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PM_{2.5} exposure in utero contributes to neonatal cardiac dysfunction in mice

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Abstract

Objective—Exposure of fine particulate matter (PM_{2.5}) to pregnant dams has been shown to be strongly associated with adverse cardiovascular outcomes in offspring at adulthood, however, effects evident during neonatal periods are unclear. We designed this study to examine cardiac function of neonatal mice (14 days old) exposed to in utero PM_{2.5}.

Methods—Pregnant FVB female mice were exposed either to filtered air (FA) or PM_{2.5} at an average concentration of 91.78 µg/m³ for 6h/day, 5 days/wk (similar to exposure in a large industrial area) throughout the gestation period (20 days). After birth, animals were analyzed at day 14 of life.

Results—Fourteen day old mice exposed to PM_{2.5} during in utero period demonstrated decreased fractional shortening (%FS, 41.1±1.2% FA, 33.7±1.2% PM_{2.5}, p<0.01) and LVEDd (2.87±0.08 mm FA, 2.58±0.07 mm PM_{2.5}, p<0.05) compared to FA exposed mice. Contractile kinetics and calcium transients in isolated cardiomyocytes from PM_{2.5} exposed mice illustrated reduced peak shortening (%PS, 16.7±0.5% FA, 14.7±0.4% PM_{2.5}, p<0.01), negative contractile velocity (-dL/dT, -6.91±0.3 µm/s FA, -5.46±0.2 µm/s PM_{2.5}, p<0.001), increased time to relaxation 90% (TR90, 0.07±0.003 s FA, 0.08±0.004 s PM_{2.5}, p<0.05), decreased calcium transient amplitude (340/380, 33.8±3.4 FA, 29.5±2.8 PM_{2.5}) and slower fluorescence decay rate (τ, 0.72±0.1 s FA, 1.16±0.15 s PM_{2.5}, p<0.05). Immunoblotting studies demonstrated alterations

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in expression of Ca^{2+} handling proteins- SERCA-2A, p-PLN, NCX and $\text{Ca}_v1.2$ in hearts of 14 day old in utero $\text{PM}_{2.5}$ exposed mice compared to FA exposed hearts.

Conclusion— $\text{PM}_{2.5}$ exposure during the critical in utero period adversely affects the developing mouse fetus leading to functional cardiac changes that were evident during the very early (14 days) stages of adolescence. These data demonstrated that exposure to $\text{PM}_{2.5}$ during the gestation period significantly impacts cardiovascular outcomes early in life.

Graphical abstract

Keywords

particulate matter; in utero; cardiovascular function; myocyte; calcium signaling; heart failure; adolescence

Introduction

It has been estimated that environmental factors account for significant risks of developing cardiovascular diseases (CVDs)¹. Ambient particulate matter (PM) pollution has gained significant attention as a global health concern in recent years after being ranked as the 9th cause of overall disease burden². In particular, $\text{PM}_{2.5}$ (ambient particles with diameters of $<2.5 \mu\text{m}$, $\text{PM}_{2.5}$) exposure contributes to significant CVD burden³. $\text{PM}_{2.5}$ exposure is associated with CVDs, including arrhythmias, hypertension, myocardial infarction and remodeling^{4, 5}. Since $\text{PM}_{2.5}$ inhalation adversely impacts cardiovascular function, it is becoming increasingly crucial to gain understanding of the effects of $\text{PM}_{2.5}$ on the developing fetus. Significant evidence has accumulated concerning the adverse pregnancy outcomes and infant health following air pollution exposure^{6, 7}. An understanding of developmental plasticity provides a conceptual basis for these observations⁸. There is also substantial evidence suggesting a positive correlation between particulate air pollution exposure and adverse cardiovascular outcomes during the postnatal periods^{9, 10}. In another study, cardiomyocytes cultured in media from PM-treated lung epithelial cells demonstrated contractile dysfunction¹¹. The multitude of $\text{PM}_{2.5}$ effects (both direct and indirect) on the cardiovascular system are also well reviewed by others^{12, 13}. These studies are suggestive that $\text{PM}_{2.5}$ exposure is associated with critical biological and cardio-developmental

mechanisms leading to harmful effects later in life. However, this is the first study demonstrating the potential association between in utero exposure to ambient air pollution and neonatal cardiac function.

In our previous work, we demonstrated the role of PM_{2.5} exposure during developmental periods in influencing an adult disease phenotype¹⁴, however, it did not determine whether PM_{2.5} exposure during the in utero period is equally detrimental to neonatal cardiac function. Here, we hypothesized that PM_{2.5} in utero exposure can influence cardiac function in neonatal mice (14 day old). The purpose of the present study was to examine the impact of in utero PM_{2.5} exposure on cardiac function in neonatal mice and to further study potential cellular and molecular mechanisms mediating these harmful cardiac effects.

Materials and Methods

Animals and PM_{2.5} exposure

All animal experiments were approved and performed according to NIH guidelines under an Institutional Animal Care and Use Committee (IACUC) protocol (IACUC#: 2013A00000074) at The Ohio State University, Columbus, Ohio. Male and female mice of the FVB strain were housed for 1 week in our facility before breeding and the presence of a vaginal plug was considered as confirmation of pregnancy. Once pregnancy was confirmed, we exposed pregnant dams to either PM_{2.5} or FA for 6 hrs/day, 5 days/week throughout pregnancy (20 days). The aerosol concentration system located at the Ohio State University⁵ was used for the concentrated PM_{2.5} exposure from the Columbus, OH region. The average PM_{2.5} concentration that the dams were exposed to was 91.78 µg/m³. Since the exposures were performed for 6 h/day, 5 days/week, the 24-h average PM_{2.5} concentration was 22.94 µg/m³, which was below the daily national ambient air quality standard (NAQS) of 35 µg/m³ set by the current U.S. Environmental Protection Agency (U.S. EPA, 2012). An identical system was used for the FA exposure except that a HEPA filter at the inlet to the system was used to remove all ambient particles. After birth, both group of pups were placed in room air until day 14. At 2 weeks of age, all experiments were conducted on male mice as described below.

Echocardiographic assessment

At 14 days of age, *in vivo* cardiac function was determined (40 MHz transducer, Vevo 2100; Visualsonics *In Vivo* Imaging System (Toronto, ON, Canada). Internal body temperature was maintained at 37°C under 1.5-2% isoflurane (delivered in 100% O₂) anesthesia supplied through a nose cone. Following induction of anesthesia, the chest fur was removed using a hair removal cream. Ultrasound gel (prewarmed) was placed on the chest and a 15 MHz (optimized for mice) probe placed in the parasternal, short axis orientation. Three cine loops of M mode data were captured per mouse and analyzed for LV end-systolic and end-diastolic internal dimensions (LVESd and LVEDd), as well as systolic and diastolic posterior wall thickness (PWTs and PWTd). Percent fractional shortening (%FS) was calculated using the equation: %FS = [(LVEDd - LVESd)/LVEDd * 100]. LV end diastolic volume (LVEDV) was calculated from LVEDd ($7/(2.4 + LVEDd) * LVEDd^3$). LV end systolic volume (LVESV) was calculated from LVESd ($7/(2.4 + LVESd) * LVESd^3$) and ejection fraction (EF = (LVEDV -

LVESV)/LVEDV*100). Stroke volume was calculated by subtracting LVESV from LVEDV (LVEDV-LVESV). Cardiac output was calculated from stroke volume multiplied by heart rate (SV*HR). An average of three cardiac cycles per mouse was used for data interpretation. Analyses of all the parameters were performed according to the AHA technique by an investigator blinded to group assignment.

In vitro assessment of cardiomyocyte function

At 14 days of age, mice were euthanized and hearts were harvested for myocyte isolation using enzymatic digestion (Liberase; Roche, Indianapolis, IN) through coronary retrograde perfusion via the aorta using a standard protocol and contractile kinetics were assessed using a Myopacer Field-Stimulator system (IonOptix, Milton, MA) as described previously⁵. The following functional indices were determined: basal sarcomere length, % peak shortening normalized to sarcomere length (%PS; in vitro equivalent of %FS), time-to-90% PS (TPS) and time-to-90% relengthening (TR90), maximal velocities of shortening (+dL/dt) and relengthening (-dL/dt).

Measurement of intracellular Ca²⁺ transient

Ca²⁺ transients were measured to identify alterations in Ca²⁺ cycling using the Ionoptix system as listed in our previous publication⁵. Fura-2 fluorescence was used to monitor basal Ca²⁺, the amplitude of the Ca²⁺ transient (340/380), time to peak transient, and the maximum rate of rise and decay of the Ca²⁺ transient. Fluorescence decay rate (tau) was used as an indication of the intracellular Ca²⁺ clearing rate.

Quantitative Real-Time PCR

Total RNA was isolated from snap frozen heart tissue of 14 day old neonates using the RNeasy kit (Qiagen, Hilden, Germany) and concentration was determined using a NanoDrop 2000c (ThermoScientific, Wilmington, DE). 1 ng of RNA was reverse transcribed to generate cDNA using the iScript Supermix kit (Bio-Rad, Hercules, CA) on a CFX96 Thermocycler (BioRad, Hercules, CA). Primers were used at a final concentration of 0.25-0.5 µM for target genes and normalized to *GAPDH* expression. Relative gene expression levels were quantified using the formula 2^{-Ct} ¹⁵. Gene-specific primer sequences are presented in Table 1. The following three step amplification protocol was used for amplification: denaturation step at 95°C for 10 minutes followed by 45 cycles of denaturation (95°C, 1 second), annealing (65°C, 10 seconds), and extension (72°C, 20 seconds).

Western blotting

Frozen tissues from 14 day old hearts were homogenized in lysis buffer similar to our previously published work⁵. Tissue homogenates were spun at 14,000 rpm for 10 minutes and supernatants were collected as total protein samples. 20 µg of total protein was loaded per well. Proteins were separated by SDS-PAGE gel and transferred to nitrocellulose membranes. Primary antibodies recognizing Na⁺/Ca²⁺ exchanger (NCX) and sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA-2A) (Thermo Fisher Scientific, MA3-926 and MA3-919 Waltham, MA, respectively), -phosphorylated phospholamban (p-PLN) (Santa

Cruz Biotechnology, sc-12963, Dallas, TX) and L-type voltage-dependent calcium channel, alpha 1C subunit (Ca_v1.2) (Millipore, MAB 13170, Temecula, CA) were used. Band density of the protein of interest was normalized to beta actin (Sigma, A1978, St.-Louis, MO). A signal was detected using the enhanced chemiluminescence method (PIERCE, SuperSignal West Pico, Chemiluminescent Substrate, CA). Films were scanned and analyzed using Image Lab software (Bio-Rad, Hercules, CA, version 4.1).

Statistical analyses

All data were analyzed for statistical significance using GraphPad Prism 5 (GraphPad Software, Inc.; San Diego, CA, USA). Differences between two groups were determined using a Student's t-test (two-tailed) with an α -value of 0.05. All error bars in the figures represent standard error of the mean; *, **, *** represent significant differences of $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

Results and Discussion

PM_{2.5} exposure in utero results in altered birth weight and body weight in neonatal mice

We assessed maternal weight gain and fetal birth weight to evaluate the effects of PM_{2.5} exposure on fetal development. We found no difference in weight gain (throughout pregnancy) or body weight of dams exposed either to FA or PM_{2.5} after 20 days of pregnancy. We found lower birth weights in pups that were born to PM_{2.5} exposed dams compared with pups born to FA exposed control dams, however, this was not significant. There was no significant difference in absolute heart weight between both groups at day 14 (Table 2). Birth weight is an important indicator of offspring health¹⁶, and low birth weight (LBW) is often associated with higher mortality risk, childhood and adulthood morbidity and diseases¹⁷. In agreement with our result, there were various epidemiological studies that have also linked PM exposure to LBW¹⁸⁻²¹. However, at 14 days of age pups that were born to PM_{2.5} exposed dams had significantly higher body weights compared to pups born to FA exposed dams. This observation is in agreement with our previous work where we demonstrated that mice exposed during the combined in utero and perinatal periods to PM_{2.5} were born with LBW, but had significantly higher body weights at adulthood⁹. Similarly, it has also been shown that infants exposed to traffic-related pollution during early life gained postnatal weight despite reduced fetal growth⁷. In response to any harmful pollutant exposure during fetal life, growth restriction manifested as LBW is a common observation. After birth, when maternal exposure is discontinued, the neonatal body tries to recover from the compromised state resulting from adverse in utero exposure. The observed higher body weight in PM_{2.5} pups could be a result of this compensation and there is a possibility that group differences in birth weight may fade with time as neonates grow into adults. On the contrary, it has also been shown that there was no significant reduction in body weight of two day old pups born to mothers exposed to diesel exhaust during gestation²², however, at 6 and 8 weeks of age body weights were significantly lowered.

Neonatal mice exposed in utero to PM_{2.5} exhibited in vivo cardiac dysfunction

Fourteen day old mice exposed to in utero PM_{2.5} in utero demonstrated reduced %FS and LVEDd compared to FA exposed mice (Fig.1C and 1D). We did not observe any differences

in LVESd, PWTs and PWTd (Fig. 1E-1G). Further, we observed significant decrease in LVEDV, percent ejection fraction and cardiac output but no difference in the heart rates between both groups (Table 3). The magnitude of cardiac dysfunction observed in in utero PM_{2.5} exposed neonatal mice was similar to that seen in adult animals exposed to PM_{2.5} during the combined in utero and perinatal periods⁹. In utero exposure to higher particulate concentrations of diesel exhaust can also increase susceptibility to heart failure as shown previously^{23, 24}. Decreased LVEDd indicated ventricular stiffness or altered distensibility commonly seen in heart failure with preserved ejection fraction²⁵. Myocardial stiffness can either be due to inability of the left ventricle to fully relax during diastole or composition and thickness of the ventricular wall.

***In utero* exposure to PM_{2.5} impairs cardiomyocyte function, calcium transient amplitude and fluorescence decay rate in neonatal mice**

We next analyzed isolated cardiomyocyte function in order to examine cell-specific mechanisms. Cardiomyocytes isolated from fourteen day old mice exposed to PM_{2.5} in utero showed changes in sarcomere function evident at the cellular level, as indicated by a reduction in %PS (Fig. 2A), -dL/dT (Fig. 2C) and increased TR90 (Fig. 2E). +dL/dT and TPS90 were not different between groups (Fig. 2B and 2D). Further, we also observed delayed calcium transient amplitude (340/380, Fig. 3A) and fluorescence decay rate as indicated by increased tau (T) duration compared with the FA group (Fig. 3B).

Thus, data obtained at the cardiomyocyte level showed compromised contractile function confirming early contractile changes in line with the in vivo echocardiography data. These results demonstrate functional consequences of intracellular calcium handling within the contractile machinery of the heart. The prolongation in TR90, tau (T) and reduced calcium transient amplitude further indicated diastolic dysfunction. In failing or stressed myocardia, these effects are observed when there are alterations in calcium handling proteins in human and animal models of cardiac diseases.

***In utero* PM_{2.5} exposure is not associated with an inflammatory or pro-fibrotic response in neonatal mice**

In order to study the role of inflammation and fibrotic pathways in the current exposure paradigm, we analyzed gene markers for cardiac inflammation and fibrosis at fourteen days of age in mice exposed to FA or PM_{2.5} *in utero*. Analysis of real-time quantitative PCR of 14 day old mouse hearts exposed to PM_{2.5} revealed no differences in inflammatory markers such as interleukin-6 & 1 β (*IL-6* & *IL-1 β*), p-selectin (*CD62P*) and e-selectin (*CD62E*) (Fig. 4A-D) or fibrotic markers such as collagen-1 (*Col-1*), matrix metalloproteases 9 & 13 (*MMP-9* & *MMP-13*) (Fig. 4 E-G).

***In utero* PM_{2.5} exposure alters expression of calcium handling proteins in neonatal mouse hearts**

Our in vivo and in vitro functional results demonstrated cardiac dysfunction. Because of the potential importance of Ca²⁺ handling proteins in maintaining efficient myocardial function, we analyzed their protein expression levels. Western blot analyses showed that cardiac sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA-2A) and phosphorylated phospholamban (p-

PLN) were slightly increased, whereas sodium-calcium exchanger (NCX), L-type voltage-dependent calcium channel, and the α_1C subunit ($Ca_v1.2$) were significantly decreased in fourteen day old neonatal in utero $PM_{2.5}$ exposed mice compared to FA exposed mice (Fig.5A & 5B).

It is well known that the contractile machinery of cardiac muscle is activated by calcium that is released from the sarcoplasmic reticulum (SR) on muscle stimulation²⁶. The transient rise in intracellular Ca^{2+} during muscle contraction is the result of the release of Ca^{2+} from the SR, its binding to various intracellular constituents and its resequestration by the SR²⁷. The contractile response of the cardiomyocyte is therefore linked to the intracellular Ca^{2+} transient, such as the peak amplitude and/or the duration of the Ca^{2+} transient. In animal and human models of heart failure, expression of SERCA-2A and p-PLN was decreased²⁸⁻³². In our study, we observed modest increased SERCA-2A expression and simultaneous increased SERCA-2A inhibitory protein PLN suggesting that both proteins regulate each other, as previously reported³³. Similar results were also observed in a model of maternal inflammation and neonatal hyperoxia³⁴. In general, PLN regulated increased SERCA-2A expression, resulting in improved calcium cycling and optimal cardiomyocyte functioning. However, the modest increase in the expression of these proteins was not sufficient to maintain optimum cardiac function in neonatal mice. The altered functional parameters can further be explained on the basis of two other important calcium handling proteins-NCX and $Ca_v1.2$.

NCX is an integral cellular mechanisms for removing Ca^{2+} from the cell in exchange for sodium³⁵⁻³⁷. We speculate that decreased NCX expression impairs this Na^+-Ca^{2+} exchange leading to accumulation of intracellular calcium and hence impaired relaxation as observed in in utero $PM_{2.5}$ exposed neonates. The decrease in NCX expression is also a common feature in conditions of increased myocardial stress, as previously reported^{35, 38, 39}. Another key regulatory protein involved in maintaining Ca^{2+} homeostasis is $Ca_v1.2$, which passes inward Ca^{2+} current and triggers calcium release from the SR^{40, 41}. Previous studies have shown that decreased expression of $Ca_v1.2$ is associated with cardiac hypertrophy and heart failure⁴²⁻⁴⁴. We also observed decreased expression of $Ca_v1.2$ in neonatal hearts exposed in utero to $PM_{2.5}$ suggesting decrease calcium influx in the cell. This further led us to speculate that both of these proteins are attempting to maintain calcium homeostasis by regulating each other's expression in order to restore intracellular calcium. Therefore, we further speculated that increases in SERCA-2A and p-PLN might also be a result of direct activation of compensatory mechanisms to increase SR calcium uptake in response to elevated intracellular calcium concentration. Despite compensatory increases in SERCA-2A and decreases in $Ca_v1.2$ expression, we observed delayed calcium reuptake (measured by τ as fluorescence decay rate) suggesting impaired ability to remove elevated intracellular calcium. Therefore, overall alterations in the calcium handling proteins can be related to altered cardiac function observed in the present study.

The mechanism by which inhaled $PM_{2.5}$ can elicit alterations in calcium homeostasis proteins remains unclear. There are various regulatory mechanisms such as transcriptional and post-translational modifications, protein-protein interactions, and microRNAs which are known to regulate expression of calcium regulatory proteins. Transcription of the *ATP2a2*

gene is essential for SERCA-2A protein expression and several transcription factors bind to the *ATP2a2* promoter region to properly regulate its transcriptional activity^{45, 46}. In addition, it is well established that the activity of SERCA-2A is regulated by PLN. Interestingly, heat shock protein 20 is found to be a novel regulator of SERCA-2A by targeting PLN⁴⁷. Further, studies have shown that protein kinase C (PKC) and A (PKA) family of kinases also play essential roles in the regulation of PLN⁴⁸ and Cav1.2⁴⁹ in the heart. Besides PLN, sarcolipin is also known to regulate SERCA-2A⁵⁰. Various posttranslational modifications are also known to regulate SERCA-2A protein activity⁵¹. Recent studies have identified microRNAs as novel regulators of post-transcriptional gene expression and protein translation, and their role in CVDs is becoming increasingly evident. Comprehensive analysis of miRNA regulation of Ca²⁺ handling processes is reviewed by Harada et al., 2014⁵². In our system, it remains to be determined whether these mechanisms are involved. Future research is therefore needed that will elucidate particular mechanisms that cause alterations in protein expression in response to particulate matter exposure.

In conclusion, our results indicate that neonatal mice exposed during the in utero period to PM_{2.5} at an average concentration of 91.78 µg/m³ for 6h/day, 5 days/wk during gestation developed cardiac dysfunction owing to alterations in calcium handling proteins. While our previous studies were aimed at studying the effects of early life PM_{2.5} exposure on adult cardiac function⁹, the present study has shown that in utero PM_{2.5} exposure is equally detrimental to neonatal cardiac function, through a mechanism that involves calcium deregulation. The presented mechanisms are suggestive of the fact that neonates are equally and adversely affected by air pollution. The neonatal susceptibility to developing adulthood diseases needs to be given close consideration when air pollution regulations are designed in the favor of public health protection. Our results further provide a conceptual basis for susceptibility of adults to developing cardiac disease very early in life, thereby emphasizing the need for the maintenance of acceptable level of air pollution worldwide.

The study has notable strengths in its exposure modeling (mimicking exposure in a large metropolitan cities). The PM_{2.5} concentration achieved in this study is below the current EPA acceptable level. Interestingly even at this concentration, PM_{2.5} can elicit adverse cardiac effects. Most notably, in the highly polluted cities of Latin America, China and India, the daily PM_{2.5} levels range from 100 to 150 µg/m³ which is approximately 10- to 15-fold higher than concentrations found in metropolitan US cities⁵³⁻⁵⁵. Therefore, we believe our results provide substantial information on PM_{2.5} levels which can prove beneficial in making future regulatory guidelines addressing environmental exposures and human health worldwide. However, it is difficult to comment completely on what an alteration in cellular mechanisms means in a neonate owing to the complexity of ongoing postnatal developmental mechanisms hence recognizing the need for further research.

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Highlights

- PM_{2.5} exposure during the *in utero* period exerts harmful effects on the developing fetus.
- This study is the first to study effects of *in utero* PM_{2.5} exposure on neonatal mice.
- Exposure to *in utero* PM_{2.5} leads to neonatal cardiac dysfunction.
- *In utero* PM_{2.5} exposure alters Ca²⁺ handling protein expression leading to functional impairments.

Figure 1.

Transthoracic echocardiography demonstrating cardiac function in fourteen day old mice that were exposed to FA or PM_{2.5} in utero. Representative M mode images obtained from FA-exposed mice (A) and PM_{2.5}-exposed mice (B). C. % Fractional shortening (%FS), D. LV end-diastolic dimension (LVED_d), E. LV end-systolic dimension (LVES_d), F. Posterior wall thickness during diastole (PWT_d), G. Posterior wall thickness during systole (PWT_s). Data are collected from 5-8 mice in each group. Five beat cycles were captured and three loops averaged per assessment. Results are mean $\pm$ S.E.M. ** $p < 0.01$ vs FA controls.

Figure 2.

In vitro cardiomyocyte functional parameters obtained from fourteen day old mice that were exposed to FA or PM_{2.5} in utero. Graphs depicted are representative of A. %Peak shortening (%PS), B. Positive velocity (+dL/dT), C. Negative velocity (-dL/dT), D. Time-to-peak shortening (TPS90), E. Time-to-90% relengthening (TR90). Data are collected from 40-45 cardiomyocytes isolated from 5 mice per group taken from individual litters. Results are mean ± S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs FA controls.

Figure 3.

Calcium transient amplitude and fluorescence decay rate (Tau) of isolated cardiomyocytes from fourteen day old mice that were exposed to FA or PM_{2.5} in utero. A. Calcium transient amplitude (Δ340/380), B. Fluorescence decay rate (τ). Data are collected from 40-45 cardiomyocytes isolated from 5 mice per group taken from individual litters. Results are mean ± S.E.M. *p<0.05 vs FA controls.

Figure 4.

Quantitative polymerase chain reaction (qPCR) analyses of RNA samples obtained from fourteen day old FA and PM_{2.5} *in utero* exposed mouse hearts (n=5 mice from each group). A. IL-6; B. IL-1 β ; C. CD62P; D. CD62E; E. Col-1; F. MMP-9; G. MMP-13. Results are mean $\pm$ S.E.M.

Figure 5.

A. Representative immune blots of Ca^{2+} handling proteins showing expression of NCX, $\text{Ca}_v1.2$, SERCA-2A and p-PLN. B. Representative graph showing quantification of NCX, $\text{Ca}_v1.2$, SERCA-2A and p-PLN relative to actin protein levels. Data are collected from 7-9 whole heart homogenate obtained from fourteen day old mice that were exposed to FA or $\text{PM}_{2.5}$ in utero.

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Table 1
Primer sequences used for PCR amplification

Gene	Forward Primer	Reverse Primer
<i>Gapdh</i>	CTCACTCAAGATTGTCAGCAATG	GAGGGAGATGCTCAGTGTGG
<i>IL-1β</i>	CCAAGAGGTGAGTGCTTCCC	CTGTTGTTTCAGACTCTCTCCCT
<i>IL-6</i>	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG
<i>CD62P</i>	CATCTGGTTCAGTGCTTTGATCT	ACCCGTGAGTTATTCCATGAGT
<i>CD62E</i>	ATGAAGCCAGTGCATACTGTC	CGGTGAATGTTTCAGATTGGAGT
<i>COL-1</i>	GCTAACGTGGTTCGTGACCGTG	GGTCAGCTGGATAGCGACATC
<i>MMP9</i>	CAGGAGTCTGGATAAGTTGGGTC	GGTACTGGAAGATGTCGTGTGAG
<i>MMP13</i>	GACAGATTCTTCTGGCGCCT	CATAACTCCACACGTGGTTCTCAG

Table 2
Parameters from mice exposed to FA or PM_{2.5} in utero

	FA	PM _{2.5}	<i>P</i> value
Birth weight, g	1.45±0.18	1.36±0.18	0.20
Body weight (14 day), g	8.08±0.43	8.49±0.63	0.05
Heart weight, mg	0.48±0.13	0.54±0.04	0.11

Table 3
Echocardiographic parameters of 14 day old in utero FA and PM_{2.5}

exposed mice	FA	PM _{2.5}	P Value
IVS _D (mm)	1.05±0.14	1.18±0.15	0.607
IVS _S (mm)	1.51±0.17	1.36±0.15	0.560
LVEDV (μl)	92.17±8.62	63.26±5.44	0.045
LVESV (μl)	15.01±2.31	14.63±0.92	0.910
Ejection fraction (%)	73.5±1.45	64.6±1.38	0.003
Stroke volume (μl)	77.16±6.59	48.63±4.77	0.016
Cardiac output (ml/min)	34.52±2.85	21.45±2.00	0.011
Heart rate (bpm)	448.25±3.21	442.2±3.24	0.276

FA, filtered air; PM_{2.5}, particulate matter (<2.5 μm diameter); IVS_D, Interventricular septal end diastole diameter; IVS_S, Interventricular septal end systole diameter; LVEDV, Left ventricular end diastolic volume; LVESV, Left ventricular end systolic volume. Data are expressed as mean±SEM.