

**Particulate matter induces prothrombotic microparticle shedding
by human mononuclear and endothelial cells**

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1 Abbreviations: phosphate buffered saline (PBS); tissue factor (TF); phosphatidylserine
2 (LPS); microparticles (MP); particulate matter (PM); peripheral blood mononuclear cells
3 (PBMC); human umbilical vein endothelial cells (HUVEC); standard reference material
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5 1648a Urban Particulate Matter (SRM1648a)
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12 Keywords: microparticles; thrombosis; tissue factor; particulate matter; endothelial cells;
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14 mononuclear cells
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Abstract

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2 Particulate airborne pollution is associated with increased cardiopulmonary morbidity.

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4 Microparticles are extracellular vesicles shed by cells upon activation or apoptosis involved
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7 in physiological processes such as coagulation and inflammation, including airway
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10 inflammation. We investigated the hypothesis that particulate matter causes the shedding
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12 of microparticles by human mononuclear and endothelial cells.

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14 Cells, isolated from the blood and the umbilical cords of normal donors, were cultured in
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16 the presence of particulate from a standard reference. Microparticles were assessed in the
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18 supernatant as phosphatidylserine concentration. Microparticle-associated tissue factor was
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21 assessed by a on-stage clotting assay. Nanosight technology was used to evaluate
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24 microparticle size distribution.

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26 Particulate matter induces a dose- and time- dependent, rapid (1 h) increase in
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29 microparticle generation in both cells. These microparticles express functional tissue
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32 factor. Particulate matter increases intracellular calcium concentration and phospholipase
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35 C inhibition reduces microparticle generation. Nanosight analysis confirmed that upon
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38 exposure to particulate matter both cells express particles with a size range consistent with
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40 the definition of microparticles (50-1000 nm).

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42 Exposure of mononuclear and endothelial cells to particulate matter upregulates the
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45 generation of microparticles at least partially mediated by calcium mobilization. This
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48 observation might provide a further link between airborne pollution and cardiopulmonary
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50 morbidity.

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1. Introduction

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2 Particulate matter (PM) is a complex mixture of small particles and liquid droplets. Particle
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4 pollution is made up of a number of components, including acids (such as nitrates and
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6 sulfates), organic chemicals, metals, and soil or dust particles. The size of particles is
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8 directly linked to their potential to cause health problems, as it is in turn directly linked to
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10 the ability of particles to penetrate more or less deeply into the respiratory system. PM
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12 exposure has wide effects on health (Alfaro-Moreno et al., 2007) and a large body of
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14 evidence has consistently shown that both short and long term exposure to PM are
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16 associated with increased cardiovascular and pulmonary related morbidity and mortality
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18 [see (Pelucchi et al., 2009) and (Franklin et al., 2015) for comprehensive reviews].
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24 However, the mechanisms behind this association are not fully understood.

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26 Microparticles (MP), also referred to as microvesicles or ectosomes, are small (diameter
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28 0.05-1 μm) vesicles that originate from the cell surface of most (if not all) cell types during
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30 activation or apoptosis. MP are heterogeneous in nature, varying in both size and content,
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32 and present cell surface markers and cytoplasmic components of the parent cells from
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34 which they originate. MP are involved in numerous physiologically relevant processes,
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36 such as blood coagulation (Falati et al., 2003, Celi et al., 2004) and inflammation (Celi et
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38 al., 2004, Leroyer et al., 2010, Ardoin et al., 2007, Distler et al., 2006), including airway
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40 inflammation (Cerri et al., 2006, Neri et al., 2011, Cordazzo et al., 2014). Accordingly, MP
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42 have recently gained attention as both biomarkers and effectors in human diseases,
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44 including cardiac and pulmonary diseases (Takahashi et al., 2014, Takahashi and Kubo,
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46 2014, Amabile et al., 2014, Hu et al., 2014, Duarte et al., 2013).
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53 Two well-known cellular processes lead to the formation of MP, i.e. cell activation, either
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55 chemical or physical, by agonists or shear stress respectively, and apoptosis, through the
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57 action of growth factor deprivation or apoptotic inducers (VanWijk et al., 2003, Mostefai et
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59 al., 2008). We have previously demonstrated that a number of agonists, including two
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different peroxisome proliferator-activated receptor- γ agonists, angiotensin II and cigarette smoke extract cause a rapid, calcium dependent, upregulation of MP shedding from leukocytes and that different calcium inhibitors largely prevented MP shedding (Neri et al., 2012, Cordazzo et al., 2013, Cordazzo et al., 2014). Since PM induces cytosolic calcium mobilization in monocytes (Brown et al., 2007), we speculated that PM, more specifically PM₁₀, induces the generation of MP by peripheral blood mononuclear cells (PBMC) and human umbilical vein endothelial cells (HUVEC), thus providing a novel link between airborne pollutants and cardiopulmonary risk.

2. Materials and Methods

2.1 Reagents and kits

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, fetal bovine serum, trypan blue, phosphate buffered saline, Ficoll-Hystopaque, dextran T500 and U73122 were obtained from Sigma (Milano, Italy). Thromboplastin standard was obtained from Beckman Coulter (Milano, Italy). Human anti-tissue factor (TF) antibody was obtained from America Diagnostica (Instrumentation Laboratory, Milano, Italy). The Zymuphen MP-Activity kit was obtained from Hyphen BioMed (Neuville-sur-Oise, France). The Fluo-4 NW Calcium Assay kit was obtained from Molecular Probes (Invitrogen, Milano, Italy). Standard reference material 1648a Urban Particulate Matter (SRM1648a) was obtained from National Institute of Standard Technology (Gaithersburg, MD, USA).

2.2 Cell isolation and culture

2.2.1 HUVEC

HUVEC were isolated from umbilical vein cords as described (Del Fiorentino et al., 2010) by digestion with 0.1% collagenase (specific activity: 316 U/mL, Gibco, Invitrogen) and grown to confluence at 37°C in 5% CO₂ humidified incubator, on 25-cm² tissue culture flasks previously coated with 1% gelatine in supplemented culture medium (M199 with

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10% heat inactivated foetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM l-glutamine, 10mM HEPES pH 7.4, heparin 12 IU/mL, 1% retinal derived growth factor, all from Sigma). Following trypsin treatment, the cells were detached from the flasks and final mono-layers were prepared by seeding HUVEC on gelatine-precoated culture plates and then incubated for 24–48 h to ensure confluence. HUVEC were identified by their typical cobblestone morphology and immunofluorescence staining by monoclonal antibodies against von Willebrand Factor (Immunotech, Milano, Italy). Cells up to the fourth passage were used for all experiments.

The investigation conformed with the principles outlined in the declaration of Helsinki for use of human tissue.

2.2.2 PBMC

PBMC were isolated either from fresh buffy coats obtained from the local blood bank or from the peripheral blood of normal volunteers as described (Cerri et al., 2006). Briefly, after obtaining the necessary consent from the donor, a fresh buffy coat was mixed gently with an equal volume of 2,5% Dextran T500, and left for 40 minutes for erythrocyte sedimentation. Ten mL of leukocyte-rich supernatant was recovered and layered over 5 mL of Ficoll-Hystopaque and centrifuged for 30 minutes at 350 x g at 4°C. The PBMC-rich ring was recovered and washed twice in phosphate buffered saline. PBMC were then resuspended in RPMI supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM l-glutamine and allowed to adhere for 30 minutes at 37°C on 24-well plates (2x10⁶ cells/well). Then the cells were washed two times with pre-warmed phosphate buffered saline and resuspended in RPMI, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM l-glutamine, 5%FBS and incubated overnight at 37°C.

All procedures related to the use of human blood and tissues were approved by the ethical committee of the Azienda Ospedaliero-Universitaria Pisana, Pisa, Italy (protocol number 16715).

2.3 MP generation and purification

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2 PBMC and HUVEC were washed twice with pre-warmed phosphate buffered saline. For
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4 MP release analysis, SRM1648a was resuspended in cell media and added; after the time
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6 of incubation indicated in the relevant figures at 37°C the supernatants were recovered,
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8 cleared by centrifugation at 14,000 x g for 5 min at room temperature to remove dead cells
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10 and big cell fragments that might have detached during the stimulation and immediately
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12 used for further experiments. In selected experiments, MP were further purified by
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14 ultracentrifugation (100,000 x g for 2 hours, 4°C); the pellet was resuspended in 250 µL of
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16 normal saline and used in a one-stage clotting assay to measure TF-dependent
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18 coagulation. For Nanosight analysis, an aliquot of cell medium was centrifuged at 4°C at
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20 1000g for 15 minutes, at 2000g for 15 minutes and then at 3000g for 15 minutes.
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22 Supernatants were then submitted to ultracentrifugation at 110,000 x g for 2 hours at 4°C.
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2.4 Measurement of MP-associated phosphatidylserine (PS)

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30 PS-positive MP in each sample were detected using the Zymuphen MPactivity kit (Hyphen
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32 BioMed, Neuville-sur-Oise, France) according to the manufacturer's instructions and
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34 expressed as PS concentration (nM PS).
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2.5 Nanosight detection of MP

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40 The number and dimension of MP were assessed by nanoparticle tracking analysis (NTA).
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42 Using a Nanosight LM10-HS system (NanoSight Ltd., Amesbury, UK), MP were visualized
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44 by laser light scattering. Briefly, MP-enriched pellets were resuspended in 300 µL of 0.1
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46 µm filtered sterile phosphate buffered saline and five recordings of 30 seconds were
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48 performed for each sample. Collected data were analyzed with NTA software, which
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50 provided high-resolution particle size distribution profiles and concentration measurements
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52 of the vesicles in solution.
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2.6 Measurement of intracellular calcium concentration

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60 Molecular Probes Fluo-4 NW Calcium Assay kit was used to measure the
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changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) of PBMC and HUVEC.

Prewashed PBMC (0.33×10^6 cells/well) or HUVEC (15×10^3 cells/well) were loaded with 100 μ L of the dye loading solution containing Fluo-4 NW dye and probenecid, according to the manufacturer's instructions. The 96-well plate was incubated at 37 °C for 45-60 min in the dark and SRM1648a (500 μ g/mL) was added to the cells. The changes in Fluo-4 NW fluorescence were measured by the Wallac 1420 Victor 2 (PerkinElmer, Milan, Italy) at λ_{ex} 494 nm and λ_{em} 516 nm. Calcium mobilization was observed over time (up to 150 sec) and analyzed by the Wallac 1420 Software version 3 (PerkinElmer Life and Analytical Sciences, Wallac, Milan, Italy). The increase in $[Ca^{2+}]_i$ fluorescence was expressed as relative fluorescence units (RFU).

2.7 Assessment of MP-bound TF activity

TF activity was measured in MP generated in vitro from PBMC and HUVEC by a one-stage clotting time assay as described, except that the normal human plasma was made MP-poor by ultracentrifugation (100,000xg for 2 h, 4°C). Briefly, disrupted MP (100 μ L) were mixed with 100 μ L of MP-poor normal human plasma at 37°C; 100 μ L of 25 mM $CaCl_2$ at 37°C was added to the mixture and the time to clot formation was recorded. The results were expressed in arbitrary milliunits of procoagulant activity by comparison with a standard curve obtained using a human brain thromboplastin standard. This preparation was assigned a value of 1,000 arbitrary mU for a clotting time of 30 s. An anti-human TF antibody (American Diagnostica, Instrumentation Laboratory, Milano, Italy; 30 μ g/mL) largely inhibited the procoagulant activity, thus confirming its identity with TF (Neri et al., 2012) (data not shown).

2.8 Data presentation and statistical analysis

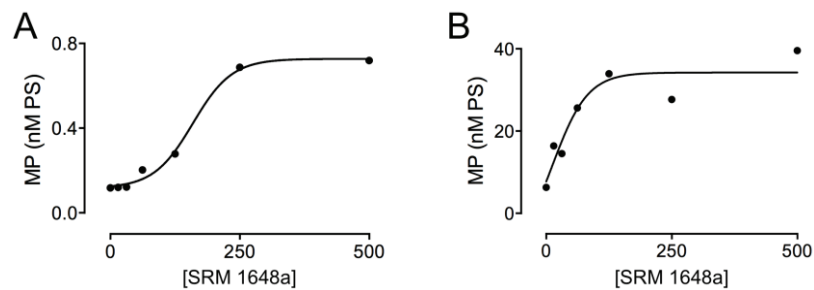
Unless otherwise indicated, data are shown as mean \pm SEM from n independent, consecutive experiments; comparisons among groups were made by either ANOVA for repeated measures followed by Tukey's analysis or Student's paired t-test, as appropriate,

1 using Prism Software (GraphPad, San Diego, CA, USA). Values of $p < 0.05$ were
2 considered statistically significant.
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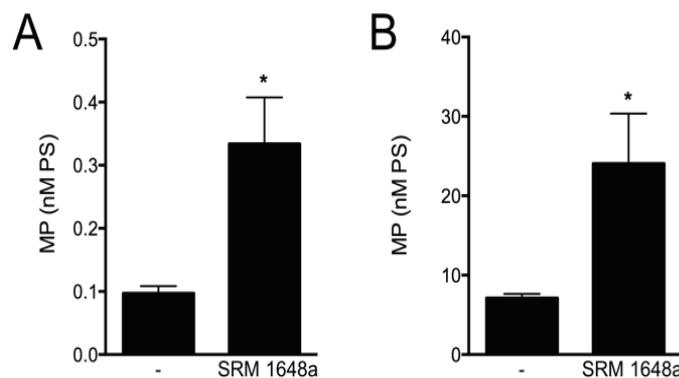
7 **3. Results**

8 **3.1 PM induces MP generation by PBMC and HUVEC**

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11 To investigate whether PM induces the release of MP, PBMC and HUVEC were stimulated
12 with increasing concentrations of SRM1648a for 1 hour. Both PBMC and HUVEC
13 generated MP upon stimulation with SRM1648a. The effect was concentration dependent
14 and reached a plateau at approximately 250 and 125 $\mu\text{g/mL}$ for PBMC (2A) and HUVEC
15 (2B), respectively (figure 1). Due to the relatively high dispersion of data,, we used 500
16 $\mu\text{g/mL}$ SRM1648a, a concentration that caused a consistent increase in MP generation,
17 for all subsequent experiments with both cell types. As shown in figure 2, the increase in
18 MP generation was statistically significant with both PBMC (2A) and HUVEC (2B).
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3.2 Kinetics of MP generation upon exposure to PM

MP generation can be caused by different mechanisms; accordingly, the kinetics vary depending, for example, on whether calcium mobilization, cell activation or apoptosis is involved (VanWijk et al., 2003). We treated cells with SRM1648a and assessed MP generation at different time points. As shown in figure 3, MP generation increased steadily with time both in the absence and in the presence of the agonist; however, the difference between SRM1648a treated and untreated cells tended to level off and was maximal at early time points (1 h) for both PBMC (3A) and HUVEC (3B).

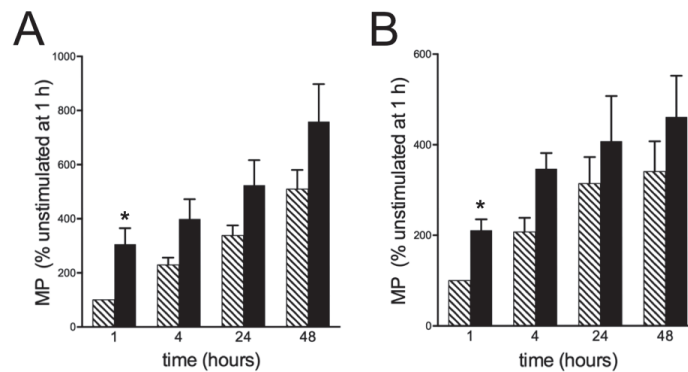


figure 3

3.3 MP analysis by Nanosight

MP released in medium after SRM1648a treatment and in untreated control were isolated and their number and size distribution analyzed by Nanosight analysis. As shown in figure 4, both PBMC (4A) and HUVEC (4B) stimulated with SRM1648a for 1 h showed an increased production of MP. In particular, for PBMC, the mean number of particles (mean of ten replicates) was $2.56 \cdot 10^8$ /mL in untreated controls and $8.54 \cdot 10^8$ /mL in SRM1648a stimulated cells while, for HUVEC cells, the mean number of particles was $11.44 \cdot 10^8$ /ml and $22.25 \cdot 10^8$ /ml for control and SRM1548a stimulated cells, respectively. MP size peaked at 120.6 nm and at 132.3 nm for control and SRM1648a-treated PBMC, and at 160.3 and at 156.1 nm for control and SRM1648a-treated HUVEC.

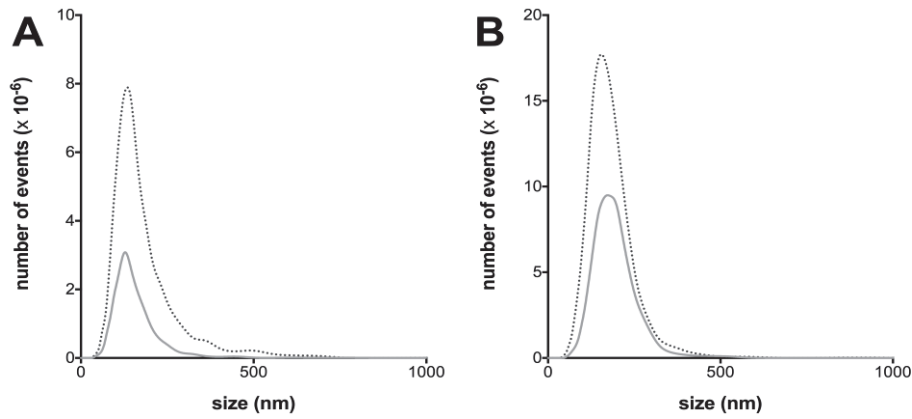


figure 4

3.4 PM induces the mobilization of intracellular calcium in PBMC and HUVEC

PM has been shown to induce an increase in intracellular calcium concentration (Brown et al., 2007). Because calcium mobilization is involved in rapid MP generation (Neri et al., 2012, Cordazzo et al., 2013, Cordazzo et al., 2014) we investigated whether SRM1648a increases $[Ca^{2+}]_i$ in our experimental conditions. Figure 5 confirms that SRM1648a (500 $\mu\text{g/mL}$) induces a rapid and significant increase in $[Ca^{2+}]_i$ in PBMC (5A) and HUVEC (5B).

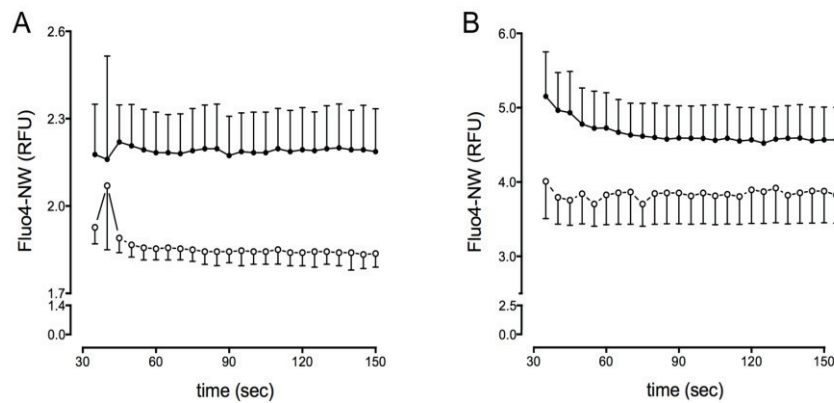


figure 5

3.5 PM-induced MP generation is mediated through phospholipase C

To further characterize the role of calcium in the induction of MP by SRM1648a-stimulated cells, we used the phospholipase C inhibitor, U73122, to investigate the role of calcium ions stored in the endoplasmic reticulum. Cells were pre-treated with U73122 (1 μ M) for 30 minutes prior to stimulation with SRM1648a for 1 h. Figure 6 shows that U73122 inhibits SRM1648a-induced MP generation in PBMC (A) and HUVEC (B).

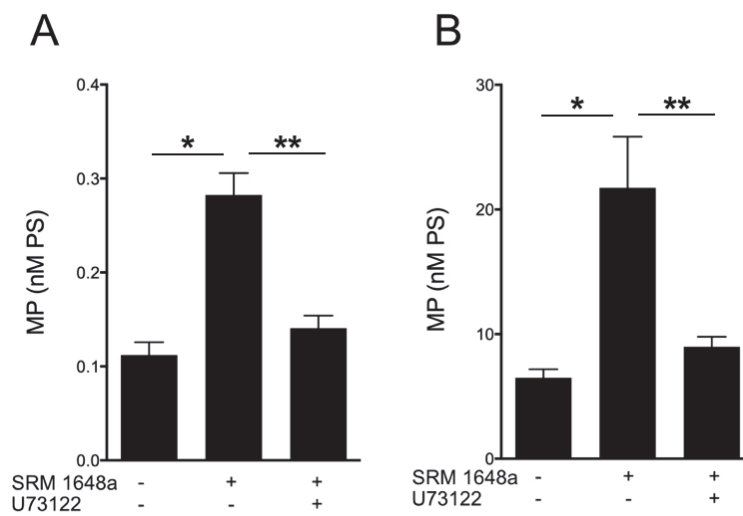


figure 6

3.6 SRM 1648a induces the expression of MP-bound TF

The procoagulant activity of MP is due to the exposure of PS on their surface and is also enhanced by the presence of functional TF (reference). To evaluate whether SRM1648a induces the generation of TF-bearing MP by PBMC and HUVEC, we analyzed the procoagulant activity of purified MP released by SRM1648a treated (1 h) and untreated cells through a one-stage clotting test. As shown in Figure 7A and 7B respectively, SRM1648a induces a 2- to 2.5-fold increase in procoagulant activity of MP by PBMC and 3- to 3.5-fold increase in procoagulant activity of MP by HUVEC. A monoclonal antibody to TF (epitope specific for amino acids 1–25; American Diagnostica, Stamford, CT, USA; 30

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µg/mL) inhibited most of the procoagulant activity (not shown), confirming its identity with TF.

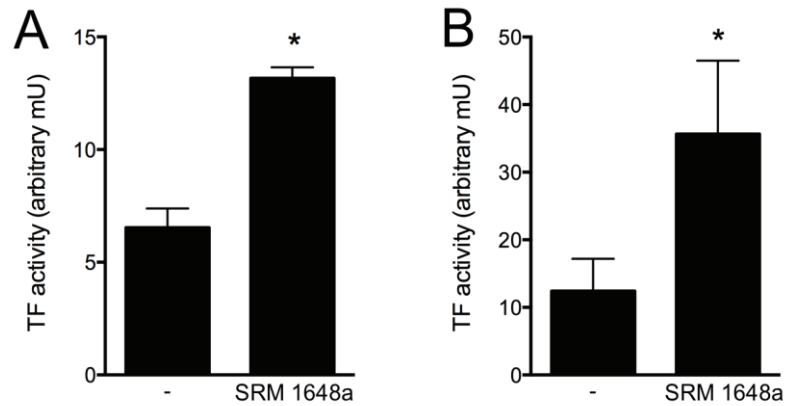


figure 7

4. Discussion

The present study aimed at investigating the possible role of PM on the generation of MP by PBMC and HUVEC. Our data demonstrate that SRM1648a, a standard reference source of PM, is able to induce the generation of MP in both PBMC and HUVEC. The observation that SRM1648a causes an increase in $[Ca^{2+}]_i$ in both cell lines is consistent with the hypothesis that PM stimulates MP shedding through calcium mobilization. The demonstration that the phospholipase C inhibitor, U73122, largely inhibits the effect suggests a scenario whereby MP shedding requires calcium mobilization from intracellular storage pools that takes place via phosphatidylinositol (3,4,5) trisphosphate (Fruhbeck, 2006).

MP were detected and enumerated through the ability of the negatively charged phospholipids expressed on the outer leaflet of their membrane to activate the prothrombinase complex. Therefore, these MP are by definition procoagulant. However, the procoagulant potential of MP of different origin may be increased by the expression of TF on their membrane (Owens and Mackman, 2011). Our data demonstrate that MP

1 generated upon exposure of PBMC and HUVEC to SRM1648a carry functionally active TF
2 and can therefore trigger the activation of the extrinsic pathway of blood coagulation, thus
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4 contributing to thrombotic disorders. Of potential interest in this context, an abnormal
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6 activation of the coagulation process has also been described in idiopathic pulmonary
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8 fibrosis (Chambers, 2008) and we have recently described a potential role for
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10 procoagulant, TF bearing MP in this disease (Novelli et al., 2014).
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13 Flow cytometry is a well established method of MP analysis and has been used
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15 successfully by many Authors, including ourselves (Cordazzo et al., 2013, Amabile et al.,
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17 2014, Thomashow et al., 2013). The approach, however, carries significant theoretical
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19 problems due to the small size of the vesicles that are of the same order of magnitude as
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21 the incident wavelength (Zwicker et al., 2009, Headland et al., 2014), and it is likely that
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23 only bigger MP are accurately recognized. Furthermore, in the experiments reported in this
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25 work large PM could not be consistently removed from the samples and clogging of the
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27 flow cytometer was observed. For these reasons, we used nanosight technology as an
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29 alternative approach to confirm the main data. The results indicate that both PBMC and
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31 HUVEC shed vesicles with a diameter that ranges from 50 to 500 nm, therefore consistent
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33 with MP, and that exposure to PM increase the number of events.
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37 Experimental evidence indicates that peaks of particulate air pollution correlate with
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39 increased morbidity and mortality from respiratory and cardiovascular causes (Pelucchi et
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41 al., 2009), (Franklin et al., 2015). Epidemiological studies have shown an increase in
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43 circulating procoagulant and proinflammatory MP after exposure to airborne pollutants
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45 (Emmerechts et al., 2012). Our data provide mechanistic bases to the observation.
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49 Furthermore, a better understanding of the mechanisms of MP generation might prove
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51 useful for a pharmacological modulation of their detrimental effects.
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4. Legends to the figures

1
2 Figure 1 - Dose response curve of MP generation by PBMC (A) and HUVEC (B) upon
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4 incubation with SRM1648a (1 h). Data from one experiment representative of three for
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6 each cell type.
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9 Figure 2 - MP generation by PBMC (A) and HUVEC (B) upon incubation with SRM1648a
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11 (500 µg/mL; 1 h). *p<.05 for SRM1648a-treated cells compared with baseline (ANOVA);
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13 n=4.
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16 Figure 3 - Time-response curve for MP generation by PBMC (A) and HUVEC (B) upon
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18 incubation with SRM1648a (500 µg/mL). Hatched bars: unstimulated cells; solid bars: PM
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20 stimulated cells. *p<.05 for SRM1648a-treated cells compared with baseline (ANOVA);
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22 n=4
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25 Figure 4 - MP size measurement and quantification. A) size distribution of PBMC MP
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27 assessed by Nanosight in untreated control and after SRM1648a stimulation (1h). B) size
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29 distribution of HUVEC MP assessed by Nanosight in untreated control and after
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31 SRM1648a stimulation (1 h). Solid line: unstimulated cells; dotted line: PM stimulated cells.
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35 Figure 5 - Comparison of intracellular calcium concentration in PBMC (A) and HUVEC (B)
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37 in baseline conditions (open circles) and after SRM1648a (500 µg/mL) treatment (solid
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39 circles), as assessed by Fluo4-NW incorporation (RFU: relative fluorescence units); n= 3
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43 Figure 6 - MP generation, expressed as PS concentration, by PBMC (A) and HUVEC (B)
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45 incubated in the absence and in the presence of SRM1648a (1 h; 500 µg/mL) and U73122
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47 (1 µM; 30 minutes preincubation). * p<.05 for SRM1648a treated cells in the presence of
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49 U73122 compared with SRM1648a-treated cells in the absence of the inhibitor; (ANOVA
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51 analysis with Tukey post-test); n=4.
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54 Figure 6 - SRM1648a induces the generation of TF bearing MP by PBMC (A) and HUVEC
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56 (B). Cells were incubated with SRM1648a (500µg/mL; 1 h).The supernatants were then
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tested for TF activity with a one-stage clotting assay. *** $p < .001$ for SRM1648a-treated cells compared with baseline, $n=3$ for PBMC and $n=6$ for HUVEC (Student's paired t-test).

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- Exposure of cells to particulate causes shedding of prothrombotic microparticles
- The phenomenon is mediated by calcium mobilization
- Microparticles could link exposure to particulate to cardiopulmonary morbidity

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