Pro-thrombotic effect of exercise in a polluted environment: a P-selectin- and CD63-related platelet activation effect

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Summary
Exposure to diesel exhaust is an important cardiovascular risk factor and may promote atherothrombotic events. Some data suggest that polluted air exposure could affect haemostasis through platelet activation. The aim of the study was to investigate the effects of acute exposure to diesel exhaust on platelet activation and platelet function. We tested the hypothesis in a randomised, crossover study in 25 healthy men exposed to ambient and polluted air; 11 of the subjects also performed exercise during exposure sessions. Platelet activation was evaluated by surface expression of CD62P (P-selectin) and CD63 (dense granule glycoprotein) using flow cytometry of labelled platelets. Platelet function was measured using the PFA-100 platelet function analyser and by Multiplate whole blood impedance platelet aggregometry. Acute diesel exhaust exposure had no effect on platelet activation at rest, but exercise in polluted air increased the collagen-induced expression of CD62P and CD63 (both p<0.05). The increase in the expression of CD62P and CD63 was related to the total amount of PM2.5 inhaled during the exercise sessions (r=+0.58 and +0.60, respectively, both p<0.05). Platelet aggregation was not impaired after polluted air exposure at rest or during exercise. In conclusion, in healthy subjects, diesel exhaust exposure induces platelet activation as illustrated by a dose-response increase in the release of CD62P and CD63. This platelet priming effect could be a contributor to the triggering of atherothrombotic events related to air pollution exposure.

Keywords
Environmental (risk) factors, atherothrombosis, platelet glycoproteins

Introduction
Air pollution is a growing public health issue, especially in developing countries, and is responsible for more than 3 million deaths per year worldwide (1). A large proportion of this burden is the result of increased cardiovascular mortality, as demonstrated in large epidemiological studies (2, 3). In addition to the long-term consequences of air pollution exposure, elevated ambient concentrations of particulate matter (PM) and traffic exposure have been strongly associated with new onset of cardiac atherothrombotic events within minutes to days after exposure (4, 5). A recent meta-analysis emphasised that acute exposure to air pollution is one of the most important triggers of myocardial infarction (6).

Air pollution includes gaseous pollutants and PM. PM < 2.5 μm in diameter (PM2.5), which are able to reach the lower respiratory tract, are considered to be the main toxic component of air pollution (7). PM2.5 mainly originate from combustion of fossil fuels as emissions from industrial and diesel engine activity. We recently demonstrated that acute experimental exposure to diesel exhaust impaired endothelial vasomotor function through decreased nitric oxide (NO) bioavailability and increased production of reactive oxygen species (ROS) (8). Mechanisms involved in the cardiovascular toxicity of PM, especially those related to how PM exposure can promote atherothrombotic events, are still under investigation. Indeed the effects of PM exposure on haemostasis remain largely unknown, but platelets may play an important role (9). In hamsters, intratracheal instillation of diesel exhaust particles induced thrombus formation through platelet activation, as shown by decreased closure time of the Platelet Function Analyser (PFA-100) (10). In healthy humans, controlled exposure to diesel exhaust increased thrombus formation and platelet aggregates with neutrophils and mastocytes within 2-6 hours (h) after exposure (11). Furthermore, population studies have demonstrated that ambient air pollution exposure induces a prothrombotic ten-
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dency, as shown by decreased PFA-100 closure time, in diabetic patients (12). In patients with coronary artery disease, controlled polluted air exposure affected especially the late phase of the coagulation cascade by inhibiting endogenous fibrinolytic capacity (13). All these observations are related to platelet aggregation ability and, consequently, to the late phase of haemostasis. No data are available on the early phases of haemostasis. Indeed, prior to aggregation, platelets are activated by expressing surface markers following granular release. This activation, also referred to as a platelet “priming state”, contributes to platelet recruitment (9). This initial step has important clinical significance in acute atherothrombotic events and has been shown to be present in patients with high cardiovascular risk (14, 15).

We, therefore, designed a study to test the hypothesis that acute exposure to diesel exhaust in healthy subjects would induce immediate platelet activation as an early step of the haemostasis cascade. The aims of the present study were to determine whether: 1) acute exposure to diesel exhaust promotes immediate activation of platelets through overexpression of surface markers; 2) air pollution increases platelet aggregation at the same time as platelet activation; and 3) platelet activation and aggregation are further enhanced when ventilation is increased during physical exercise performed in a polluted environment.

Materials and methods

Subjects

Twenty-five healthy, non-smoker male subjects (mean age 23.0 ± 0.4 years, body mass index [BMI] 22.8 ± 0.4 kg/m²) with a normal physical examination were enrolled in the study. The Ethical Committee of Erasme Hospital approved the study protocol (reference P2010/086) and written informed consent was obtained from each subject.

Study design

All subjects abstained from meals for 12 h and from alcohol and coffee beverages for at least 48 h prior to each exposure session. The volunteers were asked not to take non-steroidal anti-inflammatory drugs for at least seven days before each visit.

Diesel exhaust exposure

The diesel exhaust was generated by a PSA DW10 engine, frequently encountered in Europe, diluted with ambient air in the conduction system, and sent to a dedicated room, as described previously (8).

Diesel exhaust was delivered to obtain a PM2.5 concentration of 300 µg/m³ as previously described (8, 13). The PM concentration was measured by photometry using a GRIMM Laser Aerosol Spectrometer 1109 (GRIMM Aerosol Technik GmbH & Co, Ainring, Germany). Concentrations of NO, nitric dioxide (NO₂), nitrous oxides (NOx), and carbon monoxide (CO) and body temperature were monitored by electrochemical sensors (Multilyser NG, KWE Technologies Group, Waterloo, ON, Canada). We obtained recordings of blood pressure (BP), heart rate (HR), oxygen saturation (SpO₂) (Compaq, Datex Ohmeda, Madison, WI, USA) and minute ventilation (Pneumotrace, Medical Electronic Construction, Belgium). The University's Service for Protection and Prevention at work (SIPP-ULB) has approved the safety of the room and the equipment.

Figure 1: Study design.
Blood sampling

Blood samples were collected just before and immediately after exposure by venipuncture with a 21G needle, without use of a tourniquet and always by the same practitioner. The samples were stored in ambient air before standardised processing. Blood concentrations of E-selectin were obtained using commercially available cytokine array ELISA kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions.

Complete blood count

Complete blood count and differential were performed with EDTA anticoagulated blood on DxH 800 haematology analysers (Beckman Coulter, Brea, CA, USA) according to the manufacturer’s instructions.

Platelet activation analysis

Platelet activation analysis was assessed through alpha granule and dense granule secretion functions and was analysed by flow cytometry. We used four markers of platelet activation: CD62P (P-selectin), CD63 (dense granule glycoprotein), CD42 (GpIb) and PAC-1 (activated conformation of GpIIbIIIa). Resting platelets and platelets activated by collagen (12 µg/ml final concentration; chronolog, NOBIS Labordiagnostica GmbH, Endingen am Kaiserstuhl, Germany), ADP (5 µM final concentration, Roche, Vilvoorde, Belgium) or TRAP-6 (25 µM final concentration; Bachem, Bubendorf, Switzerland) were analysed using a Navios 10-colour flow cytometer (Beckman Coulter). Five microliters of citrated whole blood were mixed within minutes after sampling with 5 µl of two different antibody combinations and 3.3 µl of each agonist or PBS-1 % BSA. The first combination contained CD62P-FITC, CD63-PE and CD41 (2–2–1 µl); the second used PAC-1 FITC, CD42-PE and CD41-PerCp (2–2–1 µl) as a control for agonist activation. All antibodies came from Becton Dickinson (Franklin Lakes, NJ, USA). Blood, agonists and antibodies were gently mixed for 5 seconds and incubated at 37°C for 10 min in the dark. Activation and staining were stopped after exactly 10 min incubation with 1 ml PBS-1 % albumin-1 % paraformaldehyde. Platelets were gently mixed using vortex and immediately analysed with the flow cytometer. Ten thousand events fitting the platelet FSC in log scale and CD41 expression were acquired and stored. Platelet-monocyte and platelet-neutrophil aggregates were defined as monocytes or neutrophils expressing CD42α. Analysis of the acquisition files (LMD type) was performed using Kaluza (Beckman Coulter). Median fluorescence intensity in absolute units (MFI) was used for statistical analysis.

Platelet aggregation analysis

Platelet aggregation was studied by whole blood impedance aggregometry. Hirudin anticoagulated whole blood was distributed in five reactive test cells provided by the manufacturer. Four agonists (ADP, ASPI [arachidonic acid; thromboxane A2 inductor], collagen and TRAP-6 [mimicking thrombin activation]) were studied as well as self-aggregation. Platelet aggregation was plotted using a Multiplate analyser (Roche Diagnostics). The procedure was performed according to the manufacturer’s instructions without adaptation or modification.

Closure time measurement at high shear stress of citrated whole blood was performed on each sample using the PFA-100 (Siemens Healthcare Diagnostics, Marburg, Germany). For each sample, the two cartridges, collagen and epinephrine (CEPI) and collagen and ADP (CACP), were used according to the manufacturer’s instructions.

Data analysis

Diesel exhaust exposure was expressed in total amount of inhaled PM2.5 calculated from the mean minute ventilation and the mean PM2.5 concentration in the exposure room. All measurements were analysed in a blinded fashion. Physiological parameters (BP, HR, SpO2 and VE) are expressed as the mean of all measurements performed during each session. Hematological parameters are expressed as the difference between the samples collected after and before the exposure.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using SPSS (SPSS 16.0, Chicago, IL, USA). Pollution parameters and complete blood count were compared using a paired Student’s t-test. Platelet activation and aggregation testing results, expressed as difference between the samples collected after and before the exposure, were compared using a paired Student’s t-test. Correlation analyses using the Pearson correlation coefficient were performed. Statistical significance was assumed when p was < 0.05.

Results

Exposure data

Compared to ambient air exposure, the total amount of PM2.5 inhaled by the subjects during polluted air exposure increased from

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Table 1: Experimental pollution parameters.

<table>
<thead>
<tr>
<th>Pollution parameters</th>
<th>Ambient air</th>
<th>Polluted air</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM10 (µg/m³)</td>
<td>24 ± 0</td>
<td>318 ± 2 *</td>
</tr>
<tr>
<td>PM2.5 (µg/m³)</td>
<td>13 ± 0</td>
<td>309 ± 2 *</td>
</tr>
<tr>
<td>PM1 (µg/m³)</td>
<td>10 ± 0</td>
<td>305 ± 2 *</td>
</tr>
<tr>
<td>NO (ppb)</td>
<td>61 ± 3</td>
<td>919 ± 30 *</td>
</tr>
<tr>
<td>NO₂ (ppb)</td>
<td>44 ± 1</td>
<td>2160 ± 70 *</td>
</tr>
<tr>
<td>NOₓ (ppb)</td>
<td>105 ± 4</td>
<td>3079 ± 100 *</td>
</tr>
<tr>
<td>T (°C)</td>
<td>25.7 ± 0.1</td>
<td>25.9 ± 0.1</td>
</tr>
</tbody>
</table>

PM10: particulate matter <10 µm; PM2.5: particulate matter <2.5 µm; PM1: particulate matter <1 µm; NO: nitric oxide; NO₂: nitric dioxide; NOₓ: nitrous oxides; T: temperature. *: p<0.001.
Figure 2: Count of CD62P and CD63 labelled platelets during exercise sessions in subject 2. A) Count of CD62P-labelled platelets before and after ambient air exposure in subject 2. B) Count of CD62P-labelled platelets before and after polluted air exposure in subject 2. C) Count of CD63-labelled platelets before and after ambient air exposure in subject 2. D) Count of CD63-labelled platelets before and after polluted air exposure in subject 2.

Figure 3: CD62P and CD63 expression on labelled platelets during exercise sessions. A) Changes in CD62P expression on labelled platelets after exposure to ambient or polluted air. B) Correlation analysis between changes in CD62P expression and inhaled PM2.5 (the triangle point represents the mean of all values from the ambient air exposure). C) Changes in CD63 expression on labelled platelets after exposure to ambient or polluted air. D) Correlation analysis between changes in CD63 expression and inhaled PM2.5 (the triangle point represents the mean of all values from the ambient air exposure).
16 ± 2 to 312 ± 29 µg at rest (p<0.001) and from 27 ± 2 to 575 ± 50 µg during exercise (p<0.001). Other exposure data are summarised in Table 1.

Complete blood count
Acute exposure to diesel exhaust did not modify the complete blood count during rest or during exercise sessions. Polluted air exposure did not modify E-selectin concentration neither during rest nor exercise sessions.

Platelet activation
Acute exposure to diesel exhaust did not modify platelet activation in resting conditions. During exercise, air pollution exposure increased CD62P and CD63 expression on platelets labelled with collagen agonist from -0.46 ± 0.18 to 0.22 ± 0.19 MFI (Figure 3A) and from -0.09 ± 0.03 to 0.06 ± 0.04 MFI (Figure 3C), respectively (both p<0.05). The increases in CD62P and CD63 expression are both correlated between them (r=+0.97, p<0.001). Moreover, the increases in CD62P and CD63 expression were correlated with the total amounts of PM2.5 inhaled (respectively, r=+0.58 (Figure 3B) and r=+0.60 (Figure 3D), both p<0.01). Air pollution exposure did not alter PAC1 and CD42 expression during exercise sessions. Concentrations in platelet-monocyte or platelet-neutrophil aggregates were not modified after polluted air exposure neither during resting nor exercise conditions. See also Figure 2, Table 2 and Table 3.

Platelet aggregation
Polluted air exposure did not modify platelet aggregation as assessed either by PFA-100 or Multiplate with any of the aggregant agents used. Air pollution did not affect aggregation during rest or during exercise (see also Table 2 and Table 3).

Discussion
The main new findings of our study can be summarised as follows: 1) acute exposure to diesel exhaust increases platelet activation, during exercise session, by overexpression of the platelet activation markers, CD62P and CD63, following a linear dose-response pat-

Table 2: Effects of exposure to diesel exhaust at rest on platelet activation and aggregation.

<table>
<thead>
<tr>
<th>Platelet markers</th>
<th>Rest AA</th>
<th>Rest PA</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD62P control (MFI)</td>
<td>0.08 ± 0.09</td>
<td>0.08 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>CD62P collagen (MFI)</td>
<td>-0.13 ± 0.16</td>
<td>-0.33 ± 0.48</td>
<td>NS</td>
</tr>
<tr>
<td>CD63 control (MFI)</td>
<td>0.45 ± 0.39</td>
<td>0.04 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>CD63 collagen (MFI)</td>
<td>0.30 ± 0.30</td>
<td>-0.19 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>PAC-1 control (MFI)</td>
<td>0.10 ± 0.27</td>
<td>-0.02 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>PAC-1 collagen (MFI)</td>
<td>-0.22 ± 0.10</td>
<td>-0.89 ± 0.59</td>
<td>NS</td>
</tr>
<tr>
<td>CD42 control (MFI)</td>
<td>1.27 ± 1.41</td>
<td>-1.32 ± 3.98</td>
<td>NS</td>
</tr>
<tr>
<td>CD42 collagen (MFI)</td>
<td>2.85 ± 0.90</td>
<td>3.86 ± 1.92</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Platelet aggregation tests</th>
<th>Exercise AA</th>
<th>Exercise PA</th>
<th>Paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL/EPI (sec)</td>
<td>-22.4 ± 6.2</td>
<td>-1.1 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>COL/ADP (sec)</td>
<td>-8.2 ± 3.4</td>
<td>-5.2 ± 3.0</td>
<td>NS</td>
</tr>
<tr>
<td>M ADP (AUC)</td>
<td>-12.2 ± 3.5</td>
<td>-5.6 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>M ASPI (AUC)</td>
<td>-2.5 ± 5.1</td>
<td>-1.5 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>M COL (AUC)</td>
<td>-3.3 ± 4.6</td>
<td>0.6 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>M TRAP (AUC)</td>
<td>-7.2 ± 5.9</td>
<td>-6.3 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>M SPONT (AUC)</td>
<td>-3.3 ± 2.9</td>
<td>-2.8 ± 2.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

AA: Ambient air; PA: Polluted air; MFI: Median Fluorescence Unit; COL/EPI: PFA100 with collagen and epinephrine; COL/ADP: PFA100 with collagen and ADP; M ADP: Multplate with ADP; M ASPI: Multplate with ASPI; M COL: Multplate with collagen; M TRAP: Multplate with TRAP; M SPONT: Multplate without aggregant agent. NS: not significant.
ter; 2) acute exposure to diesel exhaust did not modify platelet aggregation immediately after exposure.

**Air pollution related platelet priming and atherothrombotic events**

Platelet activation, as identified by overexpression of surface proteins, is strongly associated with cardiovascular risk factors and various pathological cardiovascular conditions (14, 15, 17). Among the different platelet markers, diesel exhaust preferentially induces overexpression of CD62P and CD63. Overexpression of P-selectin and CD63 induces a activation state of the platelets. This observation has important clinical significance. Indeed, platelet activation is observed in various conditions in which thrombotic risk is increased, for example, in patients with coronary heart disease, diabetes and atrial fibrillation (14, 17, 18). P-selectin expression is also increased in hypertensive patients with no other cardiovascular risk factors (15). By demonstrating immediate platelet activation, we highlight the initial step of the pro-thrombotic state induced by diesel exhaust exposure. This early systemic reaction to polluted air may play a major role in the acute onset of myocardial infarction. Indeed very short-term variation in air pollution within only 1 h has been associated in epidemiological studies with the occurrence of ST elevation myocardial infarction (19). Changes in platelet aggregation function occur later in the time course of the haemostatic cascade. In our study, blood samples were collected immediately after a 2-h exposure session, which may explain the lack of effect of air pollution on platelet aggregation. Indeed, other studies focusing on the later phases of haemostasis were designed differently, with blood samples taken between 2 and 6 h after the pollution stimulus (11, 12).

Platelets can make specific connections with soluble elements and cells through the surface expression of various receptors. Studying platelets by the expression of various markers emphasises their multiple roles and interactions. In our study, E-selectin concentration remains unaffected, which is not in favour of an endothelial activation after polluted air exposure. However, the endothelial cells dysfunction plays a major role in the air pollution pathogenesis (8). P-selectin, which is overexpressed after air pollution exposure, also has a central role in the genesis of atherosclerosis, by promoting platelet–leukocyte cross-talk. P-selectin mediates the initial contact and rolling of leukocytes along the vessel wall (20). This initial step is required to ensure stable leukocyte adhesion, a key element in the inflammatory development of the atherosclerotic plaque (9). By inducing P-selectin overexpression, exposure to diesel exhaust not only triggers an acute atherothrombotic process but may also promote early stages of a chronic atherosclerotic process.

**Platelet function after exercise in a polluted environment**

In our study, increasing the level of diesel exhaust exposure during exercise was needed to identify platelet activation after air pollution exposure. At rest the pollution stimulus is likely not sufficient to trigger pro-thrombotic changes in the haemostasis cascade. Acute physical exercise is known to trigger cardiovascular events and to induce platelet activation, mainly by overexpression of P-selectin through increased calcium flux (21, 22). This effect was not observed in our study since moderate exercise alone, without air pollution, did not affect platelet marker overexpression in our young, healthy subjects. However, synergetic combination of exercise with increased inhaled PM clearly unmasked the platelet priming effect of air pollution exposure.

Consequently, exercising in a polluted environment may represent a public health issue for people living in urban areas (23). The onset of an acute atherothrombotic event is characterised by a combination of several elements, including a pro-thrombotic state, vasoconstrictor and endothelial dysfunction and increased O₂ consumption. All these conditions are met during physical exercise in a polluted environment. Our results are in line with a previous study and underscore the importance of avoiding exercise in highly polluted environments, especially for patients at increased cardiovascular risk (13).

**From smoke to cellular reactions**

Our study clearly demonstrates that platelet activation is a consequence of air pollution exposure. The mechanisms linking pollution inhalation and cardiovascular dysfunction are still misunderstood but may be dependent on an inflammatory reaction. However, pulmonary inflammation is associated with late events in the time course of the haemostatic effects of diesel exhaust exposure (24). It may be reasonable to consider that non-inflammatory-dependent reactions are responsible for the acute pro-thrombotic effect of air pollution.

In a similar study on air pollution, we demonstrated acute microvascular dysfunction because of decreased NO bioavailability. We also observed increased ROS production in endothelial cells rather than an acute increase in systemic markers of inflammation. We concluded that diesel exhaust exposure induces an oxidative stress reaction responsible for decreased NO bioavailability (8). In addition to its vasomotor function, NO also acts as an anti-thrombotic mediator and enhances the prostacyclin effect, which counteracts platelet activation (25). The platelet activation and vasomotor dysfunction observed in our studies may be the result of expression of the same pathway, which primarily involves NO. Furthermore, P-selectin expression has a common pathway with NO production (8). Both pathways depend on Ca²⁺ release (9, 26). Studying the effect of air pollution exposure on Ca²⁺ release remains a challenging and interesting pathway to explore and could help identify a common cellular mechanism for vascular and platelet dysfunction.

Significant relationships between PM inhalation and platelet activation were established. However, number of subjects included in this experimental standardised exposure study may not be sufficient to address less marked changes, especially in platelet aggregation tests. Further studies are needed to strengthen our suggested mechanistic hypothesis involving an acute oxidative stress reaction followed by platelet activation. The use of a greater range...
What is known about this topic?

- Air pollution exposure is associated with new onset of cardiac atherothrombotic events.
- Human studies on air pollution toxicity demonstrate mainly an effect on the late phase of the coagulation cascade.

What does this paper add?

- Acute polluted air exposure during physical exercise induces an immediate overexpression of CD62P and CD63.
- This acute platelet activation is related to the total amount of particles inhaled by the subjects.

of concentrations for the various agonists could be helpful to define more precisely the pathways involved. Furthermore we observed inter-individual variation in the platelet response to agonists after air pollution exposure. This finding may be associated to an individual susceptibility to air pollution haemostatic effect, which also needs further investigation to be confirmed. Although investigators were blinded to experimental conditions, and a careful standardisation were achieved especially on noise and room air temperature, inherent airway irritation related to particle exposure may have interfered with participant blinding.

In conclusion, for the first time in human, we have identified a platelet priming state following acute exposure to polluted air. This very early platelet activation is related to the amount of PM2.5 inhaled and is the result of a synergetic release of P-selectin and CD63. Endothelial and platelet reactions secondary to diesel exhaust exposure may share a common pathway related to NO bioavailability. Further research is needed to specifically address how diesel exhaust exposure may trigger a systemic pro-oxidative and inflammatory reaction.

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Author contributions

A.W., S.B., O.P. and J.F.A. designed research; P.v.d.B. obtained funding; A.W., F.E., I.B., W.W. and O.P. collected data; A.W., I.B. and O.P. performed statistical analysis; A.W. drafted the manuscript; and all authors interpreted data, critically revised the manuscript, and reviewed and approved the final version of the manuscript.

Conflicts of interest

None declared.

References


