



Inducible nitric oxide synthase inhibition attenuates lung tissue responsiveness and remodeling in a model of chronic pulmonary inflammation in guinea pigs[☆]

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ABSTRACT

We evaluated the influence of iNOS-derived NO on the mechanics, inflammatory, and remodeling process in peripheral lung parenchyma of guinea pigs with chronic pulmonary allergic inflammation. Animals treated or not with 1400 W were submitted to seven exposures of ovalbumin in increasing doses. Seventy-two hours after the 7th inhalation, lung strips were suspended in a Krebs organ bath, and tissue resistance and elastance measured at baseline and after ovalbumin challenge. The strips were submitted to histopathological measurements. The ovalbumin-exposed animals showed increased maximal responses of resistance and elastance ($p < 0.05$), eosinophils counting ($p < 0.001$), iNOS-positive cells ($p < 0.001$), collagen and elastic fiber deposition ($p < 0.05$), actin density ($p < 0.05$) and 8-iso-PGF2 α expression ($p < 0.001$) in alveolar septa compared to saline-exposed ones. Ovalbumin-exposed animals treated with 1400 W had a significant reduction in lung functional and histopathological findings ($p < 0.05$). We showed that iNOS-specific inhibition attenuates lung parenchyma constriction, inflammation, and remodeling, suggesting NO-participation in the modulation of the oxidative stress pathway.

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1. Introduction

Although asthma is defined as a chronic airway disease, recent investigations have emphasized the role of lung parenchyma alterations in its pathophysiology. In patients with severe asthma particularly the alterations in small airways as well as in lung tissue contribute to the functional impairment (Kraft, 1999; Mauad et al., 2004). It has been shown that peripheral lung parenchyma of asthmatic patients presents eosinophilic inflammation, overexpression of IL-5 and extracellular matrix remodeling (Bousquet et al., 2000; Mauad et al., 2004; De Magalhães et al., 2005). These alterations have also been reproduced in experimental animal models of chronic allergic pulmonary inflammation (Rocco et al., 2001; Xisto et al., 2005; Lanças et al., 2006).

Different inflammatory mediators and modulators released in the inflammatory milieu, such as nitric oxide (NO), are responsible for many of the structural and functional lung alterations observed in asthmatic patients and in animal models of chronic pulmonary allergic inflammation (Bousquet et al., 1992; Boulet et al.,

1995; Meurs et al., 2000; Holgate, 2001). Nitric oxide derived either from constitutive isoforms (nNOS and eNOS) or from other NO-adduct molecules (nitrosothiols) is able to modulate bronchomotor and vascular tone. On the other hand, NO derived from inducible isoenzyme (iNOS) seems to be mainly involved in the immunomodulation (Belvisi et al., 1991; Koarai et al., 2002; Ricciardollo, 2003; Prado et al., 2005a).

iNOS has been found in several types of inflammatory cells, such as eosinophils, neutrophils and macrophages, as well as in respiratory epithelial cells, and the large production of NO from this isoenzyme is related to the amplification of the inflammatory reaction (Ricciardollo et al., 2004; Prado et al., 2005a, 2006). In addition, considering that the interaction between NO and superoxide leads the peroxynitrite formation and generation of isoprostanes, as 8-iso-PGF2 α , some authors suggested that oxidative stress pathway could also be involved in the modulation of lung tissue constriction (Ricciardollo et al., 2006b; Jourdan et al., 1997). It is important to note that the isoprostanes are a biomarker of oxidative stress.

Therefore, NO derived from iNOS contributes to airway constriction, inflammation and remodeling (Eynott et al., 2002; Bhandari et al., 2002; Prado et al., 2006), and it has been proposed that specific inhibition of iNOS-derived NO as a future therapeutic strategy for inflammatory diseases such as asthma, sepsis and acute lung inflammation (Garvey et al., 1997; De Boer et al., 1999; Koarai et al., 2002; Ricciardollo et al., 2006a).

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Considering that the nitrergic nerve density seems progressively reduced throughout the bronchial tree (Dupuy et al., 1992), NO modulation in asthma pathophysiology has been intensively investigated in airways (Belvisi et al., 1991; Prado et al., 2005a). The alterations in tissue resistance in response to agonists may be determined by the heterogeneity of airway constriction (Lutchen et al., 1996a,b), but increases in tissue R may also reflect a direct role of parenchyma tissue in the overall lung response.

Lung parenchyma strips represent the distal units of lung tissue and offer a better assessment of pure tissue properties. For this reason, studies using this technique have been performed to evaluate the mechanical and pharmacological properties of lung periphery (Nagase et al., 1995; Rocco et al., 2001; Lanças et al., 2006).

In conclusion, a plausible hypothesis is that iNOS-derived NO modulates the lung parenchyma alterations induced by chronic ovalbumin exposures in guinea pigs. We investigated the effects of the highly selective inhibitor of iNOS activity (N-(3(Aminomethyl) Benzyl) Acetamide, 1400-W), in lung tissue mechanics, inflammation and extracellular matrix remodeling in a well-established model of chronic pulmonary allergic inflammation.

2. Materials and methods

Guinea pigs received humane care in compliance with the “Guide for care and use of laboratory animals” (NIH publication 85-23, revised 1985), and experiments described in this study were previously approved by the institutional review board of the University of São Paulo (São Paulo, Brazil).

2.1. Induction of chronic pulmonary allergic inflammation

Male Hartley guinea pigs, weighing 300–400 g, were placed in a plexiglass box (30 cm × 15 cm × 20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of ovalbumin

(OVA, Grade V, Sigma Chemical Co., Saint Louis, MO, USA) diluted in 0.9% NaCl (normal saline) was prepared. The animals received seven inhalations during 4 weeks with increasing concentrations of OVA (1–5 mg/mL) in order to counteract tolerance (Fig. 1A). Control animals received aerosolized normal saline. The solution was continuously aerosolized into the environment until respiratory distress (sneezing, coryza, cough or retraction of the thoracic wall) occurred, or until 15 min had elapsed, as previously described (Tibério et al., 1997; Leick-Maldonado et al., 2004; Prado et al., 2005a). The time during which the animals were in contact with the aerosol was called inhalation time. The observer who made the decision to withdraw the guinea pig from the inhalation box was blinded to the treatment status of the animal.

2.2. 1400 W administration

1400 W (Tocris Bioscience, Ellisville, MO, USA) (a specific and high selective iNOS inhibitor) in the dose of 2 mg/kg/animal/day was given i.p., for 4 days (OVA-W or NS-W groups, see below), beginning 30 min before the 7th inhalation of either ovalbumin or normal saline. This approach was chosen based on previous data (Garvey et al., 1997; Prado et al., 2006). Since high doses of 1400 W (50 mg/kg i.p.) can cause toxic effects, we chose a lower dose, of which the therapeutic efficacy has been previously demonstrated in experimental models (Garvey et al., 1997; Prado et al., 2006). Controls received the same volume of i.p. normal saline.

2.3. Experimental groups

- (a) Aerosolized saline, and vehicle of 1400 W (NS, $n=10$);
- (b) Aerosolized ovalbumin and vehicle of 1400 W (OVA, $n=10$);
- (c) Aerosolized saline and 1400 W treatment (NS-W, $n=12$); (d) Aerosolized ovalbumin and 1400 W treatment (OVA-W, $n=12$).

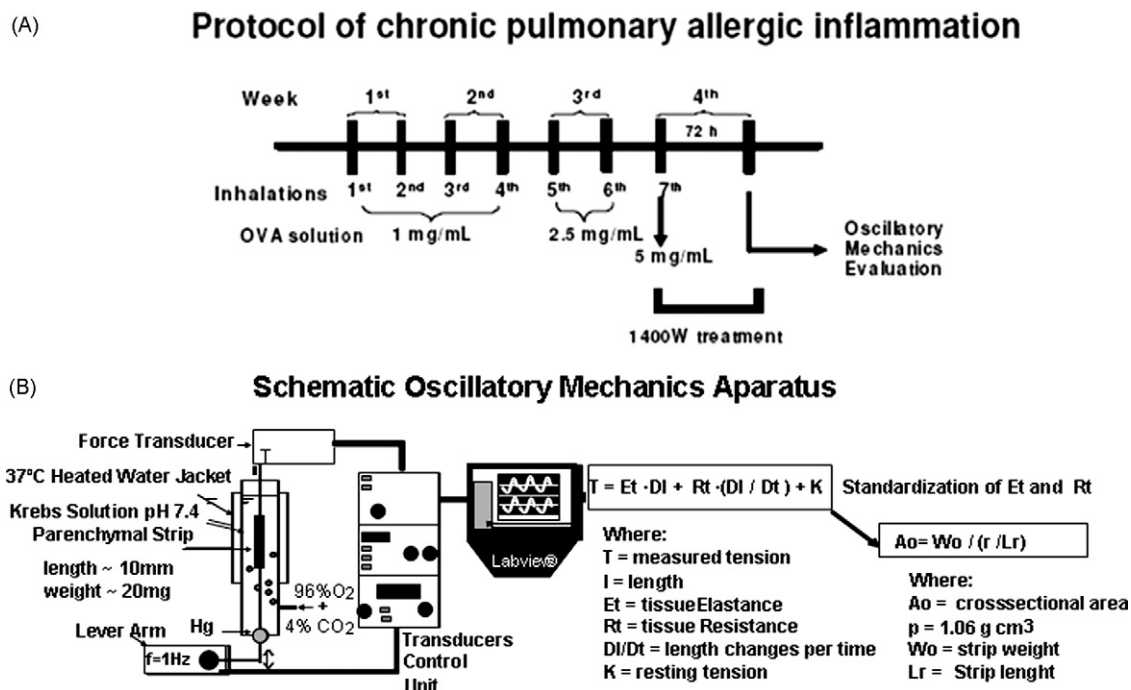


Fig. 1. (A) Experimental protocol. In (A) the guinea pigs were submitted to 7 inhalations (2 per week with 2- to 3-day intervals, during 4 weeks) with aerosols of normal saline or ovalbumin solution with increasing doses of antigen. From the 1st to the 4th inhalations, the dose used was 1 mg/mL of ovalbumin (2 weeks). In the 5th and 6th inhalations (3rd week), animals inhaled 2.5 mg/mL of ovalbumin and in the 7th inhalation (beginning in the 4th week) of 5 mg/mL of antigen was used. Treatment with 1400 W started 30 min before the 7th inhalation and was given daily until the oscillatory mechanics assessment. The solution of ovalbumin or normal saline was continuously aerosolized for 15 min or until respiratory distress occurred (sneezing, coryza, cough or retraction of the thoracic wall). Seventy-two hours after 7th inhalation, animals were anesthetized, exsanguinated, the lungs were removed and submitted to the experimental protocol of mechanics measurements, as demonstrated in B. (B) Schematic setup used for tissue mechanics measurements.

2.4. Oscillatory mechanics measurements

Seventy-two hours after the 7th inhalation, animals were anesthetized with thiopental (50 mg/kg i.p.), tracheostomized, and exsanguinated after thoracotomy. Heart and lungs were excised *en bloc* and the lungs were infused with Krebs solution (in mM: NaCl, 118; KCl, 4.5; NaHCO₃, 25.5; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; glucose 10; Sigma Chemical). Sagittal slices of both lungs (here defined as total lung) were covered with optimum cutting temperature (OCT) compound (Reichert-Jung, Heidelberg, Germany) and cooled in liquid nitrogen for subsequent immunohistochemistry detection of iNOS expression. Subpleural parenchymal strips of the lower lobes (10 mm × 2 mm × 2 mm) were cut, and the resting length (L_r) and wet weight (W_0) of each strip were measured. Metal clips were glued to either end of the tissue strip with cyanoacrylate. Steel wires (0.5-mm diameter) were attached to the clips; one side was connected to a force transducer (model 404A; Cambridge Technologies, Ontario, Canada) and the other side was connected to a servo-controlled lever arm (model 300B; Cambridge Technologies). The lever arm was capable of peak-to-peak length excursions of 8 mm with resolution of 1 μ m. It was connected to a function generator (model 3030; BK Precision, Chicago, IL, USA), which controlled the frequency, amplitude, and waveform of the oscillation.

The resting tension (T_0) was set by movement of a screw thumb wheel system, which effected slow vertical displacements of the force transducer. Length and force signals were converted from analog-to-digital with an analog-to-digital converter and recorded by a compatible computer (see Fig. 1B). The strips were preconditioned three times by slowly cycling tension from 0 to 2 g. After the third cycle, they were fixed at 1 g (Lanças et al., 2006; Dolhnikoff et al., 1998) and maintained in the organ bath containing aerated Krebs solution (95% of O₂, 5% of CO₂) changed every 15 min for 50 min to allow stress relaxation. The frequency of oscillation was 1 Hz and the amplitude was 2.5% L_r (Lanças et al., 2006). Measurements of tissue resistance (R_t) and elastance (E_t) were obtained at baseline for 5 min and the maximal responses of R_t and E_t were collected 10 min after a specific antigen challenge with OVA (0.1%) in the bath (Nagase et al., 1995). R_t and E_t were estimated by the recursive least-squares algorithm to the equation of motion (Lauzon and Bates, 1991):

$$T = E\Delta l + R \left(\frac{\Delta l}{\Delta t} \right) + K$$

where T is the measured of tension, Δl is the measured of length change, $\Delta l/\Delta t$ is the length change per unit of time, and K is a constant reflecting resting tension. To avoid the interference of the unstressed cross-sectional area (A_0) of the strip was obtained from the formula:

$$A_0 (\text{cm}^2) = \frac{W_0}{p \times L_r}$$

where p is the mass density of the tissue taken as 1.06 g/cm³, W_0 is the wet weight in grams, and L_r is the resting length in centimeters.

After that, the values of R_t and E_t were multiplied by L_r/A_0 and the results were standardized for strip size.

We analyzed the percentage of increase in R_t (% R_t) and E_t (% E_t) in relation to baseline (Lanças et al., 2006). Afterwards, the strips were fixed in 10% formalin for 48 h and embedded in paraffin for histological analysis.

2.5. Morphometric analysis

The homogeneity of the strip samples used was assured by measuring the fractional area of tissue constituents using the point-counting method (Weibel, 1990), using a 100-point grid with a known area (62,500 μ m² at 400× magnification) attached to the

ocular microscope. We measured the fractional area of bronchial wall (BW), blood vessel wall (BVW), and alveolar wall (AW) as the number of points that fell in either BW, BVW and AW divided by the total number of points that fell in the strip tissue. Measurements were performed in 10 fields per slide, at 400× magnification.

2.6. Measurement of eosinophil density

Five micrometers thick slides of lung strips were stained with hematoxylin and eosin and Luna staining (Watanabe et al., 2004; Luna, 1986). We analyzed the density of eosinophils within the alveolar septa of the lung strips, at a magnification of 1000× (10⁴ μ m²). We counted the number of points hitting alveolar tissue in each field and of eosinophils within the alveolar septa. Eosinophil density was determined as the number of eosinophils in each field divided by alveolar tissue area. Measurements were expressed as cells/ μ m². Counting was performed in 10 microscopic fields of lung strip for each animal.

2.7. Measurement of iNOS-positive cells

Total lung slices covered with OCT and cooled in liquid nitrogen were used for iNOS detection. Cryostat sections (Leica CM1850; Leica, Nofloch, Germany) were mounted on glass slides pre-coated with aminopropyltriethoxysilane (Sigma Chemical Co.) and fixed in chloroform–acetone (Merck, Rio de Janeiro, Brazil) vol/vol for 10 min at room temperature. Immunohistochemistry analysis was performed as previously described (Coers et al., 1998; Prado et al., 2005a). Sections were then incubated for 30 min at room temperature with a blocking solution containing normal mouse serum (Dako Corp., Carpinteria, CA, USA). Monoclonal antisera raised in mouse against iNOS (IgG2a - iNOS/NOS Type II - N32020-BD Transduction Laboratories, San Diego, CA, USA) (Coers et al., 1998; Prado et al., 2005a, 2006) was used as primary antisera (incubation overnight at room temperature, 1:5 dilution in Tris buffer). After three 5-min washes in Tris-buffered saline (TBS), sections were incubated with a secondary antibody (LSAB + AP Link Universal, Dako Corp.) for 30 min at 37 °C in a humid chamber. Slides were given three more 5-min washes in TBS and were coverslipped with pre-diluted (for 30 min) alkaline phosphatase (LSAB + AP - Streptavidin AP - Dako Corp.). This was followed by incubation with substrate Fast Red TR (Sigma Chemical Co.) for 6 min and light hematoxylin counterstaining for 1 min. Ten fields were analyzed per lung at a magnification of 1000× (10⁴ μ m²), using the same morphometric method described above in eosinophil density measurements. The iNOS-positive cells were expressed as number of cells/ μ m² (Prado et al., 2005b, 2006).

2.8. Measurement of collagen and elastic fiber content

Five micrometers thick slides of lung strips were stained for collagen fibers by Sirius-Red (Direct Red 80, C.I. 35780, Aldrich, Milwaukee, WI, USA) and for elastic fibers by Weigert's Resorcin-Fuchsin. The volume proportion of collagen and elastic fibers in the alveolar tissue of lung strips was determined (10 fields, 400×) by dividing the number of points hitting collagen or elastin by the total number of points hitting alveolar septa. Results were expressed as percentage (Prado et al., 2005b).

2.9. Measurement of isoprostane-8

Immunohistochemical staining was performed using antibody to Anti-8-epi-PGF2 α (Oxford Biomedical Research, Rochester Hills, MI, USA) (Megha et al., 2006) at a 1:500 dilution. Sections were deparaffinized and washed, 5 min for 7 times with H₂O₂ 10 V 3% to inhibit endogenous peroxidase activity. After washes in phosphate-buffered saline (PBS) and water, the antigen retrieval

was performed with trypsin for 20 min. Afterward, 3 washes in PBS were performed for 3 min each. Sections were incubated with Anti-8-epi-PGF2 diluted in bovine serum albumin (BSA) overnight. After washes in PBS, Vectastain ABC Kit (Vector Elite PK-6105, Burlingame, CA, USA) was used as secondary antibody and 3,3 Diaminobenzidine (DAB) (Sigma Chemical Co.) was used as chromogen. The sections were counterstained with Harris hematoxylin (Sigma Chemical Co.). The analysis was performed in the slides stained for 8-isoprostane by applying the same point-counting technique described before. We determined the volume proportion of 8-isoprostane in the alveolar septa as the relation between the number of points falling on 8-isoprostane-stained tissue and the total number of points hitting the alveolar tissue (Montuschi et al., 1999). Measurements were carried out at 400 $\times$ magnification in 10 fields in each slide.

2.10. Measurement of actin

Lung strips were also submitted to immunohistochemistry with anti-smooth muscle actin. The sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 min. Sections were incubated with Anti-Human Smooth Muscle Actin, 1A4, Dako, Denmark overnight at 4 °C. LSAB[®] Plus-HRP kit (K-0690 DAKO Corporation, Carpinteria, EUA) was used as secondary antibody and 3,3 DAB (Sigma Chemical Co.) was used as chromogen. The sections were counterstained with Harris hematoxylin. The volume proportion of actin-positive cells in the lung strips was determined by dividing the number of points hitting actin-positive cells by the total number of points hitting the tissue. Measurements were performed in 15 fields of lung strip in each animal at a magnification of 200 $\times$. Results were expressed as percentage (Lanças et al., 2006).

All morphometric analyses described in this section were performed by individuals blinded to the protocol design.

2.11. Data analysis

Values are expressed as mean $\pm$ standard error (SEM). Statistical analysis was performed using SigmaStat software (SPSS Inc., Chicago, IL, USA). Data were evaluated by two-way analysis of variance (sensitization and status of 1400 W treatment) and multiple comparisons were made using Holm-Sidak method. Spearman Rank Order correlation was used to compare the iNOS expression in alveolar septa and the other morphometric and functional parameters. A p value < 0.05 was considered significant (Zar, 1984).

3. Results

3.1. Oscillatory mechanics measurements

There was no difference in baseline tissue resistance and elastance among the experimental groups (Fig. 2, Panels A and C). Fig. 2B and D shows the mean and SEM values of the percentage of increase in tissue resistance and elastance, respectively, after OVA challenge in relation to baseline. The ovalbumin-exposed animals presented an increase in the %Rt (85.28 ± 19.37) and %Et (76.25 ± 14.45) compared to the saline-exposed ones ($p < 0.05$ for all comparisons) after OVA challenge. The 1400 W administration in ovalbumin-exposed animals reduced the %Rt (51.77 ± 9.58) and %Et (35.39 ± 8.73) compared to ovalbumin-exposed and vehicle-treated animals ($p < 0.05$).

3.2. Morphometric analysis

Lung strip were mainly represented by alveolar septa (% of alveolar septa in the four experimental groups: NS: $87.98 \pm 4.10\%$; OVA:

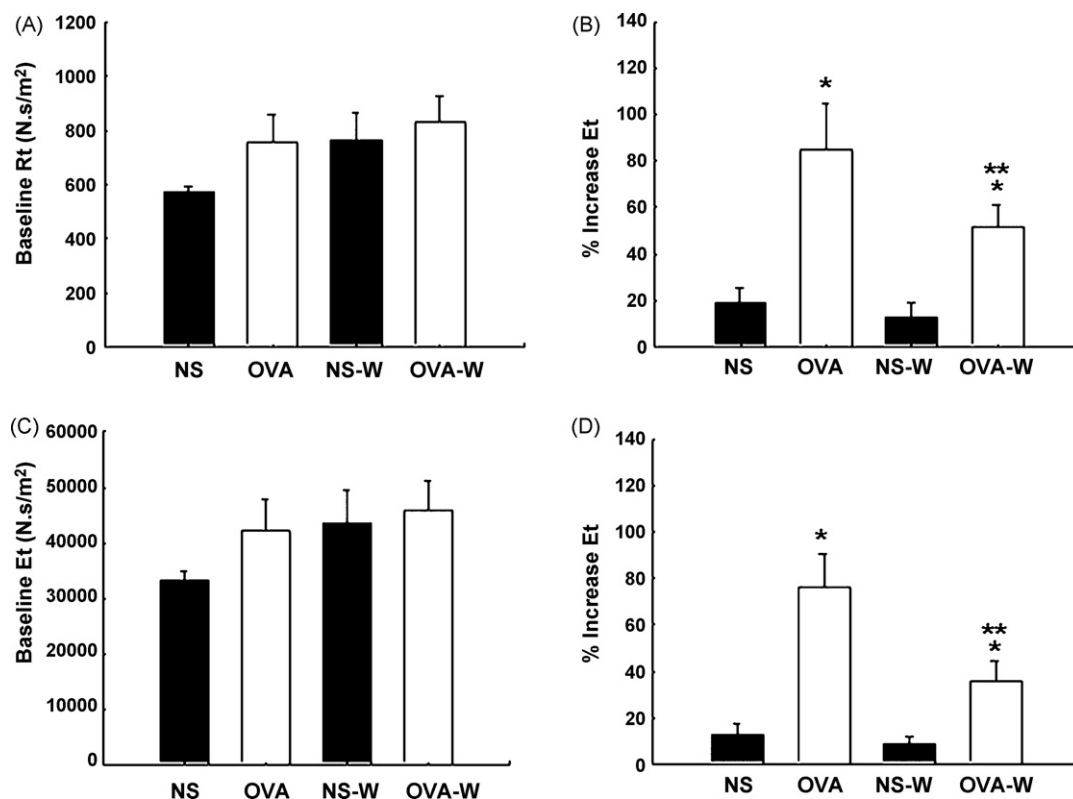


Fig. 2. Mean $\pm$ SEM of baseline values of tissue resistance (A) and elastance (C) and post-ovalbumin challenge values (percentage of increase) of tissue resistance (B) and elastance (D) in guinea pigs previously exposed to seven inhalations with ovalbumin or normal saline and chronically treated with 1400 W or vehicle. * $p < 0.05$ compared to saline-exposed animals (NS and NS-W groups) and ** $p < 0.05$ compared to ovalbumin-exposed animals that received vehicle of 1400 W (OVA group).

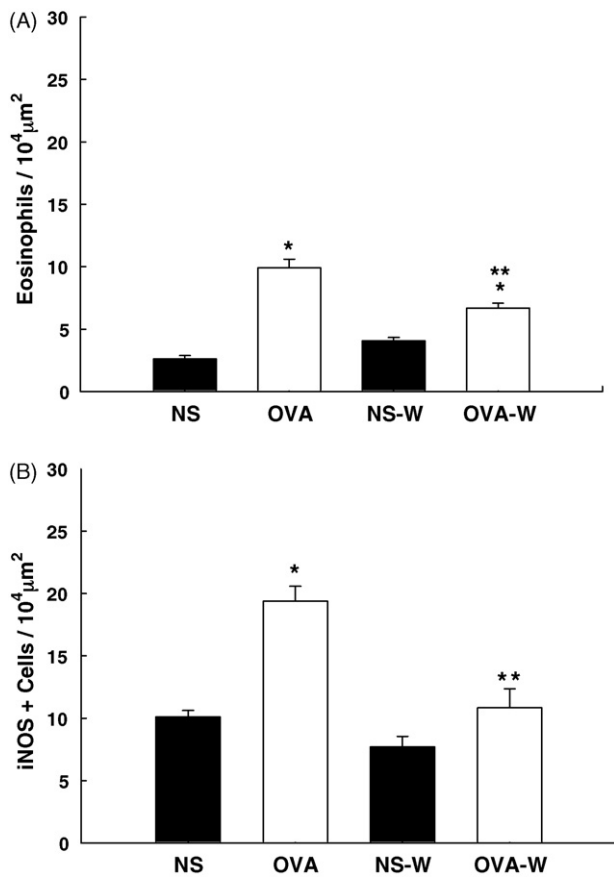


Fig. 3. Mean ± SEM of eosinophil density (A) and iNOS-positive cells (B) in lung tissue of guinea pigs previously exposed to seven inhalations with ovalbumin or normal saline and then treated with 1400 W or vehicle; * $p < 0.001$ compared to saline-exposed animals (NS and NS-W groups) and ** $p < 0.001$ compared to ovalbumin-exposed animals that received vehicle of 1400 W (OVA group).

87.52 ± 6.91%; NS-W: 89.32 ± 3.20%; OVA-W: 89.50 ± 3.20%). There were no significant differences between the contents of alveolar septa, airways, and vessels among the experimental groups.

Considering that after the fourth inhalation ovalbumin-exposed animals have already presented high titles of IgG1 antibody (1:320) (Leick-Maldonado et al., 2004; Ruiz Schütz et al., 2009), we have also evaluated the inflammatory and remodeling alterations, in ovalbumin and saline groups ($n = 4$ per group) at that moment, in order to clearly established if the 1400 W treatment was able to reverse or to avoid the morphological alterations. Compared to saline, the ovalbumin-exposed animals presented an increase in collagen content [(median (25–75%), OVA: 13.81 (8.01–21.83), NS: 7.96 (5.31–11.20) ($p < 0.001$)] and eosinophils [OVA: 7.14 (3.85–14.29), NS: 0.0 (0.0–2.15) ($p < 0.001$)]. Considering the elastic fibers content, ovalbumin-exposed animals presented similar values compared to saline [OVA: 7.36 (5.0–12.98), NS: 9.09 (7.14–13.51), ($p = 0.13$)].

3.3. Measurements of eosinophil density

Fig. 3A presents the mean and SEM values of eosinophil density in the alveolar septa. There was an increase in the eosinophil density in ovalbumin-exposed animals (9.91 ± 0.70 cells/ $10^4 \mu\text{m}^2$) compared to the saline-exposed ones (2.62 ± 0.26 cells/ $10^4 \mu\text{m}^2$, $p < 0.001$). In ovalbumin-exposed animals, the 1400 W treatment reduced the number of eosinophils in the alveolar septa (6.68 ± 0.40 cells/ $10^4 \mu\text{m}^2$) compared to those that received vehicle of 1400 W ($p < 0.001$). There was no significant difference between the saline-exposed groups.

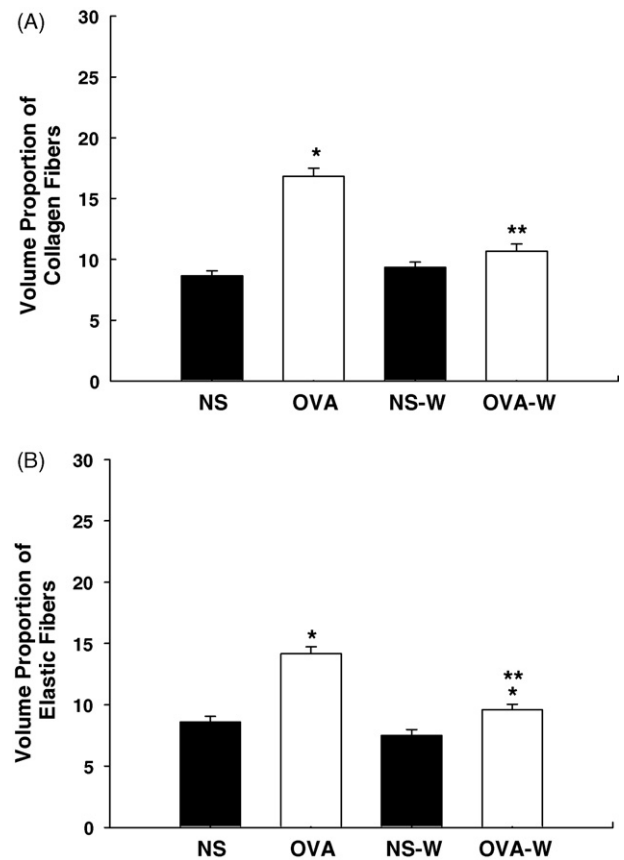


Fig. 4. Mean ± SEM values of collagen (A) and elastic fiber (B) content in guinea pigs previously exposed to seven inhalations with ovalbumin or normal saline and then treated with 1400 W or vehicle. * $p < 0.05$ compared to saline-exposed animals (NS and NS-W groups) and ** $p < 0.001$ compared to ovalbumin-exposed animals that received vehicle of 1400 W (OVA group).

3.4. Measurements of iNOS-positive cells

Considering iNOS expression in inflammatory cells (Fig. 3B), ovalbumin-exposed guinea pigs showed an increase in iNOS-positive inflammatory cells in the alveolar septa (19.38 ± 1.21 cells/ $10^4 \mu\text{m}^2$) compared to saline-exposed animals (9.96 ± 0.51 cells/ $10^4 \mu\text{m}^2$, $p < 0.001$). The 1400 W treatment reduced the iNOS-positive inflammatory cells in ovalbumin-exposed animals (OVA-W group: 8.74 ± 1.36 cells/ $10^4 \mu\text{m}^2$) compared to animals exposed to ovalbumin that received vehicle of 1400 W ($p < 0.001$). There was no difference in saline-exposed animals.

3.5. Measurements of collagen and elastic fiber content

Fig. 4 shows the collagen (panel 4A) and the elastic fiber content (panel 4B) in the alveolar septa. There was an increase in both collagen ($16.84 \pm 0.67\%$) and elastic fiber ($14.16 \pm 0.56\%$) content in alveolar septa in ovalbumin-exposed animals compared to saline-exposed ones (8.65 ± 0.43 and $8.58 \pm 0.47\%$, respectively, $p < 0.05$). The ovalbumin-exposed animals that received 1400 W treatment (OVA-W) presented lower amounts of collagen ($10.68 \pm 0.58\%$) and elastic fibers ($9.59 \pm 0.45\%$) in lung tissue compared to those that received vehicle of 1400 W (OVA group, $p < 0.001$). There was no difference in saline-exposed groups.

3.6. Measurement of isoprostane

The 8-isoprostane expression is shown in Fig. 5. We observed an increase in 8-isoprostane expression in the alveolar septa of

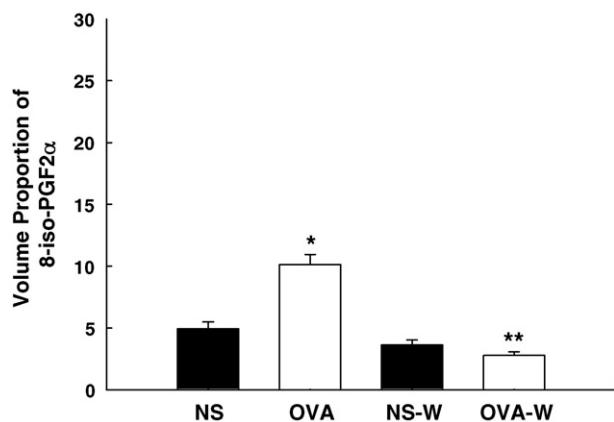


Fig. 5. Volume proportion of 8-isoprostane in the alveolar septa of guinea pigs exposed to seven inhalations with ovalbumin or normal saline and that received 1400 W treatment or vehicle. * $p < 0.001$ compared to saline-exposed animals (NS and NS-W groups) and ** $p < 0.001$ compared to ovalbumin-exposed animals that received vehicle of 1400 W (OVA group).

ovalbumin-exposed guinea pigs ($10.15 \pm 0.80\%$) compared to normal saline-exposed ones ($3.69 \pm 0.42\%$). The 1400 W treatment reduced this response in ovalbumin-exposed animals (OVA-W group: $2.79 \pm 0.28\%$) compared to animals exposed to ovalbumin that received vehicle of 1400 W ($p < 0.001$). There was no significant difference in saline-exposed animals.

3.7. Measurements of actin

Fig. 6 shows the volume proportion of actin. There was an increase in the actin content of the alveolar septa in ovalbumin-exposed animals compared to saline-exposed ones (8.93 ± 0.51 and $6.91 \pm 0.33\%$, respectively, $p < 0.05$). The ovalbumin-exposed animals that received 1400 W treatment (OVA-W) presented a lower amount of actin ($7.22 \pm 0.33\%$) in lung tissue compared to those that received vehicle of 1400 W ($p < 0.001$). There was no significant difference in saline-exposed animals.

In Fig. 7, we can observe the photomicrographs from control, ovalbumin-exposed animals that received vehicle or 1400 W treatment. In ovalbumin-exposed guinea pigs there was an increase in eosinophilic infiltration (panel B) within the alveolar septa, including those positive for iNOS expression (panel E), an increase in the amount of collagen (H), elastic fibers (K), and actin (Q), and in the

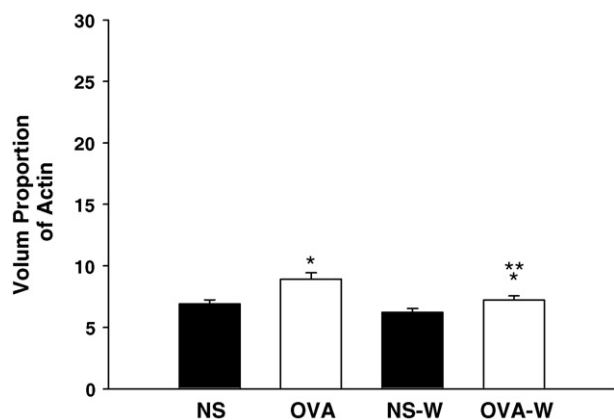


Fig. 6. Volume proportion of smooth muscle-specific actin in the alveolar septa of animals exposed to ovalbumin or normal saline and that received 1400 W treatment or vehicle. * $p < 0.05$ compared to saline-exposed animals (NS and NS-W groups) and ** $p < 0.001$ compared to ovalbumin-exposed animals that received vehicle of 1400 W (OVA group).

content of 8-isoprostane-positive tissue (N) (see arrows) compared to control group. All these alterations were reverted by 1400 W treatment (third column).

The correlations were presented in Fig. 8. There was a positive and significant correlation between the number of iNOS-positive cells and %Rt ($R = 0.481$, $p < 0.05$), %Et ($R = 0.50$, $p < 0.05$), collagen content ($R = 0.528$, $p < 0.05$), elastic fiber content ($R = 0.735$, $p < 0.001$) and 8-isoprostane expression ($R = 0.525$, $p < 0.05$).

4. Discussion

Although many studies have demonstrated the effects of NO in the proximal and distal airways (Belvisi et al., 1991; Dupuy et al., 1992; Prado et al., 2005b), the role of nitric oxide in the modulation of lung parenchyma alterations has not been described. Considering that iNOS was detected in inflammatory cells including macrophages, neutrophils (Blackford et al., 1994), eosinophils, mast cells (Gilchrist et al., 2002), lung fibroblasts (Romanska et al., 2002), and alveolar type II epithelial cells (Warner et al., 1995) we hypothesized that this isoenzyme could also affect lung parenchyma responses.

We demonstrated in this study that iNOS activation contributes to lung parenchyma inflammatory and remodeling alterations in guinea pigs with chronic pulmonary allergic inflammation. 1400 W, an iNOS-specific inhibitor, diminished lung tissue elastance and resistance, as well as the eosinophilic infiltration, collagen and elastic fiber content and the volume proportion of actin in lung tissue. To our knowledge, the present study has provided the first evidence of the effects of iNOS inhibition on distal lung parenchyma. In addition, the specific iNOS blockage also reduced 8-isoprostane expression in the alveolar septa, previously increased by repeated ovalbumin exposures. Several authors have discussed that NO release by iNOS activation also contributes to the oxidative stress, amplifying the deleterious and harmful effects on lungs (Ricciardollo et al., 2006b; Koarai et al., 2002; Muijers et al., 1997). These findings suggest that, at least partially, the effects of iNOS-derived NO in lung parenchyma depend on the activation of the oxidative stress pathway.

It has been shown that NO derived from constitutive isoenzymes acts in smooth muscle cell tone and that NO derived mainly from iNOS regulates the inflammatory responses (De Sanctis et al., 1999; Koarai et al., 2002; Eynott et al., 2002). It had been previously demonstrated, in this animal model, an increase in the number of eosinophils that express both nNOS and iNOS around airways. In addition, the treatment with 1400 W reduced airway responsiveness, eosinophilic infiltration and extracellular remodeling induced by chronic allergic inflammation (Prado et al., 2005b, 2006).

1400 W presents an *in vivo* selectivity of at least 100-fold greater than other NOS inhibitors, such as aminoguanidine (Garvey et al., 1997). We also confirmed the efficacy of this protocol, as there was a significant reduction in the number of iNOS-positive cells in lung tissue from animals exposed to ovalbumin that were treated with 1400 W ($p < 0.001$).

Although asthma is considered an airway disease, the contribution of lung parenchyma alterations to the total asthmatic response has been proposed (Montuschi et al., 1999; Dolhnikoff et al., 1999; Maud et al., 2004). The role of the peripheral tissue in the total lung response to agonists is controversial. It has been shown that changes in tissue resistance after induced constriction are a consequence of the airway inhomogeneities (Lutchen et al., 1996a,b; Ito et al., 2007). Conversely, there is evidence that, even in inhomogeneous lungs, the lung periphery is the mainly site of bronchoconstrictive responses to agonists and inflammation (Tomioka et al., 2002).

In this context, a few years ago, Kraft et al. (1996) observed the presence of alveolar eosinophilic infiltration in patients with

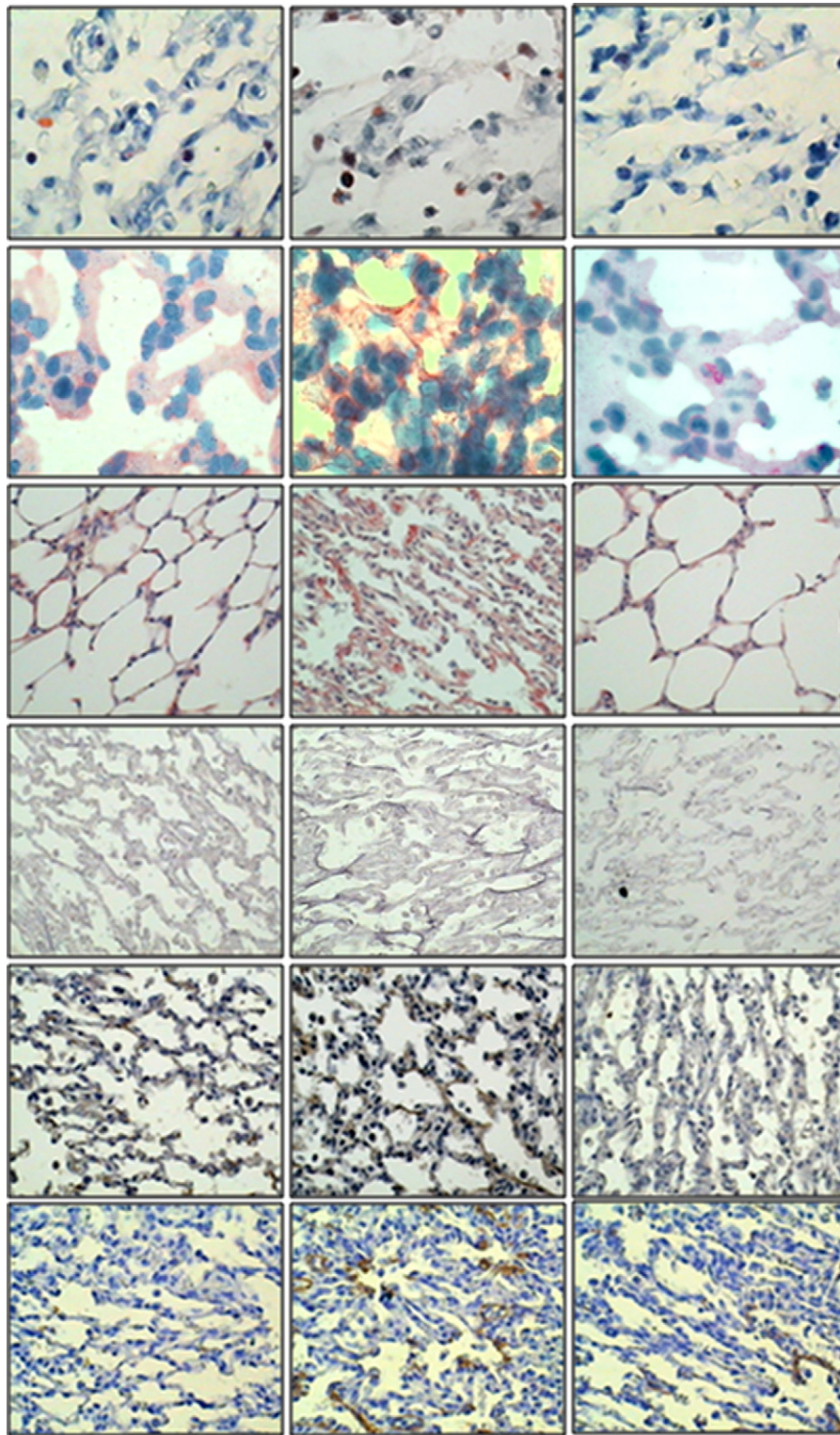


Fig. 7. Guinea pig lung tissue samples obtained from controls (panels A, D, G, J, M, and P), OVA-exposed-vehicle (B, E, H, K, N, and Q) and OVA-exposed-1400W treated (C, F, I, L, O, and R), stained with LUNA (panels A to C - $\times 1000$), immunohistochemistry for iNOS detection (panels D to F - $\times 1000$), Picrosirius (panels G to I - $\times 400$), Weigert Resorcin-Fuchsin (panels J to L - $\times 400$), 8-isoprostane detection (panels M to O - $\times 400$) and actin (P to R - $\times 400$). Control groups show scanty amounts of collagen fibers, elastic fibers and actin content in alveolar tissue sections, coincident with the maintenance of the histoarchitecture of the alveolar septa, a scarce number of eosinophils and iNOS-positive inflammatory cells. In contrast, the distal lung parenchyma of OVA-exposed-vehicle-treated animals shows intense eosinophilic infiltration (panel B) within the alveolar septa, including those positive for iNOS expression (panel E), an increase in the amount of collagen (H), elastic fibers (K), and actin (Q), and in the content of 8-isoprostane-positive tissue (N) (see arrows). The 1400W treatment in ovalbumin-exposed animals reduced all these parameters.

nocturnal asthma. In addition, Kraft (1999) also discussed that improvements in distal lung function correlated with improvements in asthma symptoms. It was also demonstrated that there are abnormal alveolar attachments with decreased elastic fibers

in distal lung from patients who died of fatal asthma (Mauad et al., 2004). Collectively, these studies suggest that tissue response has an important role in the contractile response of asthmatic humans.

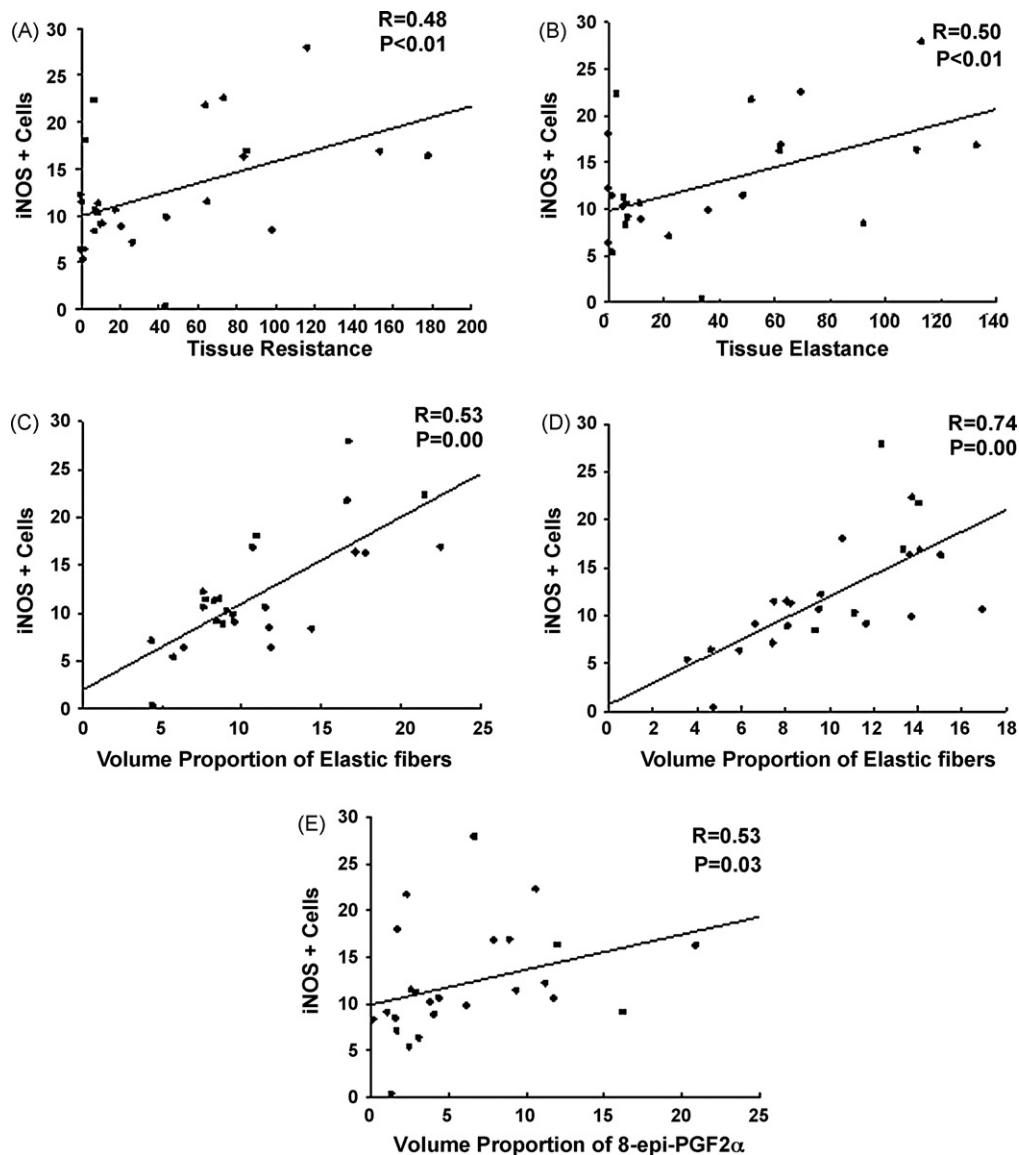


Fig. 8. Correlation between morphological and functional parameters.

There were no significant differences in baseline lung tissue resistance and elastance among the experimental groups. Considering the functional pulmonary alterations induced by chronic inflammation after specific antigen challenge, we observed that 1400 W treatment decreased both the maximal percentage of tissue resistance and elastance in ovalbumin-exposed animals, suggesting that iNOS-derived NO contributes to the modulation of lung parenchyma specific hyperresponsiveness in this experimental model. It is possible that the attenuation of lung tissue mechanics could be related to a combination of several mechanisms, such as decrease of iNOS-derived NO production due to a reduction in the number of iNOS-positive cells, attenuation of oxidative stress responses, and a reduction in the number of eosinophils and actin content in lung tissue of guinea pigs treated with 1400 W.

The mechanism by which the iNOS-derived NO production leads to the potentiation of lung tissue mechanics is poorly known, but it may be related to the promotion of peroxynitrite production (Ricciardollo et al., 2006b). This oxidant agent is formed by NO and superoxide interaction, which leads to lipid peroxidation and generation of isoprostanes (8-epi-PGF2 α). Using anti-8-isoprostane, a biochemical marker of oxidative stress, we stained the 8-epi-PGF-2 α , the main member of the family of isoprostanes, produced

through the peroxidation of arachidonic acid. Isoprostanes contribute to smooth muscle contractions acting through tyrosine kinase, Rho and Rho kinase leading to decreased activity of myosin light-chain phosphatase, increasing the level of phosphorylated myosin light-chain and contraction (Janssen, 2001). It is noteworthy that there was a significant decrease in 8-epi-PGF2 α density in lung tissue in guinea pigs repeatedly exposed to ovalbumin and treated with 1400 W. These findings corroborate that iNOS-derived NO production increases the oxidative stress pathway, which may have detrimental effects on lung tissue structure and function.

We found that specific iNOS inhibition with 1400 W reduced eosinophilic recruitment. Although other authors had also shown that iNOS-derived NO production contributes to eosinophilic airway recruitment (Koarai et al., 2002; Eynott et al., 2002; Prado et al., 2006), other studies were unable to detect this effect (Birrell et al., 2003). These apparently contradictory results may be due to the concentration, flux, and source of NO influencing eosinophilopoiesis, eosinophilic recruitment, and apoptosis, with either anti- or pro-apoptotic properties (Taylor et al., 2003).

In this study, the measurements of collagen and elastic fiber content were carried out to investigate whether iNOS inhibition would

affect tissue remodeling. We observed that ovalbumin-exposed animals presented an increase in collagen and elastic fiber content in the alveolar septa and that the treatment with the iNOS inhibitor 1400 W attenuated these responses. We have previously evaluated animals 24 h after the fourth inhalation and it is important to note that they had already presented an eosinophilic inflammation and collagen and elastic fibers deposition in airways (Ruiz Schütz et al., 2009). In the present study, we have also evaluated the inflammation and the remodeling in lung parenchyma after the fourth inhalation. We observed at that moment that there was an increase in the eosinophilic and collagen density in ovalbumin-exposed animals suggesting that iNOS inhibition was able to modify these parameters.

Considering the elastic fibers deposition, one possible explanation for the inhibition of the elastic fiber content in the lung parenchyma may be related to the effects of oxidant agents derived from oxidative stress, acting as modulators for the elastase-mediated breakdown of elastic fibers (Cantor et al., 2006). We observed an important reduction in 8-epi-PGF-2 α in ovalbumin-exposed animals treated with 1400 W when compared with the non-treated ones. Corroborating these assumptions, we observed that there was a significant positive correlation between elastic fiber content and the number of iNOS-positive cells ($R=0.735$, $p<0.001$), as well as with the volume proportion of 8-epi-PGF-2 α ($R=0.525$, $p<0.05$) in lung tissue.

We demonstrated that 1400 W treatment reduced the collagen content in the lung tissue of guinea pigs with chronic pulmonary allergic inflammation. In fact, we observed a positive and significant correlation between the number of iNOS-positive cells and the collagen content in the lung tissue ($R=0.528$, $p<0.05$). Nitric oxide has been investigated as a modulator of collagen synthesis acting on the cGMP-dependent pathway (Perkins et al., 1998). Hsu et al. (2007) demonstrated that iNOS inhibition by 1400 W in pulmonary fibroblasts in diffuse pulmonary fibrosis by lipopolysaccharide in rats has a protective role in the development of NO-induced pulmonary fibrosis. There is evidence that the NO signal pathway enhances the expression of TGF- β 1, TIMP-1 and HSP47 in pulmonary fibroblasts, which collectively activate the SMAD-signal cascade, stimulating the remodeling process. Prado et al. (2006) also demonstrated that the NO inhibition by 1400 W treatment reduced the extracellular collagen in the airways. Other mechanisms may also be involved in these responses, such as the enhanced bioavailability of L-arginine to NO constitutive forms in animals treated with iNOS inhibitor (Meurs et al., 2000) and the effects of nitric oxide on metalloproteinases (Ohbayashi and Shimokata, 2005).

Finally, we also observed an increase in the volume proportion of actin in the lung tissue of guinea pigs submitted to repeated ovalbumin exposures. The inhibition of NO production derived from iNOS activation reduced this content. These results might suggest an iNOS-derived effect on the myofibroblasts, which were believed to be the major cells responsible for the extracellular matrix production and for the parenchyma contraction (Hsu et al., 2007).

Collectively, our data suggest that the reduction of iNOS attenuates lung tissue constriction, eosinophil, collagen, elastic fiber and actin contents in the lung parenchyma. Considering that our results may have some future therapeutic implications, some limitations also need to be considered in order to clarify some relevant mechanisms that may be involved in these responses. We did not evaluate the TH2 cytokine expression in inflammatory cells as well as the sub-populations of lymphocytes. Although we can not directly transpose our data to human beings, our results reinforce the importance for further human studies using specific iNOS inhibitors as a chronic treatment for asthmatic patients in order to control distal functional and extracellular matrix pulmonary alterations, mainly observed in a group of severe asthma.

We conclude that the specific inhibition of NO production by 1400 W, in guinea pigs with chronic pulmonary allergic inflammation, attenuates the lung tissue constriction, as well as eosinophil, collagen, elastic fiber and actin contents in the lung parenchyma. Our results also support the idea that at least part of the functional and morphological effects on lung tissue modulated by iNOS inhibition is related to the reduction in the oxidative stress pathway.

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