ROS generation in RPT cells after PM2.5 exposure.



RPT cell and THP-1 cell, a clonal monocyte cell line, were co-cultured in a co-culture system with PM2.5 treatment. After PM2.5 exposure (100μg/ml) for 24 hours, removal of THP-1 cells, and then ROS in RPT cells was checked by DCFH-DA (2,7-dichloro-dihydro-fluorescein diacetate, a ROS fluorescent probe) staining. The ROS levels were quantified as fluorescent intensity of DCFH-DA. (*P<0.0001 vs. control, #P<0.0001 vs. THP-1/RPT co-culture cells, n=4 in each group).