Cardiovascular Toxicology (2019) 19:575–587
https://doi.org/10.1007/s12012-019-09530-z

Direct and Indirect Effect of Air Particles Exposure Induce Nrf2-Dependent Cardiomyocyte Cellular Response In Vitro

N. S. Orona[1,2] · F. Astort[1,2] · G. A. Maglione[1,3] · J. S. Yakisich[4] · D. R. Tasat[1,3]

Published online: 30 May 2019

Abstract
Air particulate matter has been associated with adverse effects in the cardiorespiratory system leading to cytotoxic and pro-inflammatory effects. Particulate matter-associated cardiac effects may be direct or indirect. While direct interactions may occur when inhaled ultrafine particles and/or particle components cross the air–blood barrier reaching the cardiac tissue, indirect interactions may occur as the result of pulmonary inflammation and consequently the release of inflammatory and oxidative mediators into the blood circulation. The aim of the study is to investigate the direct or indirectly the effect of Urban Air particles from downtown Buenos Aires (UAP-BA) and residual oil fly ash (ROFA), a surrogate of ambient air pollution, on cardiomyocytes (HL-1 cells). HL-1 cultured cells were directly exposed to particulate matter [UAP-BA (10–200 µg/ml), ROFA (1–100 µg/ml)] or indirectly exposed to conditioned media (CM) from particle-exposed alveolar macrophages (AM). Metabolic activity, reactive oxygen species (ROS), and Nrf2 expression were assessed by MTT, DHR 123, and immunocytochemistry techniques, respectively. We found that direct exposure of cardiomyocytes to UAP-BA or ROFA increased ROS generation but the oxidative damage did not alter metabolic activity likely by a concomitant increase in the cytoplasmic and nuclear Nrf2 expression. However, indirect exposure through CM caused a marked reduction on cardiac metabolic activity probably due to the rise in ROS generation without Nrf2 translocation into the cell nuclei. In this in vitro model, our results indicate both direct and indirect PM effects on cardiomyocytes cells in culture. Our findings employing lung and cardiomyocytes cells provide support to the hypothesis that particle-induced cardiac alteration may possibly involve lung-derived mediators.

Keywords Cardiomyocytes · Air particles · Nrf2 · Lung-derived mediators

Introduction

Cardiovascular diseases are leading causes of morbidity and mortality worldwide. Although various factors contribute to the etiology of cardiovascular diseases, recent data suggest that air particle pollution, may have a significant role in triggering numerous cardiovascular complications [1, 2]. Epidemiologic studies have shown that increased particulate matter (PM) concentrations are associated with adverse effects in the respiratory and cardiovascular systems [3–7]. In this sense, PM has been linked with several clinical manifestations of cardiovascular diseases including myocardial infarction, stroke, heart failure, arrhythmias, and venous thromboembolism [8]. Recent studies indicate that PM–cardiovascular diseases relationship is likely more complex than a mere quantitative association between overall PM and disease risk. In fact, it is known that PM-associated cardiac effects could be direct or indirect. Direct interactions

Handling Editor: John Allen Crow.

✉ N. S. Orona
naorona@gmail.com

[1] Universidad Nacional de San Martín, Escuela de Ciencia y Tecnología, Centro de Estudios en Salud y Medio Ambiente, Martín de Irigoyen 3100, 1650 San Martín, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[3] Universidad de Buenos Aires, Facultad de Odontología, Cátedra de Histología y Embriologia, Buenos Aires, Argentina

[4] Department of Pharmaceutical Sciences, School of Pharmacy, Hampton University, Hampton, VA, USA

occur when inhaled ultrafine (nano) particles and/or particle components cross the air–blood barrier, enter the macro and/or microcirculation [9–11], reach the cardiac tissue, and negatively impact cardiovascular function. Indirect effects may occur by the release into the blood circulation of lung inflammatory and oxidative mediators as result of pulmonary inflammation [12, 13]. Indeed, it has been shown that exposure to air pollution can lead to oxidative stress and/or systemic inflammation, which in turn could be responsible for the increased risk of cardiorespiratory diseases [12, 14–16]. Since stress response pathways often cross-talk or synergize with one another, mechanism by which PM exerts deleterious effect on the cardiovascular system is poorly understood. For instance, inflammation can induce oxidative stress responses but in turn, oxidative stress can induce inflammation eventually causing DNA damage as well as other types of cellular damage leading to cell death.

The nuclear factor E2-related factor 2 (Nrf2) has emerged as a key response element against oxidative stress and toxicants [17]. Nrf2 is a redox-sensitive transcription factor pivotal in anti-inflammation response and oxidative stress that regulates the expression of antioxidant/detoxification enzymes [18]. Recently, Jiang et al. [19] indicated that Nrf2 plays a basic protective role in cardiovascular diseases by attenuating oxidative stress, mitochondrial dysfunction, and inflammation.

We have previously characterized the acute effect of Urban Air particles from downtown Buenos Aires (UAP-BA) (a Latin American megacity) on the respiratory tract in an in vivo animal model [20]. We demonstrated that UAP-BA are mostly fine (diameter < 2.5 μm) and ultrafine particles (diameter < 0.1 μm) with no metallic traces. UAP-BA was able to generate lung inflammation and oxidative metabolism imbalance, probably due to the high content of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) adsorbed to their carbon core [20, 21]. Residual oil fly ash (ROFA), widely used as a surrogate of ambient pollution, is a PM that results from oil combustion which contains metallic traces such as vanadium, aluminum, silica, and iron [22–24]. We have shown that ROFA exerts an inflammatory response in both lung and heart, by inducing the release of pro-inflammatory cytokines. Furthermore, we found that due to its high metallic content (V, Al and Si), ROFA induces the generation of reactive oxygen species (ROS) and consequently a redox metabolism imbalance [20, 21, 25, 26].

Lung and heart are target organs that can respond to stressors or toxins, and cultured cells from these organs are commonly used as model systems to ascertain toxicity mechanisms. Given that PM size and composition are relevant to subsequent cytotoxicity, herein we have evaluated the effect of ROFA and UAP-BA, two distinct air particle pollutants on lung and cardiac cells. As macrophages play an important role in the pathogenesis of toxicants in the lung, we exposed mice-cultured alveolar macrophages to ROFA and UAP-BA and characterized their responses in vitro. In vivo small particles and lung mediators are plausible to cross the air–blood barrier therefore, seeking for possible indirect effects, we analyzed not only cardiomyocytes biological response after direct exposure to both PM particles, but also the indirect exposure to conditioned media (CM) from particle-exposed alveolar macrophages (AM).

This work presents an in vitro evaluation of the hypothesis that mediators derived from alveolar macrophages exposed to air pollution particulate matter could induce indirect effects in cardiomyocytes.

Materials and Methods

ROFA and UAP-BA Particle Sampling

Urban Air Particles from downtown Buenos Aires (UAP-BA) were collected in an area characterized by high population density with a high exposure to diesel exhaust mainly due to cars and buses [21]. A MiniVolTM Portable Air Sampler (Airmetrics, OR, USA) with 2.5 μm cut-point impactors using a flow rate of 1.8 L min^{-1} was employed [27]. The samples were collected on Teflon filters (47 mm, Sartorius, 0.7 μm pore size), and each filter was placed in a clean plastic cassette during transport and storage. The filters were weighed (after moisture equilibration) before and after sampling to determine the net particulate mass gain with a microbalance (Mettler M3, weighing accuracy of 1 μg), using an alpha source to remove the electrostatic charge. ROFA collected from the Mystic Power Plant, CT, USA, was employed as a recognized surrogate ambient particulate matter and was kindly provided by J. Godleski (Harvard School of Public Health, Boston, MA, USA). "Stock suspensions" of UAP-BA were prepared by sonicating the filters five times for 5 min (Astrason, Misonix) in an appropriate volume of 1× phosphate buffer solution (PBS). Stock suspensions of ROFA were prepared by diluting particles in an appropriate volume of PBS 1×.

ROFA and UAP-BA Particle Characterization

Both particulate matter, ROFA and UAP-BA, were morphologically and chemically characterized employing scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX), respectively. For SEM observations, collected particles were coated with gold by direct current sputtering. Stub preparations were examined in a quanta SEM FEG-S50 (SEI, Oregon, USA). Chemical composition was analyzed with a Phillips SEM 505 SEM (Philips

Electron Optics, NL, USA) coupled to a EDX dispersion detection unit (EDAX Inc., NJ, USA).

Animals

Male BALB/c mice (2–3 months old) were obtained from the animal facilities of the School of Natural and Exact Sciences of the University of Buenos Aires. Animals were housed according to the NIH Guide for the Care and Use of Laboratory Animals at the breeding facility of the School of Science and Technology, University of San Martin and fed a normal protein diet and water ad libitum. All experiments complied with local ethical guidelines.

Alveolar Macrophage Isolation and Culture

Fully differentiated mouse AM were obtained by bronchoalveolar lavage (BAL) as described elsewhere [28]. Briefly, the thoracic cavity was partly dissected and the trachea was cannulated with an 18-gauge needle. Lungs were gently massaged and infused 12 times with 1 ml of cold sterile phosphate-buffer saline (PBS, Ca^{2+} Mg^{2+} free, pH 7.2–7.4). BAL was immediately centrifuged at 800×*g* for 10 min at 4 °C and the total cell number was determined using a Neubauer chamber. Based on morphological criteria, control animals showed > 95% of AM. The cells were cultured in RPMI 1640 supplemented with penicillin (100 UI/ml), streptomycin (10 µg/ml), and 10% fetal calf serum. In all cases, 125,000 cells were seeded per well (24 well plate). After 20 min incubation, cultures were carefully washed to remove any non-adherent cells leaving an enriched AM population. The AM were incubated overnight at 37 °C in a 5% CO_2 atmosphere.

HL-1 Mouse Cardiomyocytes Culture

HL-1 cells, a cardiac cell line derived from the AT-1 mouse atrial myocyte tumor lineage, were kindly provided by Dr. William Claycomb (Department of Biochemistry and Molecular Biology, School of Medicine, New Orleans, LA) [29]. HL-1 cells were grown at a density of 80,000 cells/ml in Claycomb medium supplemented 10% FBS, penicillin/streptomycin 100 U/ml and 10 µg/ml, respectively, and 2 mM L-glutamine during 24 h. Cardiomyocytes cells showed a typical cardiac phenotype and were able to proliferate and to be repeatedly passaged throughout the whole experiment.

AM and HL-1 Cells Exposure to Particulate Matter

Exposure to either ROFA (1, 10 and 100 µg/ml) or UAP-BA (10, 100 and 200 µg/ml) was always performed after 24 h in culture. The selected PM doses fall within concentration ranges used in in vitro studies and are considered to be relevant for high-dose exposure levels in urban environments [30–32]. Final concentrations, of particle suspensions for either ROFA or UAP-BA, were prepared by resuspending aliquots from our ''stock suspension'' in supplemented RPMI-1640 or Claycomb media for AM or HL-1 cells, respectively. Prior to use, all suspensions were always sonicated for 10 min to disrupt possible particle aggregates or agglomerates.

HL-1 Cells Exposure to Conditioned Media

Conditioned media were obtained by collecting media from AM cell cultures exposed to ROFA or UAP-BA. Centrifugation (12,000×*g*, 10 min) was performed in order to remove particles and cell debris [33]. The supernatants were mixed with fresh supplemented Claycomb media (75:25) and added to HL-1 cell cultures during 24 h.

The experimental methodology described above is illustrated in Fig. 1. Cultured HL-1 cells exposed directly to particulate matter are shown in Fig. 1a. Figure 1b depicts the indirect exposure of cardiomyocytes particle through PM-alveolar macrophages conditioned medium (CM).

Cell Metabolism

Macrophage and cardiomyocyte metabolism was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described elsewhere [34, 35]. Briefly, AM and HL-1 cells were seeded as described before. Medium from control and directly or indirectly PM-exposed cultures was removed and 0.5 ml fresh complete growth medium supplemented with 50 µl MTT (4 mg/ml in PBS) was added for 3 h. Immediately after incubation, 10% SDS was added to stop the MTT reaction and to solubilize the formazan precipitate. The optical density (OD) of the final solution was measured at 570 nm in a spectrophotometer (Shimadzu UV-1201V).

Pro-inflammatory Cytokine Secretion in Mice Alveolar Macrophage Culture

Macrophage secretion of both pro-inflammatory cytokines, Tumor Necrosis Factor alpha (TNFα) and Interleukin 6 (IL-6), was determined in the culture media collected from control and PM-exposed cells by using specific enzyme-linked immunosorbent assay (ELISA), following manufacturer's instructions (BD Biosciences). Culture media was frozen at − 20 °C until use. Absorbance was measured at 450 nm (wavelength correction at 660 nm) on a microplate reader (BioRad, Benchmark).

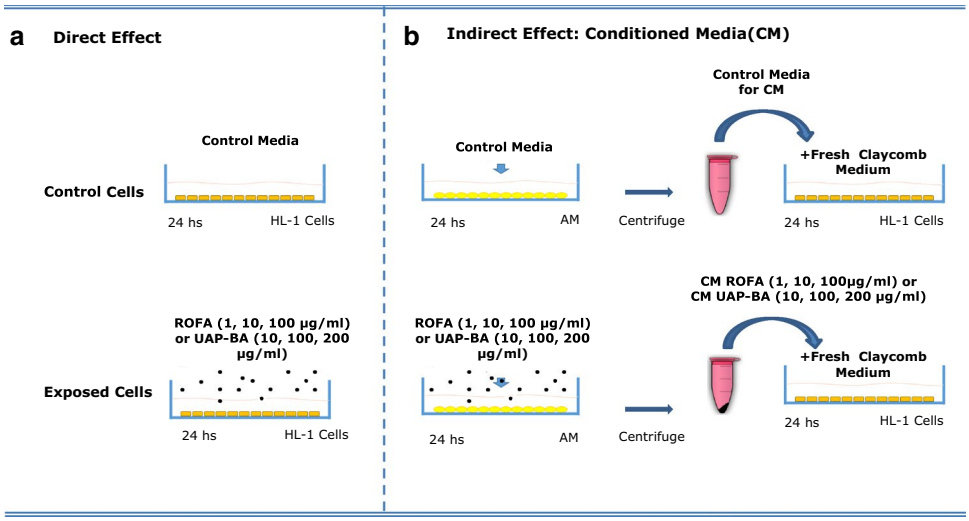

Fig. 1 Experimental methodology for cultured HL-1 cells exposed to particulate matter: direct and Indirect effects. **a** Direct effect: cultured HL-1 cells were incubated for 24 h with media alone (control) or media containing ROFA or UAP-BA particles. **b** Indirect effect: alveolar macrophages were incubated with media alone (control) or exposed to ROFA or UAP-BA during 24 h. Then, media was collected and centrifuged. Supernatants "conditioned media" were mixed with fresh supplemented Claycomb media (75:25) and added to HL-1 cell cultures during another 24 h. All HL-1 cultured cells (incubated with particles or CM) were assayed for metabolic activity, ROS generation, and Nrf2 determinations

Reactive Oxygen Species Production

Superoxide Anion Generation in Cultured Mice Alveolar Macrophages

The release of intracellular superoxide anion (O_2^-), a main reactive oxygen species (ROS) generated during the respiratory burst, by macrophage cells was evaluated by using the NBT reduction test [36]. All wells were incubated with nitroblue tetrazolium (NBT), for 45 min at 37 °C. The percentage of reactive and non-reactive cells was evaluated by light microscopy as described elsewhere [37]. Cells showing a blue formazan precipitate were considered reactive, whereas those without precipitate were scored as non-reactive.

Total Reactive Oxygen Species in Cultured Mice Cardiomyocytes

Total ROS were determined in HL-1 cells directly or indirectly exposed to ROFA or UAP-BA. ROS was detected using an intracellular ROS dye, Dihydrorhodamine 123 (DHR123). The non-fluorescent DHR 123 after loading is converted to the fluorescent product rhodamine 123 by an interaction with reactive oxygen intermediates [38]. Briefly, 10 mM DHR123 stock solution (in methanol) was diluted in culture medium to yield a 20-µM working solution. HL-1 cell cultures were washed twice with PBS, incubated in 1 ml working solution of DHR123 at 37 °C for 1 h. Cells were visualized under fluorescent light microscopy and total intensity of microphotograph was analyzed using Image Pro Plus. The fluorescence mean value was calculated after analyzing ten fields selected at random per well. Results were expressed as fluorescence arbitrary units.

Immunocytochemical Determination of Nuclear Factor Erythroid 2-Related Factor (Nrf2) in Cardiomyocytes

Nrf2 nuclear translocation was determined by immunofluorescence in HL-1 cells directly or indirectly exposed to ROFA or UAP-BA. After 24 h of exposure, cells were fixed with 4% paraformaldehyde for 10 min., permeabilized with 1% Triton X-100 at 25 °C for 30 min., rinsed with PBS, and incubated with anti-Nrf2 antibody (1:250 diluted in BSA, for 2 h) and FITC-Goat anti-Rabbit IgG (H + L) secondary antibody during 1 h. Nuclei counterstaining was done using DAPI fluorescent dye.

Statistical Analysis

Statistical analysis was performed employing ANOVA in conjunction with Bonferroni or Dunnett post-test. Statistical significance was set at $p < 0.05$. All endpoints were run in quadruplicates for each experimental condition and three independent experiments were performed. Data are expressed as the mean ± SD. Statistical analysis was performed with GraphPad Prism, Inc (GraphPad Software).

Results

ROFA and UAP-BA Morphochemical Characterization

ROFA particles were found to be heterogeneous both in size and shape with a particle mean aerodynamic diameter of 2.06 ± 1.57 µm (data not shown). UAP-BA particles were found to be composed of homogeneous ultrafine spherical particles depicting a mean aerodynamic diameter < 0.2 µm (data not shown). These morphological characterizations of ROFA and UAP-BA particles are in agreement with the previous work from our laboratory [20, 33, 39]. Regarding their chemical composition, ROFA is mainly composed of inorganic material (90%), mostly vanadium (V), nickel (Ni), and iron (Fe) metals traces and sulfur (S), sodium (Na), silica (Si), aluminum (Al), and calcium (Ca), while UAP-BA presented no detectable inorganic composition but PAHs and PCBs adsorbed to the particle-carbon core.

Cardiomyocyte Response to Direct ROFA or UAP-BA Particle Exposure

Direct Exposure of HL-1 Cardiomyocytes to ROFA and UAP-BA Does not Affect Metabolic Activity

HL-1 cardiomyocytes were directly exposed (see Fig. 1a for experimental design) to either ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml). The metabolic activity and cell morphology were evaluated by the MTT assay and by inverted phase-contrast microscope, respectively. As shown in Fig. 2a, neither ROFA nor UAP-BA significantly altered the metabolic activity of HL-1 cells. While the morphology of HL-1 cells exposed to UAP-BA was similar to control cells, HL-1 cells exposed to ROFA showed changes in cell shape in a dose-dependent manner (Fig. 2b). HL-1 culture images from ROFA 1 and 10 µg/ml exposed cells showed a slight increase in the number of rounded cells revealing reduction of cell volume while, on the other hand, ROFA 100 µg/ml provoked a drastic reduction on cell size.

Alveolar Macrophage Response to PM

We next characterized the response of alveolar macrophages to ROFA or UAP-BA particles and found that:

(1) Exposure of alveolar macrophages to ROFA but not UAP-BA significantly affects metabolic activity

AM exposed to ROFA or UAP-BA particles for 24 h elicited a different response regarding metabolic activity. ROFA particles were able to provoke a drastic reduction in AM metabolic activity only at the highest (100 µg/ml) concentration (Fig. 3a). On the contrary, UAP-BA did not modify this cell parameter at any concentration employed in this study,

(2) Exposure of alveolar macrophages to ROFA or UAP-BA significantly increased superoxide anion (O_2^-) generation

Most cells from control cultures were colorless non-reactive cells, with only 30% being reactive. Conversely, a large proportion of cells from AM cultures exposed to air particles exhibited an intense dark blue color reaction as result of the formazan precipitation. Particularly, exposure to 1 µg/ml and 10 µg/ml ROFA induced a significant O_2^- increase (Fig. 3b). It is noteworthy that the highest ROFA particle concentration induced a marked reduction of O_2^- generation. On the contrary, a dose-dependent response was observed after exposure to UAP-BA in the range of 10–200 µg/ml. Figure 3b shows a significant increase for UAP-BA 100 µg/ml and 200 µg/ml on the percentage of reactive positive cells as compared to untreated controls.

(3) Exposure of alveolar macrophages to high ROFA or UAP-BA concentrations significantly increased interleukin 6 (IL-6) release

ROFA (1–100 µg/ml) and UAP-BA (10–200 µg/ml) particles, irrespective of their different chemical compositions, induced a dose-dependent response on the release of IL-6 cytokine being only significant for the highest concentration with respect to controls (Fig. 3c). No differences between controls and PM-exposed cultures were found when TNF-α was evaluated by ELISA (data not shown).

Cardiomyocytes Response to Indirect ROFA or UAP-BA Particle Exposure

To test the hypothesis that particles deposited in the lung indirectly stimulate cardiac cells via the release of mediators from particle-activated macrophages, we used CM from ROFA or UAP-BA-exposed alveolar macrophages to treat cardiomyocyte cells (see Fig. 1b for experimental design). Our data show that:

(1) Indirect exposure of HL-1 cardiomyocytes to ROFA and UAP-BA significantly affects metabolic activity

Treatment with the CM from ROFA or UAP-BA-exposed AM had great impact on HL-1 cardiomyocyte metabolic activity in comparison with CM from control non-exposed AM (Fig. 4a). CM-ROFA or CM-UAP-BA exposure resulted in a significant decline in metabolic activity as detected when impaired mitochondrial activity was evaluated by the MTT assay. Nevertheless, HL-1 biological response to CM-ROFA and CM-UAP-BA revealed a distinct pattern, whereas

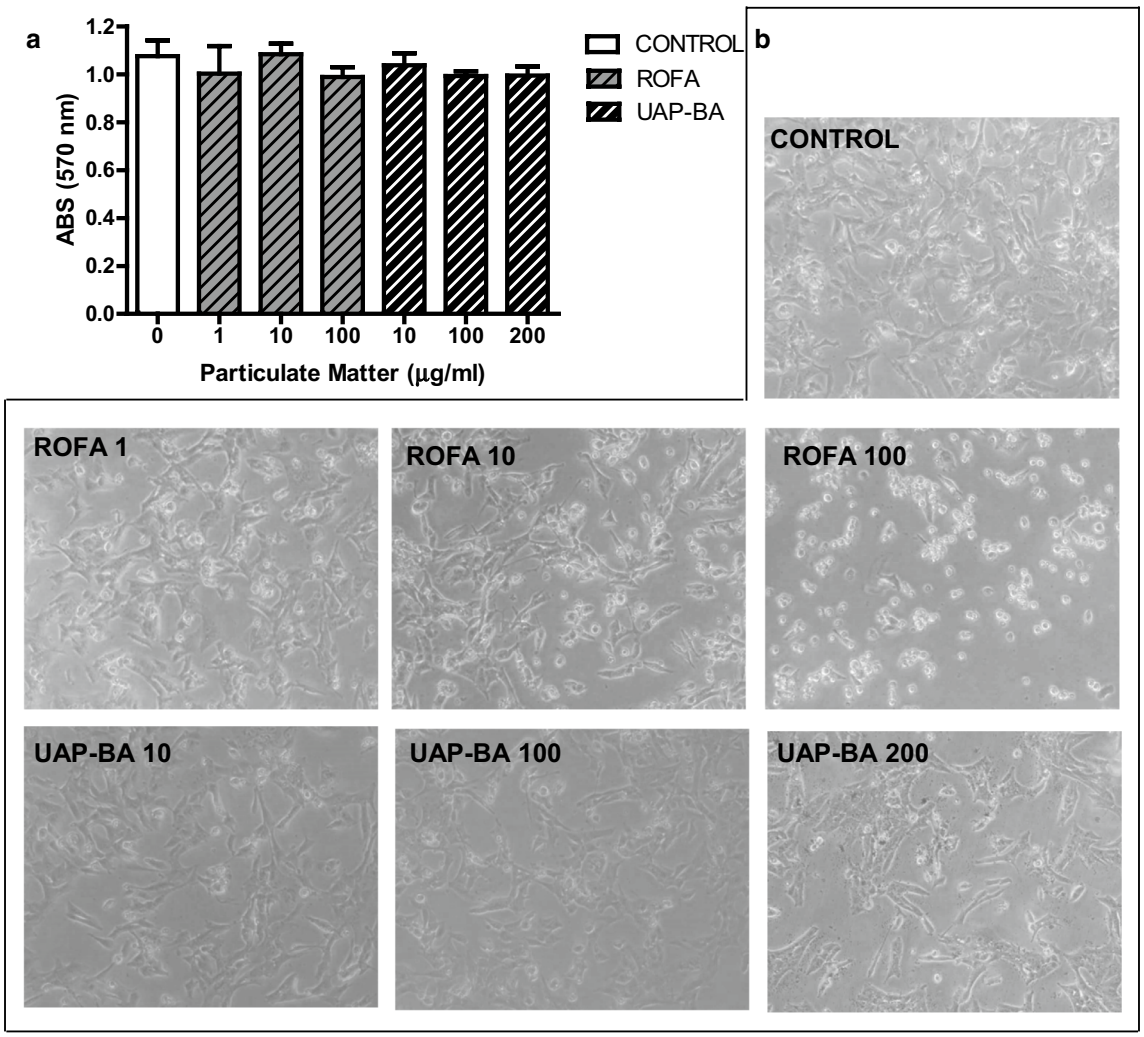

Fig. 2 Direct effect of ROFA and UAP-BA treatment on HL-1 cardiomyocytes. HL-1 cells were exposed to ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml) for 24 h. **a** Metabolic activity: metabolic activity was spectrophotometrically measured by the MTT colorimetric assay. Each bar represents the mean ± SD, $n = 3$-independent experiments, with 3 to 4 replicates within each experiment. **b** Cell morphology: microphotographs show HL-1 control and particle-exposed cells (×200)

UAP-BA induced irrespective of the particle concentration employed, a reduction of metabolic activity by 50%, ROFA provoked a dose-dependent response on cardiomyocyte metabolic activity. The lowest CM-ROFA concentration employed (1 µg/ml) reduced metabolic activity to 80% while the highest concentration (100 µg/ml) was able to decrease it up to a 30–40%. Morphological features from HL-1 cells exposed during 24 h to CM-ROFA and CM-UAP-BA displayed several alterations such as shrinkage and cell rounding thus, revealing a clear cytotoxic effect (Fig. 4b). It is worth to note that even when cultured media for CM-Control HL-1 cells is a mixture of specific MA (RPMI-1640) and cardiomyocytes (Claycomb) cultures medium, they presented normal cardiac phenotype similar to control untreated cells shown in Fig. 2b.

(2) Indirect exposure of HL-1 cardiomyocytes to ROFA or UAP-BA significantly increases total reactive oxygen species generation

Figure 5a, b shows cardiomyocytes ROS generation determined by means of a fluorescent probe after being exposed directly or indirectly (CM) to ROFA and UAP-BA. Control and CM-Control cells displayed very low levels of DHR-123-dependent fluorescence. HL-1 culture cells directly exposed to 1–100 µg/ml ROFA exhibited a dose-dependent increase in ROS generation with a much stronger signal for the highest concentration, while UAP-BA did not significantly modify

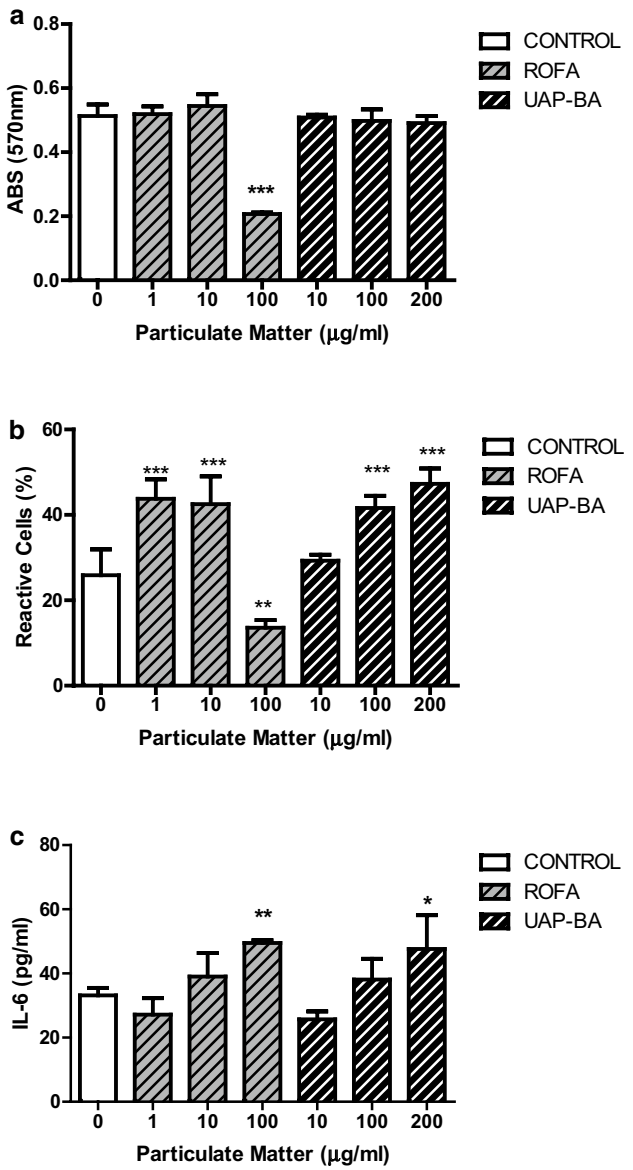

Fig. 3 Characterization of alveolar macrophage response to ROFA or UAP-BA particles. AM were exposed to ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml) for 24 h. **a** Metabolic activity. Metabolic activity was spectrophotometrically measured by the MTT colorimetric assay. **b** Superoxide anion generation: NBT colorimetric assay was used to quantitatively detect superoxide anion generated in control and particle-exposed AM in culture. **c** Generation of IL-6: cytokine IL6 level was quantified in supernatants from cultures' AMs by ELISA. Each bar represents the mean ± SD, three independent experiments, with 3 to 4 technical replicates within each experiment. Statistically significant differences between exposed and control cells $^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA followed by the Dunnett post hoc test

this parameter at any of the doses assayed in this study (Fig. 5a).

Indirect exposure to both PM provokes increase in ROS generation (Fig. 5b). Noteworthy, even though cultured HL-1 cells did not respond to direct UAP-BA exposure, the exposure to CM-UAP-BA (indirect effect) elicited a significant increase in ROS generation.

(3) Indirect exposure of HL-1 cardiomyocytes to ROFA and UAP-BA significantly increases Nrf2 cytoplasmic expression—with no nuclear translocation

Figure 6 shows nuclear factor Nrf2 expression and cell localization after direct (6a) and indirect (6b) exposure to ROFA or UAP-BA. Figure 6a shows Nrf2 basal cytoplasmic expression in control HL-1 cells. ROFA particles (10 and 100 µg/ml) caused a dose-dependent rise in Nrf2 expression revealed by a marked fluorescent intensity. Moreover, these ROFA concentrations induced nuclear translocation of Nrf2 in HL1 cardiomyocytes. Similarly, UAP-BA (100 and 200 µg/ml) direct exposure induced Nrf2 augmentation and nuclear translocation. Resembling control cultures (Fig. 6a), CM controls showed a basal cytoplasmic Nrf2 expression (Fig. 6b). All CM-ROFA induced in HL-1 cells a substantial fluorescent cytoplasmic increase, nevertheless, only 10 µg/ml CM-ROFA provoked translocation of Nrf2 to the nucleus. Stimulation of Nrf2 cytoplasmic expression with no nuclear translocation was observed for all CM-UAP-BA treatments.

Discussion

Among all body organs, the heart is the organ with the highest O_2 consumption [40] and thus, maintaining functional redox signaling is crucial to protect living cells from various pathological insults such as inflammation, oxidative stress, and toxic chemical exposure. In vivo studies revealed that direct acute and chronic PM exposures induce inflammation and oxidative stress in rodent cardiorespiratory system [26, 41–43]. In this study by using CM obtained after incubating alveolar macrophages with PM (ROFA and UAP-BA, see Fig. 1), we evaluated the hypothesis that mediators derived from AMs exposed to air pollution PM could induce indirect effects in cardiomyocytes.

Indeed, CM obtained after incubation of AMs 24 h with either ROFA or UAP-BA 1) decreased the metabolic activity and altered the morphology of cardiomyocytes (Fig. 4) and significantly increased total reactive oxygen species generation (Fig. 5). Since the increased pro-oxidant environment promotes nuclear accumulation of Nrf2 protein and activate Nrf2 signaling [44, 45], we investigated the direct and indirect effect of PM exposure on modulation of Nrf2-dependent antioxidant expression in HL-1 cardiomyocytes. When direct PM effect on cardiomyocytes was analyzed, we found increments in ROS generation and Nrf2 cytoplasmic and nuclear expression with no changes in cell metabolic activity. Regarding indirect effect in cardiomyocyte, the

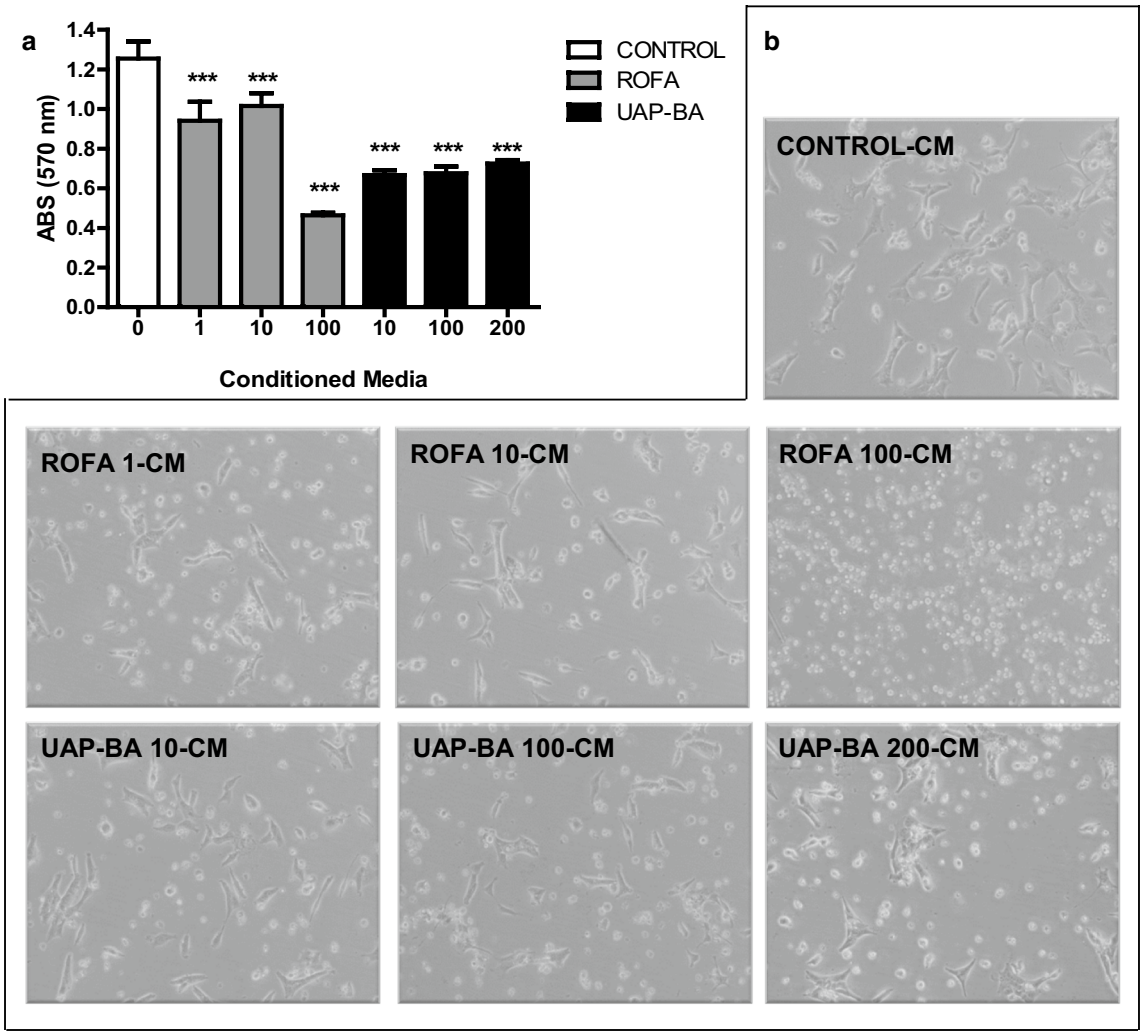

Fig. 4 Indirect effect of ROFA and UAP-BA treatment on HL-1 cardiomyocytes. HL-1 cells were incubated for 24 h with CM generated by macrophages in response to ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml). **a** Metabolic activity: metabolic activity was spectrophotometrically measured by the MTT colorimetric assay. Each bar represents the mean ± SD, three-independent experiments, with 3 to 4 replicates within each experiment.***$p < 0.001$, one-way ANOVA followed by the Dunnett post hoc test. **b** Cell morphology: microphotographs show control or exposed to CM-ROFA (1, 10, 100 µg/ml) or CM-UAP-BA (10, 100, 200 µg/ml) HL-1 cultures (×200)

CM only increase of cytoplasmic Nrf2 with absence to the cell nuclei translocation (Fig. 6). This differential response between the direct and indirect effects of PM on cardiomyocytes suggests that directly exposed to MP, Nrf2-protective signaling pathway is responsible for at least partially neutralizing the oxidative damage. Total protection was not observed since ROFA was still able to alter cell morphology by reducing its cell volume these parameters being possible indicators of cytotoxicity. The lack in the OD decrease from the MTT test in ROFA-exposed cultures with respect to controls could reflect cellular-altered metabolic activity, but neither cell number nor cell viability reduction. This cell volume reduction could be indicative of the initiation of an apoptotic process [46]. Therefore, the light microscopic images are likely a better approximation of the impact of ROFA particles on cardiomyocyte cells. Furthermore, when we tested the effect of CM from particle-exposed alveolar macrophages our results regarding cell metabolic activity are in agreement with the altered cell morphology observed for all treated CM-ROFA or CM-UAP-BA cardiac cells, thus revealing a clear cytotoxic effect. Consequently, through the evaluation of PM direct and indirect effect on cardiomyocytes, we propose that only in response to direct PM exposure, Nrf2 nuclear translocation might suppress cell death. Moreover, we observed that cardiac cell cytotoxicity in response to lung-derived mediators occurs irrespective of the particle concentration.

Given that CM is a complex mixture triggering an altered HL-1 cells response, it is important to determine what factors or mediators in the CM are responsible for the

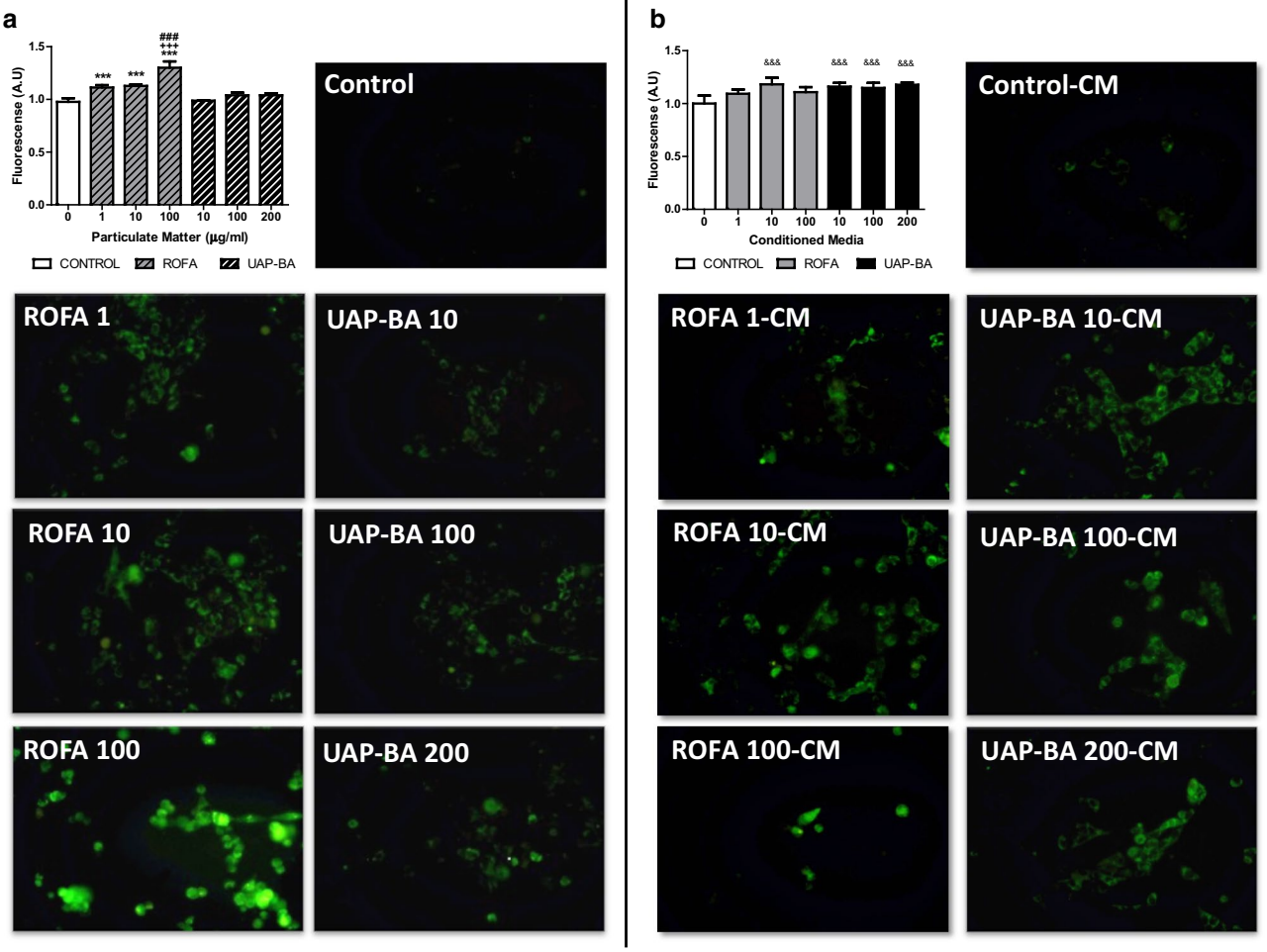

Fig. 5 Cardiomyocytes total reactive oxygen species generation after ROFA or UAP-BA direct or indirect exposure. Total ROS generation in HL-1 cardiomyocytes exposed to **a** ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml) (direct effect) or **b** the condition media (CM) generated by macrophages in response to ROFA or UAP-BA (indirect effect). ROS generation was detected using an intracellular ROS dye, dihydrorhodamine 123 (DHR123). Histograms represent the fluorescence mean value calculated after analyzing ten field selected at random per well per experiment. Representative immunofluorescence microphotographs of HL-1 control and particle-exposed cells (×200). Results were expressed as fluorescence arbitrary units. Data are representative of three independent experiments. Statistically significant differences between exposed and control cells (***) $p < 0.001$, ROFA 1 and ROFA 100 exposed cells $^{+++}p < 0.001$, ROFA10 and ROFA 100 exposed cells $^{###}p < 0.001$ and CM-exposed and CM-control cells $^{&&&}$) $p < 0.001$-one-way ANOVA followed by the Bonferroni post hoc test

significant effects on cell metabolism which are not observed when cells are directly exposed to particles. Assuming that ROFA or UAP-BA exposure in macrophages causes increased superoxide anion generation and IL-6 production, we propose that indirect changes in cardiomyocyte metabolism may by through oxidative and pro-inflammatory mediators released from particle-loaded macrophages and then be released into the CM. Inflammation is a central response of the organism against various adverse stimuli and, when it occurs with a high intensity or for long periods of time, it could trigger cardiorespiratory diseases. In the lung, this process is characterized by the release of pro-inflammatory mediators (cytokines, chemokines, reactive oxygen species, etc.) by AM and pulmonary epithelial cells. In fact, it has been reported that chronic PM exposure in humans induces airway inflammation [47]. Lung inflammation may result in an inflammatory systemic response, causing endothelial dysfunction that can subsequently contribute to cardiovascular effects [48]. In humans, increased circulating level of pro-inflammatory cytokines such as CRP, IL-6, IL-8, and IL-1β was observed after ambient PM exposure [49]. Similar results have been reported both in vivo animal models and in vitro cellular models [42, 50]. In addition, ROS-dependent mechanism was shown to be involved in the PM-triggered pro-inflammatory pathway [51]. Nevertheless, further studies, such as inhibition of ROS or IL-6, should be done to

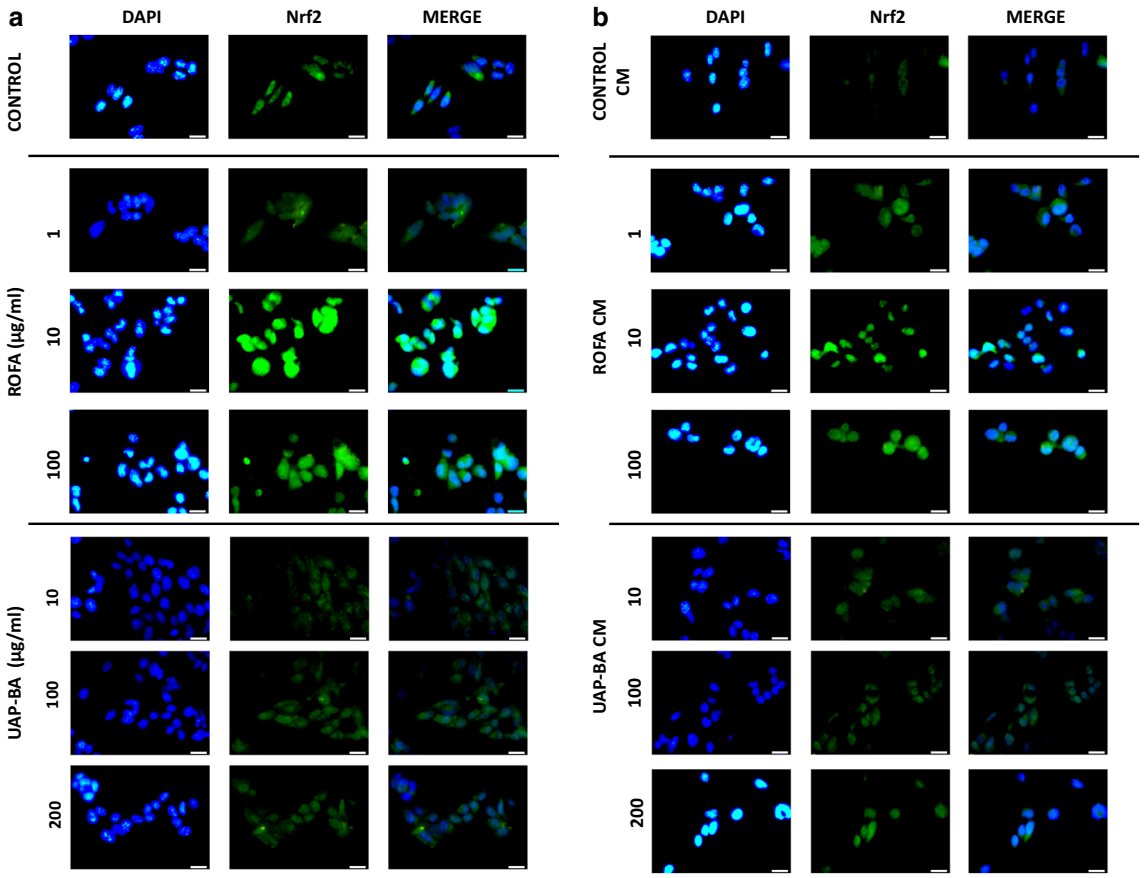

Fig. 6 Cardiomyocytes Nrf2 expression and nuclear translocation after ROFA or UAP-BA direct or indirect exposure. Nrf2 nuclear expression and cellular localization in HL-1 cardiomyocytes exposed to **a** ROFA (1–100 µg/ml) or UAP-BA (10–200 µg/ml) (direct effect) or **b** the CM generated by macrophages in response to ROFA or UAP-BA (indirect effect). Representative immunofluorescence microphotograph (×200) of control and exposed cells showing Nrf2 nuclear localization. DAPI fluorescent dye was used as a nuclear counterstain. Data are representative of three independent experiments

identify if ROS or IL-6 are critical mediators of the indirect PM effects.

Our experimental design (Fig. 1) rules out the possibility that cellular soluble products produced by AM but unrelated to PM (e.g., cellular waste) may affect cardiomyocytes since CM obtained from AMs in the absence of PM has no effect compared to CM obtained from AMs exposed to PM. We are also aware that the observed indirect effect could be due to the presence of soluble factors dissolved from the PM during AM exposure. However, this possibility is unlikely since (i) we have seen no response when cardiomyocytes were exposed directly to the particle, where the particle soluble fraction is present and, (ii) in accordance, we have previously shown that when exposed to UAP-BA or ROFA soluble fraction, the viability of A549 human epithelial lung cells was not altered [33]. Therefore, we assume that particle soluble fraction would not have any effect on cardiomyocyte metabolic activity exposed to CM. In agreement with this interpretation, Totlandsdal et al. [32] observed in vitro that mediators released by epithelial lung cells after ultrafine carbon black particles and urban particles SRM1648 exposure stimulate and exacerbate the inflammatory response in cardiac cells. Even more, recently Gorr et al. [52] showed that both direct and indirect diesel exhaust particles exposure induced cardiomyocyte dysfunction in culture. Extrapolated to the in vivo situation, our in vitro findings suggest that particle-induced lung-derived mediators are more important in the cardiac response than inhaled particles that may reach the cardiac tissue via the systemic circulation. Furthermore, it has to be pointed out that ultrafine particles, like UAP-BA, at high concentrations have a tendency to aggregate and thus in vivo translocation to systemic circulation could be potentially reduced. Hence, indirect particle via lung-derived mediators could be the main pathway leading to cardiac-altered response. Nonetheless, it must be considered that cross-talk between direct and indirect pathways could take place in vivo. Finally, the inability of Nrf2 to translocate into the nucleus suggests that this pathway plays an important role linking the depositions of inhaled particles in the lung and the initiation of adverse events in the heart and

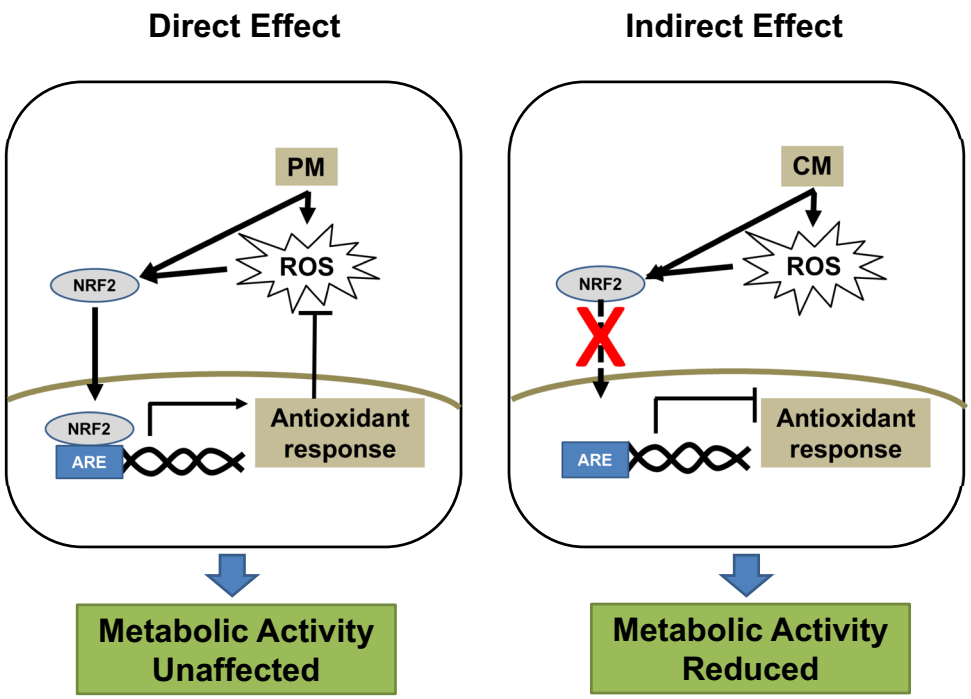

Fig. 7 Proposed model for the direct and indirect effect of PM on metabolic activity of cardiomyocytes. Right panel: direct exposure of HL-1 cells to PM increase ROS and Nrf2 expression (directly or via ROS) and translocation into the nucleus inducing a protective antioxidant response. Left panel: indirect exposure of HL-1 cells to PM through the exposure to CM generated by macrophages in response to ROFA or UAP-BA increase ROS and Nrf2 cytoplasmic expression (directly or via ROS) without Nrf2 translocation into the nucleus

vasculature [53]. Figure 7 shows a proposed model for the molecular mechanism by which indirect exposure to PM (CM) increases ROS levels and Nrf2 cytoplasmic expression but decreases metabolic activity in HL-1 cells by the failure of Nrf2 to translocate into the nucleus.

Conclusion

Our in vitro findings provide support to the hypothesis that particle-induced cardiac alterations may involve lung-derived mediators possibly through Nrf2 inactivation. Further studies are warranted to explore the potential use of Nrf2 activators as chemopreventive agents to reduce the adverse of inhaled PM on cardiomyocytes.

Acknowledgements The authors would like to thank Dr. W. Claycomb for the cardiomyocyte cell line and A. Perez de la Hoz for his assistance and technical expertise with the collector sampler.

Funding This work was partially supported by Grants A147 and SJ10/54 from Universidad Nacional de San Martín Grants 2010-1661 and 2012-0328 from ANPCyT-PICT.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research Involving Human and Animal Participants This article does not contain any studies with human participants performed by any of the authors.

References

1. Brook, R. D., Rajagopalan, S., Pope, C. A., 3rd, Brook, J. R., Bhatnagar, A., Diez-Roux, A. V., et al. (2010). Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation, 121*(21), 2331–2378. https://doi.org/10.1161/CIR.0b013e3181dbece1.
2. Lee, B. J., Kim, B., & Lee, K. (2014). Air pollution exposure and cardiovascular disease. *Toxicological Research, 30*(2), 71–75. https://doi.org/10.5487/TR.2014.30.2.071.
3. Brook, R. D., Franklin, B., Cascio, W., Hong, Y., Howard, G., Lipsett, M., et al. (2004). Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation, 109*(21), 2655–2671. https://doi.org/10.1161/01.CIR.0000128587.30041.C8.
4. Pope, C. A., 3rd, & Dockery, D. W. (2006). Health effects of fine particulate air pollution: Lines that connect. *Journal of the Air and Waste Management Association, 56*(6), 709–742.
5. Peters, A., Dockery, D. W., Muller, J. E., & Mittleman, M. A. (2001). Increased particulate air pollution and the triggering of myocardial infarction. *Circulation, 103*(23), 2810–2815.
6. Analitis, A., Katsouyanni, K., Dimakopoulou, K., Samoli, E., Nikoloulopoulos, A. K., Petasakis, Y., et al. (2006). Short-term effects of ambient particles on cardiovascular and respiratory mortality. *Epidemiology, 17*(2), 230–233. https://doi.org/10.1097/01.ede.0000199439.57655.6b.
7. Pope, C. A., 3rd, Burnett, R. T., Thurston, G. D., Thun, M. J., Calle, E. E., Krewski, D., et al. (2004). Cardiovascular mortality

and long-term exposure to particulate air pollution: Epidemiological evidence of general pathophysiological pathways of disease. *Circulation, 109*(1), 71–77. https://doi.org/10.1161/01.CIR.0000108927.80044.7F.

8. Martinelli, N., Olivieri, O., & Girelli, D. (2013). Air particulate matter and cardiovascular disease: A narrative review. *European Journal of Internal Medicine, 24*(4), 295–302. https://doi.org/10.1016/j.ejim.2013.04.001.
9. Nemmar, A., Hoet, P. H., Vanquickenborne, B., Dinsdale, D., Thomeer, M., Hoylaerts, M. F., et al. (2002). Passage of inhaled particles into the blood circulation in humans. *Circulation, 105*(4), 411–414.
10. Oberdorster, G., Sharp, Z., Atudorei, V., Elder, A., Gelein, R., Lunts, A., et al. (2002). Extrapulmonary translocation of ultrafine carbon particles following whole-body inhalation exposure of rats. *Journal of Toxicology and Environmental Health Part A, 65*(20), 1531–1543. https://doi.org/10.1080/00984100290071658.
11. Wallenborn, J. G., McGee, J. K., Schladweiler, M. C., Ledbetter, A. D., & Kodavanti, U. P. (2007). Systemic translocation of particulate matter-associated metals following a single intratracheal instillation in rats. *Toxicological Sciences: An Official Journal of the Society of Toxicology, 98*(1), 231–239. https://doi.org/10.1093/toxsci/kfm088.
12. Brook, R. D. (2008). Cardiovascular effects of air pollution. *Clinical Science, 115*(6), 175–187. https://doi.org/10.1042/CS20070444.
13. Mills, N. L., Donaldson, K., Hadoke, P. W., Boon, N. A., MacNee, W., Cassee, F. R., et al. (2009). Adverse cardiovascular effects of air pollution. *Nature Clinical Practice Cardiovascular Medicine, 6*(1), 36–44. https://doi.org/10.1038/ncpcardio1399.
14. Miller, M. R. (2014). The role of oxidative stress in the cardiovascular actions of particulate air pollution. *Biochemical Society Transactions, 42*(4), 1006–1011. https://doi.org/10.1042/BST20140090.
15. Yang, W., & Omaye, S. T. (2009). Air pollutants, oxidative stress and human health. *Mutation Research, 674*(1–2), 45–54. https://doi.org/10.1016/j.mrgentox.2008.10.005.
16. Valavanidis, A., Vlachogianni, T., Fiotakis, K., & Loridas, S. (2013). Pulmonary oxidative stress, inflammation and cancer: Respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. *International Journal of Environmental Research and Public Health, 10*(9), 3886–3907. https://doi.org/10.3390/ijerph10093886.
17. Zhang, H., Davies, K. J. A., & Forman, H. J. (2015). Oxidative stress response and Nrf2 signaling in aging. *Free Radical Biology & Medicine, 88*(Pt B), 314–336. https://doi.org/10.1016/j.freeradbiomed.2015.05.036.
18. Kaspar, J. W., Niture, S. K., & Jaiswal, A. K. (2009). Nrf 2:INrf2 (Keap1) signaling in oxidative stress. *Free Radical Biology & Medicine, 47*(9), 1304–1309. https://doi.org/10.1016/j.freeradbiomed.2009.07.035.
19. Jiang, S., Yang, Y., Li, T., Ma, Z., Hu, W., Deng, C., et al. (2016). An overview of the mechanisms and novel roles of Nrf2 in cardiovascular diseases. *Expert Opinion on Therapeutic Targets, 20*(12), 1413–1424. https://doi.org/10.1080/14728222.2016.1250887.
20. Martin, S., Dawidowski, L., Mandalunis, P., Cereceda-Balic, F., & Tasat, D. R. (2007). Characterization and biological effect of Buenos Aires urban air particles on mice lungs. *Environmental Research, 105*(3), 340–349. https://doi.org/10.1016/j.envres.2007.04.009.
21. Martin, S., Fernandez-Alanis, E., Delfosse, V., Evelson, P., Yakisich, J. S., Saldiva, P. H., et al. (2010). Low doses of urban air particles from Buenos Aires promote oxidative stress and apoptosis in mice lungs. *Inhalation Toxicology, 22*(13), 1064–1071. https://doi.org/10.3109/08958378.2010.523030.
22. Figueroa, D. A., Rodriguez-Sierra, C. J., & Jimenez-Velez, B. D. (2006). Concentrations of Ni and V, other heavy metals, arsenic, elemental and organic carbon in atmospheric fine particles (PM2.5) from Puerto Rico. *Toxicology and Industrial Health, 22*(2), 87–99. https://doi.org/10.1191/0748233706th247oa.
23. Huang, Y. C., & Ghio, A. J. (2006). Vascular effects of ambient pollutant particles and metals. *Current Vascular Pharmacology, 4*(3), 199–203.
24. Dreher, K. L., Jaskot, R. H., Lehmann, J. R., Richards, J. H., McGee, J. K., Ghio, A. J., et al. (1997). Soluble transition metals mediate residual oil fly ash induced acute lung injury. *Journal of Toxicology and Environmental Health, 50*(3), 285–305.
25. Marchini, T., Magnani, N. D., Paz, M. L., Vanasco, V., Tasat, D., Gonzalez Maglio, D. H., et al. (2014). Time course of systemic oxidative stress and inflammatory response induced by an acute exposure to residual oil fly ash. *Toxicology and Applied Pharmacology, 274*(2), 274–282. https://doi.org/10.1016/j.taap.2013.11.013.
26. Orona, N. S., Ferraro, S. A., Astort, F., Morales, C., Brites, F., Boero, L., et al. (2016). Acute exposure to Buenos Aires air particles (UAP-BA) induces local and systemic inflammatory response in middle-aged mice: A time course study. *Environmental Pollution, 208*(Pt A), 261–270. https://doi.org/10.1016/j.envpol.2015.07.020.
27. Baldauf, R. W., Lane, D. D., & Marote, G. A. (2001). Ambient air quality monitoring network design for assessing human health impacts from exposures to airborne contaminants. *Environmental Monitoring and Assessment, 66*(1), 63–76.
28. Tasat, D. R., & de Rey, B. M. (1987). Cytotoxic effect of uranium dioxide on rat alveolar macrophages. *Environmental Research, 44*(1), 71–81.
29. Claycomb, W. C., Lanson, N. A., Jr., Stallworth, B. S., Egeland, D. B., Delcarpio, J. B., Bahinski, A., et al. (1998). HL-1 cells: A cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. *Proceedings of the National academy of Sciences of the United States of America, 95*(6), 2979–2984.
30. Longhin, E., Holme, J. A., Gualtieri, M., Camatini, M., & Ovrevik, J. (2018). Milan winter fine particulate matter (wPM2.5) induces IL-6 and IL-8 synthesis in human bronchial BEAS-2B cells, but specifically impairs IL-8 release. *Toxicology in Vitro: An International Journal Published in Association with BIBRA, 52,* 365–373. https://doi.org/10.1016/j.tiv.2018.07.016.
31. Li, N., Hao, M., Phalen, R. F., Hinds, W. C., & Nel, A. E. (2003). Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clinical Immunology, 109*(3), 250–265.
32. Totlandsdal, A. I., Refsnes, M., Skomedal, T., Osnes, J. B., Schwarze, P. E., & Lag, M. (2008). Particle-induced cytokine responses in cardiac cell cultures—The effect of particles versus soluble mediators released by particle-exposed lung cells. *Toxicological Sciences: An Official Journal of the Society of Toxicology, 106*(1), 233–241. https://doi.org/10.1093/toxsci/kfn162.
33. Orona, N. S., Astort, F., Maglione, G. A., Saldiva, P. H., Yakisich, J. S., & Tasat, D. R. (2014). Direct and indirect air particle cytotoxicity in human alveolar epithelial cells. *Toxicology In Vitro: An International Journal Published in Association with BIBRA, 28*(5), 796–802. https://doi.org/10.1016/j.tiv.2014.02.011.
34. Morgan, D. M. (1998). Tetrazolium (MTT) assay for cellular viability and activity. *Methods in Molecular Biology, 79,* 179–183.
35. Molinari, B. L., Tasat, D. R., Palmieri, M. A., O'Connor, S. E., & Cabrini, R. L. (2003). Cell-based quantitative evaluation of the MTT assay. *Analytical and Quantitative Cytology and Histology, 25*(5), 254–262.
36. Segal, A. W. (1974). Nitroblue-tetrazolium tests. *Lancet, 2*(7891), 1248–1252.

37. Molinari, B. L., Tasat, D. R., Fernandez, M. L., Duran, H. A., Curiale, J., Stoliar, A., et al. (2000). Automated image analysis for monitoring oxidative burst in macrophages. *Analytical and Quantitative Cytology and Histology, 22*(5), 423–427.
38. Bueb, J. L., Gallois, A., Schneider, J. C., Parini, J. P., & Tschirhart, E. (1995). A double-labelling fluorescent assay for concomitant measurements of $[Ca^{2+}]i$ and O_2 production in human macrophages. *Biochimica et Biophysica Acta, 1244*(1), 79–84.
39. Ferraro, S. A., Yakisich, J. S., Gallo, F. T., & Tasat, D. R. (2011). Simvastatin pretreatment prevents ambient particle-induced lung injury in mice. *Inhalation Toxicology, 23*(14), 889–896. https://doi.org/10.3109/08958378.2011.623195.
40. Shanmugam, G., Narasimhan, M., Sakthivel, R., Kumar, R. R., Davidson, C., Palaniappan, S., et al. (2016). A biphasic effect of TNF-alpha in regulation of the Keap1/Nrf2 pathway in cardiomyocytes. *Redox Biology, 9,* 77–89. https://doi.org/10.1016/j.redox.2016.06.004.
41. Kodavanti, U. P., Moyer, C. F., Ledbetter, A. D., Schladweiler, M. C., Costa, D. L., Hauser, R., et al. (2003). Inhaled environmental combustion particles cause myocardial injury in the Wistar Kyoto rat. *Toxicological Sciences: An Official Journal of the Society of Toxicology, 71*(2), 237–245.
42. Astort, F., Sittner, M., Ferraro, S. A., Orona, N. S., Maglione, G. A., De la Hoz, A., et al. (2014). Pulmonary inflammation and cell death in mice after acute exposure to air particulate matter from an industrial region of Buenos Aires. *Archives of Environmental Contamination and Toxicology, 67*(1), 87–96. https://doi.org/10.1007/s00244-013-9975-4.
43. Riva, D. R., Magalhaes, C. B., Lopes, A. A., Lancas, T., Mauad, T., Malm, O., et al. (2011). Low dose of fine particulate matter (PM2.5) can induce acute oxidative stress, inflammation and pulmonary impairment in healthy mice. *Inhalation Toxicology, 23*(5), 257–267. https://doi.org/10.3109/08958378.2011.566290.
44. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., & Yamamoto, M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms, 8*(4), 379–391.
45. Muthusamy, V. R., Kannan, S., Sadhaasivam, K., Gounder, S. S., Davidson, C. J., Boeheme, C., et al. (2012). Acute exercise stress activates Nrf2/ARE signaling and promotes antioxidant mechanisms in the myocardium. *Free Radical Biology & Medicine, 52*(2), 366–376. https://doi.org/10.1016/j.freeradbiomed.2011.10.440.
46. Maeno, E., Ishizaki, Y., Kanaseki, T., Hazama, A., & Okada, Y. (2000). Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proceedings of the National academy of Sciences of the United States of America, 97*(17), 9487–9492. https://doi.org/10.1073/pnas.140216197.
47. Ling, S. H., & van Eeden, S. F. (2009). Particulate matter air pollution exposure: Role in the development and exacerbation of chronic obstructive pulmonary disease. *International Journal of Chronic Obstructive Pulmonary Disease, 4,* 233–243.
48. Hamanaka, R. B., & Mutlu, G. M. (2018). Particulate matter air pollution: Effects on the cardiovascular system. *Frontiers in Endocrinology, 9,* 680. https://doi.org/10.3389/fendo.2018.00680.
49. van Eeden, S. F., Tan, W. C., Suwa, T., Mukae, H., Terashima, T., Fujii, T., et al. (2001). Cytokines involved in the systemic inflammatory response induced by exposure to particulate matter air pollutants (PM(10)). *American Journal of Respiratory and Critical Care Medicine, 164*(5), 826–830. https://doi.org/10.1164/ajrccm.164.5.2010160.
50. Sijan, Z., Antkiewicz, D. S., Heo, J., Kado, N. Y., Schauer, J. J., Sioutas, C., et al. (2015). An in vitro alveolar macrophage assay for the assessment of inflammatory cytokine expression induced by atmospheric particulate matter. *Environmental Toxicology, 30*(7), 836–851. https://doi.org/10.1002/tox.21961.
51. Gurgueira, S. A., Lawrence, J., Coull, B., Murthy, G. G., & Gonzalez-Flecha, B. (2002). Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environmental Health Perspectives, 110*(8), 749–755. https://doi.org/10.1289/ehp.02110749.
52. Gorr, M. W., Youtz, D. J., Eichenseer, C. M., Smith, K. E., Nelin, T. D., Cormet-Boyaka, E., et al. (2015). In vitro particulate matter exposure causes direct and lung-mediated indirect effects on cardiomyocyte function. *American Journal of Physiology Heart and Circulatory Physiology, 309*(1), H53–H62. https://doi.org/10.1152/ajpheart.00162.2015.
53. Brook, R. D., & Rajagopalan, S. (2010). Particulate matter air pollution and atherosclerosis. *Current Atherosclerosis Reports, 12*(5), 291–300. https://doi.org/10.1007/s11883-010-0122-7.