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Extracellular histones mediate the effects of metal-rich air particles on blood coagulation

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ABSTRACT

Background: Epidemiological studies have shown associations of particulate matter (PM) exposure with hypercoagulability and thrombosis. Extracellular circulating histones have recently been identified as novel mediators of inflammatory and procoagulant responses. The potential roles of extracellular histones in PM-related hypercoagulability have yet not been investigated.
Objectives: In 63 steel workers, we evaluated the effects of exposure to PM and PM metal components on two extracellular histone modifications (H3K4me3 and H3K9ac); and the association of H3K4me3 and H3K9ac with coagulation markers.
Methods: Extracellular H3K4me3 and H3K9ac were determined in plasma through enzyme-linked immunosorbent assays. Coagulation markers included endogenous thrombin potentials (ETPs), tissue-type plasminogen activator antigen (t-PA) and D-dimer. Exposure to PM with aerodynamic diameters <1 μm (PM_1) or <10 μm (PM_{10}) and PM_{10} metal components were estimated for each participant.
Results: The coagulation marker ETP, measured in the presence of soluble thrombomodulin (ETP TM+), showed significant positive associations with PM_1 ($\beta=107.84$, $p=0.03$), PM_{10} ($\beta=83.06$, $p=0.02$), and zinc ($\beta=75.14$, $p=0.03$); and a marginal association with iron ($\beta=122.58$, $p=0.07$). Additional PM effects were observed on t-PA, D-dimer, and ETP TM+. PM_1 exposure was associated with increased plasma H3K4me3 and H3K9ac ($\beta=0.20$, $p=0.02$; $\beta=0.16$, $p=0.05$, respectively). H3K4me3, but not H3K9ac, was associated with zinc ($\beta=0.13$, $p=0.03$) and iron ($\beta=0.32$, $p=0.01$) contained in PM. ETP TM+ was increased in association with higher plasma H3K4me3 ($\beta=0.50$, $p=0.05$) and H3K9ac ($\beta=0.54$, $p=0.05$).
Conclusions: This observational study suggests potential roles of extracellular histones in PM-induced hypercoagulability. Experimental studies are warranted to further characterize these findings.

1. Introduction

Epidemiology investigations have repeatedly linked environmental exposure to particulate matter (PM) with increased incidence and mortality from myocardial infarction (D'ippoliti et al., 2003) and ischemic stroke (O'donnell et al., 2011). Recent studies also suggest a relationship between PM exposure and risk of venous thrombosis (Baccarelli et al., 2008; Dales et al., 2010). The mechanisms linking inhalation of air particles to cardiovascular disease exacerbation are not completely understood (Brook et al., 2010). The aerodynamic diameter of air particles is critical to determine their health effects as shape and size are primary determinants of where inhaled particles are deposited throughout the respiratory tract (Brook et al., 2010). Fine particles (PM_1), but not larger particles, can reach the alveolar epithelium and cross over into the bloodstream, thus enhancing systemic inflammation (Ljungman, 2009). The roles of finer particles in determining cardiovascular effects have been pointed out by several

Abbreviations: BMI, body mass index; ETP TM+/ETP TM−, endogenous thrombin generation—in the presence/absence of soluble thrombomodulin; ETP, endogenous thrombin potential; ELISA, enzyme-linked immunosorbent assay; H3K4me3, H3 Lysine 4 trimethylated; H3K9ac, H3 Lysine 9 acetylated; PM, particulate matter; PM1, particulate matter with aerodynamic diameters <1 μm; PM10, particulate matter with aerodynamic diameters <10 μm; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; TM, thrombomodulin

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epidemiological studies (Brook et al., 2010). PM components, as well as metals with strong pro-inflammatory actions, have been also shown to play key roles in determining PM-related cardiovascular effects (Zhang et al., 2009). PM-induced systemic inflammation has been suggested as a primary mediator of PM effects (Seaton et al., 1995; Ghio et al., 2003; Baccarelli et al., 2007). Finally, Bonzini et al. (2010) have recently shown that PM exposure is associated with increased coagulation activity, as reflected in shorter prothrombin time (PT), increased endogenous thrombin generation – measured in the presence of soluble thrombomodulin (ETP TM+) – and higher tissue-type plasminogen activator antigen (t-PA).

Work conditions may cause exposures to indoor PM considerably higher than outdoor concentrations. In modern steel plants, workers are still exposed to inhalable PM at levels well above concentrations found in ambient outdoor air, which also contain a large proportion of potentially toxic metal components (Alley et al., 2009; Fang et al., 2009). In epidemiological studies, steel workers have been found at higher risk for cardiovascular disease (Andjelkovich et al., 1990). Several metals have been demonstrated to have procoagulant effects; iron and zinc, the two metals usually found at highest concentrations in foundry PM, have been shown to have the greatest toxicity (Arslan et al., 2010; Sangani et al., 2010). Several prothrombotic modifications have been described after exposure to iron and zinc, such as alteration of clotting factors activity, increased platelet aggregation, reduced clotting time, and higher expression of pro-coagulant genes and proteins (Gilmour et al., 2006). Treatment with intravenous iron can be complicated by venous thrombosis (Seftel, 1965) and iron overdose can cause coagulopathy (Cheney et al., 1995).

Histones are nuclear proteins which organize themselves into nucleosomes containing two copies each of the core histones H2A, H2B, H3, and H4, in addition to the DNA wrapped around the histone octamer. Nucleosomes can be released in the extracellular matrix during apoptosis or can be actively secreted by inflammatory cells (Jahr et al., 2001). A recent study demonstrated that histones H3 and H4 are increased and mediate pro-inflammatory signaling in plasma samples from human individuals with sepsis, a condition associated with subclinical activation of blood coagulation (Semeraro et al., 2010). Extracellular histones also have strong affinity for circulating fibrinogen and prothrombin, which precipitate following the addition of histones, thus triggering clot formation (Fuchs et al., 2010; Pemberton et al., 2010). Recently, Ammollo et al. (2011) demonstrated that histones induce plasma thrombin production by reducing thrombomodulin-dependent protein C activation. Taken together, these findings indicate histones as emerging bridging molecules with biological significance for coagulation (Pemberton et al., 2010).

Deligezer et al. (2008) have recently shown specific post-translational modifications in plasma samples that were independent of the amounts of circulating nucleosomes. The presence of specific histone modifications in plasma has been suggested to contribute to selective activation of immune pathways and inflammatory responses (Dieker et al., 2007). In the same population of steel workers investigated in the present work, we have previously reported alterations of histone modifications (H3 Lysine 4 trimethylated [H3K4me3] and H3 Lysine 9 acetylated [H3K9ac]) in blood leukocytes following long-term PM exposure and related with specific PM metal-component levels (Cantone et al., 2011). Growing evidence suggests that extracellular histones may influence coagulation due to their strong tendency to form aggregates and precipitate with plasma proteins (Pemberton et al., 2010). In particular, histone aggregation with fibrinogen and prothrombin may enhance the effects of procoagulant factors on blood clotting (Fuchs et al., 2010; Semeraro et al., 2010; Ammollo et al., 2011). However, whether PM exposure induces changes in extracellular circulating histones, and whether extracellular histones are linked to disease-related biological outcomes has not yet been evaluated.

In the present study on steel workers with a wide range of exposure to PM, we investigated whether PM and metals in inhalable particles determined changes in two activating extracellular histone modifications – histone H3K4me3 and H3K9ac – in plasma. We also evaluated whether the observed modifications in extracellular histones were correlated with blood coagulation function.

2. Materials and methods

2.1. Study participants and exposure assessment

We investigated 63 male healthy workers, free of cancer and cardiopulmonary disease, who had been working in a steel production plant in Brescia, Northern Italy for at least one year. Individual written informed consent and approval from the local Institutional Review Board were obtained before the study. The study subjects worked in 11 different areas within the plant, which were selected to provide a wide contrast of exposures between the study participants. The exposure of each of the study participants in the plant was monitored for the first three working days of a work week. We obtained blood samples for coagulation and other biomarker measurements at two different times: time 1, in the morning of the first day of the workweek (following two days off work) before the beginning of any work activity; time 2, in the morning of the fourth day of the same workweek, following three consecutive days of work.

We used EDTA tubes to collect 7 mL whole blood that was promptly (within 30 min) centrifuged on site at 2500 rpm for 15 min. A whole blood aliquot (300 μL) was used for a complete blood count with differential. A plasma aliquot (200 μL) was separated and transferred in a cryovial, immediately stored at −80 °C until use. A self-administered questionnaire was used to collect detailed information on smoking habits, drug use (including NSAID), medical conditions, Body Mass Index (BMI) and education. Personal exposures during the working particle mass (PM with aerodynamic diameters <1 μm [PM_1] and <10 μm [PM_{10}]) and particle metal components (aluminum, manganese, nickel, zinc, arsenic, lead, iron) were estimated as previously described (Bonzini et al., 2010). In the plant, exposure levels have shown very little variability over time, as measures repeated over three years in a subset of the study population showed very high correlations ($r^2 > 0.90$). Therefore, the time-weighted levels of metals and particles represented, in addition to the exposure during the week of the study, also a measure of the usual exposure of the study participants (Tarantini et al., 2009).

2.2. Coagulation tests

We collected peripheral blood for coagulation testing into vacuum tubes (Becton Dickinson, Meylan, France) with 0.109 M trisodium citrate at a 9:1 blood/anticoagulant ratio. We determined t-PA and the fibrin degradation product that contains two cross-linked D fragments of the fibrin protein (D-dimer) using commercially available enzyme-linked immunosorbent assay (ELISA) kits (ELISA-Zymutest; HYPHEN BioMed, Neuville-Sur-Oise, France). According to Hemker et al. (2003), as described by Chantarangkul et al. (2003), we assessed thrombin generation potential (ETP) as a global measure of coagulation activation. The test was based on the activation of coagulation in platelet poor plasma with calcium chloride. We performed the test in the presence and in absence of soluble rabbit thrombomodulin (ETP TM+ and ETP TM− respectively) (ICN Biomedicals, Aurora, OH, USA) as activator of protein C, added to the reaction mixture at a final concentration of 4 nM. Continuous registration of the generated thrombin was achieved with a fluorogenic synthetic substrate (Z-Gly-Gly-Arg-AMC-HCl; Bachem, AG, Bubendorf, Switzerland) added to the test system at a final concentration of 417 μM. The procedure was carried out with an automated fluorometer (Fluoroskan Ascent; ThermoLabsystem, Helsinki, Finland). We automatically recorded readings from the fluorometer and analyzed results with dedicated software (THROMBINOSCOPE; Thrombinoscope BV, Maastricht, The Netherlands), which displays thrombin generation curves [nM thrombin vs. time (min)] and calculates the area under the curve. ETP represents the plasma balance between procoagulants and anticoagulants agents.

2.3. Histone modification analysis

In each run, 20 μL of plasma was analyzed by solid-phase sandwich enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies to detect H3K4me3 acetylated H3K9ac (EpiQuik Assay Kit, Epigentek, Brooklyn, NY, USA) (Deligezer et al., 2010). We used a Synergy HT-BioTek spectrophotometer to read 490 nm absorbance (OD), which was assumed proportional to the concentration of modified histones (Deligezer et al., 2010). The reliability coefficient of single ELISA