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The Rat Ear Vein Model for Investigating *In Vivo* Thrombogenicity of Ultrafine Particles (UFP)

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Recent studies in rodents indicate that intravenous or intratracheal administration of ultrafine particles (UFP) increases thrombogenesis in a surgically exposed peripheral vein after photodynamic excitation of intravenously injected rose bengal (RB). We sought to adapt the invasive peripheral vein RB model to a noninvasive monitoring of ear veins under an inverted microscope. Animals received one of the following: an intraperitoneal, intravenous bolus, or intravenously infused dose of RB. An ear vein was illuminated by a green laser, and formation of a thrombus was captured with a digital camera. Only continuous intravenous infusion produced a steady-state RB plasma level and reproducible thrombus responses in different ear veins of the same rat. This system was then used to study the thrombogenic effects of iv-administered positively or negatively charged 60-nm ultrafine polystyrene particles (PSP). Significant dose-dependent enhancement of thrombus formation was found, as indicated by decreased laser illumination time to 33% of baseline values at 0.5 mg/kg. Negatively charged PSP of the same size failed to affect thrombus formation. We also studied the thrombogenic effect of PSP without the use of RB. The findings were the same as with RB, although the illumination time had to be increased. When 0.5 mg/kg was instilled intratracheally, the laser illumination time to form a thrombus was decreased to 42% of the baseline value, suggesting translocation of UFP into the bloodstream. These results are consistent with previous findings using the invasive model, and they validate the use of this non-invasive ear vein model to evaluate thrombogenic effects of UFP deposition in the respiratory tract.

Key Words: **ultrafine particles; polystyrene particles; thrombosis; ear vein; rose bengal.**

INTRODUCTION

Several epidemiological studies have shown associations of ambient ultrafine particles (UFP; <100 nm in diameter) with adverse respiratory and cardiovascular effects resulting in morbidity and mortality in susceptible parts of the population (Dockery *et al.*, 1993; Pope *et al.*, 2002; Wichmann and Peters, 2000; Wichmann *et al.*, 2000). The mechanisms for the cardiovascular effects are not completely understood. However, it has been suggested that the ultrafine component of particulate matter can mediate these effects because these particles have high deposition efficiency in all areas of the respiratory tract (Frampton, 2001; Oberdörster *et al.*, 1995). Numerous mechanisms for the cardiovascular effects have been proposed, including alterations in the autonomic control of the heart, ischemic responses in the myocardium, and inflammatory responses triggering endothelial dysfunction and thrombosis (Utell *et al.*, 2002). However, an emerging idea is that, because of their small size, UFP can translocate from the lungs into the circulation and affect cardiovascular end points like blood coagulation more directly (Berry *et al.*, 1977; Nemmar *et al.*, 2004; Oberdörster *et al.*, 2002). In support of this hypothesis, studies by Nemmar *et al.* (2002; 2003a) showed that intratracheally (IT) instilled and intravenously (iv) administered ultrafine (60 nm) amine-modified polystyrene particles could increase thrombus formation. The same group also showed that more environmentally relevant pollutants like diesel exhaust particles (0.5 μg/ml) can promote femoral venous thrombosis in a dose-dependent manner when intratracheally instilled into hamsters (Nemmar *et al.*, 2003a, 2003b).

Understanding the mechanism by which UFP interact with the vascular system requires the use of animal models of endothelial injury. In fact, several such models has been developed, including those in which injuries are produced mechanically (Stockmans *et al.*, 1991), electrically (Carmeliet *et al.*, 1997), chemically with $FeCl_3$ (Farrehi *et al.*, 1998; Kurz *et al.*, 1990) and with lasers and photochemicals (Kawasaki *et al.*, 1999, 2000; Kochevar *et al.*, 1994; Nemmar *et al.*, 2002; Roesken *et al.*, 1998) in large vessels such as the femoral vein or carotid artery. Of particular interest to us was the Rose Bengal (RB) thrombosis model. This model is based on the local photodynamic generation of singlet molecular oxygen from systemically injected RB that is illuminated with green light (GL, 540 nm) (Umemura *et al.*, 1990). Peroxidative damage to endothelial membranes provides the initial stimulus for platelet adhesion and eventual formation of thrombi (Kochevar *et al.*, 1994; Saniabadi *et al.*, 1995). Evaluation of thrombogenesis in this system requires that the vessel of interest be mounted on

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a transilluminator, which allows visualization and imaging of the growing thrombus. Factors affecting thrombus formation include plasma RB concentration, time post RB administration, and total exposure time to green light (Kawasaki *et al.*, 1999; Saniabadi *et al.*, 1995). Also, manipulation of the vessel itself can affect RB-induced endothelial responses, which can contribute to increased variability of the results when using this invasive technique. Therefore, the major objectives of this article are (1) to adopt a noninvasive model without the disadvantages associated with previous models and (2) to validate this model for evaluating particle-induced thrombogenic effects by confirming the results of Nemmar *et al*. (2002) indicating that iv and intratracheal administration of UFP accelerates venous thrombus formation. This article presents a modified version of the RB model in which the rat ear vessel is used rather than exposing and manipulating the femoral vein. A similar technique has been reported by Rosen *et al*. (2001) in which the ear vessels of C57BL6 mice were imaged *in situ* to study differences between the RB- and laser-induced vascular damage. These experiments demonstrated that this noninvasive technique requires minimal handling and preparation of the vessel, thereby reducing unwanted vascular effects. Also, because the local injuries generated are small, there is no major perturbation of the coagulation system and no significant consumption of coagulation factors. Thus, multiple injuries can be induced and recorded in a single animal (Rosen *et al.*, 2001), which allows for reduced numbers of animals per experiment. In addition, the ear section can be excised, fixed, and prepared for histology and immunohistochemical analyses of local endothelial dysfunction markers such as fibrinogen, endothelin, von Willebrand factor (Muller and Griesmacher, 2000), and P-selectin (Becker, 2001). More importantly, as our data show, the ear vein model is useful in studying the thrombogenic effects of particles administered to the lungs.

METHODS

Animals. Specific pathogen-free male Fisher 344 rats weighing 250–300 g were group-housed in plastic cages with paper shavings (Alphadry) in our AAALAC accredited vivarium, where they had free access to rodent chow and water. Room temperature was maintained at 23°C with a 12 h light/dark cycle. All protocols involving animals were approved by the University of Rochester Committee on Animal Resources.

Rose Bengal route of administration. Animals received either intraperitoneal (ip, 200 mg/kg), iv bolus (10 mg/kg), or iv infused (24 mg/kg/h) doses of RB in different experiments to characterize the kinetics of plasma RB accumulation after each route of administration. For this purpose, blood was collected at different times from the tail vein (125 μl) for up to 90 min after dosing. Briefly, a small incision was made on the tail vein, from which half of a 250 μl heparinized blood collecting tube was filled (VWR International, West Chester, PA). Bleeding was stopped by applying pressure to the vessel. The same site was used for subsequent collections. Blood was then transferred into a 0.250-ml microcentrifuge tube covered with aluminum foil and centrifuged at 10,000 × g for 10 min to obtain plasma. Rose Bengal was quantified in plasma via its absorbance at 549 nm. The goal of these experiments was to select a method of RB administration that achieves and maintains a stable RB plasma level.

Rose Bengal thrombosis model. Rats were anesthetized with sodium pentobarbital (30 mg/kg, ip) and placed supine on a heating pad (37°C). A depilatory was used to remove hair from the ears. Both femoral veins were exposed and cannulated with a 15-gauge metal catheter with a Silastic tubing tip (0.025 in. inner diameter and 0.47 in. outer diameter). One femoral vein was used for RB administration; the other, for intravenous administration of the ultrafine particles. Animals were then placed on an inverted microscope stage (Olympus 1X51, Olympus America Inc., Melville, NY) such that one ear rested on a glass slide (1-mm-thick) in the optical path so that the blood vessels and flow could be visualized with a digital camera (Spot Camera Software version 4, Spectra Services, Webster, NY). The ear was kept moist with saline throughout the observation period, and body temperature was maintained between 37°C and 38°C with the help of a heating lamp. A 2-mm green laser beam (532 nm, 100 mW, Extreme Lasers, Seabrook, TX) was introduced to the optical system from the back of the microscope through the 10× objective to be focused on a small secondary or tertiary ear vein (70–90 μm). To facilitate focusing on the vein without inducing damage to the vessel, a neutral filter was placed between the laser and the microscope objective. Light intensity at this spot was measured with an optical power meter (Model 1815-C, Newport Corporation, Irvine, CA) and was found to be close to 0.0 mW with the filter and between 13 and 14 mW without the filter. At that time, separate sections of the target ear vessel were illuminated (for 60–240 s) with the green light (GL, 13.5 mW) alone to determine the laser effect on the vessel, or the baseline value. Rose Bengal was administered 5 to 10 min later, and the vessel was illuminated (for 30–120 s) in different sections to determine the effect of RB and GL on blood coagulation. In this way, each animal served as its own control. Then UFP were either administered iv or instilled intratracheally (see below under *Aminated and Carboxylated Polystyrene Particles*). After the particles were in the system for 2 min, the vessel was illuminated (for 15–60 s) repeatedly for 1 h, in different sections of the same vessel or of another vessel of the same size, to study the effects of UFP on thrombus induction. In other experiments, rats did not receive the photosensitive dye (Rose Bengal) prior to particle administration, and local endothelial activation/injury was induced with green laser illumination only.

To determine if the baseline value (effect of GL alone) changes with time, a group of animals received iv saline instead of UFP, and illumination time required to induced thrombus formation was assessed 10, 20, 30, and 60 min thereafter. The same experiment was repeated 2 and 3 days after the initial exposure in the same rat.

Thrombus formation is defined and evaluated as follows: A thrombus should occur within a maximum of 30 s after GL exposure for the determination of green laser illumination time. This illumination time is defined as the thrombus-inducing time (TIT). Reproducibility of TITs was verified in a different spot on the vein. Because TIT in the presence of RB (RB-TIT) is shorter than baseline-TIT, RB-TIT was determined by starting with half of baseline-TIT; if a thrombus was induced, the time was halved again, and so on. Likewise, this principle of halving TIT was also applied after UFP dosing to determine particle–induced-TIT (UFP-TIT). In contrast, if no thrombus was induced, illumination time was doubled until UFP-TIT could be defined.

Aminated and carboxylated polystyrene particles. To validate the ear vein model, experiments were performed using both ultrafine (60 nm) aminated (positively charged) and carboxylated (negatively charged) polystyrene particles (Bangs Laboratories, Fishers, IN). Particles were sonicated for 15 min and vortexed prior to suspension in saline, as well as before iv and IT administration to rats. To study their effects on coagulation as described above, different doses of aminated (0.02, 0.5, and 50 mg/kg) and carboxylated (0.1 and 50 mg/kg) particles were given in a volume of 250 μl after a steady level of RB was reached by iv infusion. In experiments where RB was not used, 0.5 mg/kg dose of aminated particles was administered iv or IT 10 to 15 min after baseline-TIT was determined.

Data analysis. Because our baseline-TIT measurements showed large variability between different rats but were reproducible in the same individual animal, we used the ratios of RB-TIT to baseline TIT or UFP-TIT

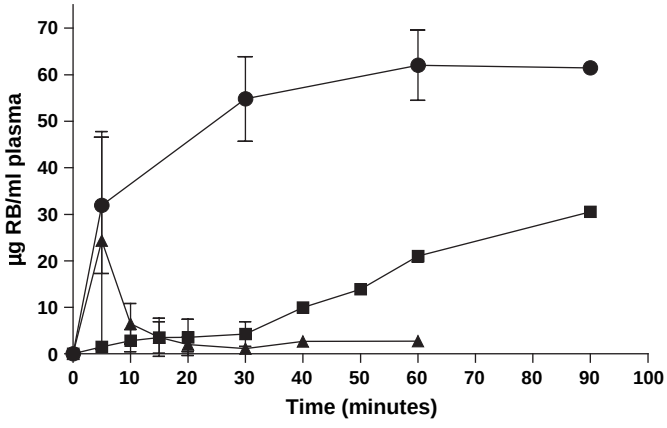

FIG. 1. Plasma Rose Bengal (RB) concentration. Animals received either ip (200 mg/kg, squares), iv bolus (10 mg/kg, triangles) or iv-infused (24 mg/kg/h, circles) RB in different experiments. Blood was collected at different times from the tail vein for up to 90 min; plasma RB was then quantified with a spectrophotometer (549 nm). Data are expressed as mean ± S.D. with $n = 3$–4 (ip and iv bolus) and $n = 4$–5 (iv infused).

to RB-TIT to assess the effects. Thus, a ratio of 1 would indicate no effect, and a ratio of 0.5 a 50% reduction of illumination time to induce thrombus formation. In experiments where rats did not receive RB prior to particle administration, the effect was calculated as the ratio of UFP-TIT to baseline-TIT. Results were analyzed for statistical differences by one-way analysis of variance (ANOVA). Differences between groups were further analyzed with the Tukey-Kramer multiple comparison test. Such differences were considered significant when $p \leq 0.05$.

RESULTS

Initial experiments were aimed at optimizing RB plasma levels and keeping them stable for an extended period. As shown in Figure 1, the iv bolus dose resulted in a plasma RB concentration that decreased rapidly 5 min after injection, while the ip dose caused a continuous increase in plasma, even up to 90 min after RB administration. Thus, for either of these two routes of administration, thrombus formation was not reproducible 20 to 30 min after the initial injury (data not shown). In contrast, a steady-state level of 60 µg/ml RB in plasma was reached after 30 min of iv infusion and was maintained for up to 90 min. Furthermore, the vascular injury was reproducible up to 60 min after reaching steady state. Therefore, subsequent experiments were performed using iv infusion of RB.

Figure 2 illustrates how the ear vein is targeted at its center with the green laser, resulting in the formation of an occlusive thrombus after 120 s of GL illumination 40 min after the beginning of RB infusion. Previous experiments showed that when the laser power was 80 mW, most of the thrombi generated were occlusive after 30 s of illumination with GL. The occlusion caused cessation of blood flow, and therefore the vessel could not be used for further studies. In contrast, illumination with lower power (5 mW) did not produce thrombus even after 10 min of illumination. However, when the laser was slightly increased to 13.5 mW, injuries were mostly sub-occlusive and different sections of the vessel upstream from the injury could be used for other exposures.

When reproducibility of baseline TIT was studied in animals injected with saline, it was observed that although the baseline values between rats can be variable (from 30 s to 240 s), the initial illumination time required to induce a thrombus after saline injection did not change for at least 1 h (Table 1). This shows that the system is stable and that experiments with particles can be conducted within the hour. We further observed that the baseline-TIT for a given rat did not change significantly between ears of the same rat, even 2 days after its first determination (data not shown).

The model was then used to study the effect of UFP on thrombogenesis. Ultrafine (60 nm) aminated-PSP—administered intravenously 30 min after start of the RB infusion and after RB-TIT was determined—significantly shortened the illumination time to induce thrombus (UFP-TIT) when compared to RB-TIT (Fig. 3). There was a dose-dependent decrease from 0.02 to 0.5 mg/kg PSP, with a 67% reduction in RB-TIT for the latter. This dose-dependent effect is in agreement with the results of Nemmar *et al*. (2002); however, at a higher dose

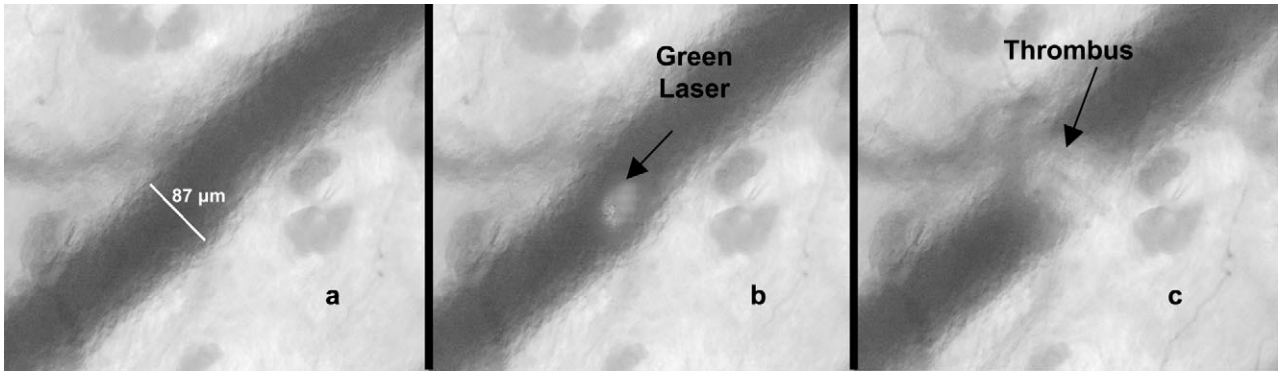

FIG. 2. Rose Bengal–induced endothelial damage to the ear vein of a Fischer 344 rat. To allow visualization of the ear vein, animals were anesthetized and placed on the stage of an inverted microscope. (a) The vessel prior to illumination with laser; (b) green laser aiming at the vein;(c) vessel immediately after the 120 s illumination with green light.

TABLE 1
Stability of Baseline Values for Illumination Time (without rose bengal)

		After saline			
Rat	Baseline-TIT (s)	10 min	20 min	30 min	60 min
1	30	30	30	30	30
2	180	180	180	180	180
3	240	240	240	240	240

Note. To allow visualization the vessel of interest, animals were anesthetized and placed on the stage of an inverted microscope. The vessel was illuminated with green light to determine the baseline TIT value. After 10–15 min, rats received iv saline (250 μl), and thrombus formation was assessed 10, 20, 30, and 60 min thereafter to determine if elapsed time affects baseline value. It was observed that although the baseline values between rats can be very variable (from 30 s to 240 s), the initial illumination time required to induce thrombogenesis does not change for at least 1 h after the first illumination.

of 50 mg/kg iv the thrombogenic response was lower, although still significant (Fig. 3), indicating that the thrombus-inducing mechanism was somehow affected but is not fully operational at this extremely high dose. In contrast, neither 0.1 nor 50 mg/kg of carboxylated PSP administered iv affected thrombus formation, indicating that the thrombogenic effect of iv-administered UFP depends on particle surface properties (charge) and that excessively high doses are less effective. These results confirm

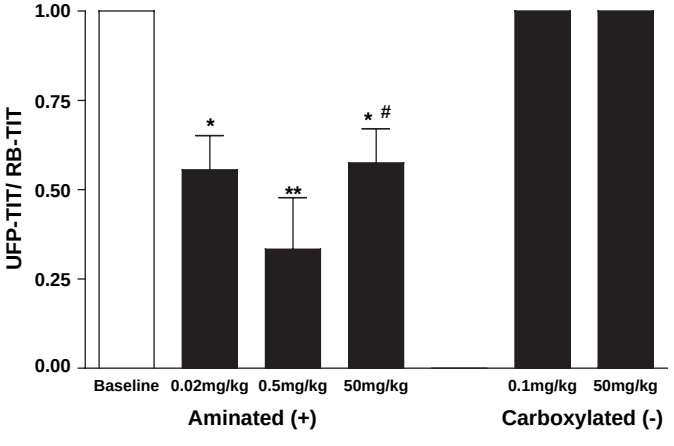

FIG. 3. Effect of intravenous ultrafine (60 nm) polystyrene particles in the rose bengal (RB) iv infusion thrombosis model. Animals received different doses of aminated (0.02, 0.5, and 50 mg/kg) and carboxylated (0.1 and 50 mg/kg) particles 30 min after the start of RB iv infusion. Two minutes after administration, the vessel was illuminated (for 15 s to 60 s) repeatedly for 1 h in a different section of the same vessel or another vessel of the same size. To determine particle effects, a ratio of UFP-TIT to RB-TIT was calculated. Thus, a ratio of 1 would indicate no effect and a ratio of 0.5 a 50% reduction of illumination time to induce a thrombus. Data are expressed as mean ± S.D., with $n = 3$ for the 0.02 and 0.5 mg/kg doses and $n = 4$ for the higher dose. *Significantly different from baseline, $p < 0.01$. #Significantly different from 0.5 mg/kg dose, $p < 0.05$.

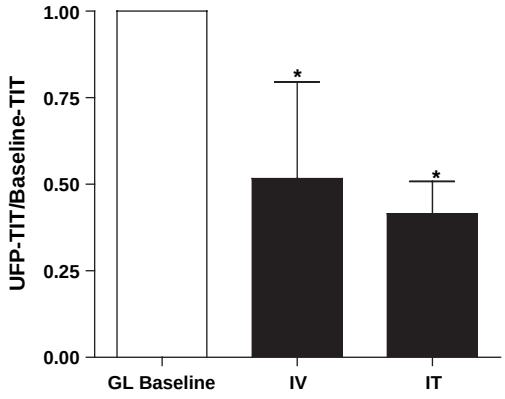

FIG. 4. Effects of iv-administered and IT-instilled UF aminated-polystyrene particles (0.5 mg/kg) on ear vein thrombosis without rose bengal. Data are expressed as mean ± S.D. with $n = 3$. *Significantly different from baseline, $p < 0.001$.

data by Nemmar *et al.* (2002) and validate the use of the ear vein model to study UFP-induced thrombogenesis.

To simplify this model further, the effect of animated ultrafine PSP on coagulation was studied without RB. Because of the absence of RB in this system, UFP-TIT was slightly longer than with RB. As depicted in Figure 4, iv administration of 0.5 mg/kg aminated PSP led to a significantly shorter (50% reduction) UFP-TIT than baseline-TIT, similar to the results obtained in the presence of RB. Likewise, IT instillation of 0.5 mg/kg aminated PSP resulted in a UFP-TIT 42% shorter than baseline-TIT ($p < 0.001$).

DISCUSSION

Recent epidemiological studies associate people with diabetes, congestive heart failure, and myocardial infarction with a greater risk of adverse events when exposed to airborne particles (Bateson and Schwartz, 2004; Peters *et al.*, 2001; Zanobetti and Schwartz, 2001, 2002). The literature indicates that susceptibility of these individuals might be related to the fact that these diseases have dysfunctional endothelia as a common denominator (Becker et al., 2001; Verma *et al.*, 2004). This condition will predispose the vessel wall to vasoconstriction, increased leukocyte adherence, platelet activation, vascular activation, and thrombosis.

In vivo experimental models of thrombosis are essential for understanding the mechanisms involved in thrombogenesis. Most of the available models require extensive surgical procedures that may inadvertently cause activation of the coagulation system in the vessel of interest, thus affecting data interpretation. Therefore, the aim of this study was to develop an alternative model that is less invasive and that is independent of RB, and to test its utility in the evaluation of previously reported thrombogenic effects of UFP. We took advantage of a recently published injury model in which damage was

induced photochemically by illuminating the ear of C57BL6 mice with a green laser after injection with RB (Rosen *et al.*, 2001). In this model as well as ours, it was possible to focus the laser beam through the objective of an inverted microscope to create the injury on specific sections of the ear vessels.

The architecture of the ear vasculature varies among rats. However, there are generally one or two primary veins proximal to the head with a diameter of approximately 250 to 350 μm. This primary vein is fed by secondary veins of 60 to 200 μm, which in turn are fed by tertiary vessels of 60 to 90 μm. The smaller secondary vessels (70–90 μm) were used to induce injury in this investigation to maintain anatomically similar vascular parameters between experiments. The use of microscopy allowed monitoring of thrombogenesis by trans-illumination in real time (Gavins and Chatterjee, 2004; Rosen *et al.*, 2001; Stockmans *et al.*, 1991).

The ear vein technique has several advantages. First, the target vessel is easy to identify and does not have to be isolated via invasive procedures. For instance, our approach only requires that the ear of an anesthetized rat rests flat on a glass slide to be imaged by a digital camera, thus avoiding unwanted manipulation of the vessel. A drop of saline or distilled water is enough to keep the ear flat for long periods of time. According to Rosen *et al.*, 2001, the ear can also be taped to the slide for easy imaging. However, this may potentially induce some pressure on ear vessels, and we did not find this to be necessary. It is also important to mention that because of the noninvasive nature of this technique, there is the additional benefit of better animal care. In addition, because the injuries generated are small, multiple injuries can be induced in both ears of the same animal. We also found that baseline-TIT did not change in vessels of both ears of the same rat, even 2 to 3 days after the first determination. This will result in a reduced number of animals used per experiment. Because fluctuations in RB plasma concentration affect the severity of the endothelial response (Saniabadi *et al*., 1995), another advantage of our model is that RB iv infusion produces a steady-state plasma level of the photosensitive dye within 30 min. Our data show that this steady-state concentration of RB (60 μg/ml) produces a constant and reproducible vascular response within the same animal even 60 min after the start of the iv infusion.

To validate our model for its use in the study of UFP-induced thrombosis, we used surface-modified polystyrene particles (PSP) 60 nm in diameter. Although not environmentally relevant, PSP offer the advantage of being well characterized in size, are not cytotoxic (Nemmar *et al.*, 2002), and may represent a model for engineered nanoparticles. The thrombogenic potential of these particles has previously been documented in a peripheral model of experimental thrombosis in hamsters (Nemmar *et al.*, 2002). In these studies, iv-administered positively charged PSP enhanced thrombus formation by 219% (50 μg/kg) and 307% (500 μg/kg) when compared to saline controls, whereas negatively charged particles at similar doses did not affect thrombus formation. Our results confirm the data of Nemmar *et al.*, because iv administration of different doses of aminated PSP induced dose-dependent significant reductions in RB-TIT, indicating accelerated development of thrombosis in the presence of these UFP. It appears, though, that the dose-dependent thrombogenic effect of positively charged PSP is limited to a certain dose range. At a very high dose of 50 mg/kg this effect was weaker, although still significant (Fig. 3). A likely explanation is that at this high dose, administrated in a small volume of 250 μl, PSP agglomerate quickly to form larger clusters which may not be as effective in inducing thrombi. Indeed, Nemmar *et al*. (2003a) have reported that larger 400-nm particles were not thrombogenic in their model.

Thus, our results using the noninvasive ear vein model and those of Nemmar *et al*. (2002; 2003a) using the femoral vein model agree quite well with each other with respect to the thrombus-inducing potential of positively charged PSP, as well as with respect to lack of such potential for negatively charged PSP. This agreement between our results and those of Nemmar *et al*. (2002) demonstrates the usefulness of our noninvasive model of *in vivo* thrombus formation.

However, quantification of thrombus formation as we assessed it can still be improved in the ear vein model. As described earlier under *Methods*, thrombus formation is determined as a fraction of RB-TIT based on an immediate response after green laser illumination; *i.e.*, laser illumination time is changed. In contrast, Nemmar and colleagues measured thrombus development for a longer period (40 min) without changing laser illumination time, and they used computer software to quantify the intensity of the injury. Our laboratory is working on the development of a procedure to quantify the damage in our ear model using a similar computerized image-processing system and keeping the laser illumination time constant.

In an effort to further simplify our model and reduce the number of variables in each experiment, we explored the effect of UFP on thrombus formation after direct laser injury without the use of RB. We observed that illumination time in this system was slightly higher than with the RB model. With this model, rats then received 0.5 mg/kg aminated-PSP iv, and thrombus formation was assessed. It was found that thrombogenesis in the presence of UFP was significantly increased (51% reduction in TIT) after administration of these positively charged PSP (Fig. 4). This response was comparable to the earlier studies in our RB ear model in which aminated-PSP reduced illumination time to 33% of baseline-TIT. Together, these data indicate that the thrombogenic effect of these particles is independent of RB. With respect to the mechanism of thrombus induction by GL alone, Rosen *et al*. (2001) showed that direct green laser illumination without RB induces significant endothelial injury (highly vacuolated cells) without denuding the vessel, leading to adhesion of numerous platelets and polymorphonuclear leukocytes (PMNs). Our data showing that IT instillation of aminated PSP induces a significant reduction in TIT in much the same way that iv injected PSPs

do support the hypothesis that ultrafine particles translocate out of the lung and can directly affect thrombus formation. The time course of response (*i.e.*, thrombus induction very soon after IT administration) adds further support to this hypothesis. If a lag in thrombus induction after IT administration were observed, this would suggest that inflammatory mediators are required for the effect instead of direct contact of the particles with endothelial cells, platelets, and/or circulating inflammatory cells. This has also been suggested by Nemmar *et al.* (2004). We conclude that this simplified version of the rat ear vein model, without the use of RB, has a number of advantages and is a valid alternative to the classical femoral vein model.

Further studies are needed to determine the mechanism of UFP-induced thrombogenesis. Interestingly, an article by Berry *et al*. (1977) presented the first evidence that UFP can indeed translocate from the lung into the bloodstream and accumulate inside blood platelets. In their studies, IT-administered ultrafine (30 nm) colloidal gold particles were identified inside blood platelets in alveolar capillaries of Wistar rats as soon as 30 min after instillation, indicating that platelets may be important target cells for UFP translocating across the alveolar-capillary barrier. The importance of platelets in thrombogenesis induction by airborne ambient UFP has further been demonstrated by showing that platelet activation time was significantly shortened after adding diesel exhaust particles to untreated hamster blood (Nemmar *et al.*, 2002). Thus, we hypothesize that once UFP reach platelets they can induce platelet activation, resulting in their aggregation after interaction with injured or activated endothelial cells. Ultrafine particles could, for example, induce expression of CD40 ligand (CD40L), a transmembrane protein expressed in activated platelets and endothelial cells that has been associated with increased risk of cardiovascular events (Vishnevetsky *et al.*, 2004).

Thus, the objective of our future studies will be to determine the contribution of inhaled environmental UFP in thrombus induction in the noninvasive ear vein model.

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