Pharmacological Stabilization of Mast Cells Abrogates Late Thrombotic Events Induced by Diesel Exhaust Particles in Hamsters

Abderrahim Nemmar, DVM, PhD; Peter H.M. Hoet, PhD; Jos Vermylen, MD, PhD; Benoit Nemery, MD, PhD; Marc F. Hoylaerts, PhD

Background—Particulate air pollution is associated with cardiovascular diseases and myocardial infarction (MI).

Methods and Results—We investigated the relationship between airway inflammation and thrombosis 24 hours after intratracheal (IT) instillation of diesel exhaust particles (DEP; 50 μg/hamster). Mild thrombosis was induced in the femoral vein by endothelial injury, and the consequences of airway inflammation on thrombogenicity were studied via online video microscopy. Lung inflammation and histamine analysis in bronchoalveolar lavage (BAL) and plasma were performed after pretreatment with dexamethasone (DEX) or sodium cromoglycate (SC). DEP induced airway inflammation and histamine release in BAL and in plasma, and increased thrombosis, without elevating plasma von Willebrand factor (vWF) levels. The IT instillation of 400-nm positively charged polystyrene particles (500 μg/hamster), serving as particles that do not penetrate into the circulation, equally produced airway inflammation, histamine release, and enhanced thrombosis. Histamine in plasma resulted from basophil activation. Intraperitoneal (IP) pretreatment with DEX (5 mg/kg) abolished the DEP-induced histamine increase in BAL and plasma and abrogated airway inflammation and thrombogenicity. The IT pretreatment with DEX (0.5 mg/kg) showed a partial but parallel inhibition of all of these parameters. Pretreatment with SC (40 mg/kg, IP) strongly inhibited airway inflammation, thrombogenicity, and histamine release.

Conclusions—Our results are compatible with the triggering of mast cell degranulation and histamine release by DEP. Histamine plays an initial central role in airway inflammation, further release of histamine by circulating basophils, and peripheral thrombotic events. Antiinflammatory pretreatment can abrogate the peripheral thrombogenicity by preventing histamine release from mast cells. (Circulation. 2004;110:1670-1677.)

Key Words: lung ■ respiration ■ thrombosis ■ air pollution

Histamine plays a major role in the pathophysiology of inflammation. Its main source is basophils and mast cells, which are distributed throughout the body. Histamine is a powerful coronary vasoconstrictor; it induces proinflammatory cytokine production from endothelial cells, upregulates P-selectin on the endothelial cell surface, and is able to induce intimal thickening in a mouse model of thrombosis. Histamine also can activate platelets, and it potentiates the aggregatory response of other agonists including adrenaline, 5-hydroxytryptamine, and thrombin. Moreover, histamine is able to modulate the activity of inflammatory cells such as neutrophils, monocytes, and eosinophils.

In urban environments, diesel exhaust particles (DEP) are one of the main contributors of atmospheric particulate matter of diameter <2.5 μm (PM2.5). Epidemiological studies reported that exposure to PM2.5 increases susceptibility to ischemia and the occurrence of myocardial infarction (MI). It is currently established that acute events of atherosclerosis such as MI result from thrombosis at the site of plaque rupture, but the mechanisms linking atmospheric particulate matter to MI are not fully understood.

Even though the elevation of histamine levels has been correlated with the onset of MI, the evidence linking exposure to DEP and release of histamine is more abundant in the context of airway allergic and inflammatory processes. Thus, DEP were reported to increase the severity of clinical symptoms to allergen exposure by enhancing mast cell degranulation and to enhance interleukin-4 and histamine production in basophils. In addition, an increase in mast cell numbers has been reported in the submucosa and elevated histamine levels were found in nasal and bronchoalveolar lavage (BAL) fluid after exposure to DEP in humans. We recently showed in hamsters that DEP cause lung inflammation and enhance the occurrence of arterial and venous
thrombosis$^{24}$ and that these effects persisted up to 24 hours.$^{25}$ An instrumental role of histamine in these processes was evidenced by pretreating hamsters with a histamine H1 receptor antagonist, which inhibited both lung inflammation and thrombotic events without affecting the histamine production itself.$^{25}$

The aim of this study was to further investigate the links among histamine release, pulmonary inflammation, peripheral thrombogenicity, and vascular inflammation. To this end, the consequences of systemic and local pulmonary anti-inflammatory pretreatments by glucocorticoids and sodium cromoglycate (SC), a well-established stabilizer of mast cell and basophil degranulation, were investigated on a number of inflammatory parameters. These studies revealed a primary role for mast cell degranulation in lung inflammation, which in turn triggered inflammation-related peripheral thrombotic events.

**Methods**

The experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee of the Katholieke Universiteit Leuven.

**Particles**

DEP (SRM 1650) obtained from the National Institute of Standards and Technology were suspended in sterile pyrogen-free saline (NaCl 0.9%) containing Tween 80 (0.1%). Polystyrene particles with a uniform diameter of 400 nm amine modified (Bangs Laboratories) were suspended in saline. These particles are positively charged at physiological pH.$^{26}$ To minimize their aggregation, particle suspensions were always sonicated (Branson 1200, VEL) for 15 minutes and vortexed immediately (<1 minute) before dilution and before intratracheal (IT) administration. Control animals received saline alone or saline containing Tween 80 (0.1%), as appropriate.

**Intratracheal Instillation of Particles**

Male and female hamsters (Pfd Gold, University of Leuven, Belgium) weighing 100 to 110 g were used. The animals were anesthetized intraperitoneally (IP) with sodium pentobarbital (60 mg/kg). The tracheal zone was shaved and disinfected with ethanol (70%), and the trachea was exposed for the IT administration of 120 μL of vehicle or DEP (50 μg/animal) or 400-nm polystyrene particles (500 μg/animal).

**Experimental Thrombosis Model**

In vivo thrombogenesis was assessed 24 hours after the IT instillation of DEP or vehicle, as recently described.$^{26,27}$ Briefly, a surgically exposed segment of the femoral vein was illuminated with green (540 nm) light for 2 minutes after the IV injection of Rose Bengal (20 mg/kg; Sigma), thus producing a free radical–mediated endothelial lesion with subsequent platelet-rich thrombosis. The kinetics of thrombus generation were monitored for 40 minutes with the use of an online microscope video camera, and thrombosis was quantified via image analysis. The size of the thrombus is measured as the total area under the curve during plots of the light intensity (expressed in arbitrary units, AU) versus time.

**Analysis of BAL Fluid**

Animals were killed with an overdose of sodium pentobarbital 24 hours after the IT instillation of DEP or vehicle. BAL was performed by cannulating the trachea and lavaging the lungs with 4.5 mL (3 times 1.5 mL) of sterile NaCl 0.9%; BAL cells were counted and identified with Diff-Quik stain.

**Determination of Histamine in BAL and Plasma**

The histamine content was determined by a radioimmunoassay kit (Immunotech) in BAL and plasma obtained after centrifuging (1000g×10 min, 4°C) venous blood collected from the abdominal vena cava on ethylenediaminetetraacetic acid. The BAL and plasma samples were stored at −20°C.

**Determination of Plasma von Willebrand Antigen Levels**

Plasma von Willebrand factor (vWF) antigen levels were measured by an enzyme-linked immunosorbent assay (ELISA) technique, as previously described,$^{28}$ and expressed as a percentage of the absorbance value for a normal plasma pool obtained from 10 untreated hamsters.

**Determination of Total Histamine After Cell Lysis**

To assess the contribution of basophils to the appearance of histamine in plasma, we performed histamine release experiments separately in whole blood collected from hamsters IT instilled with vehicle or DEP. The total histamine in cell suspensions was quantified after cell lysis in distilled water and twice freeze-thawing, according to the manufacturer’s recommendation (Immunotech). Histamine was measured as described above and compared with histamine levels in the corresponding plasma.

**Pretreatment With Dexamethasone or Sodium Cromoglicate**

Hamsters were pretreated with dexamethasone (DEX; Sigma), injected either IP (5 mg/kg) or IT (0.1 or 0.5 mg/kg), or with SC (Sigma) given IP (40 mg/kg$^{29}$), 1 hour before DEP or vehicle instillation. Animals were then allowed to recover. They were subjected to either experimental thrombosis induction or the analysis of BAL and plasma parameters 24 h later, as outlined above. In addition, in the experiments involving pretreatment with SC, platelet function was assessed ex vivo in the platelet function analyzer PFA-100 (Dade Behring),$^{30}$ with venous blood collected from the abdominal vena cava on heparin (20 μg/mL) and supplemented with 0.4% citrate. The closure time reflects platelet aggregate formation in a shear stress–dependent manner.$^{30}$

**Statistics**

Data are mean±SEM. Comparisons between groups were performed by an unpaired Student t test or a 1-way analysis of variance and then by the Newman-Keuls test.

**Results**

**Histamine Release and Lung Inflammation**

The total cell count in BAL increased to 189.7±28.9×10$^4$/mL as compared with control (2.5±0.4×10$^4$/mL, P<0.0001) 24 hours after the IT instillation of DEP (50 μg/animal). The IT instillation of 400-nm amine polystyrene particles (500 μg/animal) resulted in a less pronounced but nevertheless marked cellular influx (25.0±4.4×10$^4$/mL versus 2.3±0.6×10$^4$/mL in controls, P<0.005). Figure 1a shows that the IT instillation of DEP or 400-nm particles led to significant increases in polymorphonuclear neutrophil (PMN) numbers in BAL, reaching similar proportions in both treatments (34% and 39% of the total cell number, respectively). The rest of the cells were mainly macrophages, the proportion of lymphocytes being below 2%.

Histamine concentrations in BAL increased significantly in the DEP and 400-nm particle groups as compared with their respective controls, and the average increase (7-fold) was similar for both types of particles, despite differences in infiltrating cell numbers (Figure 1b).
Experimental thrombus formation in vivo was strongly enhanced by the IT instillation of DEP or 400-nm particles, and the magnitude was similar for both types of particles (Figure 2a). The concentrations of histamine in plasma also increased (2-fold) after DEP or 400-nm particle instillation (Figure 2b). No effect of DEP or 400-nm particle administration was observed on plasma vWF concentrations (Figure 2c).

**Origin of Plasma Histamine**

Table 1 compares the concentrations of histamine after cell lysis in vitro in whole blood and plasma histamine in vivo after the IT administration of vehicle, DEP, or 400-nm polystyrene particles. In the vehicle-instilled groups, the difference between the total blood histamine concentration (blood cells and plasma) after cell lysis and plasma histamine concentration was 35±6 nmol/L (saline-treated animals) or 36±5 nmol/L (animals treated with saline containing Tween 80). The corresponding values found in the groups treated with 400-nm amine particles and DEP were 12.2±1.8 nmol/L (P=0.01) and 17.8±1.5 nmol/L (P<0.05), respectively. These lower values compared with the vehicle groups show that blood basophils had been at least partially degranulated in both cases. This result pleads in favor of a role for basophils in the release of histamine in the circulation.

**Intraperitoneal DEX Prevents Inflammation, Thrombotic Events, and Histamine Release**

The IP pretreatment of control hamsters with DEX (5 mg/kg) did not significantly affect the total cell numbers in BAL (1.8±0.5×10⁴/mL) or the proportion of PMN as compared with nontreated controls (Figure 3a). The pretreatment did strongly reduce the DEP-induced rise of total BAL cells to control numbers (2.3±0.8×10⁴/mL, P<0.001) and the proportion of PMN (8%, Figure 3a). The histamine concentra-
tions in BAL also were strongly lowered (Figure 3b). Similarly, the IP administration of DEX neutralized the DEP-induced thrombus formation in vivo almost completely (Figure 4a); likewise, histamine levels in plasma were normalized (Figure 4b).

Dose-Dependent Inhibition by Intratracheal DEX on Lung Inflammation, Thrombotic Events, and Histamine Release

The IT pretreatment of control hamsters with DEX did not affect the total cell numbers in BAL. The total number of cells found in BAL were $2.0\pm0.2\times10^4$/mL and $1.7\pm0.4\times10^4$/mL after the IT administration of DEX at 0.1 and 0.5 mg/kg, respectively. The IT pretreatment with 0.1 mg/kg of DEX was ineffective in reducing the DEP-induced increase of total cells in BAL ($230.0\pm47.0\times10^4$/mL) and the corresponding PMN influx (38%; Figure 5a), whereas 0.5 mg/kg of DEX did reduce the DEP-induced increase of total cells in BAL (to $19.5\pm3.3\times10^4$/mL, $P<0.001$) as well as the proportion of PMN (to 17%; Figure 5a). Similarly, the histamine accumulation in BAL induced by DEP was not prevented by the IT instillation of 0.1 mg/kg of DEX, but it was partially prevented by IT pretreatment with 0.5 mg/kg of DEX (Figure 5b).

No inhibition of thrombus formation was recorded after the IT instillation of 0.1 mg/kg of DEX, but 0.5 mg/kg of DEX partially inhibited in vivo thrombus formation (Figure 6a). No effect on plasma histamine concentrations was observed after pretreatment with 0.1 mg/kg of DEX (Figure 6b); however, after the administration of 0.5 mg/kg of DEX, the DEP-induced histamine increase in plasma was reduced, although the drop failed to reach statistical significance (Figure 6b).

Table 1. Histamine Concentrations in Plasma and Whole Blood After Cell Lysis

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>400-nm Particles</th>
<th>Vehicle</th>
<th>DEP</th>
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<td>Plasma</td>
<td>21.6±3.3</td>
<td>37.6±2.9*</td>
<td>23.6±3.7</td>
<td>43.5±3.9*</td>
</tr>
<tr>
<td>Whole blood</td>
<td>57.0±7.0</td>
<td>49.8±3.1</td>
<td>59.5±9.2</td>
<td>61.3±3.4</td>
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In distilled water and twice freeze-thawing, after IT instillation of vehicle or 400-nm polystyrene amine-modified particles (500 μg/animal) or DEP (50 μg/animal).

*P<0.01, values significantly different from vehicle group. Data are mean±SEM (each group, n=4).

Figure 3. PMN influx (a) and histamine release (b) in BAL after IP DEX. Data obtained 24 h after IT instillation of vehicle or DEP (50 μg/animal) with or without IP pretreatment with DEX (5 mg/kg), as indicated. PMN expressed as % total cell numbers. Mean±SEM (each group, n=4 to 7). Statistical analysis by Newman-Keuls test. Data for non–DEX-pretreated animals same as Figure 1.

Figure 4. Thrombosis (a) and histamine release in plasma (b) IP injection of DEX. Data obtained 24 h after IT instillation of vehicle or DEP (50 μg/animal) with or without IP pretreatment with DEX (5 mg/kg), as indicated. Mean±SEM (each group, n=4 to 8). Cumulative thrombus size expressed in AU as total light intensity over 40 min after mild photochemical damage to femoral vein. Statistical analysis by Newman-Keuls test. Data for non–DEX-pretreated animals same as Figure 2.
SC Inhibition of Histamine Release, Lung Inflammation, and Platelet Activation

The IP pretreatment of control hamsters with SC (40 mg/kg) did not significantly affect the total cell numbers in BAL (2.5 ± 0.4 × 10^4/mL) or the proportion of PMN as compared with nontreated controls (Figure 7a); however, this pretreatment strongly reduced the DEP-induced increase of total BAL cell numbers to 4.0 ± 0.4 × 10^4/mL (P < 0.001) and the proportion of PMN to 12% (Figure 7a). Likewise, SC pretreatment reduced the histamine concentrations in BAL to control values (Figure 7b). The IP administration of SC strongly reduced histamine release in plasma (Figure 7c). Platelet activation, as analyzed ex vivo, also was potently inhibited (Table 2).

Discussion

Short-term increases in particulate air pollution are associated with acute cardiovascular events in people with preexisting vascular disease and diabetes mellitus and in older adults.^{14,31–33} Ultrafine particles (UFPs; ie, particles of diameter <0.1 μm) can translocate rapidly from the lungs into the systemic circulation in humans^{34} and hamsters.^{35} We previously demonstrated that the IV or IT administration of positively charged ultrafine (60 nm) polystyrene particles not only causes rapid lung inflammation but also enhances thrombotic events.^{26,36} We also provided evidence that DEP cause marked pulmonary inflammation and could aggravate thrombosis up to 24 hours after their deposition in the lungs.^{25}

Pretreating hamsters with a histamine H1 receptor antagonist strongly reduced lung inflammation at all time points investigated (ie, 1 h, 6 h, 24 h). Such pretreatment reduced the thrombotic events at 6 and 24 hours but not at 1 hour after DEP administration. These findings are compatible with platelet activation being caused by direct passage of the ultrafine fraction of DEP (or their associated constituents) at 1 hour and by lung inflammation at later time points.^{25}

In contrast to DEP that contain UFP (or their associated components) capable of translocation into the blood^{34,35,37} and capable of directly affecting the vascular end points, large particles such as 400-nm polystyrene particles are unlikely to cross the lung–blood barrier to any significant extent;^{38,39} neither do they possess associated components that can leak and pass into the blood. We have already shown that 1 hour after their IT instillation, positively charged amine-modified 400-nm particles (500 μg/hamster) did not cause any thrombotic events, although they did trigger lung inflammation. In contrast, positively charged amine particles of 60 nm (UFP) were prothrombotic, this effect being presumably related to their passage into the blood. In the present study, we wanted to assess the link between lung inflammation and systemic prothrombotic effects independent of any direct passage from the lung into the bloodstream. Therefore, we IT instilled positively charged 400-nm polystyrene particles and DEP and studied their late effects on lung inflammation, histamine release, thrombotic events, and endothelial activation in hamsters.

DEP and 400-nm Polystyrene Particles Cause Lung Inflammation, Thrombotic Events, and Histamine Release

Our present results show that 24 hours after IT instillation of DEP, histamine was elevated in BAL and plasma in association with airway inflammation and thrombosis. Compared with DEP, the 400-nm amine polystyrene particles produced less cellular influx, which presumably is related to the different size and surface chemistry for these particles. The concentrations of histamine released in BAL or plasma were comparable for both types of particles, suggestive of a maximal degranulation of mast cells by both types of particles. No changes in plasma vWF concentration were observed. vWF is synthesized by endothelial cells, stored in the Weibel-Palade bodies, and secreted on endothelial activation. Increased plasma vWF levels have been associated with cardiovascular disease. The lack of endothelial vWF release 24 hours after DEP or 400-nm particle administration in the present study suggests that the prothrombotic tendency developed without endothelial activation. In addition, we showed previously that no activation of the intravascular coagulation occurred.^{26}
Histamine H1 receptor antagonism strongly reduced DEP-induced inflammation as well as systemic platelet activation and prothrombotic tendency. In the present study, we investigated further the relative role of histamine levels in the lung and blood by targeting its cellular sources (ie, mast cells and basophils), and we assessed its role in the inflammatory and thrombotic processes accompanying DEP exposure.

Origin of Plasma Histamine
We verified whether histamine in plasma originated from basophil degranulation or whether it resulted from diffusion, after pulmonary mast cell degranulation, from the lungs into the bloodstream. To this end, we compared histamine concentrations before and after cell lysis in whole blood in vitro, which reflects the in vivo release from basophils into the circulation. We found lower histamine concentrations contained within basophils after cell lysis in both 400-nm particle- and DEP-exposed groups, as compared with vehicle groups. In other words, this analysis revealed that the increased plasma histamine concentrations after the IT instillation of DEP or 400-nm polystyrene particles resulted from the activation of blood basophils. This finding is in agreement with the study of Devouassoux et al, who showed that DEP enhance histamine production from basophils in vitro. It also follows from this analysis that the peripheral events leading to basophil degranulation are secondary to lung inflammation because they also occurred with the 400-nm particles, which stay in the lung. A possible mechanism for this activity is the release of platelet activating factor into the circulation, thus causing basophil activation, but this possibility still needs to be verified.

Effect of DEX on Lung Inflammation and Thrombotic Events
The prothrombotic “enhancement” of peripheral vascular thrombosis after endothelial damage constitutes a realistic experimental approach for human thrombosis underlying MI. The dose of DEP used here to trigger such thrombotic events is within realistic exposure during peak levels of air pollution. We pretreated hamsters with DEX, a synthetic glucocorticoid (GC), which is a potent antiinflammatory agent known to exert cardiovascular protective effects. The pharmacological actions of GC often are species specific or cell-type specific. In rodents, it has been reported that in vitro and in vivo pretreatment with GC inhibits the release of mast cell mediators, including histamine. In humans, DEX exerts direct inhibitory effects on human mast cell maturation and inhibits histamine release from human basophils.

DEX at 5 mg/kg is effective in inhibiting silica-induced pulmonary inflammation in rats. Here, IP administration of DEX strongly inhibited histamine release in BAL and plasma and reduced the DEP-induced PMN influx by 76% and thrombosis in vivo by 60%. These reductions are comparable to those

Figure 6. Thrombosis (a) and histamine release in plasma (b) after IT DEX. Data obtained 24 h after IT instillation of vehicle or DEP (50 μg/animal) with or without IT pretreatment with DEX (0.5 or 0.1 mg/kg), as indicated. Cumulative thrombus size expressed in AU as total light intensity over 40 min after mild photochemical damage to femoral vein. Mean±SEM (each group, n=4 to 8). Statistical analysis by Newman-Keuls test. Data for non-DEX-pretreated animals same as Figure 2.
observed after pretreatment with the H1 receptor antagonist diphenhydramine during the DEP-induced PMN influx. This result suggests a key role for histamine in triggering cellular inflammation in the lung and associated thrombotic events.

Inspired by the observations that the IT instillation of DEX decreases inflammatory cell influx and protein levels in BAL in oleic acid–induced lung injury and because pulmonary inflammation precedes the systemic effects triggered by DEP, we used this approach to modulate the thrombotic response to DEP exposure via antiinflammatory treatment. Although 0.1 mg/kg IT of DEX failed to affect histamine levels and thrombotic and inflammatory events, 0.5 mg/kg of DEX partially reduced the airway inflammation and subsequent thrombosis after DEP instillation to a comparable degree.

### DEP-Triggered Mast Cell Degranulation and Histamine Release Initiate Lung Inflammation and Subsequent Thrombotic Events

DEX affects the release of cytokines and other inflammatory mediators from various types of cells. Mast cell degranulation releases not only histamine but also serotonin, proteases, and various cytokines. Even though we already documented a major role for histamine in the observed effects, it remained to be shown whether the effects of DEX resulted from inhibiting histamine release or whether they resulted from any other antiinflammatory action. We used SC, a well-known stabilizer of mast cells and basophil degranulation.

Using the platelet function analyzer ex vivo, we recently confirmed the relationship between platelet activation measured ex vivo and the development of thrombotic events in vivo after DEP exposure.

Our results show that pretreatment with SC strongly inhibited the DEP-mediated histamine release in BAL and plasma and the neutrophil influx in BAL and peripheral platelet activation. The parallelism between antiinflammatory pretreatment and SC on histamine levels, in association with our earlier findings using the H1 receptor antagonist diphenhydramine, pleads in favor of a key role for mast cells’ degranulation and histamine release in initiating inflammatory effects in the lung and subsequent peripheral prothrombotic events. We can conclude therefore that the prevention of inflammatory and prothrombotic events by DEX probably stems from the stabilization of mast cells.

### Conclusion

Our data provide evidence for a direct link between the DEP-induced release of histamine and pulmonary inflammation. Lung inflammation in turn causes peripheral systemic responses and platelet activation. Mast cell stabilization and antiinflammatory pulmonary treatment abrogate the histamine production in the lung and are capable of reducing the inflammation-induced thrombotic events in the circulation.

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### References

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