Diesel Exhaust Particles in Lung Acutely Enhance Experimental Peripheral Thrombosis

Abderrahim Nemmar, DVM, PhD; Peter H.M. Hoet, PhD; David Dinsdale, PhD; Jozef Vermeylen, MD, PhD; Marc F. Hoylaerts, PhD; Benoit Nemery, MD, PhD

Background—Pollution by particulates has consistently been associated with increased cardiovascular morbidity and mortality, but a plausible biological basis for this association is lacking.

Methods and Results—Diesel exhaust particles (DEPs) were instilled into the trachea of hamsters, and blood platelet activation, experimental thrombosis, and lung inflammation were studied. Doses of 5 to 500 μg of DEPs per animal induced neutrophil influx into the bronchoalveolar lavage fluid with elevation of protein and histamine but without lactate dehydrogenase release. The same doses enhanced experimental arterial and venous platelet-rich-thrombus formation in vivo. Blood samples taken from hamsters 30 and 60 minutes after instillation of 50 μg of DEPs yielded accelerated aperture closure (ie, platelet activation) ex vivo, when analyzed in the Platelet Function Analyser (PFA-100). The direct addition of as little as 0.5 μg/mL DEPs to untreated hamster blood significantly shortened closure time in vitro.

Conclusions—The intratracheal instillation of DEPs leads to lung inflammation as well as a rapid activation of circulating blood platelets. The kinetics of platelet activation are consistent with the reported clinical occurrence of thrombotic complications after exposure to pollutants. Our findings, therefore, provide a plausible explanation for the increase in cardiovascular morbidity and mortality accompanying urban air pollution. (Circulation. 2003;107:1202-1208.)

Key Words: thrombosis • lung • inflammation • air pollution

Urban pollution, especially by particulates, contributes to respiratory and cardiovascular morbidity and mortality. To a large extent, the increase in mortality linked to particulate matter <10 μm in diameter is attributable to cardiovascular diseases. Recent epidemiological studies demonstrate that cardiovascular functional parameters are disturbed by particulate pollutants. Although these clinical and epidemiological observations are strong and consistent, the underlying mechanisms responsible for the cardiovascular toxicity of particulate matter are still largely unknown.

Studies suggest that fine particles with a diameter <2.5 μm (PM2.5) have an important role in triggering biological responses. These particles, and particularly the ultrafine fraction (<100 nm), remain airborne for long periods of time; penetrate deeply into the respiratory tract; and can carry large amounts of toxic compounds, such as hydrocarbons and metals, on their surfaces.

In urban areas, diesel engines are considered to be the major source of PM2.5. Salvi et al10 reported that short-term exposure of humans to diesel exhaust particles (DEPs) induces inflammatory responses not only in the airways but also in the peripheral blood. In vitro, DEPs are taken up by airway epithelial cells and stimulate the release of proinflammatory cytokines.

Myocardial infarction results from rupture of an atherosclerotic plaque in the coronary artery, followed by rapid thrombus growth as a consequence of exposure of highly reactive subendothelial structures to circulating blood, thus leading to additional or complete obstruction of the blood vessel. Thromboembolic disease is a major cause of morbidity and mortality in the elderly, ie, the fraction of the population that is most susceptible to the adverse effects of air pollution. Recently, Peters et al10 have shown that exposure to PM2.5 for as little as 2 hours increases the risk of myocardial infarction.

We have shown that ultrafine particles (UFPs) diffuse rapidly, ie, within 1 hour, from the lungs into the systemic circulation in hamsters and in humans. We have also demonstrated that intravenously and intratracheally administered positively charged polystyrene particles enhance experimental thrombosis in hamsters, a phenomenon demonstrated to result from particle-facilitated blood platelet activation.

We have now extended these studies to relevant pollutant particles, ie, DEPs, to investigate further the epidemiological association between particulate air pollution and cardiovascular morbidity and mortality. The intratracheal instillation of DEPs in hamsters not only leads to the rapid induction of...
pulmonary inflammation but also enhances both arterial and venous thrombosis after minor vessel injury, in association with circulating platelet activation.

Methods

Particles

We used DEPs (SRM 1650) from the US National Institute of Standards and Technology. DEPs were suspended in saline (NaCl 0.9%) containing Tween 80 (0.1%). To minimize aggregation, particle suspensions were always sonicated (Branson 1200) for 15 minutes and vortexed immediately (<1 minute) before their dilution and before intratracheal administration. Controls received saline containing Tween 80 (0.1%).

For electron microscopy, droplets (10 μL) of a suspension of 1 mg of DEPs in 500 μL were placed on matured formvar/carbon film for 30 seconds. The samples were then drained and inverted onto droplets of ultrapure water for 1 hour before being drained, dried, and examined in a Zeiss 902A electron microscope. Further samples were examined, using a dialyzed suspension or films wetted with bacitracin, and produced similar results.

Experimental Arterial and Venous Thrombosis Model

This study was reviewed and approved by the Institutional Review Board of the University of Leuven, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

The technique used to induce and monitor mural thrombosis has been described.18 Male and female hamsters (Pfd Gold, University of Leuven, Belgium) weighing 100 to 110 g were anesthetized with sodium pentobarbital (60 mg/kg, ip.). The animals were placed in a supine position on a heating pad (37°C). A 2F venous catheter (Portex) was inserted in the right jugular vein, and the right femoral vein or artery was exposed and mounted on a transilluminator. The tracheal zone was shaved and disinfected with ethanol (70%), and the trachea was exposed for the intratracheal administration of 120 μL of vehicle or DEPs (5, 50, or 500 μg per animal). Ten minutes later, Rose Bengal (Sigma) was administered in the jugular vein (20 mg/kg body weight), and the exposed segment of femoral vein or artery was irradiated, for 2 or 1.5 minutes, respectively, with green light (wavelength 540 nm) using a xenon lamp (L4887, Hamamatsu Photonics) equipped with a heat-absorbing filter and green filter. This protocol produced mild but measurable thrombosis induction.

The formation and embolization of the thrombus were continuously monitored for 40 minutes under a microscope at 40 times magnification. The change over time of light transmission through the blood vessel at the site of the vascular injury was recorded through a microscope-attached camera. Images were recorded at 10-second intervals. Image analysis (Optimas, 6.5) was used to quantify thrombus intensity and calculate the kinetics of thrombus formation. Thrombus size was expressed in arbitrary units as the total area under the curve, when the light intensity is plotted against time. Light intensity is proportional to the thrombus size intensity (rich in colorless platelets) and is expressed as the product between the number of pixels selected in the thrombus area and the mean light intensity for all pixels selected (Figure 1). The animals were euthanized at the end of the recording.

Bronchoalveolar Lavage Fluid Analysis

In separate experiments, 1 hour after intratracheal instillation of DEPs (0, 5, 50, or 500 μg per animal), animals were killed with an overdose of sodium pentobarbital. Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and lavaging the lungs with sterile saline (3×1.5 mL). The recovered fluid aliquots were pooled and centrifuged (1000g for 10 minutes, 4°C). Cells were counted in a Thoma hemocytometer after resuspension of the pellets and staining with 1% gentian violet. Cells were differentiated on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade).
In supernatants, lactate dehydrogenase (LDH) activities were determined on fresh samples using standard clinical laboratory methods, whereas total protein (BioRad München) and histamine concentrations (radioimmunoassay kit, Immunotech) were measured after storage at –20°C.

**Platelet Function Analysis**

*Ex Vivo*

Hamsters were anesthetized and intratracheally instilled with DEPs (0 or 50 μg per animal) as described above. Five, 15, 30, and 60 minutes later, venous blood was collected from the abdominal vena cava on hirudin (20 μg/mL) and supplemented with 0.4% citrate. Platelets were counted (Cell Dyn 1300, Abbott), and platelet function was assessed in the Platelet Function Analyser PFA-100 (Dade-Behring, Marburg, Germany). The PFA-100 test cartridge consists of a capillary, a blood sample reservoir, and a membrane coated with collagen/epinephrine with a central aperture. Blood is aspirated through the capillary and the aperture thus exposing platelets to high shear rates (5000/second), causing platelet activation. A platelet thrombus forms at the aperture, thus gradually diminishing and finally arresting blood flow. The time from the start of aspiration until the aperture completely occludes, ie, the closure time, reflects platelet aggregation in a shear stress–dependent way.

*In Vitro*

Saline or saline containing DEPs (0.1, 0.5, 1, and 5 μg/mL) was added to venous blood from untreated hamsters, and closure time was measured in the PFA-100 after 5 minutes.

**Statistics**

For all experiments at least one saline-treated animal was studied every day. Data are expressed as mean±SEM. Comparisons between groups were performed by one-way ANOVA, followed by the Dunnett multiple-range test or unpaired Student t test. Probability values <0.05 are considered significant.

**Results**

Transmission electron microscopy of the DEPs showed numerous small aggregates of carbonaceous particles 20 to 50 nm in diameter (Figure 2a). A few (<5%) of these aggregates were up to 10 μm across but most were <2.5 μm in largest diameter (Figure 2b).

In control hamsters there were only ~300 polymorphonuclear neutrophils (PMNs) per milliliter BAL fluid (1% of 32,300 total cells/mL), the remaining cells being macrophages. Intratracheal instillation of DEPs resulted in a marked cellular influx at all three doses. Mean total cell numbers in BAL increased to 862 000, 873 000, and 977 000 cells/mL at 5, 50, and 500 μg per animal, respectively. Figure 3a shows that PMN numbers were increased, on average, to ~100 000 cells/mL (10 to 12% of total cells), thus implying that macrophage numbers were also increased. This was accompanied by dose-dependent increases in BAL protein (Figure 3b) and histamine concentrations (Figure 3c), without significant changes in LDH (Figure 3d).

Figures 4a and 4b illustrate that the intratracheal instillation of DEPs induced an increase in the cumulative mass of thrombus formed in vivo over a 40-minute interval in a photochemically injured vessel. DEPs induced a dose-dependent enhancement of (platelet-rich) venous thrombus generation at a dose of only 5 μg per animal (+235%), and at 50 μg per animal (+503%, P<0.05) and 500 μg per animal (+720%, P<0.01) (Figure 4a). Similarly, DEPs...
significantly increased arterial thrombus formation at 50 μg per animal (+278%, P=0.01) (Figure 4b). The inserted frames show representative pictures of the segment of femoral vein (Figure 4a) or femoral artery (Figure 4b). The white sections represent the intravascular thrombus attached to the subendothelium. The arterial thrombus forms and embolizes more rapidly, which is compatible with the more pulsatile and intense flow in arteries, whereas the venous thrombus appears and disappears more gradually.

Figure 5 shows a progressive shortening of closure time, when applying the PFA-100 test to blood taken from animals instilled with 50 μg of DEPs. The shortening, which reflects platelet activation, was significant at 30 minutes and was even more pronounced at 60 minutes. Platelet counts in blood were not affected.

Adding DEPs to untreated hamster blood also caused platelet activation, as reflected by significant dose-dependent shortenings of the PFA-100 closure times (Figure 6).

**Discussion**

In this study we provide the first experimental evidence that DEPs can enhance peripheral vascular thrombosis, and that they do so in association with platelet activation. The thrombus enhancement occurs within 1 hour of particle exposure. We have also confirmed that such particles trigger lung inflammation evaluated by several BAL fluid parameters.

Urban air pollution consists of a complex mixture of gaseous and particulate agents. Although gaseous pollutants, such as ozone, play an important role, respirable particles constitute the unifying element in most studies of the adverse effects of air pollution on health.
Figure 5. Ex vivo closure time in the Platelet Function Analyser (PFA-100) in blood obtained from hamsters 5, 15, 30, and 60 minutes after intratracheal instillation of DEPs (50 μg per animal) or saline (control). Right y axis shows platelet count. Data are mean±SEM (n=3) for each time point. **P<0.001 and *P<0.05 compared with corresponding controls and †P<0.005 compared with 30-minute DEP group by unpaired Student t test.

Figure 6. In vitro closure time in the PFA-100 5 minutes after addition of DEPs to blood from untreated hamsters. Data are mean±SEM (n=3). ‡P<0.01 and †P<0.05 by Dunnett multiple-comparison test.

health effects of urban air pollution.\textsuperscript{1,20} PM\textsubscript{2.5} were consistently associated with increased cardiovascular mortality\textsuperscript{1,2} and morbidity\textsuperscript{21} in numerous American and European cities. However, the biological mechanisms underlying these associations remain unknown.\textsuperscript{5,22}

Currently, it is well recognized that thrombosis underlies most acute complications of atherosclerosis, such as acute myocardial infarction.\textsuperscript{12} Recently, it has been shown that exposure to PM\textsubscript{2.5} for as little as 2 hours increases the risk of myocardial infarction.\textsuperscript{6} In the present study, we have therefore directly addressed the question whether pollutant particles such as DEPs, which are a major contributor to urban ambient PM\textsubscript{2.5}, have an impact on this endpoint by studying thrombosis shortly after exposure to these particles. To this end, we used a recently established and validated model of acute thrombosis in the hamster.\textsuperscript{18} This photochemical injury model causes mild damage to endothelial cells and leads to the development of platelet-rich thrombi, which resemble clinical thrombi by electron microscopic analysis. This model is based on the photodynamic generation of singlet oxygen from systemically injected Rose Bengal after excitation with green light of the blood vessel. Peroxidative damage to the endothelial membrane provides the initial stimulus for platelet adhesion and thrombus formation.\textsuperscript{23,24}

The time point investigated here (1 hour) is shorter than the 6 hours\textsuperscript{9} or 20 to 24 hours\textsuperscript{25,26} in other studies of the acute effects of particles, but it may well be particularly relevant for understanding pollutant-promoted myocardial infarction.\textsuperscript{6} The choice of 1 hour was based on our recent work that showed that UFPs diffuse rapidly (within minutes) from the lungs into the systemic circulation in hamster\textsuperscript{14} and in humans.\textsuperscript{15}

Most of the world’s megacities have very high concentrations of particulates in ambient air, with annual averages of 200 to 600 μg/m\textsuperscript{3}, and peak concentrations frequently exceed 1000 μg/m\textsuperscript{3}.\textsuperscript{27} For an adult, assuming a total inhaled air volume of 20 m\textsuperscript{3} over 24 hours, >20 000 μg of particles can be inhaled during peaks of particulate air pollution. Consequently, for a hamster of 100 g, this would correspond to >30 μg. Therefore, the range of particle doses investigated here is probably relevant.

The intratracheal instillation of a bolus of particles could be criticized as being nonphysiological, but this method of delivery has been shown to be a convenient and valid, though admittedly not perfect, mode of administration of foreign compounds into the airways.\textsuperscript{28} Advantages of instillation are that the actual dose delivered to the lungs of each animal can be established accurately and that the technique is simpler than inhalation, thus permitting the introduction of a range of doses to the lung in a short time.\textsuperscript{29} Disadvantages include the essentially nonphysiological nature and the administration as a bolus as opposed to a period of several hours. The consequence of the latter on our findings remains to be established using exposure by inhalation.

Effects of DEPs on Lung Inflammation

Pulmonary inflammation within 1 hour of DEP exposure has not, to our knowledge, been reported previously. Our data show that DEPs cause a neutrophil influx in the alveoli and an increase in total protein, reflecting increased epithelial permeability.\textsuperscript{29} We have recently made similar observations after intratracheal instillation of positively charged polystyrene UFPs.\textsuperscript{17} Analogous findings have been reported in rats 6 hours after intratracheal installation of ultrafine carbon black particles.\textsuperscript{30} In humans, Salvi et al\textsuperscript{9} found an increase in the number of neutrophils in the bronchial submucosa and epithelium 6 hours after exposure to DEPs for 1 hour. We did not find changes in LDH, but increased LDH activity, suggestive of cytolysis, has been described in the BAL of rats 24 hours after intratracheal instillation of DEPs.\textsuperscript{31} Boland et al,\textsuperscript{11} using a human bronchial epithelial cell line, have shown an effect on LDH activity at 48 hours but not at 24 hours. Histamine, an endogenous mediator of many (patho)physiological processes, is synthesized and released by mast cells in the airway wall and by circulating and infiltrating basophils. Histamine is able to modulate the cytokine network\textsuperscript{32} and to upregulate P-selectin on endothelial cells.\textsuperscript{33} Our data showing that DEPs cause a dose-related release of histamine in BAL corroborate findings of Salvi et al,\textsuperscript{9} who have shown an increase in mast cell numbers in the submucosa and elevated
Effects of DEPs on Thrombosis

We found that intratracheal instillation of DEPs promotes femoral venous thrombosis in a dose-dependent manner, already starting at a dose of 5 μg per animal. We did most of our experiments with femoral vein because a more stable thrombus is produced in conditions of low blood flow encountered in veins. However, we showed that thrombosis is also enhanced in femoral artery. Many epidemiological studies have shown associations between pollution and cardiovascular morbidity and mortality, which are mainly driven by thrombotic events in arteries. There is little information regarding deep venous thrombosis or pulmonary emboli, although one study reported an association with the occurrence of “embolism and thrombosis.” However, the relevance of our vascular thrombosis model does not depend primarily on the type of vessel that is used, but on the fact that a platelet-rich thrombus is produced in a (photochemically) injured vessel.

The prothrombotic effects of DEPs corroborate our observations that intratracheal instillation of positively charged polystyrene particles (60 nm), taken as a model for UFPs, caused up to a 5-fold enhancement of thrombus formation in the same model. We attributed this rapid effect to the passage of particles into the circulation. Because electron microscopy of our DEPs showed a substantial fraction of small aggregates of particles only 20 to 50 nm in diameter, it seems reasonable to postulate passage of these DEPs into the systemic circulation as we have demonstrated to occur for UFPs in hamsters and in humans. Others have also found that the primary particles in diesel aggregates range from 10 to 40 nm. Xiong and Friedlander reported that the primary particle size of atmospheric aggregates emitted from combustion processes such as diesel engines ranges from 6 to 100 nm.

Our model of thrombosis depends on platelet activation and aggregation. Accordingly, in vivo platelet activation, as assessed ex vivo by the platelet function analyzer, was apparent 30 minutes after instillation of DEPs. The addition of DEPs to hamster blood in vitro caused platelet activation within 5 minutes with as little as 0.5 μg of DEPs/mL, which is a concentration that can presumably be achieved in the circulation after intratracheal instillation of 50 μg per hamster. All these data suggest that DEPs are prothrombotic through particle-induced platelet activation. It remains to be established which constituents are responsible for the effect and what mechanism is involved.

Our data provide novel evidence that, within an hour after their deposition in the lungs, DEPs cause pulmonary inflammation and aggravate arterial and venous thrombosis, findings we could substantiate by in vivo, ex vivo, and in vitro observations. Our findings provide a plausible explanation for the acute increases in cardiovascular morbidity and mortality caused by air pollution.


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