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Impaired cardiac mitochondrial function and contractile reserve following an acute exposure to environmental particulate matter

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ABSTRACT

Background: It has been suggested that mitochondrial function plays a central role in cardiovascular diseases associated with particulate matter inhalation. The aim of this study was to evaluate this hypothesis, with focus on cardiac O_2 and energetic metabolism, and its impact over cardiac contractility.
Methods: Swiss mice were intranasally instilled with either residual oil fly ash (ROFA) (1.0 mg/kg body weight) or saline solution. After 1, 3 or 5 h of exposure, O_2 consumption was evaluated in heart tissue samples. Mitochondrial respiration, respiratory chain complexes activity, membrane potential and ATP content and production rate were assessed in isolated mitochondria. Cardiac contractile reserve was evaluated according to the Langendorff technique.
Results: Three hours after ROFA exposure, tissue O_2 consumption was significantly decreased by 35% (from 1180 ± 70 to 760 ± 60 ng-at O/min g tissue), as well as mitochondrial rest (state 4) and active (state 3) respiration, by 30 and 24%, respectively (control state 4: 88 ± 5 ng-at O/min mg protein; state 3: 240 ± 20 ng-at O/min mg protein). These findings were associated with decreased complex II activity, mitochondrial depolarization and deficient ATP production. Even though basal contractility was not modified (control: 75 ± 5 mm Hg), isolated perfused hearts failed to properly respond to isoproterenol in ROFA-exposed mice. Tissue O_2 consumption rates positively correlated with cardiac contractile state in controls ($r^2 = 0.8271$), but not in treated mice ($r^2 = 0.1396$).
General Significance: The present results show an impaired mitochondrial function associated with deficient cardiac contractility, which could represent an early cardiovascular alteration after the exposure to environmental particulate matter.

1. Introduction

Epidemiological studies have shown a positive correlation between decreased air quality levels and adverse health effects [1]. Increased cardiovascular morbidity and mortality rates have been found to be associated not only with chronic air pollution exposures [2], but also with short-term daily exposures as well [3]. Interestingly, although the complex nature of air pollution and the coexistence of many compounds which may together contribute to the observed negative health impact, substantial epidemiological data point out that particulate matter (PM) is the main responsible for the health outcomes [4].

PM is comprised of a heterogeneous mixture of solid and liquid particles suspended in air, varying in size, chemical composition and sources of origin [5]. Anthropogenic emissions are the main contributors to environmental PM burden and consist mainly of motor vehicle emissions and fossil fuel combustion during power generation and industrial processes [6]. 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) [7]. 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 [8]. Therefore, ROFA is the most frequently used combustion-derived particle in order to evaluate the contribution of transitions metals in the biological effects of PM inhalation [9]. Moreover, ROFA particles often present an aerodynamic diameter smaller than 2.5 μm ($PM_{2.5}$), a size that have been

Abbreviations: m-CCCP, carbonyl cyanide m-chlorophenylhydrazone; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; ISO, isoproterenol; LVDP, left ventricular developed pressure; NAO, 10-*N*-nonyl acridine orange; PM, particulate matter; ROFA, residual oil fly ash; RCR, respiratory control ratio

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shown to be more closely associated with PM adverse health effects than coarser particles ($PM_{10-2.5}$) [5].

Among adverse cardiovascular effects triggered by PM exposure, daily changes in PM concentration have been associated with increased hospitalizations due to several cardiovascular events such as heart failure, arrhythmias, ischemic heart disease, cerebrovascular disease and peripheral vascular disease [10]. Increased production of reactive O_2 species leading to oxidative damage have been suggested to significantly contribute to the cardiopulmonary toxicity of PM inhalation [11]. Numerous studies in humans and animal models have shown a pulmonary and systemic inflammatory response and oxidative stress associated with PM exposure which can, in turn, alter heart O_2 metabolism and cardiovascular function [12]. Although most of these studies are focused on PM mass, in the last years increased awareness turned to PM chemical composition owing to the relevance of certain PM constituents (e.g. transition metals, organic redox-active compounds and endotoxins) in promoting cardiovascular diseases [4]. It is worth noting that, even though the link between air pollution PM inhalation and cardiovascular adverse effects is quite established, the underlying molecular mechanisms are poorly understood.

Given that mitochondria play an essential role in cellular O_2 and energetic metabolism, several authors suggested that mitochondrial dysfunction is a key feature in the development of cardiac alterations during the exposure to air pollution PM [13,14]. Cardiac contraction and relaxation have a continuous energy requirement, consuming more energy than any other organ. Because of a mismatch in ATP supply and demand, decreased levels of high-energy phosphates have been reported in the failing human heart [15] hampering with the transference of chemical energy to contractile work [16]. Most of this energy is produced in the mitochondria by oxidative phosphorylation, a process that involves electron-transfer reactions through the mitochondrial respiratory chain complexes at the inner mitochondrial membrane. In this context, any alteration triggered by PM inhalation in these multienzymatic complexes, in the electrochemical H^+ gradient that they generate across the inner membrane or in F_0–F_1 ATP synthase activity, could lead to a deficient ATP production resulting in a bioenergetic dysfunction and organ failure [17].

Taking into account that inhaled PM could alter heart oxidative metabolism, the need of an adequate energy supply to sustain proper contractile work, and the crucial role of mitochondria in both O_2 and energetic metabolism, the aim of this work was to evaluate cardiac O_2 metabolism and contractile function, focused on mitochondrial function, in a mice model of acute exposure to PM. The obtained findings could give new insights to the understanding of the biochemical basis of the observed PM-associated cardiovascular effects.

2. Materials and methods

2.1. 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). Mitochondrial fluorescent probes were provided by Molecular Probes (Eugene, OR, US).

2.2. Experimental model

2.2.1. 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). ROFA samples from this source have been previously characterized in terms of elemental composition and particle size [18]. Vanadium, nickel and iron are the predominant metals present as water-soluble sulfates, and particle mean aerodynamic diameter is 2.06 ± 1.57 μm. 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.

2.2.2. Animal exposure

Female Swiss mice weighing 20–25 g were anesthetized by an intraperitoneal (i.p.) injection of ketamine (10 mg/kg body weight) and xylazine (0.1 mg/kg body weight), and exposed to ROFA particles (1.0 mg/kg body weight) or saline solution (control group) by intranasal instillation in a single dose. 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 sacrificed and heart samples were collected. Control mice were handled in parallel, instilled with 50 μL of sterile saline solution, and sacrificed 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 [19]. The selected dose falls within the range of concentrations consistently used in several animal studies [11,20,21]. Animal treatment was carried following the 6344/96 regulation of the Argentinean National Drug, Food and Medical Technology Administration (ANMAT) guidelines.

2.3. Tissue samples

2.3.1. Heart tissue cubes

Heart samples were kept in Krebs buffer solution [118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$ and 10 mM glucose (pH 7.4)] at 4 °C. After being washed and weighted, 1 mm^3 tissue cubes were cut by the use of a scalpel [22].

2.3.2. Mitochondrial isolation and preparation of mitochondrial membranes

Heart mitochondrial purified fractions were obtained from tissue homogenates by differential centrifugation in a Sorvall RC5C centrifuge (Sorvall, Buckinghamshire, England). Two mouse hearts were pooled, washed and minced in ice-cold STE buffer [250 mM sucrose, 5 mM Tris–HCl and 2 mM EGTA (pH 7.4)]. A brief digestion was performed in STE medium supplemented with 0.5% (w/v) fatty acid-free BSA, 5 mM $MgCl_2$, 1 mM ATP and 2.5 UI/mL type XXIV bacterial proteinase. After 4 min at 4 °C, samples were homogenized in 1:10 STE buffer with a Potter Elvejhem glass homogenizer and centrifuged at 8000 *g* for 10 min. The obtained pellet was resuspended in ice-cold STE buffer and centrifuged at 700 *g* for 10 min. The sediment was discarded and mitochondria were pelleted from the supernatant by two centrifugation steps at 8000 *g* for 10 min each. Finally, the pellet was washed, rinsed and resuspended in 500 μL of STE buffer. The whole procedure was carried out at 0–4 °C [23]. Purity of isolated mitochondria was assessed by determining lactate dehydrogenase activity; only mitochondria with less than 5% impurity were used. Mitochondrial membranes were obtained by three freeze–thaw cycles of the mitochondrial preparation, followed by a homogenization step by passage through a 29 G hypodermic needle [24]. Protein concentration was measured by the Lowry assay [25] using BSA as standard.

2.4. Oxygen consumption by tissue cubes

A Clark-type O_2 electrode (Hansatech Oxygraph, Hansatech Instruments Ltd, Norfolk, England) for high resolution respirometry was used. Reaction buffer consisted of 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$ and 5.5 mM glucose (pH 7.4). After an initial stabilization period, tissue cubes were added to the reaction chamber and O_2 consumption rates

were recorded at 30 °C. Results were expressed as ng-at O/min g tissue [26].

2.5. Mitochondrial function

2.5.1. Mitochondrial respiration

Mitochondrial O_2 consumption was followed polarographically with a Clark-type O_2 electrode (Hansatech Oxygraph, Hansatech Instruments Ltd, Norfolk, England) for high resolution respirometry at 30 °C. Freshly isolated heart mitochondria (0.15 mg protein/mL) were incubated in respiration buffer [120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 3 mM HEPES and 1 mg/mL fatty acid-free BSA (pH 7.4)] supplemented with 8 mM succinate. An initial rest state respiration (state 4) was established under these conditions, which was then switched to active state respiration (state 3) by the addition of 125 μM ADP. Respiratory control ratio (RCR) was calculated as state 3/state 4 respiration rates. Oligomycin (2 μM) was used as a F_o–F_1 ATP synthase inhibitor and carbonyl cyanide m-chlorophenylhydrazone (m-CCCP) (2 μM) as a protonophore. Results were expressed as ng-at O/min mg protein [27].

2.5.2. Mitochondrial respiratory chain complexes activity

The enzymatic activity of mitochondrial respiratory complexes I, II and IV was evaluated by a colorimetric assay in a Beckman DU 7400 diode array spectrophotometer (Beckman Coulter Inc., CA, US). Mitochondrial membranes (0.25 mg protein/mL) were incubated at 30 °C in 100 mM PBS (pH 7.2). For complexes I and II, reaction buffer was supplemented with 0.2 mM NADH or 5 mM succinate as substrates, respectively, plus 25 μM cytochrome c^{3+} and 0.5 mM KCN. Cytochrome c^{3+} reduction rate was followed at 550 nm ($\varepsilon = 19\ mM^{-1}\ cm^{-1}$) and results were expressed as nmol reduced cytochrome c^{3+}/min mg protein. For complex IV, reaction buffer was instead supplemented with 60 μM cytochrome c^{2+}. In this case, cytochrome c^{2+} oxidation rate was calculated from the pseudo-first reaction constant (k') and expressed as k'/mg protein. Cytochrome c^{2+} was freshly prepared by reduction of cytochrome c^{3+} with $Na_2S_2O_4$, followed by Sephadex-G25 exclusion chromatography [28].

2.5.3. Mitochondrial membrane potential

Freshly isolated heart mitochondria (25 μg protein/mL) were incubated with the potentiometric cationic probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (30 nM) in respiration buffer. The procedure was performed in the dark at 37 °C for 20 min. After the incubation period, mitochondria were acquired by a Partec PAS-III flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488-nm argon laser. To exclude debris, samples were gated based on light-scattering properties and 30,000 events per sample within this gate (R1) were collected. 10-*N*-nonyl acridine orange (NAO) (100 nM) was used to selectively stain mitochondria and to evaluate their purity, due to its ability to selectively bind to cardiolipin at the inner mitochondrial membrane [29]. In order to quantify the resulting changes in membrane potential after the addition of 8 mM succinate (state 4) and 125 μM ADP (state 3) to the reaction mixture, $DiOC_6$ signal was analyzed in the FL-1 channel with Cyflogic software (CyFlo Ltd, Turku, Finland), and the arithmetic mean values of the median fluorescence intensities (MFI) were obtained. Total depolarization induced by m-CCCP (2 μM) was used as a positive control. Mitochondrial preparations that showed no changes in membrane potential under this condition were discarded [30].

2.5.4. Mitochondrial ATP content, ATP production rate and P/O ratio

A chemiluminescent assay based on the luciferin–luciferase system was used [31]. Mitochondrial ATP content was assessed in freshly isolated heart mitochondria (0.25 mg protein/mL) incubated in respiration buffer supplemented with 150 μM di(adenosine) pentaphosphate, 40 μM D-luciferin and 0.05 μg/mL luciferase. ATP production was triggered by the addition of 8 mM succinate and 125 μM ADP to the reaction well. Chemiluminescence emission was followed as a function of time in a LabSystems Luminoskan EL microplate reader (LabSystems, MN, US) at 30 °C. Oligomycin (2 μM) was used as a negative control in order to establish basal signal levels. A calibration curve was performed using ATP as standard. ATP content and ATP production rates were expressed as nmol ATP/mg protein and nmol ATP/min mg protein, respectively. The number of phosphorylated ADP molecules per oxygen atom (P/O ratio) was calculated as mitochondrial ATP production/state 3 O_2 consumption rates.

2.6. Isolated heart perfusion

Mice were anesthetized by an i.p. injection of sodium pentobarbital (150 mg/kg body weight) and sodium heparin (500 UI/kg body weight). After ensuring sufficient depth of anesthesia, hearts were excised and the aorta was immediately cannulated with a 21 gauge cannula. Afterwards, hearts were perfused according to the Langendorff technique with Krebs medium equilibrated with 95% O_2 and 5% CO_2 at 37 °C. A small latex fluid-filled balloon connected via a thin plastic catheter (P50) to a Deltram II pressure transducer (Utah Medical System, UT, US) was inserted into the left ventricle via the left atrium. The catheter with the transducer was positioned in such a way that it secured the position of the balloon in the left ventricle. Two electrodes were sutured and connected to a pacemaker in order to induce a constant heart rate of 470 ± 30 beats/min. Coronary perfusion pressure (CPP) was recorded through a pressure transducer connected to the perfusion line. Hearts were perfused at constant flow in order to obtain a CPP of 73 ± 3 mm Hg during the initial stabilization period and then maintained constant throughout the experiment. Left ventricular developed pressure (LVDP) was calculated as the difference between peak systolic pressure and left ventricular end-diastolic pressure. Isovolumic relaxation (lusitropism) was analyzed as the time required for the left ventricular pressure to fall up to 50% from the peak of LVDP (t_{50}) [32]. Inotropic and lusitropic reserve was evaluated as LVDP and t_{50}, before and after a β-adrenergic stimulus through the addition of isoproterenol (ISO) (1 μM) to the perfusion line [33].

2.7. 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 Student–Newman–Keuls test was performed to analyze differences between more than two groups. Statistical significance was considered at $p < 0.05$.

3. Results

3.1. Oxygen consumption by tissue cubes

The time course after the delivery of the ROFA suspension or saline solution to experimental animals is shown in Fig. 1. Intranasal instillation with ROFA induced a significant decrease in heart O_2 consumption by 23% in comparison with saline-instilled mice, 1 h after the exposure. Moreover, a 35% decrease was observed when comparing O_2 consumption rates of saline- and ROFA-exposed mice hearts at 3 h after the treatment. No significant differences were observed neither between saline- and ROFA-exposed mice at 5 h after the treatment, nor among saline-exposed mice after 1, 3 or 5 h. Since the most significant decrease in tissue O_2 consumption was observed at 3 h after the ROFA exposure, this time point was selected for further analysis.

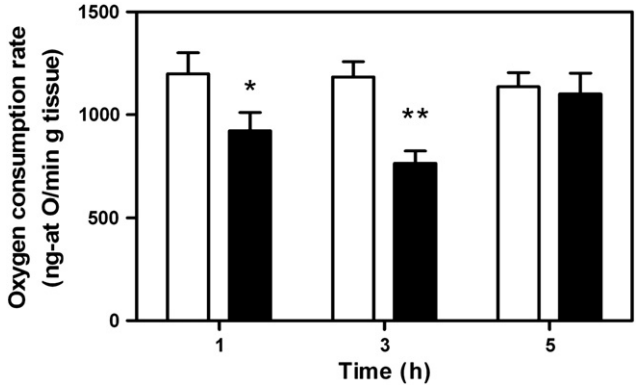

Fig. 1. Oxygen consumption rates by 1 mm^3 heart tissue cubes. Mice were intranasally instilled with a single dose of either the ROFA suspension (black bars) or saline solution (white bars). Hearts were excised 1, 3 or 5 h after the exposure. Results are expressed as mean ± SEM of at least 6 animals per group. $^*p<0.05$ compared with control 1 h and $^{**}p<0.01$ compared with control 3 h.

3.2. Mitochondrial function

3.2.1. Mitochondrial respiration

The analysis of O_2 consumption by isolated mitochondria is the classical approach to characterize mitochondrial function. Fig. 2 shows a representative measurement in samples from saline- and ROFA-exposed mice; the trace also shows the effect of oligomycin (2 μM) and m-CCCP (2 μM) on mitochondrial respiration. As it is shown in Table 1, mitochondrial rest state (state 4) was significantly decreased by 30% in ROFA-exposed mice in comparison with the control group at 3 h after the treatment. Likewise, mitochondrial metabolic state 3, the active respiration state yielding ATP, was significantly decreased by 24% in mice instilled with the ROFA suspension. No changes were observed in the RCR between both groups. Further analysis of mitochondrial respiration in the presence of the F_o–F_1 ATP synthase inhibitor oligomycin and the protonophore m-CCCP was also performed. State 3 O_2 consumption was first switched to a slower respiration rate (state 4_O) by inhibiting ATP synthesis with oligomycin. Afterwards, mitochondrial uncoupled respiration (state 3_U) was triggered by the addition of m-CCCP to isolated mitochondria incubated in state 4_O respiration. Values corresponding to the ratio between state 3_U and state 4_O rates showed no differences with RCR values from saline- and ROFA-exposed mice, indicating maintenance of mitochondrial membrane integrity. State 3_U is exclusively dependent on substrate oxidation and electron transfer rates, and represents the maximal O_2 consumption rate that the mitochondria can sustain [34]. State 3_U was significantly decreased by 51% in ROFA-exposed mice in comparison with the control group, indicating an inhibition at the mitochondrial electron transport chain.

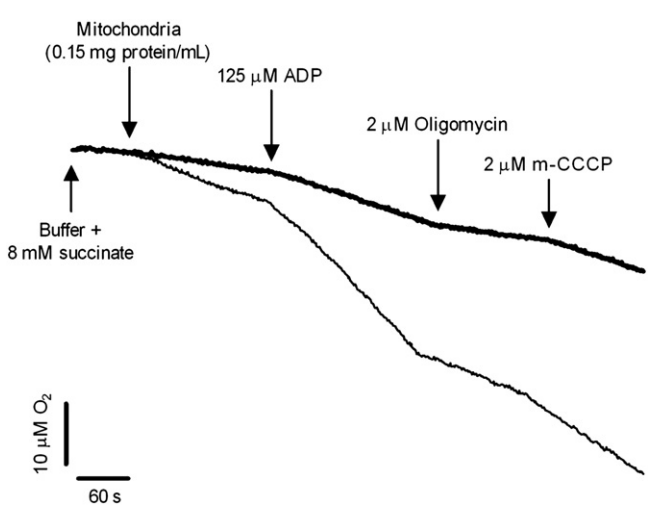

Fig. 2. Representative traces obtained during the assessment of mitochondrial O_2 consumption in rest (state 4) and active (state 3) metabolic state, and after the addition of 2 μM oligomycin (state 4_O) and 2 μM m-CCCP (state 3_U) to the reaction chamber, in control conditions (thin line) and in ROFA exposed mice (thick line).

Table 1
Oxygen consumption rates by freshly isolated mice heart mitochondria. Measurements were performed 3 h after an acute exposure to ROFA. Succinate (8 mM) was used as substrate to establish state 4 respiration rate. State 3 was triggered by the addition of ADP (125 μM) to the reaction chamber. RCR was calculated as state 3/state 4 respiration rates. State 4_O was induced by oligomycin (2 μM), while m-CCCP (2 μM) was used to establish state 3_U respiration. Results are expressed as mean ± SEM of at least 6 independent experiments. $^*p<0.05$ and $^{**}p<0.01$ compared with the control group.

Metabolic state	Control	ROFA
	(ng-at O/min mg protein)	
State 4	88 ± 5	61 ± 4**
State 3	240 ± 20	180 ± 20*
RCR	2.8	3.0
State 4_O	90 ± 10	50 ± 5**
State 3_U	250 ± 20	190 ± 10**
State 4_O/State 3_U	2.8	3.8

3.2.2. Mitochondrial respiratory chain complexes activity

The observed alteration in heart mitochondrial function after the exposure to ROFA particles was further investigated by the single assessment of mitochondrial respiratory chain complexes activities. As it is shown in Table 2, mitochondrial respiratory complex II activity was found to be significantly decreased by 25% in ROFA-exposed mice when compared with the control group at 3 h after the treatment, while complexes I and IV activities remain unchanged after the treatment.

3.2.3. Mitochondrial membrane potential

The electrochemical H^+ gradient across the inner mitochondrial membrane is the driving force for ATP synthesis, thus defining another key feature of mitochondrial function [17]. Mitochondria were selected from background based on light-scattering properties (SSC vs FSC, Fig. 3A) and events within gate R1 were chosen for analysis. In every analyzed preparation, >95% of the events within R1 were NAO-positive (R2) compared with unstained control samples (Fig. 3B), showing that contamination with other subcellular constituents throughout the isolation procedure was kept at minimum. As it is shown in the overlaid histograms (Fig. 3C), ROFA exposure induced a decrease in FL-1 $DiOC_6$ fluorescence signal in both mitochondrial sate 4 and state 3 in comparison with the control group at 3 h after the treatment, indicating mitochondrial depolarization. As expected, a strong depolarization was induced by the addition of m-CCCP in both saline- and ROFA-exposed mice. Quantification of $DiOC_6$ fluorescence as MFI (Fig. 3D) showed that the exposure to ROFA particles

Table 2
Colorimetric assessment of heart mitochondrial respiratory chain complex activities. Measurements were performed 3 h after an acute exposure to ROFA. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{**}p<0.01$ compared with the control group.

	Complex I (nmol/min mg protein)	Complex II (nmol/min mg protein)	Complex IV (k'/mg protein)
Control	310 ± 10	87 ± 5	24 ± 2
ROFA	320 ± 20	65 ± 5**	23 ± 1

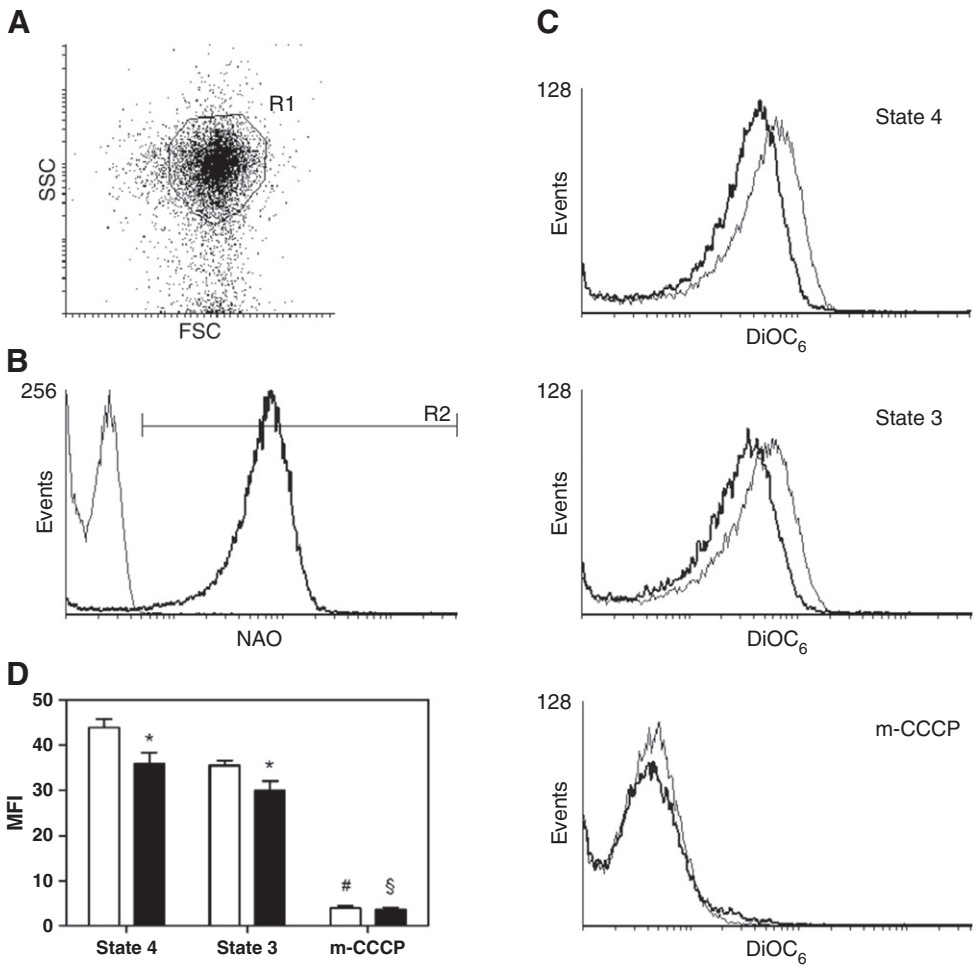

Fig. 3. Evaluation of heart mitochondrial membrane potential by flow cytometry. Measurements were performed 3 h after an acute exposure to ROFA. (A) Mitochondria were selected based on light scattering properties and 30,000 events within R1 were collected. (B) Purity of mitochondrial preparations was checked by selective staining with NAO (thick line) compared with an unstained control sample (thin line); >95% of gated (R1) events were NAO-positive (R2) in every analyzed sample. (C) Overlaid histograms of gated (R1) mitochondrial events versus $DiOC_6$ fluorescence intensity. State 4 and state 3 metabolic states were evaluated in isolated mitochondria from saline- (thin line) and ROFA-exposed mice (thick line). Large depolarization induced by m-CCCP (2 μM) was used as a positive control in both experimental groups. (D) $DiOC_6$ fluorescence quantification of gated (R1) histograms, indicating heart mitochondrial membrane potential in saline- (white bars) and ROFA-exposed mice (black bars). Results are expressed as mean ± SEM of at least 6 independent experiments. $^{*}p<0.05$ compared with the corresponding control group, $^{\#}p<0.001$ compared with control state 4 and $^{\S}p<0.001$ compared with ROFA state 4.

decreased mitochondrial membrane potential in state 4 and in state 3 by 18 and 15%, respectively, in comparison with the control group.

3.2.4. Mitochondrial ATP content, ATP production rate and P/O ratio

Impaired heart mitochondrial function can compromise ATP synthesis at F_o–F_1 ATP synthase, leading to a deficient ATP production and a bioenergetic dysfunction. Despite mitochondrial ATP content was not changed after the treatment, ATP production rate was found to be significantly decreased by 52% in ROFA-exposed mice in comparison with the control group at 3 h after the treatment (Table 3). As a consequence, the calculated P/O ratio decreased from a value of 2.3 for control animals to 1.6 for mice exposed to the ROFA suspension, indicating decreased oxidative phosphorylation efficiency in ROFA-exposed mice.

Table 3
Chemiluminescent determination of heart mitochondrial ATP content and ATP production rate, and calculated P/O ratios. Measurements were performed 3 h after an acute exposure to ROFA. Results are expressed as mean ± SEM of at least 6 independent experiments. $^{*}p<0.05$ compared with the control group.

	ATP content (nmol/mg protein)	ATP production rate (nmol/min mg protein)	P/O ratio
Control	110 ± 10	560 ± 20	2.3
ROFA	100 ± 10	290 ± 20*	1.6

3.3. Isolated heart perfusion

3.3.1. Cardiac contractile reserve

In order to evaluate if the observed alterations in heart O_2 metabolism and mitochondrial function have any impact over cardiac function, cardiac contractility and diastolic function were assessed either at the baseline or after a β-adrenergic stress. On the one hand, no significant differences were observed when comparing basal LVDP between saline- and ROFA-exposed mice at 3 h after the treatment. On the other hand, as expected for a normal myocardium, LVDP was significantly increased by 53% in control mice when comparing basal and ISO-induced LVDP. Nevertheless, this increase in cardiac contractility as a response of a β agonist was attenuated in mice exposed to the ROFA suspension (Fig. 4). Lusitropic reserve was not altered after the treatment (data not shown), suggesting a dissociation between systolic and diastolic myocardial reserve in ROFA-exposed mice.

3.3.2. Oxygen consumption by tissue cubes

After the evaluation of cardiac contractility, hearts were removed from the Langendorff perfusion apparatus and O_2 consumption rates were assessed in tissue cubes. Although ISO perfusion induced a significant increase in tissue O_2 consumption in comparison with basal conditions in both experimental groups, this response was reduced

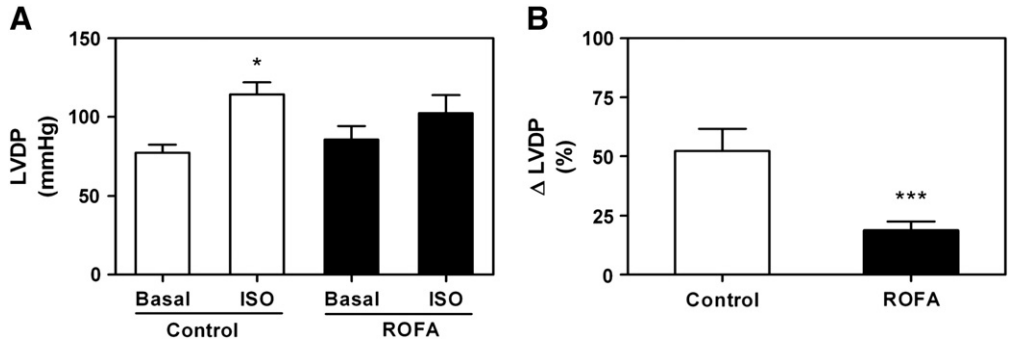

Fig. 4. Evaluation of cardiac contractile reserve according to the Langendorff technique at constant flow. Measurements were performed 3 h after an acute exposure to ROFA, in basal conditions and after a β-adrenergic stimulus induced by isoproterenol (ISO) (1 μM). Results are expressed as mean ± SEM of 6 independent experiments. (A) LVDP of isolated perfused hearts from saline- (white bars) and ROFA-exposed mice (black bars). $^{*}p<0.05$ compared with control basal LVDP. (B) Increase in LVDP (ΔLVDP), shown as the difference (%) between basal and ISO-stimulated LVDP. $^{***}p<0.001$ compared with the control group.

in mice exposed to the ROFA suspension (44 and 33%, respectively). Moreover, heart tissue O_2 consumption rate in control animals after the β-adrenergic stimuli was 60% higher than those from ROFA-exposed mice (Fig. 5).

4. Discussion

Lung injury and local oxidative stress have been described after ROFA instillation in different animal models [9]. ROFA samples used in this study already proved to induce several pulmonary [35–37] and cardiovascular alterations [11,38], both *in vitro* and in rodents. Previous results of our laboratory, using the same experimental model, showed a pulmonary oxidative metabolism imbalance, with a peak at 1 h after the instillation of the ROFA suspension [39]. In contrast, the most significant alterations in this study were found 3 h after the exposure. This difference in the induction time for the observation of cardiac effects, in comparison with the earlier alterations found within the lung, suggests that cardiac impaired mitochondrial function and contractile reserve might be secondary to the pulmonary dysfunction triggered by PM inhalation. Consistently with our findings, a similar lag phase was described for the increase in steady-state concentrations of reactive oxygen species in mice lung and heart after the exposure to PM [11]. The release of pro-oxidative and/or pro-inflammatory mediators from the lung into systemic circulation following PM inhalation could mediate the observed cardiovascular effects [4]. Of note, a direct effect of ROFA particles and/or its soluble constituents over the heart cannot be completely discarded. It has been postulated that nano-scale particles could be

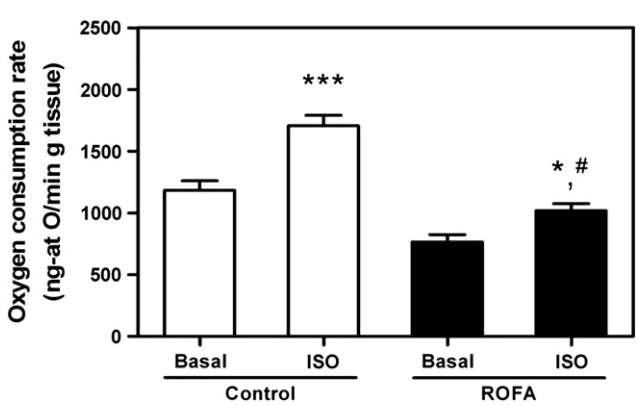

Fig. 5. Tissue O_2 consumption rates in basal conditions and after a β-adrenergic stimulus induced by isoproterenol (ISO) (1 μM). Measurements were performed 3 h after the ROFA exposure, and after the evaluation of cardiac contractile reserve by the Langendorff technique. Results are expressed as mean ± SEM of 6 independent experiments. $^{***}p<0.001$ and $^{*}p<0.05$ compared with the corresponding value in basal conditions and $^{\#}p<0.001$ compared with control + ISO.

able to break through the respiratory epithelia, reach systemic circulation, and exert direct cardiac effects within minutes to hours [40,41]. Moreover, the mentioned pathways are not mutually exclusive and may temporally overlap or be activated at different time points, contributing to the observed cardiovascular alterations triggered by PM inhalation [12].

We and others have previously shown that the observed oxidative metabolism imbalance within the lung is positively correlated with a mitochondrial dysfunction [39,42]. However, little is known about this association at the cardiac level and the potential impact of this scenario over cardiac contractile function. As a first approach in order to evaluate cardiac O_2 metabolism after an acute ROFA exposure, heart O_2 consumption was assessed in tissue cubes. The linear rates obtained represent the result of mitochondria population oscillating between states 4 and state 3, in a proportion estimated to be 68 and 32%, respectively, for cardiomyocytes under physiological conditions [43]. In these conditions, heart O_2 consumption was found to be significantly decreased by 23% as early as 1 h after the ROFA instillation, with a minimum at 3 h after the treatment, indicating deficient tissue O_2 uptake. The observed decrease seemed to be not only time-dependent but also reversible, since O_2 consumption rates of ROFA-exposed mice were not significantly different from control animals at 5 h after the treatment.

Under physiological conditions, 85–90% of tissue O_2 uptake is consumed by mitochondria in the oxidative phosphorylation process [43]. Therefore, the evaluation of mitochondrial respiration was carried out in order to clarify the observed alteration in tissue O_2 consumption. Results presented in Table 1 suggest that the altered mitochondrial function accounts for the decrease in tissue O_2 consumption, and indicates a decreased mitochondrial function due to an inhibition of the electron transport chain with maintenance of mitochondrial membrane integrity. This was confirmed by *in vitro* determination of respiratory chain complexes activities in mitochondrial membranes, where complex II activity was significantly decreased by 25% in ROFA-exposed mice in comparison with the control group. In agreement with these results, impaired mitochondrial function (resulting in a substantial reduction of cardiac O_2 consumption) is a common finding in the early steps of cardiovascular diseases which are associated with PM exposure, such as congestive heart disease and heart failure [16].

Several studies have shown a decreased mitochondrial membrane potential triggered by environmental PM [44] and PM-associated transition metals [45] in human alveolar epithelial A549 cells. To our knowledge, the present study is the first in which such functional alteration is described in isolated mitochondria from mice heart after an acute exposure to air pollution PM. In this case, mitochondrial depolarization was observed in both rest and active state in ROFA-exposed mice. Deficient substrate oxidation by the electron transport chain could be responsible for the observed decrease in

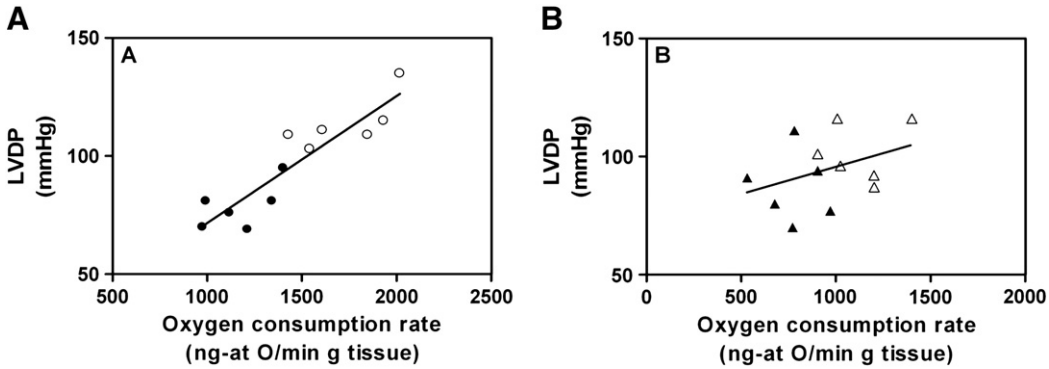

Fig. 6. Linear correlation between heart O_2 consumption and LVDP measurements. (A) Control animals in basal conditions (●) and after a β-adrenergic stimulus (○) ($r^2 = 0.8271$, $p < 0.0001$). (B) ROFA-exposed mice in basal conditions (▲) and after a β-adrenergic stimulus (△) ($r^2 = 0.1396$).

the inner mitochondrial membrane potential. Since electrochemical H^+ gradient is the driving force for ATP synthesis at F_0–F_1 ATP synthase, mitochondrial depolarization could contribute to a decreased cellular ATP supply after PM inhalation, leading to a cardiac bioenergetic dysfunction and organ failure.

Cardiac energetic state is not simply defined by ATP steady sate concentration (≈10 mM), but by the amount of ATP synthesized and used per minute (turnover). Given that cellular ATP pool is small compared with cellular energetic demands (as much as 10000 times higher), heart must continually re-synthesize ATP in order to maintain cardiac homeostasis [46]. Indeed, besides the observed decrease in mitochondrial respiration and membrane potential, ATP content was not modified after the acute ROFA exposure. ATP loss by a failing myocardium has been shown to be slow and progressive (≈0.35% of ATP pool per day) [47], meaning that a decrease in ATP content would not be detected until the heart is in severe failure. However, ATP production rate was found to be decreased by 52% in ROFA-exposed mice. These results indicate that, even though heart non-mitochondrial ATP production pathways (i.e. glycolysis and the creatine kinase system) may significantly contribute to maintain ATP concentration constant, ATP supply by oxidative phosphorylation is limited in mice exposed to the ROFA suspension. Accordingly, not all the O_2 consumed by the mitochondria may be properly converted into chemical energy in the form of ATP as suggested by the smaller P/O ratio calculated for this group. In ROFA-exposed mice, although ATP concentration may be sufficient to sustain cardiac function in normal conditions, energy production could be a limiting step for cardiomyocyte function when work output increases. Such alterations have been observed in rat heart cells loaded with iron, a transition metal present in high proportion in ROFA particles, where the decrease in respiratory chain complexes activities closely paralleled with ATP levels at conditions formerly shown to induce severe abnormalities in cardiac contractility [48].

ROFA exposure was found to be not only associated with heart bioenergetic alterations, but also with a decrease in cardiac contractile reserve. The analysis of the data presented in Fig. 4 indicate that, even though basal contractility seems to be preserved after an acute PM exposure, the heart fails to properly respond to an increased work output. This conclusion is supported by the observation that the ISO-induced increase in heart O_2 consumption in control animals was attenuated in mice exposed to the ROFA suspension. Interestingly, the positive linear correlation between tissue O_2 consumption and LVDP observed for the control group is lost in ROFA-exposed mice (Fig. 6). This means that changes in heart O_2 uptake has a direct impact on cardiac contractility, which is disrupted in ROFA-exposed mice due to the described impaired mitochondrial function. In this group, deficient mitochondrial ATP supply leads to a bioenergetic dysfunction that hampers cardiomyocytes to sustain contractile work when cardiac output increases.

As mentioned, the mitochondrial population of cardiac cells under physiological conditions oscillates between state 4 and state 3, in an intermediate state often closer to state 4 [43]. Consequently, mitochondria use only a fraction of their maximal respiratory capacity (equivalent to O_2 consumption in state 3) in basal conditions. The difference between both states is termed mitochondrial bioenergetic reserve capacity and is available in cardiomyocytes for the maintenance of organ function when energy demands are increased [49]. Given that cardiac contractile function is conserved in basal conditions but not after a β-adrenergic stimulus, and taking into account the findings on mitochondrial function assessment, a loss of mitochondrial reserve capacity in ROFA-exposed mice may explain the observed impaired cardiac function when work output increases. Depletion of mitochondrial bioenergetic reserve was also described in other experimental models such as rat [49] and mice cardiomyocytes [50] exposed to 4-hydroxynonenal, a byproduct of lipid peroxidation that accumulates in the ischemic [51] and failing heart [52]. These observations emphasize the importance of a maintained mitochondrial reserve capacity in situations of high cardiac output. The presented condition may comprise an early step of cardiomyocyte alterations observed after the exposure to PM, a pathological state that, if maintained in time, could contribute to the onset and progression of PM-associated cardiovascular diseases.

5. Conclusion

Taken together, the present findings suggest that an acute PM exposure triggers a cardiac O_2 metabolism imbalance induced by mitochondrial dysfunction and decreased succinate dehydrogenase activity, mitochondrial membrane potential and impaired oxidative phosphorylation. Interestingly, the observed changes in cellular O_2 and energetic metabolism were found to be associated with a deficient ventricular function probably due to a decreased mitochondrial reserve capacity, which can contribute to the understanding of the increase in cardiovascular morbidity and mortality mediated by environmental PM exposure.

Conflict of interest statement

Authors declare that there are no conflicts of interest.

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