Toxicology in Vitro 27 (2013) 551–559

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Season linked responses to fine and quasi-ultrafine Milan PM in cultured cells

E. Longhin [a,*], E. Pezzolato [a], P. Mantecca [a], J.A. Holme [b], A. Franzetti [a], M. Camatini [a], M. Gualtieri [a]

[a] Polaris Research Center, Department of Environmental Science, University Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy
[b] Division of Environmental Medicine, Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, N-0403 Oslo, Norway

ARTICLE INFO

Article history:
Received 8 February 2012
Accepted 23 October 2012
Available online 16 November 2012

Keywords:
PM
A549
THP-1
ROS
DNA damage

ABSTRACT

Exposure to urbane airborne particulate matter (PM) is related to the onset and exacerbation of cardiovascular and respiratory diseases. The fine (PM1), and quasi-ultrafine (PM0.4) Milan particles collected during different seasons have been characterised and the biological effects on human epithelial lung A549, monocytes THP-1 cells and their co-culture, evaluated and compared with the results obtained on the PM10 and PM2.5 fractions. Chemical composition and transmission electron microscopy (TEM) analysis of PM0.4 showed that this fraction was very similar to PM1 for biological responses and dimension. All the winter fractions increased within 1 h the level of reactive oxygen species (ROS), while only summer PM2.5 had this effect on A549 cells. The phosphorylation of H2AX (γH2AX), a marker of double strand DNA breaks (DSBs), was increased by all the winter fractions on A549 and THP-1 cells while summer PM samples did not induced this effect. PM0.4 and PM1 biological effects are partly similar and related to the season of sampling, with effects on ROS and DNA damage induced only by winter PM fractions. The winter PM damaging effect on DNA correlates with the presence of organic compounds.

1. Introduction

The relationship between the exposure to airborne particulate matter (PM) and the onset of adverse effects on human health has been widely described in many epidemiological studies on the increased rates of mortality and morbidity in the population for cardiovascular and respiratory diseases (Brook et al., 2010; de Kok et al., 2006); moreover there is increasing evidence that PM2.5 exposure increases lung cancer mortality (Pope et al., 2011; Turner et al., 2011). Pollution-related cardio respiratory effects have been linked to the presence of a pulmonary and systemic inflammatory status, evidenced by the augmented levels of pro-inflammatory cytokines, in the circulating blood of PM exposed population (Pope and Dockery, 2006). *In vitro* studies on various lung cell lines have used different biological endpoints to demonstrate such correlation. It has been demonstrated that PM from different sources and places can promote the release of inflammatory mediators (Alfaro-Moreno et al., 2002; Hetland et al., 2004), genotoxic effects (Billet et al., 2008; Don Porto Carero et al., 2001; de Kok et al., 2005) and cell death (Alfaro-Moreno et al., 2002; Hsiao et al., 2000). Cell viability, pro-inflammatory proteins release, ROS production and DNA DSB induction have been here analysed to evaluate the effects produced by exposure to different PM fractions, variable for dimension and season of sampling.

In fact, the structure and composition of PM are complex and heterogeneous, and influence the biological properties of particles. The chemical composition varies with season and region of sampling, photochemical-meteorological conditions and sources of emissions (Brüggemann et al., 2009; Perrone et al., 2010; Pey et al., 2010). The winter Milan PM chemical composition is characterised by high levels of polycyclic aromatic hydrocarbons (PAHs), whereas summer PM is rich of elements such As, Cr, Cu, Al and Zn (Perrone et al., 2010). These data are rather representative of urban air particles: Brüggemann et al. (2009) reported a higher organic carbon (OC) concentration during winter in comparison with summer PM samples and explained the presence of such element as the contribution of domestic heating and of a lower atmospheric mixing layer.

PM size is another important parameter influencing biological effects. PM can be classified as PM10 (particles with an aerodynamic diameter less than 10 μm), PM2.5 (Ø < 2.5 μm) and PM1 (Ø < 1 μm). The fine fractions (PM2.5 and PM1) are dominated by combustion derived particles, consisting mainly of organic and inorganic elements adsorbed onto the surface of a carbonaceous core (Brüggemann et al., 2009; Zerbi et al., 2008). The coarse fraction (PM10) contains a major part of mineral compounds and some adsorbed endotoxins (Pérez et al., 2007; Schins et al., 2004).

The PM coarse fraction (2.5–10 μm) has been associated with pro-inflammatory and cytotoxic effects (Gualtieri et al., 2010; Hetland et al., 2005; Monn and Becker, 1999; Schins et al., 2002). The presence of endotoxins (Soukup and Becker, 2001; Schins et al., 2004; Becker et al., 2005; Camatini et al., 2010) in summer

* Corresponding author. Tel.: +39 0264482928; fax: +39 0264482996.
E-mail address: e.longhin1@campus.unimib.it (E. Longhin).

0887-2333/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2012.10.018

PM10 is partly responsible of the inflammation processes, even the release of inflammatory mediators occurs also in cells exposed to fine PM. This event has been explained with the large surface area of these small particles (Hetland et al., 2004; Donaldson et al., 2001, 2002; Oberdörster, 2001).

The PM fine fraction has been associated mainly to a higher genotoxic potential (Billet et al., 2008; de Kok et al., 2005), with the winter samples being more potent (Binková et al., 2003; Chakra et al., 2007) in relation to higher PAHs and metal contents. Moreover, positive relationships were found between the formation of reactive oxygen (ROS) and nitrogen (RNS) species and the induction of DNA damage (de Kok et al., 2006; Gualtieri et al., 2011). Depending on the amount formed, ROS may result in necrosis as well as apoptosis. The most severe DNA lesions produced by ROS are double strand breaks (DSB; Mills et al., 2003), which may be detected by the phosphorylation of histone 2AX (γH2AX; Albino et al., 2009).

Besides the increasing epidemiological data on particles with a diameter less than 1 μm (quasi-ultrafine), there are still few data on the chemical composition (Kudo et al., 2011; Chen et al., 2010) and biological effects (Steenhof et al., 2011; Val et al., 2011; Jalava et al., 2007) of the ultrafine fraction (Ø < 0.1 μm) for the difficulty in sampling such particles (Furuuchi et al., 2010).

In order to fulfil this gap of knowledge on the biological responses triggered by the finest PM fractions on human cell lines, we extended our study to the effects produced by Milan summer and winter quasi-ultrafine PM0.4 and PM1 on A549 and THP-1 cells and on the co-culture of these two lines.

2. Materials and methods

2.1. PM sampling, physical and chemical characteristics

PM particles were collected at Milan Torre Sarca, a representative background site for air quality, during summer (June and July) and winter (January and February) of 2008–2009. A low volume gravimetric sampler (EU system, FAI Instruments, Rome, Italy) was used to collect PM samples on Teflon or quartz filter for biological analysis and chemical characterisation respectively. PM fractions were defined by sampling device cut-point convention, that means particles with an aerodynamic diameter lower than the indicated value (e.g. PM1 is constituted by particles smaller than 1 μm; EPA, 2004). Particles were characterised for elements (mineral dust and trace elements), polycyclic aromatic hydrocarbons (PAHs), inorganic ions and total carbon as previously described (Perrone et al., 2010; Camatini et al., 2010; Gualtieri et al., 2009).

Particles to be used for biological experiments were extracted from Teflon filters by sequential sonications in a Soltec water-bath (SONICA, four cycles of 20 min each) in sterile water. This procedure allows the mechanical detachment from filters of the particles together with the adsorbed compounds, regardless of their solubility in water, assuring the similarity among the extracted particles and the pristine ones. The efficiency of PM extraction from filters (PM mass extracted compared to the PM total mass) was approximately 75% and this high value sustains the similarity of the extracted particles to the original ones.

Particle suspensions were dried into a desiccator, weighed and stored at −20 °C until use and re-suspended in sterile water at a final concentration of 2 μg/μl, just before use.

2.1.1. Particle morphological characterisation

Extracted particles (PM0.4 and PM1) were prepared for TEM as reported (Gualtieri et al., 2009). Briefly, aliquots of resuspended particles were pipetted onto Formvar® coated 200mesh copper grids and air-dried. Grids were analysed by a Jeol JEM-1220 TEM equipped with a CCD camera and digital images taken at a magnification of 20k. A total of 300 among single particles and particles aggregates for each sample were measured and particle size distribution was obtained. Particles were counted in the form they appeared, this means as single particles if they were alone or as aggregates when they appeared clustered. When considering particles aggregates, the major length of the aggregate was measured and used to develop the size distribution graph.

2.2. Endotoxin content and microbiological molecular characterisation

Endotoxin content in PM was determined by a quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (International P.B.I. S.p.A., Italy) following the manufacturer's instructions. Briefly, PM extracts were diluted in apyrogenic water to the concentration of 10 μg/ml, mixed with pyrochrome LAL reagent and incubated at 37 °C for 40 min. The reaction was then stopped with 5% acetic acid and the absorbance of the samples was determined by a spectrophotometer (Ascent scan multiplate reader, Thermo Scientific) at 405 nm. The concentration of endotoxin was calculated from a standard curve of LPS (*Escherichia coli*). The endotoxin concentrations were expressed as endotoxin units per milligram (EU/mg) of tested particles.

The molecular characterisation of the microbial community associated to a representative summer PM10 sample collected in September 2009 was carried out by 16S rRNA-tag sequencing using Illumina GA IIx. Details of the experimental procedures and sequence data are provided as Supplementary data.

2.3. Cell culture and treatment

The human lung type II pneumocytes, A549 cell line, and the human monocytes, THP-1 cell line (ATCC, Rockville, MD, USA), were maintained in Opti-MEM medium (Invitrogen) supplemented with 10% foetal bovine serum (FBS), 100 μg/ml penicillin and 100 U/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. A549 were seeded at a concentration of 70,000 cell/well in 12 well-plates (or 150,000 cells/well in 6 well-plates). The next day, 80% sub-confluent cells were treated with 10 μg/cm^2 of PM. THP-1 cells (150,000 cells/well) were seeded in 12 well-plates and immediately treated with 10 μg/cm^2 of PM. Co-culture experiments were performed in 12 well-plates, THP-1 (150,000 cells/well) were added 24 h after seeding A549 cells and treatment performed afterwards. For each experiment, untreated control cells were carried together.

The exposure dose here used was selected considering the lowest effect dose, as previously reported (Gualtieri et al., 2009).

2.4. Cytotoxicity

Cell membrane integrity was used as a marker of cytotoxicity and measured after 24 h of treatment by LDH release in the culture medium. The LDH colorimetric assay (TOX-7, Sigma Aldrich; Italy) was performed following the manufacturer's instructions: 100 μl of culture medium were added to 200 μl of LDH substrate and incubated at room temperature for 30 min in the dark. The reaction was terminated by the addition of 30 μl of 1 N HCl to each well. Samples were read by the Multiscan Ascent multiplate reader at 490 nm. The maximum LDH release was measured by lysing the cells for 15 min with the lysis solution provided by the LDH kit, and this value was used to calculate the percentage of living cells according to the kit instructions.

2.5. Cytokine release

After 24 h of treatment, cell culture media were collected and centrifuged for 10 min at 250 g to remove cell debris and floating