Abstract: Objective
Air pollution with fine particles (PM10 and PM2.5) is associated with an increased incidence of cardiovascular events. The proposed mechanisms are still controversial and include indirect proinflammatory and procoagulant mechanisms including the activation of pulmonary macrophages, of endothelial cells and of the TNF/TF pathway. Others have reported on direct procoagulant effects. Preliminary observations from our laboratory suggested an interference with the platelet response to collagen after exposure to diesel exhaust particles (DEP).

Hypothesis
We hypothesized that DEP might directly interfere with the platelet collagen interaction by selectively inducing the cleavage of platelet receptor GPVI via metalloproteinases, which would represent a novel mechanism of DEP on platelets.

Methods
Citrated blood from healthy volunteers was exposed to highly standardized DEP at concentrations of 0.1 and 2.5 µg/ml and analysed for activated partial thromboplastin time (aPTT), fibrinogen and fibrin d-dimers. Equivalent experiments were performed with ultrafine carbon black (ufCB). Closure times with the PFA-100 device were deter-mined and platelet aggregation in response to a variety of agonists was monitored. Interleukins 1-beta and IL-8 were determined by ELISA and soluble P-selectin by the Luminex bead assay. Thrombin was measured as the endogenous thrombin potential (ETP) by fluorescence spectrometry. Soluble GPVI and GPIbα ectodomain fragments (glycocalcin) were measured by ELISA. ADAMTS13 activity was determined by the FRETS-VWF73 assay and plasmin activity using Spectrozyme PL.

Results
Aggregation assays with low dose DEP and ultrafine carbon black (ufCB) revealed a slight but significantly increased response with fibrillar collagen as agonist (p<0.05, n=5). At higher doses, however, collagen induced aggregation was suppressed by DEP: with 2.5 µg/ml, the inhibition was
34±12% (p<0.01, n=10). The GPVI specificity was confirmed by aggregations with cross-linked collagen related peptide (CRPxl), convulxin and with the monoclonal antibody 9O12.2 (all known to stimulate GPVI specifically). Ristocetin, arachidonic acid and ADP, however, were insensitive at these DEP concentrations. No cleavage of GPVI was found by immunosorbent assay of the fragment (soluble GPVI 27.8±3 vs 28±4 µg/ml mean±SEM, n=12); surprisingly, however, we found a significant cleavage of GPIbα as measured by an increase in plasma glycocalicin upon diesel exposure (2.58±0.11 vs 2.28±0.03 µg/ml p<0.01, n=10). ADAMTS13 and plasmin activity remained unaffected by DEP.

Conclusions
In agreement with our hypothesis, DEP specifically and directly interferes with platelet-collagen interactions. The functional consequences are biphasic and include platelet activation at lower DEP concentrations and inhibition at the higher dose. Our data indicate that the mechanisms are directed against platelet receptor GPVI, but - in contrast to our hypothesis - do not involve this receptors cleavage. A significant clipping of platelet GPIb, however, could be observed while a large array of plasma and platelet parameters remained normal including PFA-100 and soluble P-selectin, thus underlining the specificity.
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Baden, 11.10.2011 be-mb

**Diesel Exhaust Particles Impair Platelet Response to Collagen and are Associated with GPIbα Shedding**

Dear Dr. Dietrich,

Enclosed please find our manuscript entitled as stated above, which we would like to submit for publication in Toxicology *in-vitro*. It describes the response of blood coagulation, with special focus of the human platelets to diesel exhaust particles (DEP) and ultrafine carbon black (ufCB) and analyzes the mechanisms involved.

We felt that our findings are of interest to the readers of the Journal since a number of publications have pointed out the clinical association of pollution and cardiovascular events.

All the authors have read and approved the final form of the manuscript and it has not been published previously nor is it being considered for publication by any other peer-reviewed journal.

Thank you for taking our manuscript into consideration for publication.

On behalf of all the authors

Kind regards

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Disclosure of Conflicts of Interest

None of the authors of the manuscript "Diesel Exhaust Particles Impair Platelet Response to Collagen and are Associated with GPIbα Shedding" state that they have a conflict of interest with regard to publication of this article. There were no funding bodies or sponsors involved in its conception or execution with such a conflict. Accordingly, there is no stakeholder conflict, either.
Diesel Exhaust Particles Impair Platelet Response to Collagen and are Associated with GPIbα Shedding

Running title: collagen-induced platelet aggregation is suppressed at higher doses of DEP in human whole blood exposed in-vitro.

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Parts of this work were presented at the 47th annual meeting of the American Society of Hematology (ASH), Dec. 2005 in Atlanta, GA (abstract #2640) and at the 2010 meeting of the Swiss Society for Internal Medicine (FM62) in Basle, Switzerland. They were published in abstract form (Blood, Swiss Medical Weekly).

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Abstract (472 words)

Objective

Air pollution with fine particles (PM\textsubscript{10} and PM\textsubscript{2.5}) is associated with an increased incidence of cardiovascular events. The proposed mechanisms are still controversial and include indirect proinflammatory and procoagulant mechanisms including the activation of pulmonary macrophages, of endothelial cells and of the TNF/TF pathway. Others have reported on direct procoagulant effects. Preliminary observations from our laboratory suggested an interference with the platelet response to collagen after exposure to diesel exhaust particles (DEP).

Hypothesis

We hypothesized that DEP might directly interfere with the platelet collagen interaction by selectively inducing the cleavage of the GPVI receptor for collagen via metalloproteinases, which would represent a novel mechanism for DEP action on platelets.

Methods

Citrated blood from healthy volunteers was exposed to highly standardized DEP at concentrations of 0.1 and 2.5 µg/ml and analysed for activated partial thromboplastin time (aPTT), fibrinogen and fibrin d-dimers. Equivalent experiments were performed with ultrafine carbon black (ufCB). Closure times with the PFA-100 device were determined and platelet aggregation in response to a variety of agonists was monitored. Interleukins 1-beta and IL-8 were determined by ELISA and soluble P-selectin by the Luminex bead assay. Thrombin was measured as the endogenous thrombin potential (ETP) by fluorescence spectrometry. Soluble GPVI and GPIb\textalpha ectodomain fragments (glycocalicin) were measured by ELISA. ADAMTS13 activity was determined by a FRETs based assay and that of plasmin with Spectrozyme PL.

Results

Aggregation assays with low dose DEP and ultrafine carbon black (ufCB) revealed a slight but significantly increased response with fibrillar collagen as agonist (p<0.05, n=5). At higher doses, however, collagen induced aggregation was suppressed by DEP: at 2.5 µg/ml, the inhibition was 34±12% (p<0.01, n=10). The GPVI specificity was confirmed by aggregations with cross-linked collagen related peptide (CRPxl),...
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Conclusions

In agreement with our hypothesis, DEP specifically and directly interferes with platelet-collagen-interactions. The functional consequences are biphasic and include platelet activation at lower DEP concentrations and inhibition at the higher dose. Our data indicate that the mechanisms are directed against platelet receptor GPVI, but – in contrast to our hypothesis - do not involve this receptors cleavage. However, a significant cleavage of platelet GPIb could be observed while a large array of plasma and platelet parameters remained normal including PFA-100 and soluble P-selectin, thus underlining the specificity.

Keywords: platelets; aggregation; diesel exhaust particles; carbon black; glyco genetic
Introduction

Cardiovascular incidents are the leading cause of death in Western countries and airborne particulates contribute gravely to these by affecting a vast number of individuals (Huang and Brook, 2011). For several decades, evidence accumulated on the detrimental health effects of airborne pollutants (Donaldson et al., 2001; Mannucci, 2010; Mittleman, 2007; Simkhovich et al., 2008). The often cited heavy London smog in 1952, which was caused by a prolonged aerial temperature inversion, led to a thorough re-examination of the death records and to the re-evaluation of autopsies in order to gain a better understanding for the cause of a transient rise of fatalities suspected to be linked to this episode (Hunt et al., 2003). The findings pointed to a possible role for heavy metals such as lead, zinc and iron in cardiovascular and pulmonary complications.

With today’s knowledge on one of the relevant mechanisms of haemostasis – the integrin αIIβ3 receptor controlling platelet aggregation – a link between elevated manganese in the circulation by exposure to fuel by-products and enhanced platelet activation based on this receptors conformational change to a proaggregatory state induced by Mn2+ is simple to draw (Bunch et al., 2006; Smith et al., 1994). Accordingly, a recent report on manganese load related to proximity to gas stations in Taiwan may represent an interesting clinical model (Lin et al., 2011).

Presently, there is a broad consensus about the adverse effects of air pollutants on our health (Nemmar et al., 2006; Simkhovich et al., 2008). However, is this the result of just some constituents (e.g. O3 or O3 in combination with particulate matter [PM]) or do some others exhibit even ‘protective’ effects (Jensen et al., 2003)? Questions like these are subject to intense discussions. PM were found to stimulate a vascular proinflammatory response (Becker et al., 2005; van Eeden et al., 2001) and they have also been shown to decrease heart rate variability – suggesting effects on the vascular autonomous nervous system as well. In particular, a role for compromised (sub-)endothelial cells and alveolar macrophages was recognized to mediate proinflammatory responses via the release of cytokines, among them interleukin-1 (and -6) that in turn will stimulate the procoagulant tissue factor response (TF, (Szotowski et al., 2005). PM may also stimulate nuclear factor kappa B (NFκB) upon
exposure of airway endothelial cells to PM$_{10}$ by an iron-dependent mechanism (Jiménez et al., 2000).

Clinical manifestations include higher frequencies of deep vein thromboses (DVT, (Baccarelli et al., 2008)), and myocardial infarctions (MI, five cities study, (von Klot et al., 2005)) and this association is well documented.

Vehicular emissions, such as diesel exhaust particles (DEP), also featured prominently in the quest for causative agents of airborne PM (Morawska et al., 2004). As an interesting side note, the examination of above mentioned historical smog episode (London, 1952) revealed that city’s bus fleet had been converted to diesel fuel just prior to that extreme air pollution incident.

In 2003, Nemmar and coworkers published their report on direct prothrombotic effects of diesel exhaust particles (DEP) in a hamster model of arterial thrombosis (Nemmar et al., 2003). Thrombi in femoral arteries of PM-exposed animals increased in size and PFA-100 closure times progressively shortened ex vivo in whole blood, i.e. without contact with activated endothelial layers or other cells such as alveolar or vascular macrophages. More recently, exposure of healthy volunteers to high levels of DEP (~350 µg/m$^3$) while subjecting them to physical exercise led to increased thrombus and platelet aggregate formation (Lucking et al., 2008).

Based on the reported findings in rodents and on our own preliminary data (Forestier et al., 2006), we hypothesized that a direct effect by DEP (and possibly by ultrafine carbon black (ufCB)) at daily respirable doses could be measured in human whole blood in-vitro. The effects on platelet collagen interactions were of particular interest since our preliminary data let us further postulate that DEP might interfere with platelet glycoprotein (GP) receptors GPVI and/or GPIb-IX-V which play an important role in hemostasis by adhesion of platelets to collagen and von Willebrand Factor (VWF, (Coller et al., 1989; Gardiner et al., 2010)).
Materials and Methods

The study was approved by the IRB of the hospital and was strictly performed *in-vitro*. Written informed consent was obtained from healthy blood donors of up to 50 years of age with no known CVD history who had not taken any medication for 10 days prior to analysis.

Blood was drawn by venipuncture with a 19G needle (Butterfly-19, Abbott AG, Cham, Switzerland) and the S-Monovette system (Sarstedt AG, Sevelen, Switzerland). The first 2.5 ml of blood were discarded, then, 20-40 ml were collected into 10.6 mM of trisodium citrate as anticoagulant. To obtain platelet-rich plasma (PRP), the samples were centrifuged at 100 x g at room temperature for 10 min and then the platelet count was adjusted to 250,000/ µl with platelet-poor plasma (PPP was obtained at 1,500 x g for 15 min).

Particulate Matter Samples and Dose Calculation

Ultrafine carbon black (ufCB) was provided by Degussa GmbH as Printex-90, mean diameter 14 nm per particle and prepared in 0.9% NaCl, 0.1% Tween-20 for optimal dispersion by sonication (Branson type 250 sonifier with small tip, 60 sec. with 60% output, 50% duty cycle, Danbury CT, U.S.A.). Standardized Diesel exhaust particles (Standard Reference Material SRM 1650 from NIST) were a gift from Dr. Sauvain, Institut Romand de la Santé au Travail, Lausanne, Switzerland. This material also contains 19 ppm nitropyrene and 63-79 ppm of phenanthrene (Datasheet by NIST). Control samples received an equal volume of vehicle. The use of Tween-20 detergent proved indispensable for complete mass transfer to solution due to static adherence of dry DEP and ufCB to plastic material and sonicator metal.

An estimate of a daily absorbable dose for the PM surrogate was calculated, assuming a 0.5 liter respiratory volume for 17 cycles per minute (for an adult at rest). This daily respiratory volume of 12.24 m³ will yield a total burden of >0.5 mg per day if the WHO air quality limit for PM₁₀ of 50 µg/m³ is assumed. With a typical blood volume of 5,000 ml, this burden correlates to a rough estimate of an achievable concentration of at least 0.1 µg PM per ml (which is dependent on the distribution volume and cumulative effects over time and the clearance). This reference concentration of
0.1 µg per ml was chosen for our in-vitro experiments and was later raised to 2.5 µg/ml as a proof of principle. Concentrations can easily reach 10x higher levels as it was observed in some cities (Venners et al., 2003) previously. Incubations were performed in whole blood for one to maximally four hours in order to comply with the short functional stability of isolated platelets.

Concentrations of ufCB or DEP of up to 5 µg/ml did not interfere with optical platelet function measurements (not shown).

**Platelet Aggregation**

Thrombocyte aggregation in PRP in response to collagen, adenosine diphosphate (ADP), arachidonic acid (AA) or ristocetin (risto) were determined in a PACKS-4 lumi-aggregometer (Helena Diagnostika, Hartheim, Germany). Donor responses to threshold levels of agonists were established for each individual as follows: A target of 70% aggregation response was sought in order to capture inhibitory actions and a target of ≤50% was used to discern further activation. We found that these aims required 1-5 µg/ml of collagen and 0.5-5 µM of ADP (Helena Biosciences, Tyne and War, United Kingdom). Ristocetin- and arachidonic acid-dependent aggregations were measured at 1.2 mg/ml and 0.5 mg/ml, respectively. The GPVI-specificity of the aggregation response was tested using convulxin (15-50 ng/ml) and the GPVI activating antibody 9O12.2 (2.5 µg/ml), kindly provided by M. Jandrot-Perrus (Inserm Paris, France). Crosslinked collagen-related peptide (CRPx1) (monomer sequence: GCP-[GPO]10-GCPG), was provided by R. Farndale (University of Cambridge, Cambridge, UK), prepared using N-succinimidyl 3-[2-pyridyldithio]propionate (Pierce, Thermo Inc.) as crosslinker and extensively dialysed against 0.9% NaCl before use. This reagent efficiently aggregated platelets at concentrations of 5-50 ng/ml.

**Thrombin Generation (Endogenous Thrombin Potential, ETP)**

Plasma thrombin generation was monitored by calibrated automated thrombography (CAT assay) as previously described (Hemker et al., 2002)). Briefly, PPP was mixed with relipidated human recombinant tissue factor (hrTF, 5 pM final) and a fluorogenic substrate for thrombin (Z-gly-gly-arg-aminomethylcoumarin, Bachem, Basel, Switzerland), followed by recalcification. For PRP, induction was performed with hrTF (0.5 pM final) devoid of phospholipids. Thrombin generation was measured on a Fluoroskan Ascent reader (Thermo Labsystems, Helsinki, Finland) and corrected for
substrate turnover by thrombin- $\alpha_2$-macroglobulin using the Thromboscope software (Thromboscope BV, Maastricht, the Netherlands).

**Platelet Function Analyzer (PFA-100)**

Pore closure times with collagen / ADP (CADP) and collagen / epinephrine (CEPI) cartridges were recorded with the PFA-100 apparatus (Dade Behring, now Siemens healthcare, Eschborn, Germany). In this test, whole blood is drawn through a small capillary ending in a disc coated with equine collagen and a second agonist (ADP or Epinephrine). The time needed to arrest blood flow by closure of a pore in the disc serves as a parameter for hemostasis under high shear by platelet plug formation.

**Coagulation Measurements**

Routine coagulation determinations were performed on an STA-compact (Roche Diagnostics, Rotkreuz, Switzerland) using manufacturer-recommended reagents and procedures. Fibrinogen and d-dimers were determined using a modular P800/E170 instrument (Roche Diagnostics, Basel Switzerland).

**Cytokine and soluble P-Selectin Determinations**

Blood samples were apportioned to tubes containing 1 or 10 µg/ml lipopolysaccharide (LPS, strain *Salmonella abortuus equi*) in RPMI medium and incubated for 8 or 24 hours at 37° C in air containing 5% CO₂. After incubation, cells were pelleted by centrifugation at 2,000 x g and the supernatant frozen for determination of IL-1β and IL-8 as well as TNFα and IFN-γ via commercial EIA-kits with appropriate standards (Langezaal et al., 2001). The absence of LPS as a boosting substance resulted in lower and potentially more sensitive conditions, which however did not reveal any differences between treatment groups.

Soluble P-selectin (sP-selectin) as a sensitive parameter for platelet activation was measured by the Luminex bead assay (Cytolab Ltd., Dällikon, Switzerland) on a Millipore / Guava flow cytometer with commercial standards. Whole blood was exposed for 1 and 4 hours, then supernatants were obtained and frozen for later determination.
Glycocalicin (GC, Cleaved CD42b)

Glycocalicin, the soluble extracellular part of GPIbα, the receptor for von Willebrand factor (VWF), was determined using a sandwich ELISA developed by us and described earlier (Beer et al., 1994a). Briefly, 96-well polystyrene microtiter plates (Costar, Corning B.V., Amsterdam, the Netherlands) were coated with 6D1 antibody (kindly provided by B.S. Coller, the Rockefeller University, New York). Captured antigen was detected with the CD42b specific antibody Ib-23 (a gift from B. Steiner, Hoffman-La Roche preclinical research, Basel Switzerland), which was biotinylated with sulfo-NHS-LC-biotin (Pierce Chemical, Lausanne, Switzerland) and quantified with streptavidin-horseradish peroxidase conjugate (Calbiochem/Merck, Zürich, Switzerland) and ABTS substrate (Roche Diagnostics) on a Vmax reader (Molecular Dynamics, Sunnyvale CA, USA). Purified GPIb/IX was prepared as described (Beer et al., 1994a) from platelet lysates by sequential affinity chromatography using thrombin- and wheat germ agglutinin-sepharose. Aliquots of this preparation were used to obtain a standard curve in every assay. Inter-assay deviations were adjusted for by inclusion of an external standard derived from pooled plasma samples, which was determined to be at 2.1±0.3 µg/ml of GC. All samples were determined in duplicates at two dilutions each.

Soluble GPVI Measurements (sGPVI)

Plasma sGPVI was measured by ECL-based ELISA essentially as described (Al-Tamimi et al., 2009). Briefly, wells of 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with a rabbit polyclonal anti-GPVI IgG at 1 µg/ml, blocked with 1% (w/v) BSA (Sigma, St Louis, MO, USA) in PBS buffer and then 10% diluted plasma samples were added in triplicate wells. Murine anti-GPVI monoclonal antibody, 1A12 was added at 1 µg/ml, followed by addition of an HRP-conjugated secondary anti-mouse antibody (Chemicon, Melbourne, Australia). Signal was developed by adding 100 µl of a one-half dilution of SuperSignal ELISA Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and light emission measured using a Wallac-Victor2 luminescence plate reader (PerkinElmer, Waltham, MA, USA). Each plate routinely included a standard curve generated using serially diluted recombinant GPVI ectodomain in 10% GPVI-depleted plasma in PBS.
Plasmin Activity

Fibrinolysis by plasmin was determined with a peptide-pNA substrate (Spectrozyme PL, American Diagnostica, Stamford CT, USA) and purified, active Lys-plasmin (American Diagnostica) in a reaction containing 300 µM Spectrozyme PL and 0.3 µM Lys-plasmin in HBS buffer, pH 7.4. Substrate conversion was monitored in triplicates at 405 nm in a Vmax reader for 2-15 min after a 15 min preincubation (+DEP and Lys-plasmin, without Spectrozyme PL) at room temperature. After addition of substrate, values (mOD/min) were read from the linear part of the curve.

ADAMTS13 Assay

To evaluate a potential interference of fine and ultrafine particles with the processing of VWF by inhibition of the ADAMTS13 metalloprotease (larger VWF multimers may lead to thrombotic events), ADAMTS13 activity was determined under static conditions by a FRETS-VWF73 assay on a TECAN fluorescence reader as described (Kokame et al., 2005) and slightly modified (Kremer Hovinga et al., 2006). Briefly, the VWF73 peptide (D833 to R905 of VWF, Peptides International Inc.) was used as a substrate. Recombinant human (rh) ADAMTS13 (provided by F. Schleiflinger, Baxter, Vienna) was incubated with 5, 10, 20 and 40 µg/ml of DEP in carrier solution (0.9% NaCl, 0.1% Tween20 and 2mg/ml of BSA) for 15 minutes at room temperature. Incubation with DEP carrier solution only served as a negative control. Rh ADAMTS13 with or without DEP was diluted at 1-4 ng/ml into heat-inactivated normal human plasma (NHP) devoid of endogenous ADAMTS13 activity. Of this post-exposure mix, 5 µl was diluted into 120 µl of FRETS assay buffer (5mM Bis-Tris, 20 mM CaCl2, 0.005% Tween-20, pH 6.0) containing 1mM Pefabloc SC (Boehringer, Mannheim, Germany). An aliquot of 100 µl was incubated at 37°C for 10 min., then 100 µl of FRETS-VWF73 substrate (4 µM in assay buffer) were added to each well. A 1:25 dilution of NHP served to calibrate the assay (~100% activity) with further dilutions to 0, 1, 2, 5, 10, 25, and 50% of ADAMTS13 activity used to make a standard curve. After addition of the fluorogenic VWF73 peptide (2 µM final concentration), the reaction rate was calculated by linear regression analysis of fluorescence over time from 5 minutes to 4 hours.
Statistical Analysis

Values are indicated as mean±S.E.M. unless otherwise noted. Significance (p<0.05) was calculated by the paired student’s t-test with Microsoft Excel’s advanced analytical function and confirmed with SPSS V. 10 (Chicago IL, U.S.).
Results

1. Global Parameters of Coagulation (Prothrombin Time, aPTT, Fibrinogen, d-Dimers)

The global tests of hemostasis are summarized in Table 1. No effect of DEP or ufCB was evident at the concentration of 0.1 µg/ml, nor at the 25-fold higher concentration (2.5 µg/ml), on prothrombin time, aPTT, fibrinogen or d-dimers for either PM (not shown).

2. Analysis of Platelet Plug Formation in-vitro by the PFA-100 System

Our data obtained with the PFA-100 device are summarized in Table 1. Human blood was exposed to 0.1 µg/ml of either DEP or ufCB for one hour then assayed with CEPI and CADP cartridges. No significant change in rate of closure was observed for either PM treatment. In contrast to a report using hamster blood, (Nemmar et al., 2003) this outcome was consistently observed even at 25-50 fold higher concentrations of either material.

3. Markers of Inflammation

Since acute exposure to low doses of fine PM is known to induce inflammation and oxidative stress (Nemmar et al., 2011; Riva et al., 2011), we investigated whether exposure of whole blood to ufCB or DEP increased levels of proinflammatory cytokine IL-1β and ROS-sensitive IL-8 in blood samples from healthy donors. Incubation periods were adjusted for each cytokine in order to capture early and late responses. As expected, the values were higher for samples containing more LPS but interestingly, they did not differ significantly from their vehicle-treated counterparts (Table 2). A 25-fold higher dose of DEP (2.5 µg/ml) did not induce a significant difference among treatment groups either (not shown). In contrast to the findings of others, (van Eeden et al., 2001) our data indicate that IL-8 and IL-1β levels are not affected under the experimental conditions utilized here. It is possible that PBMC cannot offset for endothelial / perivascular cells with respect to the ex-vivo procoagulant response triggered by proinflammatory agents (Rauch et al., 2000).
4. Platelet-Dependent and –Independent Contributions to Thrombin Generation (CAT assay)

The capacity to generate thrombin (the ETP) was assessed when whole blood was challenged with 0.1 µg/ml of DEP or ufCB. Small but distinct differences between control and treated samples were observed for both materials (Table 1): An increase with ufCB and a slight reduction with diesel (Table 1, line ‘Thrombin-(ETP)-PRP’). These effects are platelet dependent and this is demonstrated by the absence of this difference when platelet-poor plasma (PPP) was analyzed (Table 1, line ‘Thrombin-PPP’).

5. DEP Causes a Biphasic Effect on Collagen-Induced Platelet Aggregation

This first clue to an altered platelet-specific reactivity induced by DEP or ufCB followed from the data of platelet aggregation studies. At the low concentration (0.1 µg/ml), we observed a consistent increase in the platelet response to collagen from 38.5±10.7% to 57±9.1% in the presence of a low dose of ufCB (p<0.05, n=5) with collagen as platelet agonist. This rise was also evident for DEP (increasing from 45.8±1.7% to 64.8±1.6%, p<0.02, n=5), again recorded with collagen as the agonist.

Raising the dose of DEP to 2.5 µg/ml however completely changed the picture. At the higher concentration, DEP instead caused an important, significant and selective decrease in aggregation by 34±12% (Fig. 1A, p<0.01, n=10), but with collagen as agonist, only (see below). The finding is corroborated by the fact that it was obtained from blood by ten individual donors. Some individuals seemed to be particularly sensitive and were almost completely inhibited by a low daily respirable dose of 0.1 µg/ml of DEP (Fig. 2). Within this group, consisting of 40% of all donors, the inhibition amounted to 41±15% (Fig. 1B). Interestingly, their platelets still underwent a shape change at all DEP concentrations as evident by the initial rise and dip of the aggregation tracing.
6. Aggregation Response to Convulxin, Collagen Related Peptide and the Specific Antibody 9O12.2 Directed Against GPVI

The decrease in aggregation was only observed in the presence of collagen, while other agonists such as ADP, arachidonic acid and ristocetin were insensitive to both DEP and ufCB, even at the high concentrations of 2.5-5.0 µg/ml (Fig. 1B). This surprising finding suggests that either a mechanism involving GPIb-IX-V/VWF, GPVI or integrin \( \alpha_2\beta_1 \) (GPIla/IIla) could be involved. We therefore analyzed two other GPVI-specific agonists in aggregation studies: By using mAb 9O12.2, an antibody which acts as an agonist on GPVI at high concentrations, we confirmed the findings observed with collagen (Fig. 3). The dose-dependent inhibition by DEP was equally recorded for convulxin and the crosslinked collagen related peptide (CRPxl, Fig. 3).

7. Exposure to DEP Leads to Increased Shedding of GPIb\( \alpha \), but not of GPVI

To evaluate whether the altered GPVI-related function of platelets exposed to DEP was due to increased release of the ectodomain of GPVI we measured the soluble GPVI in a series of exposed and control PRP-plasma samples. Exposures for 4 hours were found necessary to discern any effects for this slowly evolving parameter. The values indicated that under the experimental conditions used, the loss of GPVI function was not due to increased release of soluble GPVI (Fig. 4B).

However, Fig 4A shows that an increased release of glycocalicin (GC) from the same experimental samples was observed: (+20% for the DEP treated group, \( p<0.05 \)), possibly indicating an enhanced cleavage of GPIb\( \alpha \) by TACE (Bergmeier et al., 2004) induced by DEP.

8. Soluble P-Selectin in Plasma is not Increased After Exposure to DEP

Plasma levels of sP-selectin indicated no change upon whole blood exposure to diesel particles (65±53 ng/ml with 2.5 µg/ml of DEP vs 51±25 (ctr, n.s.). n=10, Fig. 4C).
9. Exposure to DEP Affects neither Plasmin nor ADAMTS-13 Activity

To detect a potential interference of DEP with enzymes of the thrombolytic pathway or those regulating VWF multimer size, plasmin and ADAMTS13 activities were determined. We did not detect any change in plasmin activity in a direct *in-vitro* assay using pure and active Lys-plasmin: the rate of substrate conversion remained unaltered by DEP addition (not shown). Likewise, the activity of ADAMTS13 towards a 73 amino acid peptide (spanning the enzyme’s cleavage site at Y842-M843) was unchanged at all concentrations of DEP under static conditions (not shown). However, this is not to say that an effect of DEP on full-length VWF or under shear stress could not occur.
Discussion

In this work, we describe the response of human whole blood and in particular of the platelets, to the addition of suspensions of DEP (as NIST standard reference material SRM1650).

In line with our hypothesis and preliminary observations, we found that the platelets’ capacity to aggregate is effectively diminished by DEP after stimulation with a single class of agonist – collagen and closely related compounds, each with specificity towards the collagen receptor GPVI. Secondly and in contrast to our hypothesis, we show that this specificity for platelet GPVI is not mediated by increased shedding of this receptor nor is it the consequence of platelet activation: sP-selectin remained unchanged. Instead, DEP exposure of whole blood leads to an increase in glycocalicin. Therefore, it is GPIbα, a shear-force dependent receptor (acting together with allosterically altered VWF) which undergoes enhanced cleavage.

Diesel fuel is increasingly being used for cargo transport and commuter mass transit which has made it the subject of a number of studies (Morawska et al., 2004), one of which was a revealing post-mortem analysis of victims of the particularly deadly smog episode in London in 1952 (Hunt et al., 2003). Air pollution is problematic in many countries and the pollutant burden often exceeds air control limits. For example, the PM load in Switzerland has exceeded 150 µg/m³ at lower elevations and remained above air control limits for up to 66 days in Winter of 2006 (SDA news briefing for Switzerland, January 2006).

While the US has managed to continuously lower their PMs according to the clean air act (Brook et al., 2004; Samet, 2011), substantially higher concentrations have been measured in other parts of the world, e.g. average particle loads even in excess of 600 µg/m³ have been reported in Chongqing, the People’s Republic of China (Venners et al., 2003). This would equate to a dose of approximately 1.2 µg per ml of blood for an adult, according to our calculation. We have chosen typical concentrations of 0.1 – 2.5 µg/ml of ufCB and DEP as the lower concentrations are likely to represent the airborne pollutant burden of a typical Western country. This conservative estimate is based on the complete absorption of inhaled PM and its transfer to the blood, and represents an experimental simplification (e.g. the
clearance and the volume of distribution are not known), that does not take into account the cumulative absorption over longer periods and the role of the different body compartments and the mobilization of particles stored within the body (both of which will likely lead to accumulation and higher values). Our initial dose of DEP used (0.1 µg/ml) is similar to the one proposed in another recent study on the subject (Khandoga et al., 2010). However, since particle loads in excess of 600 µg/m³ would translate to 1.2 µg/ml of blood, our higher concentration chosen seems not to be far fetched. We are well aware that our assumptions represent an undue experimental simplification but the actual systemic burden of airborne PM are difficult to estimate at present (T. Stoeger, Helmholtz Center for Lung Transplantation, Munich, personal communication).

A number of pathophysiological mechanisms induced by DEP have been proposed: Hunt et al (Hunt et al., 2003) found an unexpectedly high metal content of the debris in the victim’s lungs, e.g. of Zn, Sn, Pb and Mn. Divalent cations have long been recognized to mediate platelet activation (Riondino et al., 2001; Smith et al., 1994) Kroll. Heavy metal ions (Siegel et al., 2004) induce the formation of reactive oxygen species (ROS), leading to a proinflammatory response, the release of procoagulant tissue factor (TF) and promote arterosclerosis (Szotowski et al., 2005). The acceleration of the development of pre-existing atherosclerotic lesions by DEP is an accepted concept (Araujo et al., 2008; Yamawaki and Iwai, 2006). Interestingly, a novel factor (TRAF-4) was recently identified to bind to the GPIb-IX-V as well as the GPVI receptors and to be involved in signaling induced by oxidative stress (Arthur et al., 2011). In addition, organic extracts of DEP were found to reduce transglutaminase-2 in rodent alveolar epithelial cells (Koike et al., 2004). This regulator of coagulation could itself lead to a procoagulant response by influencing the gla-domains in many important (pro)enzymes of coagulation.

The clinical responses to aerosol particles have been intensively studied (Bhaskaran et al., 2011; Brook et al., 2002; Mills et al., 2005); those stemming from combustion processes of diesel fuel were recently identified as responsible for the adverse vascular effects (Mills et al., 2011).

Our present work provides a detailed analysis on the direct effects of DEP on blood coagulation and on platelets. The data clearly show no direct influence on the plasmatic coagulation, neither in global clotting tests nor in the more refined and
sensitive analysis of thrombin generation as examined in a broad concentration range. Several studies (Blomberg et al., 2005; Carlsten et al., 2007) are in agreement with our findings on plasmatic coagulation. The insensitivity to DEP however parallels earlier observations in alveolar macrophages (Becker et al., 2005) and might thus be explained by the absence of any compromised vessel lining / airway tissue in our experimental setting.

With regard to fibrinolysis, we specifically wanted to test alternative pathways that could be affected similarly to pathways already described (Furuyama et al., 2006; Gilmour et al., 2005). However, plasmin activity was unaffected by DEP treatment as was ADAMTS-13 activity towards a linear VWF peptide under static conditions. The latter would potentially be reflected in the PFA-100 analysis, since an increased contents of ultra long VWF multimers would lead to an acceleration of platelet aggregation, shortening the PFA-100 closure times. Therefore, it was important to rule out a direct involvement of ADAMTS-13 in the pathophysiology of DEP.

A direct inflammatory response as measured by a LPS driven, sensitive cytokine stimulation assays was not found, suggesting that the blood monocytes are not directly involved. In accordance with our hypothesis, further analyses revealed that the observed effects are platelet mediated. While the PFA-100 as a global test of platelet function remained unchanged – interestingly in contrast to the findings in the animal model (Nemmar et al., 2003) – several lines of evidence point to the direct and relevant involvement of platelets: a) In the global test of thrombin generation, ufCB increased and DEP decreased significantly the thrombin generated only in the presence of platelets, while the result remained unchanged in platelet-poor plasma (Table 1) b) DEP specifically interfered with the platelet response to collagen: At lower concentrations of DEP, it stimulated aggregation while higher exposures significantly diminished this response. c) Further analysis with agents specific for platelet GPVI suggested that this collagen receptor might be a target of DEP. d) In contrast to our hypothesis, the interference with GPVI was not due to increased cleavage of this ectodomain, a mechanism which has been well established (Gardiner et al., 2004). A cleavage- independent mechanism must be operative. e) Platelet collagen-interactions can be mediated indirectly (Coller et al., 1989) by the von Willebrand Factor-GPIb pathway, since VWF binds to collagen and is operative at high shear conditions.
Interestingly, the ectodomain cleavage of GPIbα is significantly induced by DEP and could account in part for the phenomenon observed. While being small, the change measured for glycocalicin apparently occurred without an increase in P-selectin, e.g. it proceeds independently from platelet activation. Polgar et al claimed that both P-selectin discharge and tissue factor procoagulant activity can occur independently from one another (Polgar et al., 2005). Interaction of platelets with von Willebrand factor is enhanced in situations of reduced ADAMTS13 activity. In our analysis under static conditions, ADAMTS13 was unchanged by DEP as tested with a linear VWF peptide.

How would our data of a specific interference of DEP with platelet-collagen-interactions fit with the concept of a prothrombotic mechanism in subjects exposed to DEP? First, it exhibited a biphasic response: At low dose, platelet aggregation increased while at the higher doses, a collagen dependent inhibition occurred. Second, the platelets will undergo enhanced clearance from the circulation when they loose GPIb as a neuraminic acid rich ectodomain, accelerating platelet turnover. As a consequence, there will be a higher proportion of fresh, reticulated platelets in the circulation leading to a higher probability for coagulation to occur. Third, while platelet aggregation to collagen was reduced, we cannot exclude increased platelet adhesion to other scaffold molecules of the subendothelium.

In conclusion, our work supports the concept of a direct platelet effect by DEP which is mediated by a specific inhibition of the collagen receptor GPVI and a significant cleavage of the von Willebrand receptor GPIbα. No other direct effects on whole blood in-vitro could be observed thus supporting the indirect proinflammatory mechanisms.
Acknowledgements

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Legends to figures

Table 1. Global parameters of coagulation. Citrated human whole blood was exposed for 1 hour to 0.1 µg/ml of DEP and ufCB (Printex-90), each. *: p<0.05, n=5.

Table 2. Determination of cytokines IL-1β and IL-8. Human blood was exposed to DEP and ufCB (n=5, each) as described under Methods. Pilot studies, performed without LPS boosting, resulted in no qualitative differences between these (not shown).

Figure 1 A) DEP exposure inhibits platelet aggregation in an agonist-specific manner. Collagen agonist exhibits the only significant change **: p<0.01 (n=10). Aggregation induced by standard doses of ADP, arachidonic acid or ristocetin is unchanged (n=5) B) The decrease in collagen-induced platelet aggregation by DEP is even stronger in a subgroup of individuals tested (solid bars: all individuals, hatched bars: highly sensitive donors).

Figure 2. Pronounced sensitivity towards DEP. Small dose of diesel particles is sufficient for essentially complete aggregation inhibition in this donor. The shape change (dip) persists at all doses, demonstrating maintained platelet fitness.

Figure 3. Inhibition of aggregation by DEP is dose-dependent. Furthermore, it is also observed with a number of additional agonists specific for GPVI. C is control, 2.5 and 5.0 denote DEP concentrations (as in Fig. 2). Typical tracings of at least triplicate measurements from different donors are shown. Agonist concentrations stated in panel heading.

Figure 4. A) DEP (2.5 µg/ml) promotes GPIbα clipping. Consequently, glycocalicin is elevated whereas B) soluble GPVI does not change. **: p<0.01 (n=10). C) sP-selectin does not increase in blood exposed to 2.5 µg/ml of DEP (n=10). Box borders denote 25th and 75th percentiles; the crossing line is the median. Group 1=Controls, group 2=DEP-exposed. Outliers are indicated by small circles.
References


Furuyama, A., Hirano, S., Koike, E., Kobayashi, T., 2006. Induction of oxidative stress and inhibition of plasminogen activator inhibitor-1 production in endothelial cells following exposure to organic extracts of diesel exhaust particles and urban fine particles. Arch Toxicol 80, 154-162.


### Table 1 (Forestier et al.)

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<td>-CEPI</td>
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Table 2
Figure 1. (Forestier et al.)

**Figure 2.**
Figure 3. (Forestier et al.)

Figure 4.
**Highlights** (of Forestier et al., “Diesel Exhaust Particles Impair Platelet Response to Collagen and are Associated with GPIbα Shedding”)

- we describe an enhancement of platelet aggregation in blood exposed to DEP or ufCB at daily inhalable doses
- this stimulation however only occurs when using collagen type I as the agonist
- an inhibition of collagen-induced aggregation occurred at 25-fold higher dose of DEP
- further agonists specific to the collagen receptor GPVI confirmed the finding but unexpectedly GPIb was increasingly cleaved, not GPVI