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ORIGINAL ARTICLE

Platelet adhesion and fibrinogen deposition in murine microvessels upon inhalation of nanosized carbon particles

A. KHANDOGA,*†[1] T. STOEGER,‡[1] A. G. KHANDOGA,* P. BIHARI,* E. KARG,‡ D. ETTEHADIEH,‡ S. LAKATOS,§ J. FENT,§ H. SCHULZ‡[2] and F. KROMBACH*[2]

**Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians-Universität München, München; †Department of Surgery-Großhadern, Ludwig-Maximilians-Universität München, München; ‡Comprehensive Pneumology Center (CPC), Institute of Lung Biology and Disease, Helmholtz Zentrum München, Neuherberg, Germany; and §Military Health Center, Hungarian Defence Forces, Budapest, Hungary*

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Summary. *Background*: The translocation of nanoparticles in the lung toward effector organs via the circulation is considered an important direct pathway for systemic effects of nanoparticles after inhalation. Recently, we have reported that a moderate dose of systemically administered nanosized carbon black particles exerted thrombogenic effects in hepatic microvessels of healthy mice. *Objectives*: This study addresses the questions of whether similar thrombogenic effects are also evoked upon inhalation of nanosized carbon particles (NCP) and whether NCP-induced hepatic platelet accumulation is associated with pulmonary or systemic inflammation. *Methods*: Two and 8 h after a 24-h exposure to either filtered air or to NCP, intravital fluorescence microscopy of the hepatic microcirculation was performed in C57Bl/6 mice. Parameters of pulmonary or systemic inflammatory response were determined in bronchoalveolar lavage and blood/plasma samples. *Results*: Inhalative exposure to NCP caused platelet accumulation in the hepatic microvasculature, whereas leukocyte recruitment and sinusoidal perfusion did not differ from controls. Fibrinogen deposition was detected by immunohistochemistry in both hepatic and cardiac microvessels from NCP-exposed mice. In contrast, inhalation of NCP affected neither the plasma levels of proinflammatory cytokines nor blood cell counts. Moreover, the bronchoalveolar lavage data indicate that no significant inflammatory response occurred in the lung. *Conclusions*: Thus, exposure to NCP exerts thrombogenic effects in the microcirculation of healthy mice independent of the route of administration (i.e. inhalation or systemic intra-arterial administration). The NCP-induced thrombogenic effects are not liver specific, are associated with neither a local nor a systemic inflammatory response, and seem to be independent of pulmonary inflammation.

Keywords: hepatic microcirculation, nanoparticles, platelets, pulmonary inflammation, thrombogenic effects, ultrafine particles.

Correspondence: Andrej Khandoga, Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians-Universität München, Marchioninistr. 27, D-81366 Munich, Germany.
Tel.: +49 89 2180 75538; fax: +49 89 2180 76532.
E-mail: Andrej.Khandoga@med.uni-muenchen.de

[1]Both authors contributed equally to this work.
[2]These authors share joint last authorship.

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Introduction

Epidemiological studies have shown that exposure to ambient ultrafine particles (diameter ≤ 0.1 μm) is associated with increased cardiovascular morbidity and deaths from myocardial ischemia [1], arrhythmia [2], ischemic stroke [3], and increased risk of deep vein thrombosis [4].

Although the mechanisms behind these consistent observations have not yet been determined, two hypotheses are discussed in the recent literature [5]. The first hypothesis proposes that inhaled particles provoke an inflammatory response in the lungs with subsequent release of prothrombotic and proinflammatory cytokines into the circulation. The second hypothesis suggests that inhaled insoluble nanoparticles rapidly translocate into the circulation and migrate to other organs including the liver, the spleen, the kidneys, the heart and the brain, where they may be deposited with the potential for direct effects on hemostasis and (cardio)vascular integrity. Once in the circulation, nanoparticles can potentially interact with vascular endothelial cells as well as with blood cells. These interactions might result in prothrombotic but also proinflammatory effects.

Recently, we have reported that a moderate dose of systemically administered nanosized carbon black particles (10^7 particles, 60% $<$ 100 nm, in buffer + 0.15% HSA as bolus over 5 min) exerted thrombogenic effects in healthy mice, that is, gpIIb/IIIa-dependent platelet adhesion and fibrinogen deposition on the endothelium of hepatic microvessels, whereas endothelial P-selectin expression or leukocyte-endothelial cell interactions were not induced [6].

In the present study, we addressed the following questions: (i) are similar thrombogenic effects also evoked upon inhalation of nanosized carbon particles and, if yes, (ii) is the platelet accumulation in the liver induced by inhalation of nanoparticles associated with pulmonary or systemic inflammation?

Methods

Animals

Female C57BL6 mice (Charles River, Sulzfeld, Germany) ranging in age from 5 to 7 weeks were used for this study. All experiments were carried out according to the German legislation on protection of animals and approved by the Bavarian Animal Research Authority.

Exposure to nanosized carbon particles (NCP)

Mice were exposed to NCP via inhalation. For inhalation, a NCP aerosol was generated by spark discharge with a commercially available but modified generator (Palas, GFG1000 [7]). These NCP are soot-like agglomerates composed of spherical primary particles (7–12 nm in diameter); they consist of > 96% elemental carbon and have a BET surface area of 800 $m^2\ g^{-1}$ (the BET method assesses the surface areas of solids by physical adsorption of gas molecules). Mice were exposed to either filtered air or NCP for 24 h using the whole body exposure chamber described by Andre *et al*. [8]. The exposure was performed at an average number and mass concentration of 440 $\mu g\ m^{-3}$ (= 0.35 mm^2(BET) cm^{-3}) and $6.4 \times 10^6\ cm^{-3}$ as determined by gravimetric analysis and a condensation nucleus counter (TSI 3022, operated in the single count mode), respectively. The count median (mobility) diameter and geometric standard deviation were 48 nm and 1.59, respectively (scanning mobility particle sizer, TSI 3071), which implies that the nanosized particle segment (diameter < 100 nm) accounts for about 80% of the total particle mass (inferred from the density-weighted number size distribution using the size-dependent density of soot-like agglomerates ($\sim d^{-1}$).

The inhaled particle number concentration was adjusted so that the estimated dose of translocated particles resembles those previously infused intra-arterially [6], namely 5×10^7. Briefly, based on experimental particle deposition and translocation data [9,10] the alveolar mass burden of the nanosized carbon particles at the end of the 24-h exposure amounts to 5 μg. Considering a translocated fraction of 0.2% NCP, the number of particles translocated into the vascular compartment is estimated to be 5×10^7 particles.

Experimental groups

Set I: microvascular effects upon NCP inhalation

The following groups were investigated (n = 6 each): two groups undergoing inhalation of NCP for 24 h followed by either 2 or 8 h post-exposure time and two groups undergoing inhalation of filtered air for 24 h followed by either 2 or 8 h post-exposure time.

Set II: systemic and pulmonary inflammation upon NCP inhalation

For this purpose, bronchoalveolar lavage (BAL) data, hematological parameters and levels of serum cytokines were obtained at 2 h after exposure to either filtered air or NCP (n = 8 each group).

Set I: microvascular effects upon NCP inhalation

Hepatic microcirculation The hepatic microcirculation was analyzed by *in vivo* fluorescence microscopy at either 2 or 8 h after exposure to NCP using a Leitz-Orthoplan microscope with a 100 W HBO mercury lamp (Leitz, Wetzlar, Germany) as described previously [6,11]. Shortly, under inhalation anesthesia with isoflurane-N_2O, a polyethylene catheter was inserted into the left carotid artery for measurement of mean arterial pressure and application of fluorescence dyes. The mean arterial pressure was continuously controlled in each experiment.

Platelets were isolated from syngeneic mice, labeled *ex vivo* with rhodamine 6G and infused intra-arterially as described previously [12]. Platelet-endothelial cell interactions were quantitatively analyzed in hepatic sinusoids, terminal arterioles and postsinusoidal venules. Rolling platelets were defined as cells crossing an imaginary perpendicular line through the vessel at a velocity markedly lower than the centerline velocity in the microvessel. Their numbers are given as cells per second per vessel cross-section. Platelets firmly attached to the endothelium for more than 20 s were counted as permanently adherent cells and expressed as number of cells per square millimeter endothelial surface. In sinusoids, the number of accumulated platelets was counted in the scanned acini and is given in [1/acinus].

Leukocytes were labeled by an intravenous application of rhodamine 6G (0.1 mL, 0.05%; Sigma-Aldrich, Deisenhofen, Germany) and visualized in postsinusoidal venules using a N2 filter block (excitation, 530–560 nm; emission, > 580 nm; Leitz).

In an attempt to evaluate the severity of perfusion injury, sinusoidal perfusion was analyzed within five to seven acini after intravenous application of FITC-labeled dextran (MW 150 000; 0.1 mL, 5%; Sigma-Aldrich) using an I2/3 filter block (excitation, 450–490 nm; emission, > 515 nm; Leitz). In each visualized acinus, the total number of sinusoids as well as the number of non-perfused sinusoids within the same acinus was counted. The results are presented as percentage of perfused sinusoids within an acinus. *In vivo* microscopic images were transferred to a video system (S-VHS Panasonic AG 7330; Matsushita Electric Ind., Tokyo, Japan) using a CCD video camera (FK 6990, Cohu; Prospective measurements, San Diego, CA, USA). All videotaped images were evaluated

off-line using a computer-assisted image analysis program (CAPIMAGE®; Dr Zeintl, Heidelberg, Germany).

Immunostaining for fibrin(ogen) Samples of liver and heart tissue were taken at the end of intravital microscopy. Paraffin sections (6 μm) were quenched with 0.5% H_2O_2 methanol solution to block production of endogenous peroxidase, incubated in 1.5% goat serum to block non-specific binding, and later incubated with primary antibodies as described previously [6]. Rabbit anti-mouse fibrinogen monoclonal antibodies (Becton Dickinson GmbH, Heidelberg, Germany) and a commercially available immunohistochemistry kit (Vectastain; Camon, Wiesbaden, Germany) were used. Control sections were incubated with an isotype-matched primary antibody. An easily detectable reddish-brown end product was obtained by development in H_2O_2/3-amino-9-ethylcarbazol. The sections were counterstained with Mayer's hemalaun. In each experimental group, six sections from six individual animals (10 observation fields per section) were examined by light microscopy (magnification × 400 and × 200).

Set II: systemic and pulmonary inflammation upon NCP inhalation

Bronchoalveolar lavage (BAL) In a separate set of experiments, we addressed the question of whether inhalation of NCP induces a pulmonary or systemic inflammatory response. BAL was performed by cannulating the trachea and infusing the lungs 10 times with 1.0 mL of PBS without Ca^{2+} and Mg^{2+}. The BAL fluids from lavages one and two, as well as those from lavages three to ten were pooled and centrifuged (4256 ×*g* for 20 min at room temperature). The cell-free supernatant from lavages one and two was used for biochemical measurements. For each animal, the 10 cell pellets were unified and resuspended in 1 mL of RPMI 1640 medium (BioChrome, Berlin, Germany) supplemented with 10% fetal calf serum (Seromed, Berlin, Germany), and the number of living cells was determined by the trypan blue exclusion method. Cell differentials were performed on cytocentrifuge preparations (May–Gruenwald–Giemsa staining). Polymorphonuclear leukocytes were counted as a parameter of pulmonary inflammation [8].

Hematology Blood withdrawal from the retroorbital sinus was collected in Microvette EDTA blood collection tubes (Sarstedt, Nuembrecht, Germany) and analysis was conducted on the Advia 120 Hematology System (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Multiplex detection of protein markers in plasma To quantify cytokine levels in plasma samples, multiplex suspension protein arrays were performed using the Luminex 100 device (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's instructions. The following panel of mouse cytokines (BioPlex Mouse Cytokine Array; Bio-Rad Laboratories) was selected: IL-1 alpha, IL-1 beta, IL-2, IL-6, IL-10, CXCL1, CXCL2. Sensitivity of the cytokine kits varied between 2 and 10 pg mL^{-1}.

Effect of NCP on platelet activation in vitro Human blood was collected from the cubital vein of healthy volunteers. All samples were obtained with the approval of the local ethical committee after the donor had given informed consent. NCB stock solution was prepared at a concentration of 0.23 mg mL^{-1} in distilled water using sonication with 4.2×10^5 kJ m^{-3} specific energy. Thereafter, human serum albumin was given to the dispersion (final concentration 1.5 mg mL^{-1}) before addition of PBS solution. The final concentration of nanoparticles in the dispersion was 0.2 mg mL^{-1}. Vehicle was prepared in the same way, but instead of nanoparticle dispersion the same volume of distilled water was added to PBS and albumin solution.

Samples of citrated blood (100 μL) were incubated with either NCP dispersion (100 μL, 0.2 mg mL^{-1}) or vehicle for 10 min at room temperature ($n = 26$ each group). As a positive control, samples were incubated with 1 μM ADP. Expression of P-selectin (CD62P) on platelets, a marker of platelet activation, was determined using flow cytometry (FACScan; Becton Dickinson GmbH) as described [13]. Whole blood was incubated with PE-labeled anti-CD62P antibodies (Dako, Glostrup, Denmark) and FITC-labeled anti-CD41 antibodies (Immunotech, Marseilles, France). The CD41 platelet marker was used as a trigger signal for data collection, the platelets were gated on the FS-SS dot plot and the mean fluorescence intensities (MFI) of CD62P were measured for CD41-positive events. To exclude non-specific binding, appropriate isotype-matched control antibodies were used. To minimize the spontaneous activation of platelets, no washing steps were used.

Statistics Data analysis was performed with a statistical software package (SIGMASTAT; Jandel Scientific, Erkrath, Germany). The Mann–Whitney test was used for two-group comparison. Mean values ± SEM are given. P values < 0.05 were considered significant.

Results

Hepatic microcirculation

As detected by *in vivo* microscopy, only a few adherent platelets were observed in hepatic microvessels of mice undergoing exposure to filtered air. In contrast, inhalation of NCP caused a markedly enhanced platelet adherence in arterioles and venules as well as platelet accumulation in sinusoids (Fig. 1). This increase was significant at 2 h after exposure to NCP in arterioles, at 8 h in venules, and at both postexposure time points in sinusoids. Interestingly, platelet adherence in arterioles and venules was not preceded by platelet rolling. Rolling platelets were almost absent in all experimental groups (data not shown).

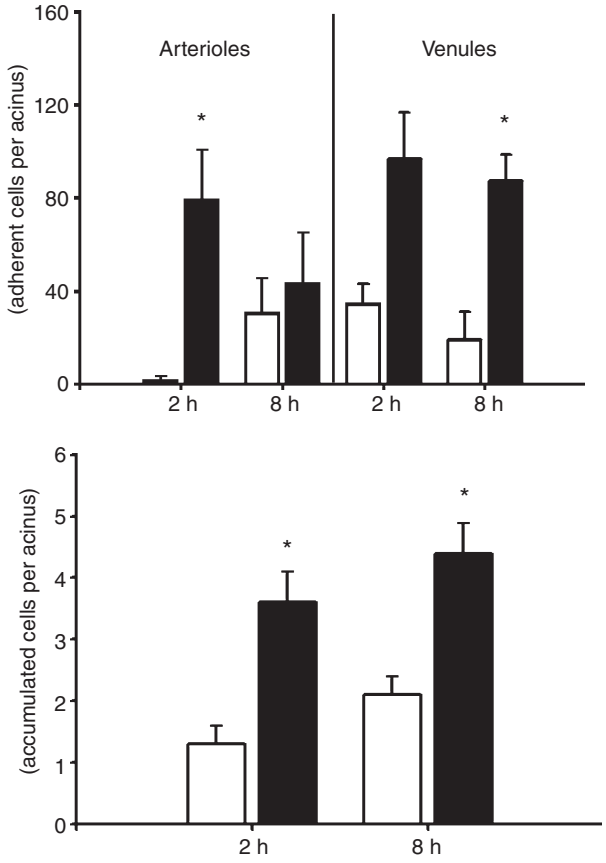

Fig. 1. Platelet-endothelial cell interactions. Numbers of platelets adherent in terminal arterioles and postsinusoidal venules as well as number of platelets intravascularly accumulated in sinusoids were quantitatively analyzed using *in vivo* video fluorescence microscopy in mice exposed for 24 h to either filtered air (open bars) or nanosized carbon particles (closed bars). Post-exposure time was 2 and 8 h. n = six animals per group, mean ± SEM, $*P < 0.05$ vs. control (filtered air).

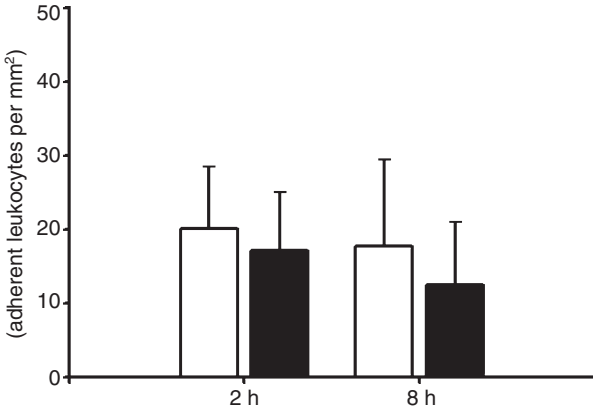

Fig. 2. Leukocyte-endothelial cell interactions. Numbers of leukocytes adherent in postsinusoidal venules were quantitatively analyzed using *in vivo* video fluorescence microscopy in mice exposed for 24 h to either filtered air (open bars) or nanosized carbon particles (closed bars). Post-exposure time was 2 and 8 h. n = six animals per group, mean ± SEM.

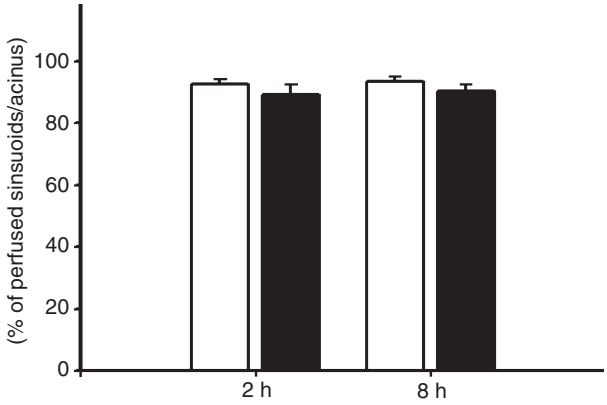

Fig. 3. Sinusoidal perfusion. Sinusoidal perfusion was analyzed as a parameter of microvascular hepatic injury using *in vivo* video fluorescence microscopy in mice exposed for 24 h to either filtered air (open bars) or nanosized carbon particles (closed bars). Post-exposure time was 2 and 8 h. n = six animals per group, mean ± SEM.

In an attempt to characterize the initial inflammatory response in the liver, leukocyte-endothelial cell interactions were analyzed in hepatic postsinusoidal venules. As shown in Fig. 2, the numbers of adherent leukocytes did not differ between animals undergoing inhalation of NCP or filtered air. Moreover, hardly any rolling leukocytes were registered in all experimental groups (not shown).

Sinusoidal perfusion was determined as a measure of microvascular hepatic injury. In mice inhaling filtered air, about 90% of all sinusoids were perfused. After NCP inhalation, sinusoidal perfusion was 89 ± 1% after 2 h and 90 ± 1% after 8 h. These changes did not reach the level of significance as compared with the controls (Fig. 3).

Fibrin(ogen) deposition

As demonstrated in our previous studies, platelet accumulation requires deposition of fibrin (or fibrinogen) in hepatic microvessels, most probably serving as a bridging molecule between gpIIb/IIIa molecules on platelets and CD54 on endothelial cells [11]. Thus, we performed staining for fibrin(ogen) in paraffin-fixed tissue sections. In line with the data on platelet recruitment, fibrin deposition was strongly detected at 2 and 8 h after exposure to NCP. In contrast, no staining was observed in both control groups (Fig. 4). Similarly, staining for fibrin(ogen) was registered in the hearts of mice exposed to NCP. This result suggests that the NCP-induced thrombogenic effects are not liver specific and might occur also in other organs.

BAL

In the next set of experiments, we addressed the question of whether prothrombotic effects observed after NCP inhalation are associated with pulmonary and/or systemic inflammation. Bronchoalveolar lavage analysis revealed a significant increase in total BAL protein concentration at 2 h after exposure, indicating a moderate alveolar-vascular barrier leakage. Total

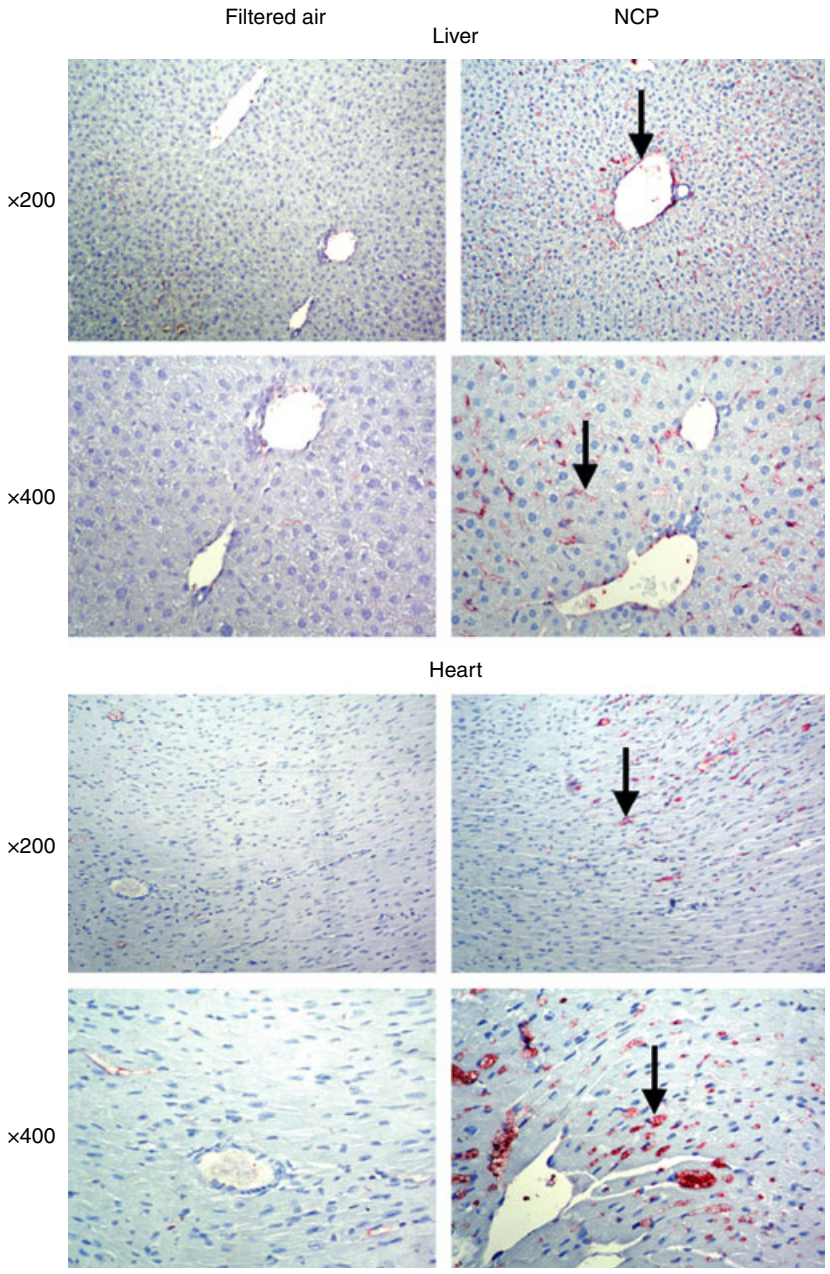

Fig. 4. Deposition of fibrin(ogen) in the hepatic microvasculature. Immunostaining for fibrin(ogen) was performed in paraffin-fixed sections of the liver and the heart of mice exposed for 24 h to either filtered air (open bars) or nanosized carbon particles. Arrows depict endothelial deposition of fibrin(ogen). Post-exposure time was 2 h. Magnification 200 × and 400 ×.

lavage cell numbers were all unchanged and neutrophil granulocyte counts also did not indicate any cellular inflammatory response in the lungs (Table 1).

Blood cytokines

To assess a systemic response the levels of seven relevant proinflammatory cytokines (IL-1alpha, IL-1beta, IL-2, IL-6, IL-10, CXCL1, and CXCL2) were measured in the serum of animals exposed to either NCP or filtered air for 24 h. The measurements were performed in blood taken 2 h after exposure. As shown in Table 2, the cytokine levels were comparable in mice exposed to NCP or filtered air, suggesting that inhalation of moderate doses of NCP does not induce systemic inflammation in C57BL6 mice.

Hematology

The hematological analysis did not give any evidence for exposure-related systemic inflammatory effects: white blood cell counts (neutrophils, lymphocytes, monocytes, basophils and eosinophils) did not differ between NCP- or filtered

Table 1 Impact of nanosized carbon particles (NCP) inhalation on bronchoalveolar lavage (BAL) parameters

	Weight (g)	Macrophages (10^6)	Lymphocytes (10^3)	PMN (10^3)	Total BAL cells (10^6)	BAL protein ($\mu g\ mL^{-1}$)
Filtered air	18.1 ± 0.3	0.4 ± 0.1	5.2 ± 1.3	1.8 ± 0.4	0.4 ± 0.1	145 ± 19
NCP	18.9 ± 0.2	0.2 ± 0.1	5.5 ± 1.1	2.0 ± 0.5	0.3 ± 0.1	205 ± 12.0*

Parameters of pulmonary inflammation were measured in BAL of mice exposed for 24 h to either NCP or filtered air. Post-exposure time was 2 h. n = eight animals per group, MW ± SEM. *$P < 0.05$ vs. control (filtered air).

Table 2 Impact of nanosized carbon particles (NCP) inhalation on cytokine levels in serum

	IL-1α	IL-1ß	IL-2	IL-6	IL-10	CXCL1	CXCL2
Filtered air	10 ± 1	30 ± 6	12 ± 3	22 ± 3	59 ± 23	27 ± 4	15 ± 3
NCP	8 ± 1	46 ± 3	18 ± 1	25 ± 2	31 ± 4	26 ± 1	10 ± 1

Cytokine levels [$pg\ mL^{-1}$] were measured in serum of mice exposed for 24 h to either NCP or filtered air. Post-exposure time was 2 h. n = eight animals per group, MW ± SEM.

air-exposed mice (Table 3). However, even though total platelet numbers were unchanged, some parameters for platelet morphology were significantly changed upon exposure to NCP. The number of large platelets was increased and accordingly also the platelet component distribution width (PCDW) was slightly elevated upon exposure to NCP ($P = 0.01$). Both parameters indirectly characterize a shape change of platelets and have been reported to be useful for screening of platelet activation.

Effect of NCP on platelet activation in vitro

In a separate set of experiments, we assessed whether a co-incubation of NCP with human blood can trigger platelet activation *in vitro*. Expression of platelet P-selectin was measured by flow cytometry to analyze direct effects of NCP on platelet activation. As shown in Fig. 5, the presence of nanoparticles in the human whole blood induced a significant increase in CD62P expression as compared with the control group. The extent of platelet activation by NCP was comparable with that after platelet incubation with ADP.

Discussion

Inhalation of NCP is associated with an increased morbidity and mortality from cardiovascular diseases [14]. The mechanisms underlying the cardiovascular effects of particulate air pollution are not fully elucidated. Several potential pathways are suggested. (i) Nanoparticles are able to translocate from lungs into the systemic circulation, accumulate in peripheral organs, and interact with endothelial cells as well as blood cells. Moreover, translocated particles trigger a systemic inflammatory reaction that is associated with a cytokine burst and an enhanced procoagulatory state. (ii) Inhaled particles accumulate in the lung and induce local inflammation, leading to a systemic release of inflammatory mediators, independent of whether or not they migrate into the circulation.

It is well known that nanosized particles migrating into the circulation accumulate predominantly in the liver. In a previous study, we analyzed the effects of intra-arterial infusion of moderate doses of NCP as a surrogate for translocated particles in the hepatic microcirculation. We have demonstrated that systemically administered NCP induced gpIIb/IIIa-dependent intravascular platelet accumulation, fibrin deposition and vWF upregulation in healthy mice at 2 h after infusion. An intriguing finding was that these prothrombotic effects were associated with neither local inflammation (no leukocyte activation, no CD62P expression) nor tissue injury (no perfusion disturbances, no apoptotic cells) or Kupffer cell activation [6]. In the present study, we addressed the question of whether these thrombogenic effects also occur after inhalation of moderate doses of NCP. Similar to the data on infusion of NCP, an increased platelet adherence was observed in hepatic microvessels at 2 h after inhalation of NCP. Interestingly, the platelet attachment detected in all segments of the hepatic microcirculation did not require a rolling phase (as usually found in inflammatory settings). The same phenomenon was observed after intra-aortal infusion of NCP in our previous study, suggesting that the mechanism of platelet activation is the same in both settings. In addition, platelet accumulation was associated with fibrin deposition in hepatic microvessels.

The link between exposure to nanoparticles and thrombosis was shown in studies by Nemmar *et al*., who described effects on thrombus formation in a hamster model after intravenous and intratracheal administration of unmodified polystyrene, carboxylate-polystyrene, or amine-polystyrene particles. While unmodified particles had no effect on thrombosis and carboxylate-polystyrene particles attenuated thrombus formation, positively charged amine-polystyrene particles rapidly (1 h) induced platelet aggregation *in vitro*, strongly increased ADP-triggered aggregation in a dose-dependent manner, and enhanced thrombus generation [15,16]. These findings could be reproduced after intratracheal instillation of diesel exhaust particles, which induced a progressive shortening of closure

Table 3 Impact of nanosized carbon particles (NCP) inhalation on hematological parameters

	RBC ($\times 10^6$ cells μL^{-1})	HCT (%)	WBC Counts ($\times 10^3$ cells μL^{-1})	NEU (%)	LYM (%)	MON (%)	BAS (%)	EOS (%)	Platelets Counts ($\times 10^3$ cells μL^{-1})	PCDW (pg)	Large platelets ($\times 10^3$ cells μL^{-1})
Filtered air	9.7 ± 0.2	53.6 ± 1.3	3.3 ± 0.5	8.7 ± 0.7	85.3 ± 1.9	1.6 ± 1.1	0.3 ± 0.1	2.1 ± 0.6	864.0 ± 58.3	6.7 ± 0.1	6.4 ± 0.6
NCP	9.9 ± 0.2	53.0 ± 1.5	3.2 ± 0.6	7.2 ± 0.6*	89.9 ± 0.8	0.4 ± 0.1	0.3 ± 0.1	1.6 ± 0.3	1014.2 ± 26.2 *	7.2 ± 0.1*	12.8 ± 0.7*

Hematological parameters were measured in mice exposed for 24 h to either NCP or filtered air. Post-exposure time was 2 h. n = eight animals per group, MW ± SEM. RBC, red blood cells; HCT, hematocrit; WBC, white blood cells; NEU, neutrophils; LYM, lymphocytes; MON, monocytes; BAS, basophils; EOS, eosinophils; PCDW, platelet component distribution width. *$P < 0.05$ vs. control.

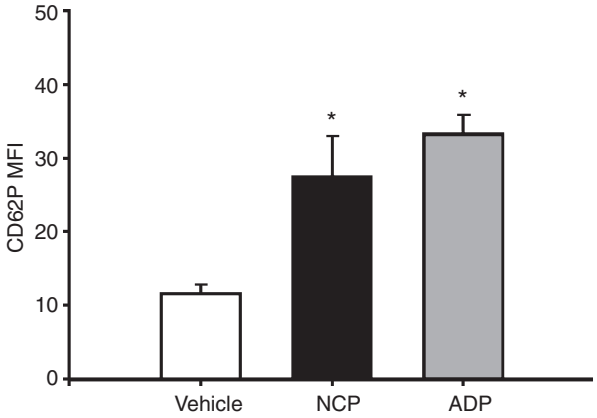

Fig. 5. CD62P expression on platelets. Samples of human blood were incubated either with PBS (vehicle), nanosized carbon particles (0.2 mg mL^{-1}) or with ADP (1 μM) for 10 min at room temperature. Mean CD62P fluorescence intensities (MFI) of CD41-positive events are plotted (*$P < 0.05$ vs. vehicle).

time and an increase in the cumulative mass of thrombus formed *in vivo* in photochemically injured vessels, both indicating platelet activation [17]. In line with these data, we have shown in our previous study that amine-modified polystyrene nanoparticles injected into the systemic circulation reduced and carboxyl-modified polystyrene nanoparticles increased the time until complete vessel occlusion in small mesenteric arteries occurred. Furthermore, amine-modified polystyrene nanoparticles activated platelets as measured by increased expression of platelet P-selectin and elevated numbers of platelet–granulocyte complexes in whole blood [13].

Interestingly, shortening of closure time was also observed after adding diesel exhaust particles to untreated hamster blood, suggesting the possibility of a direct activation of platelets by ultrafine particles [18]. This aspect was supported by another study, which demonstrated that diesel exhaust particles lead to a significant thrombotic tendency as early as 1 h after intratracheal instillation, and that this tendency persisted for 24 h [17]. More recently, Mutlu *et al.* showed that exposure to NCP triggers IL-6 production by alveolar macrophages, resulting in reduced clotting times, intravascular thrombin formation, and accelerated carotid artery thrombosis [19]. Interestingly, also quantum dots were able to cause pulmonary vascular thrombosis, most likely by activating the coagulation cascade via contact activation [20]. Here, we show *in vitro* that nanoparticles are able to activate platelets directly in whole blood and that the extent of such activation is comparable with that after platelet treatment with ADP. This finding corroborates our data demonstrating that inhaled nanoparticles induce platelet accumulation in extrapulmonary organs (liver, heart) without endothelial injury.

Because it has recently been shown that upon inhalation NCP are rapidly distributed throughout the body [10], we addressed the question of whether NCPs trigger prothrombotic effects in the liver only or also in other organs. Hence, staining for fibrin(ogen) was performed also in the heart. Fibrin(ogen)

deposition was clearly registered also in myocardial tissue, demonstrating that the effects of inhaled NCP are not liver specific. In line with the data on NCP infusion, inhalation of NCP did not induce any significant inflammatory reaction in the liver, because leukocyte rolling and adhesion as well as sinusoidal perfusion remained on the same level as observed in control animals. Taken together, both systemic infusion [6] and inhalation of moderate doses of NCP induce platelet recruitment and fibrin(ogen) deposition in the liver without inducing local inflammation. NCP-induced prothrombotic changes are not liver specific and observed also in the heart.

In the next part of our study, we focused on the question of whether the NCP-induced microvascular effects are associated with a systemic and/or pulmonary inflammatory response. There is a large body of evidence in the literature that nanosized particles can cause local (pulmonary) and/or systemic inflammation in animal models upon intrapulmonary instillation or inhalation at high dosage [21,22]. In clinical studies, evidence of pulmonary inflammation has been demonstrated after inhalation of both concentrated ambient particular matters [23] and dilute diesel exhaust [24]. Such exposures led to elevated plasma concentrations of cytokines [25], release of bone-marrow-derived neutrophils and monocytes into the circulation [26], evidence of an acute phase response [27], and altered leukocyte expression of adhesion molecules [28]. In the present study, we clearly show that inhalation of moderate doses of NCP does not cause systemic inflammation in C57BL6 mice, at least during the first 2 h after exposure, because no increase in the levels of proinflammatory cytokines was detected in the serum of animals exposed to NCP. Furthermore, the BAL data, except the slightly increased epithelial/endothelial leakage, clearly indicate that no significant inflammatory response occurred in the lungs of C57BL6 mice after inhalation of NCP.

Blood cell counts did not reveal any indices of an inflammatory reaction. However, the effect of NCP exposure on platelet morphology, that is, increased size variation, might be interpreted as an indirect sign of platelet activation.

The lack of pulmonary inflammation after the NCP dose applied is in agreement with earlier studies, where the even more susceptible (with respect to pulmonary inflammation [29]) mouse strain BALB/cJ, showed at similar exposure conditions only mild inflammatory responses in the lungs. For BALB/cJ, the BAL PMN numbers increased only 1.6-fold after 24 h inhalation of 7.7×10^6 cm^{-3} NCP [8]. Alessandrini *et al*. did not detect signs of pulmonary inflammation after inhalation of 8.8×10^6 cm^{-3} NCP [30]. In the present study, the alveolar mass burden of the nanosized carbon particles at the end of the 24 h exposure to 6.4×10^6 cm^{-3} particles amounts to about 5 μg, according to particle deposition data recently determined [9]. This lung dose is close to the 'no observed adverse effect level' we determined in instillation exposures with the same nanosized carbon particles in BALB/cJ mice [31]. This was true for the number of polymorphonuclear lymphocytes (PMN) as well as for the cytokines IL-1ß and MIP2 determined in bronchoalveolar lavage fluid. However, BAL cytokine levels are to the best of our knowledge less sensitive in detecting pulmonary inflammation in comparison to BAL PMN numbers. It is noteworthy that, in contrast to ambient PM, the NCP applied in our study represent highly pure carbon particles, which are to the greatest extent free of metallic or organic contaminations, a feature necessary to exclude toxicity of soluble compounds.

Taken together, the findings of this and our previously published [6] study suggest that exposure to moderate doses of NCP exerts thrombogenic effects in the microcirculation of healthy mice independent of the route of administration (i.e. inhalation or systemic intra-arterial administration as a surrogate for translocated particles). The NCP-induced thrombogenic effects (i) are not liver specific and also observed in the heart, (ii) are associated with neither a local nor a systemic inflammatory response and (iii) seem to be independent of PMN-associated pulmonary inflammation.

Addendum

A. Khandoga and T. Stoeger designed research, performed experiments, analyzed data and wrote the manuscript. A.-G. Khandoga participated in carrying out of *in vivo* experiments. P. Bihari, S. Lakatos and J. Fent performed *in vitro* experiments on platelet activation. E. Karg was responsible for the NCP inhalation procedure. D. Ettehadieh participated in carrying out cytokine measurements. H. Schulz and F. Krombach directed the study, designed research and participated in writing the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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