Research Article

iNOS Inhibition Reduces Lung Mechanical Alterations and Remodeling Induced by Particulate Matter in Mice

Carla Máximo Prado,1 Renato Fraga Righetti,2,3 Fernanda Degobbi Tenorio Quirino dos Santos Lopes,2 Edna Aparecida Leick,2 Fernanda Magalhães Arantes-Costa,2 Francine Maria de Almeida,2 Paulo Hilário Nascimento Saldiva,2 Thais Mauad,2 Iolanda de Fátima Lopes Calvo Tibério,2 and Milton de Arruda Martins2

1Department of Bioscience, Federal University of São Paulo, Santos, Rua Silva Jardim 136, 11015-020 Santos, SP, Brazil
2Faculdade de Medicina FMUSP, Universidade de São Paulo, Av. Dr. Arnaldo 455, Sala 1210, 01246-903 São Paulo, SP, Brazil
3Hospital Sírio-Libanês, Rua Adma Jafet 115, 01308-060 São Paulo, SP, Brazil

Correspondence should be addressed to Carla Máximo Prado; cmaximoprado@gmail.com

Received 29 August 2018; Accepted 7 February 2019; Published 11 March 2019

Academic Editor: Kazuyoshi Kuwano

Copyright © 2019 Carla Máximo Prado et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Background. The epidemiologic association between pulmonary exposure to ambient particulate matter (PM) and acute lung damage is well known. However, the mechanism involved in the effects of repeated exposures of PM in the lung injury is poorly documented. This study tested the hypotheses that chronic nasal instillation of residual oil fly ash (ROFA) induced not only distal lung and airway inflammation but also remodeling. In addition, we evaluated the effects of inducible nitric oxide inhibition in these responses. For this purpose, airway and lung parenchyma were evaluated by quantitative analysis of collagen and elastic fibers, immunohistochemistry for macrophages, neutrophils, inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and alveolar septa 8-iso prostaglandin F2α (8-iso-PGF-2α) detection. Anesthetized in vivo (airway resistance, elastance, H, G, and Raw) respiratory mechanics were also analyzed. C57BL6 mice received daily 60ul of ROFA (intranasal) for five (ROFA-5d) or fifteen days (ROFA-15d). Controls have received saline (SAL). Part of the animals has received 1400W (SAL+1400W and ROFA-15d+1400W), an iNOS inhibitor, for four days before the end of the protocol. A marked neutrophil and macrophage infiltration and an increase in the iNOS, nNOS, and 8-iso-PGF2α expression was observed in peribronchiolar and alveolar wall both in ROFA-5d and in ROFA-15d groups. There was an increment of the collagen and elastic fibers in alveolar and airway walls in ROFA-15d group. The iNOS inhibition reduced all alterations induced by ROFA, except for the 8-iso-PGF2α expression. In conclusion, repeated particulate matter exposures induce extracellular matrix remodeling of airway and alveolar walls, which could contribute to the pulmonary mechanical changes observed. The mechanism involved is, at least, dependent on the inducible nitric oxide activation.

1. Background

In the last few years, special attention has been devoted to the associations between levels of ambient particulate matter (PM) and health effects [1]. It is known that people that live in high polluted places may develop exacerbations of respiratory diseases [2]. However, less is known about the pathophysiology of chronic effects of high PM exposures, although such exposures are also associated with respiratory hospitalizations, including increases in cardiopulmonary mortality and in the rates of lung cancer and in respiratory exacerbations [3, 4].

Significant risks for the development of chronic bronchitis and obstructive airways disease were associated with increased exposure to ambient PM<10 and PM<2.5 μm in diameters (PM10 and PM2.5, respectively) [5]. In addition, Liu et al. [6] demonstrated that PM2.5 exposure decreased small airway function in asthmatic children and...
inhaled corticosteroid reverted this response improving lung function.

Accordingly, several animal models that mimic susceptible segments of the population are currently being used to elucidate physiological mechanisms [7]. Experimental studies have shown effects of PM exposure to lung function [8, 9]; however, most of them focused in acute effects of PM in the lung. In addition, scarce studies evaluated the effects of air pollution on lung remodeling [10, 11]. Although the extensive studies have evaluated effects of air pollution in health, the mechanisms involved in the lung injury induced by particulate matter were poorly investigated.

Most of the effects of air pollution, including particulate matter exposures, were related to oxidative stress activation [12, 13]. It is also known that a lot inflammatory cascade and modulators released in the inflammatory process are responsible for connective tissue and functional lung alterations observed in an experimental model of chronic lung inflammation and patients with asthma [14–17]. Moreover, is important to note that efficacy of current pharmacological treatments, including inhaled glucocorticoids and cysteinyl leukotriene type I receptor antagonists, is partial effective in patients with asthma [18].

Nitric oxide (NO) was associated with a modulation of many physiological effects. NO can be derived either from neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), or from other NO-adduct molecules (nitrosothiols) and it is associated with the airway and vascular tone control. On the other hand, NO derived from inducible nitric oxide synthase (iNOS) seems to be mainly involved in a modulation of immune system [19].

During inflammation, iNOS expression has been described in several types of cells, such as macrophages, neutrophils, and eosinophils, as well as in airway epithelial cells [20, 21]. NO derived from iNOS contributes to airway and distal lung parenchyma response, inflammatory process and extracellular matrix remodeling [22, 23]. Some evidence suggest that nitric oxide can control airway remodeling by interaction with some proteases and antiproteases, such as MMP (metalloproteinase)-12, MMP-9, or growth factors such as TGF (transforming growth factor)–β [24, 25], mediators strongly involved in lung remodeling.

Our hypothesis is that repeated PM could induce structural pulmonary changes related to inflammatory response, extracellular matrix remodeling, and oxidative stress activation. In addition, we considered that these alterations are modulated by iNOS activation. Also considering the health effects of air pollution above described, the clinical relevance of this study is related to the possibility that nitric oxide derived from iNOS is involved on lung injury associated with chronic particulate matter exposures since it may represent an important mechanism.

Therefore, our study aims in evaluate the effects of repeated nasal instillations of low dose of residual oil fly ash (ROFA), a concentrate of air pollution resulting from the burning of oil, on pulmonary mechanics and lung inflammation and remodeling. In order to investigate the effects of iNOS activation, we evaluated the effects of the treatment with a highly selective inhibitor of iNOS activity, 1400W, in pulmonary alterations induced by repeated ROFA instillations.

2. Methods

All mice received humane care in compliance with the “Guide for Care and Use of Laboratory Animals” (NIH publication 85-23, revised 1985). Animals were housed (12-h light/dark cycle) in plastic cages and received food and water ad libitum. All protocols performed in this study were approved by the institutional review board of University of São Paulo (São Paulo, Brazil).

2.1. Particulate Material (PM). Residual oil fly ash (ROFA) was collected from a solid waste incinerator, which is powered by combustible oil, from University Hospital from School of Medicine of University of São Paulo. Characterization of ROFA particles used in this investigation was previously performed [10, 26] by neutron activation to determine the elemental composition, as well as by gas chromatography and high performance liquid chromatography for organics. It was previously determined and detailed described in previous studies [10, 26]. Presence of toxic elements, such as As, Co, Li, and Zn, and several polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, acenaphthylene, fluorene, acenaphthen, anthracene, fluoranthene, pyrene, B[a]anthracene, B[k]fluoranthene, B[a]pyrene, DB[ah]anthracene, B[ghi]pyrenylene, and ind[123cd] were detected and previously detailed [10, 26]. The ROFA used is homogenous in relation to the particles diameter, since more than 80% of the material has particles less than 2.5 μm of diameter [26].

2.2. ROFA Instillation. All animals have received 10μL of ROFA solution (concentration of 6mg/mL) daily during the day 1 to 15th by intranasal instillations (Final dose of 60μg/animal/daily) [10]. This dose mimics a mouse exposed to 24 hs in a high pollution day of a city as São Paulo, Brazil. Control animals received the same volume of saline intranasal. We did only the control group with 15 days of saline since we have unpublished data showing that five or fifteen instillation of saline did not affect lung inflammation.

2.3. 1400W Treatment. To investigate the effects of iNOS inhibition in lung inflammation induced by ROFA instillations, animals were treated for four days with 1400W i.p., in a dose of 2mg/Kg/daily, as previously described [25]. This approach was chosen based in previous studies that have shown that this dose is effective and selective for iNOS and did not cause toxic effects [25, 27]. Control animals were treated with saline solution.

2.4. Experimental Design. To investigate the time effects of air pollution in lung, mice received either

(a) intranasal ROFA daily for five days (ROFA-5, n=8);
(b) intranasal ROFA daily for fifteen days (ROFA-15, n=8);
(c) intranasal saline for fifteen days (SAL, n=8);
(d) intranasal ROFA daily for fifteen days and 1400W treatment (ROFA-15+1400W, n=8);

2 Pulmonary Medicine
(e) intranasal saline for fifteen days and 1400W treatment (SAL+1400W, n=8).

2.5. Pulmonary Mechanics Evaluation. Twenty-four hours after the last instillation, the animals were anesthetized with thiopental sodium (80 mg.kg⁻¹, i.p.), tracheostomized, and connected to a ventilator to small animals (FlexiVent, SCIREQ, Montreal, Canada). Animals were ventilated at 100 breaths/min with a tidal volume of 20 mL.kg⁻¹. This high value of tidal volume is to avoid inefficient ventilation due to an increase in the anatomic death space induced by the inhalation apparatus attached to the ventilator. A dose response curve was performed using 6.125; 12.5; 25; and 50 mg/mL of methacholine inhalation. Methacholine was inhaled for 1 minute and the data was collected 30 sec after the end of inhalation. There was an interval of 3 minutes between the administrations of each dose. The impedance of respiratory system (Zrs) was calculated for each animal and the perturbation volume of 16 sec was used. The same perturbation volume was used after each inhalation. Mechanical ventilation was stopped only to the perturbation application. The cylinder position (Vcyl) and the internal pressure of the cylinder (Pcyl) were registered during the 16 sec of perturbation. We used a perturbation of 16 sec, composed by assertions of solenoids with frequencies between 0.25 and 19.625 Hz, avoiding the harmonic distortion [23]. To avoid the lost related to gases, some corrections were performed. Vcyl was correct to obtain a volume that really arrives in the animals (V) and Pcyl was correct to the values of Pao, open airways pressure. By the derivation in the time of V, we get the flow (V'). To analyze the impedance, we used the model of constant phase: where the Raw is the airway resistance, law is the inertance, G characterizes the energy dissipated to the lung tissue, H characterized the energy accumulated in the lung tissue, and f is the frequency. Results were expressed as percentage of maximal increase (%) after methacholine challenge [23].

2.6. Lung Histology. At the end of pulmonary mechanics, the anterior chest wall was opened, animals were exsanguinated via the abdominal aorta and lungs were removed en bloc. The lungs were fixed in 10% formaldehyde for 24h in a constant pressure of 20 cmH2O. Sections representing peripheral areas of the lung were cut and processed for paraffin embedding. Five micron sections were stained with Sirius Red collagen and Weigerts technique for elastic fibers quantifications. To analyze collagen and elastic fibers content in airways and lung parenchyma, we measured the total area of airway wall or lung parenchyma tissue, and the collagen or elastic fibers (μm²) area in a nine-ten airways or lung parenchyma fields, at a magnification of x10 for airways and x40 for lung parenchyma, in an image analysis system (Image ProPlus 4.6v). Collagen or elastic content (%) in peribronchiolar and alveolar wall was expressed as a relation between the area of collagen or elastic fibers in a specific frame and the total area of the frame [17].

Immunohistochemistry was performed with the following antibodies: Anti-Macrophage-2 (Clone M3/M38, Cedarlane Lab, Burlington, Canada), anti-Neutrophils (MCA771G, Abd Serotec, Oxford, UK), anti-nNOS (nNOS/NOS type I-N31020; BD Transduction Laboratories, San Diego, CA), anti-iNOS (IgG2a–iNOS/NOS type 2–N32020; BD Transduction Laboratories, San Diego, CA) anti-actin (nNOS/NOS type I-N31020; BD Transduction Laboratories, San Diego, CA), anti-8-iso-PGF2α (Oxford Biomedical Research, Oxford, UK), goat polyclonal anti-mouse MMP12 (1:200, Santa Cruz Biotechnology, USA), and rabbit polyclonal anti-transforming growth factor-β (TGF-β1) IgG (1:1200, Santa Cruz Biotechnology, USA). Secondary antibodies anti-goat, rat, or rabbit antibodies (Vectastain Abc Kit, Vector Laboratories, USA) were used in accordance with the manufacturer's instructions. Sections were counterstained with hematoxylin.

With a 50-line and 100-point grid connected to the ocular of the microscope, peribronchiolar and alveolar wall density of macrophages, neutrophils, iNOS, and nNOS, TGF-β and MMP-12 positive cells were assessed using a point counting technique [28]. Counting was performed in 5 airways (peribronchiolar area) and in 10 parenchyma fields (area of alveolar septa) in each animal at x1,000 magnification by a blinded investigator. The results were expressed as cell/10⁶μm². The 8-iso-PGF2α area was evaluated by image analysis as described above for collagen and elastic fibers. The 8-iso-PGF2α-positive area was expressed as a percentage of the total area in both peribronchiolar and alveolar wall [28].

2.7. Statistical Analysis. Statistical analysis was performed using SigmaStat software (SPSS Inc., Chicago, IL). Data were analyzed with One-way Analysis of Variance (ANOVA) followed by the Holm-Sidak method for multiple comparisons and data are presented as mean ± SE. To evaluate the effects of iNOS inhibition in ROFA-induced lung alterations, we used Two-way Analysis of Variance, considering one factor the ROFA and the other the 1400W treatment. P<0.05 values were considered significant.

3. Results

3.1. ROFA Increased the Lung Mechanics Alterations. We noted an increase in the maximal percentage of increase of Rs and Ers as well as in Gs and Hs in both ROFA-5 and ROFA-15 compared to saline (P<0.05) as shown in Table 1. There were no significant differences between ROFA-5 and ROFA-15 in all the mechanical parameters.

3.2. ROFA Increased Lung Inflammation. ROFA instillations (ROFA-5 and ROFA-15) induced an increase in the number of neutrophils in both peribronchiolar and alveolar wall compared to saline (P<0.05) (Table 2). The number of macrophages was increased only in ROFA-15 in both bronchiolar and alveolar wall compared to saline (P<0.05).

3.3. ROFA Increased Oxidative Stress. ROFA instillations (ROFA-5 and ROFA-15) increased the number of iNOS and nNOS-positive cells and the expression of fractional area of 8-iso-PGF2α in both peribronchiolar and alveolar wall compared to saline (P<0.05). The expression of 8-iso-PGF2α was increased in ROFA-15 group only at airway level (Table 3). Although the iNOS and nNOS-positive cells in airways were
increased in ROFA-15 compared to saline group, these values were lower than those obtained in ROFA-5 group (P<0.05). There were no differences in nNOS, iNOS-positive cells and 8-iso-PGF2α in alveolar wall between ROFA 5 and ROFA-15 groups.

3.4. ROFA Increased Extracellular Matrix Remodeling. Collagen and elastic fibers were increased only in ROFA-15 compared to saline in peribronchiolar and alveolar wall compartments (P<0.001). We also analyzed the number of metalloproteinase 12 (MMP-12) and TGFβ-positive cells in the lung. There was an increase in TGFβ-positive cells in both peribronchiolar and alveolar wall in ROFA-5 group and ROFA-15 group compared to saline group (P<0.001). There was a decrease in the expression of TGFβ in peribronchiolar wall in ROFA-15d group compared to ROFA-5d group. Considering MMP-12-positive cells, the expression of MMP-12 was higher in AW only in ROFA-15 and in DLP in both ROFA-5 group and ROFA-15 group compared to saline (P<0.001) (Table 4).

3.5. Effects of 1400W in the Lung Mechanics Alterations Induced by Fifteen Instillations of ROFA. The 1400W treatment in animals that received ROFA instillations for 15 days reduced the maximal percentage of increase of Rs and Ers compared to those received the vehicle (ROFA-15-W compared to ROFA-15d, P<0.05), as shown in Figure 1. There were no significantly effects of 1400W treatment in ROFA-induced an increase in Gtis [ROFA-W: 115.77 ± 32.22] and Htis [ROFA-W: 77.43 ± 20.88] values compared to those animals that received ROFA and vehicle (ROFA-15, values of ROFA-15 were shown in Table 1).

3.6. Effects of 1400W in the Lung Inflammation Induced by Fifteen Instillations of ROFA. The iNOS inhibition in ROFA-instilled animals (ROFA-15-W) reduced the number of neutrophils and macrophages in both peribronchiolar and alveolar wall compared with those animals that received vehicle (P<0.05) (Figure 2).

3.7. Effects of 1400W in the Oxidative Stress Induced by Fifteen Instillations of ROFA. We observed that in ROFA-15-W there was a decrease in the number of iNOS-positive cells in peribronchiolar and alveolar wall compared to ROFA-15 (P<0.05). As expected, the iNOS inhibition did not affect the nNOS-positive cells in animals that received ROFA-1400W compared to those that received vehicle (Figures 2(a) and 2(b)). The iNOS inhibition reduced the 8-iso-PGF2α content in ROFA-15 animals only in airways (P<0.05) (Figure 3).

3.8. Effects of 1400W in the Extracellular Matrix Remodeling by Fifteen Instillations of ROFA. Considering pulmonary remodeling (Figure 4), iNOS inhibition reduced both collagen and elastic content in airways and in lung distal parenchyma comparing to those animals that received vehicle (P<0.05). The iNOS inhibition reduced the TGFβ (Figure 5) and MMP-12 (Figure 6) positive cells only in peribronchiolar area, not affecting the alveolar wall positive cells (P<0.05).
Table 4: ROFA-induced extracellular matrix remodeling; after both five (ROFA-5d) and fifteen instillations (ROFA-15d), mice presented an increase in the number of neutrophils and inflammatory cells that express nNOS and iNOS around airways and in distal lung parenchyma. The macrophages were significantly increased only in ROFA-15d groups compared to saline. * P<0.05 compared to saline; ** P<0.05 compared to ROFA-5d.

|                | Collagen fibers | Elastic fibers | MMP-12 | TGF-β | Collagen fibers | Elastic fibers | MMP-12 | TGF-β |
|----------------|-----------------|----------------|--------|-------|-----------------|----------------|--------|-------|
| SAL            | 15.7±2.9        | 13.5±1.6       | 29.5±4.4 | 9.7±1.5 | 20.7±2.5        | 9.5±0.7       | 17.4±5.4 | 18.1±3.2 |
| ROFA-5d        | 13.9±3.2        | 11.1±1.7       | 46.6±17.4* | 34.6±3.7* | 20.9±2.6        | 17.4±1.6       | 55.7±10.1* | 46.3±4.3* |
| ROFA-15d       | 26.7±1.9*, **   | 25.4±2.4*, **  | 79.5±5.3* | 37.2±3.2* | 37.2±3.2*, **   | 23.9±3.4*      | 36.7±4.4* | 30.1±2.5*, ** |

3.9. Qualitative Analysis. Figure 7 shows representative photomicrographs of airway stained with immunohistochemistry to detect neutrophils, iNOS, isoprostane, and collagen fibers in all experimental groups. Peribronchial of ROFA-15 animals presented a prominent neutrophil infiltration including iNOS-positive cells and fractional area of the 8-iso-PGF2α and collagen fibers. Peribronchial sections from animals instilled with ROFA-15 and treated 1400W presented attenuation in neutrophil infiltration including iNOS-positive cells and fractional area of the 8-iso-PGF2α and collagen fibers (Scale bar = 30 μm).

4. Discussion

In this study we showed that repeated exposures to levels of PM induced significant changes on lung mechanics, lung inflammation, and extracellular matrix remodeling which occurred in both distal airways and lung parenchyma. The inflammatory response was characterized by macrophages and neutrophils and could be observed early after five instillations, while extracellular matrix remodeling associated with increase in MMP-12 and TGF-β expression occurred only after fifteen instillations. We also showed that repeated instillations of ROFA induced an increment in the number of nNOS and iNOS-positive inflammatory cells as well as in 8-iso-PGF2α expression around peribronchiolar and alveolar wall. This was a feasible and reproducible experimental model for studies of pathophysiological mechanisms involved in lung injury induced by air pollution. The effects of inhaled toxicants and allergen challenge on airway inflammation and oxidative stress has also been studied in well established in vitro experimental animal models [29, 30].

Several pathophysiological mechanisms may be involved in lung injury induced by air pollution [31, 32]. In order to investigate the hypothesis of NO involvement in the pathophysiology of lung inflammation induced by particulate matter, we evaluated the effects of specific iNOS inhibitor in this experimental model. We used 1400W, a highly selective and specific iNOS inhibitor, as previously shown in other experimental studies [25, 27, 33]. The effectiveness of this treatment was assessed by immunohistochemical detection of both nNOS and iNOS. We observed that 1400W treatment only reduced the number of iNOS-positive cells, not affecting the nNOS expression. The most important finding was related to the fact that NO, mainly originated from iNOS activation, was involved in the inflammatory and extracellular matrix remodeling, acting as proinflammatory modulator, since the treatment with 1400W, a high specific inhibitor, reduced the most of the features observed in this model. There were few studies evaluating experimental models of inflammation induced by repeated exposure to air pollution that had mapped the changes induced by chronic exposure of particulate matter not only in the lung parenchyma, but also in the airways [34]. Most of them have combined air pollution exposure to allergic inflammation or tobacco exposure.

It is well known that repeated exposures to irritants such as particulate matter, diesel, cigarette smoke results in bronchial epithelial damage, mucous hypersecretion, fibrosis and narrowing of the airways, destruction of parenchyma and vascular changes [35]. ROFA (residual oil fly ash) is a metal-rich particulate material, with little organic component derived from combustion processes at high temperatures [36]. Due to its high toxicity, it is used as a substitute for experimental environmental studies to assess the biological effects of particulate matter [37–39]. The element composition of ROFA used in the present study was previously characterized [26].

Repeated intranasal instillation of ROFA in mice induced an increased resistance and elastance of the respiratory system. This result is consistent with previous studies that reported increased airway resistance and elastance in response to particulate matter exposure [37–39]. The increase in RSA and ERS in ROFA-treated mice compared to control also suggests that ROFA exposure induces a chronic inflammatory response in the airways, which can lead to airway remodeling and structural changes that impair lung function. The observed changes in RSA and ERS in this study are likely due to the inflammatory and oxidative stress effects of ROFA exposure, which can cause airway epithelial damage and increased mucus secretion, leading to airway obstruction. The effects of ROFA on airway mechanics are likely to be due to the presence of metal and other toxic components in the particulate matter, which can activate inflammatory signaling pathways and release proinflammatory mediators such as cytokines and chemokines. These findings emphasize the importance of considering the toxicological and pathophysiological effects of environmental particulate matter exposure, particularly in the context of air pollution and respiratory health.
system as well as the strength and elasticity of lung tissue. Other authors had already demonstrated that ROFA exposures affected bronchial responsiveness. In this sense, Gavett et al. [40] demonstrated that single intratracheal instillation of ROFA in Balb/C altered the total resistance of the respiratory system in response to low doses of methacholine, and induced an accumulation of neutrophils in airways and alveolar walls. Arantes-Costa et al. [10] demonstrated that sensitized animals worsened mucus production when received ROFA after the sensitization period.

The alterations in lung function observed in animals that received ROFA may be secondary to pulmonary morphological alterations found. It is well known that airway extracellular matrix remodeling seen over the years may be associated with the irreversibility of pulmonary function, at least in asthmatic patients [41]. Thus, inflammation and extracellular matrix remodeling observed in ROFA-instilled group may, by itself, lead to increased responses of pulmonary mechanics. Inflammatory cell recruitment is associated with a release of several mediators/modulators, among them nitric oxide, which are also involved in the control of airway smooth muscle contractility and lung parenchyma mechanical responses [16, 25, 42].

Figure 2: Lung inflammation and nitric oxide synthase expression. This figure represents the mean and SEM values of macrophages, neutrophils, nNOS, and iNOS-positive cells in peribronchiolar (a) and alveolar (b) wall. The animals that received fifteen instillations of ROFA presented higher values of all cells, and 1400W reduced inflammation and iNOS-positive cells, not interfering with nNOS expression. * p<0.05 compared with saline groups; # p<0.05 compared with animals that received ROFA and vehicle treatment.

Figure 3: Oxidative stress: this figure represents the mean and SEM values of 8-iso-PGF-2a expression (%) in peribronchiolar and alveolar wall. The ROFA instillations increased the positive area and the iNOS inhibition reduced it only around bronchioles. * p<0.05 compared with saline groups; ** p<0.05 compared with animals that received ROFA and vehicle treatment.
FIGURE 4: Lung remodeling: this figure represents the mean and SEM values of both collagen and elastic fibers content around airways (a) and in alveolar wall (b). The ROFA instillations increased the airway and parenchyma extracellular matrix fibers deposition compared to control, and the iNOS inhibition reduced these responses. *p<0.05 compared with saline groups; **p<0.05 compared with animals that received ROFA and vehicle treatment.

FIGURE 5: TGF-β expression: this figure represents the mean and SEM values of TGF-β expression in both peribronchiolar (a) and alveolar (b) wall. The ROFA instillations increased the number of TGF-β-positive cells in both airways and alveolar wall and iNOS inhibition reduced this response only in airways. *p<0.05 compared with saline groups; **p<0.05 compared with animals that received ROFA and vehicle treatment.

In addition, an increased 8-iso-PGF-2α in peribronchiolar and alveolar wall could be associated with the responsiveness observed. 8-iso-PGF2α concentrations were measurable in exhaled breath condensate (EBC) (Montuschi et al., 2010), a noninvasive technique for sampling airway secretions, and it has been found elevated in patients with COPD compared with healthy ex-smokers [43]. Although previous studies have evaluated the effects of PGE2, which was more potent as a constrictor than PGF2α, the latter isoprostane is considered the predominant form generated during free radical attack of cell membranes [44]. Jourdan et al. [45] showed that L-NAME treatment greatly inhibits 8-iso-PGF2α and also that pulmonary artery smooth muscle can release this isoprostane. This class of substances induces contractions of the smooth muscle of airways and vessels operating in the Rho tyrosinase and Rho-kinase, leading to increased activity of phosphorylated myosin [16, 22, 46]. Shiraki et al. [47] showed studying tracheal smooth muscle
contraction that 8-isoPGF2α increased the isometric tension and they concluded that 8-isoPGF2α causes airway smooth muscle contraction. However, few studies have evaluated the specific effects of 8-isoPGF2α in smooth muscle contraction. Our group also have demonstrated that the inhibition of 8-isoPGF2α by different agents reduced the airway hyperresponsiveness [48–50].

Furthermore, collagen and elastic fibers remodeling in the airways and distal lung parenchyma occurred only in ROFA-15 day group. Alterations in the elastic fibers content are known to occur in the pathophysiology of lung disease such as asthma and COPD, a major shortfall of elastic fibers followed by loss of elasticity and elastic recoil occurs in a second stage regeneration of these fibers, making them thicker. It would be expected that an increase in neutrophilic recruitment may reduce the elastic fiber content. However, corroborating our results, other authors have found an increase in elastic fibers [42, 49], probably because of the turnover processes of elastic fibers that involves the function of smooth muscle promoting elastin formation and the elastolytic effects of inflammation, particularly related to neutrophils, which was prominent in both peribronchiolar and alveolar wall in the present study.

In an attempt to understand some of the mechanisms involved in these responses, we evaluated the 8-iso-PGF-2α expression in the lung, a marker of oxidative stress pathway activation. The formation of peroxynitrite leads to lipid peroxidation and generation of isoprostanes such as 8-iso-PGF2 α, which is one of the predominant forms generated during the attack of free radicals in the cell membrane [50]. After both 5 and 15 days of ROFA exposures there was an increase in the oxidative stress pathway activation in the lung and an increase in the number of nNOS and iNOS-positive inflammatory cells in peribronchiolar and alveolar wall. As NO has a short half-life, the reactive oxygen (ROS) and reactive nitrogen species (RNS) can act as NO carriers, culminating with oxidative stress activation.

In addition, it is well known that NO modulates bronchial smooth muscle contractility and also lung parenchyma. In this regard, 1400W treated animals had a significant reduction in the resistance and elastance of the respiratory system compared to vehicle treated groups, suggesting an effect of this inhibitor attenuating the response of lung mechanics induced by ROFA in proximal and distal airways. However, there were no differences in Htis and Gtis, which reflects more lung distal parenchyma response.

Donors of nitric oxide (nitrovasodilator) are relaxing the smooth muscles, including airway smooth muscle [51, 52]. The effects of NO as a bronchodilator is well recognized, however various studies emphasized that the effects of NO on bronchodilation depends on the enzyme type that is producing NO. When NO was produced by iNOS in high quantities, it could induce bronchoconstriction [24]. Besides the direct effects of NO in smooth muscle is important to note that the control of inflammation and remodeling by the treatment of iNOS inhibition can also contribute to the improvement of lung function.

We found that iNOS inhibition reduced the infiltration of neutrophils and macrophages and deposition of collagen and elastic fibers. This effect was similar in airways and lung parenchyma. Some mechanisms may explain how nitric oxide acts on inflammatory cells. High levels of NO act as effectors molecule of the immune system and can inhibit DNA synthesis by inactivation of ribonucleic reductase and by direct deamination of DNA [24]. The activation of apoptosis can be induced by nitric oxide since it can influence DNA fragmentation and prolong cell survival [53]. However, Chung et al. [54] suggest that NO may have effects, both apoptotic and antiapoptotic, and that these responses depend on nitric oxide levels and also the producing cell.
Corroborating the present findings, we recently showed that iNOS inhibitor attenuates lung vascular remodeling in an asthma model [24]. Some evidence tried to explain how NO affect the collagen deposition. Horstman et al. [55] suggested that nitric oxide may attenuate vascular remodeling secondary to hypoxia in rats. Aristoteles et al. [25] studied a model of chronic allergic inflammation and demonstrated that nitric oxide inhibition derived from the inducible isoform modulates the extracellular matrix remodeling.

Other possible explanations are the role of nitric oxide in the modulation of metalloproteinases synthesis and activation as well as in their inhibitors [56]. Metalloproteinases are families of enzymes that are involved in the degradation of collagen and other extracellular matrix proteins [16].

We evaluated the MMP-12 since this metalloproteinase is able to degrade extracellular matrix components and is involved in lung tissue remodeling observed in inflammatory pulmonary diseases such as chronic obstructive pulmonary diseases (COPD). We observed that the number of MMP-12-positive cells was increased in both peribronchiolar and alveolar wall after fifteen ROFA instillations. Corroborating the idea that nitric oxide influences metalloproteinases, we observed that 1400W treatment reduced the number of MMP-12 positive cells only around peribronchiolar area,
suggesting that iNOS is involved in MMP-12 upregulation induced by particulate matter, and it could be one of the mechanisms in which iNOS acts in lung remodeling. These results are in accordance with previous studies [17, 27, 57].

Another important mechanism involved in lung remodeling is the profibrotic cytokine TGF-β. It is produced by a number of cells, including macrophages, epithelial cells, and fibroblasts and is involved in most of the cellular biological processes of airway remodeling [58]. In the present study, we observed that both five and fifteen instillations of ROFA induce an increase in both peribronchiolar and alveolar wall TGF-β-positive cells. The iNOS inhibition reduced this response only in peribronchiolar cells, not affecting alveolar wall.

1400W treatment reduces the 8-iso-PGF-2α only around airways, suggesting that oxidative stress is one important mechanism involved in air pollution-induced lung alterations, as extensively discussed in the literature [59–61]. Other studies of our group have shown this same effect of iNOS inhibition in oxidative stress using an animal model of chronic allergic lung inflammation [62, 63].

The major limitation of the present study was related to the fact that ROFA is a tool to study the effects of particulate matter in lung and may be not representative of particulate matter present in air pollution. However, its exact composition is known and makes the experiments reproducible which is important to elucidate mechanisms involved. The clinical relevance is related to two important views: First, we developed an experimental model to study air pollution with repeated instillations that can be used to investigate physiopathological mechanisms. Second, we clearly showed that iNOS is involved in lung inflammation induced by particulate matter. Further studies involved other mechanics such as the role of oxidative stress, IL-17 and cholinergic anti-inflammatory system, as recent studies suggested [64].

Collectively, ROFA instillation induced lung mechanics, inflammatory, and extracellular matrix remodeling alterations in lungs of C57BL mice. These changes are associated with an increased in the oxidative stress pathway, metalloproteinase, and TGF-β activation. NO, derived mainly from iNOS, is involved in proinflammatory and profibrotic alterations induced by particulate matter.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and HC-FMUSP, Brazil.

### References

[1] K. Adams, D. S. Greenbaum, R. Shaikh, A. M. van Erp, and A. G. Russell, "Particulate matter components, sources, and health: Systematic approaches to testing effects," Journal of the Air & Waste Management Association, vol. 65, no. 5, pp. 544–558, 2015.

[2] M. Lippmann, K. Ito, A. Nádas, and R. T. Burnett, "Association of particulate matter components with daily mortality and morbidity in urban populations," Research Reports Health Effects Institute, vol. 95, pp. 5–72, 2000.

[3] A. Valavanidis, K. Fiotakis, and T. Vlachogianni, "Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms," Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicty Reviews, vol. 26, no. 4, pp. 339–362, 2008.

[4] A. Gawda, G. Majka, B. Nowak, and J. Marcinkiewicz, "Air pollution, oxidative stress, and exacerbation of autoimmune diseases," Central European Journal of Immunology, vol. 42, no. 3, pp. 305–312, 2017.

[5] S. Ling and S. F. van Eeden, "Particulate matter air pollution exposure role in the development and exacerbation of chronic obstructive pulmonary disease," International Journal of Chronic Obstructive Pulmonary Disease, vol. 4, pp. 233–243, 2009.

[6] L. Liu, R. Poon, L. Chen et al., "Acute effects of air pollution on pulmonary function, airway inflammation, and oxidative stress in asthmatic children," Environmental Health Perspectives, vol. 117, no. 4, pp. 668–674, 2009.

[7] X. Lu, H. Fu, F. Han et al., "Lipoxin A4 regulates PM2.5-induced severe allergic asthma in mice via the Th1/Th2 balance of group 2 innate lymphoid cells," Journal of Thoracic Disease, vol. 10, no. 3, pp. 1449–1459, 2018.

[8] X. Zhang, W. Zhong, Q. Meng et al., "Ambient PM2.5 exposure exacerbates severity of allergic asthma in previously sensitized mice," Journal of Asthma, vol. 52, no. 8, pp. 785–794, 2015.

[9] H. Liu, X. Fan, N. Wang, Y. Zhang, and J. Yu, "Exacerbating effects of PM2.5 in OVA-sensitized and challenged mice and the expression of TRPA1 and TRPV1 proteins in lungs," Journal of Asthma & Allergy Educators, vol. 54, no. 8, pp. 807–817, 2017.

[10] F. M. Arantes-Costa, F. D. T. Q. S. Lopes, A. C. Toledo et al., "Effects of residual oil fly ash (ROFA) in mice with chronic allergic pulmonary inflammation," Toxicologic Pathology, vol. 36, no. 5, pp. 680–686, 2008.

[11] X. Xu, H. Wang, S. Liu et al., "TP53-dependent autophagy links the ATR-CHEK1 axis activation to proinflammatory VEGFA production in human bronchial epithelial cells exposed to fine particulate matter (PM2.5)," Autophagy, vol. 12, no. 10, pp. 1832–1848, 2016.

[12] P. Montuschi, J. A. Nightingale, S. A. Kharitonov, and P. J. Barnes, "Ozone-induced increase in exhaled 8-isoprostane in healthy subjects is resistant to inhaled budesonide," Free Radical Biology & Medicine, vol. 33, no. 10, pp. 1403–1408, 2002.

[13] G. D. Thurston, H. Kipen, I. Annesi-Maesano et al., "A joint ERS/ATS policy statement: what constitutes an adverse health effect of air pollution? an analytical framework," European Respiratory Journal, vol. 49, no. 1, Article ID 1600419, 2017.

[14] P. Montuschi, "Leukotrienes, antileukotrienes and asthma," Mini-Reviews in Medicinal Chemistry, vol. 8, no. 7, pp. 647–656, 2008.
[15] M. L. Manni, J. B. Trudeau, E. V. Scheller et al., “The complex relationship between inflammation and lung function in severe asthma,” *Mucosal Immunology*, vol. 7, no. 5, pp. 1186–1198, 2014.

[16] R. F. Righetti, P. A. da Silva Pigati, and S. S. Possa, “Effects of Rho-kinase inhibition in lung tissue with chronic inflammation,” *Respiratory Physiology & Neurobiology*, vol. 192, pp. 134–146, 2014.

[17] L. Camargo, R. F. Righetti, L. R. Arístoteles et al., “Effects of anti-IL-17 on inflammation, remodeling, and oxidative stress in an experimental model of asthma exacerbated by LPS,” *Frontiers in Immunology*, vol. 8, article 1835, 2018.

[18] P. Montuschi and P. J. Barnes, “New perspectives in pharmacological treatment of mild persistent asthma,” *Drug Discovery Therapy*, vol. 16, no. 23–24, pp. 1084–1091, 2011.

[19] U. Förstermann and W. C. Sessa, “Nitric oxide synthases: regulation and function,” *European Heart Journal*, vol. 33, no. 7, pp. 829–837, 2012.

[20] S. S. Possa, E. A. Leick, C. M. Prado, M. A. Martins, and I. F. L. C. Tiberio, “Eosinophilic inflammation in allergic asthma,” *Frontiers in Pharmacology*, vol. 4, article 46, 2013.

[21] G. G. King, A. James, L. Harkness, and P. A. B. Wark, “Pathophysiology of severe asthma: We’ve only just started,” *Respirology*, vol. 23, no. 3, pp. 262–271, 2018.

[22] P. A. Pigati, R. F. Righetti, S. S. Possa et al., “Y-27632 is associated with corticosteroid-potentiated control of pulmonary remodeling and inflammation in guinea pigs with chronic allergic inflammation,” *BMC Pulmonary Medicine*, vol. 15, article 85, 2015.

[23] O. A. Theodoro-Junior, R. Fraga Righetti, R. Almeida-Reis et al., “A plant protein inhibitor from enterolobium contortisiliquum attenuates pulmonary mechanics, inflammation and remodeling induced by elastase in mice,” *International Journal of Molecular Sciences*, vol. 18, no. 2, article 403, 2017.

[24] C. M. Prado, M. A. Martins, and I. F. Tiberio, “Nitric oxide in asthma physiopathology,” *ISRN Allergy*, vol. 2011, Article ID 832560, 2011.

[25] L. R. Aristoteles, R. F. Righetti, N. M. Pinheiro et al., “Modulation of the oscillatory mechanics of lung tissue and the oxidative stress response induced by arginase inhibition in a chronic allergic inflammation model,” *BMC Pulmonary Medicine*, vol. 13, no. 1, article 52, 2013.

[26] R. Carvalho-Oliveira, M. Saiki, R. C. Pires-Neto, G. Lorenzi-Filho, M. MacChione, and P. H. N. Saldiva, “Anti-oxidants reduce the acute adverse effects of residual oil fly ash on the frog palate mucociliary epithelium,” *Environmental Research*, vol. 98, no. 3, pp. 349–354, 2005.

[27] C. M. Prado, E. A. Leick-Maldonado, L. Yano et al., “Effects of nitric oxide synthases in chronic allergic airway inflammation and remodeling,” *American Journal of Respiratory Cell and Molecular Biology*, vol. 35, no. 4, pp. 457–465, 2006.

[28] M. I. Bittencourt-Mernak, N. M. Pinheiro, F. P. R. Santana et al., “Prophylactic and therapeutic treatment with the flavonone sakuranetin ameliorates LPS-induced acute lung injury,” *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 312, no. 2, pp. L217–L230, 2017.

[29] G. Ciabattoni, P. Montuschi, D. Curró, and P. Preziosi, “In vitro testing for lung toxicity,” *Toxicology in Vitro*, vol. 7, no. 5, pp. 581–585, 1993.

[30] G. Ciabattoni, P. Montuschi, D. Curró, G. Togna, and P. Preziosi, “Effects of vasoactive intestinal polypeptide on antigen-induced bronchoconstriction and thromboxane release in guinea-pig lung,” *British Journal of Pharmacology*, vol. 109, no. 1, pp. 243–250, 1993.

[31] P. Montuschi, P. Del Cecato, and G. Ciabattoni, “In vitro testing for lung toxicity: A method for distinguishing between immune- and non-immune-mediated reactions to xenobiotics,” *Environmental Toxicology and Pharmacology*, vol. 2, no. 2–3, pp. 201–205, 1996.

[32] S. Esposito, R. Tenconi, M. Lelii et al., “Possible molecular mechanisms linking air pollution and asthma in children,” *BMC Pulmonary Medicine*, vol. 14, no. 1, article 31, 2014.

[33] M.-Y. Lee, K.-H. Sun, C.-P. Chiang et al., “Nitric oxide suppresses LPS-induced inflammation in a mouse asthma model by attenuating the interaction of IKK and Hsp90,” *Experimental Biology and Medicine*, vol. 240, no. 4, pp. 498–507, 2015.

[34] M. S. Happo, R. O. Salonen, A. I. Hlinen et al., “Inflammation and tissue damage in mouse lung by single and repeated dosing of urban air coarse and fine particles collected from six European cities,” *Inhalation Toxicology*, vol. 22, no. 5, pp. 402–416, 2010.

[35] F. Xu, M. Luo, L. He et al., “Necroptosis contributes to urban particulate matter-induced airway epithelial injury,” *Cellular Physiology and Biochemistry*, vol. 46, no. 2, pp. 699–712, 2018.

[36] A. J. Ghio, H. B. Suliman, J. D. Carter, A. M. Abushamaa, and R. J. Folz, “Overexpression of extracellular superoxide dismutase decreases lung injury after exposure to oil fly ash,” *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 283, no. 1, pp. L21–L28, 2002.

[37] G. M. Carvalho, L. K. Nagato, S. d. Fagundes et al., “Time course of pulmonary burden in mice exposed to residual oil fly ash,” *Frontiers in Physiology*, vol. 5, article 366, 2014.

[38] F. Gao, A. Barchowsky, A. A. Nemec, and J. P. Fabisiak, “Microbial stimulation by Mycoplasma fermentans synergistically amplifies IL-6 release by human lung fibroblasts in response to residual oil fly ash (ROFA) and nickel,” *Toxicological Sciences*, vol. 81, no. 2, pp. 467–479, 2004.

[39] T. Marchini, D. Wolf, N. A. Michel et al., “Acute exposure to air pollution particulate matter aggravates experimental myocardial infarction in mice by potentiating cytokine secretion from lung macrophages,” *Basic Research in Cardiology*, vol. 111, no. 4, 2016.

[40] S. H. Gavett, S. L. Madison, M. A. Stevens, and D. L. Costa, “Residual oil fly ash amplifies allergic cytokines, airway responsiveness, and inflammation in mice,” *American Journal of Respiratory and Critical Care Medicine*, vol. 160, no. 6, pp. 1897–1904, 1999.

[41] H. Fehtenbach, C. Wagner, and M. Wegmann, “Airway remodeling in asthma: what really matters,” *Cell and Tissue Research*, vol. 367, no. 3, pp. 551–569, 2017.

[42] C. P. P. Sakoda, A. C. de Toledo, A. Perini et al., “Sakuranetin reverses vascular peribronchial and lung parenchyma remodeling in a murine model of chronic allergic pulmonary inflammation,” *Acta Histochemical*, vol. 118, no. 6, pp. 615–624, 2016.

[43] G. Santini, N. Mores, R. Shohreh et al., “Exhaled and non-exhaled non-invasive markers for assessment of respiratory inflammation in patients with stable COPD and healthy smokers,” *Journal of Breath Research*, vol. 10, no. 1, article 017102, 2016.

[44] P. Montuschi, P. J. Barnes, and G. Ciabattoni, “Measurement of 8-isoprostane in exhaled breath condensate,” *Methods in Molecular Biology*, vol. 594, pp. 73–84, 2010.

[45] K. B. Jourdan, J. A. Mitchell, and T. W. Evans, “Release of isoprostanes by human pulmonary artery in organ culture:..."
A cyclo-oxygenase and nitric oxide dependent pathway,” Biochemical and Biophysical Research Communications, vol. 233, no. 3, pp. 668–672, 1997.

[46] S. S. Possa, H. T. Charafeddine, R. F. Righetti et al., “Rho-kinase inhibition attenuates airway responsiveness, inflammation, matrix remodeling, and oxidative stress activation induced by chronic inflammation,” American Journal of Physiology-Lung Cellular and Molecular Physiology, vol. 303, no. 11, pp. L939–L952, 2012.

[47] A. Shiraki, H. Kume, T. Oguma et al., “Role of Ca2+ mobilization and Ca2+ sensitization in 8-iso-PGF2α-induced contraction in airway smooth muscle,” Clinical & Experimental Allergy, vol. 39, no. 2, pp. 236–245, 2009.

[48] T. M. Dos Santos, R. F. Righetti, L. d. Camargo et al., “Effect of Anti-IL17 antibody treatment alone and in combination with rho-kinase inhibitor in a murine model of asthma,” Frontiers in Physiology, vol. 9, article 1183, 2018.

[49] A. C. Toledo, C. P. P. Sakoda, A. Perini et al., “Flavonone treatment reverses airway inflammation and remodeling in an asthma murine model,” British Journal of Pharmacology, vol. 168, no. 7, pp. 1736–1749, 2013.

[50] T. J. van’t Erve, F. B. Lih, M. B. Kadiiska, L. J. Deterding, T. E. Eling, and R. P. Mason, “Reinterpreting the best biomarker of oxidative stress: The 8-iso-PGF2α / PGF2α ratio distinguishes chemical from enzymatic lipid peroxidation,” Free Radical Biology & Medicine, vol. 83, pp. 245–251, 2015.

[51] J. F. Perez-Zoghbi, Y. Bai, and M. J. Sanderson, “Nitric oxide induces airway smooth muscle cell relaxation by decreasing the frequency of agonist-induced Ca2+ oscillations,” The Journal of General Physiology, vol. 135, no. 3, pp. 247–259, 2010.

[52] B. S. Steinhorn, J. Loscalzo, and T. Michel, “Nitroglycerin and nitric oxide—a rondo of themes in cardiovascular therapeutics,” The New England Journal of Medicine, vol. 373, no. 3, pp. 277–280, 2015.

[53] F. L. M. Ricciardolo, P. J. Sterk, B. Gaston, and G. Folkerts, “Nitric oxide in health and disease of the respiratory system,” Physiological Reviews, vol. 84, no. 3, pp. 731–765, 2004.

[54] H. T. Chung, H. O. Pae, B. M. Choi, T. R. Billiar, and Y. M. Kim, “Nitric oxide a bioregulator of apoptosis,” Biochemical and Biophysical Research Communications, vol. 282, no. 5, 2001.

[55] D. J. Horstman, L. G. Fischer, P. C. Kouretas, R. L. Hannan, and G. F. Rich, “Role of nitric oxide in heparin-induced attenuation of hypoxic pulmonary vascular remodeling,” Journal of Applied Physiology, vol. 92, no. 5, pp. 2012–2018, 2002.

[56] T. Ichikawa, H. Sugiuara, A. Koarai et al., “TLR3 activation augments matrix metalloproteinase production through reactive nitrogen species generation in human lung fibroblasts,” The Journal of Immunology, vol. 192, no. 11, pp. 4977–4988, 2014.

[57] B. T. Martins-Olivera, R. Almeida-Reis, O. A. Theodorro-Júnior et al., “The plant-derived Bauhinia bauhinoides kalliakrin proteinase inhibitor (rBbK1) attenuates elastase-induced emphysema in mice,” Mediators of Inflammation, vol. 2016, Article ID 3346574, 12 pages, 2016.

[58] R. Halwani, S. Al-Muhsen, H. Al-Jahdali, and Q. Hamid, “Role of transforming growth factor-β in airway remodeling in asthma,” American Journal of Respiratory Cell and Molecular Biology, vol. 44, no. 2, pp. 127–133, 2011.

[59] L. J. Jansen, “Isoprostanes: an overview and putative roles in pulmonary pathophysiology,” American Journal of Physiology-Lung Cellular and Molecular Physiology, vol. 280, no. 6, pp. L1067–L1082, 2001.

[60] P. Montuschi, D. Currò, E. Ragazzoni, P. Preziosi, and G. Ciabattoni, “Anaphylaxis increases 8-iso-prostaglandin F2α release from guinea-pig lung in vitro,” European Journal of Pharmacology, vol. 365, no. 1, pp. 59–64, 1999.

[61] P. J. C. Biselli, F. D. T. Q. S. Lopes, H. T. Moriya et al., “Short-term exposure of mice to cigarette smoke and/or residual oil fly ash produces proximal airspace enlargements and airway epithelium remodeling,” Brazilian Journal of Medical and Biological Research, vol. 44, no. 5, pp. 460–468, 2011.

[62] P. Angeli, C. M. Prado, D. G. Xisto et al., “Effects of chronic L-NAME treatment lung tissue responses induced by chronic pulmonary inflammation mechanics, eosinophilic and extracellular matrix,” American Journal of Physiology-Lung Cellular and Molecular Physiology, vol. 294, no. 6, pp. L1197–L1205, 2008.

[63] C. M. Starling, C. M. Prado, E. A. Leick-Maldonado et al., “Inducible nitric oxide synthase inhibition attenuates lung tissue responsiveness and remodeling in a model of chronic pulmonary inflammation in guinea pigs,” Respiratory Physiology & Neurobiology, vol. 165, no. 2-3, pp. 185–194, 2009.

[64] F. P. Santana, N. M. Pinheiro, M. I. Bittencourt-Mernak et al., “Vesicular acetylcholine transport deficiency potentiates some inflammatory responses induced by diesel exhaust particles,” Ecotoxicology and Environmental Safety, 2019.