

ABSTRACT

Knuckles, Travis Lee. *In Vitro* Cardiotoxicity of Residual Oil Fly Ash. (Under the direction of Kenneth B. Adler and Kevin L. Dreher). Epidemiological studies have shown an association between air pollution particulate matter (PM) and adverse cardiovascular effects. Although numerous mechanisms have been proposed, the actual mechanism(s), as well as emission sources and associated causal properties by which PM affects the cardiovascular system, remain elusive. At least some adverse PM health effects can be attributed to bioavailable constituents, most notably the transition metal content of the particles. Toxicological studies in rats using residual oil fly ash (ROFA) combustion source particles show extrapulmonary effects ranging from thermoregulatory alterations to fatal cardiac arrhythmias. Exposure of rats to ROFA via intratracheal instillation lead to a rapid and transient increase in plasma metal content as early as 15 minutes post-exposure, suggesting that PM constituents are bioavailable to both the systemic circulation and perfused organs. However, the impact of this systemic exposure on extrapulmonary organs at the cellular and molecular levels is unknown. In this study, cardiomyocytes derived from 1-day-old rat pups were exposed in order to determine the direct effects of a particle-free residual oil fly ash leachate (ROFA-L). Using concentrations of leachates relevant to amounts that were found in the plasma of rats following pulmonary deposition, this study has shown that ROFA bioavailable constituents induce cytotoxicity in cardiomyocyte cultures in a dose response relationship from 25µg/mL to 1.56µg/mL of ROFA-L. The cardiomyocyte cytotoxicity due to ROFA-L exposure was not found to be mediated by the major metal constituents nor was the cytotoxicity oxidant dependant at lower doses. The cytotoxic effects of ROFA-L

were enhanced with the addition of a tyrosine kinase inhibitor, genistein. Acute non-cytotoxic doses of ROFA-L altered global gene expression consistent with cardiac myocyte electrophysiological remodeling, oxidative stress, and cell survival. Genomic alterations were found to correlate with changes in transcription factor activation. These acute changes in both transcription factor activation and gene expression could lead to possible chronic consequences for the cardiac myocyte.

***In Vitro* Cardiotoxicity of Residual Oil Fly Ash.**

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A dissertation submitted to the Graduate Faculty
of North Carolina State University
in partial fulfillment of the requirements for the
Degree of Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh

2005

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ACKNOWLEDGMENTS

A special acknowledgment is extended to my advisor Dr. Kevin L. Dreher for his advice and direction throughout my Ph.D. studies at North Carolina State University. His support, guidance, and patience are greatly appreciated.

I would also like to thank the scientists part of the Pulmonary Toxicology Branch at the USEPA for their continued support throughout my graduate endeavors.

Many thanks to my graduate committee members for their assistance during my graduate career.

I would also like to thank my wife Holly for her support, love and encouragement during these past five years.

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LIST OF ABBREVIATIONS

#5 ROFA-high sulfur #5 residual oil fly ash

ACS-American Cancer Society

ApoE-apolipoprotein E

BC-bradycardia

bp-base pair

BrdU-bromo-deoxy-uridine

CAPs-concentrated ambient fine particles

CIMT-carotid artery intima-media thickness

DMTU-dimethylthiourea

EHC-93-Ottawa dust sample

H#6 ROFA-Hickory #6 residual oil fly ash

HRV-heart rate variability

ICD- implanted cardiodefibrillator

ICP-AES-inductively coupled plasma-atomic emission spectrometry

ICP-MS-inductively coupled plasma-mass spectrometry

IGF-1-insulin-like growth factor-1

IH-inhalation

IL-2-interlukin-2

IT-intratracheal instillation

KO-knockout

LDH-lactate dehydrogenase

mins-minutes

NAAQS-National Ambient Air Quality Standards

PBS-phosphate buffered saline

PI3/AKT-phospho-inositol triphosphate/protein kinase B

PM_{10-2.5}-PM between 10-2.5µm is diameter (Coarse)

PM₁₀-PM <10µm is diameter (Thoracic)

PM_{2.5}-PM <2.5µm is diameter (Fine)

PM-air pollution particulate matter

QC-quality control

RCM-rat neonatal cardiomyocyte

ROFA-L- residual oil fly ash-leachate

ROFA-NLS- residual oil fly ash neutralized leachate supernatant

ROFA-residual oil fly ash

SD-Sprague-Dawley

SH-spontaneously hypertensive

TBE-tris-borate-EDTA

TC-tachycardia

TGF-β-transforming growth factor-β

TSP-total suspended particulates

TUNEL-terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

VEGF-vascular endothelial growth factor

VF-ventricular fibrillation

WKY-Wistar-Kyoto

INTRODUCTION

Ambient Particulate Air Pollution:

Air pollution particulate matter (PM) is minute solid particles or liquid droplets suspended in ambient air. PM is formed through various natural and anthropogenic processes yielding a physicochemically diverse and dynamic mixture. Ambient air PM contains three “modes” or size ranges of ultrafine, accumulation and coarse particles. Ultrafine particles are formed by nucleation and condensation processes and have a diameter of less than $0.1\mu\text{m}$. Accumulation mode particles occur in a range between $0.1\mu\text{m}$ and $2.5\mu\text{m}$ in diameter and are formed by accumulation and coagulation of smaller particles. Collectively, the ultrafine and accumulation modes of PM are referred to as fine-mode particles and PM with a diameter less than $2.5\mu\text{m}$ in diameter is termed $\text{PM}_{2.5}$. $\text{PM}_{2.5}$ particles are generally created by combustion of fossil fuels and secondary atmospheric transformation processes. Mechanical and natural generation of particles generally leads to larger PM of

between $2.5\mu\text{m}$ to $100\mu\text{m}$ in diameter, referred to as coarse-mode PM, and the range of particles between $2.5\mu\text{m}$ to $10\mu\text{m}$ is known as coarse PM ($\text{PM}_{10-2.5}$). All PM $<10\mu\text{m}$ in diameter is known as PM_{10} or thoracic particles (for their ability

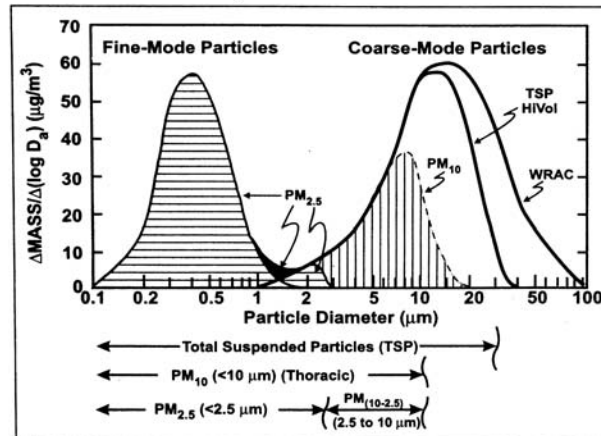


Figure 1: An idealized graph of relative amounts of particles on a mass per volume basis overlaid with various sampler penetration curves (Wilson and Suh 1997).

to penetrate the thoracic cavity). Particles separated into the fine and coarse modes are fundamentally different in their chemical speciation and formation. Formation of fine

mode particles from condensation/coagulation prevents these particles from becoming much larger than $1\mu\text{m}$ in diameter and makes these particles chemically different from coarse mode particles formed through mechanical processes. Whitby (1978) said it best:

“The distinction between fine particles and coarse particles is a fundamental one. There is now an overwhelming amount of evidence that not only are the two modes in the mass or volume distribution usually observed, but that these fine and coarse modes are usually chemically quite different. The physical separation of the fine and coarse modes originates because condensation produces fine particles while mechanical processes produce mostly coarse particles...the dynamics of fine particle growth ordinarily operate to prevent the fine particles from growing larger than about $1\mu\text{m}$. Thus, the fine and coarse modes originate separately, are transformed separately, are removed separately, and are usually chemically quite different... Thus the distinction between fine and coarse fractions is of fundamental importance to any discussion of aerosol physics, chemistry, measurement, or aerosol air quality standards.”

Table 1 compares fine mode and coarse mode particles over a wide range of physical and chemical properties.

Table 1: Comparison of PM by Mode.			
	Fine		
	Ultrafine	Accumulation	Coarse
Formation Processes:	Combustion, high-temperature processes, and atmospheric reactions		Break-up of large solids/droplets
Formed by:	Nucleation Condensation Coagulation	Condensation Coagulation Reactions of gases in or on particles Evaporation of fog and cloud droplets in which gases have dissolved and reacted	Mechanical disruption (crushing, grinding, abrasion of surfaces) Evaporation of sprays Suspension of dusts Reactions of gases in or on particles
Composed of:	Sulfate Elemental carbon Metal compounds Organic compounds with very low saturation vapor pressure at ambient temperature	Sulfate, nitrate, ammonium, and hydrogen ions Elemental carbon Large variety of organic compounds Metals: compounds of Pb, Cd, V, Ni, Cu, Zn, Mn, Fe, etc. Particle-bound water	Suspended soil or street dust Fly ash from uncontrolled combustion of coal, oil, and wood Nitrates/chlorides/sulfates from HNO ₃ /HCl/SO ₂ reactions with coarse particles Oxides of crustal elements (Si, Al, Ti, Fe) CaCO ₃ , CaSO ₄ , NaCl, sea salt Pollen, mold, fungal spores Plant and animal fragments Tire, brake pad, and road wear debris
Solubility:	Probably Less Soluble than accumulation mode	Largely soluble, hygroscopic, and deliquescent	Largely insoluble and nonhygroscopic
Sources:	Combustion Atmospheric transformation of SO ₂ and some organic compounds High temperature processes Combustion of coal, oil, gasoline, diesel fuel, wood	Atmospheric transformation products of NO _x , SO ₂ , and organic compounds, including biogenic organic species (e.g., terpenes) High-temperature processes, smelters, steel mills, etc.	Resuspension of industrial dust and soil tracked onto roads and streets Suspension from disturbed soil (e.g., farming, mining, unpaved roads) Construction and demolition Uncontrolled coal and oil combustion Ocean spray Biological sources
Atmospheric half-life:	Minutes to hours	Days to weeks	Minutes to hours
Removal Processes:	Grows into accumulation mode	Diffuses to raindrops Forms cloud droplets and rains out	Dry deposition Dry deposition by fallout Scavenging by falling rain drops
Travel distance:	< 1 to 10s of km	100s to 1000s of km	< 1 to 10s of km (small size tail, 100s to 1000s in dust storms)

(Source: US EPA 2004)

Regulation of PM by size on a mass basis began in 1971 with the NAAQS for total suspended particulates (TSP). These standards were later revised in 1987 to use PM₁₀ rather than TSP (Federal Register 1987) (Table 1). Ten years later the NAAQS were revised again to include PM <2.5µm (PM_{2.5}) in diameter based on epidemiological data using PM_{2.5} as an exposure index (Federal Register 1997). A number of legal challenges were instituted against the US EPA in regard to the PM_{2.5} standards. The PM_{2.5} standards were vacated in 1999 until a final ruling in March 2002 by the D.C. Court of Appeals in which the court ruled that the PM_{2.5} standards were not “arbitrary or capricious.” (American Trucking Associations v. EPA, 283 F. 3d 355, 369-72 [D.C. Cir. 2002, US EPA2004])(Table 2).

Table 2: PM Regulation			
Size	Year Instituted	24h Average (µg/m ³) ¹	Annual Average (µg/m ³)
TSP	1971	260	75 ¹
PM ₁₀	1987	150	50 ²
PM _{2.5}	1997 ³	65	15 ²

¹, cannot be exceed any time during the year
², based on three year average
³, Legality of PM_{2.5} standards was challenged in 1998, standards were vacated until final D.C. Court of Appeals ruling in 2002 (Source: D.C. Cir. 2002, US EPA 2004).

PM: A Public Health Risk

Particulate Matter Historical Perspectives:

The first major air pollution incident reported was in 1930 in the Meuse Valley of Belgium. During the episode, which lasted from December 1, 1930 to December 5, 1930, 60 people died and hundreds more fell ill after a weather inversion trapped emissions from local glassworks, steel works, fertilizer, and explosives plants, leading to a thick fog that settled in the valley. The deaths were directly correlated with exposure to the high particle concentrations in the air and not to infectious disease (Nemery *et al.* 2001). Later, in the United States in 1948 the town of Donora, PA was also hit by a strong weather inversion that trapped pollution from local metal works, coal-fired home and industrial facilities, coke ovens, a zinc retort refinery, and iron and steel industries. During the episode, which began on October 27, 1948 and ended on October 30, 1948, 17 people died and 3 more died within a week, which is equivalent to six times the normal death rate for Donora, PA. However, these events pale in comparison to the air pollution related disaster that occurred in 1952 in London, England. London experienced a similar incident of a dense smog that blanketed the city from December 5th to the December 9th but due to the high population density thousands died from exposure to high PM concentrations. Estimates put the excess death toll at around 12,000 resulting from PM levels approximately five times greater than current United States NAAQS for total suspended particulates (US NAAQS for TSP $260\mu\text{g}/\text{m}^3$, London fog TSP $1400\mu\text{g}/\text{m}^3$)(Bell and Davis 2001). Such events demonstrated the impact which pollution had on public health and led, ultimately, to the creation of the United States

Environmental Protection Agency, and air quality standards for gaseous air pollution and PM.

PM Epidemiology, Clinical and Panel Studies:

This dissertation will focus solely on the role of PM in exacerbation and death due to cardiovascular ailments. However, a large body of data exist for other effects of PM, most notably respiratory effects, which are beyond the scope of this work. Information on PM effects can be found in the Air Quality Criteria Document for Particulate Matter (United States Environmental Protection Agency, 2004).

Until recently, PM was not considered a public health risk after implementation and compliance to NAAQS standards until 1993 when Dockery and colleagues published the six-city study (Dockery *et al.* 1993). In this study, Dockery *et al.* associated cardiopulmonary mortality to PM_{2.5} concentrations at or below the current NAAQS standards. These associations between

PM_{2.5} and mortality were not geographically limited or gender biased and had a positive dose-response relationship. The six-city study was later confirmed by the larger American Cancer Society (ACS) study by Pope *et al.* in 1995. Figure 2 shows adjusted mortality over fine particle concentration

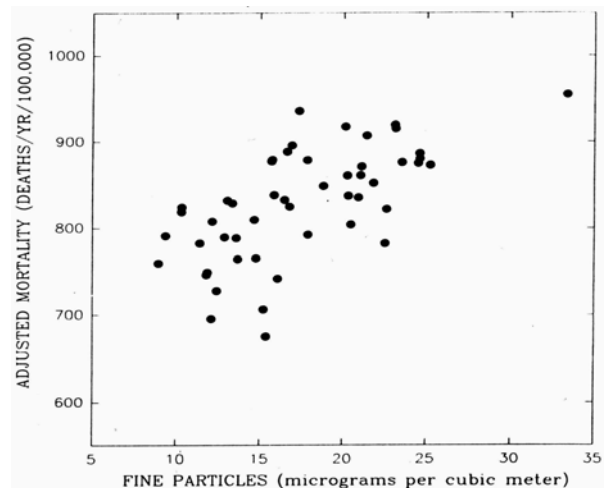


Figure 2: Adjusted mortality vs. fine particle concentration ($\mu\text{g}/\text{m}^3$). Dots represent data from metropolitan areas used in analysis (source: Pope *et al.* 1995)

in a dose dependant manner. Dots on the graph depicted in Figure 2 represent the metropolitan areas used in the study. The results of the six-city study and the ACS study

have been consistently supported by other studies as well as reanalysis of the data (Krewski *et al.* 2003, Englert 2004).

Many epidemiological studies are now focused on physiological markers for cardiac function. One such marker is autonomic nervous system control of the heart through sympathetic and parasympathetic neural stimulation. Autonomic nervous system alterations, as they pertain to particulate matter, have been well documented in the epidemiology literature (Peters *et al.* 1999, Liao *et al.* 1999, Pope *et al.* 1999, Peters *et al.* 2000, Gold *et al.* 2000, and Magari *et al.* 2001). Most of these studies have shown decreases in HRV after exposure to PM indicating an increased risk of cardiovascular related morbidity and mortality. HRV is reviewed in Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology 1996.

A study authored by Peters *et al.* in 2000 looked at 100 patient implanted cardioverter defibrillator (ICD) records. These records were used to monitor ventricular tachycardias and fibrillations where a discharge from the ICD was precipitated. Focusing in on patients with 10 or more discharges from the ICD, a positive association between PM_{2.5} or PM₁₀ and ICD intervention was seen with a 2 day lag. The authors speculated that a possible cause of the ventricular tachycardias and fibrillations were the soluble mediators produced in the lung making their way into the systemic circulation to exert their effects on the heart.

In another study by the same group in 2001, elevated levels of Black Smoke, PM_{2.5} and PM₁₀ were associated with induction of myocardial infarctions (MI). In this study 108 patient interviews were used to approximate the time at which the MI occurred.

Using this data and the daily air pollution measurements collected by the Harvard School of Public Health, they were able to correlate PM concentration with MI induction. Unlike the previous study, by Peters *et al.*, the functional cardiac effect, in this case MI, was linked to elevated PM levels after 2 and 24 hours. The authors suggested several causes for the post-exposure MI such as increases in plasma viscosity, increases in c-reactive protein and cytokine production/systemic distribution.

In 2005 a cohort study by Künzli *et al.* associated long term exposure to PM_{2.5} with atherosclerosis development. The cohort was made up of 798 individuals from two panel studies examining the effects of vitamin E or B supplementation and atherosclerosis progression. Prior to vitamin supplementation baseline measurements of carotid artery intima-media thickness (CIMT) were obtained by ultrasound of the right common carotid artery. CIMT is a well established measurement of atherosclerosis and correlates well with coronary artery atherosclerosis. PM_{2.5} concentrations were modeled for the greater Los Angeles metropolitan area based on PM monitor data from 23 sites. Increases in CIMT were found to correlate with an increase of 10µg/m³ of PM_{2.5}. This study was the first to link atherosclerosis development in humans with long term PM_{2.5} exposure.

A study by Brook *et al.* in 2002 showed that human subjects exposed to concentrated ambient fine particles (CAPs) and ozone (O₃) concurrently had a reduction in arterial diameter. The 24 subjects included in the study were given two randomized two hour exposures to either filtered air + very low or CAPs of 150µg/m³ +120ppb O₃ on two separate days. An average significant reduction in brachial artery diameter of 0.09mm was measured after exposure to CAPs and ozone when compared to control. Of

the 24 subjects, 18 (75%) of them responded to CAPs + O₃ exposure via vasoconstriction. The mechanism of vasoconstriction was proposed to be either sympathetic nervous system alterations, or systemic inflammation resulting from cytokine release in the lung.

Support for systemic distribution of cytokines can be identified in human clinical and panel studies. Many studies have shown pulmonary inflammation following exposure of an individual to PM (Salvi *et al.* 1999, Ghio *et al.* 2001, Nightingale *et al.* 2000). PM systemic human vascular effects include the release of immature neutrophils into the circulation from the bone marrow (Mukae *et al.* 2001, Tan *et al.* 2000, Salvi *et al.* 1999) and increases in blood parameters such as viscosity (Riediker *et al.* 2004). However, few studies show measurable cytokine amounts in blood post-exposure to PM (van Eeden and Hogg 2002, Gong *et al.* 2003).

Another possible cause of PM related cardiovascular morbidity and mortality is direct interactions between ultrafine particles and tissues of the heart and circulatory system. Nemmar and colleagues in 2002 showed that ultrafine particles could translocate from the lung to the circulation in humans (Nemmar *et al.* 2002a). Five healthy male non-smoking volunteers inhaled 100MBq of Technegas (5-10nm carbon black particles labeled with ^{99m}Tc). Whole body imaging was performed on the subjects and % of total lung radioactivity was calculated. After 45 minutes ~25% of the total lung radioactivity was found in the liver. An important consideration is that radioactivity in the blood was due to ^{99m}Tc bound to particles and not free ^{99m}Tc.

Source Specific PM Effects:

Mortality has been shown to be associated with PM over a broad range of geographical locations through a dose response relationship without being biased by gender. Re-evaluation of the data from the six-city study using factor analysis has associated specific sources of PM_{2.5} to increases in mortality. These sources include motor vehicle, coal, and residual oil but not particles derived from the earth's crust (Figure 3) (Laden *et al.* 2000). Tsai *et al.* (2000) also linked specific components of

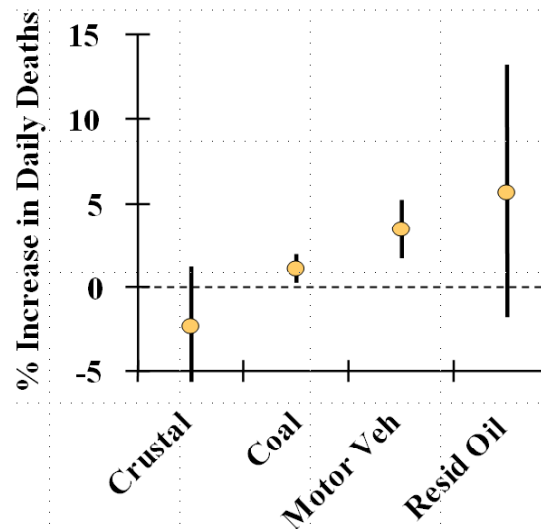


Figure 3: Reanalysis of the six-city study using factor analysis for source specific mortality. Graph displays percent increase in daily deaths per 10µg/m³ increase in PM_{2.5} (Laden *et al.* 2000).

PM_{2.5} to increases in mortality through factor analysis. Statistical associations were shown between cardio-respiratory daily deaths and motor vehicle, industrial, residual oil, and sulfate aerosol sources. In Dublin, Ireland, a ban on the sale of coal decreased the overall PM concentration by 70%. Percentages of non-trauma daily deaths decreased by 5.7% and deaths due to cardiovascular disease also declined, significantly, by 10.3% after the ban (Clancy *et al.* 2004). These studies associate combustion source particulate matter to increased risk of death.

PM Mortality Risk Factors

It has been well established that PM adversely affects the cardiovascular system. Mortality associated with PM exposure has been related to pre-existing disease including chronic lung disease, coronary artery disease, heart failure and diabetes (Goldberg *et al.*

2001a, b, Zanobetti and Schwartz 2001, Hoek *et al.* 2002, Pope *et al.* 2004b). However, it is not known how PM increases risk of death through interaction with these diseases.

PM Toxicology

With the establishment of robust relationships between PM and cardiovascular effects, many investigators have turned to animal models as a means to describe, mechanistically, how PM affects the heart and circulatory system after exposure. Many different sources as well as exposure techniques have been employed to elucidate PM effects. Outlined below are the effects and conclusions drawn from studies utilizing, ultrafine particles (PM <0.1 μ m in diameter), ambient PM (i.e. PM collected or concentrated prior to exposure) or source specific PM (e.g. residual oil, diesel, coal) following pulmonary exposure, with emphasis on cardiovascular effects.

Ultrafine Particle Translocation:

Ultrafine particle translocation has been hypothesized as one possible mechanism for the cardiovascular effects due to PM exposure. Nemmar and colleagues (2002b) exposed hamsters to a radioactive 80nm in diameter particle Nanocoll (^{99m}Tc labeled albumin colloid) via intratracheal instillation and detectable radioactivity was found in the heart, liver, kidney, spleen and brain. Using an approximation of the volume of blood in the hamster the authors estimated the total amount of radioactive particles that translocated to be 25-30% (Nemmar *et al.* 2002b). Semmler *et al.* (2004) found that ¹⁹²Ir labeled ultrafine Ir particles (15-20nm) translocated from the lung into secondary organs (i.e. liver, spleen, kidney, and brain) immediately after inhalation exposure in Wistar-Kyoto rats. However, a much smaller percentage (0.2%) of ¹⁹²Ir labeled ultrafine Ir particles was found to translocate into the circulatory system compared to Nemmar *et al.* 2002(b). These data suggest that ultrafine particles can translocate into the circulatory system and interact with perfused organs, tissues, as well as plasma proteins.

Ambient PM Cardiovascular Effects:

In 2002, Suwa *et al.* exposed hyperlipidemic rabbits to Ottawa dust (EHC-93) via intrapharyngeal instillation at 5mg/rabbit, 2-times/week for five weeks. The investigators found that EHC-93 increased progression of atherosclerotic plaques and decreased plaque stability. These results were corroborated, in part, by Chen and Nadziejko 2005. In their study, a double knockout mouse model deficient for apolipoprotein E (ApoE^{-/-}) was exposed to concentrated ambient fine particles (CAPs) at an average of 110 $\mu\text{g}/\text{m}^3$, 6h/day, 5days/week for five months by inhalation. These ApoE^{-/-} mice, which develop atherosclerosis while being fed a high lipid diet, had increased total area of atherosclerotic plaques following exposure to CAPs. Other vascular effects of ambient PM in animals include increases in circulating vasoconstrictors endothelin 1 and 3 following inhalation of EHC-93 by Wistar rats (Vincent *et al.* 2001), rat pulmonary artery vasoconstriction associated with CAPs silicon (Si) levels (Batalha *et al.* 2002), and increases in peripheral neutrophils in dogs (Clarke *et al.* 2000). These data indicate that ambient PM can alter progression of disease in animal models as well as induce systemic inflammatory effects.

Source Specific PM Cardiovascular Effects:

Various sources have been shown to play a role in cardiovascular mortality associated with PM. Combustion sources of oil, coal, and motor vehicle have been associated with increased risk of death. However the mechanism through which these sources exert their effects is unknown. A great deal of work has focused on the oil combustion source particle residual oil fly ash (ROFA).

In 1997 Costa and Dreher studied the effects of various sources of PM and discovered that particle composition played a role in the extent of lung injury induced. They also demonstrated that instillation of ROFA could increase mortality in a rat model of pulmonary hypertension. These data were consistent with others studies (Gavett *et al.* 1997, Watkinson *et al.* 1998, and Dye *et al.* 2001).

ROFA has also been shown to be arrhythmogenic in a study conducted by Watkinson *et al.* in 1998. In this study healthy and pulmonary hypertensive rats were given a low (0.25mg/rat), medium (1.0mg/rat) and high dose (2.5mg/rat) of ROFA via intratracheal instillation (IT). Arrhythmias were seen in the healthy animals but were greatly exacerbated with pre-existing pulmonary hypertension. This response has been consistently shown in other studies using the model of pulmonary hypertension and ROFA exposure (Muggenburg *et al.* 2000, Killingsworth *et al.* 1997). Also, Wichers *et al.* in 2004 showed increased arrhythmias in spontaneously hypertensive animals following exposure to a low metal content ROFA. In 2003, Kodavanti *et al.* exposed normal WKY rats to a high zinc ROFA particle via nose-only inhalation for 16 weeks at 6h/day, 1day/week exhibited necrotic lesion development throughout the ventricles and within the interventricular septum.

Gardner *et al.* 2000 showed systemic effects following ROFA exposure. In this study healthy rats were exposed to 0.3, 1.7, or 8.3mg/kg of ROFA via IT. Results indicated an increase in plasma fibrinogen in animals 24 hours post-exposure to the highest, 8.3mg/kg, ROFA dose. No other alterations in hematological parameters were detected (white blood cell count, red blood cell count, hemoglobin, hematocrit, and platelet count).

PM Mechanisms of Cardiovascular Injury

A number of mechanisms have been proposed by which PM affects the cardiovascular system (Donaldson *et al.* 2001, Uttell *et al.* 2002, Brook *et al.* 2004)

(Figure 4). The autonomic nervous system may be stimulated following an air pollution event in susceptible subpopulations. This change in autonomic stimulation to the heart results in changes in HRV (Chan *et al.* 2004, Pope *et al.* 2004a, Park *et al.* 2005).

Another mechanism is through

translocation of ultrafine particles into the circulation. This has been shown possible in controlled exposures to a radioactive particle in both humans and animals (Nemmar *et al.* 2002a, b, Oberdorster *et al.* 2004, Semmler *et al.* 2004). Ultrafine particles can then act directly on the circulatory and/or organ systems. Lung inflammation and cytokine production have been shown in exposures to particles in both animal and human models. It is believed that cytokine production in the lung could lead to a systemic inflammatory response that could then increase blood coagulability and decrease atherosclerotic plaque stability (van Eeden and Hogg 2002, Suwa *et al.* 2002). Finally, soluble constituents on particles could enter the circulatory system eliciting a systemic inflammatory response or direct cardiovascular effects (Costa and Dreher 1997, Kodavanti *et al.* 2003, Neurkowicz

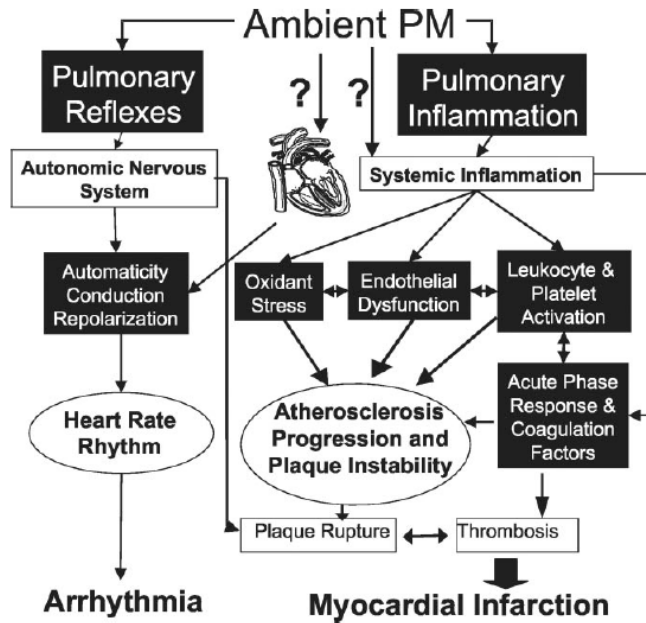


Figure 4: Diagrammatic representation of the mechanisms of PM cardiovascular effects (Source: Brook *et al.* 2004).

et al. 2004). However, the actual mechanism(s), as well as specific sources and associated causal properties by which PM affects the cardiovascular system, remain elusive. Therefore, my dissertation will focus on elucidating what mechanisms by which soluble constituents of PM affect the heart.

Experimental Model

Based on the purposed mechanisms of PM cardiovascular effects, I formulated the following hypotheses: 1) Bioavailable constituents of air pollution particles directly affect the cardiovascular system to produce its adverse health effects; 2) Sensitivity

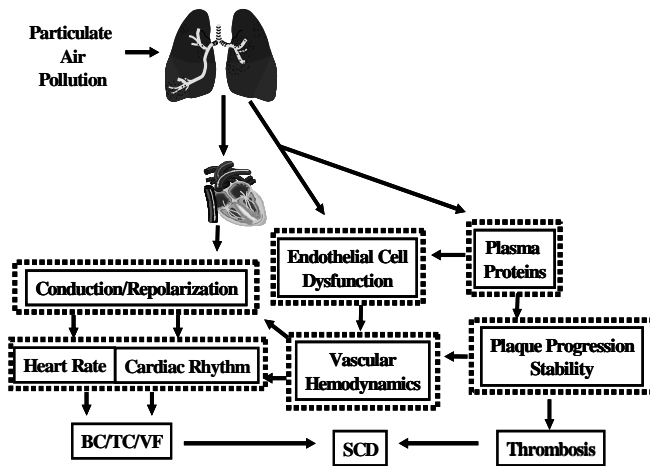


Figure 5: Mechanisms by which PM and its constituents directly affect the heart and vascular systems. Dashed boxes represent sensitivity factors that may play a role in PM induced cardiovascular effects.

factors play a critical role in modifying the adverse health effects of particulate air pollution within susceptible sub-populations. These hypotheses are outlined in Figure 5. To test these hypotheses, I used isolated rat neonatal cardiomyocytes.

For cardiac research, isolated hearts and cultured cardiomyocytes are the

most widely used models for drug toxicity studies, conduction/repolarization, and myocyte signaling in normal and disease states. Rat neonates supply an undemanding and easy procedure for isolation versus the adult heart and allow for longer term culturing (Chlopickova *et al.* 2001). Neonatal heart culture preps are not sensitive to calcium in the isolation buffer unlike adult isolations (also referred to as calcium paradox, see reference Ray *et al.* 2000 for more information). Rat neonatal cardiomyocytes also produce a very stable and sustainable phenotype that is comparable to *in situ* whole heart preparations. This is in contrast to adult cardiomyocyte cultures, which are phenotypically different than *in situ* whole heart preparations and have a limited period of use of about 24-48h after isolation (Yamashita *et al.* 1994). Experiments performed with

rat neonatal cardiomyocytes include hypoxia reperfusion, contractile force measurements, cell morphology, and cellular ionic exchange, among others (Chlopikova *et al.* 2001).

The isolated cardiomyocyte model has been used for over 40 years and much of what is known about basic cardiomyocyte structure and function, as well as signaling pathways in normal and disease states, was elucidated with cultured cardiomyocyte preparations (Harary and Farley 1963, Chlopikova *et al.* 2001). Gene expression, signaling pathways, and death have been studied extensively in isolated cardiac myocytes (reviewed in Chien *et al.* 1991, Musters *et al.* 1991, Klein *et al.* 1993, Hunter and Chien 1999, Jalili *et al.* 1999, Nicol *et al.* 2000, Bueno and Molkentin 2002, Naga Prasad *et al.* 2002, Williams and Rosenberg 2002, Gardner 2003, McGregor and Dunn 2003, Ravingerová *et al.* 2003, Pakkarainen *et al.* 2004).

Hypertrophy is considered the beginning of a downward spiral leading to cardiomyopathy and heart failure. Gene expression hallmarks of pathological hypertrophy are increased expression of fetal cardiac genes (e.g. myosin light chain-2, β -myosin heavy chain, skeletal α -actin), modulators of hypertrophy (natriuretic factors) and immediate early genes (e.g. c-fos, c-myc, egr-1) (Chien *et al.* 1991, Nicol *et al.* 2000). Cultured cardiomyocytes can be exploited, depending on the model system, to measure these gene expression hallmarks. This model system is the only way to directly assess the cellular and molecular effects of bioavailable source specific constituents of PM on cardiac myocytes.

Summary

Current epidemiological literature has associated adverse health consequences with elevated PM levels in susceptible subpopulations. PM is formed from various anthropogenic and natural sources leading to three distinct size fractions; ultrafine, accumulation and coarse. PM size modes are physicochemically diverse and produce varied biological effects impacting the respiratory and cardiovascular systems. Numerous mechanisms have been proposed by which PM affects the cardiovascular system, however the actual mechanism(s) and PM sources have yet to be elucidated. One hypothesized mechanism of PM cardiovascular effects is deposition and dissolution of PM in the lung leading to increased plasma levels of PM constituents. These constituents then impact perfused organs and tissues. The direct effects that particle constituents have on cellular and molecular processes are not known. The rat neonatal cardiomyocyte culture system offers significant advantages over *in vivo* and *ex vivo* (i.e. whole heart perfusions) models allowing the investigator to identify altered cellular function and perturbed/activated molecular pathways. The cultured cardiomyocyte system affords great flexibility to study a wide range of genomic, proteomic and physiological questions. With these ideas in mind, this study was undertaken to increase the knowledge base regarding the direct effect of PM constituents on the heart and to further substantiate epidemiological data linking PM to cardiovascular toxicology. To accomplish this task, I exposed an *in vitro* model of cardiac myocytes to bioavailable particle-free leachates derived from residual oil fly ash (ROFA-L). Using concentrations of leachates relevant to amounts that were found in the plasma of rats following pulmonary deposition, I have shown that ROFA bioavailable constituents induce cardiomyocyte cytotoxicity in a dose

response relationship from 25µg/mL to 1.56µg/mL of ROFA-L. The cardiomyocyte cytotoxicity due to ROFA-L exposure was not found to be mediated by the major metal constituents nor was the cytotoxicity oxidant dependant at lower doses. The cytotoxic effects of ROFA-L could be enhanced with the addition of a tyrosine kinase inhibitor, genistein. Acute non-cytotoxic doses of ROFA-L altered global gene expression consistent with cardiac myocyte electrophysiological remodeling, oxidative stress, and cell survival. Genomic alterations were found to correlate with changes in transcription factors.

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Chapter 1

TITLE: *In Vitro* Cardiotoxicity of Air Pollution Particles: Identification of Causal Constituents and Mechanisms of Injury.

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RUNNING TITLE: Residual Oil Fly Ash Cardiotoxicity

To be submitted to: *Cardiovascular Toxicology*

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ABSTRACT:

Identifying causal particulate emission sources and their mechanism(s) of action are critically needed in order to provide biological plausibility to adverse health effects associated with ambient air particulate matter (PM) exposure as well as to begin to understand the impact of air pollution on the cardiovascular system. This research examines the cardiotoxicity of bioavailable constituents of oil combustion particles. Pulmonary deposition of residual oil fly ash (ROFA) led to a rapid/transient increase in plasma vanadium (V) levels in the absence of any systemic inflammation. However, plasma V levels remained elevated for a significantly longer time period within cardiac compromised animals when compared to healthy animals. ROFA cardiotoxicity was evaluated using 1 day old rat cardiomyocyte (RCM) cultures exposed to particle-free leachates of ROFA (ROFA-L) at levels present in rat plasma that were found following pulmonary deposition. Significant cardiotoxicity was observed at low levels (3.13 μ g/mL) of ROFA-L at 24h post-exposure. Dimethylthiourea (28mM) inhibited ROFA-L-induced cytotoxicity at high (25-12.5 μ g/mL) but not low (3.13-6.25 μ g/mL) doses. Cardiotoxicity could not be reproduced using a V+Ni+Fe mixture, or ROFA-L depleted of these metals. Tyrosine phosphorylation inhibition by genistein (25 μ M) increased the susceptibility of RCMs to ROFA-L-induced cytotoxicity. Our studies demonstrate that oxidative stress plays a major role in ROFA-L-induced cardiotoxicity only at high doses. ROFA-L cardiotoxicity was not due to its major metal constituents. Phosphotyrosine cell signaling pathways play a critical role in regulating the susceptibility of cardiomyocytes to ROFA-L induced cellular injury. These data

demonstrate that bioavailable constituents of specific emission particles PM are capable of direct cardiac effects.

Keywords: ROFA, cardiac injury, PM, oxidative stress, cardiac toxicity

INTRODUCTION:

Current scientific literature addressing particulate matter effects has shown an increased risk of morbidity and mortality associated with exposure to ambient levels of PM. In 1993 Dockery *et al.* 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 (Pope 2000). Measured effects from ambient PM exposures in epidemiological and clinical studies include; triggering of myocardial infarction, increases in prothrombotic factors, increases in numbers of peripheral neutrophils, increases in circulating cytokine levels, decreases in heart rate variability, decreases in vasodilation, and increases in atherosclerotic plaque progression (Peters *et al.* 2000a,b, 2001, van Eeden and Hogg 2002, Brook *et al.* 2004, and Künzli *et al.* 2004). The percent of excess deaths due to PM for cardiovascular disease has been estimated to be as high as 69% (Pope 2000).

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 (Dreher *et al.* 2000). PM of an aerodynamic diameter of less than $2.5\mu\text{m}$ ($\text{PM}_{2.5}$) has been shown to be of particular concern. The composition of $\text{PM}_{2.5}$, mainly from combustion sources, has been shown to preferentially increase the risk of death associated with PM exposure (Pope 2000, Laden *et al.* 2000). Components of combustion source particles have been shown, either through direct analysis of each element present in $\text{PM}_{2.5}$, or through factor analysis, to increase daily deaths (Burnett *et al.* 2000, Laden *et al.* 2000). However, hazard

identification of causal constituents of PM and source specific effects remain to be clarified.

In rats exposed to the combustion source particle residual oil fly ash (ROFA) has been linked to increases in mortality in pulmonary hypertensive animals (Costa and Dreher 1997, Watkinson *et al.* 1998, Killingsworth *et al.* 1997), necrotic lesions development in healthy animals exposed via inhalation (Kodavanti *et al.* 2003) and reduced a vasodilation response (Neurkowitz *et al.* 2004). These effects could be attributed to the transition metal contents of the particles.

Several mechanisms have been purposed as to how PM increases morbidity and mortality due to cardiovascular disease. These include pulmonary inflammation leading to an altered autonomic nervous system response (Peters *et al.* 2000b, Clarke *et al.* 1999), systemic inflammation leading to an abnormal vascular response (Brook *et al.* 2002), and direct effects on the cardiovascular system through dissolution of soluble “bioavailable” constituents following deposition in the lungs and subsequent transport by the vasculature (Costa and Dreher 1997, Dreher *et al.* 1997, Kodavanti *et al.* 2003).

In this study we attempt to explain that some of the cardiac effects due to air pollution particulate matter can be described through direct interaction between bioavailable constituents on particles and cardiac myocytes. We show that constituents from residual oil fly ash (ROFA) can be found in the plasma of animals as little as 15 mins post intratracheal instillation (IT) or inhalation (IH) exposure without any evidence of systemic inflammation. Back calculating to amounts of vanadium (V) present in the plasma, we were able to expose cultures of rat neonatal cardiomyocytes to relevant doses of particle-free leachates generated from ROFA (ROFA-L). The *in vitro* cardiotoxic

effects of ROFA-L were not mediated by the major metal constituents (V, Ni or Fe). We also show that the antioxidant status of the myocyte cultures can modulate the amount of cytotoxic effects of ROFA-L. At more environmentally/occupationally relevant doses of ROFA, oxidative stress plays less of a role in mediating *in vitro* cardiotoxicity. In addition, alterations of certain phosphotyrosine signaling pathways can increase susceptibility of myocytes to deleterious effects of PM constituents. These data indicate a possible role of combustion source PM constituents as a mediator of cardiovascular effects.

MATERIALS AND METHODS:

Animals:

Male, 65-70 day old, Sprague-Dawley (SD) rats were used for ROFA intratracheal instillation and inhalation exposures and male, 90 day old cardiac compromised spontaneously hypertensive (SH) rats were used for ROFA intratracheal instillation exposures. Female, 60-90 day old, gestation day 19, time pregnant Sprague Dawley rats were used to obtain 1 day-old rat neonates to generate rat neonatal cardiomyocyte (RCM) cultures. All animals were obtained from Charles River Laboratory, Raleigh NC and 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 $2.5\mu\text{m}$, referred to as $\text{PM}_{2.5}$) residual oil fly ash (ROFA) employed in intratracheal instillation and *in vitro* exposures was obtained from a power plant burning low sulfur #6 residual oil and physicochemically characterized as previously described (Hatch *et al.* 1985, Dreher *et al.* 1997, Costa and Dreher 1997). This ROFA contained $35.04\mu\text{g}$ of water-soluble vanadium (V)/mg ROFA. Bulk collected $\text{PM}_{2.5}$ Hickory #6 residual oil fly ash (H#6 ROFA) was obtained from the National Risk Management Research Laboratory, US Environmental Protection Agency (US EPA 1996b). Briefly, unemulsified Hickory #6 residual oil was combusted in a North American Package Boiler and $\text{PM}_{2.5}$ ROFA was collected using a $\text{PM}_{2.5}$ cyclone. Chemical characterization of

Hickory #6 ROFA has been previously published (US EPA 1996b). Hickory #6 ROFA contained 25.10µg of water-soluble V/mg H#6ROFA

ROFA and Metal Exposures:

In vivo ROFA Exposures: SD rats were exposed to saline or ROFA at 2.5 mg/0.3 mL/rat or at 0.5 mg/0.3 mL/rat and SH rats were exposed to ROFA at 0.5mg/0.3mL/rat, both by intratracheal instillation as previously described (Dreher *et al.* 1997). In two separate studies rats were exposed by inhalation to two different residual oil fly ash samples. In one case SD rats were exposed by nose only inhalation using a string generator to bulk collected PM_{2.5} H#6 ROFA reaerosolized at a concentration of 9mg/m³ for 6h as previously described (Kodavanti *et al.* 1999). In another study, SD rats were exposed in whole-body chambers by inhalation to freshly generated PM_{2.5} residual oil fly ash produced from the combustion of high sulfur #5 residual oil (#5 ROFA) at a concentration of 1.3mg/m³ for 4h. #5 ROFA was found to contain 24µg of water-soluble V/mg ROFA (Huffman *et al.* 2000).

In vitro ROFA Exposures: All *in vitro* studies were conducted with bulk collected PM_{2.5} ROFA, as described previously, (Hatch *et al.* 1985; Dreher *et al.* 1997; Costa and Dreher, 1997). RCM cultures were exposed to various concentrations of a particle-free leachate of ROFA (ROFA-L). Briefly, a 5mg/mL stock suspension of ROFA was prepared in sterile saline and mixed for 10 mins at room temperature. The suspension was centrifuged at 17,000xg in an eppendorf microfuge. The recovered leachate was filtered through a 0.2µm PETE filter (Poretics Products). Aliquots of the 5mg/mL ROFA-L were added to RCM cultures to yield final concentrations of 1.56, 3.13, 6.25, 12.5, and 25µg/mL. ROFA neutralized leachate supernatant (ROFA-NLS) generation was

previously described in Dreher *et al.* 1997. Briefly, ROFA-L solution was neutralized to pH 6.0 with 1N NaOH. The solution was then centrifuged at 17,000xg for 15 mins and the resulting supernatant was used for metal analysis and RCM culture exposures. Cultures were exposed to surrogate metal solutions at concentrations equivalent to 12.5µg/mL ROFA-L containing V, nickel (Ni), and iron (Fe) or V+Ni+Fe (8.2µM, 11.5µM, and 7µM, respectively). These surrogate metal solutions were prepared as previously described (Dreher *et al.* 1997). RCMs were treated with genistein, a tyrosine kinase inhibitor, (Akiyama *et al.* 1987) at 25µM, while control RCMs received daidzein, an inactive analog of genistein (Akiyama *et al.* 1987), also at 25µM. 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%. Dimethylthiourea (DMTU, Sigma, St. Louis MO) was made in sterile ddH₂O and diluted to 28mM. Daidzein, genistein, and DMTU were added to the wells 30 mins 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 (US EPA 1991, Dreher *et al.* 1997). Plasma was taken from SD and SH rats at 0.25, 0.5, 1, 3, 6, 12, and 24h following intratracheal instillation with ROFA using sodium citrate (Sigma, St. Louis, MO) as the anticoagulant. Plasma metal content was determined by diluting the plasma samples with 0.025M HCl immediately prior to ICP-AES analysis (US EPA 1991). For H#6 ROFA and #5 ROFA inhalation 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) (Vanhoe *et al.* 1989, US EPA 1998).

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

Cardiomyocyte Cell Culture and Cytotoxicity Analysis:

RCMs were obtained from 1-day-old neonatal rat pups using the neonatal rat cardiomyocyte isolation kit (Worthington) with modifications as follows. Minced heart tissue fragments were incubated overnight with trypsin in a HEPES simple salt solution (120mM NaCl, 3mM KCl, 1mM NaH₂PO₄, 25mM HEPES, 0.0001% Phenol Red, in sterile ddH₂O, pH 7.35). After trituration and centrifugation, cells were resuspended in high serum media [10% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100 μ g Streptomycin (Invitrogen), 0.1mM Bromo-deoxy-Uridine (BrdU)] and preplated onto a 150cm² tissue culture flask (Becton Dickenson) for two hours at 37°C, 1-2%CO₂, to remove most of the non-myocytes. After preplating, cardiomyocytes were recovered and total cell counts and viability were determined by trypan blue dye (0.4%, Sigma) exclusion using a hemacytometer. Alive cells were plated at a density of 170,000cells/cm² in 6-well, or 35mm laminin coated tissue culture plates (BD BioCoat, Becton Dickinson) in high

serum media. On days 1-3, cells were maintained in high serum media, changing media every 24 hours. On day 4 cells were placed into low serum media [1% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100 μ g Streptomycin (Invitrogen)] with BrdU. On day 5 of culturing, cells were maintained in low serum media without BrdU. On day 6 cells were given low serum media minus BrdU three hours prior to exposure.

The quality of RCM cultures was assessed by immunohistochemistry and visual inspection of cellular beating and morphology. Fibroblast specific anti-vimentin and cardiac specific anti- α -sarcomeric actin antibodies (Sigma) were used to determine the percent cardiomyocyte content of the cultures grown on 35mm laminin coated dishes. RCMs designated for immunohistochemistry with anti-vimentin antibody were fixed with 3% paraformaldehyde (10 mins, room temperature) (Fisher), rinsed 3 times with 1X PBS, then permeablized with 0.1% Triton X-100 (15 mins, room temperature)(Fisher). For immunohistochemistry corresponding to anti- α -sarcomeric actin, RCMs were fixed with 96%ethanol, 3%glacial acetic acid with no permeabilization. After fixation and permeabilization, both sets of cells were rinsed with 0.1%Tween 20 (Fisher)/1x phosphate buffered saline (PBS), v/v (Sigma). Cells were then treated with a blocking buffer of 5% (w/v) non-fat dry milk (Carnation) in 0.1%Tween 20/1xPBS for 30 mins, to block non-specific binding. RCMs were then allowed to react with primary antibodies against vimentin (dilution 1:100) or α -sarcomeric actin (dilution 1:100) in blocking buffer for 90 mins at room temperature. Cells were then rinsed with 0.1%Tween20/PBS three times. A horseradish peroxidase conjugated secondary antibody, to IgG (1:400 dilution, Sigma) for vimentin, or IgM (1:400 dilution, Sigma) for α -sarcomeric actin, was

used to label the bound primary antibody. Cells were rinsed with 0.1%Tween20/1xPBS three times and then visualized with 3'-3'-diaminobenzimide kit (Innogenex). Cells were then counter-stained with 1% methylene green (w/v, in 0.1%Tween20/PBS, Sigma) for 3 mins. Cells were then rinsed three times with ddH₂O. Four fields per dish at 400X magnification were counted for vimentin/ α -sarcomeric actin positive cells. RCM cultures were 73.4% +/- 8.8% (n=3) negative for vimentin, 80.2% +/-9.8% (n=5) positive with α -sarcomeric actin, and 84.3% +/-8.8% (n=30) RCM by functional and morphological assessment (i.e. beating, refraction), suggesting the majority of the cell population was cardiomyocyte.

Cytotoxicity was determined by release of the enzyme lactate dehydrogenase (LDH) into the media following exposure to saline or ROFA-L. A one mL aliquot of media was taken following exposure and stored at 4°C until analyzed. RCMs were then rinsed with ice cold 1X PBS. Cells were lysed with 1ml of ice cold 1X PBS containing 0.8% Triton X-100 on ice, then scraped and collected into microcentrifuge tubes. Lysates were mixed at 4°C for 15 mins. Media and cell lysates were centrifuged at 17,000xg for 10 mins to remove debris and an aliquot immediately taken for measurement of lactate dehydrogenase (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 percent release of total LDH = [(media LDH content)/(media + lysate LDH content)] X 100%. Total cell numbers were determined by trypsinizing the cells with 0.05% trypsin/EDTA (Invitrogen) for 15 mins at 37°C. The cells were resuspended in 1X PBS and trypan blue. The cells were counted on a hemacytometer and expressed as total viable cells/well.

Data Statistical Analysis:

Data were analyzed using student's t-test in Microsoft Excel for control vs. exposed using a two tail distribution and assuming equal variance. Results with a p value <0.05 were considered significant. Cardiomyocyte immunohistochemical data is presented as the average of the total number of runs \pm the standard deviation.

RESULTS:

Dissolution of ROFA Constituents Following Pulmonary Exposure:

In order to perform relevant cardiomyocyte *in vitro* toxicity testing of oil combustion particles it was necessary to determine the fate of oil combustion particle constituents following their pulmonary deposition at doses that have been reported in the literature to alter cardiac function in rats (Costa and Dreher 1997; Watkinson *et al.* 1998). Therefore, rats were exposed to various ROFAs by either intratracheal instillation or inhalation and the plasma levels of V, a marker associated with oil combustion particles exposure, were monitored at various post-exposure times. A very rapid and transient increase in plasma V levels were observed in rats exposed by intratracheal instillation (IT) to either 2.5 or 0.5mg of ROFA, Figure 1A. Plasma V levels were found to increase at both ROFA doses as early as 15 mins post-exposure and remained elevated over the next 6h before decreasing towards control saline levels by 24h post-exposure, Figure 1A. Plasma levels of V remained elevated up to 24 hr in the SH rats exposed to 0.5mg/rat and were significantly different than SD rats given the same dose at 24h post-exposure, Figure 1A. Maximal concentrations of plasma V were found to be 833ng/mL, at 15 mins post-exposure, for the 2.5mg/rat ROFA dose, 169ng/ml of V, at 1h post-exposure, for the 0.5mg/SD rat ROFA dose, and 157ng/mL of V, at 1h pos-exposure for the 0.5mg/SH rat ROFA dose. These levels of V in the plasma of ROFA exposed rats corresponded to 16.3 μ M V, or 25 μ g/ml ROFA-L, and 3.3 μ M V, or 4.8 μ g/ml ROFA-L, for the high and low ROFA doses, respectively, based on the water soluble V content for this ROFA (Costa and Dreher, 1997). Approximately, 70% of the V within the plasma of ROFA exposed rats could be removed by dialysis against physiological phosphate buffer

suggesting that V is not strongly associated with plasma proteins (data not shown). Finally, 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. Intratracheal instillation of either 2.5mg/rat or 0.5mg/rat ROFA did not cause any changes in the levels of IL-1 β , IL-10, IL-6 and TNF- α within the plasma recovered ROFA exposed rats at 0.25, 0.5, 1, 3, 6, 12, and 24h post-exposure (data not shown, N=4-5 per time point).

Subsequent studies examined the fate of pulmonary deposited ROFA constituents following either nose-only or whole-body inhalation 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. These inhalation formats are commonly employed in *in vivo* particle pulmonary toxicology studies. Plasma V levels were significantly elevated immediately following a 6h nose-only inhalation exposure to H#6 ROFA at a concentration of 9mg/m³, Figure 1B. This exposure produced a concentration of 30ng V/ml in plasma which corresponded to a plasma V concentration of 0.6 μ M V and 1.2 μ g/ml of H#6 ROFA-L since H#6 ROFA was found to contain 25 μ g of water soluble V/mg of fly ash. Plasma V levels were also elevated immediately following a 4h whole body inhalation exposure of rats to freshly generated aerosol of ROFA generated from the combustion of #5 residual oil at a concentration of 1.3mg/m³, Figure 1B. This inhalation exposure produced a concentration of 9ng V/ml in plasma which corresponded to 0.2 μ M V and 0.4 μ g/ml of #5 ROFA-L since #5 ROFA was found to contain 24 μ g of water soluble V/mg of fly ash. Plasma cytokine levels were also measured for rats immediately following whole body inhalation exposure to freshly generated aerosol of #5

ROFA. Plasma levels for IL-1 β , IL-10, IL-6 and TNF- α were not altered following whole body inhalation exposure to #5 ROFA aerosol (data not shown).

ROFA Cardiomyocyte Cytotoxicity and Hazard Identification:

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

Mechanism of Injury and Susceptibility Associated with Cardiomyocyte Exposure to ROFA Bioavailable Constituents:

Mechanistic studies were conducted in order to determine whether oxidative stress played a role in the cardiotoxicity associated with RCM exposure to bioavailable constituents of ROFA as well as to identify molecular pathways that may regulate the susceptibility of these cells to ROFA-L induced cytotoxicity. DMTU treatment was found to significantly inhibit ROFA-L induced RCM cytotoxicity at high, 12.5 μ g/ml, but not

low, 6.25µg/ml, dose, Figure 4A. Treatment of RCMs with genistein, a phosphotyrosine kinase inhibitor, was found to significantly enhance the cardiotoxicity associated with exposure to ROFA bioavailable constituents, Figure 4B. Daidzein, the inactive analogue of genistein, was found not to affect ROFA-L induced cardiomyocyte cytotoxicity, Figure 4B.

DISCUSSION:

PM *in vivo* toxicological studies using oil combustion particles or diesel exhaust particles have noted adverse cardiac effects, but there are several unknowns that need to be resolved. Further research is needed to define a specific mechanism(s) through which PM affects the heart, identify specific components that are directly toxic to the heart, and to elucidate what susceptibility factors play a role in excess cardiovascular disease.

In this study we examined the impact of particle-free leachates, equivalent to metal concentrations found *in vivo* after intratracheal instillation or inhalation of ROFA particles, on an *in vitro* model of cardiac myocytes. *In vivo* instillation and inhalation of combustion source ROFA induced no significant elevation of cytokines in the peripheral blood of rats. The major metals of ROFA-L are not causal in regards to *in vitro* cardiotoxicity, suggesting a possible role of minor metal constituents. The mechanism through which ROFA-L induced cytotoxicity was found to be oxidant-dependent at higher concentrations but not at lower concentrations. Lastly, the phosphotyrosine status of the cell (i.e. inhibition of tyrosine kinases = reduced tyrosine phosphorylation) increases the potential of cell death due to ROFA constituent exposure.

In this study we evaluated the effects of bioavailable constituents at relevant PM lung toxicological concentrations on cardiomyocytes. Using ROFA IT exposure as reported in past combustion source studies (Dreher *et al.* 1997, Watkinson *et al.* 1998) as well as an inhalation exposure, we were able to show bioavailable constituents enter the blood stream post exposure. In cardiac compromised animals (SH), plasma V remains elevated by 75% as compared to normal animals (SD). These data suggest the possibility for a prolonged exposure to bioavailable constituents of ROFA in cardiac compromised

individuals. Using V as a guide we were able to approximate the potential amount of metal content the organ might see after a 2.5mg/rat IT of ROFA. A maximal plasma V level of 833ng/mL was measured as little as 15 mins post IT exposure, which corresponds to 16.4μM of V. Back calculating using the V ROFA-L number from Table 1 we were able to infer that plasma metal content would match 25μg/mL of ROFA-L (i.e. $[833\text{ng/mL Plasma V} * 5\text{mg/mL ROFA-L}] / 163745\text{ng/mL ROFA-L V}$). Our highest dose was set at 25μg/mL for RCM cytotoxicity measurements. Significant dose dependent cytotoxicity was observed down to 3.13μg/mL ROFA-L at 24h (Figure 1A, B). To our knowledge no other study has linked relevant PM lung toxicological study concentrations to *in vitro* RCM exposures.

In the course of addressing systemic delivery of constituents we addressed systemic inflammation as a possible mechanism of action for ROFA. Our results indicate that increased levels of metals in the plasma along with some lung injury at the high IT dose of ROFA (i.e. 2.5mg/rat) do not induce systemic inflammation as measured by plasma cytokine analysis. After IH or IT of #5 ROFA or ROFA, respectively, no increases in plasma cytokines (IL-6, IL-10, IL-1β, and TNF-α) could be measured by ELISA (data not shown). Systemic inflammation caused by PM exposure has also been measured through association of increases in peripheral blood neutrophils and band cell counts as well as increases in bone marrow mitogenic pool of neutrophils with increases in concentrations of PM (van Eeden and Hogg 2002). Gardner and colleagues found in 2000 that exposure to ROFA did not increase peripheral blood WBC counts, suggesting that this mechanism of cardiovascular effects (i.e. systemic inflammation) is not a probable cause of ROFA induced cardiotoxicity.

We have established that metal constituents are present in the plasma of animals post-exposure to ROFA either by IT or IH and that corresponding exposure of particle-free leachates of ROFA, *in vitro*, lead to excess cardiomyocyte death. However, after recreating the concentration of major metal constituents of 12.5µg/mL ROFA-L (i.e. V, Ni and Fe) we could not show any excess cardiotoxicity *in vitro* (figure 3). These data suggest a constituent(s) of ROFA-L that is precipitable by neutralization possibly a minor metal constituent of ROFA-L. Possible candidates include Zn, Cu, Al, Mn, and Pb. Single metal exposures to Zn and V, in a cardiomyocyte system similar to ours, produced no increases in % LDH release up to a final concentration of 50µM at 24h (Graff *et al.* 2004). The data for V from our study as well as Graff and colleagues confirms our assertion that V is probably not causative for the *in vitro* cardiotoxicity due to ROFA-L exposure. Kodavanti *et al.* in 2003 found that animals chronically exposed to a high Zn ROFA had areas of necrotic death within the heart. Our study, coupled with Graff *et al.* 2004, does not seem support Zn as a single causative constituent for cardiomyocytes death. Further study is needed to elucidate possible single metal effects as well as synergistic effects of these minor metal constituents.

RCM exposure to ROFA-L at higher concentrations (12.5µg/mL) suggests that oxidative stress plays a vital role in cell death but not at lower concentrations (6.25µg/mL) (figure 4a). These data indicate that as the concentration of ROFA-L bioavailable constituents approach environmental and/or occupational levels oxidative stress plays a less relevant role. This is in direct contrast to lung *in vivo* and *in vitro* studies after exposure to ROFA particles (Ghio *et al.* 2002, Roberts *et al.* 2003). Additionally, our study does not support findings by Gurgueira *et al.* 2002 that PM can

induce reactive oxygen species generation in the heart following inhalation of concentration ambient particles and ROFA. Gurgueira and colleagues used a non-specific chemiluminescent measure of oxidant generation. This, however, does not distinguish between physiological generation of oxidant radicals (e.g. local ischemia/reperfusion) versus particle constituent oxidant radical generation.

Pretreatment of cardiomyocytes with a tyrosine kinase inhibitor, genistein greatly enhances the susceptibility of RCM to cytotoxicity by ROFA-L. A recent publication by Benter *et al.* 2004 related genistein administration with excess cardiomyocyte death after ischemia/reperfusion myocardial injury. These data support the assertion that the tyrosine phosphorylation state of the cell modulates the cells susceptibility to injury.

In this study we have shown that relevant pulmonary toxicology doses lead to increases in plasma metal concentrations and recreating these metal concentrations of ROFA-L *in vitro* lead to excess cardiomyocyte death. Systemic inflammation was not detected when measured by cytokine release into the peripheral blood, suggesting that cardiovascular effects induced by ROFA particle pulmonary exposures are not due to systemic inflammation. We also provide new insights into the hazard identification of ROFA with data indicating that the *in vitro* cytotoxicity induced by ROFA-L is not due to the major metal constituents or Zn. These data are in contrast to what has been found by pulmonary toxicological studies. Our data indicate that *in vitro* cardiotoxicity due to combustion source PM is not oxidant dependent at lower more environmentally or occupationally relevant exposures. Susceptibility to ROFA-L can be greatly increased with addition of a tyrosine kinase inhibitor. These data match *in vivo* studies that examined ischemia/reperfusion injury exacerbation.

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FIGURE LEGENDS:

Figure 1: Plasma vanadium (V) content following pulmonary exposure to ROFA. A) A rapid and transient increase in plasma V levels occurred in rats following intratracheal instillation of ROFA at 2.5mg/rat (■) (N=2 per group at 0.25h-1h post-exposure; N=3 per group at 3-24h post-exposure), 0.5mg/rat SD (♦)(N=3-4 per group), and 0.5mg/rat SHR (▲)(N=3-4 per group). Control animals were given saline (●), 0.3ml/rat, by intratracheal instillation (N=3-4 per group). Maximal plasma V levels corresponding to 833ng V/ml at 15 min and 168ng V/ml at 1h were observed following exposure to the high and low ROFA doses given to SD rats, respectively. Plasma levels of V remain elevated in SH rats exposed to 0.5mg/rat up to 24h. *, $p<0.01$, significant difference between saline and ROFA-L. #, $p<0.05$ significant difference between ROFA 0.5mg/rat in SH vs. SD. B) Plasma V levels in rats immediately following either a 6h nose only inhalation exposure to Hickory #6 ROFA at $9\text{mg}/\text{m}^3$ or 4h whole body exposure to #5 ROFA at $1.3\text{mg}/\text{m}^3$. *, $p<0.05$ significant difference between air and ROFA (N=3-4 per group).

Figure 2: Cardiotoxicity of ROFA bioavailable constituents. RCM were exposed to various doses of ROFA-L ranging from 25 to $1.56\mu\text{g}/\text{ml}$ and examined for cytotoxicity at 24h exposure. Cardiomyocyte cytotoxicity was assessed by either: A) percent release of total cellular LDH; or B) total number of viable cells per well. * $p<0.01$, significant difference between saline versus ROFA-L. # $p<0.09$, between saline and $1.56\mu\text{g}/\text{ml}$ ROFA-L (sample number and number of primary cultures are indicated by parentheses).

Figure 3: Identification of causal cytotoxic ROFA bioavailable constituents. RCM were exposed to either various “forms” of ROFA, such as ROFA-L or ROFA-NLS (25 and 12.5µg/mL), or surrogate metals and metal mixtures equivalent to a dose of 12.5µg/ml ROFA-L and cytotoxicity examined 24h post-exposure in order to identify constituents responsible of ROFA-L induced cytotoxicity. *, $p<0.01$, significant difference between saline and ROFA-L. *, $p<0.01$, significant difference between saline and individual metals and metal mixtures. # $p<0.01$, significant difference between ROFA-L and ROFA-NLS (sample number and number of primary cultures are indicated by parentheses).

Figure 4: Role of oxidative stress and intracellular signaling in ROFA-L induced cardiotoxicity. A) RCMs were pretreated with DMTU (28mM) and exposed to high (12.5µg/ml) and low (6.25µg/ml) doses of ROFA-L. Cardiomyocyte cytotoxicity was measured by percent release of total LDH at 24h 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. B) RCMs were pretreated with 25µM genistein prior to exposure to ROFA-L at 12.5 and 6.25µg/ml in order to determine the role of phosphotyrosine mediated cell signaling played in ROFA-L cardiotoxicity. Cardiomyocyte cytotoxicity was measured by percent release of total LDH at 24h post-exposure. Genistein pretreatment was found to enhance ROFA-L induced cardiotoxicity, Pretreatment of RCMs with 25µM daidzein prior to exposure to 6.25µg/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 (sample number and number of primary cultures are indicated by parentheses).

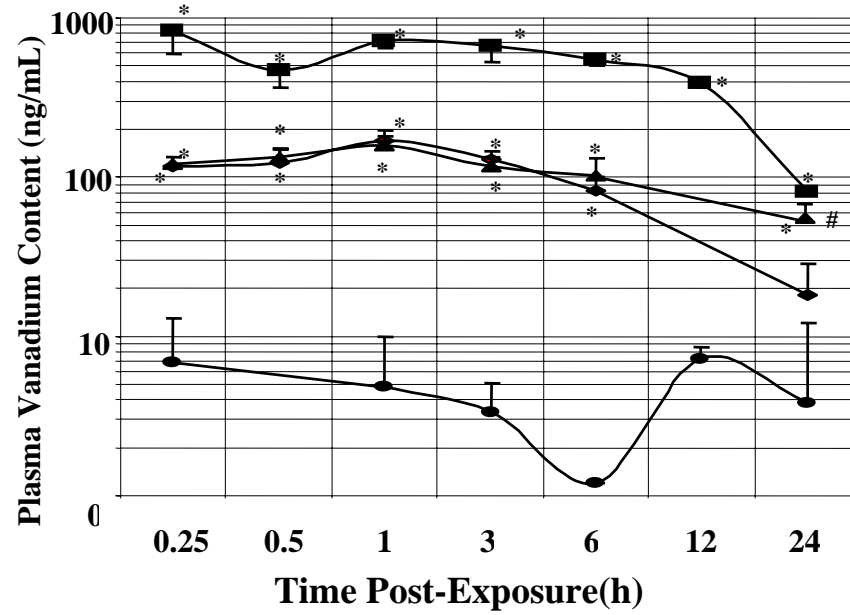
Table 1: ROFA sample metal and sulfate concentrations

Element	ROFA-L	ROFA-NLS	ROFA Neut. Pellet
Sulfate	2483266	2408262	45462
Zn	3753	0	3204
Pb	1354	690	1039
Ni	155254	67	132960
Mn	1871	12	1637
Fe	67798	0	58460
Mg	288790	200784	79005
V	163745	23220	99622
Al	7970	585	6687
Ca	49482	44469	3497
Cu	917	0	696

ICP-AES analysis for metal and sulfate from ROFA leachate (L), neutralized leachate supernatant (NLS) and neutralized pellet derived from 5mg/mL ROFA. ROFA-L, ROFA-NLS and ROFA neutralized pellet derivation is described in the methods. Percent 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.

Figure 1

A



B

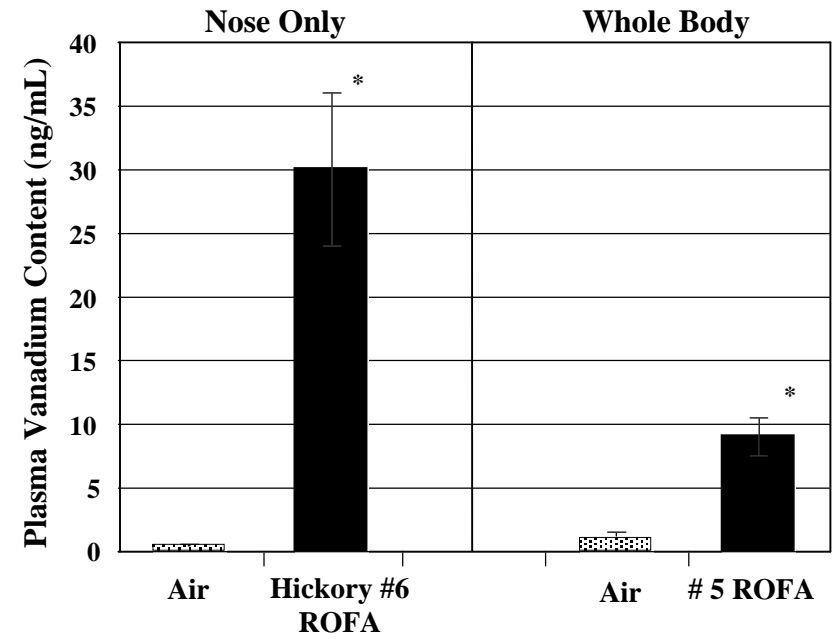


Figure 2

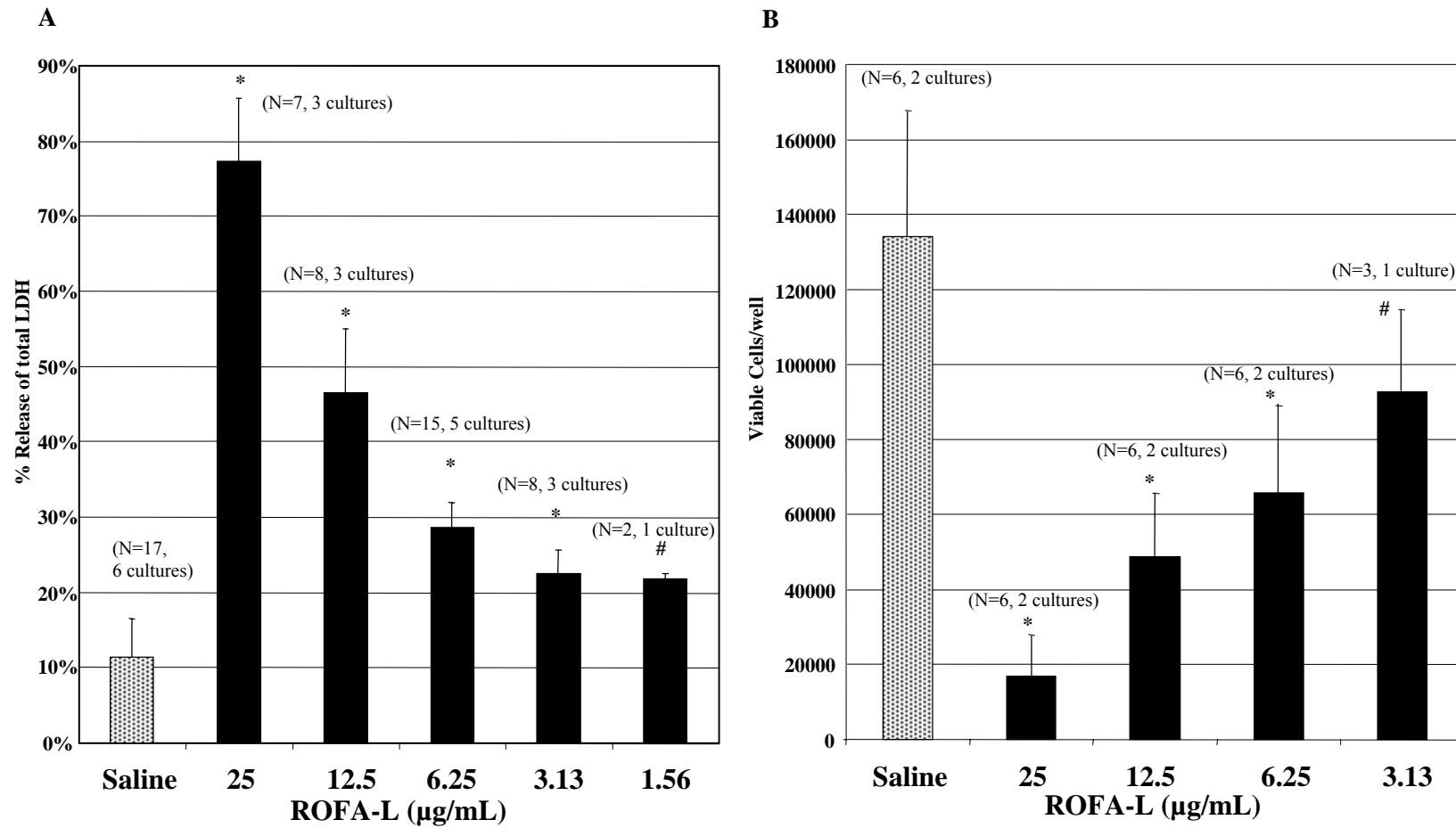


Figure 3

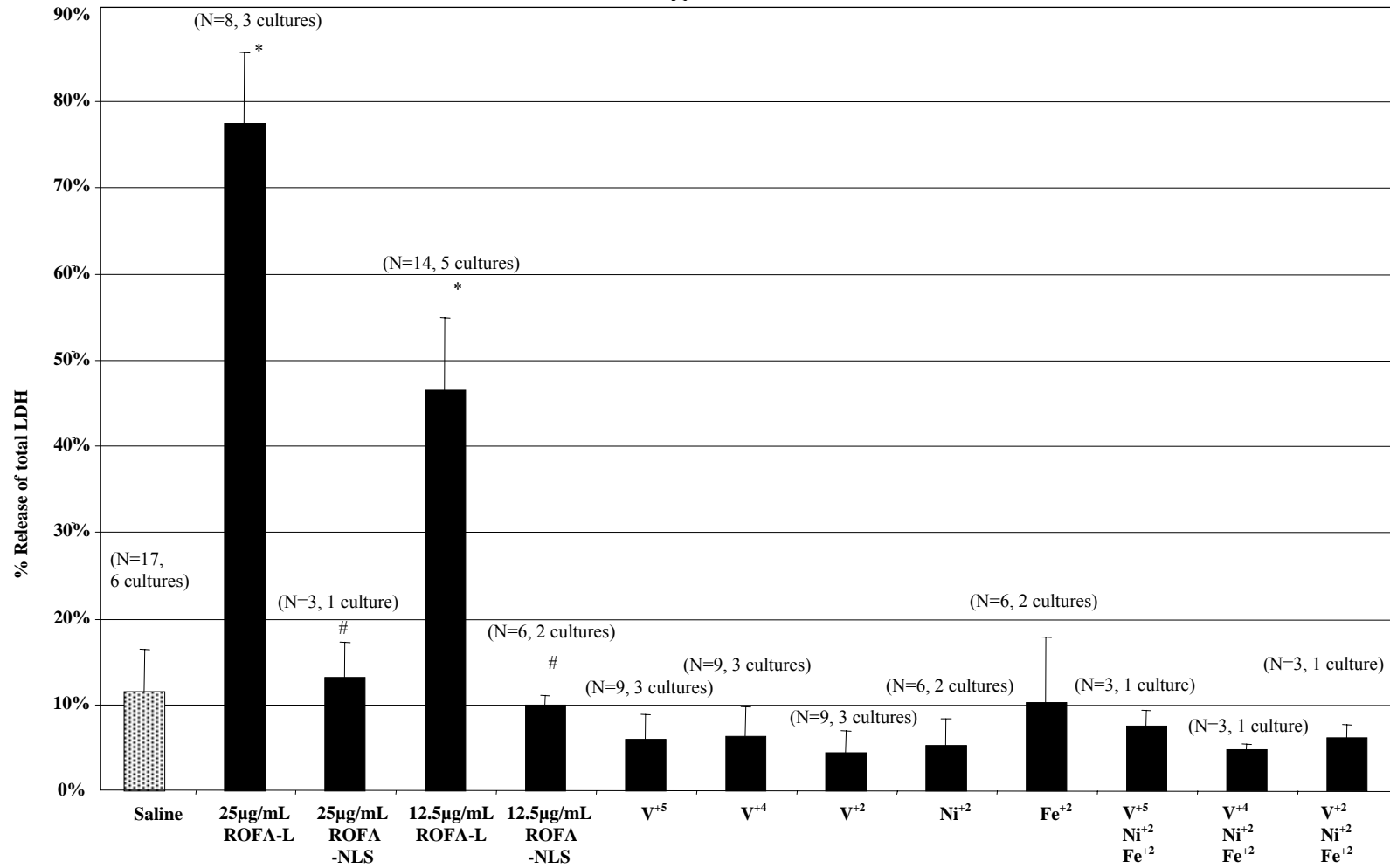
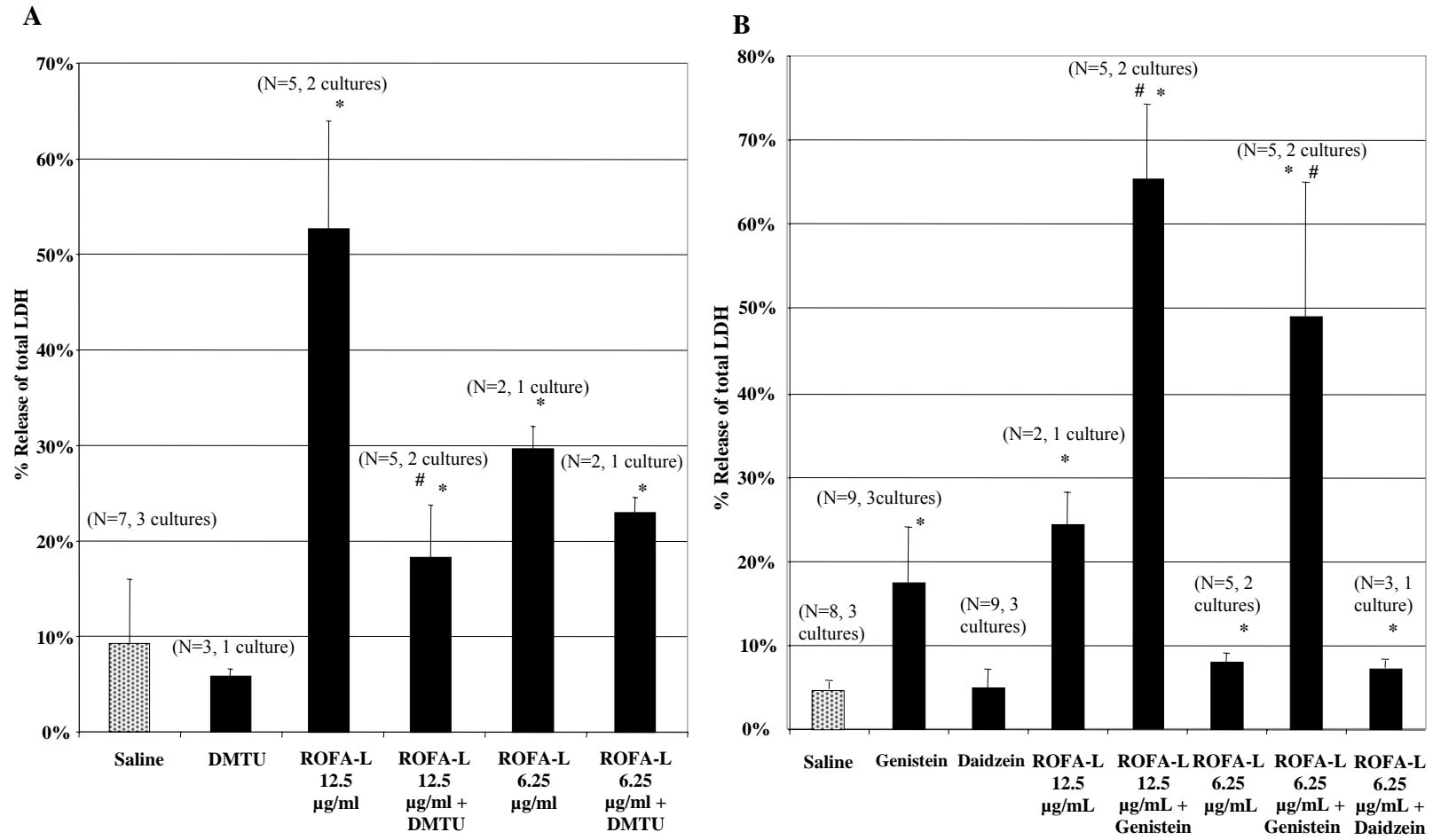


Figure 4



Chapter 2

TITLE: Functional Genomic and Proteomic Alterations in Cardiomyocytes Induced by Residual Oil Fly Ash Bioavailable Constituents

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To be submitted to: *Journal of Toxicology and Environmental Health*.

RUNNING TITLE: Residual Oil Fly Ash Induced Cardiomyocyte Transcription Factor and Gene Expression Changes.

KEYWORDS: ROFA, Microarray analysis, PM, Transcription factors

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ABSTRACT

Epidemiological studies have linked air pollution particulate matter (PM) to adverse cardiovascular endpoints in susceptible subpopulations such as pre-existing cardiovascular disease. The mechanism(s) through which PM, particularly PM $<2.5\mu\text{m}$ in diameter (PM_{2.5}), effect the cardiovascular system is currently under investigation. One proposed mechanism for how PM affects the cardiovascular system is dissolution of PM constituents in the lung following deposition and subsequent delivery to perfused organs and tissues. However, the exact mechanism(s) through which PM acts, as well as the cellular and molecular effects of systemically delivered PM constituents, remain to be elucidated. In this study, we monitored gene expression and transcription factor changes in rat neonatal cardiomyocyte (RCM) cultures following an acute exposure to constituents of the oil combustion particle residual oil fly ash (ROFA). Genomic alterations in IGF-1, VEGF, IL-2 and PI3/AKT pathways could be detected post exposure to ROFA-L. Acute non-cytotoxic doses of ROFA-L altered global gene expression consistent with cardiac myocyte electrophysiological remodeling, oxidative stress, and cell survival. Genomic alterations were found to correlate with changes in transcription factors and these acute changes in both transcription factor activation and gene expression could lead to possible chronic consequences for the cardiac myocyte.

INTRODUCTION

Recent scientific literature addressing air pollution particulate matter (PM) effects have shown a consistent and reproducible association between PM and morbidity/mortality within susceptible subpopulations (US Environmental Protection Agency 2004). One such susceptible subpopulation is individuals with pre-existing cardiovascular disease (Pope 2000). Associated acute effects of PM include increases in myocardial infarction, arrhythmogenesis, and blood neutrophils, as well as decreases in heart rate variability and vasodilation (Liao *et al.* 1999, Peters *et al.* 2000, Peters *et al.* 2001, Brook *et al.* 2002, Reidikar *et al.* 2004). Long-term exposure to PM has been related to mortality due to ischemic heart disease, cardiac arrest, or myocardial infarction (Pope *et al.* 2004). Death due to PM exposure from cardiovascular ailments has been estimated to be in the tens of thousands per year and as much as 69% of all PM related deaths (Schwartz 2001, Pope 2000).

Air pollution is a dynamic and physicochemically diverse mixture of solid and gaseous pollutants arising from various anthropogenic and natural sources (Dreher 2000). Particles of an aerodynamic diameter less than 2.5 μm (PM_{2.5}) arising mostly from combustion sources are of a particular concern. Specific sources have been associated with cardiovascular mortality (Laden *et al.* 2000, Burnett *et al.* 2000, Clancy *et al.* 2004). However, the causal PM properties and the source specific toxicological effects remain to be clarified.

The mechanism(s) by which PM affects the heart and the circulatory system are currently unknown. Several mechanisms have been proposed to explain PM related cardiovascular mortality and morbidity. These include pulmonary inflammation leading to an altered

autonomic nervous system response (Godleski *et al.* 1999, Peters *et al.* 2000), systemic inflammation leading to an abnormal vascular response (Brook *et al.* 2002), and direct effects on the cardiovascular system through dissolution of soluble “bioavailable” constituents following deposition in the lungs and subsequent transport by the circulatory system (Costa and Dreher 1997, Dreher *et al.* 1997, Kodavanti *et al.* 2003).

In this study we attempted to elucidate the potential adverse effects of bioavailable constituents of PM have on cardiac myocytes. To facilitate this objective we exposed cultures of rat neonatal cardiac myocytes (RCM) to constituents of the combustion source particle residual oil fly ash (ROFA). We then measured gene expression changes via microarray analysis after a 1h of exposure to a non-cytotoxic concentration of a particle free leachate of ROFA (ROFA-L). We also looked into whether or not gene expression changes could be correlated to activation or suppression of transcription factors in RCM cultures exposed to ROFA-L. Genomic alterations in IGF-1, VEGF, IL-2 and PI3/AKT pathways could be detected post exposure to ROFA-L. These data suggest that ROFA-L bioavailable constituent exposure can induce gene expression profiles consistent with cell growth, electrophysiological remodeling, and cell survival. This is the first study to directly link exposure to constituents of oil combustion particles to potentially adverse cardiac myocyte molecular changes. This study also demonstrates that particle constituents at lower more occupationally/environmentally relevant doses could elicit acute changes in cardiac myocyte gene expression/signal transduction pathways leading to possible chronic alterations.

MATERIALS AND METHODS

Animals:

Female 60-90 day-old Sprague-Dawley rats were purchased at gestation day 19 (Charles River, Raleigh, NC). Animals were housed in an AAALAC approved facility with water and food *ad libitum* according to federal guidelines.

Cell Culture:

RCMs were obtained from 1-day-old neonatal rat pups using the neonatal rat cardiomyocyte isolation kit (Worthington) with modifications as follows. Minced heart tissue fragments were incubated overnight with trypsin in a HEPES simple salt solution (120mM NaCl, 3mM KCl, 1mM NaH₂PO₄, 25mM HEPES, 0.0001% Phenol Red, in sterile ddH₂O, pH 7.35). After trituration and centrifugation, cells were resuspended in high serum media [10% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen), 0.1mM Bromo-deoxy-Uridine (BrdU)] and preplated onto a 150cm² tissue culture flask (Becton Dickenson) for two hours at 37°C, 1-2%CO₂, to remove most of the non-myocytes. After preplating, cardiomyocytes were recovered and total cell counts and viability were determined by trypan blue dye (0.4%, Sigma) exclusion using a hemacytometer. Alive cells were plated at a density of 170,000cells/cm² in 6-well, 100mm, or 35mm laminin coated tissue culture plates (BD BioCoat, Becton Dickinson) in high serum media. On days 1-3, cells were maintained in high serum media, changing media every 24 hours. On day 4 cells were placed into low serum media [1% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen)] with BrdU. On day 5

of culturing, cells were maintained in low serum media without BrdU. On day 6 cells were given low serum media minus BrdU three hours prior to exposure.

The quality of RCM cultures was assessed by immunohistochemistry and visual inspection of cellular beating and morphology. Fibroblast specific anti-vimentin and cardiac specific anti- α -sarcomeric actin antibodies (Sigma) were used to determine the percent cardiomyocyte content of the cultures grown on 35mm laminin coated dishes. RCMs designated for immunohistochemistry with anti-vimentin antibody were fixed with 3% paraformaldehyde (10 mins, room temperature) (Fisher), rinsed 3 times with 1X PBS, then permeabilized with 0.1% Triton X-100 (15 mins, room temperature)(Fisher). For immunohistochemistry corresponding to anti- α -sarcomeric actin, RCMs were fixed with 96%ethanol, 3%glacial acetic acid with no permeabilization. After fixation and permeabilization, both sets of cells were rinsed with 0.1%Tween 20 (Fisher)/1x phosphate buffered saline (PBS), v/v (Sigma). Cells were then treated with a blocking buffer of 5% (w/v) non-fat dry milk (Carnation) in 0.1%Tween 20/1xPBS for 30 mins, to block non-specific binding. RCMs were then allowed to react with primary antibodies against vimentin (dilution 1:100) or α -sarcomeric actin (dilution 1:100) in blocking buffer for 90 mins at room temperature. Cells were then rinsed with 0.1%Tween20/PBS three times. A horseradish peroxidase conjugated secondary antibody, to IgG (1:400 dilution, Sigma) for vimentin, or IgM (1:400 dilution, Sigma) for α -sarcomeric actin, was used to label the bound primary antibody. Cells were rinsed with 0.1%Tween20/1xPBS three times and then visualized with 3'-3'-diaminobenzimide kit (Innogenex). Cells were then counter-stained with 1% methylene green (w/v, in 0.1%Tween20/PBS, Sigma) for 3 mins. Cells were then rinsed three times with ddH₂O. Four fields per dish at 400X

magnification were counted for vimentin/ α -sarcomeric actin positive cells. RCM cultures were 73.4% \pm 8.8% (n=3) negative for vimentin, 80.2% \pm 9.8% (n=5) positive with α -sarcomeric actin, and 84.3% \pm 8.8% (n=30) RCM by functional and morphological assessment (i.e. beating, refraction), suggesting the majority of the cell population was cardiomyocyte.

RCM cultures were exposed to 3.5 μ g/mL of a particle-free leachate of ROFA (ROFA-L). Briefly, a 5mg/mL stock suspension of ROFA was prepared in sterile saline and mixed for 10 mins at room temperature. The suspension was centrifuged at 17,000xg in an eppendorf microfuge. The recovered leachate was filtered through a 0.2 μ m PETE filter (Poretics Products). An aliquot of the 5mg/mL ROFA-L was added to RCM cultures to yield a final concentration of 3.5 μ g/mL.

RNA Isolation and Quantification:

Total RNA was isolated from RCM cultures grown on 6-well laminin coated culture plates after a one hour exposure to either saline or 3.5 μ g/mL ROFA-L using Trizol Reagent (Invitrogen) according to the Trizol Reagent protocol. RNA was quantitated with the RiboGreen kit (Molecular Probes) and then DNase treated (Clontech) according to the manufacturer's directions. RNA was reisolated using Trizol Reagent and resuspended in ddH₂O containing 1Unit/ μ L of RNasin (Promega). DNA-free RNA was then requantitated using the RiboGreen kit and the quality was assessed with the Bioanalyzer 2100 eukaryotic nano RNA kit (Agilent).

Gene Expression Profiling:

The Atlas rat 4k plastic microarrays were purchased from Clontech. Messenger RNA was isolated from an equal amount of total DNase treated RNA from 2 or more

runs per sample using a total RNA isolation kit (Clontech) according to kit directions. The isolated mRNA was then labeled with 50uCi of α -dATP ^{33}P (PE Life Sciences) with an additional 25 mins labeling incubation with 2.5ul/reaction of a non-radioactive dATP buffer (2uL 5xPowerScript Buffer, 4uL ddH₂O, 3uL of Power Script reverse transcriptase, BD Biosciences Clontech, 1 μ L of 100mM dATP Sigma) and hybridized overnight according to Atlas rat 4K array kit conditions. The next day the arrays were washed and placed into a phosphorimaging screen (Molecular Probes) at room temperature for 48h. The phosphorimaging screen was scanned using the STORM 860 (Molecular Dynamics).

The array images were aligned and quantified using Atlas Image 2.7 software (Clontech). The un-normalized relative optical densities of each spot subtracted from background were used for input into GeneSpring 7.2 program (Silicon Genetics). GeneSpring allows users analyze data from multiple experiments and to perform normalizations, generate restriction lists, and group differentially expressed genes based on functional classification. Normalizations were performed in three steps: 1) “per chip normalization” each measurement was divided by the 50th percentile of all measurements in its array, 2) “data transformation normalization”, all samples values below 0.01 were set to 0.01, and 3) “data transformation normalization”, array grids that were not spotted as part of array, their median value was used to subtract from raw values of each gene for background correction. After normalizations were performed, the data from all three runs of saline and ROFA-L were filtered by expression level to eliminate genes that were expressed at or below 0.01 in three of the six samples loaded. A filtered by expression level gene list was generated and served as the list from which all comparisons were made. However, microarrays were run before additional cultures were produced to

provide replicate arrays. This led to a strong “day bias”, that was not controlled for via normalization. To control for this day bias control vs. exposed 2-fold activation or inhibition gene lists were generated from the filtered by expression level gene list for each “day” that the experiment was performed. These gene lists were then compared to each other via a Venn diagram to determine what genes were activated or inhibited by ROFA-L.

Additional analyses were performed on the internet using the proprietary software Ingenuity Pathway Analysis (www.ingenuity.com). This software allows for measurement and characterization of altered pathways within data sets. GenBank accession numbers from the 2-fold up and down-regulated gene lists were loaded into the software. Analyses of induced and suppressed gene lists were created and those analyses were also compared with one another.

Protein Isolation and Quantification:

Activation of transcription factors was determined using nuclear protein extracts from RCM cultures grown on 100mm laminin coated culture dishes exposed to either saline or ROFA-L 3.5µg/mL for 30 mins. Nuclear protein was extracted using a Nuclear Extraction Kit (Panomics) according to kit directions. Nuclear protein was quantitated using the KoneLab 30 clinical analyzer with reagents from Sigma.

Transcription Factor Analysis:

TranSignal Protein/DNA arrays 1 and 2 were obtained from Panomics (cat# MA1010, MA1011) and activation of transcription factors were determined using manufacturers instructions. Briefly, 35µg of nuclear protein for each sample in parallel was combined with TranSignal Probe Mix and DNA, protein and protein/DNA

complexes were separated on a 2% agarose gel (Sigma) in 0.5% TBE (Novex). The gel area containing the protein/DNA complexes was excised with a razor blade and protein and DNA were extracted from the gel at 60°C. The extracted DNA was bound to silica gel, the protein washed away, and the DNA eluted into deionized H₂O. The extracted probe DNA was then hybridized to a blot overnight at 42°C and then washed and reacted to Streptavidin-HRP conjugate. Each blot was washed again, stained using included substrate solution and visualized via autoradiography (X-OMAT Blue Film, Kodak).

Autoradiographs were scanned using a Personal Densitometer SI (Molecular Dynamics). The images were cropped and relative optical densities were determined using the grid method in ImageQuant 5.1. The data were exported to Microsoft Excel (Microsoft Corp.) and average values for each pair of spots were calculated. Ratios of exposed to control were calculated and ratio values above 2 or below 0.5 were considered significant.

RESULTS

ROFA-L Induced RCM Genomic Alterations:

Insight into cellular alterations of global gene expression in the heart after PM exposure is critically needed to determine the mechanism of the reported cardiovascular effects. An acute, 1h, exposure of RCM to ROFA-L at a non-cytotoxic concentration of 3.5µg/mL suppressed 37 and induced 44 out of a total of 3924 genes. Figure 1 shows a graphical representation of genes induced or suppressed by ROFA-L. Lines originating from either side of the x/y intercept at 45 angles represent 2-fold changes in expression with dots denoting genes induced or suppressed. Tables 1 and 2 list all the genes that are induced or suppressed according to GenBank accession#, common name, description and family. GenBank accession numbers from Tables 1 and 2 were loaded into the Ingenuity Pathway Analysis software to determine alterations in pathways after RCM ROFA-L exposure. Figure 2 is a graphical representation of user selected pathways found to be altered. The light grey bars represent gene pathways altered in the ROFA-L 2-fold induction gene list and the dark grey bars represent gene pathways from the 2-fold ROFA-L suppressed gene list. Significant gene pathway alterations associated with ROFA-L exposure included cardiovascular development and function, cardiovascular disease, free radical scavenging, among others. Another feature of this software is the ability to produce a significance line based on uploaded genes (i.e. canonical pathways) (Figure 3). The line represents $-\log$ of a p value of 0.05 and pathways that go above this line are considered highly significant. Six pathways were shown to be significantly altered according to this method. Genes associated with B-cell receptor signaling, IGF-1, IL-2, PI3/AKT, and TGF- β pathways were all shown to be significantly altered within the

gene list for ROFA-L suppression. The VEGF pathway was shown to be altered in the gene list from ROFA-L induction.

ROFA-L Induced Alterations in Transcription Factor Activation:

Studies were carried out to determine whether or not transcription factor activation or suppression played a role in ROFA-L induced genomic alterations. Figure 4 displays Panomics TranSignal DNA/protein array 1, and 2 platforms and general results for saline and ROFA-L (3.5µg/mL) at 30 mins exposures. Panel A, Figure 4 depicts a schematic diagram of TranSignal 1 arrays. TranSignal 1 arrays are spotted in duplicate for each transcription factor as well as a 1:10 dilution pair of spots. Panels B (saline) and C (ROFA-L) depict typical results of TranSignal 1 array experiment with generalized suppression of transcription factor activation by ROFA-L. The opposite is true for TranSignal 2 arrays, which can be viewed in Panels D and E. A schematic diagram of TranSignal 2 arrays can be viewed in Panel F of Figure 4. TranSignal 2 arrays do not contain a 1:10 dilution of each transcription factor. Quantitative results of transcription factor suppression and activation are displayed in Tables 3 and 4, respectively. Activation of transcription factors include the GATA family (GATA 1, 2, 4), and several forkhead proteins. ROFA-L exposure also led to the suppression of transcription factors of NF E1, NF E2, NFκB, Stat family (1,3,4), and p53.

DISCUSSION

PM toxicological studies have noted adverse cardiac effects, but there are several unknowns that need to be resolved. Further research is needed to understand the cellular and molecular alterations that occur within the heart following PM exposure. Previous work in our laboratory had identified the ability of ROFA constituents to make their way into the systemic circulation following pulmonary exposure and dissolution (unpublished results). In this study we examined the genomic and proteomic effects that a non-cytotoxic, acute exposure, to ROFA-L had on cultured cardiac myocytes. ROFA-L suppressed pathways relating to IGF-1, PI3/AKT, and TGF- β . Acute activation of the PI3/AKT pathway has been shown to enhance cellular survival and function of cardiac myocytes (Matsui and Rosenzweig 2004). Suppression of this pathway could lead to susceptibility to cell death. The VEGF pathway has been shown to increase expression of connexins 40 and 43. These proteins are involved in electrical syncytial properties of the heart as a communicating electrical network and alterations in connexin 40 and 43 expression have been noted in cardiac disease (e.g. heart failure, atrial fibrillation) (Dhein *et al.* 2002). ROFA-L significantly reduced expression of connexin 43 on two out of three microarrays when compared to control (data not shown), suggesting a possible alteration in cardiac conduction channels by ROFA-L. Other significant inductions in RCM gene expression include, heme oxygenase, which has been shown to be cardioprotective following ischemia/reperfusion injury (Yet *et al.* 2001), and Bcl-2, an anti-apoptotic protein.

To identify possible transcription factors that may be responsible for the altered gene expression profiles, we exposed our RCM cultures to ROFA-L at 3.5 μ g/mL for 30

mins instead of 1h. Overall, 24 out of a possible 149 transcription factors were suppressed, while 39 out of 149 were activated over control. Specific responses would include reduced levels of active p53, a known pro-apoptotic/cell cycle suppressant, and induction of GATA family of transcription factors, especially GATA-4, which is known to play a role in reduction of apoptosis (Suzuki and Evans 2004). Metal detoxification pathways have been shown to be up-regulated by MEP-1 (LaRochelle *et al.* 2001), which was activated by ROFA-L, however the response element for MEP-1, MRE, was suppressed by ROFA-L exposure.

Forkhead proteins as a group have been shown to play a role in cell cycle and death control (Bergering and Kops 2002). Especially activation of FOXO4 and down-regulation of PI3/AKT pathway has been shown to result in quiescence or apoptosis depending on other cellular signals (e.g. FasL, p130) (Bergering and Kops 2002). In this study we have shown activation of FOXO4 and alteration of the PI3/AKT pathway indicating another possible susceptibility pathway for ROFA-L induced cardiomyocyte cell death.

This study identified several possible interactions between combustion source PM and cardiac myocyte death and electrophysiological remodeling. Increased cardiomyocyte cell death in the heart has been shown to play a role in heart failure (Gill *et al.* 2002). These data show that constituents of PM could increase susceptibility to cell death and electrophysiological remodeling leading to chronic adverse health effects.

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FIGURE LEGENDS

Figure 1: ROFA-L induced alterations in the cardiomyocyte transcriptome. An image taken from GeneSpring 7.2 analysis software showing 2 fold up and down regulated genes following exposure to ROFA-L at 3.5µg/mL for 1 hour versus saline control.

Figure 2: Ontogeny of ROFA-L induced alterations in the cardiomyocyte transcriptome. Suppressed and induced gene lists were loaded into Ingenuity Pathway Analysis software. Bars represent significance of gene expressed alterations. The light grey bars represent gene pathways altered in the ROFA-L 2-fold induction gene list and the dark grey bars represent gene pathways from the 2-fold ROFA-L suppressed gene list.

Figure 3: Significantly altered pathways following ROFA-L exposure. The light grey bars represent gene pathways altered in the ROFA-L 2-fold induction gene list and the dark grey bars represent gene pathways from the 2-fold ROFA-L suppressed gene list.

Figure 4: Alterations in cardiomyocyte transcription factor activation following ROFA-L exposure. Panel A is a schematic diagram of the transcription factors on the TranSignal 1 arrays. Panels B and C depict Panomics Protein/DNA Array I images from RCM cultures exposed to either saline or ROFA-L 3.5µg/mL for 30 mins, respectively. A generalized suppression can be observed between saline and ROFA-L exposed cultures. Panels D and E depict images of Panomics Protein/DNA array 2 also exposed to saline or ROFA-L 3.5µg/mL, respectively. An overall activation of transcription factors can be

seen. Quantitative data for Panels B, C, D and E are presented in Tables 3, 4. Panel F is a schematic diagram of the transcription factors contained on TranSignal 2 arrays.

Table 1 ROFA-L Suppression of Gene Expression¹			
<i>GenBank#</i>	<i>Common</i>	<i>Gene name</i>	<i>Family</i>
M63006	Adcyap1	pituitary adenylate cyclase activating polypeptide	Adenylate cyclase
U78875	cpg20	candidate plasticity-related gene20	Cell death
M37807	Igflr; IGFIRC	insulin-like growth factor I receptor	Cell growth
X63434	Plau	urokinase-type plasminogen activator	Cell growth
AB015946	Tubg1	tubulin g1	Cytoskeletal protein
AF127798	Itsn	EH domain/SH3 domain-containing protein	Exocytosis
AF042714	Nph4	neurexophilin 4	Extracellular signaling ligand
AF141386	Slit2	Secretory ligand for roundabout (Robo) receptor	Extracellular signaling ligand
D78482	Zp3	zona pellucida 3 glycoprotein	Glycoprotein
L02926	IL-10	interleukin 10	Immune
L12458	Lyz; Lysz	lysozyme	Immune
L18891	MRP8	intercellular calcium-binding protein	Immune
Y00480	RT1.D alpha	alpha chain precursor to rat MHC class II	Immune presentation
U55192	SHIP	SH2 inositol phosphatase	Immune suppression
AJ003004	Abcb6	ATP-Binding Cassette half-transporter, UMAT	Protein trafficking
AF000973	rSK1	calcium-activated potassium channel	Ion channel
L19031	oatp	organic anion transporter	Ion transporter
J03588	Gamt	guanidinoacetate methyltransferase	Metabolism
M27812	synapsin Ia	synapsin Ia	Neurotransmitter release
M83680	RAB14	low molecular weight GTP-binding proteins	Neurotransmitter release
M25071	ErbA-beta-2	pituitary-specific thyroid hormone receptor	Receptor
M83561	GluR5-2	glutamate receptor subunit 5-2	Receptor
U36785	VN1	putative pheromone receptor VN1	Receptor
M31837	Igfbp3	insulin-like growth factor binding protein	Receptor ligand binding
AB023624	Scop	suprachiasmatic circadian oscillatory protein	Signal transduction
AF107723	AGS3	activator of G-protein signaling 3	Signal transduction
D82928	Cdipt	phosphatidylinositol synthase	Signal transduction
U21684	syk	tyrosine kinase p72	Signal transduction
U44948	SmLIM	smooth muscle cell LIM protein	Signal transduction
X96663	rab29	ras-related GTPase 29	Signal transduction
Z16415	Map2k1	mitogen activated protein kinase kinase	Signal transduction
AF087037	Btg3	rat homolog of mouse Btg3 gene	Stress response
AF220760	Txnrd1	thioredoxin reductase 1	Stress response
M64780	Agrin	agrin	Synapse development
AF012347	Madh9	Smad 8	Transcription factor
L03557	hox1.4	hox1.4	Transcription factor
X14776	TP2	transition protein 2	Unknown

¹, Suppression of gene expression is > 2fold relative to saline control.

Table 2 ROFA-L Induction of Gene Expression ¹			
<i>GenBank ID</i>	<i>Common</i>	<i>Gene name</i>	<i>Family</i>
M58590	rPK	plasma kallikrein	Blood coagulation factor
M92848	C-CAM	cell adhesion molecule	Cell adhesion
D14014	Ccnd1	cyclin D1	Cell Cycle
L14680	bcl-2	Bcl-2	Cell survival
M87634	BF-1	Brain factor-1 (a.k.a. foxg1)	Cell survival
D16339	Ttpa; TTP	alpha-tocopherol transfer protein	Cellular Maintenance
AF069525	Ank3	ankyrin	Cytoskeletal protein
J05519	LOC64300	tetrahydrofolate synthesis	Electron transport
M63510	Umod	Tamm-Horsfall protein	Electron transport
D50671	Loc192258	GABA receptor subunit rho-3	Extracellular signaling
L17080		NGF-binding Ig rearranged kappa-chain	Extracellular signaling
U93306	Kdr	VEGF receptor-2	Extracellular signaling
M81231	SP-D	surfactant protein-D	Glycoprotein
U68172	MUC2	mucin (MUC2) gene	Glycoprotein
AF233596	Ptgs2	prostaglandin synthase	immune
M80826	trefoil protein	intestinal trefoil protein	immune
X52712	Mx1	Mx2 Protein	immune
AF019043	DLP1	dynamitin like protein	Intracellular trafficking
AF036255	Trim3	ring finger protein	Intracellular trafficking
D38380	Tf	transferrin	Iron transport
J05231	nAChR-related	neuronal nicotinic acetylcholine receptor	Ion channel
L10077	ACHR	nicotinic acetylcholine receptor alpha 2	Ion channel
L39018	SCP6	voltage-gated sodium channel	Ion channel
L77929	RBGIRK3/Kir3.3	inwardly rectifying potassium channel	Ion channel
M13646	CYP3A2	cytochrome P450 isoform 3A2	Metabolism
M68965	LIMP2	Lysosomal membrane protein	Metabolism
S73583	Enpep	Aminopeptidase 1 (angiotensinase)	Metabolism
X57565	ugt2A1	UDP-glucuronosyltransferase	Metabolism
D10757	Psmb9; Lmp2	proteasome subunit	Protein turnover
K02298	chyB	chymotrypsin B	Protein turnover
AB038038	cbp	Csk binding protein	Receptors
M88592	PPAR	peroxisome proliferator activated receptor	Receptors
M93273	somatostatin receptor	somatostatin receptor	Receptors
U29339	ErbB3	Tyrosine kinase receptor ErbB3	Receptors
AB020615	PKC lambda	protein kinase C-isoform Lambda	Signal transduction
U17901	plap	phospholipase A-2 activating protein	Signal transduction
J02722	Hmox1; Heox; HEOXG	heme oxygenase	Stress response
U18913	Fra-2	transcription factor Fra-2	Transcription factor
Z17223	Meox2	Gax protein	Transcription factor
U58857	Serpib5	maspin	Tumor suppression
AF020618	Myd116	progression elevated gene 3	Tumorigenesis
L20681	Ets-1	proto-oncogene Ets-1	Tumorigenesis
M86514	PRP	proline rich protein	Unknown

¹, Induction of gene expression is > 2fold relative to saline control.

Table 3		
ROFA-L Induced Suppression of Transcription Factor Activation		
<i>Transcription Factor</i>	<i>Fold Change¹</i>	<i>Description</i>
TR	0.01	thyroid hormone receptor
Skn	0.02	octamer-binding site in epidermis (POU domain factor)
HSE	0.02	heat shock transcription factor
RAR(DR5)	0.02	RAR: retinoic acid receptor
SIE	0.04	serum inducible element responsive factor
Stat1	0.04	STAT1: signal transducer and activator of transcription 1, 84kDa/91kDa
NF-E2	0.07	NFE2: nuclear factor (erythroid-derived 2), 45kDa
NF-E1	0.07	YY1 transcription factor
Stat3	0.11	STAT3: signal transducer and activator of transcription 3
NFkB	0.12	NFkB: nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
GRE	0.14	GR: glucocorticoid receptor
MRE	0.14	Metal response factor
Smad SBE	0.14	MADH: MAD, mothers against decapentaplegic homolog
Sp1	0.17	SP1: Sp1 transcription factor
PRE	0.2	Progesterone receptor
USF-1	0.2	USF: upstream transcription factor
RXR(DR1)	0.23	RXR: retinoid X receptor
Stat4	0.26	STAT4: signal transducer and activator of transcription 4
IRF-1	0.33	IRF1: interferon regulatory factor 1
TR(DR-4)	0.35	thyroid hormone receptor
VDR(DR3)	0.4	VDR: vitamin D (1,25- dihydroxyvitamin D3) receptor
Pbx1	0.43	PBX1: pre-B-cell leukemia transcription factor 1
p53	0.47	TP53: tumor protein p53
CBF	0.49	CAAT box General

¹, Suppression of transcription factor activation by ROFA-L <0.5 relative to saline control.

Table 4		
ROFA-L Induced Activation of Transcription Factors		
<i>Transcription Factor</i>	<i>Fold Change¹</i>	<i>Description</i>
GATA-1/2	725	GATA binding protein 1/2 (globin transcription factor)
AP4(RIP60)	403	activator protein 4 binding element
ISRE(1)	261	interferon- α stimulated response element
NZF-3	200	neural zinc finger factor 3
GATA-1	84.3	GATA binding protein 1 (globin transcription factor 1)
ISRE (Trans TF)	83.7	interferon- α stimulated response element
CEF2	79.1	cTnC (Slow/Cardiac Troponin C)
Afxh (foxo4)	47.9	forkhead box O1mouse
ISRE(2)	33.8	interferon- α stimulated response element
XRE	33.6	xenobiotic response element
XPB-1	31.3	X-box binding protein 1
GATA-2	30.4	GATA binding protein 2 (globin transcription factor 2)
Freac-7	28.1	forkhead box L1
FKHR (mouse)	28.0	forkhead box O1A (rhabdomyosarcoma) human
HFH-3	27.2	HIF-1 binding site (cis-element regulating VEGF)
ANG-IRE	27.1	Angiotensinogen (ANG) insulin-response element (IRE)
HNF-3 β	23.5	hepatocyte nuclear factor 3 beta (forkhead domain)
MZF1	17.8	zinc finger protein 42 (myeloid-specific retinoic acid- responsive)
ZIC	12.4	one of four DNA binding domain on BZLF1 gene (EBV virus) promoter
MSP1	11.9	the sequences are the same as SAA except SP1 binding site is removed
Ikaros	11.8	Ikaros protein (zinc-finger protein)
HBS/xbp-1	11.1	HIF binding sequence rat, as human xbp-1
Freac-2 (1)	11.0	myeloid/lymphoid or mixed-lineage leukemia, Member of the forkhead family
MEP-1(2)	10.0	Metal element protein-1
AP-1	9.26	Activator protein-1
Freac-4	5.90	forkhead box F2 (mouse)
IRF-1/2	5.89	interferon regulatory factor 1/2 binding element
ADR1	5.47	alcohol dehydrogenase regulatory gene1binding element
MUSF1	4.84	the sequences are the same as SAA except USF binding site is removed
MT-Box	4.57	tentative new binding domain
NF-Y	4.27	nuclear Y box factor
E47	4.03	E2A immunoglobulin enhancer binding factors E12/E47
Antiox-RE	3.60	Antioxidant responsive element
c-Rel	3.23	NFkB p75kDa protein
GATA-4	3.23	GATA binding protein 4 (globin transcription factor 4)
E4F, ATF	3.19	E4F transcription factor 1
HBS	3.07	HIF-1 binding site (cis-element regulating VEGF)
Pax4	2.72	paired box gene 4
Elk1	2.68	member of ETS oncogene family
E4BP4	2.48	nuclear factor, interleukin 3 regulated

¹, Activation of transcription factors by ROFA-L >2 relative to saline control.

Figure 1

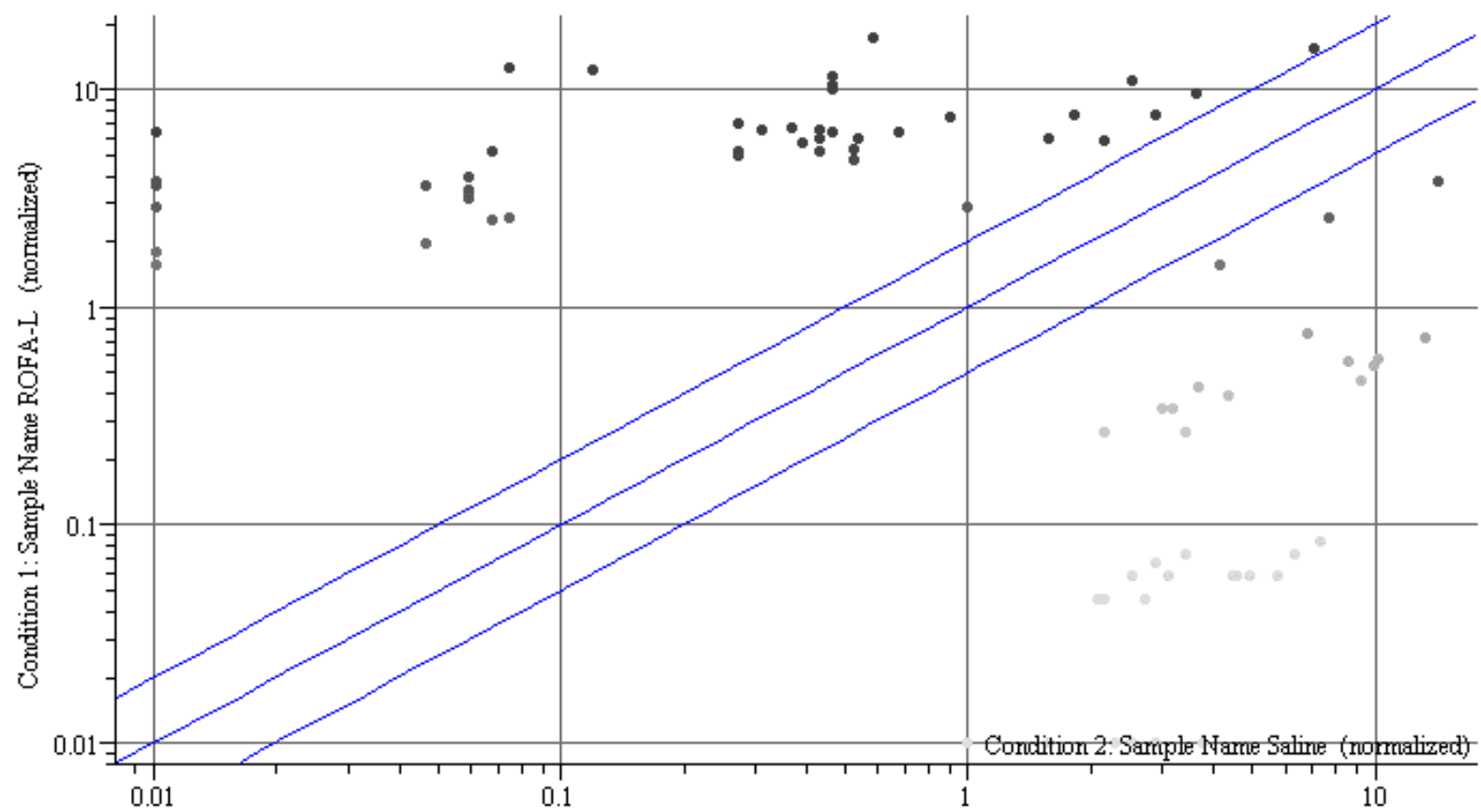


Figure 2

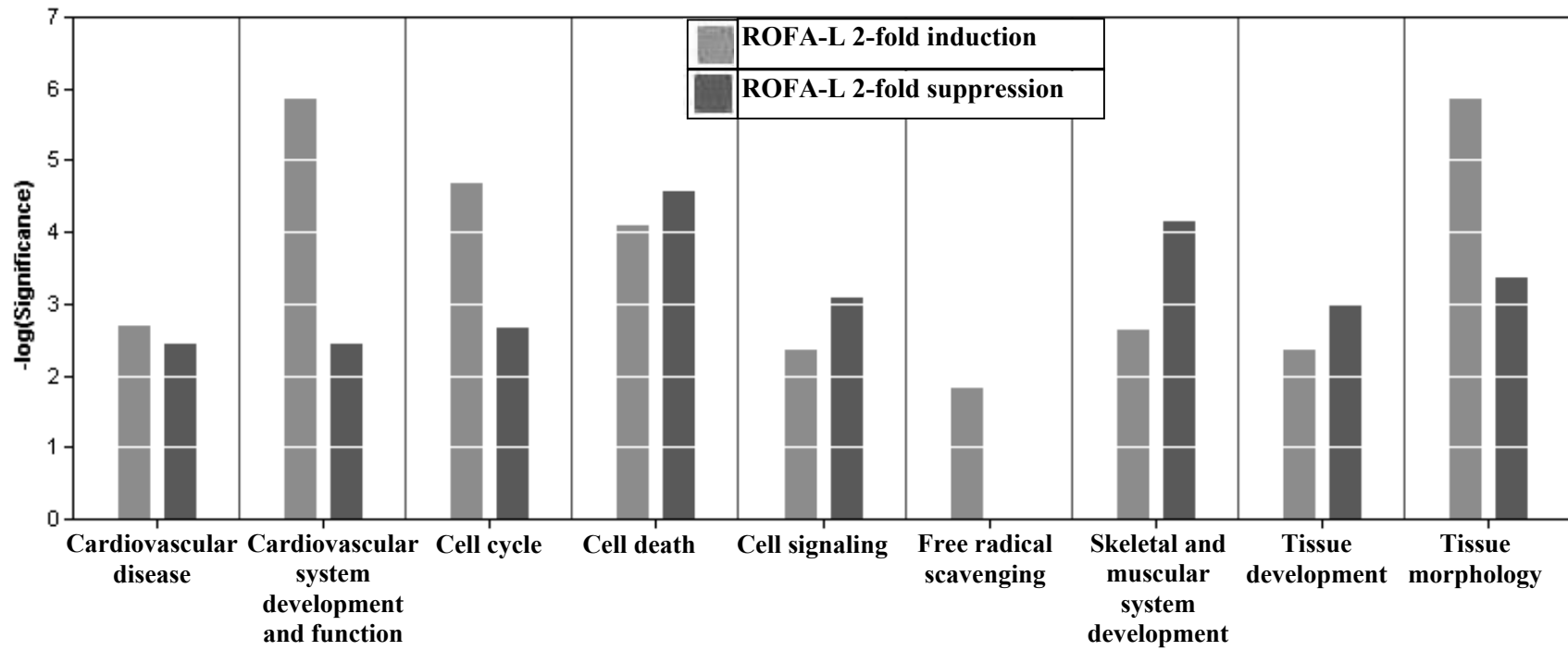


Figure 3

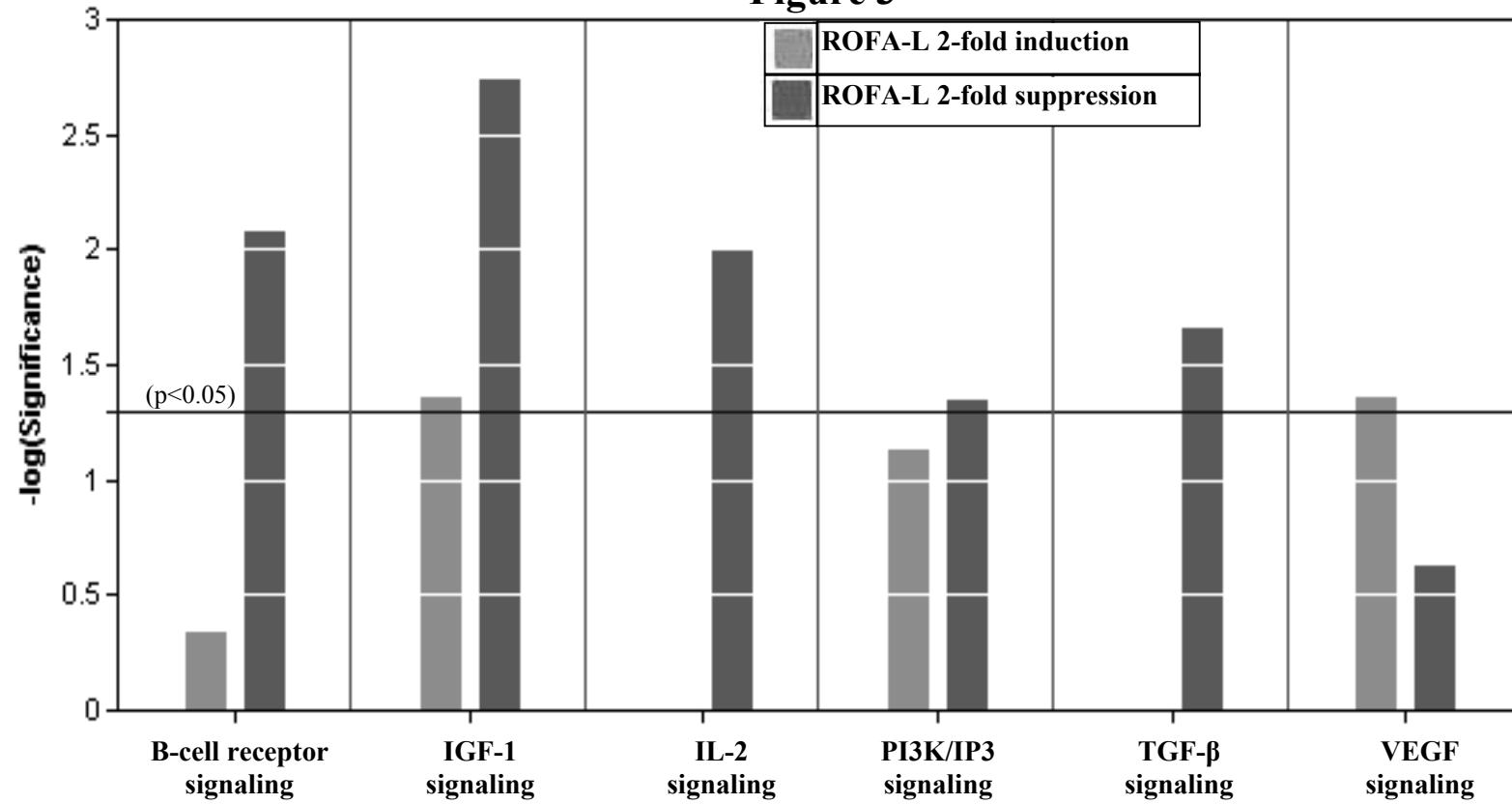
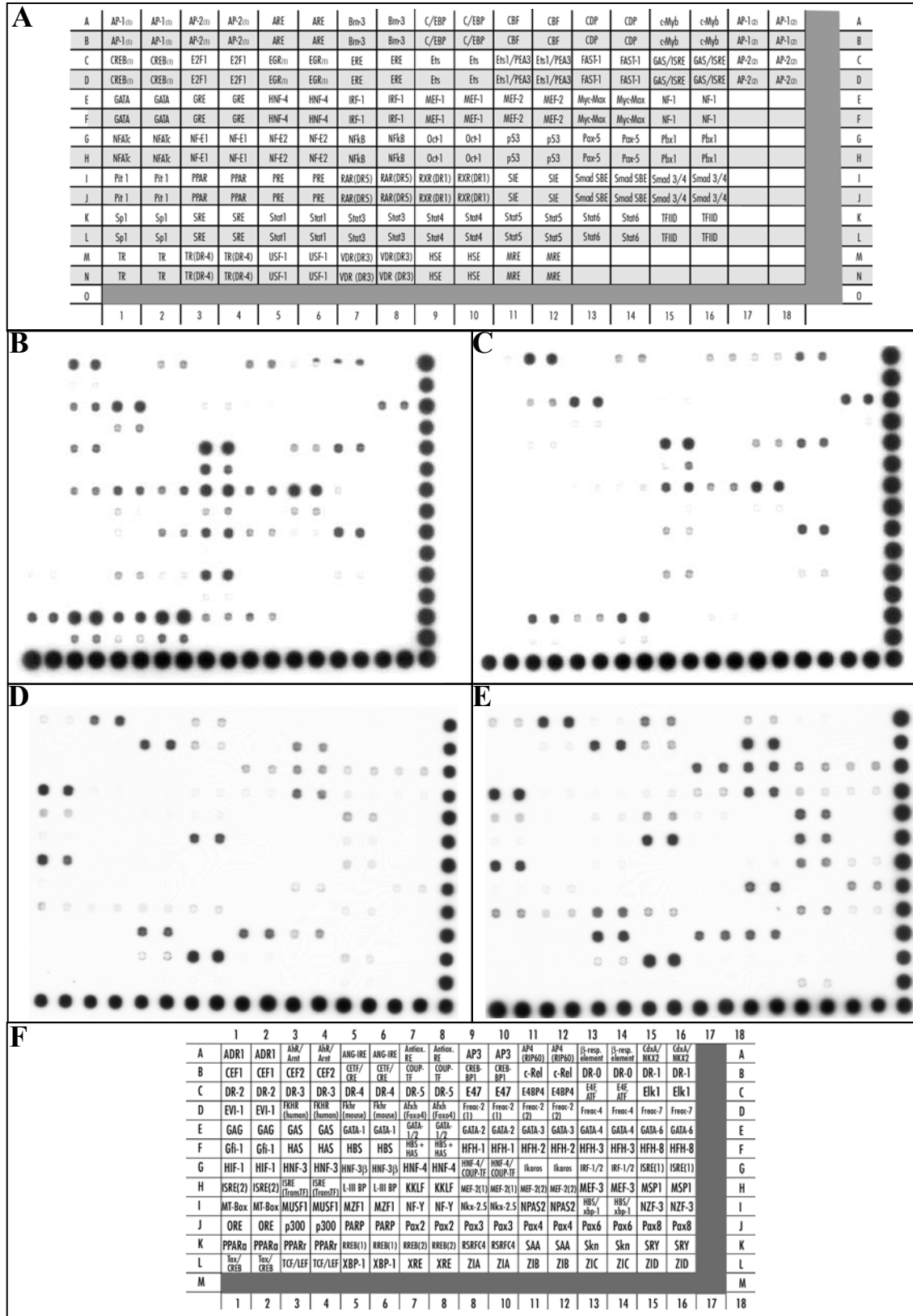


Figure 4



FINAL DISCUSSION

The studies presented in this dissertation investigated the role of bioavailable constituents of ROFA, a component of PM, on rat neonatal cardiac myocytes. This work is the first to link ROFA bioavailable constituents to adverse effects on cardiomyocytes such as cytotoxicity, and pathological alterations in gene expression and transcription factor activation. Specific findings of this work are:

1. A rapid and transient increase in ROFA associated metals were observed in rat plasma following pulmonary deposition of these particles.
2. A lack of evidence for systemic or vascular inflammation following ROFA pulmonary exposure.
3. Clearance of ROFA associated metals within plasma of SH rats was impaired when compared to normotensive rats.
4. Contrary to the causal constituents of ROFA induced lung injury, the major metal constituents of ROFA did not mediate cardiomyocyte cytotoxicity.
5. Inhibition of tyrosine kinases leads to an enhanced susceptibility to constituents of ROFA, therefore, indicating that diseases or processes that alter tyrosine kinase function could lead to an increase in susceptibility to PM.
6. Oxidative stress did not play a significant role in ROFA-L induced cardiomyocyte cytotoxicity at lower exposure concentrations.
7. Acute, non-cytotoxic exposures of cardiac myocytes to ROFA-L lead to alterations in genomic expression of pathways involved in VEGF, IGF-1,

TGF- β , and IL-2 signaling, as well as activation or suppression of transcription factors involved in cell growth, cell cycle, and metal detoxification.

The studies reported here employed an *in vitro* approach to gain insight into the cellular and molecular effects of ROFA bioavailable constituents on cardiomyocytes. The RCM model has been shown to be phenotypically stable in culture and allows for greater culturing time when compared to the adult isolated cardiac myocyte (Chlopikova *et al.* 2003). In this work we examined the impact which ROFA bioavailable constituents had on cardiomyocyte cell survival, as well as alterations in its transcriptome and proteome.

We first identified relevant toxicological concentrations of ROFA-L by extrapolating from the amount of ROFA associated metal present within the plasma of rats following pulmonary exposure to this combustion particle. This is the first study to use relevant concentrations of PM constituents found *in vivo* after pulmonary exposure as a basis for *in vitro* exposure. The highest equivalent dose of ROFA-L found in the plasma of rats post intratracheal instillation of ROFA was at 15 mins equaling 25 μ g/mL ROFA-L. Using this dose as a starting point the data show a dose response relationship between increases in cell death and increases in ROFA-L concentration, probably through a necrotic mechanism of cell death. However, the specific constituents of ROFA-L, mainly V, Ni and Fe, were found not to increase cytotoxicity. Other studies exposing RCM cultures to metals have found similar results (Graff *et al.* 2004). This is in direct contrast to data from pulmonary studies that have linked the major metal constituents of ROFA to its effects (Dreher *et al.* 1997). This study is the first to demonstrate a lack of

systemic or vascular inflammation following pulmonary deposition of ROFA, suggesting that systemic inflammatory mediators do not play a role in ROFA effects on the cardiovascular system.

Pretreatment of RCM with the tyrosine kinase inhibitor, genistein, greatly exacerbated the cytotoxicity induced by ROFA-L. These findings are similar to those of ischemia/reperfusion studies where genistein also enhances injury after reperfusion of the ischemic site (Benter *et al.* 2004, Shinji *et al.* 2004). These data show that the tyrosine phosphorylation state of the cells has a protective effect, as far as, reduction of cytotoxicity due to insult. We also found that ROFA did not induced cytotoxicity through an oxidant generative pathway at lower more occupationally and/or environmentally relevant doses. This is also in contrast to pulmonary *in vivo* and *in vitro* studies, which have linked ROFA exposure to oxidant generation (Ghio *et al.* 2002 Roberts *et al.* 2003).

Acute and non-cytotoxic exposure (i.e. 3.5µg/mL for 1h) of RCM cultures to ROFA-L show an alteration of global gene expression consistent with electrophysiological remodeling, changes in signal transduction pathways, increased ability to respond to stress, and induction of genes necessary for cell survival. Transcription factor analysis for cultures exposed for 30 mins to 3.5µg/mL of ROFA-L indicate an increase in activation of transcription factors dealing with survival of cardiac myocytes, detoxification of metals, and response to oxidative stress.

Two of the most important findings of this work are the alteration of PI3/AKT coupled with FOXO4 induction, and VEGF pathway induction. Acute activation of the PI3/AKT pathway has been shown to enhance cellular survival and function of cardiac

myocytes (Matsui and Rosenzweig 2004). Suppression of this pathway could lead to susceptibility to cell death. However, when the PI3/AKT pathway suppression is coupled with FOXO4 induction, this signals a quiescent state or apoptosis depending on other factors (e.g. FasL, p130) (Bergering and Kops 2002). These data suggest a possible susceptibility of RCM to ROFA-L induced cell death. The VEGF signaling pathway has been shown to be involved electrical remodeling of connexins 40, 43 in the heart. Alterations in the electrophysiology of connexins 40, 43 have been linked to diseases processes (e.g. heart failure, atrial fibrillation) (Dhein *et al.* 2002). In this study, we observed up-regulation of the VEGF pathway along with possible changes in connexin 43 expression.

Future directions of this work would involve identification of possible minor ROFA-L constituents that could possibly be mediating the cytotoxic response of RCM. Other sources of PM could be used as a comparative toxicological tool for assessing potentially relevant doses that induce cytotoxicity in RCM cultures. Elucidation of the exact tyrosine kinases involved in cardioprotection would prove valuable in assessing what diseases or conditions would increase susceptibility to PM induced cardiovascular effects. Elucidation of signal transduction pathways, through global expression changes and transcription factor activation, would provide critical information as to the overall susceptibility of an individual to PM effects. The ultimate goal of this work is to link sources of PM to a specific mechanism of PM induced cardiovascular effects.

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Appendix I

TOXICOLOGICAL ASSESSMENT OF RESIDUAL OIL FLY ASH LEACHATE ON CARDIOMYOCYTES USING A REPORTER-GENE TRANSFECTION BASED APPROACH

ABSTRACT

Robust relationships between air pollution particulate matter (PM) and adverse cardiovascular health effects within susceptible subpopulations have been reported by epidemiological studies. PM mortality effects have been associated with specific sources, however, the direct impact a given source has on the cardiovascular system remains to be elucidated. Several mechanisms have been proposed to explain PM effects on the cardiovascular system including; direct particle constituent effects on the heart and circulatory system after dissolution in the lung. Support for this proposed mechanism comes from animal studies that have linked constituents of PM to adverse cardiovascular health outcomes. However, the direct impact PM constituents have on the cardiac myocytes remain to be elucidated. Rat neonatal cardiac myocytes (RCM) cultures have been used extensively to study the effects of drugs, hypertrophy, myocyte death, conduction/repolarization, and ischemia/reperfusion injury. Also, RCM are very phenotypically stable in culture and are well characterized in their response to various stimuli. In this study I attempted to look at the hypertrophic effects of constituents of residual oil fly ash (ROFA) have on RCM cultures by employing plasmid constructs containing hypertrophic promoters.

INTRODUCTION

Current epidemiological literature has linked PM with excess death and exacerbation of disease in susceptible sub-populations (Brook *et al.* 2004). Ambient PM_{2.5} has been shown to increase risk of myocardial infarction, decrease heart rate variability and increase blood coagulation parameters (Peters *et al.* 2000, Liao *et al.* 1999, Reidiker *et al.* 2004). It has been hypothesized that components of PM could solubilize within the lung and make their way into blood stream and then directly impact the heart (Costa and Dreher 1997, Watkinson *et al.* 1998, Kodavanti *et al.* 2003). Support for this hypothesis is found in studies linking metal contents of combustion source PM with adverse cardiac endpoints (Campen *et al.* 2001). Also, gene expression changes in the hearts of rats have been noted following pulmonary exposure to concentrated ambient PM (Chen *et al.* 2005). However, the exact mechanism(s), causal particle properties, and emission sources remain to be identified. One potential cardiac PM constituent effect is hypertrophic stimulation. Non-physiological cardiac hypertrophy is marked temporally regulated induction of immediate-early (e.g. c-fos, c-myc), reappearance of fetal cardiac genes (e.g. atrial natriuretic factor, β -myosin heavy chain, Skeletal- α -actin), and induction of structural genes (e.g. myosin light chain-2, cardiac- α -actin). Hypertrophic stimulation eventually leads to incorporation of protein to increase cellular size without mitosis (Chien *et al.* 1991, Gillespie-Brown *et al.* 1995). Rat neonatal cardiac myocytes (RCM) have a well-characterized response to hypertrophic stimuli. Many of the growth and survival signal transduction pathways, as well as the gene expression profiles, have been elucidated with this cell type, making the RCM an ideal model for the study of cellular growth. In order to monitor hypertrophic gene

induction in cultured cardiac myocytes, plasmid gene constructs were created for transient transfection containing promoter elements of hypertrophic genes linked to an easily measured gene product (e.g. luciferase, green fluorescence protein, β -galactosidase). Gene transfer experiments using plasmids packaged in liposomes have been widely used to monitor gene induction in cardiac myocytes (Eizema *et al.* 1999). Based the availability and wide use of hypertrophic promoter constructs, as well as the well characterized hypertrophic culture system, RCM cultures appeared to be an attractive system to elucidate direct cardiac effects of PM constituents. Therefore, in this study I performed plasmid isolations and liposomal transfections of RCM cultures to determine the utility of cardiomyocyte transformation for use in toxicological studies linking particle constituent exposure to pathological gene expression.

MATERIALS AND METHODS

Animals:

Female 60-90 day old Sprague-Dawley rats were purchased from Charles River Laboratory at gestation day 19. Animals were given food and water *ad libitum*. After birth neonatal pups were used after one day. Animals were housed in an AAALAC approved facility and handled according to federal guidelines.

Cell culture:

RCMs were obtained from 1-day-old neonatal rat pups using the neonatal rat cardiomyocyte isolation kit (Worthington) with modifications as follows. Minced heart tissue fragments were incubated overnight with trypsin in a HEPES simple salt solution (120mM NaCl, 3mM KCl, 1mM NaH₂PO₄, 25mM HEPES, 0.0001% Phenol Red, in sterile ddH₂O, pH 7.35). After trituration and centrifugation, cells were resuspended in high serum media [10% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen), 0.1mM Bromo-deoxy-Uridine (BrdU)] and preplated onto a 150cm² tissue culture flask (Becton Dickenson) for two hours at 37°C, 1-2%CO₂, to remove most of the non-myocytes. After preplating, cardiomyocytes were recovered and total cell counts and viability were determined by trypan blue dye (0.4%, Sigma) exclusion using a hemacytometer. Alive cells were plated at a density of 170,000cells/cm² in 6-well, 100mm, or 35mm laminin coated tissue culture plates (BD BioCoat, Becton Dickinson) in high serum media. On days 1-3, cells were maintained in high serum media, changing media every 24 hours. On day four cells were placed into low serum media [1% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta

biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen)] with BrdU. On day five of culturing, cells were transfected in low serum media without antibiotics and BrdU using procedure outlined below.

Plasmid isolation and quantitation:

Competent *E. coli* DH-5α bacteria were obtained from Gibco-Brl. DH-5α cells were transformed with 10ng of plasmid and were grown on Lueria agar (sigma) + ampicilin at 100µg/mL (Invitrogen), Bluo-Gal plates. The plates were incubated at 25°C for 36h and positive colonies were picked and grown up in 100mL of Lueria Broth + 100µg/mL ampicilin with chloramphenicol amplification (16µg/mL) overnight at 37°C with shaking (225rpm). Plasmids were isolated by the Endotoxin free Maxi kit (Qaigen) and were checked for fidelity via restriction endonuclease digestion followed by agrose gel electrophoresis. DNA concentrations were determined either by OD260 on a Milton-Roy Spectronic 620 or by 4',6-Diamidino-2-phenylindole (DAPI, Hoechst) on a Hoefer TK-100 DNA fluorometer as described by the manufacturer's protocols.

Transfection of rat neonatal cardiomyocytes:

RCM cultures were fed as above. Transfection of myocytes was accomplished using a liposome-based method, GeneShuttle-20 (Quantum), following the manufacturer's protocol with pCMVβ (Clontech). pCMVβ is a high copy number plasmid with bacterial β-galactosidase under mammalian cytomegalovirus constitutive promoter control and was used as a positive transfection control. Cells were transfected for 6h then placed in low serum media with antibiotics.

Detection of transfected cardiomyocytes:

Twenty-four hours after transfecting, the efficiency was determined using the β -Gal staining kit (Roche) following the manufacture's directions. After fixation and staining for cells producing bacterial β -galactosidase, random areas of the culture wells were counted using a Nikon phase-contrast inverted scope and at least three fields per well were evaluated.

RESULTS

To identify hypertrophic stimulus we employed plasmid constructs containing a promoter region known to be up regulated during hypertrophy coupled with a reporter gene that produces either β -galactosidase or luciferase. These plasmid constructs are outlined in Table 1 by their source, expressed protein and hypertrophic gene group. To determine efficiency by which our plasmid constructs could be introduced in RCM, I histochemically stained the cells for β -galactosidase activity after transfection with the positive control vector pCMV β . Histochemically positive cells for β -galactosidase activity stain blue and can be viewed in Figure 1. A relatively low number of blue histochemically positive cells are surrounded by high number of unstained cardiac myocytes and fibroblasts (Figure 1). Quantitative results of transfection efficiencies at various concentrations of liposome reagent and plasmid DNA are depicted in Figure 2. Transfection efficiencies ranged from 1.74% in 1 μ g plasmid DNA/3.5 μ L of GeneShuttle-20 to 6.56% in 1 μ g of plasmid DNA/ 5 μ L of GeneShuttle-20 (similar to other studies, see Eizema *et al.* 1999). Attempts to optimize the transfection procedure through varied ratios of DNA to liposome failed to produce results higher than 6.56% in the 1 μ g of plasmid DNA/ 5 μ L of GeneShuttle-20 group (Figure 2). Percent cardiac myocyte and fibroblasts were determined functionally (i.e. beating) to be $\geq 85\%$ cardiomyocyte (data not shown).

DISCUSSION AND CONCLUSION

The percentage of transfected cells never reached above 6.56% even with attempts to optimize the gene transfer. These results are consistent with published results of a number of liposomal methods (Eizema *et al.* 1999). These data represent total transfected cells, however, while the percentage of cardiac myocytes is high, it cannot be ruled out that transfection of non-myocyte cell types had occurred. Taking into consideration the very low transfection efficiency seen and the unknown cell type, I was unable to support the utility of liposome transfection in my approach to elucidating acute PM constituent effects (i.e. hypertrophic stimulation). Hence, I decided to move on to proteomic and genomic technologies as a comprehensive means to determine global pathological effects of PM constituents on RCM cultures.

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Table 1 Hypertrophic Gene Reporter Constructs

Construct	Expressed Protein	Source (institution)	Hypertrophic Gene Group ¹
pCMV β	β -Galactosidase	Clontech	(+) Transfection control
rat ventricular-myosin light chain-2	Firefly luciferase	Dr. Chien (UCSD)	Structural
β -myosin heavy chain	Firefly luciferase	Dr. Baldwin (UC at Irvine)	Fetal cardiac
α -myosin heavy chain	Renella luciferase	Dr. Baldwin (UC at Irvine)	Fetal cardiac
rat-atrial natriuretic factor	Firefly luciferase	Dr. Chien (UCSD)	Fetal cardiac
hypoxia inducible elements	Firefly luciferase	Dr. Schmedtje (U of Texas)	Fetal cardiac
skeletal- α -actin	Firefly luciferase	Dr. Schneider (Baylor U.)	Fetal cardiac
β -myosin heavy chain	Firefly luciferase	Dr. Schneider (Baylor U.)	Fetal cardiac
fos-serum response element	Firefly luciferase	Dr. Schneider (Baylor U.)	Immediate early
delta-56-fos	Firefly luciferase	Dr. Schneider (Baylor U.)	Immediate early
¹ , hypertrophic gene group refers to genes that are temporally up-regulated following stimulation of hypertrophy (see introduction).			

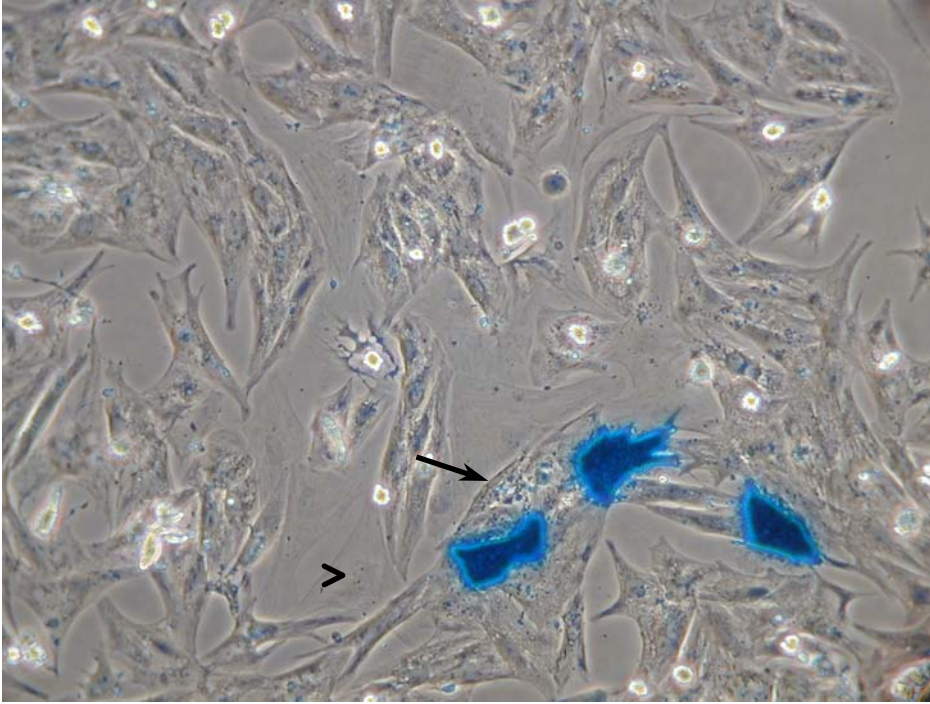


Figure 1: Transfection of RCM by pCMV β . Typical rat neonatal cardiomyocyte culture showing cells histochemically positive for β -galactosidase activity in blue. Low transfection efficiency is evident by the large numbers of unstained myocyte and non-myocyte cells. The arrow indicates a cardiac myocyte and the pointer indicates a cardiac fibroblast.

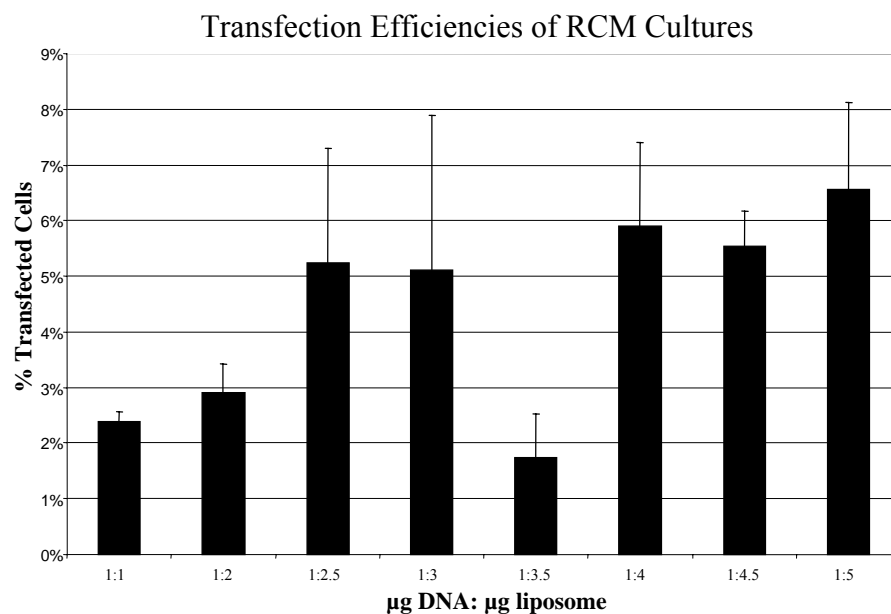


Figure 2: Percent pCMV β transfected cells. Transfection efficiencies based on β -gal positive cells of at least 100 cells per well. Ratios represent the amount of DNA in μg per μg of liposome. Control wells of liposome with no DNA were negative for staining and are not shown. Data is represented as percent-transfected \pm standard deviation.

Appendix II

MECHANISTIC INSIGHT INTO RESIDUAL OIL FLY ASH-INDUCED CARDIOMYOCYTE CYTOTOXICITY

ABSTRACT

Epidemiological studies have shown robust relationships between fine ambient air pollution particulate matter (PM_{2.5}) and cardiovascular morbidity/mortality in susceptible subpopulations. PM mortality has been associated with specific sources, however, the specific cardiovascular effects a given source has remain to be clarified. PM has been associated with alterations in autonomic control, arrhythmogenesis, and myocardial infarction. The mechanism(s) by which PM affects the cardiovascular system at the organ and cellular levels are unknown. One mechanism by which PM mediates its cardiac effects is the direct interaction between cardiac myocytes with PM constituents, which become systemically released following particle deposition in the lung. We have previously established that constituents of the combustion source particle residual oil fly ash (ROFA) can be found in the plasma of rats following pulmonary exposure, making them available to perfused tissues and organs. Using *in vitro* model of rat neonatal cardiac myocytes (RCM), we also have shown that constituents of ROFA induce cell death. However, the mechanisms responsible for cardiomyocyte cell death are unknown. Therefore in this study, I set out to determine the molecular pathway by which ROFA bioavailable constituents induce cytotoxicity in RCM. Molecular insight into the cellular effects of combustion source bioavailable constituents could provide critical insight into the mechanistic information as to how PM impacts the cardiovascular system.

INTRODUCTION

Epidemiological studies have shown an association between fine particulate matter, PM_{2.5}, and cardiovascular effects in susceptible subpopulations. Increases in daily cardiac mortality have been statistically associated to specific sources (Pope *et al.* 1996, Laden *et al.* 2000, Peters *et al.* 2004, Dockery *et al.* 2004) and long term exposure to PM_{2.5} has been associated with mortality by ischemic heart disease, heart failure, and cardiac arrest (Pope *et al.* 2004). However, the mechanism(s) by which PM affects the cardiovascular system remain to be identified (Brook *et al.* 2004). One such mechanism is direct cardiac effects by constituents of PM impacting the heart and cardiovascular system after deposition and dissolution in the lung (Costa and Dreher 1997). Animal studies have linked particle constituents to adverse cardiac endpoints such as arrhythmogenesis and cardiac pathology (Campen *et al.* 2003, Kodavanti *et al.* 2003). Based on previous work in our laboratory, we propose that soluble constituents of PM directly affect the cardiovascular system following their deposition and dissolution in the lung. The impact of this systemic exposure of PM constituents on the cardiovascular system at the cellular level is not known. We have also linked soluble constituents of ROFA to cell death in a culture model of rat neonatal cardiac myocytes. In this study, we attempted to evaluate the cellular mechanism(s) responsible for ROFA-leachate (ROFA-L) induced cardiotoxicity.

MATERIALS AND METHODS

Animals:

Female 60-90 day old Sprague-Dawley rats were purchased from Charles River Laboratory at gestation day 19. Animals were given food and water *ad libitum*. After birth neonatal pups were used after one day. Animals were housed in an AAALAC approved facility and handled according to federal guidelines.

Cell culture:

RCMs were obtained from 1-day-old neonatal rat pups using the neonatal rat cardiomyocyte isolation kit (Worthington) with modifications as follows. Minced heart tissue fragments were incubated overnight with trypsin in a HEPES simple salt solution (120mM NaCl, 3mM KCl, 1mM NaH₂PO₄, 25mM HEPES, 0.0001% Phenol Red, in sterile ddH₂O, pH 7.35). After trituration and centrifugation, cells were resuspended in high serum media [10% fetal bovine serum (Invitrogen), medium 199 with hank's salts and l-glutamine (Atlanta biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen), 0.1mM Bromo-deoxy-Uridine (BrdU)] and preplated onto a 150cm² tissue culture flask (Becton Dickenson) for two hours at 37°C, 1-2%CO₂. After preplating, cardiomyocytes were recovered. Total cell counts and viability were determined by trypan blue dye (0.4%, Sigma) exclusion using a hemacytometer. Alive cells were plated at a density of 170,000 cells/cm² in either 6-well laminin coated culture plates, 35mm laminin coated dishes (BD BioCoat, Becton Dickinson), or 2-well permanox culture slides (Nunc) coated with 1µg laminin (sigma)/cm². On days 1-3, cells were maintained in high serum media, changing media every 24 hours. On day four cells were placed into low serum media [1% fetal bovine serum (Invitrogen), medium 199 with hank's salts and

l-glutamine (Atlanta biologicals), 100 units Penicillin/100µg Streptomycin (Invitrogen)] with BrdU. On day 5 of culturing, cells were placed into low serum media no BrdU. On day six cultures were fed 3 hours prior to exposure with low serum media no BrdU.

ROFA exposure:

In vitro studies were conducted with bulk collected PM_{2.5} ROFA, as described previously, (Hatch *et al.* 1985; Dreher *et al.* 1997; Costa and Dreher, 1997). RCM cultures were exposed to various concentrations of a particle free leachate of ROFA (ROFA-L). Briefly, a 5mg/mL stock suspension of ROFA was prepared in sterile saline and mixed for 10min at room temperature. The suspension was centrifuged at 17,000xg in an eppendorf microfuge. The recovered leachate was filtered through a 0.2µm PETE filter (Poretics Products). Aliquots of the 5mg/mL ROFA-L were added to RCM cultures to yield final concentrations of 12.5, and 25µg/mL. The positive control for apoptosis induction was staurosporine, an activator of the mitochondrial pathway of apoptosis. Stock solutions of staurosporine were made in sterile DMSO at 50µM and RCM cultures were exposed to 0.5µM. Final DMSO concentrations did not exceed 0.1%.

Apoptosis detection:

After 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 in the manufacturer's documentation except for 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 agarose gel loading of the sample. DNA was visualized on a 1% agarose gel post-stained with Sybr Gold (Molecular Probes) at 1:5000 for 45min.

RESULTS

Results from RCM exposed to ROFA-L at 25, 12.5, and 6.25 μ g/mL for 24h and analyzed for evidence of apoptosis by the Quick Apoptosis DNA Ladder Detection Kit indicate DNA fragmentation (Figure 1). The presence of a 200bp banding pattern of DNA for staurosporine and ROFA-L, but not for saline is indicative that these treatments induced apoptosis. ROFA-L treatment also appears to induce apoptosis in a dose-response manner as indicated by the brighter banding in 25 μ g/ml vs. 12.5 and 6.25 μ g/mL. However, these results were found not to be reproducible since DNA fragmentation occurred in only one out of four experiments (data not shown). Additional evaluation of apoptosis was performed with a cardiac specific *in situ* terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay, CardioTACS. The results from four separate experimental exposures of saline, staurosporine, and ROFA-L at 25 and 12.5 μ g/mL at 24h can be seen in Figure 2, Panels 1-4, respectively. Nuclei positive for apoptosis stain blue. Control saline exposure (Panel 1 of Figure 2) was slightly positive for some apoptotic cell death. The positive control, staurosporine, was 100% positive for apoptotic nuclei as seen in Figure 2, Panel 2. However, RCM exposure to ROFA-L at concentrations of 25 μ g/mL and 12.5 μ g/mL show no increases in apoptotic cells as indicated by the lack of blue nuclei in Figure 2, Panels 3 and 4.

DISCUSSION AND CONCLUSION

Exposure to bioavailable constituents of the oil combustion particle ROFA induces cytotoxicity in RCM, however, the mechanism is unknown. Insight into the mechanisms of ROFA-L induced cytotoxicity would provide valuable molecular insight into how PM constituents affect the heart at the cellular level. Generally, cell death can occur by two different mechanisms, necrosis and apoptosis. Apoptosis can be detected by a variety of methods including; cellular morphology by transmission electron microscopy, DNA fragmentation at 200bp intervals by either TUNEL or agrose gel electrophoresis, caspase activation and annexin V/propidium iodide staining. In this study I examined the mechanism of ROFA-L induced cell death by an *in situ* TUNEL assay and DNA agrose gel electrophoresis. Figure 1 depicts DNA fragmentation as shown by 200bp size fragments, which are indicative of apoptosis. These data suggest that ROFA-L exposure led to an apoptotic mechanism of death. However, three additional replications of this experiment showed no positive results. This leads us to believe that the data representing Figure 1 was either incorrect or RCM cell death due to ROFA-L exposure is a mixed response containing both apoptosis and necrosis and apoptosis could not be detected on the other gels. Attempts to validate these results with an *in situ* TUNEL assay, CardioTACS, gave only negative results (Figure 2). The data presented in Figure 2 suggests that necrosis is the mechanism through which cardiac myocytes die from ROFA-L exposure. Given that only one gel out of four was positive for laddering and could not be validated by a separate assessment of apoptosis, it is concluded that the mechanism of cell death due to ROFA-L exposure is most likely

necrosis. However, more investigation is needed to definitively determine what mechanism of cell death ROFA-L induces in RCM cultures.

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