Environmental Research 110 (2010) 237–243

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

The time course of vasoconstriction and endothelin receptor A expression in pulmonary arterioles of mice continuously exposed to ambient urban levels of air pollution ☆

Giselli Matsumoto [a], Naomi Kondo Nakagawa [e], Rodolfo de Paula Vieira [b,c], Thais Mauad [a], Luis Fernando Ferraz da Silva [a], Carmen Diva Saldiva de André [d], Regiani Carvalho-Oliveira [a], Paulo Hilário Nascimento Saldiva [a], Maria Lucia Bueno Garcia [c,*]

[a] Laboratory of Experimental Air Pollution, Department of Pathology, University of Sao Paulo, Sao Paulo, Brazil
[b] COPD and Asthma Research Group, Department of Pneumology, Albert-Ludwigs University of Freiburg, Freiburg im Breisgau, Baden-Württemberg, Germany
[c] Laboratory of Experimental Therapeutics, Department of Internal Medicine, School of Medicine, University of Sao Paulo, Av. Dr. Arnaldo 455, Sao Paulo CEP: 1246-903, Brazil
[d] Mathematics and Statistics Institute, University of Sao Paulo, Sao Paulo, Brazil
[e] School of Physiotherapy, University of Sao Paulo, Sao Paulo, Brazil

ARTICLE INFO

Article history:
Received 7 May 2009
Received in revised form
6 November 2009
Accepted 13 January 2010
Available online 7 February 2010

Keywords:
Vascular constriction
Air pollution
Particulate matter
Endothelin-1
NO

ABSTRACT

The present study aimed to verify the time course of the effects of environmental levels of urban air pollution toxicity on lung arterioles. BALB/c mice (n=56) were continuously exposed to selective chambers equipped with (filtered, F) or without (non-filtered, NF) filter devices for particles and toxic gases for 24 h/day, over 14, 21, 30 or 45 days. After exposure, we evaluated the lumen–wall relationship (an estimator of arteriolar narrowing), endothelial nitric oxide synthase (eNOS) and endothelin type A receptor (ETAr) expression in the vascular wall and inflammatory influx of the peribronchiolar area. Concentrations of fine particulate matter ($PM \leq 2.5\ \mu g/m^3$), nitrogen dioxide (NO_2), black smoke (BS), humidity and temperature in both the environment and inside the chambers were measured daily. Filters cleared 100% of BS and 97% of PM inside the F chamber. The arteriole wall of the lungs of mice from NF chamber had an increased ETAr expression ($p \leq 0.042$) concomitant to a decrease in the lumen/wall ratio (p=0.02) on the early days of exposure, compared to controls. They also presented a progressive increment of inflammatory influx in the peribronchiolar area during the study (p=0.04) and decrement of the eNOS expression on the 45th day of exposure in both vascular layers ($p \leq 0.03$). We found that after 14 days of exposure, the ambient levels of air pollutants in Sao Paulo induced vasoconstriction that was associated with an increase in ETAr expression. These vascular results do not appear to be coupled to the progressive inflammatory influx in lung tissue, suggesting a down-regulation of vasoconstrictive mechanisms through an imbalance in the cytokines network. It is likely that these responses are protective measures that decrease tissue damage brought about by continuous exposure to air pollutants.

1. Introduction

Lifelong exposure to air pollution is associated with pulmonary and vascular disease (Saldiva et al., 1994; Dockery, 2001; Wong et al., 2002; Hoek et al., 2002). It is hypothesized that systemic alterations due to pollutants, especially those from vehicular sources within big cities, are dependent on pulmonary injury/inflammation (Van Eeden et al., 2005; Hogg and van Eeden, 2009) and imbalances in cytokine networks, due to a shift towards the up-regulation of inflammatory cytokines, which contributes to hypoxemia/ischemia (Calderón-Garcidueñas et al., 2003, 2008, Peretz et al., 2008). These studies suggest a close relationship between lung epithelial and endothelial pathology resulting in airway and vascular remodeling and altered repair (Kodavant et al., 2002).

The levels of some cytokines and reactive oxygen species may be augmented by exposure to pollutants (Lippmann et al., 2003; Kodavant et al., 2002), leading to release specific endothelial-derived mediators such as endothelin (ET) (Thomson et al., 2004; Chauhan et al., 2005) and nitric oxide (NO) (Knuckles et al., 2008) by endothelial and epithelial cells.

Several experimental protocols performed on animals acutely and chronically exposed to polluted areas of downtown Sao Paulo have shown an association between air pollution and lung alterations such as inflammation (Pereira et al., 1995; Saldiva,

☆This work was supported by Institute of the Laboratories of Medical Investigation, Clinical Hospital, School of Medicine, University of Sao Paulo and CNPQ.
* Corresponding author. Fax: +55 11 3064 2744.
E-mail address: gajugu@terra.com.br (M.L. Garcia).

0013-9351/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.envres.2010.01.003

2008), vasoconstriction (Rivero et al., 2005; Lemos et al., 2006) and oxidative stress dependent on the elemental chemistry of the particles (Prahalad et al., 1999; Carvalho-Oliveira et al., 2005), which can result in tissue damage. Most experimental studies, however, are designed such that they do not show the time course of these events, and therefore cannot give plausible epidemiological data.

Experimental models of acute exposure to selective concentrated air pollutants are necessary to express the effect of a specific component of atmospheric pollution. However, in "real-world" ambient, air pollution varies constantly, depending on traffic, wind directions, photochemical processes and seasons. Thus, experiments conducted using ambient air as target of study may be used to provide plausibility to the epidemiological studies of the hazardous effects of ambient airborne pollutants on individuals.

We therefore designed a murine experiment to study the mechanisms of lung vascular reactivity after exposure to real air pollution, taking into account the time course of the effects. We analyzed the inflammatory markers and vascular narrowing of the peribronchiolar vessels in the distal lungs of mice continuously exposed to the true ambient atmosphere of São Paulo, Brazil.

2. Material and methods

The Review Board for human and experimental studies of the Clinical Hospital, Medical School of the University of Sao Paulo (CAPPesq-HC-FMUSP), approved this study (research no. 1250/05).

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" published by the National Institutes of Health (NIH publication 86–23, revised 1985).

2.1. Experimental groups

Healthy male 4 weeks old BALB/c mice (n=56) weighing 25 g were randomly separated in groups of seven animals per cage and continuously submitted to exposure chambers with or without filter devices (F or NF, respectively) for particulate matter (PM $\geq$ 0.3 μm) and toxic gases for 24 h/day over 14, 21, 30 or 45 days. Food (Nuvital-Nutrients Ltd., Colombo, Brazil) and water were available *ad libitum*. After the exposure period, mice were anesthetized with sodium pentobarbital, euthanized by cervical dislocation and exsanguinated. Total body and lung weights were obtained from each animal.

2.2. Exposure site

2.2.1. Exposure site characterization

The exposure site was located on the campus of the Medical School, 20 m from the roadside, 150 m from a busy traffic crossroad in downtown Sao Paulo and 160 m from a monitoring station of Sao Paulo State Environmental Sanitary Agency (CETESB). Previous characterization of $PM_{2.5}$ (PM $\leq$ 2.5 μm) mass collected at the monitoring station and at the campus of the Medical School has shown that approximately 67% of the $PM_{2.5}$ mass is composed of vehicular sources, with a black carbon/organic carbon ratio ranging between 40% and 70% throughout the day (Ynoue and Andrade, 2004; CETESB, 2005; Mauad et al., 2008). There were no industries or significant biomass burning sources in the surrounding area.

2.3. Exposure chambers

The exposure system employed in this study has been previously described (Mohallem et al., 2005; Pires-Neto et al., 2006; Lichtenfels et al., 2007; Lemos et al., 2006, Veras et al., 2008, Akinaga et al., 2009). The two side-by-side exposure chambers consisted of cylindrical aluminum structures that measured 2.0 m in diameter and 2.15 m in height and were covered by a plastic UV film. Air entered the chamber at the base of the cylinder and was uniformly distributed throughout. The air was forced into the chamber and exited through a wide opening at the top. The chamber is a normobaric system; the pressure inside the chambers did not exceed 30 mm H_2O and had a flow rate of 20 m^3/min. The exposure chambers were either filtered (F; with less toxic gases and particulate matter above 0.3 μm) or non-filtered (NF; no filtering devices, similar to external ambient air). In the filtered system, three stages of filters were in line. The first (plain and bag filters) eliminated large particles and the second (model JFL-90) and third stages (high-efficiency particulate air, HEPA) trapped fine particles. The filters were purchased from Purafil (Sao Paulo, SP, Brazil).

Inside the chambers, animals were kept at the same ambient conditions of temperature, humidity (measured by digital thermography – Soil Control, Tecnologia Instrumentos do Solo Ltda, Brazil), light cycle and noise.

2.4. Pollutant measurements

Nitrogen dioxide (NO_2) was measured using a colorimetric method (Lodge, 1989). Black smoke (BS) was estimated by light reflectance (ABNT, 1989), and the 24-h concentration of $PM_{2.5}$ was measured gravimetrically using Harvard Impactors (Air Diagnostics, Harrison, ME) at a flow rate of 10 L m^{-1}. All pollutants were measured daily inside the exposure chambers, and the results are expressed as μg/m^3.

Approximately 160 m from the exposure chambers there is a monitoring station of the Sao Paulo State Environmental Sanitary Agency (CETESB), which measures the external environmental levels of PM_{10} (FH62 I-n Beta Attenuation Monitor, Graseby Andersen, Smyrna, GA), NO_2 (Chemiluminescence 42 Series, Thermo Scientific, Waltham, MA) and BS (reflectance method) daily, available on-line at http://www.cetesb.sp.gov.br. External $PM_{2.5}$ was calculated as 60% of measured PM_{10} to be compared to $PM_{2.5}$ measurements inside the exposure chambers (Ynoue and Andrade, 2004; Mauad et al., 2008; Akinaga et al., 2009).

2.5. Tissue sampling and morphometric analysis

The lungs were isolated and fixed for 48 h by intratracheal instillation of buffered 10% formalin solution at a pressure of 25 cm H_2O. After fixation, longitudinal sections were performed near the carina, at the level of the entrance of the main bronchus. The sections were stained with hematoxylin and eosin (H&E) to obtain the lung lumen–wall ratio and inflammatory influx analysis and immunohistochemistry for the expression of the endothelial nitric oxide synthase (eNOS) and endothelin type A receptor (ETAr) was performed.

Morphometric data were obtained from histological images made using a light microscope (Leica DMR; Leica Microsystems GmbH, Wetzlar, Germany) connected to a video camera connected to a compatible microcomputer. The images were processed using Image-Pro Plus 4.1 for Windows (Media Cybernetics, Silver Springs, MD, USA). All measurements were performed using histological images obtained with a 40× objective lens, except for leukocyte analyses that were performed at magnification of 100×. After total vascular measurements, vessels were categorized as large and small by their percentage of the lumen area.

To verify the inflammatory responses, histopathological semi-quantitative scores were done in 5 μm thick slides of lungs stained with H&E. The semi-quantitative scores were 0=0–25%, 1=focal 25–50%, 2=moderate 50–75% and 3=diffuse $\geq$75% (Nurkiewicz et al., 2006). The inflammatory histological end-point was perivascular inflammatory cellular influx in the peribronchiolar area, which was expressed as scores/area.

The lumen/wall ratio of the peribronchiolar arterioles of the lungs was assessed in transversally cut vessels (maximum and minimum diameter variation $<$10%). The areas of the lumen and of the vascular wall were determined by the point counting technique in H&E stained slides (Batalha et al., 2002; Rivero et al., 2005; Lemos et al., 2006). A grid was superimposed over the histological images on the monitor, and the points overlying the lumen or the arteriolar wall were counted separately. The lumen/wall ratio (L/W) was calculated as points overlying the lumen/points overlying the arteriolar wall (μm^2/μm^2).

2.5.1. Immunohistochemical analysis

Immunohistochemistry was performed using antibodies against eNOS and ETAr (Labvision, Neomarkes, CA, USA), which reflect ET-1 release through the biotin–streptavidin peroxidase method. Primary antibodies were applied in positive and negative controls, and secondary antibody was also applied in control animal slides. Using an image analysis program (Image-Pro Plus; Media Cybernetics, Silver Spring, Maryland, USA), each layer of the arteriolar wall in the peribronchiolar area was measured as well as the positive area for each antibody described above. Briefly, the area and perimeter of the layers of the peribronchiolar arterioles walls (adventitia, media+intima) and of the lumen of each experimental animal were outlined and quantified using a light microscope attached to an image analysis system at a 10× magnification in covered slices. The results are expressed as percentage of positive area (Cho et al., 2004).

2.6. Statistical analysis

The first approach of our data analysis was a descriptive evaluation to check the dispersion and distribution of the samples (mean, SD, SE, median, IC, max, min, 25% and 75%). The effects of chamber (filtered and non-filtered) and time of exposure (14, 21, 30 and 45 days) in the responses were first evaluated by a two-factor analysis of variance model (ANOVA) (Neter et al., 2005), followed by Tukey's post hoc test. When the residual analysis showed departures from ANOVA model, the factors Chamber and Time of exposure were combined and the Kruskal–Wallis

test was applied. If the test led to the conclusion that the means were not equal, the multiple pairwise testing procedure described by Neter et al. (2005) was applied.

So, ETAr, eNOS, inflammatory influx and lumen–wall ratio data were analyzed by Kruskal–Wallis followed by multiple pairwise testing procedure.

The Spearman's correlation coefficient was used to evaluate the time effect in each chamber for the lumen–wall ratio data after categorization as small and large vessels.

A repeated measures analysis of variance model followed by Tukey's post hoc test was used to evaluate the levels of pollutants between the two chambers and the external environmental air for the whole experimental period.

The level of significance was set at 5%. Statistical analyses were performed using the SPSS v15.0 computer package and Minitab v 15.

3. Results

There were no deaths, apparent diseases or differences in body and lung weight among experimental groups.

Table 1 presents the descriptive analyses of NO_2, BS and $PM_{2.5}$ inside the F and NF chambers and external ambient air during the study. There was no difference among NF chamber, F chamber and the external air, in terms of humidity, temperature and NO_2 concentration. As expected, the particles were non-existent in the F chamber (100% BC, 97% $PM_{2.5}$, $p \leq 0.001$). The daily fluctuation of particles during the experiment is presented in Fig. 1. Inside the NF chamber, the $PM_{2.5}$ fluctuations below 15 $\mu g/mm^3$ were coincident with power outage and were underestimated during these days. The NF chamber, therefore, presented similar values when compared to the external environment.

There was no alteration in leukocytes in lung tissue between mice from both exposure chambers when each period of the experiment was evaluated separately (14, 21, 30 and 45 days). However, after the whole experimental period, leukocytes in the peribronchiolar area increased in mice from the NF chamber in a progressive pattern when compared to those from the F chamber, which did not express any alterations during the experiment ($p=0.04$, Table 2, Fig. 5D).

Fig. 2 illustrates the lumen–wall parameters of the peribronchiolar vasculature. There was a narrowing of the total vessels in mice submitted to the NF chamber after 21 days of

Table 1
Pollutants inside and outside the exposure chambers during the experiment.

	BS	$PM_{2.5}$	NO_2
Filtered chamber	3.13 ± 2.34*	2.30 ± 1.8 *	48.57 ± 18.32
Non-filtered chamber	91.54 ± 50.38	22.08 ± 11.92	71.10 ± 14.50
External air	61.98 ± 37.54	21.30 ± 9.36	61.90 ± 24.48

BS: Black smoke; $PM_{2.5}$: particulate matter ≤ 2.5 um; NO_2: nitric dioxide.*$p \leq 0.001$. Values expressed as $\mu g/m^3$, mean $\pm$ SD.
Descriptive analysis of NO_2, $PM_{2.5}$ and black smoke (BS) measured in both exposure chambers and in the external environment during the study. There were no significant differences among the groups regarding NO_2 concentration. Black smoke and $PM_{2.5}$ had lower values inside the filtered (F) chamber when compared to the non-filtered (NF) chamber and the environment ($p \leq 0.001$).

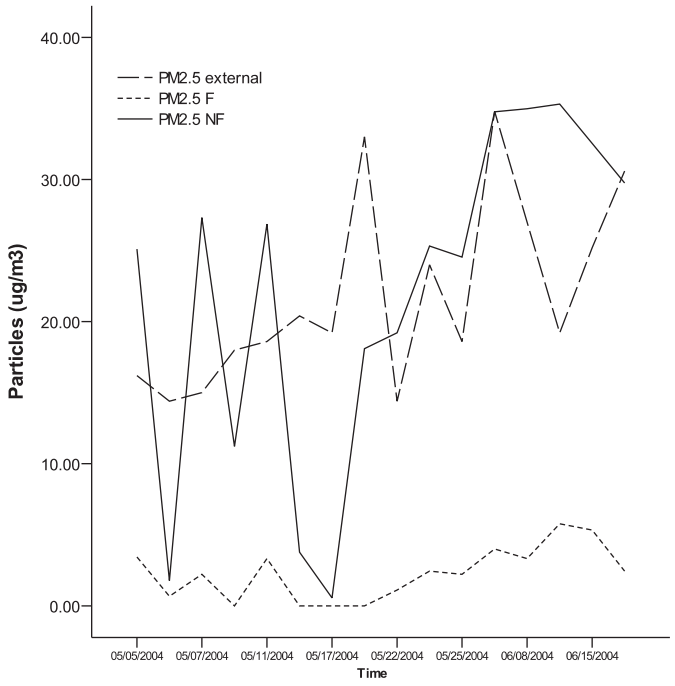

Fig. 1. Particle fluctuations inside and outside the exposure chambers during the experiment. Note that filters trapped almost all particles in the filtered (F) chamber ($p \leq 0.001$). The non-filtered (NF) chamber presented similar values to the external environment.

Table 2
Inflammatory influx.

	Leukocytes in lung tissue (%cells/μm^2)	
Time (days)	**Filtered**	**Non-filtered**
14	0.91 ± 0.29	0.91 ± 0.52
21	0.92 ± 0.27	0.96 ± 1.89
30	0.93 ± 0.25	1.08 ± 0.27
45	1.03 ± 0.18	1.17 ± 0.38
Total period	0.95 ± 0.26	1.02 ± 0.81*

Filtered: filtered chamber; non-filtered: non-filtered chamber; *$p=0.04$.
Values expressed as mean $\pm$ SD.
Inflammatory influx in the peribronchiolar area of mice exposed to the filtered (F) and non-filtered (NF) chamber for particles and toxic gases for 14, 21, 30 and 45 days. Notice that air pollutants triggered progressive leukocytes increase in lung tissue during the experiment ($p \leq 0.04$).

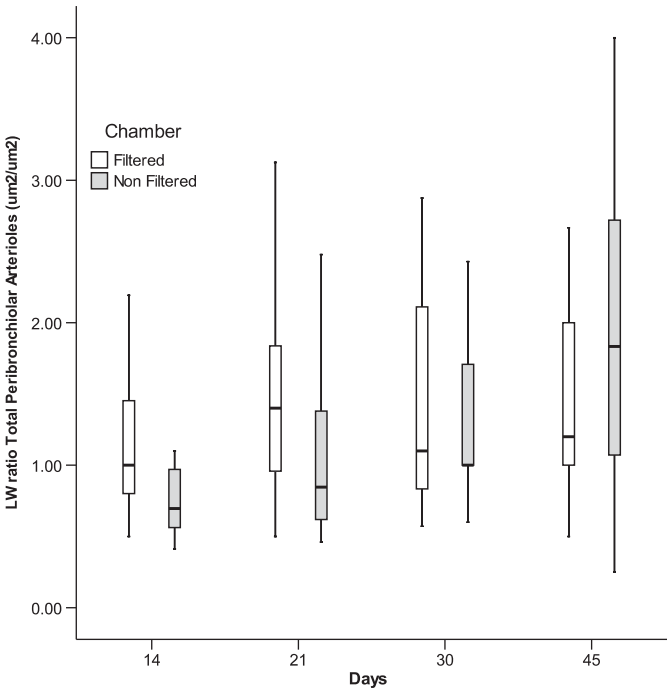

Fig. 2. Lumen–wall ratio of peribronchiolar arterioles of mice continuously exposed to selective exposure chambers with and without filters (F and NF, respectively) for particles and toxic gases for 14, 21, 30 or 45 days. Notice that mice from the NF chamber presented with the lowest values. On the 21st day, there was a narrowing of lung vessels from mice from the NF chamber compared to those from the F chamber ($p=0.023$).

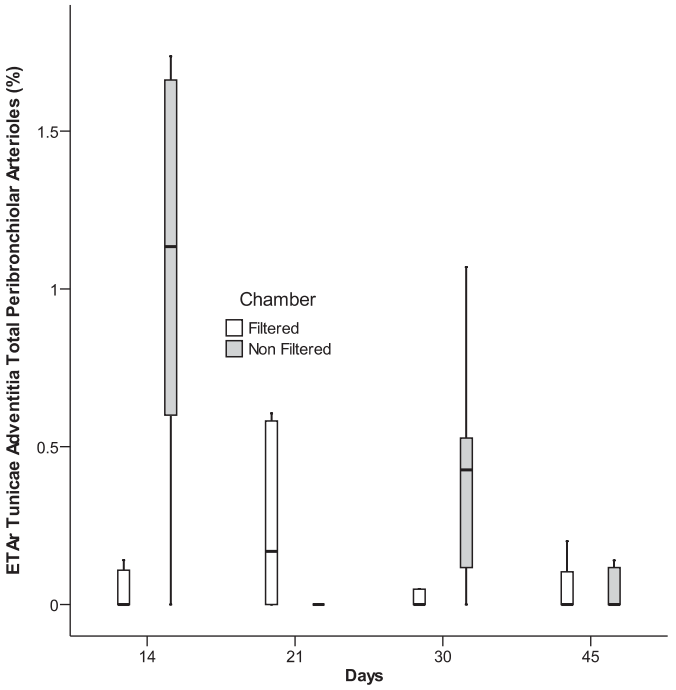

Fig. 3. Endothelin receptor detection (ETAr) in the adventitia of peribronchiolar arteriole walls of mice continuously exposed to selective exposure chambers with and without filters (F and NF, respectively) for particles and toxic gases for 14, 21, 30 or 45 days. Note that mice from the NF chamber showed an increase in ETAr detection in the adventitia layer on the 14th and 30th day of exposure ($p = < 0.001$ and 0.02, respectively).

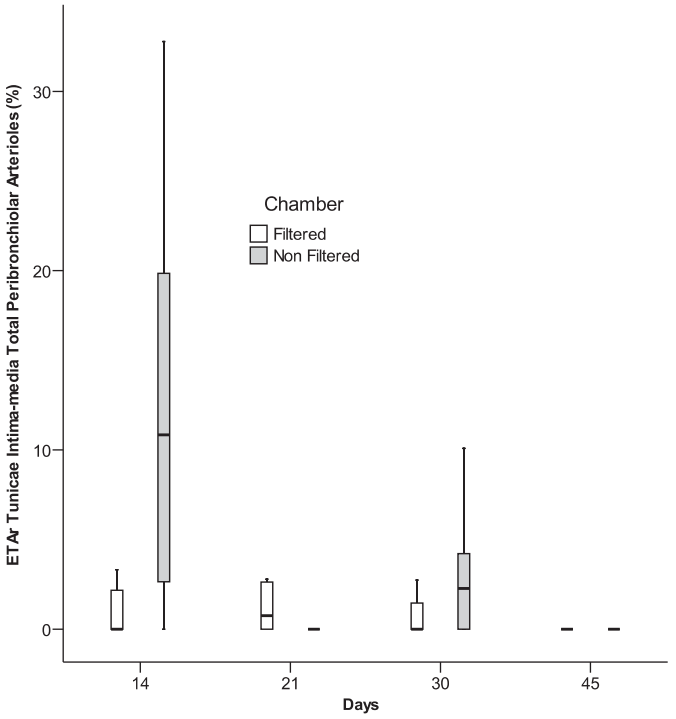

Fig. 4. Endothelin receptor detection (ETAr) in the tunicae intima/media of the peribronchiolar arteriole walls of mice continuously exposed to selective exposure chambers with and without filters (F and NF, respectively) for particles and toxic gases for 14, 21, 30 or 45 days. Note that mice from NF chamber showed an increase in ETAr expression in the intima/media layer on the 14th (p=0.003).

exposure (p=0.023) and tendency for vessel narrowing on the 14th day of exposure (p=0.07) when compared to mice from F ambient. The vascular narrowing presented a trend to decrease in small vessels (p=0.06) during the same period in the same group. Although pollutants induced LW decrement in both small and large vessels when the whole experimental period was considered (p=0.012 and 0.000, respectively, Spearman's correlation), this parameter went toward normalization at the end of experiment.

Figs. 3 and 4 show the endothelin-A receptor detection on the peribronchiolar vessels wall. After 14 days of exposure, mice from the NF chamber presented higher ETAr values in total (Figs. 3, 5A and B), as well as in large and small arterioles in both the adventitia (p=0.001 and $\leq$0.02, respectively) and in the intima-media layer (Figs. 4 and 5A and B) (p=0.003 and $\leq$0.042, respectively) when compared to mice from F ambient. On the 30th day of exposure, total and small sized vessels presented an increase in the adventitia vascular layers ($p \leq 0.02$) and in small arterioles in the tunicae intima-media (p=0.04) in the same group.

The eNOS detection in lung vessels wall of the experimental animals was very modest during the studied period. However, mice from NF chamber presented lower values of eNOS in both vascular layers after 45 days of exposure in total vessels and after categorization (tunicae intima/media: total vessels 1.9 ± 1.87 F and 0.91 ± 2.0 NF, p=0.001; small vessels 1.56 ± 1.46 F and 0.15 ± 0.52 NF, p=0.03; adventitia layer: total vessels 0.02 ± 0.03 F and 0.0 NF, p=0.01, small vessels 0.02 ± 0.05 F and 0.0 NF, p=0.02, large vessels 0.01 ± 0.002 F and 0.0 NF, p=0.03) when compared to animals submitted to the F chamber (Figs. 5C and D).

4. Discussion

Airborne pollution within Sao Paulo city, Brazil, modestly, but significantly, modulated lung vessel patency of asymptomatic healthy mice. The vascular constriction response was concomitant to the increase in ETAr detection in vessel walls and was detected in the early days of exposure (14 days), which became normalized by the end of exposure (45 days). The vascular narrowing and ETAr expression was somehow dissociated from the inflammatory influx in lung tissue, which presented a monotonic but progressive behavior during the time of the experiment.

The present protocol was chosen to combine the advantage of controlled experimental study with real air continuous exposure. We used groups of animals that were submitted to chambers with air filters under controlled conditions in an attempt to mimic the realistic environmental exposure of individuals from Sao Paulo city, Brazil. The NF exposure chamber was similar to the external air, while the F chamber was almost 100% clear of pollutants (Table 1, Fig. 1). Our results indicate that particulate matter may play an important role in lung damage, findings that are consistent with the literature (Becker et al., 2005). The ambient air, however, is a combination of primary and newly formed secondary pollutants with seasonal variations, physicochemical properties and aging that are widely dependent on their source (Becker et al., 2005; Carvalho-Oliveira et al., 2005). The implication of other components of atmospheric pollution, such as polycyclic aromatic hydrocarbons (PAH), ozone and other toxic gases, were not evaluated in this study and their influence cannot be ignored.

We opted to use isogenic Balb-c strain of mice focusing to work with a more homogenous sample. Similar previous murine experimental works developed in Sao Paulo city have shown that Swiss as well as in Balb-c mice adapted quite well in the exposure chambers and presented significant alteration in the target organs (Akinaga et al. 2009; Lichtenfels et al. 2007; Mauad et al. 2008; Veras et al. 2008; Pires-Neto et al., 2006). Ambient air pollution modulates lung alterations but these effects although continuous, are modest. So, taking this to account, by choosing a more homogenous strain of mice we expected to add more sensitiveness to the results, with less dispersion of our data.

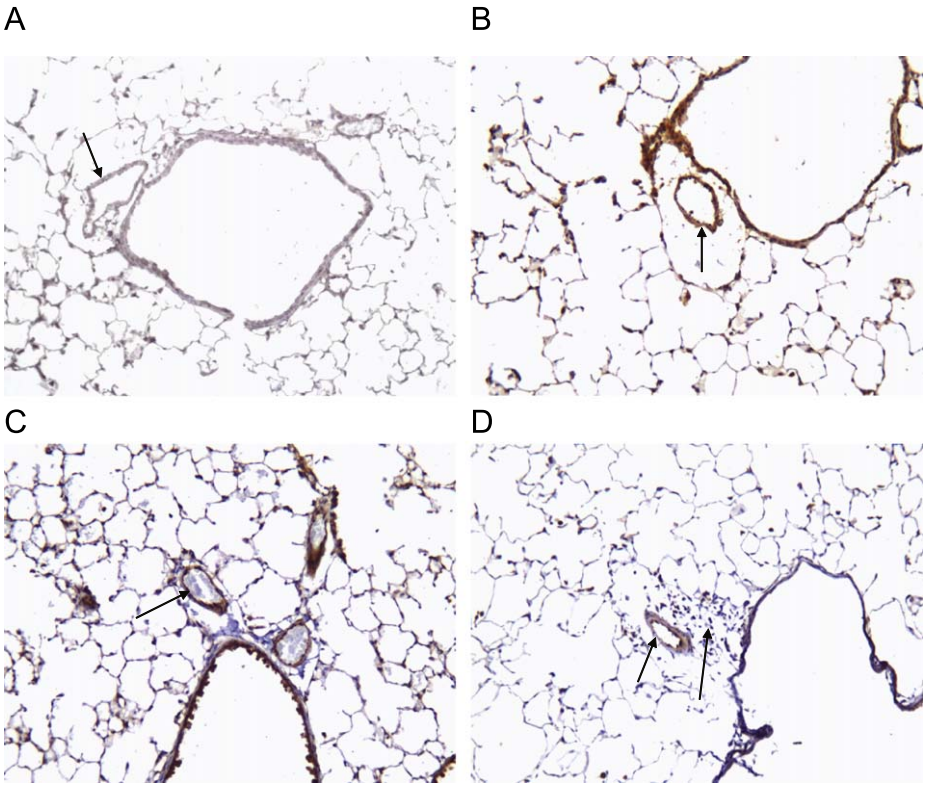

Fig. 5. Photomicrographies of peribronchiolar arterioles of mice submitted to filtered and non-filtered ambient air of Sao Paulo city. ETAr detection in mice from filtered chamber (A) and non-filtered chamber (B) on the 14th day of exposure; eNOS detection in mice from filtered (C) and non-filtered (D) chamber on the 45th day of exposure. Notice that lung vessel walls of mice exposed to non-filtered ambient air presented increment of EtAr detection (arrow, 5B) and cellular influx (arrow, 5D) as well as decrement of eNOS detetection (arrow, 5D). Immunohistochemistry staining; magnification 100 ×.

We chose to study lung vascular effects on the 14th, 21st, 30th and 45th days of exposure in order to check the time course events induced by air pollution prolonged exposure of Sao Paulo city. We opted for choosing two weeks for the adaptation of the animals to the chambers, and we would not conduct our experiment too long to have substantial seasonal variations of particle composition. In São Paulo, May and June are months when air pollution rises because of bad dispersion conditions and low rain. We decided to explore such period of the year. Published data focusing the temporal association between lung effects and pollutants exposure have also been published as acute (hours), prolonged (1–28 days) and chronic exposure (months to years). However, most of all reflected epidemiological longitudinal chronic studies (Hogg and van Eeden, 2009) or punctual acute and prolonged exposure to concentrated particles (Kodavant et al., 2002; Thomson et al., 2004, 2005; Tamagawa et al., 2008). None of them focused mechanistic pathways of the hazard effects on the lung arterioles after prolonged real continuous ambient atmospheric pollutants exposure (14–45 days) as in our study.

Breathing ambient air for 14 days induced progressive inflammatory influx in the peribronchiolar area (Table 2). These data are consistent with previous studies (Van Eeden et al., 2005; Tamagawa et al., 2008; Hogg and van Eeden, 2009), and suggest that particles and other toxic gases induce inflammatory mediators that produce an integrated local and systemic inflammatory immune response. This results in the release of leukocytes and platelets from the bone marrow and influx to the damage tissue. These studies, however, also suggest that inflammation induced by pollution modulates vasoconstriction and cytokine release (Van Eeden et al., 2005; Nurkiewicz et al., 2006, Scapellato and Lotti, 2007; Tamagawa et al., 2008; Hogg and van Eeden, 2009).

Vascular response may be modulated by cytokines and other inflammatory agents, such as reactive oxygen species (Prahalad et al., 1999; Squadrito et al., 2001; Kodavant et al., 2002; Lippmann et al., 2003), via transition metals and organic poliaromatic carbon, NOS mediators (Anazawa et al., 2004), hypoxia due to vasoconstriction, ET-1 expression and matrix metallo proteinases (MMP) (Calderón-Garcidueñas et al., 2003, 2008). Several authors have documented that NOS inhibitors may mediate vascular remodeling and vasoconstriction by regulating expression of MMP and tissue inhibitor metallopreoteinase (TIMP) in aortic tissue (Eagleton et al., 2002).

The ETAr detection, which reflects the vasoconstrictor peptide ET-1 expression, was increased in both adventitia and intima-media layers of the peribronchiolar arterioles of mice submitted to the NF chamber on the 14th and 30th days of exposure (Figs. 3–5A and B). The ETAr up-regulation expression in both layers of lung vessel walls was attenuated during the study period and was similar to controls by the end of the experiment (45 days).

Air particles, pulmonary vasoconstriction and peptide ET-1 expression present a complex toxicological interaction (Meidan et al., 2002; Chauhan, et al., 2005). The lungs are the primary source of circulating ET-1, and this cytokine is connected to hypoxia, pulmonary vasoconstriction and hypertension (Thomson et al., 2004, 2006; Calderon-Garcidueñas et al., 2007, 2008; Peretz et al., 2008; Lund et al., 2009). The epidemiological studies of Calderon-Garcidueñas have shown lung vasoconstriction and pulmonary hypertension in children exposed to high levels of ambient air particles in Mexico; in addition, the children presented with leucopenia in the blood (Calderon-Garcidueñas et al., 2007, 2008). Pulmonary smooth muscle cells submitted to hypoxia enhances lung ET-1 receptor expression response to

hypoxia manifested by exaggerated contractility (Fredenbergh et al., (2008)). In addition, it has been suggested that particles may increment ET-1 in the blood and lung tissue secondary to the up-regulation of ET-1 gene expression by epithelial and endothelial cells (Thomson et al., 2005). Furthermore, PM may affect the imbalance between endothelium-converting enzyme (ECE-1) and TIMP, which modulates ET-1 maturation and activity. Further, mRNA levels of ET-1 gene expression, ECE-1 and ET receptor subtypes A and B can be detected as early as 2 h and 1, 2, 3, 7 and 14 days after exposure, but ET-1 in the lung tissue cannot be detected after 24 h of particles exposure (Thomson et al., 2004). These data show that the lung endothelin system responds rapidly and transiently to acute inhalation of urban pollutants, which is consistent with the dynamics of ambient pollutant ischemic events reported in the human population and in this experimental protocol. The time course of cytokine expression modulated by continuous ambient air exposure, however, remains to be elucidated.

Rivero et al. (2005) and Lemos et al. (2006) in cross-sectional studies, observed that lung vasculature was markedly affected by acute and chronic exposure to concentrated fine environmental particles in the city of Sao Paulo. Interestingly, our data show a clear time course behavior of inflammation, vessels tonus and ETAr detection connected to pollutants exposure, but these events did not appear to be temporally correlated. Mice in the NF chamber expressed vasculature narrowing, which was concomitant with an increase in ETAr detection in the lungs early in the experiment but was normal by the end of the studied period (Figs. 2–5A and B). Meanwhile, the inflammatory influx in the lungs of mice submitted to air pollution (NF chamber) presented with a monotonic, modest but progressive pattern that was not associated with the vascular data (Table 2). We suggest that the time course dissociation of inflammation and vascular response and cytokine expression could be due to a protective response of the lung tissue, down-regulating the mechanistic pathways of vasoconstriction in a protective manner against damage induced by pollutants.

Our data showed little expression of eNOS in lung vessels. A decrease in eNOS was observed in mice from the NF chamber in the adventitia and muscular layer of lung vessels when compared to the group submitted to the F chamber after 45 days of exposure (Figs. 5C and D).

Oxidative stress may be secondary to inflammation due to seasonal fluctuations of ambient levels of airborne particles (Becker et al., 2005). In fact, it has been hypothesized that diesel emissions (DE) could enhance vasoconstriction in arteries and veins through the uncoupling of eNOS (Knuckles et al., 2008). The expression of eNOS mRNA, however, may be released early after 4 h of ozone and concentrated particles exposure, but their levels are transient and are not detected after 24 h of exposure (Thomson et al., 2005). The eNOS activity in our study was very low in vascular wall during the whole experimental period and even decreased significantly after 45 days of exposure. These results may be due to the study design, as the animals were exposed for a short period under real conditions of air pollution, and therefore to modest environmental seasonal fluctuations. It is possible that eNOS is transient and not a sufficiently sensitive marker to de detected in such a situation. Further, eNOS is a vasodilator and not an inducible enzyme such as inductible NOS (iNOS), which has a potent vasoconstrictor effect. It is therefore possible that eNOS simply has a modest expression or is even down-regulated during inflammation, when an imbalance of eNOS/iNOS may occur, resulting in up-regulation and a dominant expression of iNOS.

We conclude that urban air pollutants induced systemic subclinical, continuous and progressive lung inflammation. These alterations appeared to be uncorrelated with ETAr expression and vascular constriction, which were both detected from the 14th day of exposure and returned to normal by the 45th day. Further studies are necessary to clarify the mechanistic pathway of vasoconstriction over the time course of healthy individuals exposed to pollutants.

Acknowledgments

We thank to Maria Regina de Siqueira Bueno for technical support.

References

ABNT, 1989. Material particulado em suspensão na atmosfera – determinação da concentração de fumaça pelo método da refletância da luz. NBR, 10736.

Anazawa, T., Dimayuga, P.C., Li, H., 2004. Effect of exposure to cigarette smoke on carotid intimal thickening: the role of inducible NO synthase. Arterioscler. Throm. Vasc. Biol. 24 (9), 1652–1658.

Akinaga, L.M.Y., Lichtenfels, A.J., Carvalho-Oliveira, R., Caldini, E.G., Dolhnikoff, M., Silva, L.F.F., Bueno, H.M.S., Pereira, L.A.A., Saldiva, P.H.N., Garcia, M.L.B., 2009. Effects of chronic exposure to air pollution from Sao Paulo City on coronary of Swiss mice, from birth to adulthood. Toxicol. Pathol. 37 (3), 306–314.

Batalha, J.R.F., Saldiva, P.H.N., Clarke, R.W., Coull, B.A., Stearns, R.C., Lawrence, J., Murthy, G.G.K., Koutrakis, P., Godleski, J.J., 2002. Concentrated ambient air particles induce vasoconstriction of small pulmonary arteries in rats. Environ. Health Perspect. 110 (12), 191–1197.

Becker, S., Dailey, L.A., Soukup, J.M., Grambow, S.C., Devlin, R.B., Huang, Y.C., 2005. Seasonal variations in air pollution particle-induced inflammatory mediator release and oxidative stress. Environ. Health Perspect. 113 (8), 1032–1038.

Calderón-Garcidueñas, L., Mora-Tiscareño, A., Fordham, L.A., Valencia-Salazar, G., Chung, C.J., Rodriguez-Alcaraz, A., Paredes, R., Variakojis, D., Villarreal-Calderón, A., Flores-Camacho, L., Antunez-Solis, A., Henríquez-Roldán, C., Hazucha, M.J., 2003. Respiratory damage in children exposed to urbana ir pollution. Pediatr. Pulmonol. 36 (2), 148–161.

Calderon-Garcidueñas, L., Vincent, R., Mora-Tiscareño, A., Franco-Lira, M., Henríquez-Roldán, C., Barragan-Mejia, G., Garrido-Garcia, L., Camacho-Reyes, L., Valencia-Salazar, G., Paredes, R., Romero, L., Osnaya, H., Villarreal-Calderón, R., Torres-Jardón, R., Hazucha, M.J., Reed, W., 2007. Elevated plasma endothelin-1 and pulmonary arterial pressure in children exposed to air pollution. Environ. Health Perspec. 115 (8), 1248–1253.

Calderon-Garcidueñas, L., Villarreal-Calderón, R., Valencia-Salazar, G., Henríquez-Roldán, C., Gutierrez-Castellón, P., Torres-Jardón, R., Osnaya –Briziela, N., Romero, L., Solt, Reed, W., 2008. Systemic inflammation, endothelial dysfunction and activation in clinically healthy children exposed to air pollutants. Inhal. Toxicol. 20 (5), 499–506.

Carvalho-Oliveira, R., Pozo, R.M.K., Lobo, D.J.A., Lichtenfels, A.J.F.C., Martins Jr., H.A., Bustilho, J.O.W.V., Saiki, M., Sato, I.M., Saldiva, P.H.N., 2005. Diesel emissions significantly influence composition and mutagenicity of ambient particles: a case study in Sao Paulo, Brazil. Environ. Res. 98, 1–7.

Chauhan, V., Brezan, D., Thomson, E., Karthikeyan, S., Vicent, R., 2005. Effects of ambient air particles on the endothelin system in human pulmonary epithelial cells (A549). Cell Biol. Toxicol. 21 (5–6), 191–205.

CETESB. Caracterização das Estações da Rede Automática de Monitoramento da Qualidade do Ar na RMSP – Estação Cerqueira César. CETESB, 2005. Série Relatórios/Secretaria de Estado do Meio Ambiente, ISSN: 0103-4103. Available from: http://www.cetesb.sp.gov.br.

Cho, J.Y., Miller, M., Baek, K.Y., Han, J.W., Nayar, J., Lee, S.Y., Mc Elwain, K., Mc Elwain, S., Friedman, S., Broide, D.H., 2004. Inhibition of airway remodeling in IL-5-deficient mice. J. Clin. Invest. 113, 551–560.

Dockery, D.W., 2001. Epidemiologic evidence of cardiovascular effects of particulate air pollution. Environ. Health Perspect. suppl 4, 483–486.

Eagleton, M.J., Peterson, D.A., Sullivan, V.V., Roelofs, K.J., Ford, J., Stanley, J.C., Upchurch Jr, G.R., 2002. Nitric oxide inhibition increases aortic wall matrix metalloproteinase-9 expression. J. Surg. Res. 104 (1), 15–21.

Fredenbergh, L.E., Liang, O.D., Macias, A.A., Polte, T.R., Liu, X., Riascos, D.F., Chung, S.W., Schissel, S.L., Inger, D.E., Mitsialis, S.A., Kourembanas, S., Perrella, M.A., 2008. Absence of cyclooxygenase-2 exacerbates hypoxia-induced pulmonary hypertension and enhances contractility of vascular smooth muscle cell. Circulation 117 (16), 2114–2122.

Hoek, G., Brunekreef, B., Goldbohm, S., Fischer, P., Brandt, P.A., 2002. Association between mortality and indicators of traffic-related air pollution in the Netherlands: a cohort study. Lancet 360, 1203–1209.

Hogg, J.C., van Eeden, S., 2009. Pulmonary and systemic response to atmospheric pollution. Respirology 14 (3), 336–346.

Knuckles, T.L., Lund, A.K., Lucas, S.N., Campen, M.J., 2008. Diesel exhaust exposure enhances venoconstriction via uncoupling of eNOS. Toxicol. Appl. Pharmacol. 29 March [Epub. ahead of print].

Kodavant, U.P., Schladweiller, M.C., Ledbetter, A.D., Hauser, R., Christiani, D.C., McGee, J., Richards, J.R., Costa, D.L., 2002. Temporal association between pulmonary and systemic effects of particulate matter in healthy and cardiovascular compromised rats. Toxicol. Environ. Health A 65 (20), 1545–1569.

Lemos, M., Mohallen, S.V., Macchione, M., Dolhnikoff, M., Assunção, J.V., Godleski, J.J., Saldiva, P.H.N., 2006. Chronic exposure to urban air pollution induces structural alterations in murine pulmonary and coronary arteries. Inhal. Toxicol. 18, 247–253.

Lippmann, M., Frampton, M., Schwartz, J., Dockery, D., Schlesinger, R., Koutrakis, P., Froines, J., Nel, A., Finkelstein, J., Godleski, J., Kaufman, J., Koening, J., Larson, T., Luchtel, D., Liu, L., Oberdörster, G., Peters, A., Sarnat, J., Sioutas, C., Salvi, S., Blomberg, A., Rudell, B., Kelly, F., Sandstrom, T., Holgate, S.T., Frew, A., Suh, H., Sullivan, J., Utell, M., Wichmann, E., Zelikoff, J., The U.S, 2003. Environmental protection agency particulate matter health effects research centers program: a midcourse report of status, progress, and plans. Environ. Health Perspect. 111, 1074–1092.

Lichtenfels, A.J., Gomes, J.B., Pieri, P.C., El Khouri Miraglia, S.G., Hallak, J., Saldiva, P.H., 2007. Increased levels of air pollution and a decrease in the human and mouse male-to-female ratio in Sao Paulo, Brazil. Fertil. Steril. 87, 230–232.

Lodge, J.P. Jr., 1989. Methods of Air Sampling and Analysis 3rd ed. Lewis Paublic, Chelsea, Mich.

Lund, A.K., Lucero, J., Lucas, S., Madden, M.C., McDonald, J.D., Seagrave, J.C., Knuckles, T.L., Campen, M.J., 2009. Vehicular emissions induce vascular MMP_9 expression and activity associated with endothelin-1 mediated pathways. Artherioscler Thromb. Vasc. Biol. 29 (4), 511–517.

Mauad, T., Rivero, D.H.R.F., Carvalho-Oliveira, R., Lichtenfels, A.J.F.C., Guimarães, E.T., André, P.A., Kasahara, D.I., Bueno, H.M.S., Saldiva, P.H.N., 2008. Chronic exposure to ambient levels of urban particles affects mouse lung development. Am. J. Respir. Crit. Care 178 (7), 721–728.

Meidan, R., Levy, N., 2002. Endothelin-1 receptors and biosynthesis in the corpus luteum: molecular and physiological implications. Domestic Anim. Endocrinol. 23, 287–298.

Mohallem, S.V.A., Lobo, D.J.P., Pesquero, C.R., Assuncao, J.V., de Andre, P.A., Saldiva, P.H., Dolhnikoff, M., 2005. Decreased fertility in mice exposed to environmental air pollution in the city of Sao Paulo. Environ. Res. 98, 196–202.

Neter, J., Kutner, M.H., Nachtsheim, C.J., Li, W., 2005. Applied Linear Statistical Models 5th ed.

Nurkiewicz, T.R., Porter, D.W., Barger, M., Millecchia, L., Rao, K.M.K., Marvar, P.J., Hubbs, A.F., Castranova, V., Boegehold, M.A., 2006. Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure. Environ. Health Perspect. 114 (3), 412–419.

Pereira, P.M., Saldiva, P.H.N., Sakae, R.S., Böhm, G., Martins, M.A., 1995. Urban levels of air pollution increase lung responsiveness in rats. Environ. Res. 69, 96–101.

Peretz, A., Sullivan, J.H., Leotta, D.F., Trenga, C.A., Sandis, F.N., Carlsten, C., Wilkinson, C.W., Gill, E.A., Kaufman, J.D., 2008. Diesel exhaust inhalation elicits acute vasoconstriction in vivo. Environ. Health Perspect. 116 (7), 937–942.

Pires-Neto, R.C., Lichtenfels, A.J., Soares, S.R., Macchione, M., Saldiva, P.H.N., Dolhnikoff, M., 2006. Effects of São Paulo air pollution on the upper airways of mice. Environ. Res. 101, 356–361.

Prahalad, A.K., Soukup, J.M., Willis, R., Ghio, A.J., Becker, S., Gallagher, J.E., 1999. Ambient air particles: effects on cellular oxidant radical generation in relation to particulate elemental chemistry. Toxicol. Appl. Phamacol. 158 (2), 81–91.

Rivero, D.H., Soares, S.R., Lorenzi-Filho, G., Saiki, M., Godleski, J.J., Antonangelo, L., Dolhnikoff, M., Saldiva, P.H., 2005. Acute cardiopulmonary alterations induced by fine particulate matter of Sao Paulo. Brazil Toxicol. Sci. 85 (2), 898–905.

Saldiva, P.H.N., Lichtenfels, A.J.F.C., Paiva, P.S.O., Barone, I.A., Martins, M.A., Massad, E., Pereira, J.C.R., Xavier, V.P., Böhm, G.M., 1994. Association between air pollution and mortality due to respiratory diseases in children in São Paulo: a preliminary report. Environ. Res. 65, 218–225.

Saldiva, P., 2008. Air pollution and our lung disease. J. Bras. Pneumol. 34 (1), 1.

Scapellato, M.L., Lotti, M., 2007. Short-term effects of particulate matter: an inflammatory mechanism? Toxicology 37 (6), 461–487.

Squadrito, G.L., Cueto, R., Dellinger, B., Pryor, W.A., 2001. Quinoid redox cycling as a mechanism for sutained free radical generation by inhaled airborne particulate matter. Free Rad. Biol. Med. 31 (9), 1132–1138.

Tamagawa, E., Bai, N., Morimoto, K., Gray, C., Mui, T., Yatera, k, Zhang, x, Xing, L., Li, Y., Laher, I., Sin, D.D., Man, S.F., van Eeden, S.F., 2008. Particulate matter exposure induces persistent lung inflammation and endothelial dysfunction. Am. J. Physiol. Lung Cell Mol. Physiol. 295 (1), L79–L85 Epub 2008 May 9.

Thomson, E., Goegan, P., Kumarathasan, P., Vicent, R., 2004. Air pollutants increase gene expression of the vasoconstrictor endothelin-1 in the lungs. Biochim. Biophys. Acta 1689 (1), 75–82.

Thomson, E., Kumarathasan, P., Goegan, P., Aubin, R.A., Vicent, R., 2005. Differential regulation of the lung endothelin system by urban particulate matter and ozone. Toxicol. Sci. 88 (1), 103–113 Epub. 4 August 2005.

Thomson, E., Kumarathasan, P., Vicent, R., 2006. Pulmonary expression of preproET-1 and preproET-3 mRNA is altered reciprocally in rats after inhalation of air pollutants. Exp. Biol. Med. (Maywood) 231 (6), 979–984.

Van Eeden, S.F., Yeung, A., Quinlam, K., Hogg, J.C., 2005. Systemic response to ambient particulate matter: relevance to chronic obstructive pulmonary disease. Proc. Am. Thorac. Soc. 2 (1), 61–67.

Veras, M.M., Damaceno-Rodrigues, N.R., Caldini, E.G., Maciel Ribeiro, A.A., Mayhew, T.M., Saldiva, P.H., Dolhnikoff, M., 2008. Particulate urban air pollution affects the functional morphology of mouse placenta. Biol. Reprod. 79 (3), 578–584.

Wong, T.W., Tam, W.S., Yu, T.S., Wong, A.H., 2002. Associations between daily mortalities from respiratory and cardiovascular disease and air pollution in Hong Kong, China. Occup. Environ. Med. 59, 30–35.

Ynoue, R.Y., Andrade, M.F., 2004. Characteristics of urban aerosols: size-resolved mass balance of aerosol particles over the São Paulo metropolitan area of Brazil. Aerosol. Sic. Technol. 38 (S2), 52–62.