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Lung oxidative metabolism after exposure to ambient particles

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ABSTRACT

The aim of this work was to study the time course of the oxidative metabolism in mice lung after exposure to ambient particles (ROFA). Swiss mice were intranasally instilled with a ROFA suspension (0.20 mg/kg). Animals were sacrificed 1 or 3 h after the exposure. Eighty percentage of increased oxygen consumption was observed in tissue cubes after 1 h of exposure. This observation was accompanied by an increased NADPH oxidase activity (40%) and mitochondrial oxygen consumption in state 3 (19%). NO production by lung homogenates was found to be increased by 43% after 3 h of exposure. Phospholipid oxidation in lung homogenates showed a 29% increase after 1 h of exposure, while a 30% increase in the carbonyl content was found only after 3 h of exposure. Our data show the relative importance of different sources of reactive oxygen species (NADPH oxidase activity and mitochondrial respiration) to the increased tissue oxygen consumption, oxidative damage and antioxidant status observed in an acute model of ROFA particles exposure.

1. Introduction

Numerous epidemiological studies suggest an association between adverse health effects and exposure to air pollution [1,2]. Updated analyses of these data have shown an increased morbidity from pneumonia, chronic obstructive pulmonary disease (COPD), and asthma attacks, along with the aggravation of respiratory and cardiovascular diseases, mainly observed in individuals sensitive to urban air pollution, including elderly, children, and people with heart and lung diseases [3,4]. The adverse health effects were found to be significant for short term exposure, particularly for components containing several transition metals [5].

Air pollution is comprised by a wide range of chemicals and solid particles, which relative composition is different in each microenvironment. Some of the pollutants present in this mixture are free radicals (as $NO_2^{\cdot}$) or are able to drive free radical reactions, as ozone and particulate matter (PM). Consequently, the exposure to these air pollutants can cause changes in the oxidative metabolism and oxidative damage within the lung, and triggering responses that are particularly dangerous not only to susceptible but healthy members of the population as well.

Of the different air pollutants, PM seems to be the major concern from a health perspective [6]. Both organic and inorganic compounds could be found in the particle's core. Inorganic residues such as fly ashes are the result of an incomplete oxidation of carbonaceous materials. This pollutant significantly contributes to the ambient air particulate burden. Because of the unique composition of the Residual Oil Fly Ashes (ROFA), especially rich in metals, it has been useful as surrogate for ambient air PM in many biological studies. Previous data suggests that ambient air and other particles emission sources follow a comparable mechanism of action as ROFA including phosphorylation reactions, transcription factor activation, mediators release and inflammatory injury [7].

Interactions between PM and mitochondria could also generate additional oxidants species [8]. Under physiological conditions, about 85–90% of the oxygen taken up by animals is consumed by the mitochondria [9]. It is now accepted that mitochondria plays an integral role in orchestrating the cellular response to a wide variety of metabolic and environmental stressors. Therefore, the biological effects that reactive oxygen and nitrogen species evoke on this organelle, and their potential regulatory function, are of particular interest [10]. Most of the 10–15% of the oxygen taken up that is not consumed by mitochondria is used by various oxidase and oxygenase enzymes. The phagocytosis of PM can induce the assembly and activation of NADPH oxidase. This enzyme may play a role as a potent superoxide anion generating enzyme which is expressed both in phagocytic and nonphagocytic cells under inflammatory conditions [11].

Abbreviations: ROFA, Residual Oil Fly Ashes; PM, particulate matter; TBARS, thiobarbituric acid reactive species; TRAP, total reactive antioxidant potential; SOD, superoxide dismutase.

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The aim of this work is to study the oxidative mechanisms occurring in lung mice after an acute ROFA exposure, and the temporal sequence of oxidative damage after this exposure.

2. Materials and methods

2.1. Drugs and chemicals

All chemicals were purchased from Sigma–Aldrich Chemical Company (St Louis, MO, USA), except gp91ds-tat peptide that was provided by AnaSpec (Fremont, CA, USA).

2.2. Experimental model

2.2.1. Preparation of ROFA suspension

Particles were freshly prepared by suspending ROFA particles in sterile saline solution, (0.1 mg/mL) and sonicated for 10 min in an ultrasonic water bath [12].

ROFA particles were collected from Boston Edison Co., Mystic Power Plant, Mystic, CT, USA, and were kindly provided by Dr. J. Godleski (Harvard School of Public Health, MA, USA).

2.2.2. Animal exposure

Female Swiss mice, weighing 20–25 g, were exposed to ROFA particles by intranasal instillation [13]. Briefly, mice anaesthetized (i.p.) with 1 mL/kg body weight of xylazine (2%) and ketamine (50 mg/mL) were intranasally instilled with 50 μL of a ROFA suspension (0.20 mg/kg body weight). The ROFA dose selected (0.20 mg/kg) was based on results shown in previous studies [14]. Animals were sacrificed 1 or 3 h after the exposure. Animal treatment was carried out in accordance with the guidelines of the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT).

2.3. Oxygen consumption by tissue cubes

To evaluate the lung oxygen consumption, tissue cubes (1 mm^3) were used as organ sample. A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph) was used. Briefly, oxygen consumption rates were measured in a reaction medium consisting of 118 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 5.5 mM glucose at 30 °C [15]. Results were expressed as ng-at O/min g tissue.

2.4. Tissue homogenates

Lung samples (0.2 g of wet weight) were homogenized in 120 mM KCl, 30 mM phosphate buffer (pH 7.4) (1:5). The suspension was centrifuged at 600*g* for 10 min at 0–4 °C to remove nuclei and cell debris. The pellet was discarded and the supernatant was used as "homogenate" [16].

2.5. NO production

Nitric oxide production was determined by the oxidation of oxyhemoglobin to methemoglobin, and followed spectrophotometrically at two wavelengths 577–591 nm (ε = 11.2 $mM^{-1}\,cm^{-1}$) in a Beckman DU 7400 diode array spectrophotometer at 37 °C [17]. Control measurements in the presence of 2 mM N^G-methyl-L-arginine (L-NMMA) were performed to consider only L-NMMA-sensitive hemoglobin oxidation, usually 90–95% due to NO formation. Results were expressed as nmol NO/min mg protein.

2.6. NADPH oxidase activity

2.6.1. NADPH dependent superoxide anion production

Superoxide production was measured by the lucigenin-derived chemiluminescence method [18]. An aliquot of the sample was diluted in 250 μL of 50 mM phosphate buffer containing 1 mM EGTA and 150 mM sucrose. 50 μM lucigenin was added and 100 μM NADPH was used as substrate, chemiluminescence was immediately measured at 15 s intervals for 3 min in a Labsystems Luminoskan RS Microplate Reader. The specificity of the measurement was confirmed by adding superoxide dismutase (200 U/mL). Results were expressed in arbitrary units/mg protein.

2.6.2. NADPH consumption

The NADPH consumption rate was spectrophotometrically followed by measuring the decay in absorbance at 340 nm in a medium containing phosphate buffer 100 μM (pH 7.4) at 37 °C [19]. Results were expressed as nmol NADPH/min mg protein.

The specificity of both assays was tested using the NADPH oxidase inhibitor gp91 ds-tat. The peptide gp91 ds-tat was administrated (i.p) (10 mg/kg) 30 min before ROFA exposure [20].

2.7. Isolation of mitochondria

Tissues samples were homogenized in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl, and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 700*g* for 10 min to discard nuclei and cell debris, the sediment was discarded, and the supernatant was centrifuged at 8000*g* for 10 min to obtain the enriched mitochondrial fraction. Purity of isolated mitochondria was assessed by determining lactate dehydrogenase activity; only mitochondria with less than 5% impurity were used [21].

2.8. Mitochondrial respiration

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph) was used. Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 1 mM EDTA, 4 mM $MgCl_2$, and 5 mM phosphate buffer (pH 7.4), at 30 °C. Succinate 8 mM was used as substrate to measure resting respiration (state 4); 1 mM ADP was added to measure active respiration (state 3). Results were expressed as ng-at O/mg protein. Respiratory control (RC) was calculated as the relationship between state 3 respiration and state 4 respiration [22].

2.9. Protein carbonyl content

The content of carbonyl groups in oxidatively modified proteins was measured by assessing the amount of 2,4 dinitrophenylhydrazone formed upon the reaction with 2,4 dinitrophenylhydrazine (DNPH). Homogenates were treated for 1 h with 2 mM DNPH at room temperature. Proteins were precipitated with TCA, washed with ethanol/ethyl acetate (1:1), and dissolved in 6 M guanidine hydrochloride (pH 2.5). Carbonyl content was calculated from the absorbance at 360 nm [23]. Results were expressed as nmol carbonyl groups/mg protein.

2.10. Phospholipid oxidation

Phospholipid oxidation was determined as thiobarbituric acid reactive substances (TBARS) with a fluorometric assay [24]. Tissue homogenate (0.5 mL) was added to a medium consisting of 0.1 N HCl, 10% (w/v) phosphotungstic acid and 0.7% (w/v) 2-thiobarbituric acid. After incubation in boiling water for 60 min, TBARS were extracted in *n*-butanol. The fluorescence of the butanolic layer

was measured in a Perkin–Elmer LS 55 flourometer 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 pmol TBARS/mg protein.

2.11. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined spectrophotometrically by following the inhibition of the rate of adenochrome formation at 480 nm, in a reaction medium containing 1 mM epinephrine and 50 mM glycine/NaOH (pH 10.5) [25]. Enzymatic activity was expressed as SOD units/mg protein. One unit was defined as the amount of sample able to inhibit the rate of adrenochrome formation by 50%.

2.12. Catalase activity

Catalase activity was evaluated by following the decrease in absorbance at 240 nm in a reaction medium consisting of 100 mM phosphate buffer (pH 7.4) and 20 mM hydrogen peroxide [26]. Results were expressed as pmol catalase/mg protein.

2.13. Total reactive antioxidant potential

Total reactive antioxidant potential (TRAP) was measured by a chemiluminescent method [27]. Briefly, 20 mM of 2,2-azobis 2-amidinopropane (ABAP) was used as a source of free radicals, which react with luminol (40 μM), yielding a chemiluminescence signal followed in a Packard Tri Carb liquid scintillation counter. The addition of the sample decreases the chemiluminescence signal to basal levels, for a period of time which is proportional to the amount of antioxidants present in the sample (induction time). The system was calibrated against Trolox, a vitamin E hydrosoluble analog. Results were expressed as μM Trolox/mg protein.

2.14. Protein content

Protein concentration was measured by the method of Lowry et al. [28] using bovine serum albumin as standard.

2.15. Statistics

Results were expressed as mean values ± SEM. Student's *t*-test for unpaired data was used to analyze differences between mean values of two groups. ANOVA followed by Tukey test was used to analyse differences between mean values of more than two groups. Statistical significance was considered at $p < 0.05$.

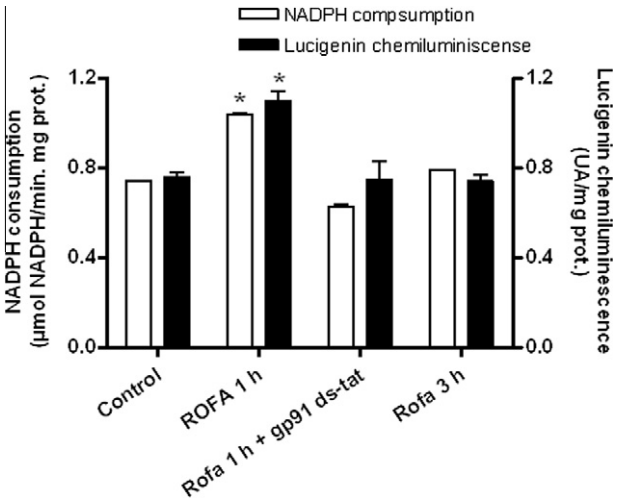

Fig. 2. NADPH oxidase activity assayed through the measurement of superoxide anion production using the chemiluminescent probe lucigenin (□) and the spectrophotometric determination of NADPH consumption (■) after exposure to ROFA. $n = 6$. $^{*}p < 0.005$ vs. control.

3. Results

3.1. Oxygen and NO metabolism

3.1.1. Oxygen consumption by tissue cubes

As shown in Fig. 1A, instillation with ROFA increased oxygen consumption in lung by 80% after 1 h of exposure. The 3 h group showed a 23% increased oxygen consumption in comparison with the control, whereas a significant decrease was observed when the same group was compared with the 1 h group (control value: 225 ± 7 ng-at O/min mg tissue; 1 h: 407 ± 33 ng-at O/min mg; 3 h: 277 ± 12 ng-at O/min mg).

3.1.2. Nitric oxide production

The production of NO by lung homogenates (Fig. 1B) was significantly increased, by 43% (control value: 0.74 ± 0.03 nmol NO/min mg prot), when compared to the control group after 3 h of ROFA exposure. No significant changes were found for the 1 h exposure to ROFA group.

3.1.3. NADPH oxidase activity

As shown in Fig. 2 and 1 h after the instillation NADPH-dependent superoxide production by lung homogenate showed a 45% increase, (control value: 0.76 ± 0.02 UA/mg prot), regarding NADPH consumption, a 40% increase was observed (control value:

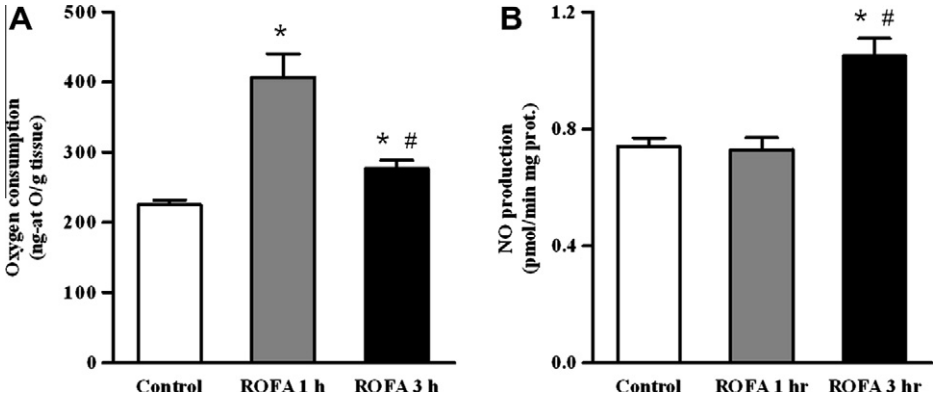

Fig. 1. Oxygen consumption by tissue cubes (Panel A) and NO production in lung homogenates (Panel B) after exposure to ROFA. $n = 6$. $^{*}p < 0.005$ vs. control; $^{\#}p < 0.005$ vs. 1 h.

0.74 ± 0.01 μmol NADPH/min mg prot). The pretreatment with NOX2 inhibitor gp91 ds-tat suppressed the increase observed in the 1 h exposure to ROFA group. No significant differences were observed for the 3 h group.

3.1.4. Oxygen consumption by lung mitochondria

Oxygen consumption by isolated mitochondria in state 4 (resting respiration), state 3 (active respiration) and the corresponding RC were obtained and the results are shown in Table 1. ROFA treatment after 1 h of exposure significantly increased mitochondrial state 3 oxygen consumption by 20%. No significant changes were observed in either state 4 respiration or RC. No changes were observed after 3 h of exposure.

3.2. Oxidative damage

3.2.1. Protein carbonyl content

Fig. 3A shows the carbonyl content of lung homogenates after the instillation with ROFA. While no statistical changes were observed after 1 h of exposure, a 30% increase was found after 3 h (control value: 2.2 ± 0.1 nmol/mg prot).

3.2.2. Phospholipid oxidation

After 1 h of exposure, a 29% increase (control value: 249 ± 5 pmol/mg prot) in phospholipid oxidation was observed (Fig. 3B). After 3 h of exposure this marker of oxidative damage to lipids returned to control values.

A positive correlation was found between phospholipid oxidation and NADPH dependent $O_2^{\cdot-}$ production, measured by lucigenin chemiluminescence, and mitochondrial oxygen consumption in state 3 as it is shown in Fig. 4.

3.3. Antioxidant status

3.3.1. Activity of antioxidant enzymes

A significant 26% increase in SOD activity was found in lung homogenates after 3 h of exposure, whereas catalase, the main detoxifying system for hydrogen peroxide, was not affected either after 1 or 3 h of exposure (Table 2).

Table 1
Oxygen consumption and respiratory control of mitochondria isolated from lung of mice exposed to Residual Oil Fly Ash (ROFA).

	Control	ROFA 1 h	ROFA 3 h
State 3 respiration (ng-at O/min mg prot.)	107 ± 4	127 ± 4*	116 ± 5
State 4 respiration (ng-at O/min mg prot.)	60 ± 4	66 ± 4	55 ± 6
Respiratory control	1.83	2.03	1.97

* $p < 0.005$.

3.3.2. TRAP levels

Total reactive antioxidant potential evaluation estimates the hydrosoluble low molecular weight antioxidants levels present in biological samples [29]. TRAP values after 3 h of exposure were 40% lower than the corresponding control value (Table 2).

4. Discussion

The present study shows different sources of reactive species contributing to the increased tissue oxygen consumption after inhalation of particulate matter (ROFA). Additionally, the time course of oxidative metabolism and oxidative damage in mice lung is presented.

The ROFA dose was selected assuming that the minute ventilation of a mouse corresponds to 30 mL, making a total ventilation of about 0.043 m^3 in 24 h. The amount of particles administered in this study was 5 μg per mouse, corresponding to an ambient concentration of particles of 116 μg/m^3 [30], which can be considered as a low dose when compared with pollution levels attained in large cities worldwide [31].

Tissue oxygen consumption was found to be significantly increased after 1 h of instillation with ROFA. With the aim of clarifying the underlying mechanisms of this observation, NADPH oxidase activity and mitochondrial oxygen consumption were measured as the main sources of oxygen consumption after ROFA exposure. The activation of NADPH oxidase (a 40% increase was observed) may be a relevant non-mitochondrial source of reactive oxygen species in this model. The inflammatory process triggered by the ROFA exposure, includes leukocyte recruitment, activation (respiratory burst) and increased alveolar macrophages count [32]. It has been shown that the NADPH oxidase homolog NOX2 expressed in macrophages is an important source of reactive oxygen species under airway inflammation elicited by environmental factors, including PM exposure [33,34]. However, through the use of NOX2 knockout mice exposed to PM, it was suggested that monocyte infiltration could also contribute to the augmented $O_2^{\cdot-}$ production [35]. Tissue oxygen consumption is related to mitochondrial energy production and reactive oxygen species release. Consequently, the increased tissue oxygen consumption observed after 1 h may also be originated by an elevated mitochondrial subpopulation in state 3 (active respiration), in order to respond to high ATP demand [36]. In agreement with these results, it has been shown that exposure to PM is able to alter mitochondrial function [8].

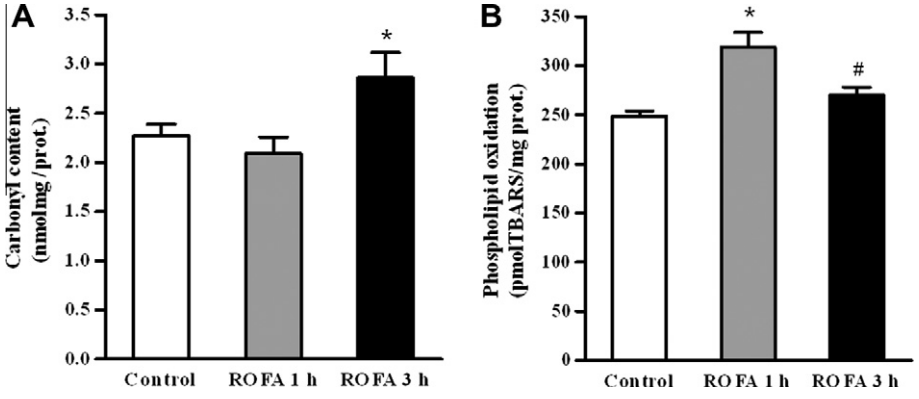

Fig. 3. Carbonyl content (Panel A) and TBARS levels (Panel B) in lung homogenates after exposure to ROFA. $n = 6$. *$p < 0.005$ vs. control by ANOVA–Tukey test; #$p < 0.005$ vs. 1 h.

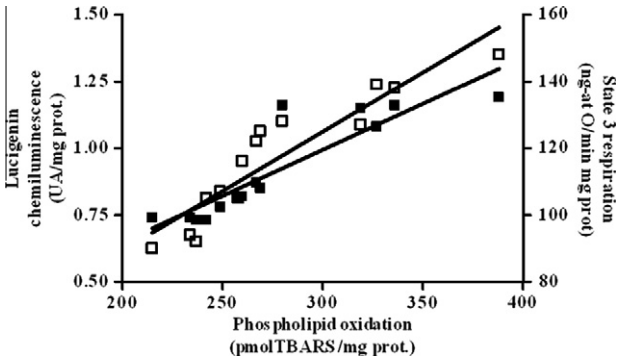

Fig. 4. Linear correlation between phospholipid oxidation assayed as TBARS content and NADPH dependent $O_2^{\cdot-}$ production, assayed by lucigenin chemiluminiscence in lung homogenate (□, $p < 0.0001$, $r^2 = 0.80$), and mitochondrial oxygen consumption in state 3 (■, $p < 0.0001$, $r^2 = 0.85$), from lung of control and ROFA treated animals.

Table 2
Activity of the antioxidant enzymes superoxide dismutase and catalase and Total Reactive Antioxidant Potential (TRAP) levels in lung homogenates of mice exposed to Residual Oil Fly Ash (ROFA).

	Control	ROFA 1 h	ROFA 3 h
Superoxide dismutase (USOD/mg prot.)	4.31 ± 0.18	4.82 ± 0.30	5.43 ± 0.25*
Catalase (pmoles/mg prot.)	1.05 ± 0.05	0.90 ± 0.15	0.96 ± 0.08
TRAP (μM Trolox/mg prot.)	14.8 ± 0.99	16.8 ± 1.25	9.43 ± 1.21*

* $p < 0.005$.

NO plays an important regulatory role on mitochondrial respiration [12,22], inflammatory responses, and may be involved in the nitroxidative damage in lung due to exposition to polluted environments [37]. The increased production of NO by lung homogenates found after 3 h of exposure is in agreement with this observation (Fig. 1B). Moreover, the increased formation of $O_2^{\cdot-}$ and its reaction with NO may account for the formation of peroxynitrite ($ONOO^-$). Peroxynitrite is a powerful oxidant that can yield the highly reactive hydroxyl radical ($HO^{\cdot}$) after homolysis by reaction with Fe^{3+}, initiating free radical chain reaction (as lipid peroxidation) [38]. This last observation is suggested in Fig. 4.

In inflammatory situations, superoxide anion mainly produced by NADPH oxidase is removed by superoxide dismutase, which is present at the surface of epithelial cells in the lung [39]. The product of this reaction is H_2O_2, which can easily diffuse through plasma membranes. It can therefore initiate intracellular signaling pathways contributing to the generation of other oxidant species or being detoxified by the cytosolic enzyme catalase [40]. The observed increase in the activity of SOD may be due to an adaptive response, since an augment in the expression and activity of the enzyme can prevent lung damage produced by the exposure to particulate matter [41]. The 36% decrease observed in TRAP values in tissue homogenates after 3 h shows a moderate consumption of antioxidants, since TRAP values are an indication of the levels of low molecular weight antioxidants (mainly glutathione, ascorbic acid and other hydrosoluble molecules capable of trapping free radicals) [29].

According to our results, the macromolecular damage showed different induction time, since there was a statistically significant increase in phospholipid oxidation after 1 h of exposure, while oxidative damage to proteins was significant only after 3 h exposure. The different time course is consistent with different susceptibility to oxidative damage of these macromolecules, as suggested by previous works [42].

In summary, our data shows for the first time the relative importance of different sources of reactive species (NADPH oxidase activity and mitochondrial respiration) to the increased tissue oxygen consumption observed in a model of exposure to ROFA particles. The usefulness of this observation is further supported by its positive correlation with the increase in the oxidative damage. This work provides new insights to the understanding of the molecular mechanisms involving free radicals in lung oxidative damage due to exposition to ambient particles as ROFA.

Conflict of interest

The authors declare that there are no conflicts of interest

Acknowledgments

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