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Traffic-Related Air Pollution Effect on Fast Glycemia of Aged Obese Type 2 Diabetic Mice

Matsuda M[1], Krempel PG[1], André PA[2], Freitas JR[3], Freitas LAR[4], Veras PST[4], Saldiva PHN[2] and Marquezini MV[2,5*]

[1]Laboratory of Investigation in Ophthalmology, University of São Paulo Medical School, São Paulo, 01246-903, Brazil
[2]Experimental Air Pollution Laboratory of Pathology Department, University of São Paulo Medical School, São Paulo, 01246-903, Brazil.
[3]Department of Pathology and Forensic Medicine, Medical School, Federal University of Bahia. Salvador, 40025-010, Brazil.
[4]Gonçalo Moniz Research Center, Oswaldo Cruz-Fiocruz Foundation and Federal University of Bahia, Salvador, 40296-710, Brazil.
[5]Pro-Sangue Foundation, São Paulo, 05403-000, Brazil

Abstract

Recent experimental data have provided associations between ambient $PM_{2.5}$ (fine particulate matter; diameter ≤ 2.5 μm) and propensity to inflammation and chronic diseases especially among susceptible groups, such as elderly people. There is cumulative evidence that type-2 diabetes mellitus is a chronic inflammatory state aggravated by factors that promote endothelium inflammation. Accordingly our hypothesis that the exposure of aged obese population to $PM_{2.5}$ might aggravate type-2 diabetes, we used a model of aged, diet-induced obese mice. C57BL6 male mice were fed with regular chow (n=30; RC) or high-fat chow (n=36; HF) during one-year and randomly assigned to filtered (FA-RC, n=16; FA-HF, n=19) or $PM_{2.5}$ concentrated air (600 μg.m^{-3}) (EXP-RC, n=14; EXP-HF, n=17) chambers to have a daily 1 hour exposition during consecutive 30- days. Fast glycemia was measured before the animals were euthanized. The Institution's Ethics Committee approved all experimental procedures. Heart mRNA content of selected migration, signalization and adhesion proteins were measured by SYBR Green fluorescence Real Time RT-PCR protocol using appropriate primers. There were no difference between RC-EXP and RC-FA nor between HF-EXP and HF-FA body weight. Regarding fast glycemia, both, RC and HF groups, were diabetic, but only the HF group was affected by acute exposure to $PM_{2.5}$ (mean ± SD, EXP-HF vs FA- HF, 172.8 ± 23.4 vs 156.7 ± 17.6, p <0.05; EXP-RC vs FA-RC, 149.8 ± 19.2, 139.7 ± 15.3, ns; ANOVA). The gene expression profile of E-selectin, IL-6, VCAM-1, ICAM-1 and MMP-9, was differently affected by $PM_{2.5}$ in heart and lung. Proteins activated by inflammatory stimuli involved in the inhibition of insulin signaling are being investigated.

Keywords: Aged; Obesity; Type 2 diabetic mice; Air pollution

Introduction

In 2011, the International Diabetes Federation reported that diabetes mellitus (DM) affects 366 million people worldwide, projecting that by 2030, to reach 566 million. Over 99% of cases represent type 2 diabetes, which is accompanied by pathophysiological abnormalities of the coronary artery and cerebrovascular system presenting, in addition, peripheral arterial disease. Thus, the increased risk of DM-2 automatically increases the risk of arterial disease [1,2].

Air-pollution exposure alters endothelial function in both animals and humans [3,4]. Alterations in endothelial function often precede changes in insulin resistance and have been implicated in reduced peripheral glucose uptake [5].

Because of the urban centers increasing growth and intensive use of vehicles engines, air pollution by particles with diameter ≤ 2.5 μm ($PM_{2.5}$), composed by sulfates, nitrates and oxides, is more frequent. This particle can reach up to the lung alveoli. Inhaling $PM_{2.5}$ contributes to increased mortality and morbidity of the population exposed, inducing lung stress [6] and causing a worsening of cardio-metabolic disease [7,8].

Aging compromises the body's ability to compensate for the effects of environmental hazards and therefore the effects of pollution are more severe in the elderly. Epidemiologic studies evidence that diabetic individuals are adversely affected by air pollution exposure [9] Obesity is the primary cause underlying the development of insulin resistance and type 2 diabetes [10] and is associated with chronically elevated plasma levels of IL-6 [11]. Studies in experimental rat models have shown that air pollution agg ravates inflammation and insulin resistance especially in obese animals [12].

Due to the thousands of people continuously exposed to $PM_{2.5}$, long term effects of this ubiquitous pollutant in the air cause major problems for the global public health [13-16].

Seeking to correlate pollution $PM_{2.5}$ with pathophysiological processes such as obesity, diabetes and aging, we chose to study the gene expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin), the extracellular matrix metalloproteinase (MMP-9) and the inflammatory cytokine interleukin-6 [17-21], in elderly and obese mice subjected to daily exposure to $PM_{2.5}$ in a controlled ambient. This set of molecules was chosen for its important role in tissue homeostasis and repair [22-24].

Materials and Methods

Animals

C57BL6 male mice were obtained and kept in the Center Gonçalo Moniz Oswaldo Cruz Foundation of Salvador – Bahia, Brazil vivarium.

After weaning, animals were divided into two groups: 1) RC, fed with a regular chow (n=30) and 2) HF, fed with high-fat diet (n=36). After a year of treatment, the aged animals, lean (group RC) and obese (HF group), were transferred to the Laboratory of Experimental Air Pollution (LPAE), FMUSP, and randomly distributed in the exposure chambers to air free of particles with filtered air (FA; n= 16, RC, n= 19, HF) or concentrate air containing $PM_{2.5}$ (EXP; n=14, RC, n=17, HF). During the exposure period, the animals were weighed daily and fasting glycemia was measured on the thirty day prior to euthanasia, on average at 14 months of age. The project was conducted in accordance with the Guide for Care and Use of Laboratory Animals (NRC, 1996) and approved by the Ethics Committee on the Use of Animals of the Oswaldo Cruz Foundation, RJ (license LW 16/09).

Exposure protocol

The animals were exposed for consecutive thirty days to 600 $\mu g.m^{-3}$ of fine inhalable particulate matter ($PM_{2.5}$) during one hour, considering the maximum load recommended by WHO (maximum daily mean concentration of 25 $\mu g.m^{-3}$) in the atmospheric particle concentrator [25] (APC) of the Medical School, USP (EXP group). As a control, another group remained under identical conditions, but with filtered air (FA), i.e., free of particles.

In the APC inlet, the external air is captured with vacuum and conditioned to remove particles over $PM_{2.5}$ by the use of a single impaction plate device with a flow of 4,000 $L.min^{-1}$. The concentration step is performed in three subsequent virtual impactors developed by the Harvard School of Public Health - United States, where the particles are accelerated while an adequate high vacuum is applied beside the flow to remove gases, but not particles due to their inertia, so the remaining air flow of 50 $L.min^{-3}$ has a high reduction on the gas volume but the same initial mass of particulate matter, increasing the concentration of $PM_{2.5}$ about 20 to 30 times the external concentration. This air flow with a high $PM_{2.5}$ concentration is then routed to feed the exposure chamber with dirty air (EXP).

Extraction of lung and heart total RNA and quantitative real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's standards. Real-time PCR was performed using the Corbett Rotor-Gene 3000 (Qiagen Valencia, CA, USA) detection system. SYBR Green I based real-time polymerase chain reaction (PCR) method was adopted for mRNA expression of migration and adhesion proteins (E-selectin, VCAM-1, ICAM-1), IL-6 (signaling protein) and MMP-9 (extracellular matrix metalloproteinase) gene expression, in heart and lung. All reactions were performed under the same condition: denatured at 95°C for 5 min, followed by 40 cycles of PCR, each cycle consisting of 95°C for 30s, 60°C for 15s, and 72°C for 30s. Table 1 describes the primers used. Each reaction was performed in triplicate and results were normalized for the expression of β-actin gene and Rotor Gene software version 6.0 was used to quantify the transcripts.

Statistical analysis

Data are expressed as mean ± CI 95% unless otherwise indicated. The results of the experiments were analyzed by one way ANOVA. A p value of <0.05 was considered statistically significant.

Results

Exposure to $PM_{2.5}$ does not alter body weight, but affects fasting glycemia

Regarding body weight, no differences were observed between FA-RC and EXP-RC nor between FA-HF and HF-EXP (Table 2 and Figure 1A).

Possibly due to age, even the lean animals exposed to filtered air showed fasting glycemia above 110 $mg.kg^{-1}$, indicating that glucose uptake by the cells was already compromised. However, only the obese animals had worsening in fasting plasma glucose after exposure to $PM_{2.5}$, i.e. the group EXP-HF presented fasting glycemia levels significantly higher than the group FA-FH (Table 2 and Figure 1B).

The gene expression profile of the proteins studied are differently affected by air pollution in heart and lung

VCAM-1 gene: Its expression was not affected in both tissues in the lean mice group (EXP- RC vs FA-RC not significant in lung and heart). However, in the obese group, we found differences between EXP-HF and FA-HF in the lung but not in the heart tissue (Figures 2B and 2A, respectively; Table 3).

Gene	Gene Bank Accession Number	Product (bp)	Forward	Reverse
VCAM-1	NM011693	75	5′ tctcccaggaatacaacgat 3′	5′ acaggtcattgtcacagcac 3′
ICAM-1	NM010493	119	5′ aaggagatcacattcacggt 3′	5′ gcctcggagacattagagaa 3′
E-Selectin	NM011345	84	5′ ccctgcccacggtatcag 3′	5′ ccttccacacagtcaaacgt3′
MMP-9	NM013599	128	5′ ggtggcagcgcacgagtt 3′	5′ ggatgccgtctatgtcgtctt 3′
IL-6	NM031168	92	5′ tttcctctggtcttctggag 3′	5′ ctgaaggactctggctttgt 3′
β-Actin	X03672	141	5′ cccaggcattgctgacagg 3′	5′ tggaaggtggacagtgaggc 3′

Table 1: Primers used in the analysis by real time RT-PCR. bp, base pair.

	Body Weight				Fast Glycemia			
	FA-RC	**EXP-RC**	**FA-HF**	**EXP-HF**	**FA-RC**	**EXP-RC**	**FA-HF**	**EXP-HF**
mean:	30.7	31.6	51.3	48.1	139.7	149.8	156.7	172.8
SD:	3.3	2.4	7.0	5.1	15.3	19.2	17.6	23.4
CI$_{95\%}$:	29.07 ; 32.26	30.33 ; 32.83	48.21 ; 54.35	45.72 ; 50.55	7.81 ; 22.80	9.12; 29. 20	9.70 ; 25.56	12.28 ; 34.53
n:	16	14	20	17	16	14	19	17

Table 2: Descriptive statistics of body weight and Fast Glycemia levels results. FA-RC, filtered air - regular chow; EXP-RC, exposure concentrate air containing $PM_{2.5}$-regular chow; FA-HF, filtered air - high-fat diet; EXP-HF, exposure concentrate air containing $PM_{2.5}$- high-fat diet.

ICAM-1 gene: Its expression was unchanged in both tissues in all experimental conditions

(Figures 2C, 2D and Table 3).

MMP-9 gene: In the heart, gene expression of MMP-9 decreased in both exposed lean and obese mice (Figure 2E). At the lung, although the mean of exposed animals were higher than their respective controls, the differences were not significant (Figure 2F and Table 3).

E-selectin gene: Its expression was not affected in both tissues of obese mice nor in the lung of lean mice. However, comparing unexposed and exposed lean mice we found an increased expression of E-selectin at heart (Figures 2G, 2H and Table 3).

Il-6 gene: Its expression was not affected in both tissues in the lean mice group. However, its expression increased in lung tissue of exposed obese animals comparing the not exposed controls, but not in the heart tissue (Figure 2J, 2I, respectively and Table 3).

Discussion

Literature data indicate that diabetic patients are more susceptible to worsening cardiovascular diseases [3,26], however, there are few experimental controlled studies on the mechanisms that lead to increased susceptibility of diabetic subjects to air pollution. We studied the effects of controlled $PM_{2.5}$ exposure in worsening type 2 diabetes, as evaluated by fast glycemia, using aged and obese C57BL6 mice as model and age matched lean animals as control. We studied the gene expression of a set of molecules that has an important role in tissue repair and homeostasis in heart and lung. We aimed to study adhesion molecules (VCAM-1, ICAM-1 and E-selectin), the extracellular matrix metalloproteinase (MMP-9), responsible for the remodeling of tissues against various attacks and the cytokine IL-6, an inflammatory marker, in old mice, obese and diabetic.

We know that the type 2 diabetes subjects present a high risk of cardiovascular disease and atherosclerosis, both closely linked to endothelium dysfunction. The pathogenesis of endothelial dysfunction is multifactorial; however, oxidative stress appears to be the common underlying cellular mechanism in the ensuing loss of vasoactive, inflammatory, haemostatic and redox homeostasis in the body's vascular system. The role of endothelial dysfunction as a pathophysiological link between early endothelial cell changes associated with cardiovascular

Figure 1: (A) Body weight as Y axis and animals group as X axis represented as FA-RC (filtered air - regular chow), EXP-RC (exposure concentrate air containing $PM_{2.5}$- regular chow), FA-HF (filtered air - high-fat diet), EXP-HF (exposure concentrate air containing $PM_{2.5}$- high-fat diet). (B) Fasting glycemia measures as Y axis and animals group as X axis. The points represent the mean and bars represent the 95% confidence interval of the mean. ns (not significant), * (p <0.05).

	FA-RC vs EXP-RC		FA-HF vs EXP-HF	
	heart	lung	heart	lung
VCAM-1	ns	ns	ns	< 0.01
ICAM-1	ns	ns	ns	ns
MMP-9	< 0.05	ns	< 0.01	ns
E-selectin	<0.05	ns	ns	ns
IL-6	ns	ns	ns	< 0.05

Table 3: Statistical analysis (ANOVA) results of gene expression comparing $PM_{2.5}$ and filtered air exposed lean and obese mice groups. FA-RC, filtered air - regular chow; EXP-RC, exposure concentrate air containing $PM_{2.5}$- regular chow; FA-HF, filtered air - high-fat diet; EXP-HF, exposure concentrate air containing $PM_{2.5}$- high-fat diet; ns, non-significant.

risk factors and the development of ischemic heart disease is of importance to basic scientists and clinicians alike [27].

Inflammation in response to $PM_{2.5}$ exposure represents a common mechanism that may interact with other pro-inflammatory influences like diet and life style to modulate susceptibility to cardiometabolic diseases [28]. Mechanisms proposed to explain associations between air pollution and cardiovascular disease, specifically, inflammation, oxidative stress, and endothelial dysfunction, are plausible mechanisms linking air pollution with the development or exacerbation of diabetic conditions. Several studies have been indicate that type 2 diabetes subjects present a greater inflammatory response to $PM_{2.5}$ exposure [29-32], but what is not known is whether diabetes is in itself an adverse outcome of air pollution [33].

Studying insulin resistance (IR) and type 2 diabetes mellitus development and its relation to air pollution may help clarify the causative relations between environmental factors and the development of cardiovascular risk [34]. Inflammation and oxidative stress pathways in disease development may provoke maladaptive responses that, in turn, adversely affect organ function and appear to play critical roles in this process as demonstrated by numerous investigations [35-38]. The effects of $PM_{2.5}$ exposure on cardiovascular and pulmonary systems have been extensively studied with both short- and long-term exposures implicated in major adverse cardiovascular events [3,14].

Exposure to high levels of air pollution exaggerates adipose inflammation and insulin resistance in a mouse model of diet-induced obesity [39]. Regarding fasting glycemia, our results are in agreement with the findings of Yan and Col [40], which showed that whole-body exposure to $PM_{2.5}$ for 6 hours/day for 5 days/week during 128 days decreases glucose tolerance compared to age matched mice exposed to filtered air. In addition, both groups showed identical chow consumption and weight gain over the duration of the entire experiment.

Chronic inhalation of $PM_{2.5}$ causes an inflammatory reaction which increases the secretion of primary pro-inflammatory cytokines, such as interleukins (IL) 1 and 6, which are responsible for stimulating the expression of vascular adhesion molecules, VCAM-1, intracellular adhesion, ICAM-1, and cell surface glycoprotein, E-selectin [41,42]. Sun et al. [24] demonstrated increased plasma levels of inflammatory biomarkers, including soluble E-selectin, ICAM-1 and IL6, in C57BL6 mice plasma rendered diabetic by hyperlipidic diet ingestion.

Our data partially agree with the above-mentioned paper: the increased gene expression of IL-6 in the lung of obese mice is accompanied by the increase in VCAM-1 gene expression. However, we found no statistically significant change with respect to ICAM-1
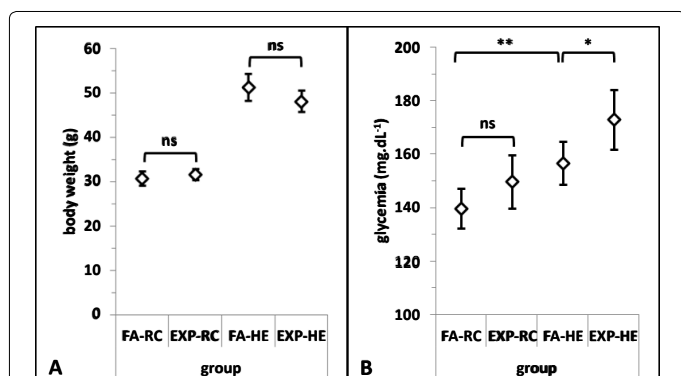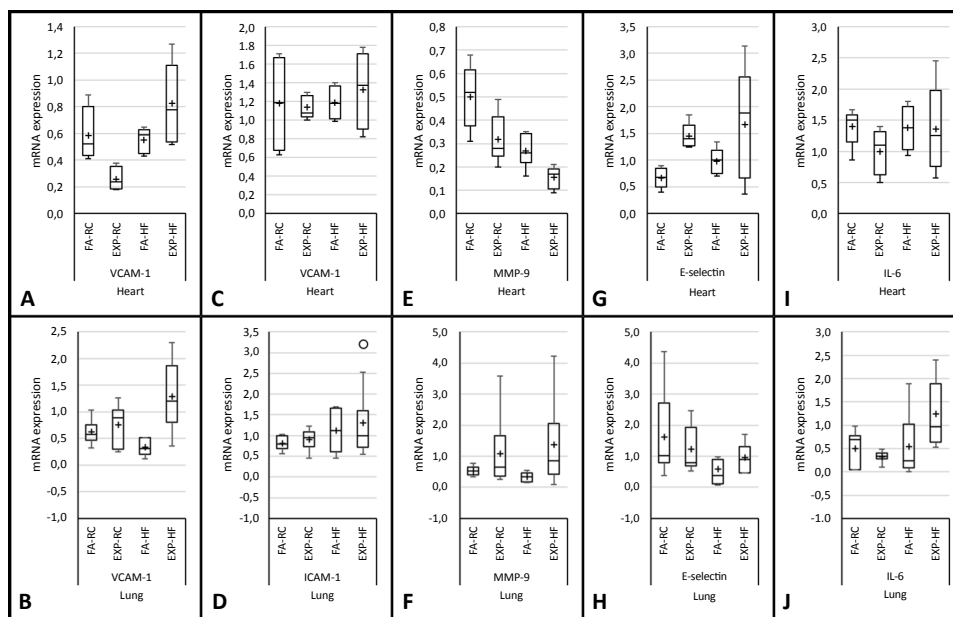
Figure 2: Gene expression results plotted as boxplot. mRNA expression results are in Y axis and samples heart and lung of the animals group amplified to VCAM-1, ICAM-1, MMP-9, E-selectin, and IL-6 genes in X axis represented as FA-RC (filtered air - regular chow), EXP-RC (exposure concentrate air containing $PM_{2.5}$- regular chow), FA-HF (filtered air - high-fat diet), EXP-HF (exposure concentrate air containing $PM_{2.5}$- high-fat diet). The cross represents the mean value of each data set. The circle represents an outlier value.

and E-selectin in the same tissue. On the other hand, E-selectin gene expression was increased in the heart of exposed lean mice.

In our model, we observed decreased gene expression of MMP-9 in the heart of both obese and in the lean mice exposed to $PM_{2.5}$. Considering that when activated MMPs can promote excessive degradation of extracellular matrix components causing thereby pathological vascular remodeling [43,44], our results may reflect an adaptive response of heart tissue in order to protect myocardium.

On the other hand, in the lung, although we observed increased gene expression of MMP-9 in the exposed groups, obese and lean when compared to the controls, the difference was not statistically significant, possibly due to the wide dispersion of results. The lung is the organ in direct contact with polluted air and although not significant, this finding may be important in elucidating the mechanism of action involved in the aggravation of respiratory diseases when individuals are chronically exposed to $PM_{2.5}$.

The literature data show that there is some evidence that the degree of pulmonary inflammation correlates with the elevation of systemic cytokines and systemic vascular dysfunction [28]. Taken together, our results indicate that exposure to $PM_{2.5}$ causes distinct inflammatory events in the heart and lung, and the obese diabetic animals are more sensitive to these changes.

The expression/activity of proteins involved in insulin signaling, activated by inflammatory stimuli, will be investigated.

Acknowledgment

Disclosures

None

Study limitations

This study was conducted under whole-body exposure conditions, i.e., animals were exposed to the particulate material through the respiratory tract. In addition, we work with the maximum daily $PM_{2.5}$ considered safe by WHO. However, since the animals were old and obese, the number of animals became reduced during the experiment.

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