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Time course of systemic oxidative stress and inflammatory response induced by an acute exposure to Residual Oil Fly Ash

T. Marchini [a], N.D. Magnani [a], M.L. Paz [b], V. Vanasco [a], D. Tasat [c], D.H. González Maglio [b], S. Alvarez [a], P.A. Evelson [a,*]

[a] Cátedra de Química General e Inorgánica, Instituto de Bioquímica y Medicina Molecular (IBIMOL UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, C1113AAB Buenos Aires, Argentina
[b] Cátedra de Inmunología, Instituto de Estudios de la Inmunidad Humoral (IDEHU UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, C1113AAB Buenos Aires, Argentina
[c] CESyMA, Facultad de Ciencia Tecnología, Universidad Nacional de General San Martín, Martín de Irigoyen 3100, 1650 San Martín, Buenos Aires, Argentina

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ABSTRACT

It is suggested that systemic oxidative stress and inflammation play a central role in the onset and progression of cardiovascular diseases associated with the exposure to particulate matter (PM). The aim of this work was to evaluate the time changes of systemic markers of oxidative stress and inflammation, after an acute exposure to Residual Oil Fly Ash (ROFA). Female Swiss mice were intranasally instilled with a ROFA suspension (1.0 mg/kg body weight) or saline solution, and plasma levels of oxidative damage markers [thiobarbituric acid reactive substances (TBARSs) and protein carbonyls], antioxidant status [reduced (GSH) and oxidized (GSSG) glutathione, ascorbic acid levels, and superoxide dismutase (SOD) activity], cytokines levels, and intravascular leukocyte activation were evaluated after 1, 3 or 5 h of exposure. Oxidative damage to lipids and decreased GSH/GSSG ratio were observed in ROFA-exposed mice as early as 1 h. Afterwards, increased protein oxidation, decreased ascorbic acid content and SOD activity were found in this group at 3 h. The onset of an adaptive response was observed at 5 h after the ROFA exposure, as indicated by decreased TBARS plasma content and increased SOD activity. The observed increase in oxidative damage to plasma macromolecules, together with systemic antioxidants depletion, may be a consequence of a systemic inflammatory response triggered by the ROFA exposure, since increased TNF-α and IL-6 plasma levels and polymorphonuclear leukocytes activation was found at every evaluated time point. These findings contribute to the understanding of the increase in cardiovascular morbidity and mortality, in association with environmental PM inhalation.

Introduction

Epidemiological studies have shown that an acute exposure to environmental particulate matter (PM) is associated with increased cardiopulmonary mortality rates (Analitis et al., 2006). Daily changes in PM concentration have also been positively correlated with increased hospitalizations due to lower respiratory diseases, ischemic cardiovascular events, arrhythmias, and heart failure (Dominici et al., 2006). In this context, oxidative stress and inflammation have been suggested to play a predominant role in cardiopulmonary PM toxicity (Brook et al., 2010; Gurgueira et al., 2002).

Oxidative stress can be defined as an increase, over physiological values, in the steady-state concentrations of reactive oxygen species (ROS). This situation may lead to changes in the levels of antioxidant defenses, which can be increased as an adaptive response, or depleted due to the action of oxidants (Sies, 1985). Systemic oxidative stress can occur following PM inhalation, leading to oxidative damage to plasma macromolecules, antioxidants depletion and inflammation. Indeed, several studies in humans have found elevated oxidative stress markers and increased circulating proinflammatory mediators in association with environmental PM levels (Brook et al., 2010). Personal exposure to ambient PM concentrations closely paralleled with increased plasma markers of protein oxidation and lipid peroxidation (Sørensen et al., 2003), and of DNA damage (Bräuner et al., 2007). Moreover, short-term PM inhalation positively correlates with increased white blood cell counts, IL-6, and TNF-α levels (Calderón-Garcidueñas et al., 2008; Riediker, 2007). Acute-phase proteins such as C-reactive protein and fibrinogen are also increased in plasma, in association with day-to-day variations in PM (Chuang et al., 2007). Interestingly, the presence of soluble transition metals as PM constituents seems to enhance the

Abbreviations: DAF-2, 4,5-diaminofluorescein; DCF, 2′,7′-dichlorofluorescein; PM, particulate matter; ROS, reactive oxygen species; ROFA, Residual Oil Fly Ash; SOD, superoxide dismutase.

* Corresponding author at: Instituto de Bioquímica y Medicina Molecular (IBIMOL UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 954, C1113AAB Buenos Aires, Argentina. Tel.: +54 011 4964-8249; fax: +54 011 4508-3646.
E-mail address: pevelson@ffyb.uba.ar (P.A. Evelson).

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inflammatory response triggered by PM, due to increased production of ROS via Fenton-like chemical reactions (Chen and Lippmann, 2009).

Airborne PM varies in size, chemical composition and sources of origin. Anthropogenic emissions are the main contributors to the environmental PM burden, and consist mainly of motor vehicle emissions and fossil fuel combustion during power generation and industrial processes (Nel, 2005). The inorganic residue that remains after the incomplete oxidation of such carbonaceous materials contributes to PM in urban air and is termed Residual Oil Fly Ash (ROFA) (Ghio et al., 2002a). Diverse PM surrogates have been assayed in different animal models in order to study the biological effects of PM exposure. Among them, ROFA has been particularly useful given that it is especially rich in soluble transition metals (namely iron, nickel and vanadium), and because of its low concentration of organic compounds (Schroeder et al., 1987). Hence, ROFA is the most frequently used combustion-derived particle in order to evaluate the contribution of such metals on air pollution toxicity (Chen and Lippmann, 2009). Moreover, ROFA particles often present an aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$), a size that has been shown to be more closely associated with PM adverse health effects than coarser particles ($PM_{10-2.5}$) (Brook et al., 2010).

We have previously reported that the NADPH oxidase homolog Nox2 is activated in mice lung after an acute ROFA exposure (Magnani et al., 2013). This condition contributes to the onset of local oxidative damage, together with direct oxidant production from transition metals coated on ROFA surface, and with an altered mitochondrial function. Moreover, impaired cardiac mitochondrial function and contractile reserve were also observed in the same animal model (Marchini et al., 2013). Of note, the most significant imbalance in lung oxidative metabolism required a shorter exposure time (1 h), in comparison with the time needed to observe those alterations in heart (3 h).

Consistently with our findings, alveolar macrophage activation (Nurkiewicz et al., 2004) and increased macrophage inflammatory protein (MIP)-2 and TNF-α levels (Ferraro et al., 2011; Ghio et al., 2002b) were observed in bronchoalveolar lavages from ROFA-exposed rodents. Increased leukocyte adhesion and rolling, and microvascular oxidative stress have been also reported to be triggered by ROFA inhalation (Nurkiewicz et al., 2006). Interestingly, extracellular superoxide dismutase (SOD) overexpression decreased both lung inflammation and oxidative damage in mice exposed to ROFA (Ghio et al., 2002b). This scenario was also attenuated in IL-$6^{-/-}$ knockout mice (Fujimaki et al., 2006), as well as in toll-like receptor 4 and Nox2 deficient mice (Kampfrath et al., 2011). These findings emphasize the tight link between PM-associated oxidative stress and inflammation in lung, which can in turn trigger a systemic response leading to the observed cardiovascular effects. However, a comprehensive analysis of the time course of the changes in plasma markers of oxidative stress and inflammation, and of the link between the systemic inflammatory response and oxidative stress triggered after an acute ROFA exposure, has not yet been performed.

Taking into account that oxidative stress and inflammation play a predominant role in cardiopulmonary PM toxicity, and the observed lag phase between the onset of pulmonary and cardiac effects, the aim of this work was to evaluate the time changes of systemic markers of oxidative stress and inflammation, in a mouse model of acute exposure to ROFA. The obtained findings could give new insights to the understanding of the biochemical basis of the observed PM-associated adverse health effects.

Methods

Drugs and chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, US), except HCl, H_2SO_4 and organic solvents which were purchased from Merck KGaA (Darmstadt, Germany), and 2′,7′-dichlorofluorescein diacetate and 4,5-diaminofluorescein diacetate which were provided by Molecular Probes (Eugene, OR, US).

Experimental model

ROFA suspension. ROFA particles were collected from Boston Edison Co., Mystic Power Plant, Mystic, CT, US and were kindly provided by Dr. J. Godleski (Harvard School of Public Health, MA, US) (Killingsworth et al., 1997). ROFA samples from this source have been previously characterized in terms of elemental composition and particle size. Vanadium, nickel and iron are the predominant metals present as water-soluble sulfates, and particle mean aerodynamic diameter is 2.06 ± 1.57 μm (Ostachuk et al., 2008). PM samples were freshly prepared by suspending ROFA particles in sterile saline solution (0.5 mg/mL), followed by a 10 min incubation in an ultrasonic water bath before use.

Animal exposure. Female Swiss mice weighting 20–25 g were anesthetized by an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (0.1 mg/kg), and exposed to ROFA particles (1.0 mg/kg body weight) or saline solution (control group) in a single dose by intranasal instillation. Mice were immobilized in a 60° inclined supine position, while 50 μL of the ROFA suspension was delivered dropwise to the nares by the use of an automatic pipette. After 1, 3 or 5 h of exposure, animals were euthanized and blood samples were collected. Control mice were handled in parallel, instilled with 50 μL of sterile saline solution, and euthanized at the same time points. Due to the presence of fluid in the mouse nasal cavity, a respiratory reflex is triggered which ensures that the maximum delivered volume reaches the lung (Southam et al., 2002). An exposure time of up to 5 h was chosen in order to focus on ROFA-associated short-term effects, and based on previous reports (Marchini et al., 2013). The selected dose falls within the range of concentrations consistently used in several animal studies (Ghio et al., 2002b; Gurgueira et al., 2002; Nurkiewicz et al., 2006, 2004), and would correspond to a weekly human exposure to certain highly polluted environments, such as atmospheric temperature inversions, peak hourly PM levels within certain megalopolis, and occupational PM levels of exposure. Animal treatment was carried following the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT) guidelines.

Blood samples. Blood samples were obtained by cardiac puncture using heparin as anticoagulant. A 10 min centrifugation at 600 g and 4 °C was carried out in a Thermo Scientific CL31R Multispeed Centrifuge (Thermo Fisher Scientific, Waltham, MA, US) in order to separate the plasma. Samples were kept at −80 °C until use.

Lung homogenates. Lung tissue samples (0.2 g of wet weight) were homogenized in ice-cold 50 mM Tris–HCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.4 mM PMSE (pH 8.0) with a Potter-Elvehjem glass homogenizer. The obtained suspension was centrifuged at 600 *g* for 10 min at 4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as tissue homogenate and kept at −80 °C until use.

Protein content. Protein concentration of plasma samples and lung homogenates was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Oxidative damage markers

Thiobarbituric acid reactive substances (TBARS) assay. Oxidative damage to plasma lipids was determined as thiobarbituric acid reactive substances (TBARSs), using a fluorometric assay (Yagi, 1976). Briefly, plasma samples (25 μL) were incubated with 200 μL of 0.1 N HCl, 30 μL of 10% (w/v) phosphotungstic acid, and 100 μL of 0.7% (w/v) 2-

thiobarbituric acid (TBA) in a boiling water bath. After 60 min, TBARSs were extracted in 500 μL of n-butanol and centrifuged at 1000 *g* for 10 min. The fluorescence of the butanolic layer was measured in a Perkin Elmer LS 55 Fluorescence Spectrometer (Perkin Elmer, Waltham, MA, US) at 515 nm (excitation) and 553 nm (emission). A calibration curve was performed using 1,1,3,3-tetramethoxypropane as standard. Results were expressed as μM TBARS.

Carbonyl groups assay. Plasma content of carbonyl groups from oxidatively modified proteins were determined by a colorimetric assay, measuring the amount of 2,4-dinitrofenylhydrazone formed upon the reaction with 2,4-dinitrofenylhydrazine (DNPH) and protein carbonyls (Levine et al., 1994). Plasma samples (200 μL of a 1/10 (v/v) dilution) were incubated with 800 μL 2 mM DNPH in the dark at room temperature. Samples were vortexed every 15 min during this period. After 60 min, proteins were precipitated with 1 mL 20% (w/v) trichloroacetic acid (TCA), incubated on ice for 10 min, and centrifuged at 10,000 *g* for 10 min at 4 °C. The obtained pellet was resuspended in 1 mL 10% (w/v) TCA, incubated on ice for 10 min, and centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was discarded and the pellet was washed three times with ethanol:ethyl acetate (1:1). After the final wash, protein pellets were resuspended in 1 mL 6 M guanidinium hydrochloride (pH 2.5) and incubated at 37 °C for 10 min. Carbonyl content was calculated from the absorbance at 360 nm ($\varepsilon = 22\ mM^{-1}\ cm^{-1}$), measured in a Hitachi U-2000 Spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). Results were expressed as nmol carbonyl groups/mg protein.

Antioxidant status

Reduced (GSH) and oxidized (GSSG) glutathione levels. Plasma samples (100 μL) were mixed with 1 M $HClO_4$–2 mM EDTA (1:1), and centrifuged at 20,000 *g* for 20 min at 4 °C. Supernatants were filtered through 0.22 μm cellulose acetate membranes (Corning Inc., NY, US), and frozen at −80 °C until use. HPLC analysis was performed in a Perkin Elmer LC 250 liquid chromatography (Perkin Elmer, Waltham, MA, US), equipped with a Perkin Elmer LC ISS 200 advanced sample processor, and a Coulochem II (ESA, Bedford, MA, US) electrochemical detector. A Supelcosil LC-18 (250 × 4.6 mm ID, 5 μm particle size) column protected by a Supelguard (20 × 4.6 mm ID) precolumn (Supelco, Bellfonte, PA, US) was used for sample separation. GSH and GSSG were eluted at a flow rate of 1.2 mL/min with 20 mM sodium phosphate (pH 2.7), and electrochemically detected at an applied oxidation potential of +0.800 V. Results were expressed as μM (Rodriguez-Ariza et al., 1994).

Ascorbic acid content. Plasma samples (90 μL) were mixed with 200 μL of 10% (w/v) metaphosphoric acid (MPA), and centrifuged at 10,000 *g* for 5 min at 4 °C. Supernatants were filtered through 0.22 μm cellulose acetate membranes (Corning Inc., NY, US), and frozen at −80 °C until use. Filtered samples (90 μL) were mixed with 300 μL of 0.8% (w/v) MPA, and subjected to HPLC separation using a Supelcosil LC-18 (33 × 4.6 mm ID, 3 μm particle size) column (Supelco, Bellfonte, PA, US). Ascorbic acid was eluted at a flow rate of 1 mL/min with 0.8% (w/v) MPA, and electrochemically detected at an applied oxidation potential of +0.600 V. Results were expressed as μM ascorbic acid (Kutnink et al., 1987).

Superoxide dismutase (SOD) activity. A colorimetric assay was used, based on the inhibition of adrenochrome formation rate at 37 °C due to the addition of increasing amounts of plasma samples, in a reaction medium consisting of 1 mM epinephrine and 50 mM glycine (pH 10.5). Measurements were performed at 480 nm in a Hitachi U-2000 Spectrophotometer (Hitachi Ltd., Chiyoda, Tokyo, Japan). Enzymatic activity was expressed as SOD U/L plasma. One SOD unit was defined as the volume of plasma needed to inhibit adrenochrome formation rate by 50% (Misra and Fridovich, 1972).

Tissue damage markers

Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activities were spectrophotometrically determined, using standard assay kits according to manufacturer's instructions. Enzymatic activities were expressed as U/L plasma.

Cytokines quantification

Cytokine levels in plasma samples and lung homogenates were determined by enzyme-linked immunosorbent assay (ELISA). The OptEIA system (BD, Franklin Lakes, NJ, US) was used for TNF-α, IL-6, IL-10 and IFN-γ according to manufacturer's instructions. Results were expressed as pg/mL for plasma samples or pg/mg of protein for lung homogenates.

Flow cytometry assessment of intravascular leukocyte activation

Leukocyte isolation. Freshly obtained blood samples (100 μL) were hemolyzed by incubation with 1:10 Red Blood Cell Lysing Buffer (Sigma) for 5 min. Afterwards, a 6 min centrifugation at 600 *g* was performed and the supernatant was discarded. The pellet was washed with PBS and resuspended in Hank's buffer solution [137 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$ and 5 mM glucose (pH 7.4)]. Cell viability was assessed by trypan blue exclusion. The whole procedure was carried out at room temperature.

Leukocyte respiratory burst activity. Isolated mice leukocytes (1×10^6 cells/mL) were loaded with 5 μM 2′,7′-dichlorofluorescein (DCF) diacetate and analyzed by flow cytometry. DCF diacetate passively diffuses into cells, where it is de-esterified by intracellular stearases and readily oxidized to the green-fluorescent product DCF upon its reaction with oxidant species, thus indicating intracellular redox status. After a 30 min incubation in the dark at 37 °C, 10,000 events per sample were acquired in a Partec PAS-III flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488-nm argon laser. The different leukocytes populations were gated based on light scattering properties. DCF signal was analyzed in the FL-1 channel with Cyflogic software (CyFlo Ltd., Turku, Finland), and quantified as median fluorescence intensities (MFI) (Walrand et al., 2003).

Leukocyte nitric oxide (NO) production. Intracellular NO production was assessed based on the ability of 4,5-diaminofluorescein (DAF-2) diacetate to permeate the plasma membrane, be cleaved by intracellular stearases, and react with NO yielding the green-fluorescent product triazolofluorescein. Isolated mice leukocytes (1×10^6 cells/mL) were incubated with 10 μM DAF-2 diacetate for 30 min in the dark at 37 °C. Afterwards, 10,000 events per sample were acquired in a Partec PAS-III flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488-nm argon laser. The different leukocytes populations were gated based on light scattering properties. DAF-2 signal was analyzed in the FL-1 channel with Cyflogic software (CyFlo Ltd., Turku, Finland), and quantified as MFI (Tiscornia et al., 2009).

Statistics

Results were expressed as mean values ± standard error of the mean (SEM) and represent the mean of at least 6 independent experiments. Unpaired Student's *t*-test was used to analyze differences between two groups. ANOVA followed by the Student–Newman–Keuls test was performed to analyze differences between more than two groups. Statistical significance was considered at $p < 0.05$.

Results

Oxidative damage markers

In order to evaluate the occurrence of systemic oxidative stress after an acute ROFA exposure, plasma markers of oxidative damage to macromolecules were assessed. The TBARS assay is one of the most frequently used screening method to evaluate the occurrence of oxidative damage to lipids; while the carbonyl groups assay is a classical approach to measure protein oxidation (Boveris and Navarro, 2008). The acute ROFA exposure triggered a significant increase in both markers, but following different kinetics. On one hand, augmented plasma lipids oxidation was observed after 1 and 3 h, by 16% and 19%, respectively (Fig. 1A). On the other hand, plasma content of oxidatively modified proteins was found to be significantly increased by 12% at 3 h after the exposure, and by 16% after 5 h (Fig. 1B). No changes were observed among control groups at the different time points in neither of the assays.

Antioxidant status

GSH and GSSG levels

GSH is a low molecular weight antioxidant which is readily oxidized to GSSG in order to detoxify a wide variety of oxidant species. A decrease in the GSH/GSSG ratio indicates a shift towards a more oxidized environment, and suggests the occurrence of oxidative stress. Therefore, the GSH/GSSG ratio is one of the most widely used parameters of redox status in cells and tissues (Franco et al., 2007). A 27% decrease in GSH plasma levels was observed in ROFA-exposed mice at 3 h in comparison with the control group. Regarding plasma GSSG levels, a time dependent increase was observed after the ROFA exposure. Statistical significance was reached at 3 and 5 h, when GSSG content was increased by 42% and 62%, respectively. As a consequence, plasma GSH/GSSG ratio decreased in ROFA-exposed mice at every evaluated time point in comparison with the control group (Table 1).

Ascorbic acid content

Ascorbic acid is a hydrosoluble low molecular weight antioxidant which has the ability to directly scavenge ROS or to regenerate other antioxidants. Ascorbic acid levels in plasma were significantly decreased in ROFA-exposed mice at 3 and 5 h by 29% and 31%, respectively, in comparison with the control group (Fig. 2).

SOD activity

SOD catalyzes the dismutation of superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Three SOD isoforms have been described in mammals: the cytosolic Cu,Zn-SOD (SOD1), the mitochondrial Mn-SOD (SOD2), and the extracellular Cu,Zn-SOD (EC-SOD). Most of the EC-SOD is anchored to the extracellular matrix, especially of blood vessel walls, except for a soluble fraction present in circulation (Petersen et al., 2010). This fraction accounts for SOD activity in plasma, which was significantly decreased in ROFA-exposed mice by 38% at 3 h in comparison with the control. Interestingly, a significant increase by 44% was observed at 5 h in this group in comparison with saline-exposed mice (Fig. 3).

Table 1
Plasmatic GSH and GSSG levels, and GSH/GSSG ratio, after the ROFA exposure. Results are expressed as mean ± SEM of at least 6 independent experiments.

Time (h)	GSH (μM)		GSSG (μM)		GSH/GSSG ratio	
	CONTROL	ROFA	CONTROL	ROFA	CONTROL	ROFA
1	6.3 ± 0.5	5.1 ± 0.4	0.60 ± 0.05	0.77 ± 0.08	10.5	6.6
3	6.4 ± 0.5	4.6 ± 0.2*	0.59 ± 0.06	0.84 ± 0.05**	10.8	5.5
5	6.3 ± 0.5	5.3 ± 0.9	0.62 ± 0.05	0.99 ± 0.09***	10.2	5.4

* $p < 0.05$ compared with GSH control 3 h.
** $p < 0.01$ compared with GSSG control 3 h.
*** $p < 0.001$ compared with GSSG control 5 h.

Tissue damage markers

Plasma CK and LDH activities were measured in order to evaluate if the acute ROFA exposure could lead to significant tissue damage, in addition to the systemic oxidative stress condition. As shown in Table 2, no significant differences were observed for both markers when comparing saline- and ROFA-exposed mice at every evaluated time point.

Cytokine quantification

In order to identify if the acute ROFA exposure could trigger a systemic inflammatory response, plasma levels of TNF-α, IL-6, and IFN-γ were measured. IL-10 levels were also assessed to evaluate a regulatory response. On one hand, TNF-α and IL-6 were found to be increased in mice exposed to ROFA at every evaluated time point, with a peak at 3 h (Figs. 4A and B). Both proinflammatory cytokines were still elevated in ROFA-exposed mice at 5 h after the treatment, but their levels were significantly lower than those at 3 h. On the other hand, a time dependent increase in IL-10 plasma levels was observed after the ROFA exposure, with a maximum at 5 h (Fig. 4C). Regarding IFN-γ, no significant differences were observed at 1 or 3 h between saline- and ROFA-exposed mice. However, a marked increase was found at 5 h in mice exposed to ROFA (Fig. 4D).

Cytokine levels in lung homogenates were also evaluated in order to study the contribution of local inflammation to the observed plasma cytokine levels. In this case, TNF-α and IL-6 were found to be increased as early as 1 h in mice exposed to ROFA, with a maximum at 3 h (Figs. 5A and B). Both cytokines were still elevated in ROFA-exposed mice at 5 h after the treatment, but IL-6 levels were significantly lower than those at 3 h. No changes were observed in IL-10 or IFN-γ levels at any of the evaluated time points (Figs. 5C and D).

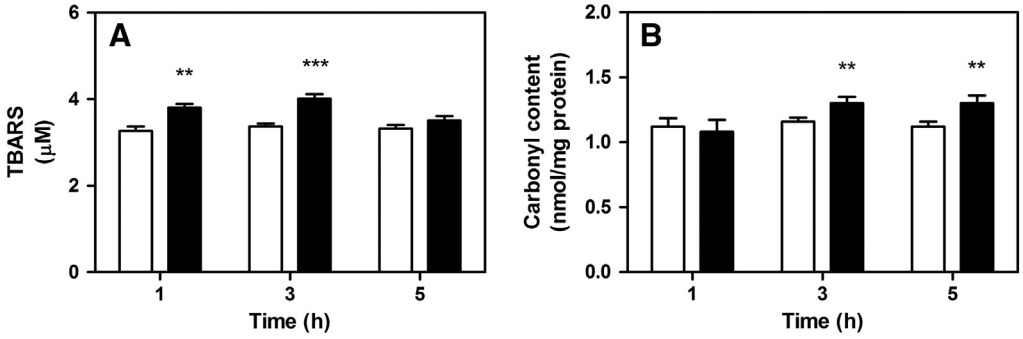

Fig. 1. Fluorometric evaluation of plasma TBARS content (A) and colorimetric assessment of plasma carbonyl content (B), in mice exposed to the ROFA suspension (black bars) or saline solution (white bars). Samples were obtained at 1, 3 and 5 h after the treatment. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding control group.

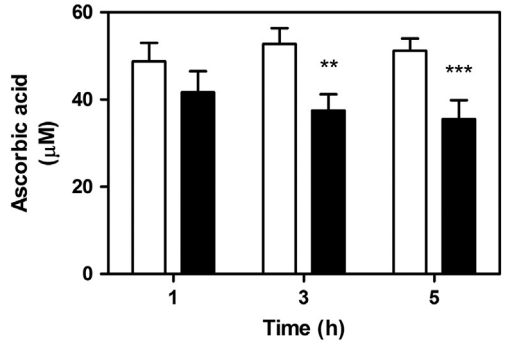

Fig. 2. HPLC determination of plasma ascorbic acid content in mice exposed to the ROFA suspension (black bars) or saline solution (white bars). Samples were obtained at 1, 3 and 5 h after the treatment. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p < 0.01$ compared with control 3 h, and $^{***}p < 0.001$ compared with control 5 h.

Leukocyte respiratory burst and NO production

Given that the acute ROFA exposure induced systemic oxidative stress and inflammation, leukocyte ROS and NO production were assessed by flow cytometry in order to evaluate intravascular activation of innate immune cells. Mononuclear (MN) and polymorphonuclear (PMN) leukocytes were selected based on light-scattering properties (SSC vs FSC, Fig. 6A), and events within gates R1 and R2 were chosen for analysis. No significant changes were observed in MN cells (R1) after the ROFA exposure (data not shown) in none of the assays. However, as shown in the overlaid histograms (Figs. 6B and C), an increase in FL-1 DCF and DAF-2 signal was found in PMN cells (R2) from ROFA-exposed mice in comparison with the control group at the different evaluated time points. Quantification of DCF fluorescence as MFI (Fig. 6D) showed that the exposure to ROFA particles induced a significant increase in DCF oxidation in PMN at 1 h (24%), 3 h (62%), and 5 h (65%), indicating the occurrence of intravascular PMN ROS production in this group. In addition, quantification of DAF-2 fluorescence as MFI showed a significant increase in PMN at 3 and 5 h by 54% and 59%, respectively, indicating increased NO production in PMN in ROFA-exposed mice.

Discussion

Lung injury and local oxidative stress have been extensively described in association with short-term PM inhalation in humans and animal models (Chen and Lippmann, 2009). Several epidemiological studies have also shown that the exposure to PM positively correlates with systemic markers of oxidative stress (Delfino et al., 2011). However, the data available is limited to susceptible populations, such as the elderly and those with preexisting cardiopulmonary diseases (Delfino et al., 2008), or due to occupational exposures (Rossner et al., 2007). ROFA samples used in this study already proved to induce pulmonary (Ferraro et al., 2011; Magnani et al., 2013; Ostachuk et al., 2008) and cardiovascular (Wellenius et al., 2002) alterations, both in vitro and in rodents. Previous results from our group in the same animal model used in this study showed a pulmonary oxidative metabolism imbalance, with a peak at 1 h after the instillation of the ROFA suspension (Magnani et al., 2011). In contrast, the most significant cardiac oxidative metabolism alterations in the same conditions were observed at 3 h after the treatment (Marchini et al., 2013). Consistently with our findings, a similar lag phase was described for the increase in steady-state concentrations of ROS in mice lung and heart after PM inhalation (Gurgueira et al., 2002). As we and others have suggested, this lag may represent the time needed for inhaled PM to induce local tissue damage, which can in turn trigger systemic oxidative stress and inflammation, leading to secondary cardiovascular effects (Gurgueira et al., 2002; Marchini et al., 2013). Therefore, in order to clarify the phenomena underlying these observations, the time course of systemic oxidative stress and inflammation after an acute exposure to PM was evaluated.

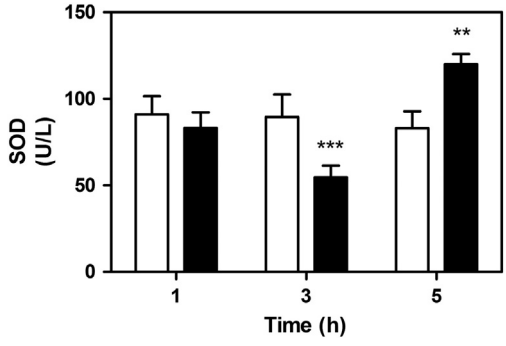

Fig. 3. Colorimetric assessment of plasma SOD activity in mice exposed to the ROFA suspension (black bars) or saline solution (white bars). Samples were obtained at 1, 3 and 5 h after the treatment. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p < 0.01$ compared with control 5 h, and $^{***}p < 0.001$ compared with control 3 h.

Table 2
Colorimetric evaluation of plasmatic CK and LDH activities after the ROFA exposure. Results are expressed as mean ± SEM of at least 6 independent experiments.

Time (h)	CK (U/L)		LDH (U/L)	
	CONTROL	ROFA	CONTROL	ROFA
1	220 ± 20	230 ± 10	150 ± 20	150 ± 10
3	230 ± 10	220 ± 10	160 ± 10	170 ± 20
5	250 ± 20	240 ± 20	150 ± 10	150 ± 20

Normalizing the surface area of the respiratory zone (Stone et al., 1992), the delivered ROFA dose to mice in the present work would equate to ≈50 mg lung burden in humans, which may be archived by one exposed to certain mild to highly polluted microenvironments (PM concentration ≈ 500 μg/m^3) within a week. As a consequence, our first finding was increased systemic markers of oxidative damage to macromolecules in ROFA-exposed mice, together with decreased plasma antioxidant status. This scenario may indicate an increased production of ROS that overwhelm antioxidant defenses, leading to oxidative damage to plasma lipids and proteins. Increased end-products of lipid peroxidation processes were the earliest modified marker after the ROFA exposure, as indicated by plasma TBARS levels at 1 and 3 h. Carbonyl groups from oxidatively modified plasma proteins were also found to be accumulated in this experimental group, but at 3 and 5 h after the treatment. This profile was also observed in lung tissue in the same animal model (Magnani et al., 2011), which may indicate that increased plasma levels of oxidative damage markers may result from some translocation of oxidation end-products from the lung to systemic circulation. Although it is feasible to occur, this mechanism is unlikely the main contributor of this finding. It has been postulated that nanoscale particles and PM water-soluble metals could be able to break through the respiratory epithelia, reaching systemic circulation within minutes to hours (Nemmar et al., 2002; Wallenborn et al., 2007). Therefore, a direct effect of ROFA particles or of its soluble constituents (namely transition metals) over plasma macromolecules may also play a role. The observed lag phase is also consistent with the different susceptibility to oxidative damage of lipids and proteins, as previously reported (Gurgueira et al., 2002). Consistently with our results, elevated markers of oxidative damage are a common finding in association with cardiovascular risk factors such as cigarette smoke, which strengthens the link between air pollutants, oxidative stress, and the onset and progression of cardiovascular diseases (Lee et al., 2012).

Regarding antioxidant defenses, changes in GSH and GSSG levels were observed to exert the earliest response. Depletion of plasma GSH, together with increased GSSG levels, led to a decreased GSH/

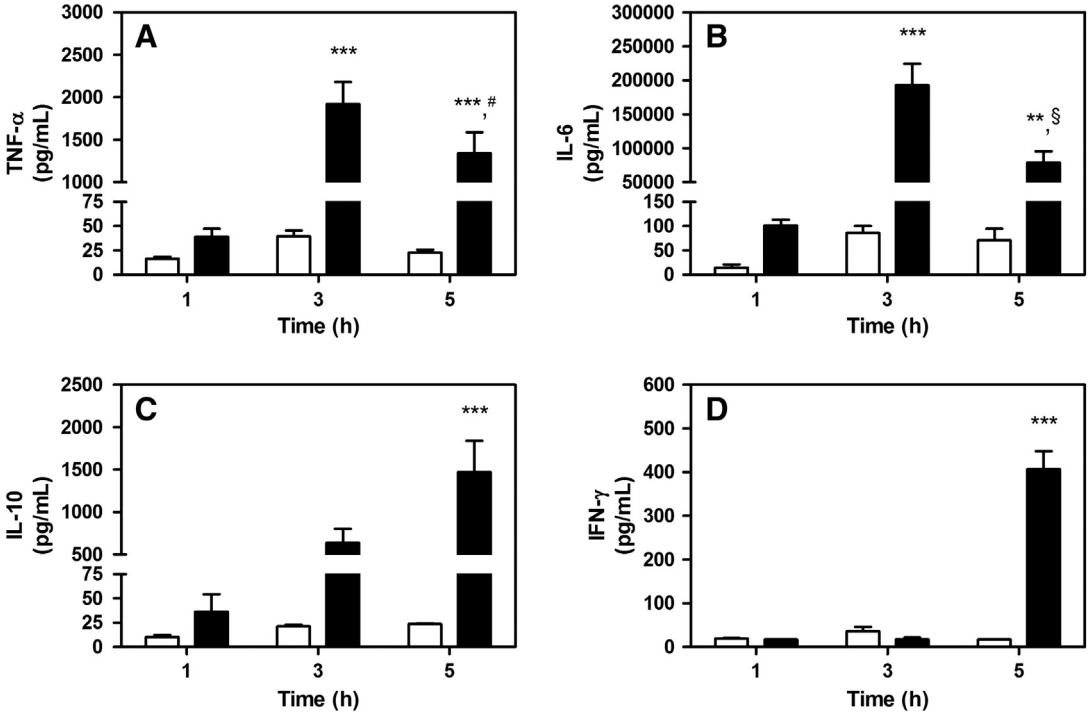

Fig. 4. Plasma cytokine profile of mice exposed to the ROFA suspension (black bars) or saline solution (white bars). TNF-α (A), IL-6 (B), IL-10 (C) and IFN-γ (D) levels were measured by ELISA. Samples were obtained at 1, 3 and 5 h after the treatment. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding control group, $^{\#}p < 0.05$ and $^{\S}p < 0.001$ compared with ROFA 3 h.

GSSG ratio in ROFA-exposed mice as early as 1 h, which remained below control values at every evaluated time point. Consistently, decreased plasmatic ascorbic acid content was also observed in ROFA-exposed mice. Both results, together with increased circulating oxidative damage markers to plasma macromolecules, indicate a shift of systemic redox status towards a more oxidized environment in mice exposed to ROFA, which is a common feature of environmental PM toxicity related to particles' oxidation potential and ability to induce an inflammatory response (Møller et al., 2010). Depletion of systemic antioxidant defenses, together with increased ROS production (as indicated by increased

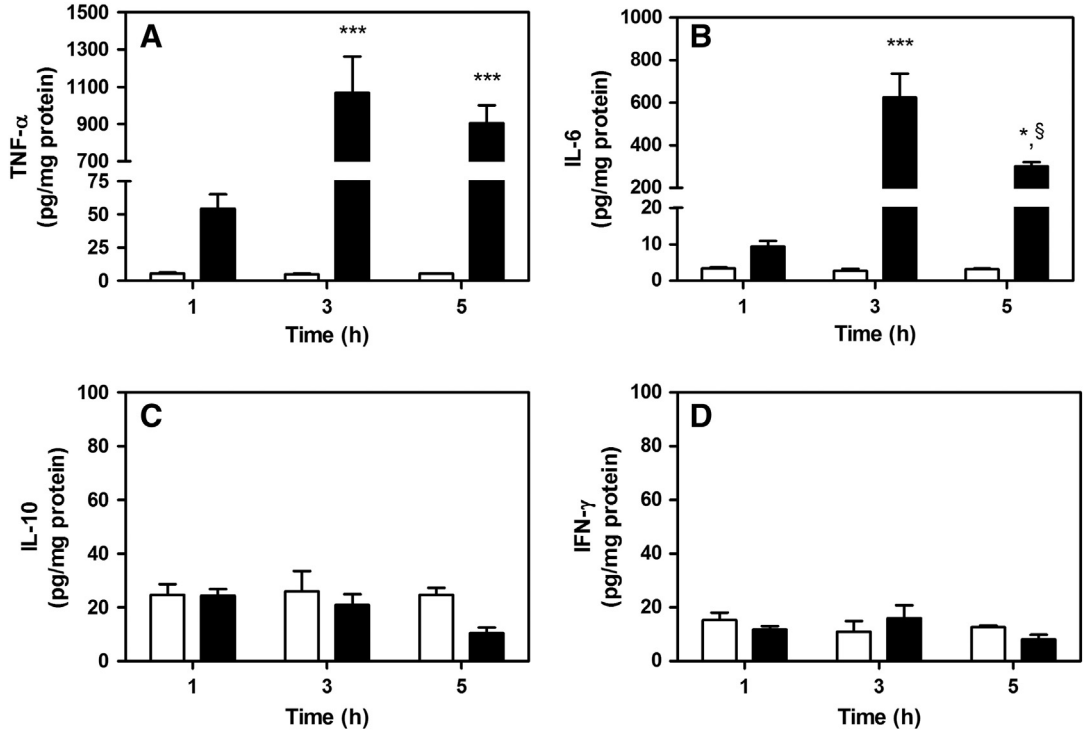

Fig. 5. Cytokine profile in lung homogenates from mice exposed to the ROFA suspension (black bars) or saline solution (white bars). TNF-α (A), IL-6 (B), IL-10 (C) and IFN-γ (D) levels were measured by ELISA. Samples were obtained at 1, 3 and 5 h after the treatment. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the corresponding control group, $^{\S}p < 0.001$ compared with ROFA 3 h.

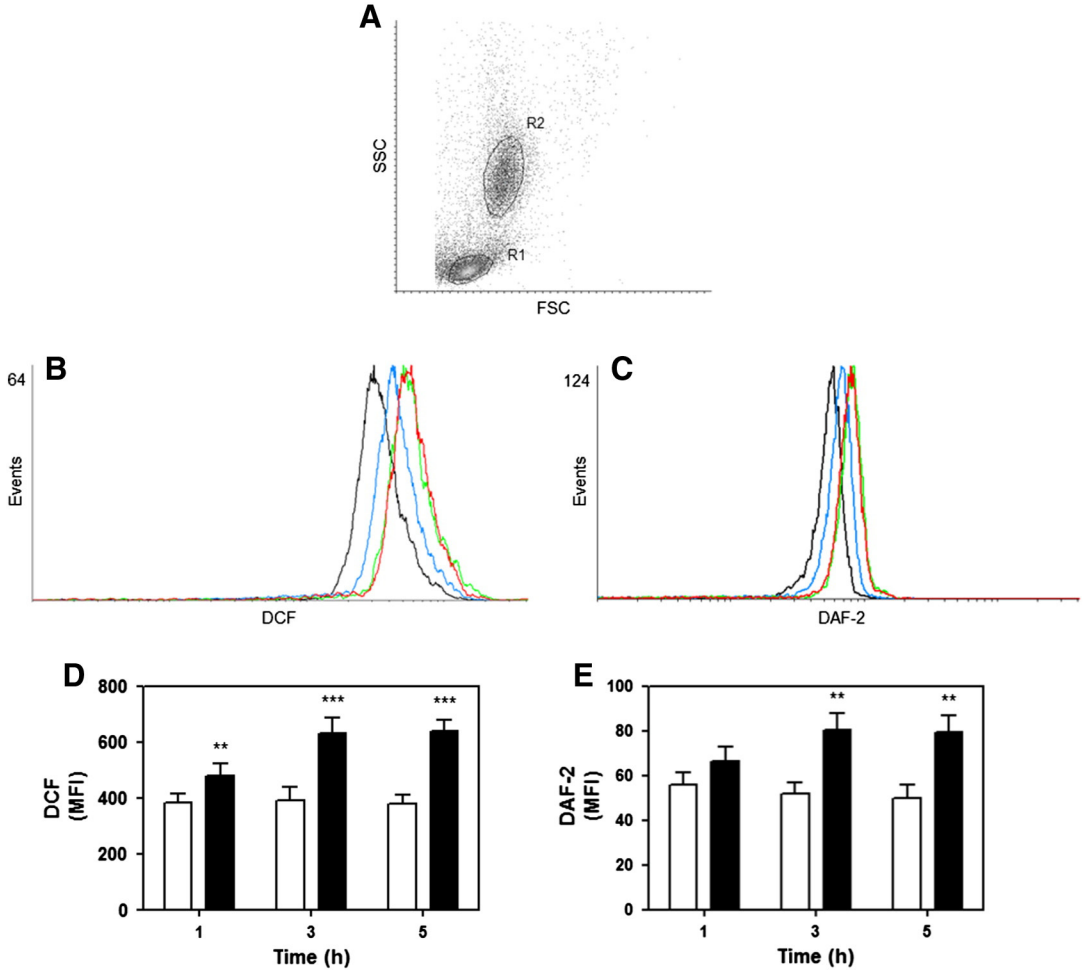

Fig. 6. Leukocyte respiratory burst and NO production assays. Isolated mice leukocytes were obtained at 1, 3, and 5 h after the treatment as described in Methods. (A) 10,000 events per sample were collected and MN (R1) and PMN (R2) cells were gated based on light scattering properties. Overlaid histograms of gated PMN (R2) events versus (B) DCF and (C) DAF-2 fluorescence intensities. Control (black), ROFA 1 h (blue), ROFA 3 h (green), and ROFA 5 h (red). (D) DCF fluorescence quantification of gated PMN cells (R2), indicating PMN activation in saline- (white bars) and ROFA-exposed mice (black bars). (E) DAF-2 fluorescence quantification of gated PMN cells (R2), indicating PMN NO production in saline- (white bars) and ROFA-exposed mice (black bars). Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding control group.

plasma oxidative damage markers), suggest that the exposure to ROFA particles triggers systemic oxidative stress. Interestingly, plasma SOD activity showed a more complex response. This antioxidant enzyme is often decreased in a wide variety of oxidative stress conditions, but also positively modulated as an adaptive response or as a consequence of a proinflammatory state (Fattman et al., 2003). On one hand, it was decreased at 3 h in ROFA-exposed mice. Such an inverse association between SOD activity and PM exposure has been previously reported (Delfino et al., 2008), which is suggested to play a central role in the progression of endothelial dysfunction in patients with coronary artery disease (Landmesser et al., 2000). On the other hand, increased SOD activity at 5 h may indicate an adaptive response to the systemic oxidative stress triggered by ROFA at earlier time points. Consistently, increased SOD activity has also been observed in lung and heart after an acute exposure to PM (Gurgueira et al., 2002; Magnani et al., 2011).

Taken together, these results indicate that the acute ROFA exposure leads to a systemic oxidative stress condition, in addition to the previously described cardiopulmonary oxidative metabolism alterations (Magnani et al., 2011; Marchini et al., 2013). In this study, significant lipid oxidation together with decreased GSH/GSSG ratio was the main modified parameter at 1 h after the treatment. At 3 h, systemic oxidative stress can be clearly observed, characterized by increased oxidative damage to lipids and proteins (as indicated by TBARS and carbonyl groups content, respectively), and decreased antioxidant status (decreased GSH/GSSG ratio, ascorbic acid content, and SOD activity). Despite increased plasma carbonyl groups content and decreased GSH/GSSG ratio and ascorbic acid content are still present at 5 h after the ROFA exposure, the onset of an adaptive response (such as increased SOD activity and decreased TBARS content) can be inferred. It is worth noting that these alterations were not associated with significant lung or heart tissue damage, as indicated by the unaltered plasma LDH and CK activities.

It is well-established that pulmonary oxidative stress triggered by PM inhalation modulates the activity of redox-sensitive transcription factors such as NFκB (Samet et al., 2002). Activated NFκB leads, in turn, to the onset of a local inflammatory response via increased transcription of several chemokines, cytokines, and acute phase proteins. Interestingly, this condition seems not to be limited to the lung, since increased circulating proinflammatory mediators after PM inhalation are indicative of a systemic response (Brook et al., 2010). Consistently with these findings, an acute exposure to ROFA particles induced an increase in circulating proinflammatory cytokines (TNF-α and IL-6), with a maximum at 3 h. At this time point, impaired mitochondrial function and contractile reserve was described in the same animal model (Marchini et al., 2013), suggesting that systemic inflammation may account, at least in part, for the observed cardiac alterations. Indeed, the role of TNF-α and IL-6 as mediators of disease progression in

the failing heart is well-documented (Hohensinner et al., 2011). Moreover, this proinflammatory state may also be related to the increased plasma SOD activity at 5 h, since this antioxidant enzyme is positively modulated in inflammatory conditions as an adaptive response. Another important finding in the evolution of the systemic inflammatory response triggered by ROFA is the late increase in IL-10 plasma levels. This anti-inflammatory cytokine is commonly up-regulated in order to limit TNF-α and IL-6 effects (Saraiva and O'Garra, 2010). In ROFA-exposed mice, IL-10 may play a role in the resolution of the immune response, leading to the observed decrease in TNF-α and IL-6 levels at 5 h. However, a late increase in IFN-γ plasma levels was observed at 5 h after the treatment. This cytokine is mainly produced by T cells and Natural Killer Lymphocytes, inducing macrophage activation and promoting the perpetuation of the immune response (Schroder et al., 2004).

Cytokines production in lung homogenates was also assessed in order to clarify the source of plasma cytokine levels. Interestingly, the time course of the changes in pulmonary TNF-α and IL-6 levels in ROFA-exposed mice paralleled with those observed in plasma. However, these increases were higher in plasma samples than in lung homogenates (in particular for IL-6), which suggests that pulmonary proinflammatory cytokine release contributes to the observed cytokine levels in plasma, yet is not the only source. Moreover, no pulmonary induction of IL-10 or IFN-γ was observed, supporting the idea of differential cytokine sources rather than solely the lung. Indeed, intravascular PMN leukocytes showed increased activity at 1, 3 and 5 h (as indicated by ROS and NO production). Given that this cell type is able to respond to several proinflammatory cytokines (e.g. macrophage-secreted TNF-α), producing a variety of soluble mediators such as chemokines, cytokines, and angiogenic factors (Mantovani et al., 2011), activated PMN may significantly contribute to the observed plasma cytokine levels. Taken together, these results indicate that the acute ROFA exposure promotes lung release of inflammatory cytokines that might reach systemic circulation and impact on other cell types, promoting more inflammation, PMN activation, and a regulatory response. In this scenario, both lung and circulatory cells activation may account for the observed increased in plasma cytokine levels in ROFA-exposed mice.

During respiratory burst, activated PMN cells produce large amounts of $O_2^{\bullet-}$, mainly due to NADPH oxidase assembly and activation. By transferring electrons from NADPH to O_2, NADPH oxidase produces $O_2^{\bullet-}$ which can, in turn, dismutate to H_2O_2 in a diffusion-controlled reaction catalyzed by SOD. Traces of soluble transition metals, such as the ones described as ROFA constituents, are able to cleave the H_2O_2 molecule, yielding the highly reactive hydroxyl radical ($^{\bullet}OH$). This pathway may account for the increased lipid peroxidation and decreased GHS/GSSG ratio, as early as 1 h after the exposure to ROFA. Alternatively, $O_2^{\bullet-}$ can react with NO to yield peroxynitrite ($ONOO^-$), a powerful oxidant and nitrating agent. Given that NO production is increased in PMN at 3 and 5 h, $ONOO^-$ may contribute to the observed oxidative damage to plasma macromolecules and depletion of antioxidant defenses at later time points. Moreover, $ONOO^-$ formation decreases NO bioavailability, which may be related to impaired NO-dependent arteriolar dilation after a ROFA exposure as previously described (Nurkiewicz et al., 2004). As mentioned, direct oxidants production from ROFA particles and/or soluble constituents may also contribute to these observations.

In conclusion, the relevance of this study relies on the unraveling of the mechanisms by which the lung and heart impaired oxidative metabolisms can develop an integrated response to an acute PM exposure, which is initiated by local tissue damage that afterwards triggers systemic oxidative stress and inflammation. The obtained findings contribute to the understanding of the increase in cardiopulmonary morbidity and mortality, in association with environmental PM inhalation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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