

# Ultrafine Particles Affect Experimental Thrombosis in an *In Vivo* Hamster Model

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Particulate air pollution is associated with cardiovascular morbidity and mortality. To investigate this association, we studied the effect of ultrafine (60 nm) polystyrene particles on thrombus formation in a hamster model after intravenous and intratracheal administration of unmodified, carboxylate-polystyrene, or amine-polystyrene particles. Unmodified particles had no effect on thrombosis up to 5 mg/kg. Carboxylate-polystyrene particles significantly inhibited thrombus formation at 500 and 100  $\mu\text{g}/\text{kg}$  body weight but not at 50  $\mu\text{g}/\text{kg}$  body weight. In contrast, amine-polystyrene particles significantly enhanced thrombosis at 500 and 50  $\mu\text{g}/\text{kg}$  body weight but not at 5  $\mu\text{g}/\text{kg}$  body weight. Similarly, the intratracheal instillation of 5,000  $\mu\text{g}$  of amine-polystyrene particles significantly increased thrombus formation. The unmodified particles and carboxylate-polystyrene particles had no effect. During platelet aggregation in human platelet-rich plasma, induced with 1.25  $\mu\text{M}$  ADP, unmodified particles had no effect up to 100  $\mu\text{g}/\text{ml}$ , and carboxylate-polystyrene particles weakly enhanced platelet aggregation at 25 to 100  $\mu\text{g}/\text{ml}$ . However, amine-polystyrene particles (50 and 100  $\mu\text{g}/\text{ml}$ ) induced platelet aggregation themselves and strongly increased the ADP-induced aggregation. We conclude that the presence of (ultrafine) particles in the circulation may affect hemostasis. The observed *in vivo* prothrombotic tendency results, at least in part, from platelet activation by positively charged amine-polystyrene particles.

**Keywords:** air pollution; cardiovascular effects; hemostasis; platelets; ultrafine particles

A major issue in modern environmental toxicology relates to finding scientific possibility for the epidemiologic effects of air pollution. Recently, Ware (1) pointed out in an editorial that we still have no explanation for the consistent association between particulate air pollution and increases in morbidity and mortality. A particularly puzzling point is that fine particulate matter affects not only respiratory morbidity and mortality but also, and perhaps even more, cardiovascular morbidity and mortality (2–5). Several research groups have begun to investigate cardiovascular endpoints to try to understand the mechanisms whereby inhaled particles may impact on extrapulmonary organs. Various hypotheses are currently being verified in humans and laboratory animals. Most research is centered around the possible consequences of particle-induced pulmonary inflammation on the heart and other

systems, such as coagulation. The rationale of these studies is that cytokines and other mediators produced in the lungs are also released in the circulation and exert extrapulmonary effects (6). Thus, in response to particle exposure, heart rate or rhythm abnormalities without hypoxia or respiratory distress, as well as an increase in neutrophils and platelets in peripheral blood have been observed (7–9).

Another line of research, that has not been pursued much so far, consists of studying the possible “direct” effects of particles that may pass from the lung into the circulation. The rationale for this approach is based on the observation that the ultrafine fraction of the particles is probably most hazardous to health (10). Ultrafine particles (UFPs), i.e., particles with a diameter less than 100 nm, deposit in greater numbers and deeper into the lungs than do larger-sized particles (11). Their small size allows them to translocate from the lung into the blood, as we recently demonstrated (12, 13).

Thromboembolic disease is a major cause of morbidity and mortality in the elderly, i.e., the fraction of the population that is most susceptible to the adverse effects of air pollution (14). Recently, it has been shown that exposure to particulate air pollution for as little as 2 hours increased the occurrence of myocardial infarction in people at risk of developing cardiovascular disease (15). The heterogeneous composition of particle pollutants has complicated identification of the particulate components underlying the observed health effects. The latter have been related either to soluble metal components (e.g., iron, vanadium, and nickel) that cause the formation of oxygen-derived free radicals (16, 17) or to biologic components (e.g., endotoxins) that cause activation of macrophages (18). Moreover, a substantial proportion of ambient particles is charged (19), and the acidic nature seems to play an important role in particle toxicity (20, 21).

To study the effects of UFPs on cardiovascular endpoints such as thrombus formation, we have used commercially available polystyrene particles with a uniform diameter of 60 nm. The particles used in this study are not typical of air pollutants. However, these model particles offer the advantage of avoiding the occurrence of complex chemical reactions arising from the solubilization of the particles themselves, from agents adsorbed on their surface, or from leaching out. We have thus used noncytotoxic polystyrene particles (Xu and coworkers, unpublished data) and have evaluated surface chemistry modifications (i.e., positively charged amine-modified particles, negatively charged carboxylate-modified particles, and unmodified particles) as a parameter possibly involved in vascular toxicity. In addition to chemical composition, these particles are well characterized in terms of size, in contrast to the commonly used complex mixtures of diesel exhaust particles or concentrated urban air particles. For our investigations, we have used a well established, small, animal model of vascular thrombosis (22).

Our findings indicate that particles, which we purposefully chose to be ultrafine, have an impact on thrombus formation depending on their surface chemistry.

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## METHODS

We used unmodified, amine-modified, or carboxylate-modified polystyrene particles (60 nm) (Bangs Laboratories, Inc., Fishers, IN). Fluorescently labeled particles were made according to the manufacturer's (Tech-Note 205) instructions. To minimize particle aggregation, particles were sonicated for 15 minutes and vortexed immediately before suspension in saline, as well as before intravascular or intratracheal administration.

Particle suspensions were examined by electron microscopy (Jeol 100 CXII, Peabody, MA). Their zeta potential was measured by Dr. B.R. Paulke (Fraunhofer-IAP, Germany). We investigated the presence of accessible charges on the surface by incubating (and then eluting) the particles with the anion exchanger Q-sepharose or the cation exchanger S-sepharose (Pharmacia, Uppsala, Sweden). The eluted fractions were measured by spectrophotometry.

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

The technique used to induce and to monitor mural thrombosis has been described by Kawasaki and coworkers (22). Hamsters (100–150 g) were anesthetized (sodium pentobarbital) and placed supine on a heating pad (37°C). A venous catheter was inserted in the right jugular vein. The right femoral vein was exposed and mounted on a transilluminator. After the intravenous administration of Rose Bengal (20 mg/kg), the segment of the femoral vein was irradiated with green light (540 nm) for 2 minutes, using an optic fiber mounted on a micromanipulator located 5 mm above the vein. The thrombus was monitored under a microscope at  $\times 40$  magnification. The change over time in light transmission through the blood vessel at the site of the trauma was recorded using a microscope-attached camera. Images were recorded at intervals of 10 seconds over a period of 40 minutes. Image analysis was used to quantitate thrombus intensity, which is expressed in arbitrary units as the total area under the curve, with light intensity plotted against time (23).

Ten minutes before Rose Bengal administration, 250  $\mu$ l of saline (for control hamsters) or particle suspension in saline was administered in the jugular vein or into the trachea. In separate experiments, histologic specimens were prepared for light microscopy after the administration of amine-polystyrene particles (500  $\mu$ g/kg) and induction of thrombosis as described previously. Positive control animals were obtained by increasing the duration of the irradiation with green light to 5 minutes, resulting in the production of major thrombosis. Histologic sections were stained with hematoxylin and eosin and by immunohistochemical staining for the detection of von Willebrand factor and fibrinogen.

Platelet-rich plasma was prepared by centrifugation of citrated human blood from normal volunteers (150 g for 10 minutes). Platelet aggregation by ADP (0.625–5  $\mu$ M) was investigated in the presence of particles (12.5–100  $\mu$ g/ml). Particles were added to platelet-rich plasma 5 minutes before induction of stirring and administration of ADP. After platelet aggregation in the presence of fluorescent particles, platelet aggregates were fixed and analyzed by confocal microscopy. The prothrombin time and the activated partial thromboplastin time were determined in normal human plasma using standard techniques.

## Statistics

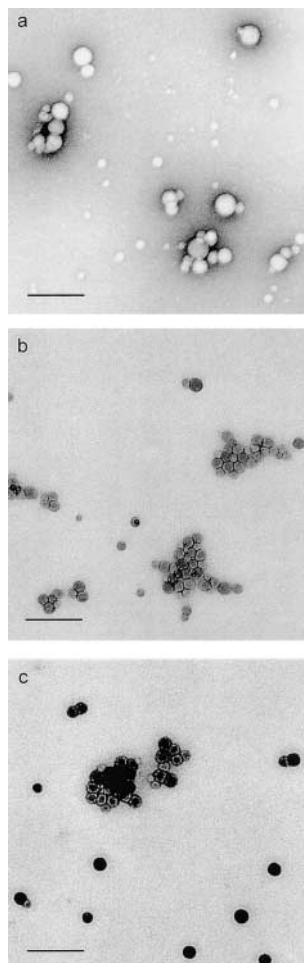
Three to four laboratory animals (undergoing different types of treatment), including at least one internal control animal (in random sequence), were studied each day. Data from control animals were pooled and used as the reference group for all treatments investigated.

Data are expressed as mean  $\pm$  SEM. Comparisons between groups were performed by one-way analysis of variance, followed by Dunnett's multiple range test. *p* Values  $< 0.05$  are considered significant.

## RESULTS

### Particle Characterization

Transmission electron microscopy of all three types of particles studied revealed a homogeneous particle size for unmodified, amine-polystyrene, and carboxylate-polystyrene particles (Fig-



**Figure 1.** Electron micrographs of negatively stained unmodified (A), carboxylate-modified (B), or amine-modified (C) particles. Bar: 250 nm.

ure 1). In all samples, both large aggregates and discrete particles were observed. It was not possible to discern whether the aggregation was present in the original suspension or, more probably, was an artifact of the drying down process during negative staining.

The zeta potential values ( $n = 3$ , mean  $\pm$  SD), measured at pH 7.4 in phosphate buffer, were  $-44 \pm 6$ ,  $-41 \pm 6$ , and  $-51 \pm 6$  mV for the amine-polystyrene particles, carboxylate-polystyrene particles, and unmodified particles, respectively. However, contrary to the  $\zeta$  potential that indicated only minor differences between the amino-polystyrene and carboxylate-polystyrene particles, sepharose chromatography yielded substantial differences between these particles. Amine-polystyrene particles attached to S-sepharose (cation exchanger) but not to Q-sepharose (anion exchanger), in agreement with the presence of positive charges on these particles. Conversely, the carboxylate-polystyrene particles attached to Q-sepharose but not to S-sepharose, whereas the unmodified particles did not interact with any resin. Moreover, the elution of amine-polystyrene and carboxylate-polystyrene particles with phosphate buffered (1M NaCl) from resins showed that the particles and resins interact in an entirely reversible way (Table 1).

### Effect of Particles on Experimental Thrombosis

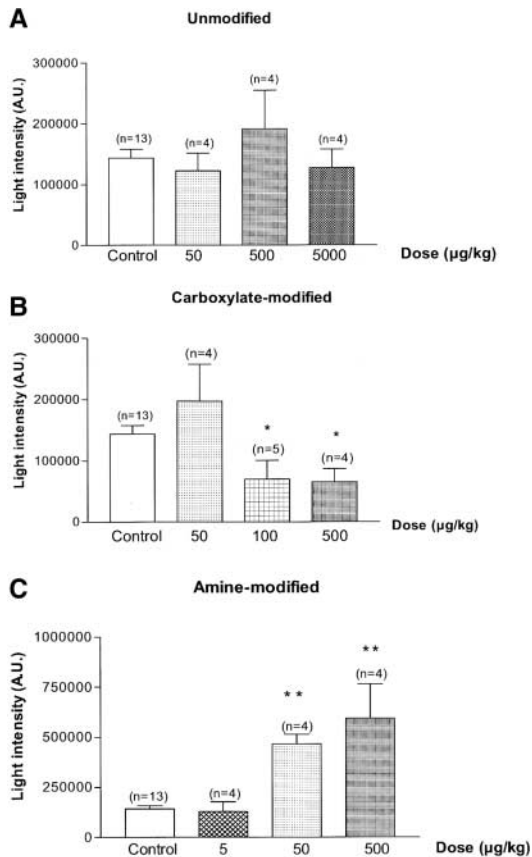
**Intravascular administration.** Figure 2A shows that the administration of unmodified particles at doses of 50, 500, and 5,000  $\mu$ g/kg in hamsters had no effect on the amount of thrombus formed compared with the thrombus observed in control hamsters. In

**TABLE 1. INTERACTIONS OF UNMODIFIED, AMINE-POLYSTYRENE AND CARBOXYLATE-POLYSTYRENE PARTICLES WITH S- AND Q-SEPHAROSE\***

Particles (50 µg)	Initial Particle Suspension	Absorbance (245 nm)			
		Q-sepharose		S-sepharose	
		Unbound	Eluted	Unbound	Eluted
Amine-modified	0.59 ± 0.03	0.51 ± 0.08	0.013 ± 0.008	0.04 ± 0.03	0.44 ± 0.09
Carboxylate-modified	0.87 ± 0.02	0.006 ± 0.007	0.74 ± 0.18	0.77 ± 0.10	0.01 ± 0.01
Unmodified	0.82 ± 0.03	0.65 ± 0.13	0.004 ± 0.003	0.73 ± 0.07	0.03 ± 0.02

\* The data are expressed as mean ± SD (n = 3). Particles were incubated with cationic (Q) or anionic (S) sepharose. The data represent values of absorbance in the supernatant after addition to the sepharose (unbound fraction) and after elution (eluted) with phosphate buffered (1M NaCl).

contrast, pretreatment of hamsters with carboxylate-polystyrene particles induced a significant decrease in thrombus formation at doses of 100 (−52%) and 500 µg/kg (−55%) compared with the thrombus in control hamsters (Figure 2B). At a dose of 50 µg/kg, the thrombus formation was not affected. Amine-polystyrene particles significantly enhanced thrombus generation in hamsters at doses of 50 (+219%) and 500 µg/kg (+307%)



**Figure 2.** Effect of unmodified (A), carboxylate-modified (B), or amine-modified (C) particles on thrombus formation. Cumulative thrombus generation, expressed as total light intensity over 40 minutes, after photochemical injury (2 minutes) to the femoral vein in control hamsters and in hamsters pretreated with an intravenous bolus of UFPs, administered 10 minutes before photochemical injury. Data are mean ± SEM for the number of hamsters indicated in parentheses. Control animals are the same in all three panels. \*p < 0.05, \*\*p < 0.01 by Dunnett's multiple comparison test. AU: arbitrary units. Note: y-axis is different in (C).

compared with control hamsters. No effect was observed after pretreatment of hamsters with a dose of 5 µg/kg (Figure 2C).

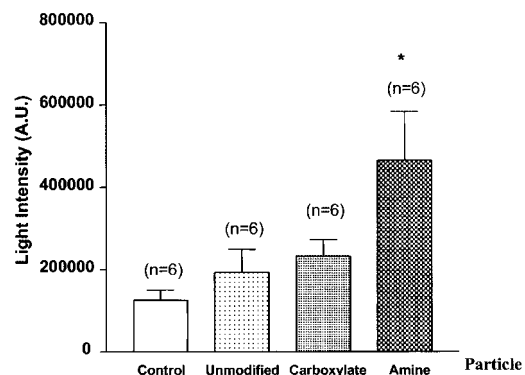
**Intratracheal instillation.** Figure 3 illustrates that the instillation of 5 mg/kg of unmodified and carboxylate-polystyrene particles did not significantly modify the intensity (+153 and +185%, respectively) of thrombus formed. In contrast, the administration of 5 mg/kg of amine-polystyrene particles induced a significant increase in thrombus formation (+368%).

### Histology of Femoral Vein Segments

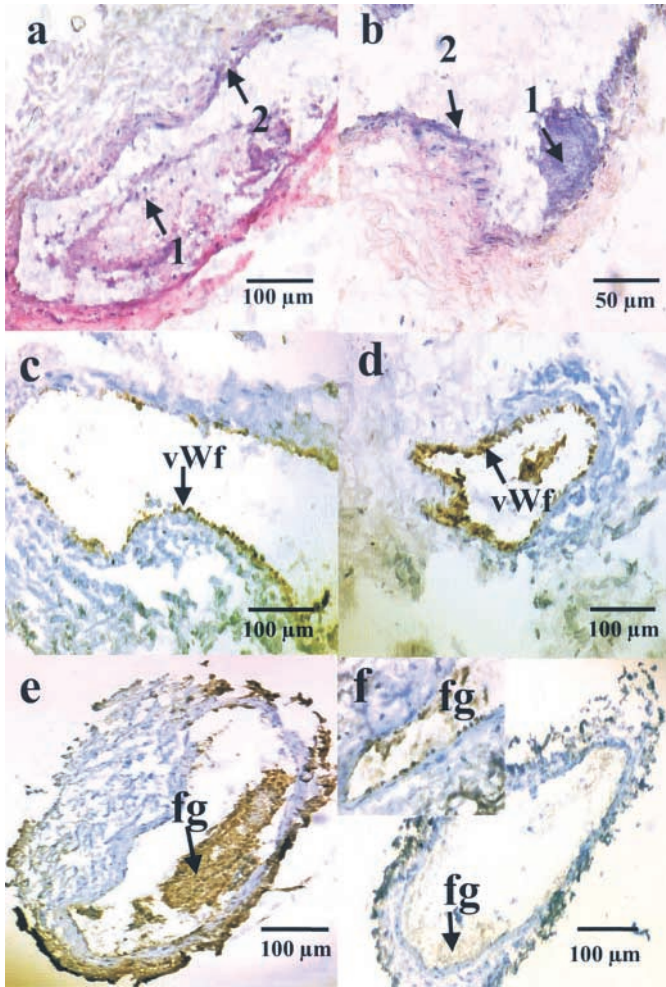
Similar to the positive control thrombus, i.e., a major thrombus obtained by a more prolonged (5 minutes) irradiation (Figure 4A), light microscopy of femoral vein cross-sections after intravascular administration of amine-polystyrene particles confirmed partial de-endothelialization at the site of trauma, accompanied by diffuse platelet-rich thrombosis (Figure 4B). The immunohistochemical staining for the von Willebrand factor confirmed the presence of residual, intact endothelium, both in the positive control hamster (Figure 4C) and in the vein of a hamster after the administration of amine-polystyrene particles (Figure 4D). The staining for fibrinogen also confirmed the presence of substantial amounts of fibrin in the positive control thrombus (Figure 4E) as well as in the thrombus formed after the administration of amine-polystyrene particles (Figure 4F). These findings substantiate that the particle-induced thrombus morphology matches that of the positive control thrombus.

### Platelet Aggregation Studies

The capacity of particles to induce platelet aggregation was investigated in the presence and absence of ADP, an agonist of plate-



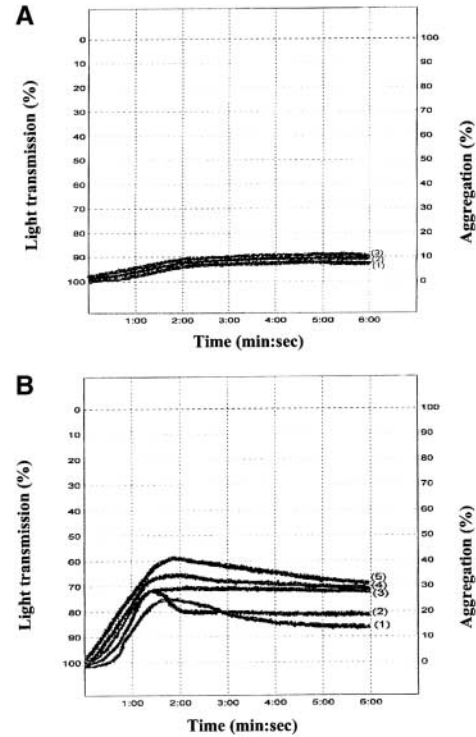
**Figure 3.** Effect of intratracheal instillation of 5 mg/kg of unmodified, carboxylate-modified, or amine-modified UFPs on thrombus formation. Procedure as described in Figure 2. Data are mean ± SEM for the number of hamsters indicated in parentheses. \*p < 0.05 by Dunnett's multiple comparison test. AU: arbitrary units.



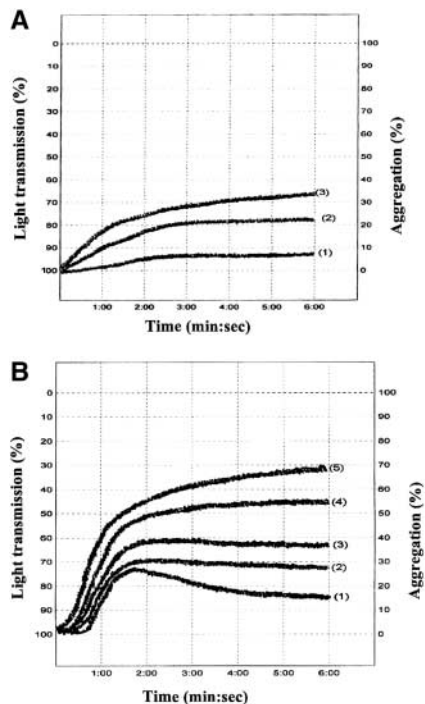
**Figure 4.** Morphology of thrombus formation in the femoral vein (a, c, e): control thrombus (5 minutes irradiation with green light); (b, d, f): particle-induced thrombus (2 minutes irradiation + 500 μg/kg of amine-modified particles). Hematoxylin and eosin staining (a, b); von Willebrand factor (vWf) staining (c, d) identifies the remaining endothelium in the irradiated vessels, and fibrin(ogen) (fg) immunohistochemistry (e, f) identifies the presence of thrombus. 1: thrombus; 2: endothelium. The inset in f shows another view of fibrin(ogen) immunohistochemistry.

let activation. In preliminary experiments, the capacity to enhance platelet activation was investigated after preincubating platelets with particles for 5 minutes, followed by induction of aggregation by different concentrations of ADP (0.625–5 μM) (not shown). On this basis, 1.25 μM of ADP was selected, this concentration inducing mild platelet aggregation by itself.

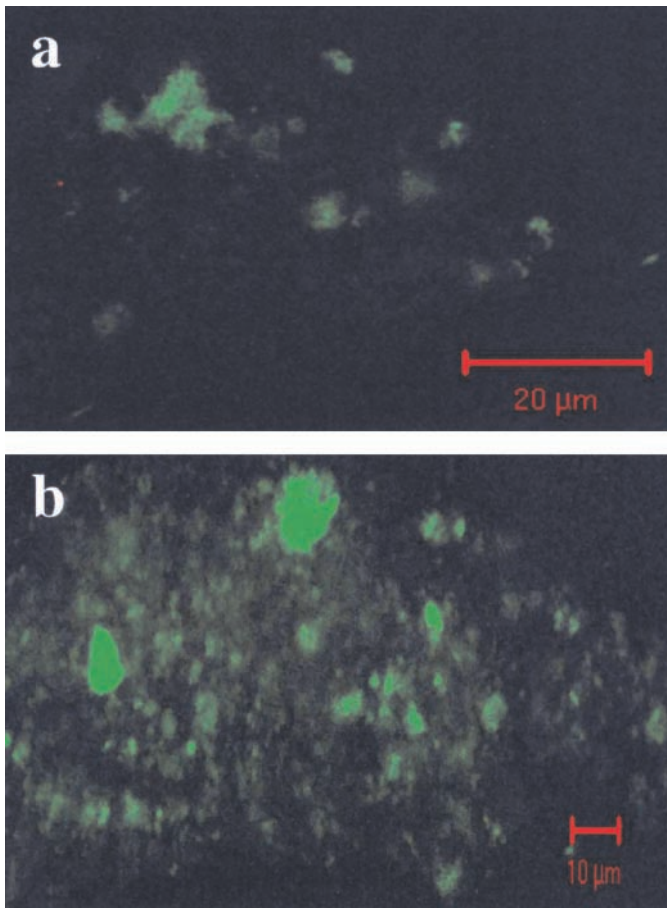
Unmodified particles neither triggered platelet aggregation on stirring nor altered the ADP-induced aggregation (not shown). Carboxylate-polystyrene particles themselves (12.5, 25, 50, or 100 μg/ml) did not induce platelet aggregation on stirring (Figure 5A). However, preincubation of platelets (5 minutes) with different doses of carboxylate-polystyrene particles led to a moderate increase in the ADP-triggered platelet aggregation (Figure 5B). Figure 6 illustrates the effect of amine-polystyrene particles on platelet aggregation. Preincubation of platelets (5 minutes) with 50 or 100 μg/ml of amine-polystyrene particles produced moderate and dose-dependent platelet aggregation when stirring was initiated (Figure 6A). Moreover, after preincu-



**Figure 5.** Effect of carboxylate-polystyrene particles on platelet aggregation. (A) Platelet aggregation tracings induced by stirring in the absence of ADP without (1) or with addition of carboxylate-polystyrene particles: 50 μg/ml (2) or 100 μg/ml (3). (B) Platelet aggregation tracings induced by stirring and 1.25 μM of ADP, without (1) and with preincubation with carboxylate-polystyrene particles: 12.5 μg/ml (2), 25 μg/ml (3), 50 μg/ml (4), or 100 μg/ml (5).



**Figure 6.** Effect of amine-polystyrene particles on platelet aggregation. (A) Platelet aggregation tracings induced by stirring in the absence of ADP without (1) or with addition of amine-polystyrene particles: 50 μg/ml (2) or 100 μg/ml (3). (B) Platelet aggregation tracing induced by stirring and 1.25 μM of ADP, without (1) and with preincubation of amine-polystyrene particles: 12.5 μg/ml (2), 25 μg/ml (3), 50 μg/ml (4), or 100 μg/ml (5).



**Figure 7.** Confocal microscopy of fixed platelet aggregates obtained after incubation with fluorescently labeled, carboxylate-modified particles (A) or amine-modified particles (B) (50 µg/ml) and ADP (1.25 µM).

bation for 5 minutes, the amine-polystyrene particles enhanced the ADP-induced platelet aggregation substantially and in a dose-dependent manner (Figure 6B).

#### Confocal Microscopy

Fluorescent labeling of particles did not interfere with their capacity to induce or enhance platelet aggregation (not shown). Confocal microscopy of fixed fluorescent platelet aggregates demonstrated larger aggregates after incubation with amine-polystyrene particles (Figure 7B) than with carboxylate-polystyrene particles (Figure 7A). Individual platelets were strongly stained by the amine-polystyrene particles but not by the carboxylate-polystyrene particles.

#### Coagulation Assays

Neither the unmodified particles nor the amine-polystyrene or carboxylate-polystyrene particles had any effect on the activated partial thromboplastin time or the prothrombin time (Table 2).

#### DISCUSSION

We found that hemostasis may be affected by the presence of (ultrafine) particles in the circulation and that this phenomenon is dependent on the surface properties of the particles. Thus, positively charged amine-polystyrene particles led to an increased prothrombotic tendency, which results, at least in part, from platelet activation.

#### Choice of Route of Administration, Types of Particles, and Thrombosis Model

The effects of UFPs have been usually studied in animals exposed to particles, either by inhalation or by intratracheal instillation (24–26). Here, we mainly used an admittedly less physiologic mode of administration, namely, the intravascular route, because we wanted to investigate the potential of particles to directly impact on hemostasis. The rationale for this approach is that UFPs may be found in substantial amounts within the blood circulation (10). We have, indeed, demonstrated that intratracheally instilled ultrafine albumin colloid particles rapidly diffuse from the lungs into the systemic circulation in the hamster (12) and that ultrafine carbon particles do so in humans too (13). Thus, we adopted a pharmacodynamic approach consisting of injecting precise amounts of well-characterized particles. However, we also verified these observations by administering particles intratracheally, and these experiments, which we intend to expand in the future, corroborate our observations.

Until now, the majority of studies have been performed with particles of various surface chemistries, such as cobalt, nickel, titanium dioxide (27), or diesel exhaust particles (28). These agents cause epithelial and endothelial damage, possibly as a result of free-radical activity at their surfaces (29).

We have used noncytotoxic (Xu and coworkers, unpublished data) polystyrene particles and have evaluated surface chemistry modifications on such particles as a parameter possibly involved in vascular toxicity. The Zeta potential of particles at physiologic pH revealed a negative absolute charge in all particles, even in the amine-modified particles, and this probably relates to the presence of sulfate in the polystyrene matrix (30). However, studies of the interactions with S- and Q-sepharose clearly showed that the amine modifications of the particles were accompanied by positive charges on the surface, whereas carboxylate modification of the particles introduced negative charges.

Although most ambient particles are charged (19), the role of charge has hitherto not received much attention in relation to particle toxicity, except regarding particle deposition (19). Even though our particles are not true pollutant particles, they offer a convenient and, we believe, adequate model to study the mechanistic effects of particles and some aspects of their surface properties on hemostatic parameters.

We made use of a recently established and validated model of acute thrombosis in the hamster (22). In this photochemical injury model, it is possible to cause mild damage to endothelial cells; developing thrombi are platelet rich, and they resemble clinical thrombi, as shown by electron microscopic analysis (31). By combining transillumination and photochemical vessel wall injury, it has become possible to link the degree of vessel wall injury with the intensity of thrombosis that develops as a consequence of endothelial cell destruction.

#### Effects of Particles on Thrombosis

Our results demonstrate that unmodified particles do not interfere with thrombus formation, even at a dose as high as 5,000 µg/kg. Thus, there is no simple “particle effect” on thrombus formation. In contrast, negatively charged carboxylate-polystyrene particles significantly inhibited thrombus generation at 100 µg/kg, whereas thrombosis was enhanced by the positively charged amine-polystyrene particles at 50 µg/kg, i.e., by as little as 5 µg for a hamster of about 100 g. Light microscopy analysis confirmed de-endothelialization of the vein at the site of trauma and confirmed that thrombi were platelet rich, as expected (23). Moreover, after intratracheal instillation, a prothrombotic effect was only recorded for positively charged amine-polystyrene particles, corroborating the results obtained after intravascular ad-

**TABLE 2. EFFECT OF UNMODIFIED, AMINE-POLYSTYRENE AND CARBOXYLATE-POLYSTYRENE PARTICLES ON PROTHROMBIN TIME AND ACTIVATED PARTIAL THROMBOPLASTIN TIME IN HUMAN PLASMA**

Dose ( $\mu\text{g/ml}$ )	PTs			aPTTs		
	Unmodified	Carboxylate	Amine	Unmodified	Carboxylate	Amine
0	11.2	11.2	11.2	32.5	31.9	29.1
0.31	11.2	11.4	11.2	32.8	32.9	32.7
0.62	11.4	11.2	11.2	32.9	32.6	33.1
1.25	11.0	11.4	11.2	32.9	32.6	32.1
2.5	11.2	11.4	11.4	33.0	31.7	32.8
5	11.2	11.4	11.2	33.0	32.2	32.8
10	11.2	11.4	11.4	32.4	32.5	32.7

Definition of abbreviations: aPTT = activated partial thromboplastin time; PT = prothrombin time.

ministration of particles. This effect, which occurred very rapidly (within 1 hour of instillation), may be the result of direct passage of particles into the circulation (12) or pulmonary inflammation and the systemic release of mediators (6), or a combination of both. Both particle injection and intratracheal instillation show a prothrombotic effect of the positively charged amine-polystyrene particles. Before administration, to minimize aggregation, the particles were sonicated and vortexed, but we do not know whether they induce their effects individually or as aggregates. We do not know the exact mechanism whereby the positive surface charge particles exert their effects on biologic surfaces (on platelets, endothelial cells, or elsewhere). The complexity of this mechanism comes mainly from the fact that electrostatic interactions and chemical structure are intimately linked. However, we believe that it is reasonable to consider that the most likely mechanism is via electrostatic interactions rather than via active chemical reactions such as condensation of amine with carboxyl groups (to yield peptide bonds) or aldehydes or other functional groups. Indeed, the particles used have rather low chemical reactivity. The main argument in favor of this is that the particles were shown to interact in an entirely reversible way with ion exchange resins (Table 1). Moreover, the particles are unlikely to be good substrates for enzymes.

Some endogenous and exogenous polycations are known to affect cellular viability and cell function, both *in vivo* and *in vitro*. Thus, polycations, such as poly-L-arginine or poly-L-lysine, alter the blood-brain barrier in rat *in vivo* after intracarotid infusion (32), and they increase the permeability of both epithelial and endothelial cells *in vitro* (33, 34). We have shown that polycationic components of air-sprayed paints exhibit cytotoxicity in various cells, including human type II pneumocytes and alveolar macrophages (35). These effects could be inhibited by polyanionic molecules such as heparin (36). Moreover, it has been shown that the partially quaternized poly[thio-1-(*N,N*-diethyl-aminomethyl)ethylene] can induce both hemagglutination and hemolysis by the disruption of the lipid bilayer of red blood cells through electrostatic interactions (37).

### Mechanism of Enhanced Thrombosis

To investigate whether particles acted in primary hemostasis, platelet aggregation studies were undertaken. Unmodified particles did not exert any effect, which is in agreement with their lack of effect on thrombus formation *in vivo*. Amine modification induced platelet aggregation and strongly increased the ADP-triggered aggregation in a dose-dependent manner, thus providing a mechanistic basis for the enhancement of thrombus formation observed *in vivo*. Carboxylate-polystyrene particles, on the contrary, did not interfere with platelet aggregation themselves and only weakly enhanced the ADP-induced platelet aggrega-

tion. The latter findings are not in complete agreement with the observed inhibitory effect of carboxylate-polystyrene particles *in vivo*. Nevertheless, confocal microscopy demonstrated larger platelet aggregates after incubation with amine-polystyrene particles than with carboxylate-polystyrene particles, and individual platelets were strongly stained by the amine-polystyrene particles but not by the carboxylate-polystyrene particles.

Platelets carry a net negative charge on their surface, mainly due to ionizable sialic acid groups (38). It is likely that positively charged amine-polystyrene particles attach to these sialic acid groups. Indeed, it has been demonstrated that the cationic poly-peptide polylysine can induce platelet aggregation, probably by reducing their surface charge and by forming bridges between platelets (39). More recently, it has been demonstrated that polylysine can activate human platelets through a specific receptor, possibly consisting of negatively charged proteoglycans present on their surfaces (40). Thus, positively charged particles would facilitate platelet-platelet interactions via negative charge neutralization and crossbridges. In this interpretation, negatively charged carboxylate-polystyrene particles would compete with platelet-ligand or platelet-platelet interactions. The possibility that particles are taken up by phagocytosis or the open canalicular system of platelets cannot be ruled out at present (41).

Further experiments showed that the effect of particles could not be attributed to activation of intravascular coagulation because neither the activated partial thromboplastin time nor the prothrombin time was affected by any type of particle. Gardner and coworkers (42) demonstrated that intratracheal instillation of oil fly ash particles to rats did not induce changes in activated partial thromboplastin time and prothrombin time indices.

We conclude that the presence of (ultrafine) particles in the circulation affects hemostasis. The increased prothrombotic tendency results, at least in part, from platelet activation by the positively charged amine-polystyrene particles. Thus, the presence of pollutant particles in blood may lead to an enhanced risk for thromboembolic disease. Our finding thus adds a much needed experimental support to an increasing body of epidemiologic evidences (5, 15, 43, 44) showing that pollution impacts on cardiovascular diseases.

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### References

1. Ware JH. Particulate air pollution and mortality—clearing the air. *N Engl J Med* 2000;343:1798–1799.
2. Dockery DW, Pope CA III, Xu X, Spengler JD, Ware JH, Fay ME,

- Ferris BG Jr, Speizer FE. An association between air pollution and mortality in six US cities. *N Engl J Med* 1993;329:1753-1759.
3. Schwartz J. What are people dying of on high air pollution days? *Environ Res* 1994;64:26-35.
  4. Peters A, Doring A, Wichmann HE, Koenig W. Increased plasma viscosity during an air pollution episode: a link to mortality? *Lancet* 1997;349:1582-1587.
  5. Samet JM, Dominici F, Curriero FC, Coursac I, Zeger SL. Fine particulate air pollution and mortality in 20 US cities, 1987-1994. *N Engl J Med* 2000;343:1742-1749.
  6. Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet* 1995;345:176-178.
  7. Salvi S, Blomberg A, Rudell B, Kelly F, Sandstrom T, Holgate ST, Frew A. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med* 1999;159:702-709.
  8. Pope CA III, Thun MJ, Namboodiri MM, Dockery DW, Evans JS, Speizer FE, Heath CW Jr. Particulate air pollution as a predictor of mortality in a prospective study of US adults. *Am J Respir Crit Care Med* 1995;151:669-674.
  9. Watkinson WP, Campen MJ, Costa DL. Cardiac arrhythmia induction after exposure to residual oil fly ash particles in a rodent model of pulmonary hypertension. *Toxicol Sci* 1998;41:209-216.
  10. Oberdorster G. Pulmonary effects of inhaled ultrafine particles. *Int Arch Occup Environ Health* 2001;74:1-8.
  11. MacNee W, Donaldson K. How can ultrafine particles be responsible for increased mortality? *Monaldi Arch Chest Dis* 2000;55:135-139.
  12. Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med* 2001;164:1665-1668.
  13. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B. Passage of inhaled particles into the blood circulation in humans. *Circulation* 2002;105:411-414.
  14. Pope CA III, Verrier RL, Lovett EG, Larson AC, Raizenne ME, Kanner RE, Schwartz J, Villegas GM, Gold DR, Dockery DW. Heart rate variability associated with particulate air pollution. *Am Heart J* 1999;138:890-899.
  15. Peters A, Dockery DW, Muller JE, Mittleman MA. Increased particulate air pollution and the triggering of myocardial infarction. *Circulation* 2001;103:2810-2815.
  16. Dreher KL, Jaskot RH, Lehmann JR, Richards JH, McGee JK, Ghio AJ, Costa DL. Soluble transition metals mediate residual oil fly ash induced acute lung injury. *J Toxicol Environ Health* 1997;50:285-305.
  17. Gilmour PS, Brown DM, Lindsay TG, Beswick PH, MacNee W, Donaldson K. Adverse health effects of PM10 particles: involvement of iron in generation of hydroxyl radical. *Occup Environ Med* 1996;53:817-822.
  18. Becker S, Soukup JM, Gilmour MI, Devlin RB. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. *Toxicol Appl Pharmacol* 1996;141:637-648.
  19. Cohen BS, Xiong JQ, Fang CP, Li W. Deposition of charged particles on lung airways. *Health Phys* 1998;74:554-560.
  20. Chen LC, Miller PD, Amdur MO, Gordon T. Airway hyperresponsiveness in guinea pigs exposed to acid-coated ultrafine particles. *J Toxicol Environ Health* 1992;35:165-174.
  21. Dockery DW, Schwartz J, Spengler JD. Air pollution and daily mortality: associations with particulates and acid aerosols. *Environ Res* 1992;59:362-373.
  22. Kawasaki T, Kaida T, Arnout J, Vermeylen J, Hoylaerts MF. A new animal model of thrombophilia confirms that high plasma factor VIII levels are thrombogenic. *Thromb Haemost* 1999;81:306-311.
  23. Stockmans F, Stassen JM, Vermeylen J, Hoylaerts MF, Nystrom A. A technique to investigate mural thrombus formation in small arteries and veins. I. Comparative morphometric and histological analysis. *Ann Plast Surg* 1997;38:56-62.
  24. Nemmar A, Delaunois A, Nemery B, Dessy-Doize C, Beckers JF, Sulon J, Gustin P. Inflammatory effect of intratracheal instillation of ultrafine particles in the rabbit: role of C-fiber and mast cells. *Toxicol Appl Pharmacol* 1999;160:250-261.
  25. Li XY, Brown D, Smith S, MacNee W, Donaldson K. Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. *Inhal Toxicol* 1999;11:709-731.
  26. Shukla A, Timblin C, BeruBe K, Gordon T, McKinney W, Driscoll K, Vacek P, Mossman BT. Inhaled particulate matter causes expression of nuclear factor (NF)- $\kappa$ B-related genes and oxidant-dependent NF- $\kappa$ B activation in vitro. *Am J Respir Cell Mol Biol* 2000;23:182-187.
  27. Zhang Q, Kusaka Y, Sato K, Nakakuki K, Kohyama N, Donaldson K. Differences in the extent of inflammation caused by intratracheal exposure to three ultrafine metals: role of free radicals. *J Toxicol Environ Health* 1998;53:423-438.
  28. Kumagai Y, Taira J, Sagai M. Apparent inhibition of superoxide dismutase activity in vitro by diesel exhaust particles. *Free Radic Biol Med* 1995;18:365-371.
  29. Lim HB, Ichinose T, Miyabara Y, Takano H, Kumagai Y, Shimojo N, Devalia JL, Sagai M. Involvement of superoxide and nitric oxide on airway inflammation and hyperresponsiveness induced by diesel exhaust particles in mice. *Free Radic Biol Med* 1998;25:635-644.
  30. Muller RH, Ruhl D, Luck M, Paulke BR. Influence of fluorescent labelling of polystyrene particles on phagocytic uptake, surface hydrophobicity, and plasma protein adsorption. *Pharm Res* 1997;14:18-24.
  31. Matsuno H, Uematsu T, Nagashima S, Nakashima M. Photochemically induced thrombosis model in rat femoral artery and evaluation of effects of heparin and tissue-type plasminogen activator with use of this model. *J Pharmacol Methods* 1991;25:303-317.
  32. Westergren I, Johansson BB. Altering the blood-brain barrier in the rat by intracarotid infusion of polycations: a comparison between protamine, poly-L-lysine and poly-L-arginine. *Acta Physiol Scand* 1993;149:99-104.
  33. Needham L, Hellewell PG, Williams TJ, Gordon JL. Endothelial functional responses and increased vascular permeability induced by polycations. *Lab Invest* 1988;59:538-548.
  34. Uchida D, Ballowe C, Larsen G, Irvin C, Cott G. Polycations decrease the transepithelial resistance of cultured tracheal epithelial cells. *Chest* 1992;101:335.
  35. Hoet PH, Gilissen LP, Leyva M, Nemery B. In vitro cytotoxicity of textile paint components linked to the "Ardystil syndrome." *Toxicol Sci* 1999;52:209-216.
  36. Hoet PH, Gilissen L, Nemery B. Polyanions protect against the in vitro pulmonary toxicity of polycationic paint components associated with the Ardystil syndrome. *Toxicol Appl Pharmacol* 2001;175:184-190.
  37. Moreau E, Ferrari I, Drochon A, Chapon P, Vert M, Domurado D. Interactions between red blood cells and a lethal, partly quaternized tertiary polyamine. *J Controlled Release* 2000;64:115-128.
  38. Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta* 1998;1376:433-453.
  39. Taketomi Y, Kuramoto A. Ultrastructural studies on the surface coat of human platelet aggregated by polylysine and dextran. *Thromb Haemost* 1978;40:11-23.
  40. Donato JL, Marcondes S, Antunes E, Nogueira MD, Nader HB, Dietrich CP, Rendu F, de Nucci G. Role of chondroitin 4-sulphate as a receptor for polycation induced human platelet aggregation. *Br J Pharmacol* 1996;119:1447-1453.
  41. White JG, Clawson CC. Effects of large latex particle uptake of the surface connected canalicular system of blood platelets: a freeze-fracture and cytochemical study. *Ultrastruct Pathol* 1981;2:277-287.
  42. Gardner SY, Lehmann JR, Costa DL. Oil fly ash-induced elevation of plasma fibrinogen levels in rats. *Toxicol Sci* 2000;56:175-180.
  43. Peters A, Liu E, Verrier RL, Schwartz J, Gold DR, Mittleman M, Baliff J, Oh JA, Allen G, Monahan K, et al. Air pollution and incidence of cardiac arrhythmia. *Epidemiology* 2000;11:11-17.
  44. Peters A, Wichmann HE, Tuch T, Heinrich J, Heyder J. Respiratory effects are associated with the number of ultrafine particles. *Am J Respir Crit Care Med* 1997;155:1376-1383.