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Biokinetically-Based In Vitro Cardiotoxicity of Residual Oil Fly Ash: Hazard Identification and Mechanisms of Injury

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Abstract Epidemiological studies have associated air pollution particulate matter (PM) exposure with adverse cardiovascular effects. Identification of causal PM sources is critically needed to support regulatory decisions to protect public health. This research examines the in vitro cardiotoxicity of bioavailable constituents of residual oil fly ash (ROFA) employing in vivo, biokinetically-based, concentrations determined from their pulmonary deposition. Pulmonary deposition of ROFA led to a rapid increase in plasma vanadium (V) levels that were prolonged in hypertensive animals without systemic inflammation. ROFA cardiotoxicity was evaluated using neonatal rat cardiomyocyte (RCM) cultures exposed to particle-free leachates of ROFA (ROFA-L) at levels present in exposed rat plasma. Cardiotoxicity was observed at low levels (3.13 μg/mL) of ROFA-L 24 h post-exposure. Dimethylthiourea (28 mM) inhibited ROFA-L-induced cytotoxicity at high (25–12.5 μg/mL) doses, suggesting that oxidative stress is responsible at high ROFA-L doses. Cardiotoxicity could not be reproduced using a V + Ni + Fe mixture or a ROFA-L depleted of these metals, suggesting that ROFA-L cardiotoxicity requires the full complement of bioavailable constituents. Susceptibility of RCMs to ROFA-L-induced cytotoxicity was increased following tyrosine phosphorylation inhibition, suggesting that phosphotyrosine signaling pathways play a critical role in regulating ROFA-L-induced cardiotoxicity. These data demonstrate that bioavailable constituents of ROFA are capable of direct adverse cardiac effects.

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Introduction

Current scientific literature addressing air pollution particulate matter (PM) effects has shown an increased risk of morbidity and mortality associated with exposure to ambient levels of PM. In 1993, Dockery et al. [1] related excess mortality to PM at or below national ambient air quality standards. Subsequent epidemiological studies have consistently identified individuals with cardiovascular disease as one susceptible subpopulation adversely impacted by air pollution [2]. Adverse health effects associated with ambient PM exposure include triggering of myocardial infarction, increases in pro-thrombotic factors, increases in numbers of peripheral neutrophils, increases in circulating cytokine levels, decreases in heart rate variability and arterial vasodilation, as well as exacerbation of atherosclerosis in humans and animal models [3–8].

Air pollution is a physicochemically diverse mixture of particles and gaseous pollutants arising from multiple sources, such as combustion of fossil fuels, secondary atmospheric transformation, and natural sources [9]. Combustion sources contribute significantly to the composition of PM <2.5 μm in aerodynamic diameter ($PM_{2.5}$) and contribute preferentially to an increase in the risk of cardiovascular death associated with PM exposure [2, 10]. Components of combustion source particles have been shown, either through direct analysis of each element present in $PM_{2.5}$ or through factor analysis, to increase daily deaths [10, 11]. However, hazard identification of causal constituents of PM and source-specific cardiovascular health effects remain to be determined. Pulmonary exposure of rats to residual oil fly ash (ROFA) has been linked to increases in mortality in pulmonary hypertensive animals [12, 13], necrotic lesion development in healthy animals [14], and reduced vasodilation response [15]. These effects could be attributed to bioavailable metal constituents associated with this type of particulate emissions.

Several mechanisms have been proposed as to how pulmonary-deposited PM increases cardiovascular disease-related morbidity and mortality. These mechanisms have been reviewed extensively by our laboratory [9, 16]. The following hypotheses have been proposed to explain the association of PM deposited in the lung with remote cardiovascular effects: PM directly/indirectly stimulates an altered autonomic nervous system (ANS) response [17, 18], PM-induced systemic inflammation [19], and direct effects on the cardiovascular system through translocation of particles or dissolution of soluble "bioavailable" constituents [13, 14, 20–22].

In the present study we determined: (1) whether cardiac toxicity of ROFA particles could be due to direct interaction between its bioavailable constituents and cardiomyocytes at concentrations observed in vivo (biokinetically-based) following their pulmonary deposition; (2) the role of oxidative stress and cellular phosphorylation in mediating cardiomyocyte cytotoxicity; and (3) the mechanism of cardiomyocyte cytotoxicity induced by ROFA bioavailable constituents.

Materials and Methods

Animals

Male, 65–70-day-old, Sprague–Dawley (SD) rats were used for ROFA intratracheal instillation (IT) and inhalation (IH) exposures. Male, 90-day-old, cardiac-compromised spontaneously hypertensive rats (SHRs) were used for ROFA IT exposures. Ninety-day-old SHRs display hypertension with cardiomyocyte hypertrophy [23, 24]. SD rats aged 60–90 days obtained at gestation day 19 were used as a source for neonatal rat cardiomyocyte (RCM) cultures. All animals were obtained from Charles River Laboratory, Raleigh, NC. All animals were maintained in an AAALAC-approved animal facility and provided food and water ad libitum. Animals were used in accordance with federal animal use guidelines.

Bulk-collected ROFAs and ROFA Characterization

Bulk-collected fine mode (particles with a mass mean aerodynamic diameter of 2.5 μm, referred to as $PM_{2.5}$) ROFA employed in IT and in vitro exposures was obtained from a power plant burning low-sulfur #6 residual fuel oil and physicochemically characterized as previously described [13, 20, 25]. This ROFA contained 35.04 μg of water-soluble vanadium (V)/mg ROFA. Bulk-collected $PM_{2.5}$ high-sulfur #6 residual oil fly ash (H#6 ROFA) and high-sulfur #5 residual oil (#5 ROFA) were obtained from a 732 kW rated fire-tube boiler located within the laboratories of the US Environmental Protection Agency in Research Triangle Park [26, 27]. Briefly, high-sulfur (2.33 %) H#6 or high-sulfur (1.73 %) #5 residual oil was combusted in a North American package boiler, and $PM_{2.5}$ ROFA was collected on Teflon-coated glass fiber filters following a $PM_{2.5}$ cyclone. Detailed physicochemical characterization of H#6 and #5 ROFA has been previously published [26–30]. H#6 ROFA contained 25.10 μg of water-soluble V/mg H#6ROFA. #5 ROFA was found to contain 24 μg of water-soluble V/mg ROFA.

ROFA and Metal Exposures

In Vivo ROFA Exposures

ROFA doses employed in either IT pulmonary or IH exposures were chosen to be compatible with previously published doses that have been shown to elicit adverse cardiac effects in vivo [31, 32]. For IT exposures, SD rats were exposed to either saline (pH 6.0) or ROFA at 2.5 mg/0.3 mL saline/rat or at 0.5 mg/0.3 mL saline/rat, while SHRs were exposed to ROFA at 0.5 mg/0.3 mL saline/rat, as previously described [20]. In two separate studies, rats were exposed by IH to two different ROFA samples. In one study SD rats were exposed by nose-only IH using a string generator to bulk-collected $PM_{2.5}$ H#6 ROFA reaerosolized at a concentration of 9 mg/m^3 for 6 h as previously described [33]. In a second study, SD rats were exposed by IH to combusted #5 residual oil and freshly generated $PM_{2.5}$ #5 ROFA using whole-body chambers at a concentration of 1.3 mg/m^3 for 4 h.

In Vitro ROFA Exposures

All in vitro studies were conducted with bulk-collected $PM_{2.5}$ ROFA, as described previously [13, 20, 25, 34]. RCM cultures were exposed to various concentrations of a particle-free leachate of ROFA (ROFA-L). Briefly, a 5 mg/mL (w/v) stock suspension of ROFA was prepared in sterile saline and mixed for 10 min at room temperature. The suspension was centrifuged at 17,000×*g* in an Eppendorf microfuge. The recovered leachate was filtered through a 0.2-μm PETE filter (Poretics Products). Aliquots of 5 mg/mL ROFA-L were added to RCM cultures to yield final concentrations of 1.56, 3.13, 6.25, 12.5, and 25 μg/mL. These particle-free aliquots represent dilutions of the original suspension. ROFA-neutralized leachate supernatant (ROFA-NLS) was generated as previously described [20]. Briefly, ROFA-L solution was neutralized to pH 6.0 with 1 N NaOH. The solution was then centrifuged at 17,000×*g* for 15 min, as described above, and the resulting supernatant was used for metal analysis and RCM culture exposures. Cultures were exposed to saline (pH 6.0) or surrogate metal solutions at concentrations equivalent to 12.5 μg/mL ROFA-L containing V, nickel (Ni), and iron (Fe) or V + Ni + Fe (8.2, 11.5, and 7 μM, respectively). These surrogate metal solutions were prepared as previously described [20]. RCMs were treated with genistein, a tyrosine kinase inhibitor [35], at 25 μM, while control RCMs received daidzein, an inactive analog of genistein [35], also at 25 μM. Stock solutions of both genistein and daidzein were made in cell culture grade DMSO (Sigma), and the final DMSO concentration in the culture media did not exceed 0.1 %. This concentration of genistein 25 μM has been shown by other laboratories to confer tyrosine kinase inhibition without conferring antioxidant properties [36] seen at higher genistein concentrations, ≥100 μM [37]. Dimethylthiourea (DMTU, Sigma, St. Louis, MO) was made in sterile ddH_2O and diluted to 28 mM. Daidzein, genistein, and DMTU were added to the wells 30 min prior to ROFA-L exposure.

Plasma Metal and Cytokine Analysis

The metal contents of H#6 ROFA, #5ROFA, ROFA-L, and ROFA-NLS were determined using a model P40 inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Perkin Elmer, Shelton, CT) as described previously [20, 38]. Plasma was taken from abdominal aorta of SD and SHRs at 0.25, 0.5, 1, 3, 6, 12, and 24 h following IT dosing with ROFA using sodium citrate (Sigma, St. Louis, MO) as the anticoagulant. Plasma metal content was determined by diluting the plasma samples with 0.025 M HCl immediately prior to ICP-AES analysis [38]. For H#6 ROFA and #5 ROFA IH studies, plasma was isolated from SD rats immediately following exposure and samples were diluted with 0.2 % nitric acid. H#6 ROFA and #5 ROFA plasma metal concentrations were determined using inductively coupled plasma-mass spectrometry (ICP-MS) [39, 40].

Plasma was collected following either IT- or IH-exposed animals at 0, 3, 6, 12, and 24 h post-exposure to ROFA samples. Control plasma was obtained from rats at corresponding time intervals following exposure to saline for IT exposure or air for IH studies. Plasma cytokine levels were determined using ELISA kits for IL-6, IL-10, IL-1β, and TNFα (Biosource) according to manufacturer's directions.

Cardiomyocyte Cell Culture

Rat cardiomyocytes were obtained from 1-day-old neonatal rat pups using the neonatal RCM isolation kit (Worthington) with modifications as previously described [34]. The quality of RCM cultures was assessed by immunohistochemistry for cardiac-specific α-sarcomeric actin, fibroblast-specific vimentin, and through visual inspection of cellular beating and morphology as previously described [34]. RCM cultures were 73.4 ± 8.8 % ($N = 3$) negative for vimentin, 80.2 ± 9.8 % ($N = 5$) positive with cardiac α-sarcomeric actin, and 84.3 ± 8.8 % ($N = 30$) RCM by functional and morphological assessment (i.e., beating, refraction), suggesting that majority of the cell population was cardiomyocyte.

Cytotoxicity and Apoptosis Analysis

Cytotoxicity was determined by release of the enzyme lactate dehydrogenase (LDH) into the media following exposure to saline or ROFA-L. A 1-mL aliquot of media was taken following exposure and stored at 4 °C until analyzed. RCMs were then rinsed with ice-cold 1× PBS. Cells were lysed with 1 mL of ice-cold 1× PBS containing 0.8 % Triton X-100 on ice, then scraped, and collected into microcentrifuge tubes. Lysates were mixed at 4 °C for 15 min. Media and cell lysates were centrifuged at 17,000×*g* for 10 min to remove debris, and an aliquot was immediately taken for the measurement of LDH activity. LDH enzyme activity was determined using kit 228 from Sigma and adapted for use on the COBAS FARA II (Roche, Indianapolis, IN) or a KoneLab 30 (Finland). Cytotoxicity was reported as percentage release of total LDH = [(media LDH content)/(media + lysate LDH content)] × 100 %. Total cell numbers were determined by trypsinizing the cells with 0.05 % trypsin/EDTA (Invitrogen) for 15 min at 37 °C. The cells were resuspended in 1× PBS and 0.4 % trypan blue in saline. Live and dead cells were counted on a hemacytometer and expressed as total viable cells/well.

After a 16-h exposure to ROFA-L or staurosporine, cultures were evaluated for apoptosis using either the Quick Apoptosis DNA Ladder Detection Kit (Biovision Research Products) or the CardioTACS: In situ apoptosis detection kit system (Trevigen Inc.). Procedures were performed as described by the manufacturer with the following modifications to the Quick Apoptosis DNA Ladder Detection Kit. After precipitation of DNA by addition of ammonium acetate and absolute ethanol at −20 °C, the longer strands of genomic DNA were removed by winding them around the tip of a sterile glass Pasteur pipette. This was done to cut down on the viscosity of the DNA solution to improve loading of the sample for analysis by agarose gel electrophoresis. DNA was visualized on a 1 % agarose gel post-stained with Sybr Gold (Molecular Probes) at 1:5,000 for 45 min. Additionally, we determined apoptosis via the Cell Death Detection ELISA (Roche). Samples were isolated as per manufacturers' protocol, and data are represented as normalized absorbance ($A = A_{405} - A_{490}$) values.

Data Statistical Analysis

Cytotoxicity data were analyzed using one-way analysis of variance (ANOVA) with a Newman–Keuls post hoc test. Plasma metal concentration data and cell death detection ELISA data were analyzed using a two-way ANOVA with a Newman–Keuls post hoc test (SigmaStat ver 3.5). Results with a p value <0.05 were considered significant. Cardiomyocyte immunohistochemical data are presented as the average of the total number of runs ± the standard deviation.

Results

Vascular Biokinetics of ROFA Constituents Following Pulmonary Deposition

In order to perform relevant cardiomyocyte in vitro toxicity testing of oil combustion particle bioavailable constituents, it was necessary to determine their fate following their

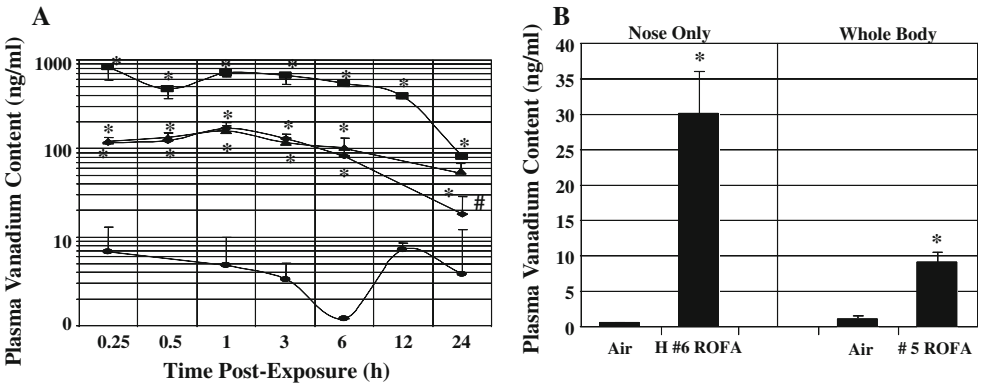

Fig. 1 Vascular in vivo biokinetics of pulmonary-deposited ROFA-associated metal. Plasma V content (ng/mL) following pulmonary exposure to ROFA. **a** IT of ROFA at 2.5 mg/rat (*square*) ($N = 2$ per group at 0.25–1 h post-exposure; $N = 3$ per group at 3–24 h post-exposure), 0.5 mg/rat SD (*diamond*) ($N = 3$–4 per group), and 0.5 mg/rat SHR (*triangle*) ($N = 3$–4 per group). Control animals were given saline (*circle*), 0.3 mL/rat, by IT ($N = 3$–4 per group). Maximal plasma V levels corresponding to 833 ng V/mL at 15 min and 168 ng V/mL at 1 h were observed following exposure to the high and low ROFA doses given to SD rats, respectively. Plasma levels of V remain elevated in SHRs exposed to 0.5 mg/rat up to 24 h. $^{*}p < 0.01$, significant difference between saline and ROFA-L. $^{\#}p < 0.05$ significant difference between ROFA 0.5 mg/rat in SH and SD (0.25–1-h samples were combined for the generation of a p value). **b** Plasma V levels in rats immediately following either a 6-h nose-only IH exposure to H#6 ROFA at 9 mg/m^3 or 4-h whole-body exposure to #5 ROFA at 1.3 mg/m^3. $^{*}p < 0.05$ significant difference between air and ROFA ($N = 3$–4 per group)

pulmonary deposition at doses that have been reported in the literature to alter cardiac and vascular function in rats [12, 13, 31, 32]. Therefore, rats were exposed to various ROFAs by either IT or IH, and plasma levels of V, a marker associated with oil combustion particle exposure, were monitored at various post-exposure times. With IT exposure, plasma V levels were found to increase in healthy and cardiac-compromised SHRs at both ROFA doses as early as 15 min post-exposure and remained elevated over the next 6 h before decreasing toward control saline levels by 24 h post-exposure (Fig. 1a). Interestingly, plasma levels of V remained elevated up to 24 h in the cardiac-compromised SHRs exposed to 0.5 mg/rat and were significantly different than in the healthy normal SD rats given the same dose at 24 h post-exposure (Fig. 1a). Maximal concentrations of plasma V were found to be 833 ng/mL at 15 min post-exposure for the 2.5 mg/rat ROFA dose, 169 ng/mL of V at 1 h post-exposure for the 0.5 mg/SD rat ROFA dose, and 157 ng/mL of V at 1 h post-exposure for the 0.5 mg/SHR ROFA dose. These levels of V in the plasma of ROFA-exposed rats corresponded to 16.3 μM V, or 25 μg/mL ROFA-L, and 3.3 μM V, or 4.8 μg/mL ROFA-L, for the high and low ROFA doses, respectively, based on the water-soluble V content for this ROFA [13].

Plasma V content was also identified following either nose-only or whole-body IH exposure to either resuspended $PM_{2.5}$ H#6 ROFA or freshly generated fly ash derived from the combustion of high-sulfur residual oil number 5 (#5ROFA), respectively. Plasma V levels were significantly elevated immediately following a 6-h nose-only IH exposure to H#6 ROFA at a concentration of 9 mg/m^3 (Fig. 1b). This exposure produced a concentration of 30 ng V/mL in plasma which corresponded to a plasma V concentration of 0.6 μM V and 1.2 μg/mL of H#6 ROFA-L since H#6 ROFA was found to contain 25 μg of water-soluble V/mg of fly ash. Plasma V levels were also elevated immediately following a 4-h whole-body IH exposure of rats to freshly generated aerosol of ROFA generated from the combustion of #5 residual oil at a concentration of 1.3 mg/m^3 (Fig. 1b). This IH exposure produced a concentration of 9 ng V/mL in plasma which corresponded to 0.2 μM V and 0.4 μg/mL of #5 ROFA-L since #5 ROFA was found to contain 24 μg of water-soluble V/mg of fly ash.

Pulmonary Deposition of ROFA did not Induce Systemic Inflammation

Cytokine levels were examined in plasma recovered from rats intratracheally instilled with either saline or ROFA in order to determine the ability of pulmonary-deposited ROFA to induce systemic inflammation. IT of either 2.5 mg/rat or 0.5 mg/rat ROFA did not cause any changes in the levels of IL-1β, IL-10, IL-6, and TNF-α within the plasma recovered from ROFA-exposed rats at 0.25, 0.5, 1, 3, 6, 12, and 24 h post-exposure (data not shown, $N = 4$–5 per time point). Plasma cytokine levels were also measured for rats immediately following whole-body IH exposure to freshly generated aerosol of #5 ROFA. Plasma levels of IL-1β, IL-10, IL-6, and TNF-α were not altered following whole-body IH exposure to #5 ROFA aerosols (data not shown).

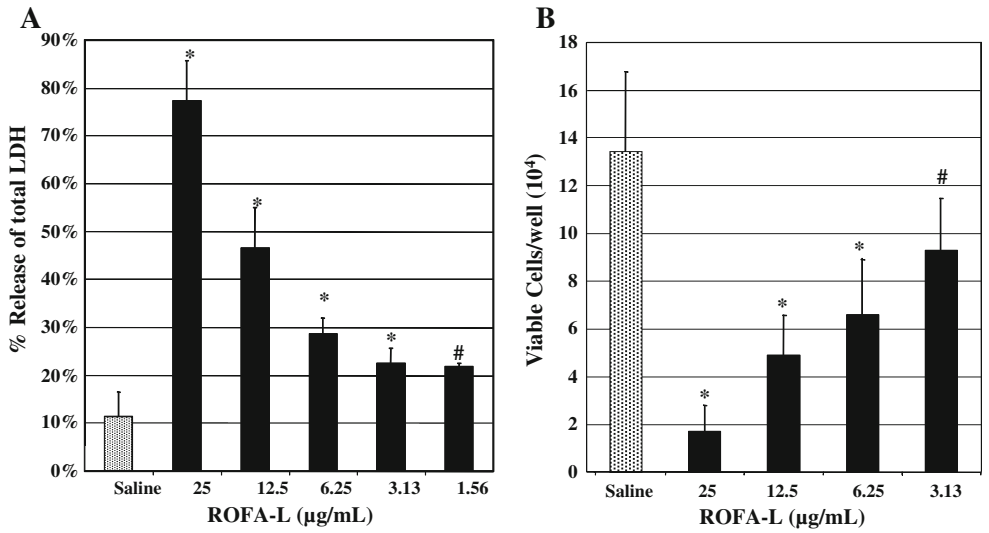

Fig. 2 Cardiotoxicity of ROFA bioavailable constituents. RCM was exposed to various doses of ROFA-L ranging from 25 to 1.56 μg/mL and examined for cytotoxicity at 24-h exposure. Cardiomyocyte cytotoxicity was assessed by either: **a** percentage release of total cellular LDH or **b** total number of viable cells per well. $^*p < 0.01$, significant difference between saline and ROFA-L, $^\#p < 0.09$, and between saline and 1.56 μg/mL ROFA-L ($N = 2$–17 per group, derived from 3 to 6 replicated cultures)

Table 1 ROFA sample metal and sulfate concentrations

Element	ROFA-L	ROFA-NLS	ROFA-neutralized pellet
Sulfate	2,483,266	2,408,262	45,462
Zn	3,753	0	3,204
Pb	1,354	690	1,039
Ni	155,254	67	132,960
Mn	1,871	12	1,637
Fe	67,798	0	58,460
Mg	288,790	200,784	79,005
V	163,745	23,220	99,622
Al	7,970	585	6,687
Ca	49,482	44,469	3,497
Cu	917	0	696

ICP-AES analysis for metal and sulfate from ROFA leachate (L), normalized leachate supernatant (NLS), and neutralized pellet derived from 5 mg/mL ROFA. ROFA-L, ROFA-NLS, and ROFA-neutralized pellet derivation is described in the methods. Percentage recovery of metal from ROFA-NLS and ROFA-neutralized pellet ranged between 74 % for Cu and 97 % for Mg. Metal concentrations are in ng/mL

ROFA Cardiomyocyte Cytotoxicity and Hazard Identification

Rat cardiomyocyte cytotoxicity studies using ROFA-L were conducted at the high dose of 25 μg/mL since rat plasma levels following IT exposure to 2.5 mg/rat ROFA for V correlate with this concentration of ROFA-L. The greatest release of LDH into the culture media observed after 24 h was 77.4 % at the 25 μg/mL dose (Fig. 2a). Significant RCM cytotoxicity response to ROFA-L was detected at the lowest concentration of 1.56 μg/mL ROFA-L with 21.9 % LDH release (Fig. 2a). ROFA-L effect on RCM viable cell number followed the same trend as LDH for the dose range of 25–3.13 μg/mL when examined at 24 h post-exposure (Fig. 2b). Removal of the major metal constituents of ROFA V, Ni, Fe, Zn, and Cu (Table 1) via neutralization was found to completely inhibit the cytotoxic response to ROFA-L at both the 25 and 12.5 μg/mL concentrations (Fig. 3). However, direct exposure of RCM cultures to V, Ni, or Fe either individually or as a three-metal mixture equivalent to 12.5 μg/mL ROFA-L produced no significant increase in cytotoxicity as measured by percentage of total LDH release (Fig. 3).

Mechanisms of ROFA-L-induced Cardiotoxicity

Mechanistic studies were conducted in order to determine to what extent molecular signaling and oxidative stress play a role in RCM cytotoxicity induced by ROFA-L. DMTU pretreatment was found to significantly inhibit ROFA-L-induced RCM cytotoxicity at high, 12.5 μg/mL,

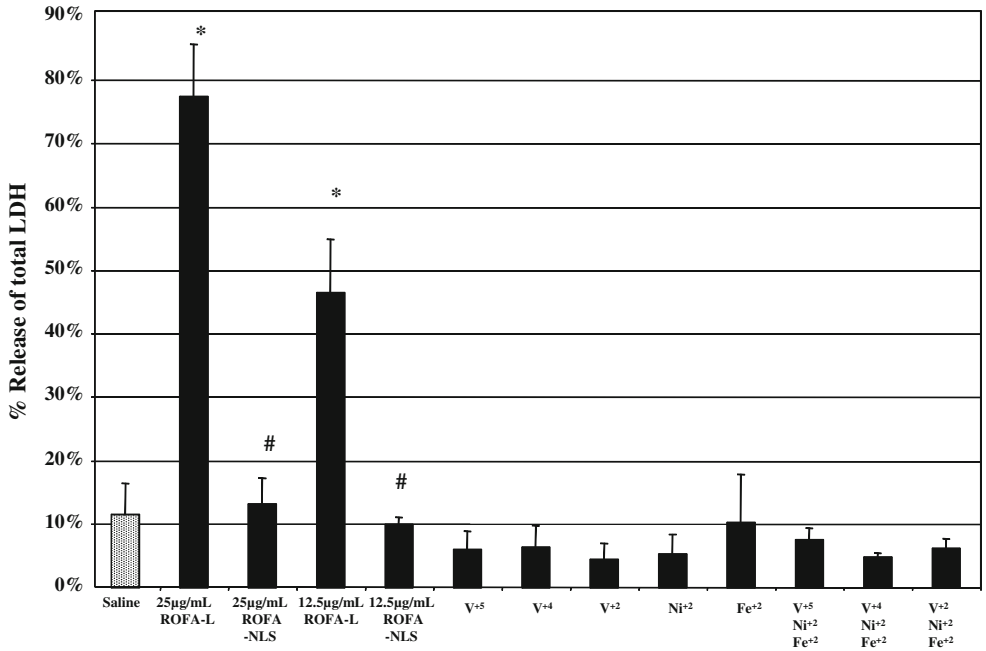

Fig. 3 Identification of causal cytotoxic ROFA bioavailable constituents. RCM was exposed to either various "forms" of ROFA, such as ROFA-L and ROFA-NLS (25 and 12.5 μg/mL), or surrogate metals and metal mixtures equivalent to a dose of 12.5 μg/mL ROFA-L and cytotoxicity examined 24 h post-exposure in order to identify constituents responsible for ROFA-L-induced cytotoxicity. $^{*}p < 0.01$, significant difference between saline and ROFA-L ($N = 2$–17 per group, derived from 3 to 6 replicated cultures), $^{\#}p < 0.01$, significant difference between ROFA-L (data from Fig. 2) and ROFA-NLS ($N = 3$–6 per group, derived from 3 to 6 replicated cultures)

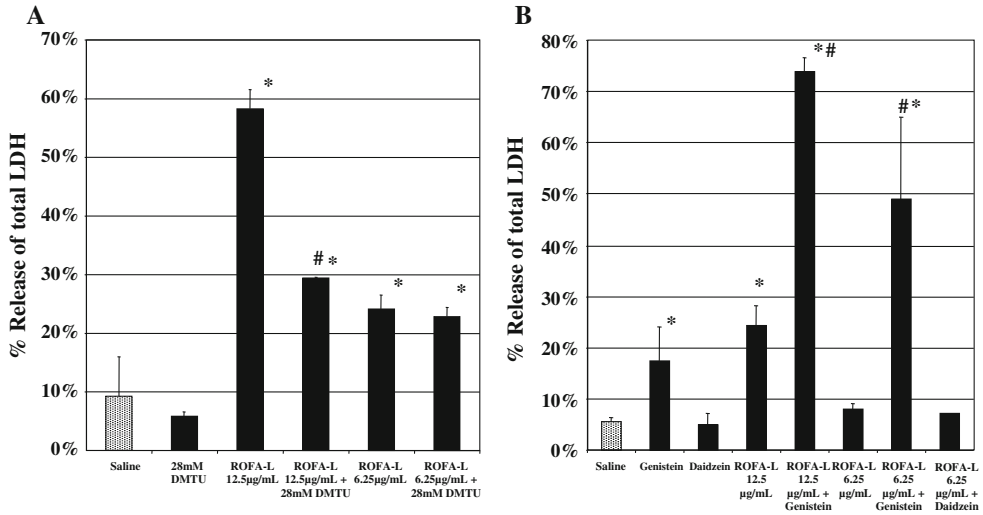

Fig. 4 Role of oxidative stress and intracellular signaling in ROFA-L-induced cardiotoxicity. **a** RCMs were pretreated with DMTU (28 mM) and exposed to high (12.5 μg/mL) and low (6.25 μg/mL) doses of ROFA-L. Cardiomyocyte cytotoxicity was measured by percentage release of total LDH at 24 h post-exposure. DMTU pretreatment of RCM was found to significantly attenuate ROFA-L-induced cardiomyocyte cytotoxicity at the high but not the low dose of ROFA-L. $^{*}p < 0.05$, significant difference between saline and ROFA-L; $^{\#}p < 0.05$, significant difference between ROFA-L + DMTU and ROFA-L ($N = 2$–7 per group, derived from 1 to 3 replicated cultures). **b** RCMs were pretreated with 25 μM genistein prior to exposure to ROFA-L at 12.5 and 6.25 μg/mL in order to determine the role of phosphotyrosine-mediated cell signaling in ROFA-L cardiotoxicity. Cardiomyocyte cytotoxicity was measured by percentage release of total LDH at 24 h post-exposure. Genistein pretreatment was found to enhance ROFA-L-induced cardiotoxicity. Pretreatment of RCMs with 25 μM daidzein prior to exposure to 6.25 μg/mL ROFA-L had no effect. $^{*}p < 0.05$, significant difference between saline and ROFA-L, and saline and ROFA-L + genistein; $^{\#}p < 0.05$, significant difference between ROFA-L + genistein and ROFA-L ($N = 2$–9 per group, derived from 1 to 3 replicated cultures)

but not low, 6.25 μg/mL, dose (Fig. 4a). The resulting reduction in fold LDH release in the 12.5 μg/mL group compared to control went from 5.6 to 2.0 with no change in the 6.25 μg/mL group. Treatment of RCMs with genistein, a phosphotyrosine kinase inhibitor, was found to dramatically enhance, in a synergistic manner, the cardiotoxicity associated with exposure to ROFA bioavailable constituents (Fig. 4b). The resulting increase in fold LDH release following genistein treatment in the 12.5 μg/mL group compared to control went from 5.2 to 14. Daidzein, the inactive analog of genistein, was found not to affect ROFA-L-induced cardiomyocyte cytotoxicity (Fig. 4b).

Rat cardiomyocyte exposed to ROFA-L at 25, 12.5, and 6.25 μg/mL for 16 h was analyzed qualitatively for evidence of apoptosis by DNA fragmentation or laddering. There was no increase in DNA laddering intensity or size in any ROFA-L-exposed RCM cultures relative to saline-exposed RCM cultures (data not shown). Additional evaluation of apoptosis was performed using a cardiac-specific in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, CardioTACS. RCM cultures were exposed to saline, staurosporine, and ROFA-L at 25 and 12.5 μg/mL, and at 16 h CardioTACS was performed (Fig. 5). Nuclei positive for apoptosis stained blue, and a pink background stain was included for contrast. Control saline exposure (Fig. 5a) was slightly positive for some apoptotic cell death. The positive control, staurosporine, was ~100 % positive for apoptotic nuclei (Fig. 5b). However, RCM exposure to ROFA-L at concentrations of 25 and 12.5 μg/mL showed no increases in apoptotic cells as indicated by the lack of blue nuclei (Fig. 5c, d). These data were further confirmed in Fig. 6. The positive control sorbitol (0.3 M) had enhanced absorbance compared to the negative control; however, none of the ROFA-L concentrations at any time point were statistically significant from control (Fig. 6).

Discussion

Cardiovascular effects of combustion source particulates have been attributed to a wide variety of fuel sources and exposure concentrations [41–44]. Furthermore, a variety of mechanisms have been proposed to explain the link between pulmonary PM exposure and cardiovascular toxicity [13, 14, 17–22]. However, the direct effect of PM and PM constituents on the heart following their pulmonary deposition is currently unclear. In this study, PM constituent concentrations were established biokinetically via plasma V concentrations following pulmonary exposure to ROFA (IT or IH). This ROFA constituent concentration (i.e., ROFA-L) was the benchmark for which to identify the

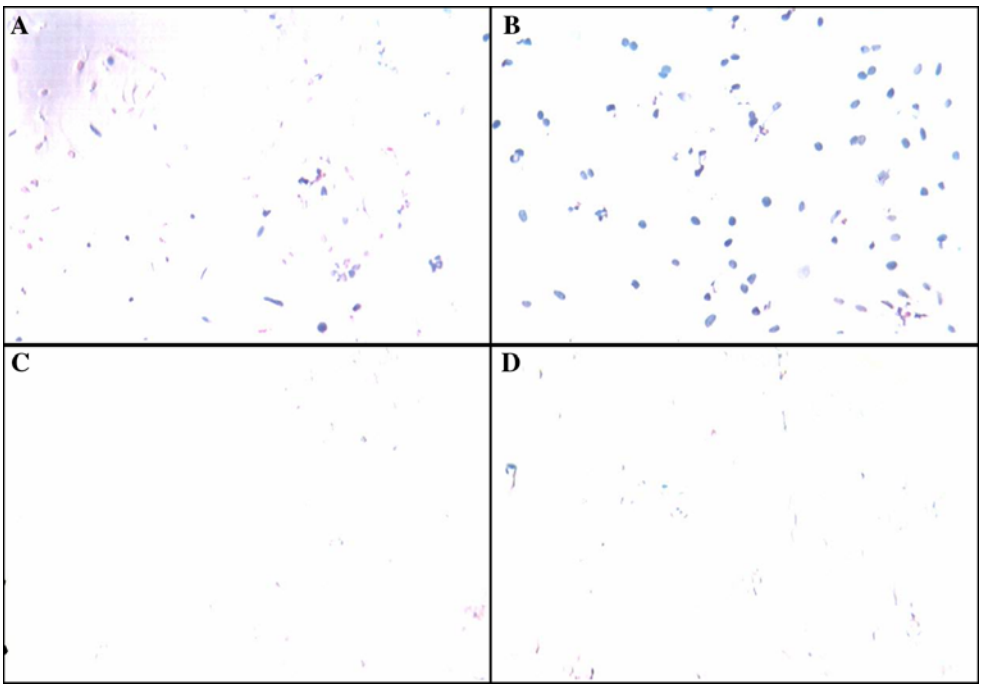

Fig. 5 Mechanism of ROFA-L-induced RCM cytotoxicity. As described in the "Materials and Methods" section, an in situ TUNEL assay was used to detect nuclear DNA fragmentation, a hallmark of apoptosis. Cells were stained with nuclear fast red giving the nucleus a red color; then DNA fragmentation was identified by in situ TUNEL and stained blue. **a** Saline-treated cells (16 h). **b** A positive control for apoptosis, RCM cells treated with staurosporine and indicated by ~100 % of the cells staining blue. **c** and **d** ROFA-L exposures at 12.5 and 25 μg/mL, respectively (16 h)

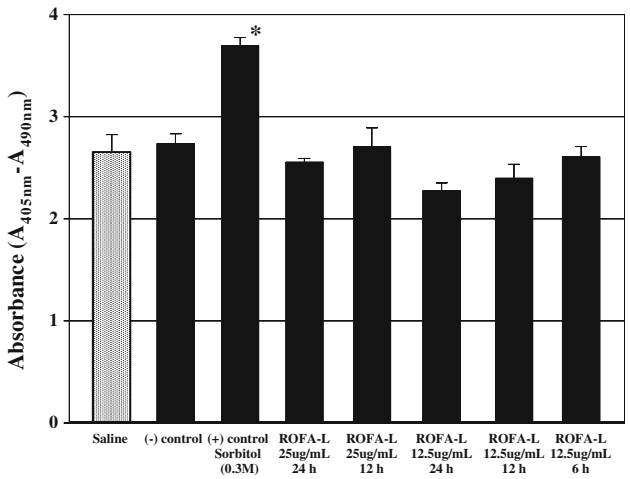

Fig. 6 ROFA-L-induced RCM cytotoxicity. Cells were treated with the concentrations of ROFA-L at 12.5 and 25 μg/mL for 24, 12, and 6 h. Sorbitol induced a significant apoptotic signal, as indicated by an increase in absorbance. However, ROFA-L at any dose or time point failed to elicit an increase in absorbance, suggesting a non-apoptotic cell death mechanism. *$p < 0.05$ significant difference between saline and sorbitol ($N = 3$ per group from 1 culture)

toxicity of circulating ROFA constituents in vitro. The corresponding cardiotoxicity of ROFA-L after 24 h was dose dependent, necrotic, exacerbated by inhibition of phosphotyrosine kinases, inhibited by antioxidant at high doses and could not be recapitulated with the major metal constituents (V, Ni, and Fe) of ROFA-L.

The biokinetics of ROFA exposure for both IT and IH demonstrated a clear translocation of soluble V into the circulation that begins to wane at ~12 h following exposure. The enhanced circulating V following ROFA exposure is consistent with previous reports in boilermakers following work cleaning an oil-fired boiler [45]. Wallenborn et al. [46] demonstrated similar results in WKY rats for HP-12, also a high transition metal content ROFA. In their study, heart tissue vanadium concentration was ~65 ng V/g of tissue with a plasma V concentration of ~325 ng V/g of plasma (~330 ng V/mL). Based on these numbers, a heart tissue concentration would be one-fifth of plasma concentration or ~162 ng V/mL based on 833 ng V/mL maximal concentration. The diluted concentration of ROFA-L would correspond to our midrange dose of ~6.25 μg/mL ROFA-L, well within the dose range employed in this study. However, it is not known whether SD rats and WKY animals have equivalent clearance mechanisms for ROFA-associated metals nor can it be assumed that the speciation and solubility of the metals associated with ROFA used in this study and HP-12 are the same. Interestingly, the V plasma concentration in SHRs showed a rapid rise, similar to that observed in healthy SD rats, with a steady concentration that continued to be

elevated out to 24 h, suggesting a lack of clearance in this cardiac-compromised animal model. This reduction in clearance of plasma V in the SHRs increases the duration of exposure, which may increase toxicity. This alteration in plasma V biokinetics could be due to increased volume retention in SHR model or a lack of clearance mechanisms due to impaired kidney function [47].

Cytokine production following exposure to PM is considered as one hypothesis to explain extrapulmonary effects of PM exposure. In several studies, investigators have found altered cytokine levels in the plasma of animals and humans following exposure to PM [19, 48]. However, in our study we did not find elevated levels of cytokines (IL-6, IL-10, IL-1β, and TNFα) in the plasma of rats following pulmonary exposure to ROFA at any time point or exposure level. Previous work with ROFA demonstrated a clear increase in plasma fibrinogen levels associated with IT exposure [49]. Furthermore, there is a clear production of cytokines in the lung following exposure [50]. However, our findings indicate that the systemic effects of ROFA are not related to induction of cytokines in the lung, which yield systemic effects but rather soluble metals that result in direct interactions with extrapulmonary tissues and their cells.

In this study, the ROFA pulmonary exposures employed were directly correlative to previous in vivo studies that were found to display pulmonary and cardiovascular toxicity or responses [12, 13, 22]. Therefore, the ROFA-L biokinetically-based in vitro cardiotoxicity reported in this study has a clear dose–response comparison with these in vivo studies. Using LDH as a biomarker, there is a clear and direct toxicity of soluble metals in the cardiomyocytes. However, we could not recapitulate the toxicity of ROFA-L with concentrations of its major metal constituents (V, Ni, and Fe). These results are consistent with previous work that demonstrated no cardiomyocyte cytotoxicity up to 50 μM with either Zn or V [51]. It is likely not the result of osmotic stress as all of the metal constituents combined would only amount to an approximate increase in osmolarity of 3.2 mOsm. Furthermore, any pH change induced by the metal constituents would be buffered by the cell culture medium as addition of the ROFA-L solutions only initially alters the phenol red indicator in the culture medium which was quickly restored to 7.4 upon placement into the incubator (personal observation). The subsequent neutralization of the ROFA-L likely mimics the in vivo particle deposition and dissolution. Hence, the observed cardiotoxicity of the soluble metal constituents of ROFA is likely the result of a minor metal constituent, the combination of a larger group of metal constituents, or the entire combination of soluble components of ROFA. This finding is in contrast to the pulmonary toxicity of ROFA, which can be recapitulated with a metal mixture of V, Fe, and Ni [20].

The cytotoxicity of ROFA-L was reduced with the addition of DMTU, an antioxidant known to scavenge both hydrogen peroxide and hydroxyl radical [52]. However, the reduction in cytotoxicity was not effective at 6.25 μg/mL of ROFA-L, which did not produce a statistically significant reduction in toxicity. This finding suggests that reactive oxidative stress may only be operative at high but not lower ROFA-L concentrations. Alternatively, the concentrations of metals may not be great enough to generate an oxidative stress response in the cell, but may induce alterations in ion channels essential to cellular survival. Alvarez-Collazo et al. [53] found that Zn levels necessary to alter ventricular cardiomyocyte L-type Ca^{2+} channels were much lower than levels that were needed for to induce redox cycling. The cytotoxicity induced at the lower concentration may be related to induction of gene expression associated with apoptosis or necrosis. Indeed, previous work from our laboratory demonstrated the induction of cell death pathways at 3.5 μg/mL of ROFA-L [34], although we did not directly test apoptosis at this level of ROFA-L exposure. In contrast to our previous transcriptomic profiling study that suggested apoptosis [34], our current study employing direct analysis suggests that ROFA-L-induced in vitro cardiomyocyte toxicity is mediated by necrosis [34]. Previous work by other laboratories focused on high-dose effects of individual metals or particle suspensions and ignored relating their findings to the effect of biokinetics on cardiomyocyte toxicity [51, 54–56]. Hence, the doses used in culture in these studies have no basis to what could be seen post-exposure to occupational or environmental exposure levels. In contrast, this study demonstrates that cardiomyocyte injury using plasma V concentrations derived from soluble metal content in the plasma of exposed rats occurs through different mechanisms depending on the dose of ROFA-L.

Tyrosine kinases are known to regulate growth and survival in cardiomyocytes [57]. Not surprisingly, the induction of in vitro cardiotoxicity could be augmented greatly with the inclusion of genistein, a soy isoflavonoid known to inhibit tyrosine kinase activity [35]. These data indicate a tyrosine-mediated survival pathway that confers protection of the cardiomyocytes during ROFA-L exposure, since the inactive analog of genistein, daidzein, did not increase cardiomyocyte toxicity. In genomic and proteomic studies, we showed that tyrosine kinase signaling through mitogen-activated protein kinases (MAPKs) was altered in the airway epithelium of animals exposed to ROFA [50]. Furthermore, downstream effector transcription factors regulated by MAPKs were activated in culture cardiomyocytes exposed to ROFA-L [34]. Additionally, vanadium has been shown to inhibit tyrosine phosphatases, leading to an increase in overall cellular protein tyrosine phosphorylation and cardioprotection [58]. However, V

inhibition of tyrosine phosphatases may require high concentrations of V, as shown by Samet et al. [59] where ROFA concentrations >50 μg/mL were required to generate statistically significant increases in protein phosphotyrosine levels in human epithelial airway cells. In addition, given that genistein treatment greatly enhanced the cytotoxicity of ROFA-L, V-induced phosphatase inhibition does not play a role in ROFA-L-induced RCM cytotoxicity.

In summary, we have presented results from the first study describing the in vitro cardiotoxicity of bioavailable components of ROFA particles using concentrations based on pulmonary deposition of this point-source particulate air pollutant (biokinetically-based). Pulmonary deposition of ROFA particles produced the same level of rapid and prolonged translocation of metals into the vasculature in either healthy or hypertensive rats. However, levels of bioavailable metals remain at higher levels longer in cardiac-compromised hypertensive rats, possibly making them more susceptible to ROFA bioavailable constituents. Unlike previous pulmonary toxicity findings, our results provide strong evidence supporting direct cardiomyocyte toxicity by a more complete mixture of ROFA bioavailable constituents being mediated by non-apoptotic and non-ROS mechanisms regulated by key tyrosine phosphorylation pathway(s). Finally, this in vitro work provides evidence that ROFA-L has direct effects on cardiomyocytes, supporting this as a mechanism of PM-associated systemic injury.

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