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### ► To cite this version:

J. Deweirdt, J.F. Quignard, B. Crobeddu, A. Baeza-Squiban, J. Sciare, et al.. Involvement of oxidative stress and calcium signaling in airborne particulate matter - induced damages in human pulmonary artery endothelial cells. *Toxicology in Vitro*, 2017, 45, pp.340-350. 10.1016/j.tiv.2017.07.001 . hal-02439655

**HAL Id: hal-02439655**

**<https://hal.science/hal-02439655>**

Submitted on 16 Nov 2022

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## Involvement of oxidative stress and calcium signaling in airborne particulate matter - induced damages in human pulmonary artery endothelial cells

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Keywords: particulate matter, PM<sub>2.5</sub>, pulmonary artery endothelial cells, free radicals, reactive oxygen species, intracellular calcium, calcium imaging

Abbreviations: PM, particulate matter; PH, pulmonary hypertension; HPAEC, human pulmonary artery endothelial cells; ROS, reactive oxygen species; PAHs, polycyclic aromatic hydrocarbons

### 1. Introduction

Many epidemiological studies have revealed an association between airborne particulate matter (PM) pollution and an excess in cardiovascular and respiratory mortality and morbidity such as asthma, chronic obstructive pulmonary disease (COPD), cancers, myocardial infarction and pulmonary hypertension (PH) (Pope et al., 2004; 2015; Chen et al., 2014; Beelen et al., 2014). Mortality and morbidity due to respiratory and cardiovascular diseases increase by 0.5 to 1.5 % for each increase of PM in the air of 5-6  $\mu\text{g}/\text{m}^3$  (Lee et al., 2014). PM is a complex mixture of particles from different sources and different sizes (coarse particles: PM<sub>10</sub> with a mean aerodynamic diameter less than 10 micron, fine particles PM<sub>2.5</sub> with a mean aerodynamic diameter less than 2.5 micron and ultrafine particles: PM<sub>0.1</sub> with a mean aerodynamic diameter less than 0.1 micron). These particles also have different chemical compositions thus leading to different toxic effects. Transition metals (such as iron and copper), organic compounds such as polycyclic aromatic hydrocarbons (PAHs), quinones... and microorganisms (bacteria, fungi ...) can be adsorbed onto pre-existing particles (Thomson et al., 2016). Traffic emissions (fossil fuel combustion from mobile sources) are considered as the major health-relevant particulate pollution sources in industrialized urban centres (Hopke et al., 2006) and recent studies have shown that the fine PM fraction of these emissions is mainly involved in the increased incidence of respiratory and cardiovascular diseases as compared to other atmospheric pollutants (Newby et al., 2015).

The respiratory tract is the first target of inhaled particles. However, the cardiovascular system and, more specifically, endothelial cells lining the inner surface of blood arteries that are involved in the vasomotricity regulation can also be a direct target of inhaled particles or some of their soluble constituents. In humans and animals, the finest inhaled particles may accumulate into pulmonary alveoli and the lung parenchyma close to pulmonary arterial wall. These particles may then cross the epithelial barriers and reach the pulmonary circulation, in particular the artery endothelial cells, where they can exert their deleterious effects on vascular function by increasing oxidative stress (Calderón-Garcidueñas et al., 2001; Kreyling et al., 2002; Nemmar et al., 2002; 2013; Mills et al., 2009). Alternatively, effects on endothelial cells can also be indirect resulting from secretions of pro-inflammatory mediators produced by bronchial epithelial cells. In previous studies, it has been shown that particulate pollution could alter the vascular reactivity by inducing impairment of endothelial NO-dependent relaxation in intrapulmonary arteries (Courtois et al., 2008, 2010; Barrier et al., 2016) that may be responsible for an increase in systemic or pulmonary blood pressure (Urch et al., 2005; Calderón-Garcidueñas et al., 2007; Mills et al., 2011; Mahne et al., 2012; Bellavia et al., 2013; Morishita et al., 2015). As a consequence, the accumulation and retention of PM could be determining factors for exacerbation of pre-existent cardiovascular diseases such as pulmonary hypertension (PH). PH is the main disease of the pulmonary circulation characterized by increase of pulmonary arterial resistances, remodeling of the pulmonary arteries, changes in pulmonary vascular reactivity, right heart hypertrophy and heart failure. Oxidative stress and alteration in calcium signaling are critical events involved in the physiopathology of PH (Touyz et al., 2004; Guibert et al., 2007; Ducret et al., 2010; Martin et al., 2012; Freund - Michel et al., 2013; Xu et al., 2014; Gilbert et al., 2014; Lai et al., 2015; Parpaite et al., 2016).

Moreover, after inhalation, particles trigger an oxidative stress and alter calcium homeostasis with overproduction of reactive oxygen species (ROS), often associated with a pulmonary pro-inflammatory response, which is also a characteristic of PH (Stone et al., 2000; Chen et al., 2011; Dubes et al., 2017). Size and chemical composition are the main determinants of the PM-induced molecular and cellular effects via oxidative stress. The finest particles are associated with an increased surface reactivity that is involved in intrinsic free radical production (Ayres et al., 2008). Transition metals and organic compounds which are present in PM are known to produce oxidative stress respectively via the Fenton reaction or by the redox cycling (such as the superoxide anion and the reactive hydroxyl radical) and can induce oxidative lesions in cellular macromolecules (Donaldson et al., 1997; Squadrito et al., 2001). Moreover, previous studies have shown, that in rat pulmonary artery smooth muscle cells (PASMC), acute or chronic exposure to nanoparticles increases the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) via activation of calcium channels at the plasma membrane (Stone et al., 2000; Chen et al., 2011; Dubes et al., 2017). Despite this major role of calcium signaling and oxidative stress in the pathogenesis of PH, the effect of airborne particulate matter on these cellular targets is poorly described particularly on pulmonary artery endothelial cells.

In this context, the objectives of the present study were to assess the effects of PM<sub>2.5</sub> exposure, in human pulmonary artery endothelial cells (HPAEC), on oxidative stress and calcium signaling, which both are deregulated in PH.

HPAEC were exposed for 4 hrs to PM<sub>2.5</sub> (5 to 15 µg/cm<sup>2</sup>). Different endpoints were studied (i) PM<sub>2.5</sub> internalization (ii) intracellular and acellular production of ROS by a fluorescent probe (H<sub>2</sub>DCF-DA) (iii) intracellular and acellular production of superoxide anion by electronic paramagnetic resonance spectroscopy (EPR) (iv) production of mitochondrial superoxide anion by MitoSOX probe (confocal microscopy) (v) nitric oxide (NO) and

peroxynitrite (ONOO<sup>-</sup>) productions by Griess reaction (vi) calcium signaling using calcium imaging (fluorescent indicator dye Fluo-4) and confocal microscopy analysis.

## 2. Materials and methods

### 2.1. PM filter samples collection

Daily (24 hrs-integrated) PM<sub>2.5</sub> was sampled continuously on 47-mm diameter Teflon filters, during September and October 2013, at a traffic urban site (“Porte d’Auteuil”) in Paris with a low volume Leckel (2.3 m<sup>3</sup>/h) air sampler (Leckel, Germany) (Sciare et al., 2011).

Samples have been taken in this period in order to target a period that is usually optimal for sampling PM emissions from traffic (high pressure systems with low precipitation) and to avoid the winter period that may contain, out of traffic emissions, an additional source of combustion aerosol (domestic wood burning).

### 2.2. Extraction of PM filter samples

PM<sub>2.5</sub> Teflon filters were extracted with methanol, sonicated (Branson Digital sonifier) during 30 sec at 50 % (200 W), reduced in volume using evaporator set (Concentrator plus, Eppendorf) at 30 °C, until about 200 µL was left, and then dried overnight at 30 °C under a constant flow of nitrogen, according to the method adapted from Janssen et al. (2014). Dry extracts were resuspended in supplemented Endothelial Cell Growth Medium (ECGM without phenol red) supplemented with streptomycin (0.1 µg/mL), penicillin (100 U/mL) and fungizone (0.25 µg/mL). PM<sub>2.5</sub> suspension stocks were stored at -20 °C until use and, immediately prior to use, stock suspensions were vortexed and freshly resuspended by sonication, 3 x 30 s (Vibracell 75186, 130 W, 56 - 60 Hz).

### 2.3. Cell culture

All experiments were done on HPAEC cultures isolated from pulmonary artery main branch of a 23-year old male Caucasian donor. HPAEC were purchased from PromoCell® (France) and grown in ECGM supplemented with a Supplement Mix [2 % fetal bovine serum (FBS), 0.4 % endothelial cell growth factor (ECGF), 0.1 ng/ml epidermal growth factor (EGF), 1 ng/ml basic fibroblast growth factor (BFGF), 90 µg/ml heparin, and 1 µg/ml hydrocortisone, PromoCell®]. Cells were seeded at 10,000 cells/cm<sup>2</sup> on 25 cm<sup>2</sup> culture flasks and were maintained at 37 °C, in a 95 % humidified air and 5 % CO<sub>2</sub>. Cell passages were performed when about 80 % confluence was achieved. All experiments were done on HPAEC from passages 2 to 8.

### 2.4. Cytotoxicity assay

Cell viability was assessed using the WST-1 (Water-Soluble Tetrazolium) cell proliferation reagent (Roche, France) according to the manufacturer’s recommendations. It is a colorimetric method for determining mitochondrial metabolic activity (viable cells) in which a succinate-tetrazolium reductase enzyme can convert a tetrazolium salt compound (WST-1) into an aqueous soluble and coloured formazan. This assay assumes that the relative number of viable cells is proportional with the mitochondrial enzymatic activity. Cells were seeded in a 96-well culture plate at 20,000 cells/cm<sup>2</sup> and cultured in supplemented ECGM for 24 hrs at 37 °C and 5 % CO<sub>2</sub>. Cells were exposed or not (control cells) to PM<sub>2.5</sub> in a concentration range from 1 to 10 µg/cm<sup>2</sup>.

After a 24 hrs-treatment with PM<sub>2.5</sub>, cells were rinsed with ECGM and incubated for 3 hrs with WST-1 reagent. The absorbance of supernatants was measured at 450 nm by spectrophotometry using a microplate spectrophotometer reader (SPECTROstarNano2.10, BMG Labtech®, Germany). The working concentrations for all further experiments were determined by this test (mortality rate less than 25 %).

## 2.5. Internalization studies

Cells were seeded at 20,000 cells/cm<sup>2</sup> on plastic Lab-Tek chambers (Dutscher, France) and cultured in supplemented ECGM, for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. Cultures were then exposed or not (control cells) to PM<sub>2.5</sub> at 7.5 µg/cm<sup>2</sup>, at 37 °C and 5 % CO<sub>2</sub>. After a 1h-exposure to PM<sub>2.5</sub>, cells were fixed with 1.6 % paraformaldehyde and 2 % glutaraldehyde in 0.08 M cacodylate buffer (pH 7.4) during 2 hrs at room temperature (RT). After washing in 0.1 M cacodylate buffer, cells were post-fixed in a mix 1 % osmium tetroxide (v/v)/ 1 % potassium ferricyanide K<sub>3</sub>Fe(CN)<sub>6</sub> (p/v) in 0.1 M cacodylate buffer during 2 hrs on ice in the dark. Then, samples were washed in water, and stained in block in 0.5 % aqueous uranyl acetate solution during 30 min at RT in the dark. Subsequently, cells were washed and dehydrated through a series of graded ethanol and embedded in a mixture of pure ethanol and epoxy resin 50/50 (v/v) (Epon 812; Delta Microscopie, Toulouse, France) during 2 hrs and then in 100 % resin overnight at RT. The polymerization of the resin was carried out during 48 hrs at 60 °C.

Samples were then sectioned using a diamond knife (Diatome, Biel-Bienne, Switzerland) on an ultramicrotome (EM UCT, Leica Microsystems, Vienna, Austria). Ultrathin sections (65 nm) were picked up on copper grids and then stained with Uranylless (Delta Microscopies, Toulouse, France) and lead citrate. Grids were examined with a Transmission Electron Microscope (H7650, Hitachi, Tokyo, Japan) at 80 kV.

## 2.6. Global reactive oxygen species production

### 2.6.1. Acellular global ROS production

The global ROS were detected using the CM-H<sub>2</sub>DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceine diacetate, acetyl ester) assay (Fisher Scientific®) according to the manufacturer's recommendations and using the method described by Foucaud et al. (2007) modified for this study.

Just before use, the CM-H<sub>2</sub>DCFDA probe must be hydrolyzed. To do so, 50 µg were resuspended with a mix of absolute ethanol and a 10 mM sodium hydroxide solution (final concentration of the probe 66 µM). After a 30 min activation in the dark, at room temperature, the chemical reaction was stopped by adding PBS 10X (final concentration of the probe 16.5 µM).

Finally, the activated CM-H<sub>2</sub>DCF probe was added into the wells of a 96-well plate containing either H<sub>2</sub>O<sub>2</sub> standard in the presence of FeSO<sub>4</sub> (10 µM) or PM<sub>2.5</sub> suspension to obtain a final concentration of 3.3 µM CM-H<sub>2</sub>DCF probe. Then, samples were incubated, for 1 hr, at 37 °C and 5 % CO<sub>2</sub>. Thus, final concentrations of H<sub>2</sub>O<sub>2</sub> standard range were 1-150 µM, and final concentrations of PM<sub>2.5</sub> were 1-50 µg/ml. Fluorescence intensity was measured by spectrofluorimetry at  $\lambda_{ex}$ =485 nm and  $\lambda_{em}$ =530 nm using a FLUOstar Omega 2.10 plate reader (BMG Labtech®) and the analyses were performed with the MARS Data Analysis Software 2.30 R3 (BMG Labtech®).

### 2.6.2. Intracellular global ROS production

Intracellular ROS production was assessed using the CM-H<sub>2</sub>DCFDA probe (Fisher Scientific®) according to the manufacturer's recommendations and using the method adapted from Wang and Joseph (1999) and Chen et al. (2010). The non-fluorescent CM-H<sub>2</sub>DCFDA reagent rapidly diffuses through the cell membrane and is hydrolyzed by intracellular esterases to a non-fluorescent oxidative sensitive form (CM-H<sub>2</sub>DCF) which is oxidized by ROS to generate a highly fluorescent adduct with a fluorescent intensity proportional to intracellular ROS production.

HPAEC cells were seeded at 50,000 cells/cm<sup>2</sup> in a 24-well plate and cultured for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. Cells were rinsed with Hank's Balanced Salt Solution (HBSS) and were pre-incubated for 20 min with CM-H<sub>2</sub>DCFDA probe (final concentration 20 µM) in HBSS. Cells were then rinsed with HBSS before a 4 hrs-exposure with PM<sub>2.5</sub>, at different concentrations (5-15 µg/cm<sup>2</sup>), at 37 °C. Fluorescence intensity was measured at  $\lambda_{ex}=485$  nm and  $\lambda_{em}=520$  nm in spectrofluorimetry by using FLUOstar Omega 2.10 plate reader and the analyses were performed with the MARS Data Analysis Software 2.30 R3 (BMG Labtech®).

## 2.7. Superoxide anion detection by electron paramagnetic resonance (EPR)-spin trapping

EPR spin-trapping is one of the specific technique used to study generation of free radicals such as the superoxide anions. However, their high activity, their very short life span and their relative low concentrations made their measurement difficult to develop. The detection of O<sub>2</sub><sup>•-</sup> was carried out using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) probe (Noxygen®) which could be oxidized by O<sub>2</sub><sup>•-</sup> to generate a stable nitroxide radical (CM<sup>•</sup>) easily detectable by EPR spectroscopy. The CMH probe has the property of being soluble and can measure intra and extracellular superoxide anion (Dikalov et al., 2011; Könczöl et al., 2012).

### 2.7.1. Acellular superoxide anion production

Superoxide anion was detected using the method described by Dellinger et al. (2001) and Nymark et al. (2014) modified for this study. A PM<sub>2.5</sub> range of 25 to 250 µg/ml was prepared in ECGM. A Krebs-HEPES Buffer solution (118.4 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 4 mM NaHCO<sub>3</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 10 mM HEPES and 6 mM D-Glucose; adjusted at pH=7.4) was prepared and just before use was filtered and put under a constant flow of nitrogen. Finally, PM<sub>2.5</sub> (1-50 µg/ml final concentration) were incubated, in a 24-well plate for a 1 hr-incubation, at 37 °C, 5 % CO<sub>2</sub>, with the spin-probe mix containing at final concentrations, CMH probe (1,25 mM), diethyldithiocarbamate (6,25 µM) and deferoxamine (31,25 µM) solubilized in the Krebs-HEPES Buffer solution. The mix was then removed in a syringe and the reaction was stopped by freezing the samples in liquid nitrogen before EPR analysis.

### 2.7.2. Cellular superoxide anion production

EPR assay was performed as previously described (Billaud et al., 2009; Deschacht et al., 2010; Dikalov et al., 2011) and was adapted for this study. HPAEC cells were seeded at 50,000 cells/cm<sup>2</sup> in a 12-well plate and cultured for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. After a 4 hrs-exposition with PM<sub>2.5</sub>, at different concentrations (5-15 µg/cm<sup>2</sup>), at 37 °C and 5 % CO<sub>2</sub>, cells were incubated for 20 min with the spin-probe mix containing CMH (1 mM), diethyldithiocarbamate (5 µM) and deferoxamine (25 µM) in Krebs-HEPES Buffer solution. Then, cells were scraped, homogenized and frozen in a syringe in liquid nitrogen before EPR analysis. All the EPR spectra were recorded using a Spectrometer X Miniscope MS200

(Magnettech®, Berlin, Germany). The following EPR conditions were used: benchtop spectrometer working at X-band (9.4 GHz) with a B0 fields 3339.4 G, B0 sweep 293.84 G, sweep time 60 sec, modulation amplitude 0.5, modulation frequency 100 kHz, modulation 5000 G, microwave attenuation 10 dB, gain 100. Spectra were quantified by peak amplitude measurement using the Miniscope software (control 6.51 and Analysis 2.02).

The results were normalized to protein quantities and expressed in EPR signal amplitude in arbitrary units (AU)/mg/ml of protein. After the reading of EPR spectra, protein quantities were measured by a Lowry-test (Lowry reagent, Sigma Aldrich) according to the manufacturer's recommendations.

## 2.8. Mitochondrial superoxide anion generation by confocal microscopy

Mitochondrial superoxide anion  $O_2^{\cdot-}$  formation was assessed using MitoSOX™ red dye (ThermoFischer®) according to the manufacturer's recommendations. MitoSOX probe oxidation by superoxide anion produces a red fluorescence. Fluorescence intensity is proportional to the rate of the probe oxidation (Tseng et al., 2015a).

HPAEC cells were seeded at 20,000 cells/cm<sup>2</sup> in a 96-well plate and cultured for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. After a 4 hrs-exposition with PM<sub>2.5</sub>, at 37 °C and 5 % CO<sub>2</sub>, at different concentrations (5 - 15 µg/cm<sup>2</sup>), cells were incubated for 10 min at 37° C, in the dark, with the MitoSOX™ probe (final concentration 5 µM) in a physiological salt solution (PSS) containing 130 mM NaCl, 5.6 mM KCl, 8 mM HEPES, 11 mM Glucose, 1 mM Mg<sup>2+</sup> and 2 mM Ca<sup>2+</sup>; adjusted at pH=7.4. In some experiments, MitoSOX™ probe was co-incubated with a mitochondrial probe (Mitotracker green - final concentration 1 µM) and a nucleus probe, Hoeschst 33342 (final concentration 2 µM) for colocalization experiments.

The plates were observed at X600 magnification with an oil immersion objective on a laser scanning confocal microscope (TE 2000, Nikon). Fluorescence intensity were measured for MitoSOX at 543/605 nm, for Mitotracker at 488/515 nm and for Hoeschst at 408/450 nm (excitation/emission). The results are expressed in fluorescence intensity in absolute values.

The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

## 2.9. Nitric oxide and peroxynitrite productions

Nitric oxide (NO) and peroxynitrite (ONOO<sup>-</sup>) productions were assessed in HPAEC culture supernatants by Griess reaction according to the manufacturer's instructions (Molecular probes). HPAEC cells were seeded, in a 24-well culture plate at 20,000 cells/cm<sup>2</sup> and cultured for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. After a 4 hrs-exposure to PM<sub>2.5</sub> (5 - 15 µg/cm<sup>2</sup>) the supernatants were collected and centrifuged at 1000 g for 15 min for the NO determination. Levels of NO were quantified by measuring the stable metabolite of nitrite with Griess reagent according to the manufacturer's instructions (Molecular probes).

Supernatants were also used for the measurement of ONOO<sup>-</sup>. As peroxynitrites are rapidly transformed to nitrates, supernatants were first incubated with nitrate reductase (Cayman chemicals) to convert nitrates into nitrites. Levels of ONOO<sup>-</sup> were then quantified from nitrites with Griess reagent according to the manufacturer's instructions (Molecular probes).

The absorbance of supernatants was measured at 550 nm by spectrophotometry using a microplate spectrophotometer reader (SPECTROstarNano2.10, BMG Labtech®, Germany).

Previously a standard curve was obtained using known concentrations of sodium nitrite (NaNO<sub>2</sub>) and results were expressed in nanogram per microliter (ng/µl).

### 2.10. Cytosolic calcium measurement by confocal microscopy

Intracellular  $[Ca^{2+}]$  was detected using the Fluo-4-AM dye (1-[2-amino-5-(2,7-difluoro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester) (ThermoFischer®). We first investigated the effect of  $PM_{2.5}$  on Fluo4 fluorescence with an acellular assay. Our results showed that  $PM_{2.5}$  did not interfere with the Fluo4 calcium assay (data not shown). Cells were seeded at 20,000 cell/cm<sup>2</sup> in a 96-well plate and cultured for 24 hrs, at 37 °C and 5 % CO<sub>2</sub>. HPAEC were exposed to the PM suspension (5-15 µg/cm<sup>2</sup>) for 4 hrs. After the exposure, cells were incubated for 30 min (in the dark) at 37 °C with 1 µM Fluo-4-AM probe in the PSS. Cells were rinsed three times with the PSS and 100 µl of this solution was added in each well for measurement by confocal laser microscope.

The plates were observed at X200 magnification with an oil immersion objective. Cells were stimulated at 488 nm and emission was recorded at 515 nm. The results are expressed in fluorescence intensity in absolute values. The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

### 2.11. Statistical analysis

Each experimental condition was independently repeated three times and for each independent experiment 3 to 8 wells per condition were done. Data were expressed as mean ± Standard Error of the Mean (SEM) for 3 independent experiments (n=3). Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by Mann-Whitney-U-test for multiple comparisons (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001) with GraphPad statistics software. P values < 0.05 were considered significant.

## 3. Results

### 3.1. $PM_{2.5}$ increased intrinsic ROS production

The ability of  $PM_{2.5}$  to generate intrinsic reactive oxygen species (ROS) was investigated through two different methods: oxidation of the CM-H<sub>2</sub>DCFDA probe detected by fluorimetry and EPR technique to specifically measure superoxide anion.

$PM_{2.5}$  induced a global ROS production in acellular condition (Fig. 1A). After a 1 hr-incubation with the probe,  $PM_{2.5}$  (1-50 µg/ml) induced, in a concentration-dependent manner, a statistical significant increase of global ROS levels from 5 µg/ml (p<0.001).

Furthermore, after a 1 hr-incubation with the spin probe,  $PM_{2.5}$  (1-50 µg/ml) also induced specifically, and with a concentration-dependent pattern, a significant increase of acellular O<sub>2</sub><sup>•-</sup> levels (p<0.001), (Fig. 1B).

### 3.2. $PM_{2.5}$ decreased HPAEC viability

To measure ROS production and intracellular calcium levels on HPAEC, we investigated first the non-toxic range of  $PM_{2.5}$  (mortality rate less than 25%) that could be used based upon the WST-1 test. Cytotoxic assay was performed after a 24 hrs-treatment with PM.

Fig. 2 shows, that exposure of HPAEC to  $PM_{2.5}$  (1 to 10 µg/cm<sup>2</sup>) for 24 hrs, decreased concentration - dependently cell viability. Statistical significant reduction of cell viability was observed from 7.5 µg/cm<sup>2</sup> (p<0.01, p<0.001). At the highest concentration (10 µg/cm<sup>2</sup>), cell viability was decreased to no more than 23.25 % of control cells.

### 3.3. $PM_{2.5}$ internalization in HPAEC



Transmission electronic microscopy (TEM) was performed to obtain information on the uptake and localization of PM<sub>2.5</sub> in HPAEC exposed for 1 hr to PM<sub>2.5</sub> (7.5 µg/cm<sup>2</sup>). Representative micrographs are shown in Fig. 3. After a rapid internalization into cells, fine particles were mainly clustered into vesicles (Fig. 3B, Fig. 3C) but were also remained free in the cytoplasm (data not shown). Internalized PM was mainly organized in aggregates of various sizes but single or isolated particles were also observed. TEM images evidenced that PM was also localized very close to mitochondria (Fig. 3C), to the nucleus (Fig. 3B) and to the cytosolic membrane (data not shown).

### 3.4. PM<sub>2.5</sub> increased HPAEC intracellular ROS production

Intracellular ability of PM<sub>2.5</sub> to generate oxidative stress in cells was investigated through four different methods: oxidation of the CM-H<sub>2</sub>DCFDA probe detected by fluorimetry for the evaluation of the global intracellular ROS production, the EPR technique to specifically determine PM<sub>2.5</sub>-induced superoxide anion generation, the MitoSOX probe to measure more specifically oxidative stress produced in particular organelles such as mitochondria and the Griess reaction for the determination of NO and reactive nitrosative species (RNS) such as ONOO<sup>-</sup>.

#### 3.4.1. Intracellular global ROS production (CM-H<sub>2</sub>DCF-DA)

Fig. 4 shows the concentration-dependent increase of ROS production by HPAEC in response to a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>). H<sub>2</sub>O<sub>2</sub> (1 mM) was used as positive control. PM<sub>2.5</sub> induced, with a concentration-dependent pattern, an increase of intracellular global ROS levels in HPAEC. At the highest concentrations (10 and 15 µg/cm<sup>2</sup>), the differences were significant as compared to control cells (respectively at p<0.01, p<0.001).

#### 3.4.2. Intracellular superoxyde anion (O<sub>2</sub><sup>•-</sup>) production (EPR)

We have previously shown that PM<sub>2.5</sub> induced intrinsic O<sub>2</sub><sup>•-</sup> production (Fig. 1B) and intracellular ROS generation (Fig. 4). We investigated here by EPR the ability of these particles to generate O<sub>2</sub><sup>•-</sup> production in HPAEC. A 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) induced, in a concentration-dependent manner, a statistical significant increase of intracellular O<sub>2</sub><sup>•-</sup> levels in HPAEC from 7.5 µg/cm<sup>2</sup> (p<0.01, p<0.001) (Fig. 5).

#### 3.4.3. PM<sub>2.5</sub> increased mitochondrial superoxyde anion O<sub>2</sub><sup>•-</sup> production (MitoSOX probe)

We assessed the effects of PM<sub>2.5</sub> on mitochondrial O<sub>2</sub><sup>•-</sup> production by MitoSOX red probe. Fig. 6A shows, in HPAEC, the concentration-dependent increase of mitochondrial O<sub>2</sub><sup>•-</sup> production by HPAEC in response to a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>). Our results show that a 4 hrs-exposure to PM<sub>2.5</sub> induced for all concentrations tested and in a concentration-dependent manner, a statistically significant rise in mitochondrial O<sub>2</sub><sup>•-</sup> production in HPAEC compared to cell controls (p<0.001). Figures 6B and 6C show HPAEC cell imaging by confocal microscopy displaying an increase of mitochondrial MitoSOX probe red oxidation compared to control cells when cells were treated, for 4 hrs, with PM<sub>2.5</sub> (10 µg/cm<sup>2</sup>). Some co-localization (yellow) between mitochondria (mitotracker green fluorescence) and O<sub>2</sub><sup>•-</sup> (mitoSOX red fluorescence) were observed (Fig. 6C). Nuclei were stained with Hoechst (blue fluorescence).

#### 3.4.4. PM<sub>2.5</sub> increased peroxynitrites production (Griess reaction)

The effects of PM<sub>2.5</sub> on NO production were first examined. Our results showed that a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) induced in HPAEC with a concentration-dependent pattern, an increase of NO levels. At the highest concentrations (10 and 15 µg/cm<sup>2</sup>), the differences were significant as compared to control cells (p<0.05\*) (Fig. 7A). We have previously shown that, after a 4 hrs-exposure, PM<sub>2.5</sub> induced intracellular O<sub>2</sub><sup>•-</sup> production in HPAEC (Fig. 6A). Since NO can be oxidized by O<sub>2</sub><sup>•-</sup> to form peroxynitrites (ONOO<sup>-</sup>), we measured here in HPAEC the peroxynitrite production by Griess reaction. Our results showed that a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) induced for all concentrations tested and in a concentration-dependent manner, a statistically significant rise in ONOO<sup>-</sup> production in HPAEC compared to control cells (p<0.05\* and p<0.01\*\*) (Fig. 7B). After a 1 hr-pre-treatment of HPAEC with antioxidants enzymes such as polyethylene glycol-superoxide dismutase (PEG-SOD, 300 U/ml) and polyethylene glycol-catalase (PEG-CAT, 600 U/ml), followed by a 4 hrs-PM<sub>2.5</sub> exposure (5-15 µg/cm<sup>2</sup>), no significant effect of PM<sub>2.5</sub> on ONOO<sup>-</sup> level was observed compared to control cells (Fig. 7B).

### 3.5. PM<sub>2.5</sub> increased HPAEC cytoplasmic calcium levels [Ca<sup>2+</sup>]<sub>i</sub> (Fluo-4-AM probe)

In a first set of experiments we investigated whether a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) could increase cytoplasmic calcium level by calcium imaging (Fluo-4-AM probe). Fig. 8 shows, in HPAEC, a statistically significant and dose-dependent increase of the basal intracellular calcium ion level [Ca<sup>2+</sup>]<sub>i</sub> (p<0.05\* and p<0.001\*\*\*). In a second set of experiments, and in order to elucidate whether the PM<sub>2.5</sub>-induced impairment of intracellular calcium homeostasis was correlated to an increase of ROS production, the same experiments were performed using PEG-SOD (300 U/ml) and PEG-CAT (600 U/ml). After a 1 hr-pre-treatment of HPAEC with both PEG-SOD and PEG-catalase, followed by a 4 hrs-PM<sub>2.5</sub> exposure (5-15 µg/cm<sup>2</sup>), no significant effect of PM<sub>2.5</sub> on basal intracellular calcium level was observed compared to control cells (Fig. 8).

Cells co-treated with PM<sub>2.5</sub> and antioxidant enzymes fully reversed the PM<sub>2.5</sub>-induced increase in intracellular calcium basal level.

## 4. Discussion

An increased level of fine particles in the atmosphere is suspected to aggravate cardiovascular diseases. Primary particles, especially those from traffic (tail-pipe) emissions which are formed by combustion processes of fossil fuel, are amongst the most harmful components of PM<sub>2.5</sub> in urban environment (Hopke et al., 2006; Newby et al., 2015). This study focused for that purpose on PM<sub>2.5</sub> sampled at a traffic site. Several studies provided evidence that ROS-induced oxidative damages in vascular cells play a major role in endothelial dysfunction and in the physiopathology of vascular diseases such as PH (Touyz et al., 2004; Cioffi, 2011; Freund - Michel et al., 2013; 2014). Finally, only a few studies have investigated the direct effect of airborne particulate matter on pulmonary artery endothelial cells and the cellular mechanisms remain unclear. In the present study, the toxic effects of PM<sub>2.5</sub> were investigated on HPAEC, which could be one of the primary target of inhaled particles, with a particular focus on (i) acellular and intracellular ROS and RNS generation, (ii) PM<sub>2.5</sub> intracellular uptake and (iii) calcium homeostasis.

ROS and RNS were measured by different methods. The fluorimetric method using the CM-H<sub>2</sub>DCFDA probe is a powerful assay, easy to carry out, and can detect different reactive oxygen species generated by particles (Cohn et al., 2008). This probe was used extensively to

analyze global acellular and intracellular oxidative stress (Foucaud et al., 2007; Chen et al., 2010; Tseng et al., 2015b) but does not allow determining specific free radicals. The EPR method is an alternative to determine specific oxygen species ( $O_2^{\cdot-}$ ,  $ONOO^-$ ,  $OH^{\cdot}$ ...) but requires an expensive instrumentation (Billaud et al., 2009; Dikalov et al., 2011; Nymark et al., 2014). As  $O_2^{\cdot-}$  can be scavenged by NO to form  $ONOO^-$ , we also measured  $ONOO^-$  levels in HPAEC with Griess reaction as an alternative method to investigate  $PM_{2.5}$ -induced  $O_2^{\cdot-}$  increase (Genet et al., 2017). To measure more specifically oxidative stress produced in mitochondria we used confocal microscopy techniques with the mitosox probe (Li et al., 2009; Tseng et al., 2015a). Combining these different methods, we demonstrated that the oxidative stress mediated by  $PM_{2.5}$  originated from several sources. Firstly we demonstrated that, in acellular conditions, different ROS and, in particular, free radical such as  $O_2^{\cdot-}$  were generated by  $PM_{2.5}$  themselves, in a concentration dependent manner, before any contact with HPAEC. These data are in line with those found in the literature for  $PM_{2.5}$  sampled at different sites or with nanoparticles (Dellinger et al., 2001; Latvala et al., 2016). Secondly, we tested the hypothesis that  $PM_{2.5}$  are able to generate an oxidative stress after they were taken up by cells (ie  $PM_{2.5}$  internalization into cells). We provided evidence that these particles were quickly (within one hour) internalized into HPAEC mainly in vesicles or free in the cytoplasm. Some of them were found very close to the cytosolic membrane, to the nucleus and to mitochondria.

We then showed, that  $PM_{2.5}$  and/or their soluble components, could induce an intracellular oxidative stress in a concentration dependant manner. In HPAEC, particles induced (i) a global overproduction of ROS such as  $H_2O_2$  mainly detected by the fluorescent probe CM- $H_2DCFDA$  (up to  $10 \mu g/cm^2$ ), (ii) an increase in some free radicals, in particular the superoxide anion specifically measured by EPR (up to  $7.5 \mu g/cm^2$ ) and (iii) an overproduction of NO and RNS such as  $ONOO^-$  measured by the Griess reaction (up to  $7.5 \mu g/cm^2$ ). These findings suggest that  $PM_{2.5}$ -induced oxidative stress could be correlated to their internalization. These data are in line with those from the literature with silica nanoparticles, diesel and urban SRM 1648-particles (Bai et al., 2001; Li et al., 2006; Guo et al., 2015).

The presence of PM near to mitochondria of exposed cells and the overproduction of intracellular ROS suggest that functions of this organelle could be disturbed. In order to confirm this hypothesis, mitochondria-specific ROS production was assessed, in HPAEC, by confocal microscopy, after exposure to  $PM_{2.5}$ . Our results showed an increase of mitochondria endogenous superoxide anion production from  $5 \mu g/cm^2$ . These results are in accordance with those reported in the literature in aortic and umbilical endothelial cells (Li et al., 2009; Tseng et al., 2015a).

Interestingly, for these concentrations,  $PM_{2.5}$  induced in HPAEC a slight decrease of mitochondrial activity (measured by the WST-1 assay) at 24 hrs which could be correlated to over production of mitochondrial superoxide anion observed after 4 hrs exposure.

Overall, these results indicate that no single assay was completely predictive of oxidative stress and confirm the interest of using various complementary assays for studying  $PM_{2.5}$ -induced ROS generation. These results also suggest that, at the lowest concentrations ( $5 \mu g/cm^2$ ), the confocal microscopy assay using MitoSOX probe was a better predictor of oxidative stress than any of the other assays. In conclusion, this study provided evidence that  $PM_{2.5}$  contain stable free radicals and could generate intracellular ROS and RNS that can easily be detected by these methods.

The detailed chemical composition of  $PM_{2.5}$  is not available. But the physicochemical properties of  $PM_{2.5}$  sampled at the same traffic site were already well characterized in previous studies (Baulig et al., 2004, Val et al., 2011). As it is a site of sampling near traffic, particles are mainly composed by tail-pipe carbonaceous material, namely elemental carbon (EC), organic matter (OM), and PAHs (mainly pyrene, benzo(e)pyrene, benzo(a)anthracene

and chrysene) but also include trace metals from non-tail-pipe (break wears) traffic emissions (mainly Fe, Cu, Mn, Pb, Mo, Sb, Sn). After PM<sub>2.5</sub> inhalation these carbonaceous and trace metal components could be solubilized by macrophages and surfactants in pulmonary alveoli and could directly alter endothelial cells by inducing ROS production after cytochrome P450 1A1 (CYP1A1)-induced metabolization. PAHs are known to specifically induce CYP1A1, a phase I xenobiotic metabolizing enzymes producing superoxide anions and reactive intermediates (Squadrito et al., 2001, Baulig et al., 2009; Val et al., 2011; Boubilil et al., 2013). Previous study showed, in bronchial epithelial cells, that the expression of the CYP1A1 gene was highly induced by PAH-containing PM<sub>2.5</sub> sampled at urban traffic sites (Val et al., 2011). Quinones were also involved in ROS generation due to redox cycling. The oxidative capacity of PM<sub>2.5</sub> could also be attributed to their transition metal constituents mainly to iron and copper that could generate ROS (O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub>, OH<sup>•</sup>) through the Fenton and Harber-Weiss reaction (Donaldson et al., 1997; 2004). In a previous study, we showed that metals and in particular iron contained in particulate matter such as SRM 1648 were involved in particles-induced vascular deleterious effects (Courtois et al., 2014). Furthermore, metals and PAHs could present additive or synergistic interactions with particle surface area. Finally, PM<sub>2.5</sub> induced-intracellular ROS production could then occur as the result of free radicals production at the surface of the particles or by soluble harmful components such as metals or organics that are likely to be involved in vascular diseases (Donaldson et al., 2004; Ayres et al., 2008; Thomson et al., 2016). Additionally, once internalized in the cells, particles could also generate oxidative stress by direct effects on cells that are not driven by intrinsic free radicals, such as depletion of antioxidants (SOD, catalase), inflammatory response, alteration in mitochondrial respiration... which could indirectly induce oxidative stress (Ayres et al., 2008). Further experiments are necessary to address the mechanisms involved in PM<sub>2.5</sub>-induced ROS production.

Several studies provided evidence that ROS/RNS-induced oxidative damages in vascular cells play a major role in endothelial dysfunction and in the physiopathology of vascular diseases. Endothelial NO is a major vasculoprotective agent and it plays a very important role as a major secondary messenger in vascular signaling pathway. NO exerts many effects on the organism, either beneficial or deleterious. Beneficial ones are mainly mediated by NO itself generated at low concentrations by endothelial NO-synthases in a regulated manner. By contrast, prolonged or high-output inducible NOS-derived NO may exert both oxidative and nitrosative stress through peroxynitrite. Therefore, we investigated, in the present study, the ability of PM<sub>2.5</sub> to impair NO signaling pathway. We demonstrated that a 4 hrs-exposure to PM<sub>2.5</sub> stimulated a concentration-dependent increase in NO content especially at the highest concentrations (10 and 15 µg/cm<sup>2</sup>). Similarly, Guo et al. (2015) demonstrated that silica nanoparticles stimulated NO release in endothelial cells. Interestingly, we also showed that the PM<sub>2.5</sub>-generated O<sub>2</sub><sup>•-</sup> rapidly reacted with NO to produce a highly reactive and cytotoxic nitrosative specie such as ONOO<sup>-</sup> and could change the balance of [NO]/[ONOO<sup>-</sup>] unfavorably leading to NO-bioavailability decrease. Indeed, PM<sub>2.5</sub>-induced ONOO<sup>-</sup> overproduction was strongly reduced by a pre-treatment with both superoxide dismutase and catalase, two antioxidant enzymes.

It is now well established that, in many vascular diseases, oxidative stress causes a [Ca<sup>2+</sup>]<sub>i</sub>-increase due to calcium entry across plasma membrane as well as calcium release from intracellular stores (Cioffi, 2011; Dubes et al., 2017). Recent studies (Simon et al., 2011; Nemmar et al., 2015; Neri et al., 2016; Dubes et al., 2017) have also reported that ultrafine particles as well as particulate matter increase intracellular [Ca<sup>2+</sup>]<sub>i</sub> although the underlying mechanisms have been poorly described in particular in pulmonary artery endothelial cells. Therefore, we investigated, in the present study, the ability of PM<sub>2.5</sub> to increase [Ca<sup>2+</sup>]<sub>i</sub> levels

via ROS production. We demonstrated that a 4 hrs-exposure to PM<sub>2.5</sub> induce a concentration-dependent increase of the basal [Ca<sup>2+</sup>]<sub>i</sub> in HPAEC. It was interesting to note that this effect was raised after a 24h-exposure of PM<sub>2.5</sub> to HPAEC (data not shown) suggesting a time-dependent response. This impairment of calcium homeostasis was strongly reduced by a pre-treatment with both superoxide dismutase and catalase suggesting that PM<sub>2.5</sub>-induced alteration of intracellular calcium homeostasis was closely correlated to an increase of O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>. Several studies performed in animal models that mimic PH, have reported that the intracellular [Ca<sup>2+</sup>]<sub>i</sub> in vascular cells was increased in this disease (Rodat et al., 2007; Ducret et al., 2010; Gilbert et al., 2014). Consequently, an additive or synergistic effect in calcium homeostasis is likely to occur after exposure to PM<sub>2.5</sub> particles in PH.

In summary, the oxidative stress mediated by PM<sub>2.5</sub> originates from several sources involving (i) intrinsic generation of ROS from the surface of particles (ii) initiation of free radical chain reaction by extracellular ROS such as lipid peroxidation (iii) internalization of PM (iv) soluble transition metals and organic compounds (v) alteration of mitochondrial function and production of superoxide anion from the mitochondrial electron transport chain. These results are summarized in Fig. 9.

In conclusion, this study provides evidence that PM<sub>2.5</sub>-induced both oxidative and nitrosative species in HPAEC causes redox imbalance and seems to be an important molecular mechanism for endothelial dysfunction leading to impairment of calcium signaling and NO bioavailability which are the main characteristics of hypertensive disease. Thus, exposure to PM<sub>2.5</sub> may be a risk factor for the exacerbation of pre-existent cardiovascular diseases such as PH.

### ***Acknowledgments***

This work was supported by the “Agence de l’environnement et de la maîtrise de l’énergie” – ADEME under grant 12 62 c 0037, the “Agence nationale de sécurité sanitaire de l’alimentation, de l’environnement et du travail” - ANSES under grant n° 2012-2-013. The PhD of Juliette DEWEIRDT is supported by the “Fondation pour la Recherche Médicale” – FRM.

### **Abstract**

Recent studies have revealed that particulate matter (PM) exert deleterious effects on vascular function. Pulmonary artery endothelial cells (HPAEC), which are involved in the vasomotricity regulation, can be a direct target of inhaled particles. Modifications in calcium homeostasis and oxidative stress are critical events involved in the physiopathology of vascular diseases. The objectives of this study were to assess the effects of PM<sub>2.5</sub> on oxidative stress and calcium signaling in HPAEC. Different endpoints were studied, (i) intrinsic and intracellular production of reactive oxygen species (ROS) by the H<sub>2</sub>DCF-DA probe, (ii) intrinsic, intracellular and mitochondrial production of superoxide anion (O<sub>2</sub><sup>•-</sup>) by electronic paramagnetic resonance spectroscopy and MitoSOX probe, (iii) reactive nitrosative species (RNS) production by Griess reaction, and (iv) calcium signaling by the Fluo-4 probe. In acellular conditions, PM<sub>2.5</sub> leads to an intrinsic free radical production (ROS, O<sub>2</sub><sup>•-</sup>) and a 4 hrs-exposure to PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>), induced, in HPAEC, an increase of RNS, of global ROS and of cytoplasmic and mitochondrial O<sub>2</sub><sup>•-</sup> levels. The basal intracellular calcium ion level [Ca<sup>2+</sup>]<sub>i</sub> was also increased after 4 hrs-exposure to PM<sub>2.5</sub> and a pre-treatment with superoxide dismutase and catalase significantly reduced this response. This study provides evidence that the alteration of intracellular calcium homeostasis induced by PM<sub>2.5</sub> is closely correlated to an increase of oxidative stress.

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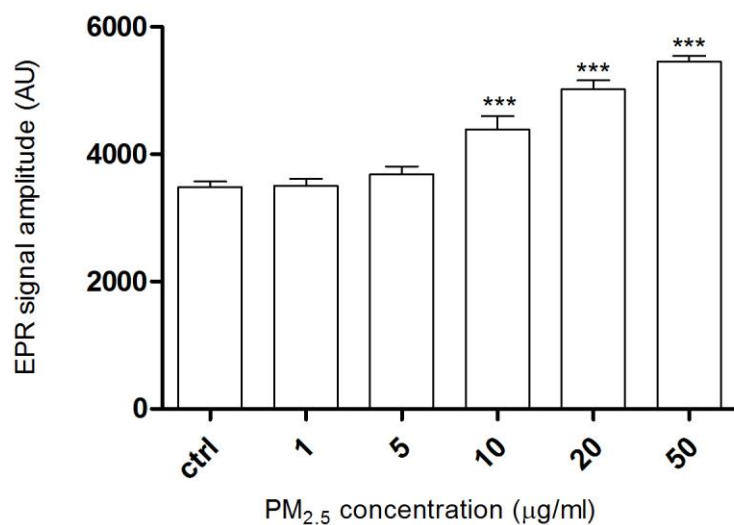
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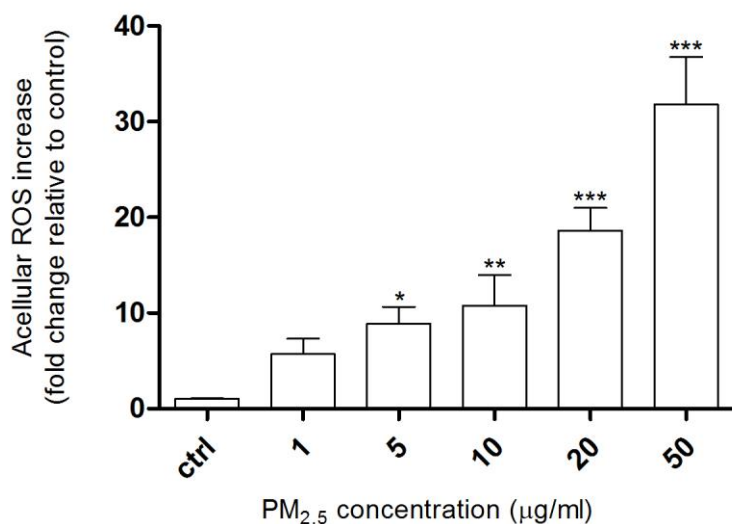
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ACCEPTED MANUSCRIPT

A



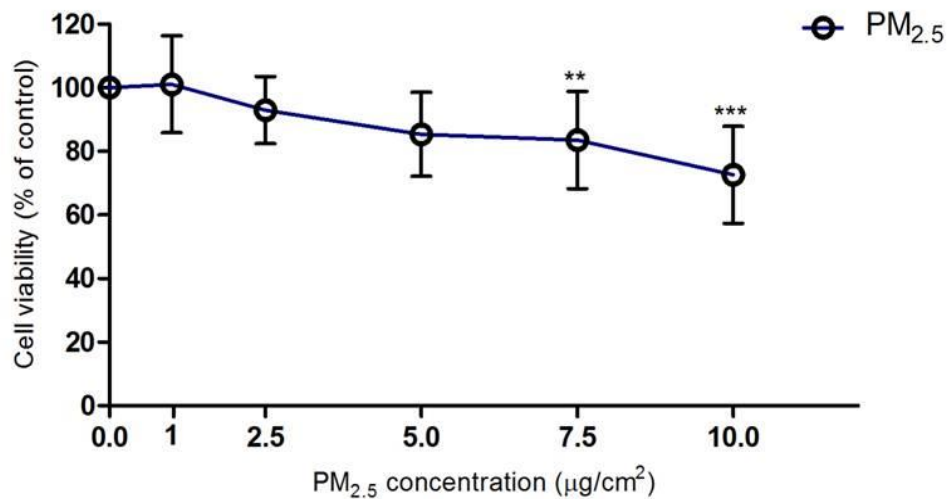
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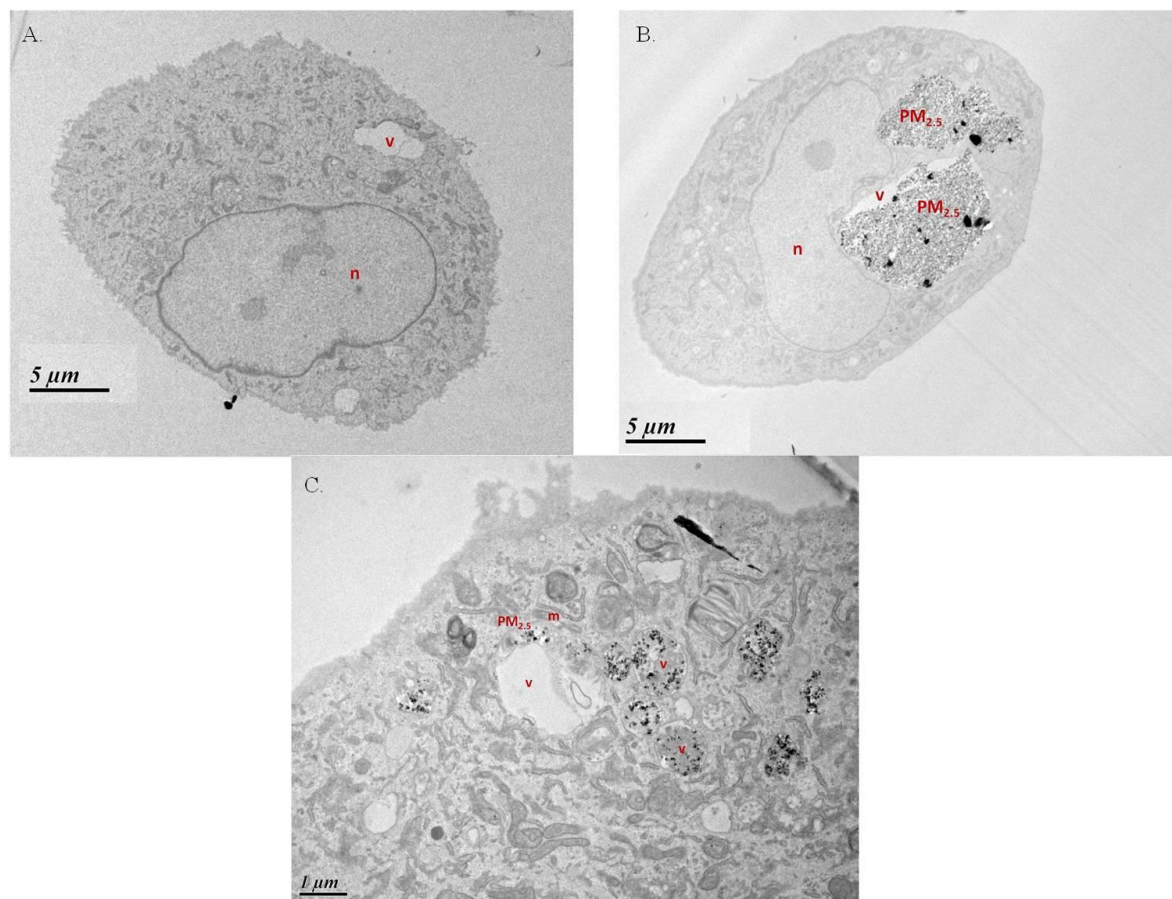
**Fig. 1.** PM<sub>2.5</sub> intrinsic ROS production (acellular condition).

A: ROS production measured by spectrofluorimetry with the CM-H<sub>2</sub>DCFDA probe assay. Water PM<sub>2.5</sub> suspensions (1 - 50 µg/ml) were incubated with the probe for 1 hr at 37°C. The fluorescence intensity was measured by fluorimetry. Results were expressed in H<sub>2</sub>O<sub>2</sub> equivalents (µM). The values were normalized to the control (ctrl) and expressed as the fold change of the CM-H<sub>2</sub>DCF fluorescence intensity relative to the control (without treatment, ctrl). Data were mean ± SEM of three independent experiments, n=3 (eight wells/concentration). Statistically significant at p<0.05 (\*), p<0.01(\*\*) and p<0.001(\*\*\*) as compared to controls, Mann-Whitney-U-test.

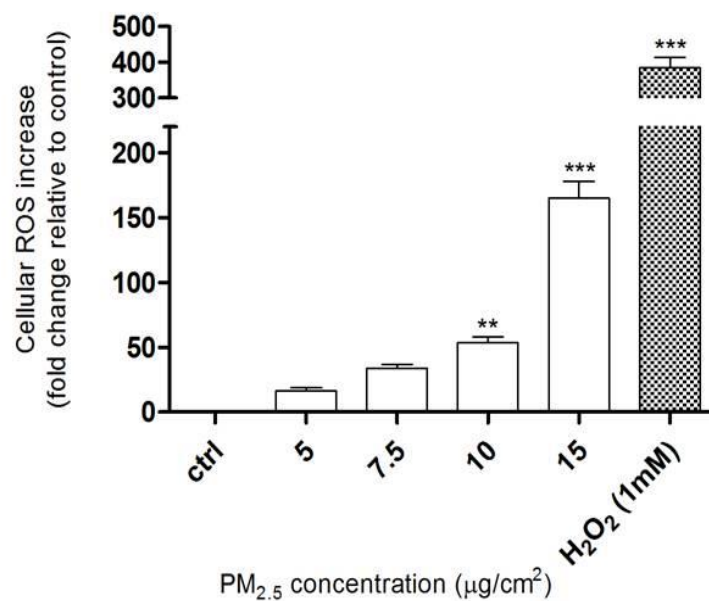
B: Superoxide anion (O<sub>2</sub><sup>•-</sup>) production measured by Electron Paramagnetic Resonance spectrometer with the CMH probe. PM<sub>2.5</sub> (1 - 50 µg/ml) were incubated with the spin-probe mix: CMH, diethyldithiocarbamate and deferoxamine in Krebs-HEPES Buffer, for 1 hr at 37°C. Results were expressed in EPR signal amplitude in arbitrary unit (AU). Data were mean ± SEM of three independent experiments (n=3) performed in triplicate. Statistically significant at p<0.001(\*\*\*) as compared to controls, Mann-Whitney-U-test.



**Fig. 2.** HPAEC viability, after a 24 hrs incubation with PM<sub>2.5</sub> (1 - 10 µg/cm<sup>2</sup>), measured by the WST-1 colorimetric assay. Cells were exposed for 24 hrs at 37°C, 5% CO<sub>2</sub> to PM<sub>2.5</sub>. Results were expressed as the percentage of cell viability in treated cells as compared to control cells (100%). Data were mean ± SEM of three independent experiments, n=3 (eight wells/concentration). Statistically significant at p<0.01(\*\*) and p<0.001(\*\*\*), as compared to controls, Mann-Whitney-U-test.

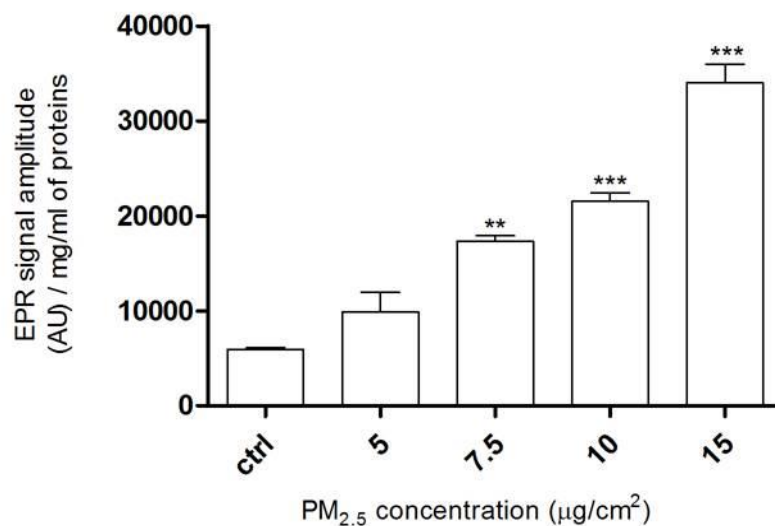


**Fig. 3.** PM<sub>2.5</sub> internalization and intracellular localization in HPAEC, observed by transmission electronic microscopy (TEM). Cells were exposed, for 1 h, at 37°C, 5% CO<sub>2</sub> with PM<sub>2.5</sub> at 7.5 μg/cm<sup>2</sup>. (A) Untreated cells, control (X2900), (B) PM<sub>2.5</sub> treated cells (X2900) and (C) PM<sub>2.5</sub> treated cells (X11000). n correspond to the nucleus, m correspond to the mitochondria and v correspond to the vesicle. Scale bars, 5 μm (A, B) and 1 μm (C).



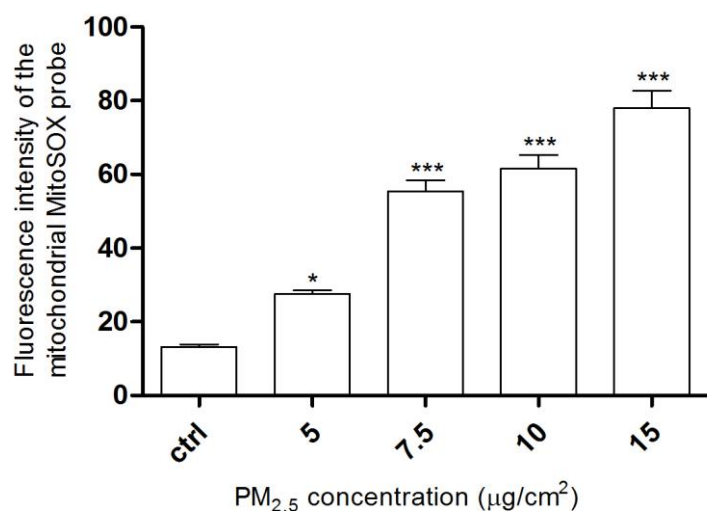
**Fig. 4.** Intracellular PM<sub>2.5</sub>-induced ROS production in HPAEC, measured by spectrofluorimetry with the CMH<sub>2</sub>DCF-DA probe assay. Cells were exposed or not (negative control) with PM<sub>2.5</sub> (5 - 15 μg/cm<sup>2</sup>) or with H<sub>2</sub>O<sub>2</sub> (1 mM, positive control) for 4 hrs at 37°C and 5% CO<sub>2</sub>. The fluorescence intensity was measured by fluorimetry. The values were normalized to the untreated cells (control, ctrl) and results were expressed as the fold change of the CMH<sub>2</sub>DCF fluorescence intensity relative to the control cells. Data were mean ± SEM of three independent experiments, n=3 (eight wells/ concentration). Statistically significant at p<0.01(\*\*) and p<0.001(\*\*\*) as compared to controls, Mann-Whitney-U-test.



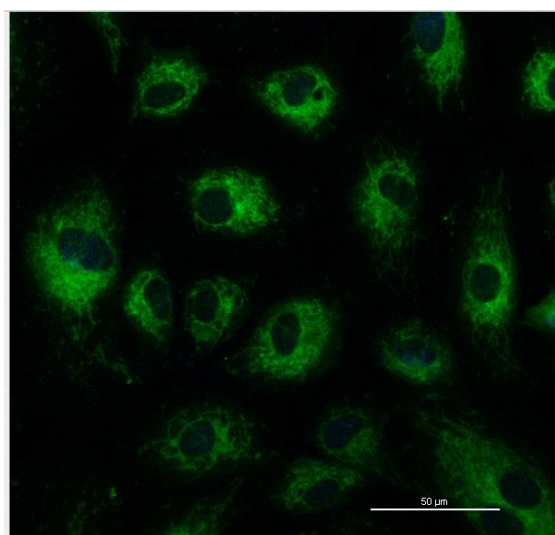


**Fig. 5.** Intracellular PM<sub>2.5</sub>-induced O<sub>2</sub><sup>-</sup> production in HPAEC, measured by EPR spectrometer with the CMH probe. Cells were exposed with PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) for 4 hrs at 37°C, 5% CO<sub>2</sub>. Then, cells were incubated for 20 min with the spin-probe mix: CMH, diethyldithiocarbamate and deferoxamine in Krebs-HEPES Buffer. Protein concentration was performed with the Lowry test. Results were expressed in EPR signal amplitude in arbitrary unit (AU) / mg/ml of proteins. Data were mean ± SEM of three independent experiments (n=3) performed in triplicate. Statistically significant at p<0.01(\*\*) and p<0.001(\*\*\*) as compared to controls, Mann-Whitney-U-test.

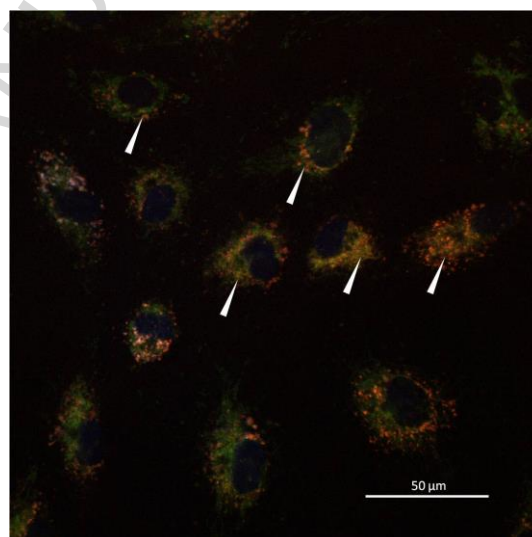
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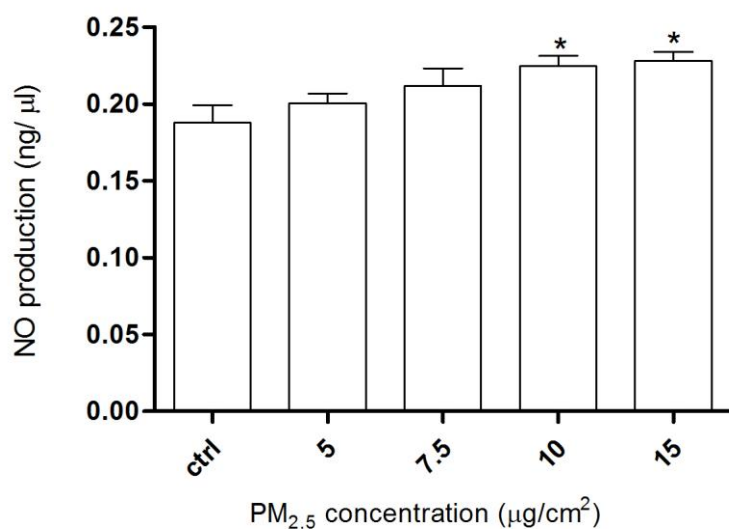


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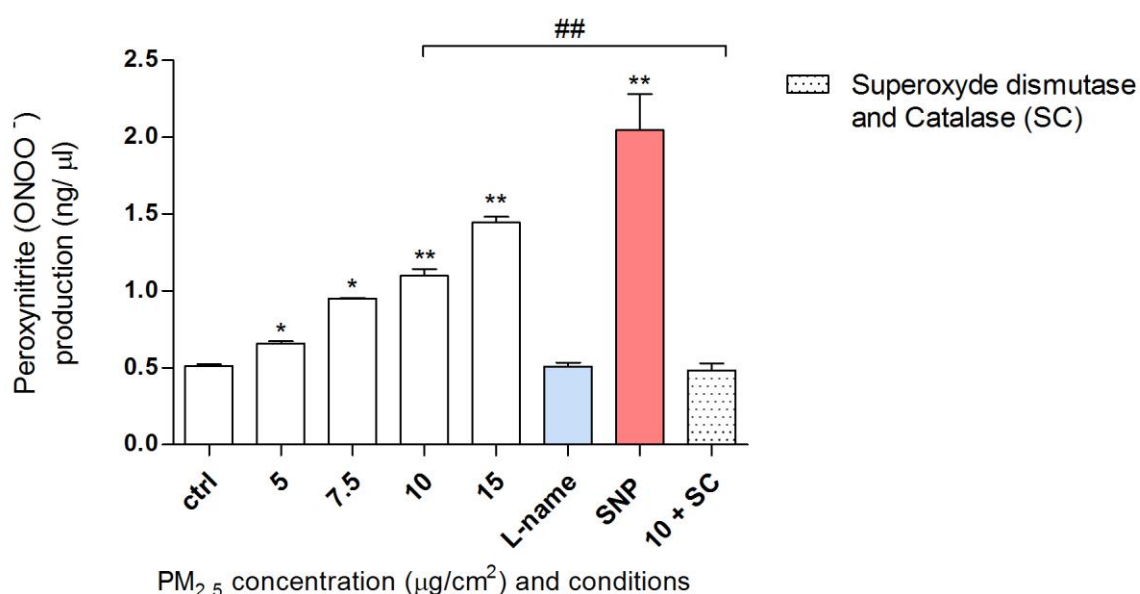


**Fig. 6.** Mitochondrial PM<sub>2.5</sub>-induced O<sub>2</sub><sup>-</sup> production in HPAEC, measured with the MitoSOX probe (5 µM), by confocal microscopy. Cells were exposed with PM<sub>2.5</sub> (5-15 µg/cm<sup>2</sup>) for 4 hrs at 37°C, 5% CO<sub>2</sub>. (A) The mitochondrial O<sub>2</sub><sup>-</sup> production was expressed as the oxidized MitoSOX fluorescence intensity and was compared to untreated cells (control, ctrl). Data were mean ± SEM of three independent experiments, n=3 (six wells/concentration). Statistically significant at p<0.05 (\*), p<0.001(\*\*\*) as compared to controls, Mann-Whitney-U-test. (B, C) HPAEC cell imaging of MitoSOX probe oxidation by confocal microscopy. Cells were exposed, for 1h, at 37°C, 5% CO<sub>2</sub> with PM<sub>2.5</sub> at 10 µg/cm<sup>2</sup> (B) untreated cells, control (X600), (C) PM<sub>2.5</sub> treated cells (X600). Nucleus were stained with Hoechst (blue fluorescence), mitochondria with Mitotracker (green fluorescence) and mitochondrial superoxide anion with MitoSOX (red fluorescence). White arrows indicated some co-localization (yellow) between mitochondria (green) and O<sub>2</sub><sup>-</sup> (red). Scale bars, 50 µm (B, C).

A

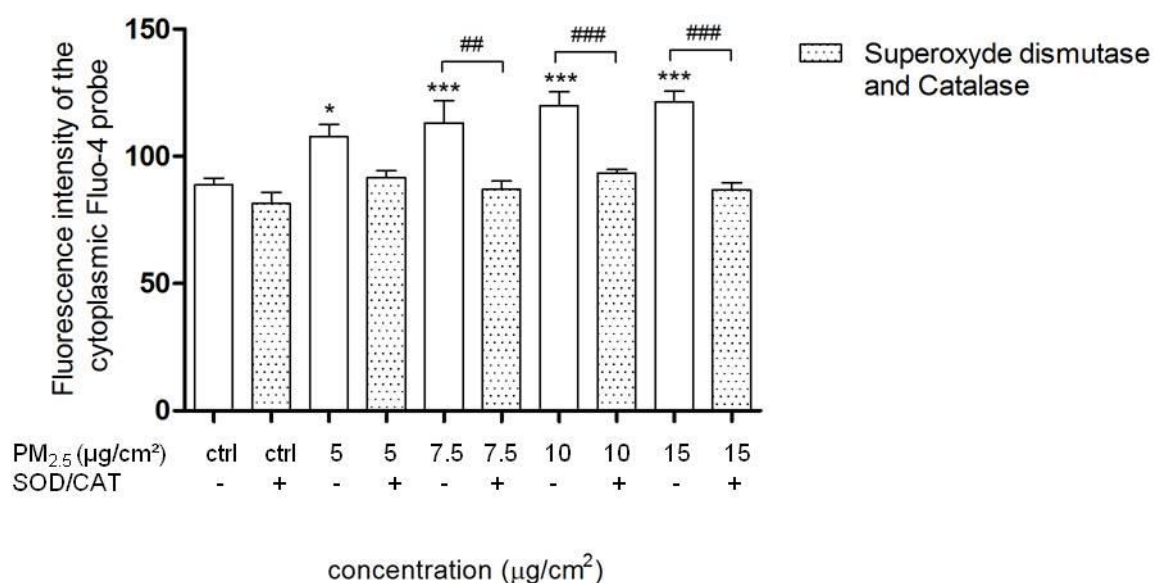


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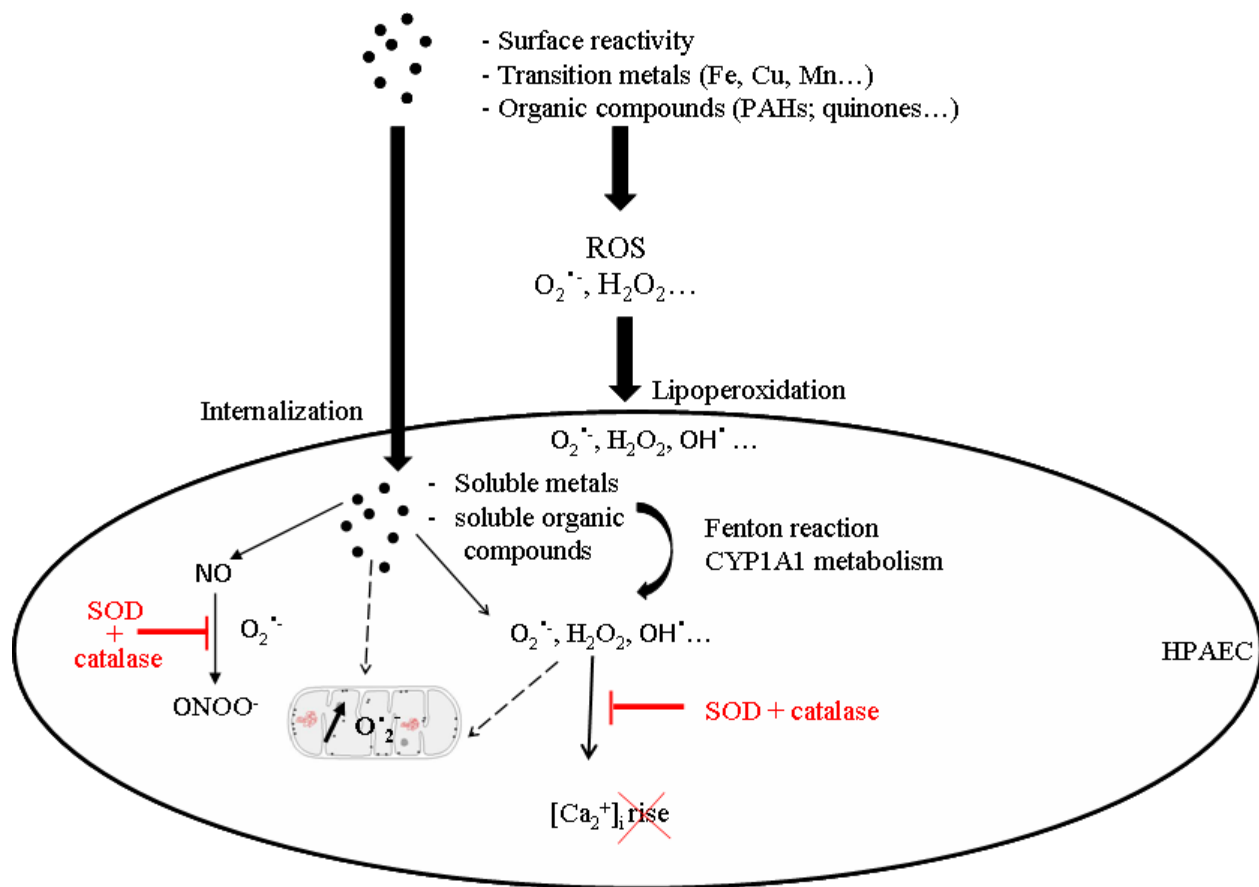


**Fig. 7.** Intracellular PM<sub>2.5</sub>-induced RNS (NO and ONOO<sup>-</sup>) production in HPAEC, measured by the Griess reaction. Cells were exposed or not (negative control) with PM<sub>2.5</sub> (5 - 15 μg/cm<sup>2</sup>) for 4 hrs at 37 °C and 5 % CO<sub>2</sub>. A: NO production in HPAEC. The results were expressed in ng/μl. Data were mean ± SEM of three independent experiments, n=3 (three wells/ concentration). Statistically significant at p<0.05 (\*) as compared to controls, Mann-Whitney-U-test.

B: ONOO<sup>-</sup> production in HPAEC after or not a 1 hr pre-treatment with PEG-SOD (300 U/ ml) and PEG-catalase (600 U/ ml). The NO-donor sodium nitroprusside (SNP, 10<sup>-4</sup> M) and the NO synthase inhibitor NG-L-Nitro Arginine Methyl Ester (L-Name, 10<sup>-4</sup> M) were used respectively as positive and as negative controls. The results were expressed in ng/μl. Data were mean ± SEM of three independent experiments, n=3 (three wells/ concentration). Statistically significant at p<0.05 (\*) and p<0.01(\*\*) as compared to controls, Mann-Whitney-U-test. Statistically significant at p<0.01(##) between cells treated with PM<sub>2.5</sub> at 10 μg/cm<sup>2</sup> and cells pre-treated with antioxidant enzymes followed by treatment with PM<sub>2.5</sub> at 10 μg/cm<sup>2</sup>, Mann-Whitney-U-test.



**Fig. 8.** Intracellular basal  $\text{Ca}^{2+}$  level in HPAEC, measured with Fluo-4-AM probe (1  $\mu\text{M}$ ), by confocal microscopy. Cells were exposed to  $\text{PM}_{2.5}$  (5-15  $\mu\text{g}/\text{cm}^2$ ) for 4 hrs at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , after or not a 1 hr pre-treatment with PEG-SOD (300 U/ ml) and PEG-catalase (600 U/ ml). The basal  $\text{Ca}^{2+}$  level was expressed as the fluorescence intensity of the Fluo-4 probe and was compared to untreated cells (control, ctrl). Data were mean  $\pm$  SEM of three independent experiments,  $n=3$  (six wells/concentration). Statistically significant at  $p<0.05$  (\*) and  $p<0.001$  (\*\*\*) as compared to controls. Statistically significant at  $p<0.01$  (##) and  $p<0.001$  (###) as compared to cells pre-treated with antioxidant enzymes, Mann-Whitney-U-test.



**Fig. 9.** Summary of hypothetical  $PM_{2.5}$  oxidative stress-mediated impairment of calcium homeostasis leading to vascular health disease

## Highlights

- PM<sub>2.5</sub> increased both reactive oxidative and nitrosative species (ROS/RNS) in HPAEC
- PM<sub>2.5</sub> impaired calcium signaling and nitric oxide (NO) bioavailability in HPAEC
- PM<sub>2.5</sub>-induced alteration of intracellular calcium homeostasis was correlated to ROS
- PM<sub>2.5</sub> exposure may be a risk factor for exacerbation of preexistent vascular disease