Air Pollution Exposure Potentiates Hypertension Through Reactive Oxygen Species–Mediated Activation of Rho/ROCK

Qinghua Sun, Peibin Yue, Zhekang Ying, Arturo J. Cardounel, Robert D. Brook, Robert Devlin, Jing-Shiang Hwang, Jay L. Zweier, Lung Chi Chen, Sanjay Rajagopalan

Objective—Fine particulate matter <2.5 μm (PM$_{2.5}$) has been implicated in vasoconstriction and potentiation of hypertension in humans. We investigated the effects of short-term exposure to PM$_{2.5}$ in the angiotensin II (AII) infusion model.

Methods and Results—Sprague-Dawley rats were exposed to PM$_{2.5}$ or filtered air (FA) for 10 weeks. At week 9, minipumps containing AII were implanted and the responses studied over a week. Mean concentration of PM$_{2.5}$ inside the chamber was 79.1±7.4 μg/m$^3$. After AII infusion, mean arterial pressure was significantly higher in PM$_{2.5}$-FA group versus FA-AII group. Aortic vasoconstriction to phenylephrine was potentiated with exaggerated relaxation to the Rho-kinase (ROCK) inhibitor Y-27632 and increase in ROCK-1 mRNA levels in the PM$_{2.5}$-FA group. Superoxide (O$_2^{•−}$) production in aorta was increased in the PM$_{2.5}$-FAII compared to the FA group, inhabitable by apocynin and L-NAME with coordinate upregulation of NAD(P)H oxidase subunits p22phox and p47phox and depletion of tetrahydrobiopterin. In vitro exposure to ultrafine particles (UFP) and PM$_{2.5}$ was associated with an increase in ROCK activity, phosphorylation of myosin light chain, and myosin phosphatase target subunit (MYPT1). Pretreatment with the nonspecific antioxidant N-Acetylcysteine and the Rho kinase inhibitors (Fasudil and Y-27632) prevented MLC and MYPT-1 phosphorylation by UFP suggesting a O$_2^{•−}$-mediated mechanism for PM$_{2.5}$ and UFP effects.


Key Words: air pollution ▪ NADPH oxidase ▪ hypertension ▪ free radicals ▪ Rho/ROCK

Fine particulate matter (aerodynamic diameter <2.5 μm, PM$_{2.5}$) in ambient air has been implicated in the pathogenesis of cardiovascular disease. Recent studies have suggested that this risk is rapid and occurs within hours to days of exposure to high levels of PM$_{2.5}$. Increases in blood pressure may represent an important mechanism through which PM$_{2.5}$ may modulate its effects. Data from recent epidemiological studies from North America and Europe are indeed consistent with this hypothesis and have associated short-term exposure to PM$_{2.5}$ with elevations in blood pressure (BP). This effect seems to be exaggerated in predisposed individuals, an observation that has also been noted in relation to the association of PM$_{2.5}$ with other chronic conditions such as atherosclerosis. Although the precise mechanisms through which PM$_{2.5}$ gains access to the systemic vasculature is still hotly debated, there is increasing evidence that particles in the fine and ultrafine range transgress into the systemic circulation and modulate vascular tone acutely, presumably through reactive oxygen species (ROS)–dependent pathways. We hypothesized that short-term (weeks) increases in PM$_{2.5}$ levels is associated with an increases in BP and that these responses are exaggerated in a model of angiotensin II–dependent hypertension through upregulation of ROS pathways.

Methods

Animals and BP Monitoring

All experimental procedures were approved by the Committees on Use and Care of Animals from New York University and Mount Sinai School of Medicine. Male Sprague-Dawley (SD) rats (500 to 650 g) were purchased from Charles River Laboratories Inc. (Wilmington, Mass). The conscious systolic, diastolic, and mean arterial
pressure (MAP) was monitored by radio-telemetry method with the Dataquest IV system (Data Sciences International).

**PM2.5 Exposure and All Infusion**
The animal exposure and the monitoring of the exposure environment and ambient aerosol were performed as previously described. Rats were randomly exposed to PM2.5 or filtered air (FA) for a total of 10 weeks. At the end of 9-week exposure, the rats were infused with 0.75 mg/kg/d of All for 7 days. PM2.5 or FA exposure continued during All infusion. Our exposure system allows for exposure to all particles <2.5 μm in diameter and thus allows for both PM2.5 and ultrafine particles (UFP, particulate matter <0.1 μm) exposure.

**Myograph Experiments**
The myograph experiments were performed with 2-mm thoracic aortic rings mounted in organ bath chambers as previously described.

**In Situ Detection and Quantification of O2− Generation**
In situ detection and quantification of O2− generation in aortic tissues were determined with dihydrouethidium (DHE, Molecular Probes, Inc) staining and a modified high-throughput lucigenin chemiluminescence assay, respectively.

**High-Performance Liquid Chromatography Analysis of Tetrahydrobiopterin (BH4)**
BH4 content was determined in the heart, mesenteric vasculature, and liver samples by a modification of the method described previously.

**Cell Culture**
Primary rat aortic smooth muscle cells (RASMCs) were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum in a humidified atmosphere at 37°C. Cells at passages 4 to 8 were used for the experiments. Cells were treated with UFP or All for the indicated time.

**RhoA Activation Assay**
RhoA-GTP levels were determined with G-LISA RhoA activation assay kit (Cytoskeleton, Inc) according to the manufacturer’s instructions.

**Quantitative RT-PCR and Immunoblotting**
Total RNA was prepared from aortic tissues and subjected to real-time quantitative RT-PCR. Whole lysates of aortic samples were prepared and subjected to immunoblotting.

**Data Analyses**
All data are expressed as mean±SE unless otherwise specified. Comparisons between groups of animals or treatments were made with 1-way analysis of variance (ANOVA). When significance was indicated, a Student-Newman-Keuls post hoc analysis was used. Significance was considered at a value of *P*<0.05. The Fishing License method was used to analyze blood pressure differences including mean arterial pressure between the PM2.5-All and FA-All groups as detailed previously.

For details, please see the supplemental materials, available online at http://atvb.ahajournals.org.

**Results**

**PM2.5 Concentrations During the Study Period**
The mean daily ambient PM2.5 concentration at the study site was 6.1±0.4 μg/m3, whereas the mean concentration inside the PM2.5 chamber was 79.1±7.4 μg/m3. During the exposure period, the outdoor mean temperature was 5.9±8.9°F (median 5.8°F), and the outdoor mean humidity was 63.2±20.6% (median 58%). Because the rats were exposed for 6 hours a day, 5 days a week, the equivalent PM2.5 concentration to which the rats were exposed to in the chamber “normalized” over the 10-week period was 14.1 μg/m3 after taking into account nonexposed time and weekends, which is well within the annual average PM2.5 National Ambient Air Quality Standard of 15.0 μg/m3 (US Environmental Protection Agency).

**BP Change**
Figure 1A displays the mean arterial pressure at baseline after implantation of the radiotelemetry device and after 9 weeks of PM2.5 exposure (Pre-All) compared to the FA exposed group. There was no change in mean arterial pressure after PM2.5 or FA exposure alone (96±3 and 98±4 mm Hg versus 101±2 and 103±3 mm Hg after PM2.5 and FA exposure, respectively). Figure 1B depicts changes in mean arterial pressure (MAP) in response to a 7-day infusion of All. MAP was significantly higher after All compared to baseline beginning at 24 hours. The MAP response was significantly different between FA-All and PM2.5-All groups, beginning at 93.0±16.7 hours and lasting until the end of the monitoring period (hour 135.8±5.2; *P*<0.0001, Figure 1B). The slopes of the BP curves were significantly different with a persis-
tently positive slope for the PM_{2.5}-AII animals compared to the FA-AII group (Figure 1B).

**Vasomotor Responses**

Figure 2A depicts responsiveness of thoracic aortic segments to the α-adrenergic agonist phenylephrine (PE). Responses to PE in the PM_{2.5}-AII group were characterized by a shift in the half-maximal dose for constriction (EC_{50}, 1.4±0.1×10^{-8} versus 2.5±0.1×10^{-8} mol/L, P<0.05) and an increase in peak constriction compared with FA-AII. Figure 2B and 2C demonstrate responses of preconstricted aortic segments to the endothelium-dependent vasodilator acetylcholine (Ach) and relaxation of aortic rings in response to endothelium dependent vasodilator acetylcholine (B) or ROCK inhibitor Y-27632 (C) (n=6). *P<0.05 vs FA-AII for peak constriction or dilation, †P<0.05 vs FA-AII for EC_{50} or ED_{50}.

**Superoxide Generation**

We used DHE staining (in situ method) and lucigenin chemiluminescence assays to determine superoxide (O_{2}^{-}) generation in the aorta. O_{2}^{-} production in aortic rings was markedly enhanced in PM_{2.5}-AII group compared with the FA-AII group. Pretreatment of aortic sections with O_{2}^{-} scavenger polyethylene glycol (PEG)-superoxide dismutase (PEG-SOD) reduced the DHE fluorescence, confirming the authenticity of the signal (data not shown). The NAD(P)H oxidases in the vasculature, are composed of 2 membrane-associated subunits p22^{phox} and gp91^{phox} (also named Nox2 [neutrophil oxidase 2] in vascular smooth muscle cells, or Nox4 in endothelial cells), and the cytosolic components p47^{phox}, p67^{phox}, and the small GTP-binding protein Rac (Ras-related C3 botulinum toxin substrate). The mRNA expression levels of both the membrane-associated subunit p22^{phox} and the cytoplasmic subunit p47^{phox} were significantly increased in the aortic tissues of PM_{2.5}-AII group compared with those of the FA-AII group (Figure 3A). No changes were observed in the expression levels of other components in these 2 groups, including p67_{phox}, Nox2 (gp91^{phox}), and other 2 members of the family of gp91^{phox}-like proteins Nox1 and Nox4 (Figure 3B). There were no differences in the expression levels of the small GTP binding cytosolic units Rac-1 or Rac-2 (data not shown).

**Tetrahydrobiopterin (BH_{4}) Levels in Response to PM_{2.5}**

Because we demonstrated L-NAME mediated inhibition of O_{2}^{-}, we investigated whether BH_{4} depletion was involved as a mechanism for eNOS uncoupling in response to PM_{2.5}-mediated oxidant stress. BH_{4} levels in the mesenteric vasculature (resistance vessels) and heart were quantified. These were 6.5±1.2 and 9.7±1.3 pmol/mg protein in the FA-AII group compared with 3.5±0.9 and 5.9±0.8 pmol/mg protein, respectively in the PM_{2.5}-AII group, which represent a 46% and 41% reduction, respectively (n=6 per group, P<0.05 for both heart and mesenteric tissue). Additionally, BH_{4} levels in the liver, an important site of BH_{4} synthesis and a highly vascular organ, were decreased in the PM_{2.5}-AII versus FA-AII (27.2±2.1 versus 15.8±3.0 pmol/mg protein, P<0.05), consistent with a systemic effect of PM_{2.5} on extrapulmonary tissues.

**NADPH Oxidase Subunit Expression**

NAD(P)H oxidases in the vasculature, are composed of 2 membrane-associated subunits p22^{phox} and gp91^{phox} (also named Nox2 [neutrophil oxidase 2] in vascular smooth muscle cells, or Nox4 in endothelial cells), and the cytosolic components p47^{phox}, p67^{phox}, and the small GTP-binding protein Rac (Ras-related C3 botulinum toxin substrate). The mRNA expression levels of both the membrane-associated subunit p22^{phox} and the cytoplasmic subunit p47^{phox} were significantly increased in the aortic tissues of PM_{2.5}-AII group compared with those of the FA-AII group (Figure 3A). No changes were observed in the expression levels of other components in these 2 groups, including p67_{phox}, Nox2 (gp91^{phox}), and other 2 members of the family of gp91^{phox}-like proteins Nox1 and Nox4 (Figure 3B). There were no differences in the expression levels of the small GTP binding cytosolic units Rac-1 or Rac-2 (data not shown).
Expression of RhoA/ROCK

To determine the effect of PM2.5 on the RhoA/ROCK expression, mRNA and protein levels of RhoA and ROCKs in aortic tissues were detected. Relative mRNA level of ROCK1 was 2.6-fold higher in the PM2.5-AII group versus the FA-AII group (Figure 3C, *P ≤ 0.05), whereas ROCK-2 level (expressed predominantly in brain and skeletal muscle) was not different between the groups (P > 0.05). No difference was found in the expression level of RhoA mRNA (Figure 3D, P > 0.05). Although the protein level of RhoA protein in aortic tissues was 2.5 times higher in the PM2.5-AII group compared with the FA-AII group, this difference was not statistically significant (Figure 3E, P > 0.05).

Ultrafine Particles Mediates Myosin Light Chain Activation Through RhoA/ROCK Pathways

To further investigate the mechanism through which PM2.5 mediates smooth muscle vasoconstriction and hypertension and the involvement of ROS in RhoA/ROCK activation, we performed an in vitro study where we exposed rat aortic smooth muscle cells (RASMCs) with UFP. We chose these as particles in this size range have been shown to transgress into the systemic circulation. Further our exposure system allows for simultaneous exposure to PM2.5 and particles in the UFP range (PM0.1). Exposure of RASMCs to 10 μg/mL of UFP rapidly and significantly induced the activation of RhoA (Figure 4A), which was similar to the activation of RhoA induced by 100 nmol/L of AII (supplemental Figure III). The effect of PM2.5 on Rho activity was considerably weaker than the effects of UFP (supplemental Figure III). Because the well known myosin phosphatase target subunit (MYPT1) is a major effector of RhoA/ROCK-mediated Ca2+ sensitization and a regulator of myosin light chain (MLC) activation for the contraction in smooth muscle cells, we tested the ability of UFP and PM2.5 exposure to phosphorylate MLC and MYPT1. Figure 4B to 4E depicts the results in response to acute exposure of UFP and PM2.5 in RASMCs. Exposure to 10 μg/mL of UFP rapidly induced the phosphorylation of MLC in RASMCs (Figure 4B). Both UFP and PM2.5 exposure exhibited similar magnitude of effect on MLC phosphorylation. These effects were not dose-dependent with the lower concentration (10 μg/mL) showing more potent effect than high concentration (50 μg/mL; Figure 4C and 4D). Moreover, both UFP and PM2.5 induced the phosphorylation of MLC and MYPT1 to the same extent of that induced by AII, a well known activator of ROCK (Figure 4C and 4E). The activation of MLC induced by UFP exposure in RASMCs was inhibited by ROCK inhibitors Y-27632 and fasudil and by the nonspecific thiol antioxidant N-acetyl-L-cysteine (NAC, Figure 4F), implicating ROS mediated ROCK activation in response to PM.

Discussion

We demonstrate in this article that 10 weeks of exposure to concentrated ambient PM2.5 potentiates hypertension in response to AII and alters vasoconstrictor/vasodilator sensitivity. These alterations were accompanied by increased NAD(P)H oxidase and NOS-dependent generation of O2·− and upregulation of the RhoA/ROCK pathway. Because exposure to PM2.5 alone did not alter BP, we did not pursue additional investigations in the PM2.5 group alone and investigated the impact of PM2.5 in conjunction with AII. An additional reason to examine the effect of PM2.5 in conjunction with AII is prior observations by us and others that suggest that PM2.5 has minimal effects by itself, but actively synergizes with other risk factors to influence out-
It also has a human of reactive oxygen species (ROS) through an NAD(P)H oxidase–dependent mechanism.19,20,21 It is well known that AII infusion as well as deoxycorticosterone acetate (DOCA) can result in O2− production through NOS uncoupling attributable to depletion of the NOS cofactor BH4.33 Thus our observations provide in vivo confirmation of PM2.5-mediated vascular effects through dysregulation of 2 major homeostatic pathways. Based on our findings, it can be hypothesized that PM2.5 exposure in the presence of AII may activate NAD(P)H oxidases which then could lead to further BH4 depletion and NOS uncoupling.

The increased activity of Rho/ROCK in this model is a new finding and one that may provide additional mechanistic basis for increase in BP seen with PM2.5 in prior studies.7–9,34 The Rho/ROCK pathway is a key regulator of vascular smooth muscle tone through its effects on calcium sensitization of the contractile apparatus.35 Blockade of Rho/ROCK signaling through the usage of the ROCK inhibitors Y-27632 or hydroxyfasudil ameliorates BP and blood flow in hypertensive animals and humans, implicating this pathway in the

**Figure 4.** PM exposure induces MLC activation through RhoA/ROCK-mediated pathways in cultured rat aortic smooth muscle cells. For all experiments, cells were serum starved and exposed to UFP or PM2.5 for the indicated time or dose. A, The level of "active" GTP-RhoA in cell lysates detected with a specific anti-RhoA antibody. Values are expressed as mean±SE (n=3) *P<0.05 vs 0 minutes. B, Time course of MLC phosphorylation with UFP. Cells were exposed to 10 μg/mL of UFP for the indicated time and MLC phosphorylation was determined by immunoblotting. C, Comparison of UFP with PM2.5 on MLC phosphorylation. Cells were exposed to 10 μg/mL of UFP or PM2.5 or to 100 nmol/L of All (positive control) for the indicated time. MLC phosphorylation was determined by immunoblotting. D, Effect of UFP and PM2.5 on MLC phosphorylation contrasted by dose. 10 or 50 μg/mL of UFP or PM2.5 were used for 5 minutes with MLC phosphorylation determined by immunoblotting. E, Effect of UFP compared with All on phosphorylation of large sub-unit of myosin phosphatase-MYPT1. Cells were treated with 10 μg/mL of UFP or 100 nmol/L of All for 3 minutes, and the phosphorylation of MYPT1 and MLC were detected. F, PM effects on MLC phosphorylation are mediated by ROS and Rho/ROCK pathways. Cells were exposed to 10 μg/mL of UFP for 3 minutes with or without pretreatment for 20 minutes with the thiol antioxidant N-acetylcysteine (NAC, 5 mmol/L), Y-27632 (10 μmol/L) and fasudil (10 μmol/L). All data shown are representative of 3 to 4 independent experiments.

Our data are consistent with this notion and suggest that although PM2.5 by itself had no discernible impact on BP, has an important effect in potentiating it, presumably by “sensitizing” the vasculature. The AII infusion model is a well characterized model of hypertension, where at least a portion of the BP elevation is related to the generation of reactive oxygen species (ROS) through an NAD(P)H oxidase–dependent mechanism.19,20,21 It also has a human analogue (renovascular hypertension) with the dose of AII used in this experiment, being comparable to that seen in these patients.22 Thus, the usage of this model to test the effects of PM2.5 exposure (a well known generator of ROS) was deliberate and planned. There is now increasing evidence that a number of components of PM2.5 may be facile mediators of redox cycling events such as polycyclic aromatic hydrocarbons, quinones, and transition metals.23 These events may be exaggerated in vulnerable patient populations such as diabetics, hypertensives, and individuals with established cardiovascular diseases.

Both animal models and human studies have demonstrated a central role for ROS in the pathogenesis of hypertension.24–26 In the vasculature, the NA(D)PH oxidase system, a prototypical electron transport chain with both membrane (p22phox, Nox-1, 2, 3, or 4, depending on the tissue and species) and cytosolic units (p47phox, p67phox, Rac-1), have been shown to be functionally important in AII-mediated O2− production and in the genesis of hypertension.20,27–29 We have shown upregulation of key components of this oxidase (p22phox and p47phox) by PM2.5. The finding that PM2.5 activates the NAD(P)H oxidase system above and beyond what one may encounter with AII alone likely represents a specific
pathogenesis of hypertension.\textsuperscript{36,37} Rho/ROCK may potentially interact with the NAD(P)H oxidase system at multiple loci. Both ROS and AII, through NAD(P)H oxidase, have been previously shown to activate Rho/ROCK.\textsuperscript{38,39} Thus PM\textsubscript{2.5} may potentially synergize with AII-derived ROS generation to upregulate calcium sensitization pathways. Our in vitro experiments, where UFP and PM\textsubscript{2.5}-derived \textsuperscript{18}O\textsubscript{2} generation activates Rho/ROCK strongly implicates ROS generation as being a proximal signaling pathway. This is consistent with prior publications suggesting that ROS (primarily NADPH oxidase--derived) is proximal and important for Rho/ROCK activation.\textsuperscript{40,41} Our findings suggest that additional sources of ROS such as uncoupled eNOS may additionally be important.

The exposure pattern in our current study is environmentally relevant and allows for exposure to PM\textsubscript{2.5} and UFP. The latter particles have been shown to transgress the pulmonary barrier and justify the use of the UFP in the in vitro study.\textsuperscript{11,12} The peak daily levels of exposure, although higher than the recently revised daily PM\textsubscript{2.5} NAAQS standards (<35 \textmu g/m\textsuperscript{3}, http://www.epa.gov/air/criteria.html) is regularly encountered in niches in urban areas or in close vicinity to automobiles and power plants. This situation at a global level is far worse as suggested by daily PM\textsubscript{2.5} levels in urban areas in developing countries such as India and China where daily PM\textsubscript{2.5} levels may exceed 200 \textmu g/m\textsuperscript{3}.\textsuperscript{42} The mean levels of exposure in our study of 14.1 \textmu g/m\textsuperscript{3} is within the annual NAAQS standards, suggesting a discernible effect of PM\textsubscript{2.5} at levels previously thought to be safe. Our findings thus have major implications for further regulations in PM levels.

In conclusion, exposure to PM\textsubscript{2.5} may potentiate hypertension through NAD(P)H oxidase and eNOS dependent ROS generation, which in turn activates the Rho/ROCK signaling pathway. These findings have important implications for PM\textsubscript{2.5}-mediated cardiovascular effects and suggest that vascular effects of PM\textsubscript{2.5} may modulate sensitivity to pressor stimuli.

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Disclosures

None.

References


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Supplemental Methods

Animal Model and Ambulatory Blood Pressure Monitoring

The Committees on Use and Care of Animals from New York University and Mount Sinai School of Medicine approved all experimental procedures. Male Sprague-Dawley (SD) rats of 500-650 g (n = 12) were purchased from Charles River Laboratories Inc. (Wilmington, MA), and maintained on regular chow for 1 week. At the end of this period telemetry transmitter probe (model TA11PA-C40, Data Sciences International, St. Paul, MN) was implanted into the rat under isoflurane anesthesia (2.0% isoflurane in 1 L/min oxygen flow), and the flexible tip of the probe positioned in the abdominal aorta, immediately below the renal arteries. The transmitter was then surgically sutured into the abdominal wall and the incision closed. The rats were allowed to recover for 6 days prior to beginning measurements. The conscious systolic, diastolic and mean arterial pressure (MAP) in unrestricted and untethered animals were monitored continuously with the Dataquest IV system (Data Sciences International), which consisted of an implanted radiofrequency transmitter and a receiver placed under each cage. The output was relayed from the receiver through a consolidation matrix to a personal computer. At the end of this period, rats were randomized to receive fine particulate matter < 2.5 μm (PM$_{2.5}$, n=6) or filtered air (FA, n = 6). Individual 10-s blood pressure (BP) and activity waveforms were sampled every 5 minutes throughout the course of the study, and daily averages were then calculated.

PM$_{2.5}$ Exposure Protocol
**Whole body exposure:** The animal exposure and the monitoring of the exposure environment and ambient aerosol were performed as previously described. Animals were exposed to PM$_{2.5}$ which was concentrated from northeastern regional background to 10x ambient concentrations, for 6 hours per day, 5 days per week for a total of 10 weeks. The FA exposure consisted of an identical protocol as that of the PM$_{2.5}$ with the exception of a high-efficiency particulate air (HEPA) filter that was positioned in the inlet position to remove all PM$_{2.5}$.

**Intranasal exposure:** C57BL/6 mice were anesthetized as stated above. One dose of either PM$_{2.5}$ particles (50 mg/kg in 200 μl normal saline) or normal saline was delivered intranasally for intravital microscopy.

**Analysis of PM$_{2.5}$ Concentration in the Exposure Chamber**

To calculate exposure mass concentrations of PM$_{2.5}$ in the exposure chambers, samples were collected on Teflon filters (Gelman Teflo, 37 mm, 2.0 μm pore; Gelman Sciences, Ann Arbor, MI) and weighed before and after sampling in a temperature and humidity–controlled weighing room using an oscillating microbalance (Tapered-Element Oscillating Microbalance, Model 1400; Ruprecht and Patashnick, East Greenbush, NY). The weight gains were used to calculate the mass concentrations that the animals were exposed to.

**Angiotensin Il Infusion**

At the end of week 9 of exposure, the rats were anesthetized as stated previously, without knowledge of the exposure groups. An Alzet osmotic pump
(model 2001, Alza Corporation, Mountain View, CA) containing angiotensin II (AII, dissolved in a solution of 0.15 mol/L NaCl and 0.01 N acetic acid at a concentration calculated to deliver 0.75 mg/kg/day of AII) was inserted into a subcutaneous pocket in the back of the rat and infused for a duration of 7 days. The dose of AII provides a plasma concentration similar to that reported in patients with renovascular hypertension and is based on prior publications.\textsuperscript{3,4}

The animals were recovered and were exposed to PM\textsubscript{2.5} beginning 24 hours after implantation. PM\textsubscript{2.5} or FA exposure continued during AII infusion. In additional experiments to explore the functional role of Rho/ROCK activation, we performed studies with C57BL/6 mice that were administered Fasudil (1 mg/kg/day, Sigma Aldrich, St. Louis, MO) or placebo (0.9% NaCl) intraperitoneally, in conjunction with the AII infusion (model\_1007D). Fasudil and its major active metabolite, hydroxyfasudil, are both specific inhibitors of Rho-kinase (ROCK).

\textit{Intravital Microscopy}

These studies were performed to assess the effect of PM\textsubscript{2.5} in modulating resistance vessel function. C57BL/6 mice were anesthetized intraperitoneally by a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) 24 hours after intranasal exposure. The cremaster muscle was exteriorized on an optically coherent mount. The tissue was first superfused with Ringers Lactate at 37°C, then superfused with acetylcholine (Ach) in Ringers Lactate (10\textsuperscript{-5}M). The arteriole diameters were measured before and after Ach superfusion on the same
selected vessels in 5-10 movies acquired with a 40x/0.80 W water immersed objective using a Nikon Eclipse FN1 microscope (Nikon, Japan) and Metamorph software (version 7.1.2.0, Metamorph, Downingtown, PA). Vessel diameters of 20 – 50 μm ranges were chosen for vascular relaxation response measurement.

**Myograph Experiments**

At the end of the exposure period, rats were euthanized by phlebotomy from the heart under isoflurane anesthesia. The ascending aortas were removed, and the 2-mm thoracic aortic rings were suspended in individual organ chambers that were filled with physiological salt solution (PSS) using methods previously described. The vessels were subjected to graded doses of vasoconstrictor phenylephrine (PE, $10^{-9}$ to $10^{-5}$ mol/L). Responses were then expressed as a percentage of the peak response to 120 mmol/L of potassium chloride (KCl) in PSS (KPSS). After a stable contraction plateau was reached with PE, which was about 50% of peak tension generated with 120 mmol/L of potassium chloride, the rings were exposed to graded doses of the endothelium-dependent agonist Ach ($10^{-10}$ to $10^{-5}$ mol/L), or Rho-kinase (ROCK) inhibitor Y-27632 ($10^{-9}$ to $10^{-5}$ mol/L). In this study, the degree of relaxation of aortic rings to increasing concentrations of Y-27632 in PE preconstricted segments was used as an index of ROCK activity. For basal NO bioavailability contribution measurement, the rings were pre-incubated with L-NMMA ($10^{-4}$ mol/L) for 30 min, and then subject to PE constrictive response.

**In situ Detection and Quantitation of O$_2^\cdot$ Generation**
O$_2^-$ generation from aortic tissue was determined using two methodologies. In the first set of experiments, in situ detection of O$_2^-$ was performed in snap-frozen aortic tissues embedded in OCT compound (Tissue-Tek®, Sakura Finetek USA Inc, Torrance, CA). Tissue samples were cryosectioned at 20 μm of thickness, collected onto Superfrost Plus slides (Fisher Scientific, Pittsburg, PA), and stored at -80°C until needed. Four slides that were randomly chosen from each rat (tissue block) were placed into phosphate buffered saline (PBS) for 30 min at room temperature and then stained with dihydroethidium (DHE, 10 μM, Molecular Probes, Inc., Eugene, OR) in PBS for 30 min in a moist chamber in the dark. The slides were rinsed extensively with PBS, coverslipped, and digitally imaged with a research microscope (Zeiss Axioskop with a Spot I digital camera, Jena, Germany). DHE is freely permeable to cells and in the presence of O$_2^-$, can undergo a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide that is trapped intracellularly by intercalation into DNA. The reaction is relatively specific for O$_2^-$, with minimal oxidation induced by cellular peroxidases and hydrogen peroxide.$^5$ For inhibitor studies, slides were incubated with 300 μM apocynin (Sigma), 10 μM diphenyliodonium (DPI, Fisher Scientific), or PEG-SOD (250 U/ml) for 30 min before being stained with DHE as described above. In the second set of experiments, quantification of O$_2^-$ production in freshly isolated aortic rings was performed in 96-well microplates using lucigenin (5 μM) chemiluminescence method, and the specific counts per minute were normalized to the dry weight of the tissue. The lucigenin method utilized in this study is a modified high-throughput method, which accommodates comparisons of multiple
Validation of the high-throughput method utilized a purified substrate/enzyme source of $O_2^-$, xanthine/xanthine oxidase, in the presence and absence of SOD. Prior studies have documented that this concentration of lucigenin allows measurement of radical species without redox cycling of the detector compound itself. Background wells contained lucigenin solution without tissue with at least one column/row of well separation to minimize crosstalk and prevent light piping between wells. The biophotonic images of the plates were acquired using the IVIS® Imaging System 200 Series (Xenogen Corporation, Alameda, CA), and analyzed using the Living Image® software (Xenogen Corporation). The software adopted a pseudo color scale to represent variations of signal intensity, and also accounted for and subtracted out background signal. Emitted light was quantified from regions of interest (ROI) sized to encompass each well. Absolute intensity calibration of the IVIS® Imaging System 200 Series was accomplished utilizing a calibrated 8-inch integrating sphere (OL Series 425 Variable Low-Light-Level Calibration Standard, Optronic Laboratories, Inc., Orlando, FL). By imaging and measuring a ROI on the integrating sphere, counts detected by the CCD camera digitizer were converted to physical units of radiance in photons/second/cm²/steradian, as described by Rice et al. A subset of rings was pre-incubated for 30 min with one of the following compounds: PEG-SOD (300 U/ml), apocynin (300 μM), L-NAME (10 μM).

High-Performance Liquid Chromatography Analysis of Tetrahydrobiopterin ($BH_4$)
BH₄ content was determined in heart, mesenteric vessel and liver samples by modification of the method described previously.⁹ At the time of analysis, samples were diluted 1:10 in ice-cold 50 mM Tris (pH 7.4) containing ascorbate to prevent BH₄ auto-oxidation. BH₄ levels were determined using a Shimadzu pump with an ESA 7400 autosampler, a Tosho Haas (Milford, MA) ODS-80Tm column, and an ESA coulochem 5600 electrochemical detector.

**Cell Culture and In-Vitro Experiments with Particles**

Primary rat aortic smooth muscle cells (RASMCs) were purchased from Cell Application, Inc. (San Diego, CA) and maintained in Dulbeco modified Eagle medium with 10% fetal bovine serum in a humidified atmosphere in 5% CO₂ at 37°C. Cells at passages 4-8 were used for the experiments. Cells were treated with or without fine (PM₂.₅) or ultrafine particulate matter particles (<0.1 μm, UFP) collected using a high-volume cascade impactor, as described by Duvall et al.¹⁰ or AII (100 nmol/L) for the indicated time. These ambient UFP were collected in Tuxedo, NY with a high-volume cascade impactor (model 2400, Thermo Scientific Inc., Waltham, MA) for 24 h/day for 4 week in September, 2006. The particles were collected on prewashed polyethylene final filter for PM₀.₁ particles. The particles were removed into sterile water by ultrasonication, lyophilized, and resuspended into cell culture media, as indicated previously.¹¹ In some of the experiments cells were pretreated with the indicated inhibitors for 20 min. The UFP was suspended in PBS at a 5 mg/ml concentration and vortexed immediately prior to adding to cell monolayers.
**RhoA Activation Assay**

RhoA-GTP levels were determined with G-LISA™ RhoA activation assay kit (Cytoskeleton, Inc., Denver, CO) according to the manufacturer’s instructions. Briefly, the RhoA G-LISA kit contains a Rho-GTP-binding protein linked to the wells of a 96-well plate. Active GTP-bound RhoA in the cell lysates will bind to the wells while inactive GDP-bound RhoA is removed during washing steps. Equal amount of the lysates were subjected to incubate in the Rho-GTP affinity plate. The bound active RhoA was detected with a specific anti-RhoA antibody and the signal was developed with colorimetric method.

**Quantitative RT-PCR and Immunoblotting**

Total RNA was prepared from aortic tissue with an RNeasy Mini kit (Qiagen, Valencia, CA). The cDNA was synthesized by a Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN). Real-time quantitative PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Sequences of primers used for real-time PCR in this study are listed in Supplemental Table.

Aortic tissues and cell samples were homogenized and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor cocktail. Whole lysates were then centrifuged at 12,000 g for 10 min at 4°C. Protein concentration of the lysates was measured by Bio-Rad protein assay reagents. Equal amounts (20 μg) of lysates were prepared,
subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and immunoblotted with antibodies against myosin phosphotase target subunit (MYPT1), phospho-MYPT1 (Upstate, Billerica, MA), phospho-myosin light chain (phospho-MLC, Cell Signaling, Danvers, MA), MLC, and RhoA (Santa Cruz Biotechnology Inc., Santa Cruz, CA). To normalize the protein levels, the blots were stripped and re-probed with anti-actin antibody (Santa Cruz Biotechnology).

**Data Analyses**
All data are expressed as mean ± SE unless otherwise specified. Comparisons between groups of animals or treatments were made with one-way analysis of variance (ANOVA). When significance was indicated, a Student-Newman-Keuls post hoc analysis was used. Significance was considered at a value of $P < 0.05$. The Fishing License method was used to analyze blood pressure differences including mean arterial pressure between the PM$_{2.5}$-AII and FA-AII groups as detailed previously.$^{12}$
Supplemental Results

**PM$_{2.5}$ Exposure Induces Vascular Tone Change Mediated by Rho/ROCK Pathways**

We examined the impact of Fasudil treatment on NO bioavailability. Fasudil improved NO bioavailability *in vivo* as evidenced by a restoration of constriction in response to L-NMMA in the Fasudil treated PM$_{2.5}$-AII group but not in the FA-AII groups (Figure I). These data provides confirmation of the involvement of Rho/ROCK in decreasing bioavailability of NO and provides confirmation of the relevance of this pathway.

**PM$_{2.5}$ Exposure on Vascular Hypertrophy**

As shown in Figure V, we observed a trend towards an increase in vascular smooth muscle hyperplasia at the end of the 7 day AII infusion between the PM$_{2.5}$ and FA groups although it was not statistically significant.

**PM$_{2.5}$ Exposure on Resistance Vascular Tone Change**

We have studied resistance vessel (arteriole) function in response to PM$_{2.5}$ exposure, and have noted changes in endothelial function as evidenced by reduction in acetylcholine (Ach) mediated dilation indicative of reduction in bioavailable nitric oxide (Figure VI).


Supplemental Table. Primers for real-time quantitative RT-PCR used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward oligonucleotides</th>
<th>Reverse oligonucleotides</th>
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<tr>
<td>p22^phox</td>
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<td>5′-ACCCTCCACAGAATCCACAC-3′</td>
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</table>
Supplemental Figure I
Effect of nitric oxidase synthase inhibition on aortic constrictive response. Pre-constricted aortic rings with PE ($10^{-7}$ mol/L) were incubated with L-NMMA ($10^{-4}$ mol/L) for 30 min to assess the contribution of basal NO bioavailability to the constrictive response. *$P<0.05$ vs FA-AII-PI, and †$P<0.05$ vs PM$_{2.5}$-All-PI. $n = 6$. 
Supplemental Figure II

A, Aortic section stained with dihydroethidium to quantify in situ $O_2\cdot$ production. Aortic sections from PM$_{2.5}$-All or FA-All groups (n= 6) were pre-incubated with vehicle (PSS), apocynin (300 $\mu$M), DPI (10 $\mu$M), or PEG-SOD (250 U/ml) for 30 min. Scale bars: 5 $\mu$m. B, Lucigenin chemiluminescence assay in aortic rings to assess PM$_{2.5}$-mediated $O_2\cdot$ production. Aortic rings (2 mm) were incubated with lucigenin (5 mM) in the presence of PSS, L-NAME (10 $\mu$M), PEG-SOD (300 U/ml), or apocynin (300 $\mu$M). Data shown as mean ± SE (n= 6). *$P < 0.001$ vs. PSS of FA-All. PSS, physiological salt solution.
Supplemental Figure III.
AII induced RhoA activation in RASMCs. RASMCs were starved and exposed to 100 nmol/L of AII for the indicated time, and the level of GTP-RhoA was determined as mentioned in “Methods”.
Supplemental Figure IV.
PM$_{2.5}$ exposure induced RhoA activation. RASMCs were starved and then exposed to 10 μg/ml of PM$_{2.5}$ for the indicated time, and the levels of GTP-RhoA were determined with G-LISATM RhoA activation assay kit (Cytoskeleton, Inc., Denver, CO) according to the manufacture’s instructions. Values are expressed as mean ± SE (n= 4).
Supplemental Figure V.
Effect of ambient PM$_{2.5}$ exposure on aortic hypertrophy in SD rats. The rats were exposed to PM$_{2.5}$ for 10 weeks and infused with AII for 7 days. The index of aortic hypertrophy was determined by the wall-to-lumen ratio. Values are expressed as mean ± SE (n= 4).
Supplemental Figure VI.
Intravital microscopic examination on cremasteric arterioles 24 hours after intranasal exposure to PM$_{2.5}$ (50 mg/kg in 200 μl normal saline) or normal saline (equal volume) only in C57BL/6 mice (n = 5) in response to acetylcholine (Ach). *P<0.05 PM$_{2.5}$ vs FA.