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Effects of inducible nitric oxide synthase inhibition in bronchial vascular remodeling-induced by chronic allergic pulmonary inflammation

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ABSTRACT

Vascular remodeling is an important feature in asthma pathophysiology. Although investigations suggested that nitric oxide (NO) is involved in lung remodeling, little evidence established the role of inducible NO synthase (iNOS) isoform in bronchial vascular remodeling. The authors investigated if iNOS contribute to bronchial vascular remodeling induced by chronic allergic pulmonary inflammation. Guinea pigs were submitted to ovalbumin exposures with increasing doses (1–5 mg/mL) for 4 weeks. Animals received 1400W (iNOS-specific inhibitor) treatment for 4 days beginning at 7th inhalation. Seventy-two hours after the 7th inhalation, animals were anesthetized, mechanical ventilated, exhaled NO was collected, and lungs were removed and submitted to picosirius and resorcin-fuchsin stains and to immunohistochemistry for matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and transforming growth factor- β (TGF- β). Collagen and elastic fiber deposition as well as MMP-9, TIMP-1, and TGF- β expression were increase in bronchial vascular wall in ovalbumin-exposed animals. The iNOS inhibition reduced all parameters studied. In this model, iNOS inhibition reduced the bronchial vascular extracellular remodeling, particularly controlling the collagen and elastic fibers deposition in pulmonary vessels. This effect can be associated to a reduction on TGF- β and on metalloproteinase-9/TIMP-1 vascular expression. It reveals new therapeutic strategies and some possible mechanism related to specific iNOS inhibition to control vascular remodeling.

KEYWORDS experimental asthma model; inducible nitric oxide; metalloproteinases; tissue metalloproteinase inhibition; vascular remodeling

Asthma is defined as an inflammatory disease associated to a hyperresponsiveness that can lead to

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structural alterations in the lung. The pulmonary remodeling, mainly characterized by peribronchial fibrosis, smooth muscle hypertrophy and collagen deposition, as well as elastic fibers system disarrangement, is associated to a decrease in the lung function observed in asthmatic patients [1]. Thus far, attention has focused primarily on the role of remodeling in airways with less attention given to the role of pulmonary vessels.

Recently, some studies have discussed the importance of lung vessels in the asthma pathophysiology [2, 3]. Early studies on the pathology of asthma showed edematous bronchial mucosa with dilated

and congested blood vessels in patients with fatal disease [4, 5]. More recent *in vivo* quantitative studies in asthmatic patients found an increase in the total number of vessels and in the vascular area of medium and small airways, when compared to control subjects [6, 7]. Lastly, an increased vascular network in the airway mucosa was observed even in early phases of chronic adult asthma [8].

It is likely that the alterations in the bronchial microcirculation may have an indirect role to amplify the inflammatory and lung mechanical responses in asthma. It may be due to the fact that bronchial vessels act as a gateway to the submucosa for inflammatory cells, and the increase in the number, size, and thickening of these vessels may contribute to the critical narrowing of the bronchial lumen, when bronchial smooth muscle contraction occurs [9].

The lung inflammation and remodeling in asthma is under the control of complex and incompletely understood mechanisms, involving the release of a variety of neurotransmitters and inflammatory mediators [10, 11], including nitric oxide (NO). NO is involved in several biological processes, including host defense, immune regulation, platelet aggregation, neurotransmission, and inflammation [12]. It could be produced by constitutive or inducible NO synthase (iNOS) isoforms. The iNOS-derived NO is stimulated by many proinflammatory cytokines and is expressed in several types of inflammatory cells [13–15]. A lot of evidence suggested the importance of NO in cellular proliferation, collagen deposition, and myofibroblast differentiation.

In the ovalbumin-induced guinea pig asthma model, repeated allergen exposure led to chronic allergic airway and distal lung parenchyma inflammation, characterized by an influx of eosinophils, mast cells, and allergen-specific T cells, a Th2-type cytokine pattern, and airway and extracellular tissue remodeling [14, 16–19]. Recently, we have shown that the iNOS inhibition reduced lung hyperresponsiveness and inflammation as well as airway and lung tissue remodeling [14, 15].

Nevertheless, so far there has been little information specifically relating to the effects of asthmatic anti-inflammatory interventions on altered bronchial vascular remodeling. Since pharmacological control of bronchial vascular remodeling may have an impact on asthmatic symptoms [2], the present study evaluated, by means of using a guinea pig chronic allergic pulmonary inflammation model, the effects of specific iNOS inhibitor treatment on vascular extracellular remodeling, particularly in collagen and elastic fibers deposition. In order to elucidate possible involved mechanisms, we have evaluated ma-

trix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and transforming growth factor- β (TGF- β) expression in airway vascular wall.

METHODS

This study was approved by the institutional review board of University of São Paulo (São Paulo, Brazil), protocol number 531–00. All animals in the study have received humane care in compliance with the *Guide for Care and Use of Laboratory Animals* (NIH publication 85–23, revised 1985).

Experimental groups

Thirty-two male Hartley guinea pigs, weighing 300 to 400 g, were divided in the following 4 groups ($n = 8$ each): (a) animals that received aerosolized saline and vehicle of 1400W (saline-Ve); (b) animals that received aerosolized ovalbumin and vehicle of 1400W (ovalbumin-Ve); (c) animals received aerosolized saline and 1400W treatment (saline-W); (d) animals received aerosolized ovalbumin and 1400W treatment (ovalbumin-W).

Induction of chronic allergic pulmonary inflammation

Our experimental protocol was previously described [14, 20]. Animals were placed in a plexiglass box (30 × 15 × 20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil) and received aerosol of a solution of ovalbumin (OVA; Grade V, Sigma Chemical, Saint Louis, MO) diluted in 0.9% NaCl (saline). Animals received 7 inhalations during 4 weeks with increasing concentrations of ovalbumin (1–5 mg/mL) in order to counteract tolerance (Figure 1). The solution was continuously aerosolized into the environment until respiratory distress (sneezing, coryza, cough, or retraction of the thoracic wall) occurred, or until 15 minutes had elapsed, as previously described. The observer who made the decision to withdraw the guinea pig from the inhalation box was blinded to the treatment status of the animal. Control animals received aerosolized saline.

1400W treatment

Each animal received 1400W (2 mg/kg/animal/day, intraperitoneal [i.p.]) for 4 days (ovalbumin-W and saline-W groups; see below), beginning 30 minutes before the 7th inhalation of either ovalbumin or normal saline. Control animals received the same volume of vehicle i.p. The choice of this dose was based on a study that found a significant reduction

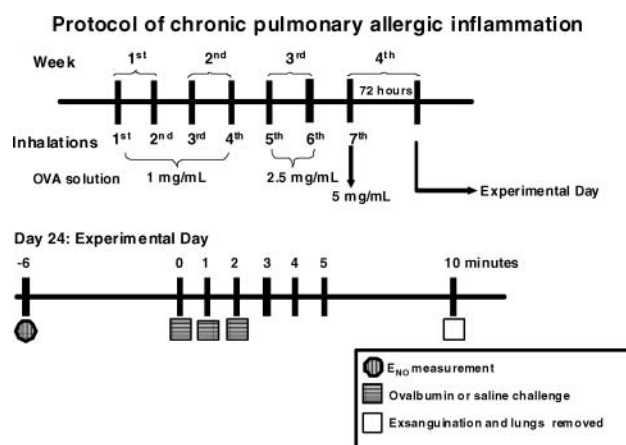


FIGURE 1 Time line of the experimental protocol. Guinea pigs received 7 inhalations (2 per week during 4 weeks) with aerosols of normal saline or ovalbumin solution with increasing doses of antigen. From the 1st to the 4th inhalations, animals received the dose of 1 mg/mL of ovalbumin (2 weeks). In the 5th and 6th inhalations (3rd week), animals inhaled with 2.5 mg/mL of ovalbumin and in the 7th inhalation (beginning of the 4th week) the dose of 5 mg/mL of antigen was used. The 1400W treatment started on the day of the 7th inhalation and was administered 30 minutes before the inhalation. The solution of ovalbumin or saline was continuously aerosolized for 15 minutes or until respiratory distress occurred (sneezing, coryza, cough, or retraction of the thoracic wall). Seventy-two hours after the 7th inhalation, all guinea pigs were anesthetized, tracheostomized, and mechanically ventilated. Expired air was collected for NO measurement for 5 minutes. Then, animals received either ovalbumin (30 mg·mL⁻¹) (OVA and OVA-W groups) or saline (NS and NS-W groups) challenges for 2 minutes. After 10 minutes, the guinea pigs were exsanguinated and the lungs removed.

in lipopolysaccharide (LPS)-induced vascular leakage using doses of 1400W within the range of 1 to 5 mg/kg [21] and on previously published studies [14, 15, 22].

Concentration of exhaled nitric oxide

Seventy-two hours after the 7th inhalation, animals were anesthetized with pentobarbital sodium (50 mg·kg⁻¹, i.p.). They were then tracheostomized and mechanically ventilated at 60 breaths/min with a tidal volume of 8 mL·kg⁻¹ using a Harvard 683 ventilator (Harvard Apparatus, South Natick, MA). Exhaled NO (E_{NO}) levels were measured at the expiratory port of the ventilator using a Mylar Bag [14, 23, 24] for 5 minutes (Figure 1). Concentrations of E_{NO} were measured by chemiluminescence using a fast-responding analyzer (NOA 280; Sievers Instruments, Boulder, CO). Prior to each measurement, the analyzer was calibrated with a certified 47 parts per billion (ppb) NO source (White Martins, São Paulo, Brazil) and zero NO filter (Sievers Instruments). In

order to avoid environment contamination, a NO filter was attached to the breathing circuit.

After E_{NO} measurement, we performed 2-minute challenges with either aerosolized ovalbumin (30 mg·mL⁻¹) or saline delivered into the breathing circuit through the air inlet of the ventilator. After 10 minutes, still under anesthesia, the anterior chest wall was opened and lungs were washed with heparinized saline solution (1:40). Guinea pigs were then killed by exsanguinated, a positive end-expiratory pressure of 5 cm H₂O was applied to the respiratory system, the airways were occluded at the end of expiration, and the lungs were removed en bloc.

Morphometric evaluation

The left lung was fixed with 4% buffered paraformaldehyde for 24 h and then transferred to 70% ethanol. Sections representing peripheral areas of the lung were cut and processed for paraffin embedding. Histological sections (5 μm in thickness) were cut and stained with hematoxylin and eosin (H&E) for vascular wall area quantification, Picrosirius (Direct Red 80; catalog no. 35780, Aldrich, Milwaukee, WI) for collagen fiber detection and resorcin-fuchsin to elastic fibers evaluation [14]. Elastic system fibers include mature elastic fibers, clausalic fibers, and oxytalan fibers [25]. These 3 distinct fibers types represent a full range of the combination between microfibrils and elastin deposition. It is well known that resorcin-fuchsin stains both elastic and clausalic fibers. In fact, considering that we did not separate these types, we called the all-fiber content measured as elastic-fiber content. Immunohistochemistry was performed with the following antibodies: anti-MMP9 (SC-6840; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TGF-β active form (SC-146; Santa Cruz Biotechnology), and anti-TIMP-1 (SC-5538; Santa Cruz Biotechnology) through the biotin-streptavidin-peroxidase method.

The vascular content of collagen and elastic fibers and the MMP-9, TIMP-1, and TGF-β expression levels were evaluated by an image analysis system (Image Pro Plus- 4.5) [14]. We analyzed only bronchiolar vessels that can be whole observed at magnification of ×200. As previously described, we measured the external muscle wall perimeter as well as the collagen and elastic fibers area and the areas positive for MMP-9, TIMP-1, or TGF-β (μm²) in 9 to 10 randomly selected bronchial vessels wall per lung. The collagen evaluation was performed using a polarized light. All parameters were evaluated at a magnification of ×200. All results were expressed as a relation between the quantity of positive area in a specific frame and the total area of the frame (%). Since

there was no difference in the vessels external perimeter, to quantify the vascular wall area, we measured the smooth muscle and adventitia walls area and results were expressed in μm^2 . To evaluate the angiogenesis, we used the point counting technique, and we counted the numbers of vessels and the number of points that were coincident with lung tissue and the results were expressed by number of vessels per area ($10^4 \mu\text{m}^2$). These techniques were performed by 2 different researchers blinded to the protocol design, as previously described [14, 26].

Statistical analysis

Values are expressed as mean $\pm$ SE. Statistical analysis was performed using SigmaStat software (SPSS, Chicago, IL). Data were evaluated by 2-way analysis of variance (sensitization $\times$ treatment status) and multiple comparisons were made using Holm-Sidak test. A *P* value of less than .05 was considered significant.

RESULTS

Concentration of E_{NO}

Values of E_{NO} were higher in the ovalbumin-Ve group (28.91 ± 1.74) compared with saline-Ve group (20.95 ± 1.53 ; *P* = .001). 1400W treatment reduced E_{NO} only in ovalbumin-exposed guinea pigs (ovalbumin-W: 18.46 ± 2.46 ; *P* = .001).

Qualitative analysis of airway vascular extracellular remodeling

Figure 2, panels A to C, show the collagenous fibers in the controls (A) and ovalbumin-exposed animals treated with vehicle (B) or with 1400W (C) stained with picrosirius and observed using polarized light. Figure 2D to F show lung tissue stained with resorcin-fuchsin to detect elastic fibers in controls (D) and ovalbumin-exposed animals that received vehicle (E) or 1400W treatment (F), at a magnification of $\times 400$. In saline-exposed guinea pigs, there was a weak red-orange birefringence in tissue sections (Figure 2A) coincident with the maintenance of the perivascular extracellular matrix architecture. In contrast, in ovalbumin-exposed animals there was a diffuse increase of birefringence (Figure 2B) in perivascular extracellular matrix (ECM) and an increase in elastic fibers content in vascular wall (Figure 2E). Figure 2C and F show that chronic 1400W treatment decreased both collagen and elastic fibers deposition around the vessel wall.

Qualitative analysis of the immunohistochemistry studies

Figure 2, panels G to O, show the immunohistochemistry to detect the TGF- β (panels G to I), MMP-9 (panels J to L), and TIMP-1 (panels M to O) in vascular wall of guinea pigs from saline-Ve group (panels G, J, and M), ovalbumin group that received vehicle (panels H, K, and N), or ovalbumin- and 1400W-treated group (panels I, L, and M). In vascular wall from ovalbumin-exposed animals that received vehicle (ovalbumin-Ve), there was a large positive stained to TGF- β , MMP-9, and TIMP-1 in vascular wall compared to lungs from saline-Ve and from ovalbumin-W groups (see arrows).

Quantitative analysis of angiogenesis and vascular wall area

We analyzed only bronchiolar vessels that can be whole observed at magnification of $\times 200$. In order to be sure that the vessels analyzed had similar size, we have measured the external muscular wall perimeter. In fact, no significant difference in muscle wall external perimeter of vessels of all groups (data not shown) was observed. Measurements of angiogenesis showed no significantly difference among experimental groups (saline-Ve: 4.19 ± 0.56 ; Saline-1400W: 4.79 ± 0.62 ; ovalbumin-Ve: 4.14 ± 0.56 ; ovalbumin-1400W: 4.98 ± 0.44). In Figure 3, the values of vascular wall area (smooth muscle and adventitia area) are presented. We note that there was an increase in vascular wall area in ovalbumin-Ve group compared to saline (*P* < .01). The 1400W treatment in ovalbumin-exposed animals reduced the vascular wall area compared to those animals that received vehicle of 140W (*P* < .01).

Quantitative analysis of airway vascular extracellular remodeling

Measurements of vessel remodeling are shown in Figure 4. Both regarding collagen (panel A) and elastic content (panel B), ovalbumin-exposed animals showed greater values of percent collagen and elastic fibers compared to saline-exposed animals (*P* < .05). The inhibition of iNOS by 1400W decreased this response compared to ovalbumin-treated and non-treated animals (*P* < .05 comparing ovalbumin-W to ovalbumin-Ve group).

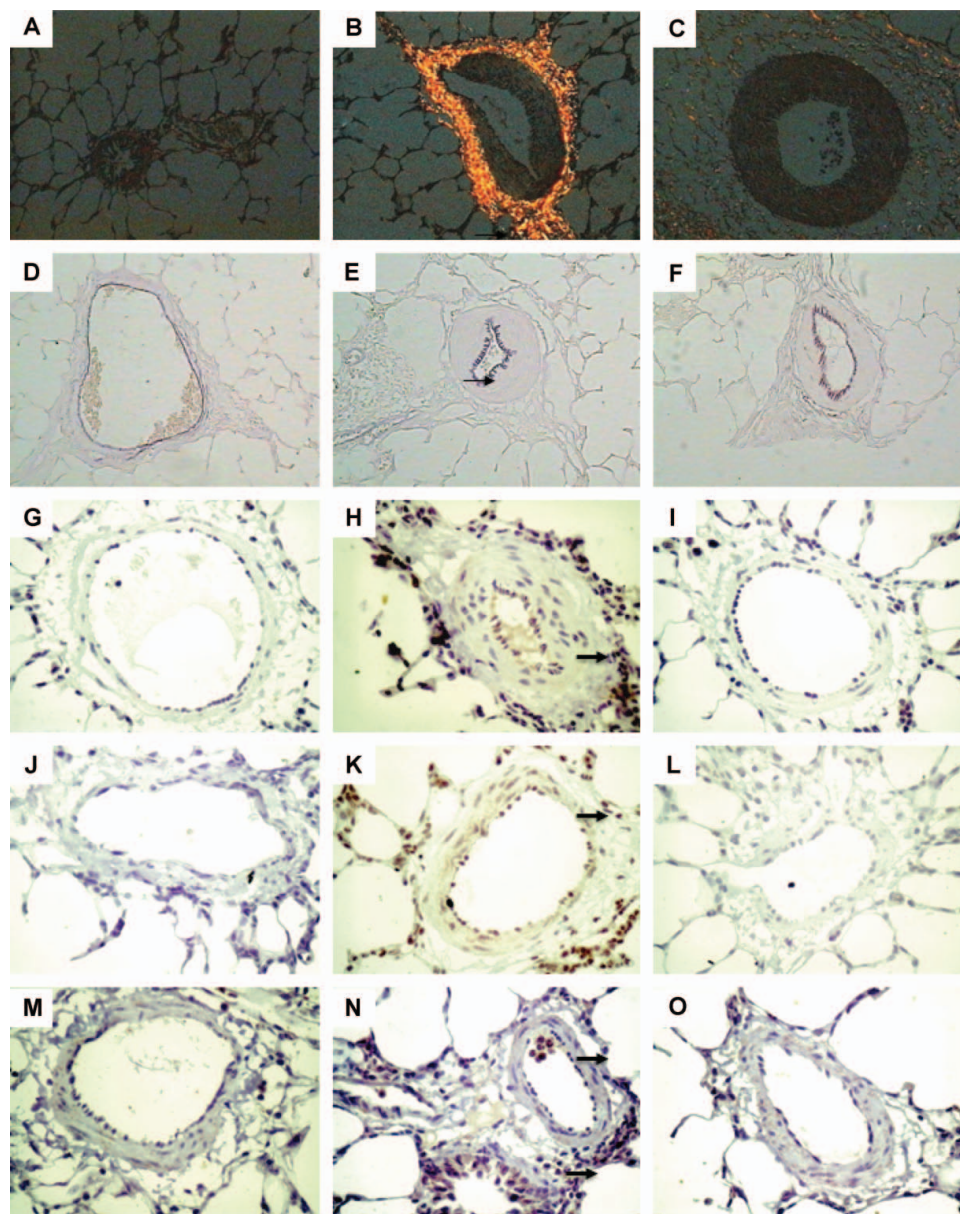


FIGURE 2 Guinea pig vessels obtained from saline-exposed (saline-Ve group: panels **A**, **D**, **G**, **J**, and **M**), ovalbumin-exposed (ovalbumin-Ve group: panels **B**, **E**, **H**, **K**, and **N**), and ovalbumin-exposed and 1400W-treated (ovalbumin-W group: panels **C**, **F**, **I**, **L**, and **O**) groups, stained with picrosirius (panels **A–C**), resorcin-fuchsin (panels **D–F**), anti-TGF- β (panels **G–I**), anti-MMP-9 (panels **J–L**), and anti-TIMP-1 (panels **M–O**) and observed under polarized light. Saline-exposed animal showed the weak yellow-greenish birefringence of the vascular walls in tissue section (**A**), coincident with the maintenance of the histoarchitecture of the extracellular matrix and scant elastic fibers (**D**). In contrast, vascular wall of OVA group show an increase of birefringence (**B**) and in elastic fibers content (**E**). These structural alterations coincides with large positive stained to TGF- β , MMP-9, and TIMP-1 (see arrows) in vascular wall. In contrast, 1400W treatment in ovalbumin-exposed animals reduced collagen (**C**) and elastic fiber (**F**) deposition in vascular wall, and the positive expression of TGF- β , MMP-9, and TIMP-1 in vascular wall (all panels observed at $\times 200$). (Color figure available online.)

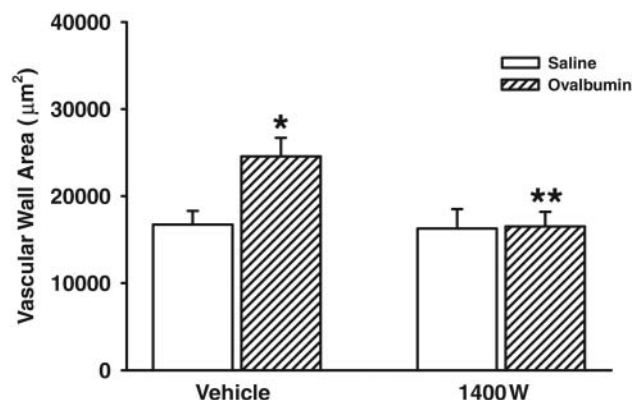


FIGURE 3 The mean values of vascular wall area (including the adventitia and smooth muscle wall). Note the ovalbumin-Ve group presents higher values of vascular wall compared to saline-Ve, and the treatment with 1400W reduced this response. * $P < .01$ compared with saline-exposed guinea pigs that received vehicle (saline-Ve group); ** $P < .01$ compared with ovalbumin-exposed group that received vehicle (ovalbumin-Ve group).

Quantitative analysis of the immunohistochemistry stain

We have quantified the expression of MMP-9, TIMP-1, and TGF- β in airway vascular wall (Figure 5). There was an increase in MMP-9, TIMP-1, and TGF- β expression in airway vascular wall in ovalbumin-exposed animals compared to saline-exposed ones ($P < .001$). The ovalbumin-exposed and 1400W-treated animals (ovalbumin-W group) presented a reduction of MMP-9, TIMP-1, and TGF- β expression in airway vascular wall compared to ovalbumin-exposed and vehicle-treated ones ($P < .05$).

DISCUSSION

In the present study, we have shown for the first time that inducible nitric oxide is involved in the airway vascular remodeling, particularly concerning the collagen and elastic fibers content, induced by allergic chronic pulmonary inflammation in guinea pigs. The effects of iNOS inhibition on the collagen and elastic fibers content in vascular wall could be attributed, at least in part, to effects of nitric oxide on metalloproteinase (MMP-9), on their respective tissue inhibitor (TIMP-1), and also on TGF- β expressed in vascular wall. This effect of iNOS reducing extracellular matrix remodeling and inflammation is not exclusively related to lung vessels, since iNOS is required for airway eosinophilia and airway remodeling in this experimental model, as previously showed [14, 15]. These

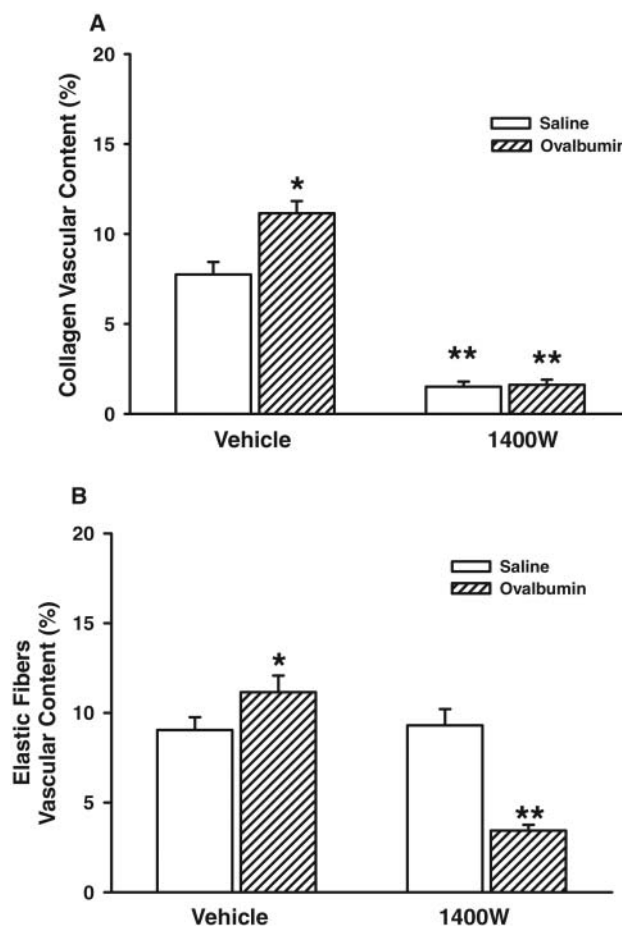


FIGURE 4 The mean values of percentage of collagen (A) and elastic fiber (B) in vascular wall. Note the ovalbumin-Ve group presents an increase in the collagen and elastic fibers content in vascular wall compared to saline-Ve, and the treatment with 1400W reduced this response. * $P < .05$ compared with saline-exposed guinea pigs that received vehicle (saline-Ve group); ** $P < .05$ compared with ovalbumin-exposed group that received vehicle (ovalbumin-Ve group).

features in airways and vessels probably were occurring concomitantly in order to counteract the lung inflammation and repair the tissue damage. Although it is clear that airway vascular remodeling may have clinical and functional implications in asthma, up to now few studies have been published on the therapeutic interventions to control these alterations [2]. The pharmacological control of bronchial vascular remodeling may be crucial for symptom control in asthmatic patients [2, 3]. In order to develop new drugs, the pathophysiological mechanism involved on bronchial vascular remodeling need to be further clarified. Taking it into focus in the present study, we intended to investigate the importance of iNOS isoform in airway vascular remodeling in an experimental model of

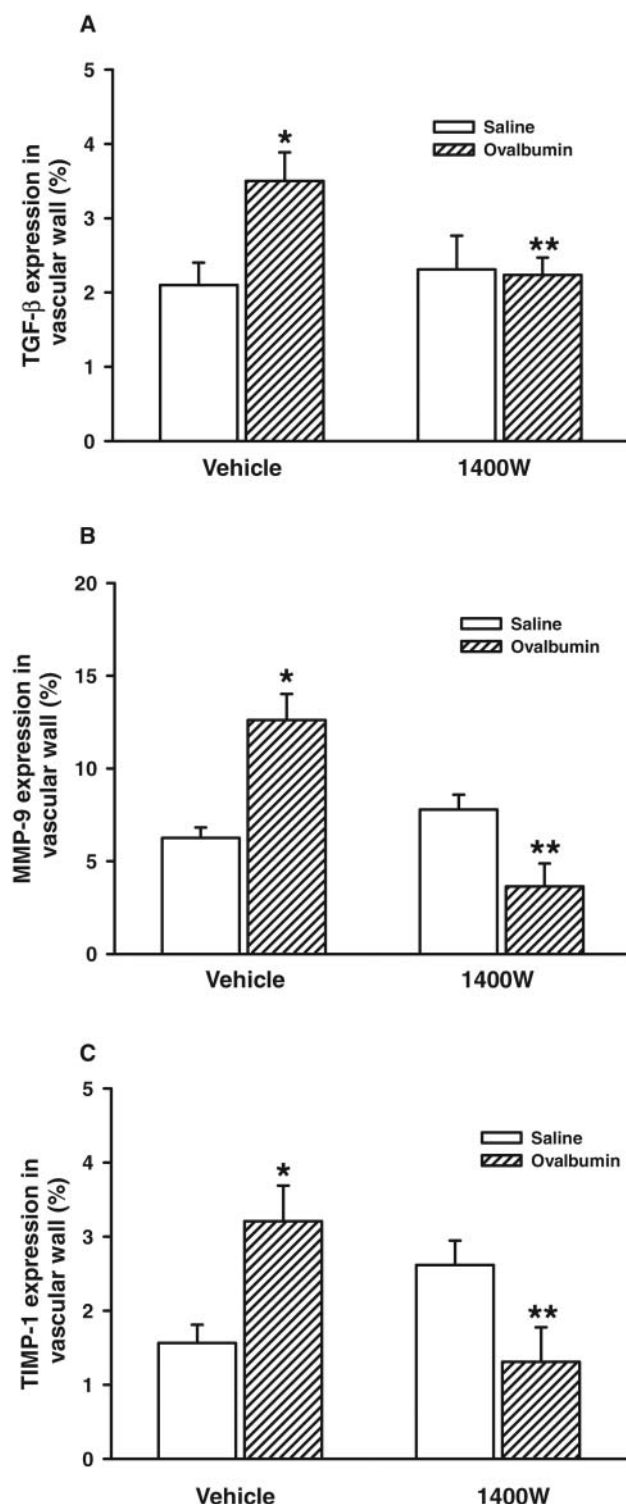


FIGURE 5 The mean values of percentage of TGF- β (A), MMP-9 (B), and TIMP-1 (C) expression in vascular wall. * $P < .001$ compared with saline-exposed guinea pigs that received vehicle (saline-Ve group); ** $P < .05$ compared with ovalbumin-exposed group that received vehicle (ovalbumin-Ve group).

chronic allergic airway inflammation in guinea pigs, also intending to make clear the possible mechanisms involved.

Nitric oxide is strongly involved in the control of lung inflammation. We have previously demonstrated that in this experimental guinea pigs model, there is an increase in iNOS expression in both lymphomononuclear cells and eosinophils around airways of ovalbumin-exposed animals [24]. In addition, we observed an increase in the content of collagen and elastic fibers around airway wall [14]. The iNOS-derived NO increases both collagen and elastic fibers content in airway wall. Additionally, it contributes to bronchoconstriction and amplifies inflammatory responses.

Concerning the choice of iNOS inhibitor, we used 1400W because it has a high specificity and selectivity for iNOS inhibition. Previous studies had demonstrated that 1400W is one of the most selective inhibitors of iNOS. In addition, 1400W presents an in vivo selectivity of at least 100-fold greater than other NOS inhibitors such as aminoguanidine [22, 27]. Other iNOS inhibitors are at best 30-fold more potent against iNOS than endothelial NOS (eNOS), whereas 1400W is over 5000- and 200-fold more potent against iNOS than eNOS and neuronal NOS (nNOS), respectively [22, 27].

Considering that the therapeutic efficacy of 1400W treatment in this guinea pig asthma model was previously obtained by the measurements of exhaled nitric oxide and also the iNOS expression in mononuclear and eosinophils in lung parenchyma and in peribronchiolar wall [14, 15], in the present study we repeated only the measurements of exhaled nitric oxide in order to confirm the above results. Our data showed that 1400W treatment reduced by approximately 40% the values obtained in ovalbumin-exposed animals (ovalbumin-W group).

Airway vascular wall remodeling is characterized by structural changes in the number and size of vessels, including the structural changes of extracellular matrix. Our study showed some important results concerning airway vascular wall remodeling process. First, iNOS inhibition reduced the vascular wall area that was increased by ovalbumin exposure. Second, iNOS inhibition contributes to the reduction of collagen and elastic fibers content in vascular wall previously increased by repeated ovalbumin exposures. One of the mechanisms involved was related to the decrease in MMP-9 and TIMP-1 expression. Second, another mechanism involved in the control of extracellular vascular matrix remodeling in ovalbumin-exposed guinea pigs may be due to the attenuation of TGF- β expression by the inhibition of iNOS.

Although there were these structural alterations in vascular wall and in extracellular matrix components, we did not observe any signs of angiogenesis, since we did not observe any difference in number of vessels per lung tissue area among the experimental groups.

Metalloproteinases are a family of Zn-containing endopeptidases that are able to degrade most extracellular matrix components [28]. The MMP-9 is a gelatinase-B that is mainly activated in any repair or remodeling process and also in inflamed situations [29]. Recent evidence has shown the importance of MMP expression in asthma pathogenesis. These endopeptidases are involved in the degradation of ECM components, inflammatory cell trafficking, host defenses, and tissue repair [30].

In the present study we observed an increase in the MMP-9 in vascular wall of guinea pigs with chronic pulmonary inflammation. Although MMP-2 is also involved in extracellular matrix proliferation and both MMP-2 and MMP-9 have the same substrate, it was previously demonstrated that MMP-2 is mainly produced by tissue structural cells, including fibroblasts and endothelial cells, and MMP-9 is produced mainly by inflammatory cells such as macrophage, neutrophils, and eosinophils [31, 32]. It has been also shown that MMP-9 would be linked to tissue remodeling induced by inflammatory process, whereas MMP-2 may be associated with an impaired tissue remodeling leading to pathological collagen deposition and pulmonary fibrosis [33].

In the current study we also observed that there is an augment in TIMP-1 in vascular wall after ovalbumin exposures. It is important to note that the specific tissue inhibitor of MMP-9, TIMP-1, is recognized as having biologic effects independent of MMPs [34, 35], such as inducing cellular apoptosis. In addition, TIMP-1 is also involved in the modulation of other MMPs, such as MMP-2 and MMP-12 [36, 37]. These results are in accordance with the idea that a process of degradation/turnover of collagen and elastic fibers in airway vascular vessels is continuously occurring, induced by chronic inflammatory stimuli, as previously suggested in other studies [38].

One limitation of our study is related to the evaluation the MMP-9 and TIMP-1 ratio. It is worthy to clarify that this ratio is of importance to better understand the counterregulatory mechanisms involved in extracellular matrix remodeling. Considering that the vessels were randomly selected, it is not possible to compare the MMP-9 and TIMP-1 contents in the same vessels. In addition, considering that the aim of the present study was related to vascular analysis, the evaluation of total lung content of these proteinases was not an adequate methodological strategy.

Even though there are no other studies evaluating the iNOS effects on airway vascular remodeling, we could conjecture some possibilities based on previous published studies that focused on the NO effects in airway remodeling. In this regard, Hogaboam *et al.* [39], in a model of nonfibrotic lung granuloma, showed that nonspecific inhibition of NO synthesis by *N*^ω-nitro-L-arginine methyl ester (L-NAME) increased the C-C chemokine receptor CCR2 and CCR3 mRNAs, reducing macrophage chemoattractant protein-1 (MCP-1) and eotaxin in isolated lung fibroblast culture. These alterations contribute to increase the collagen lung content. Meurs *et al.* [40] also suggested that increases in pulmonary arginase activity could interfere with myofibroblast differentiation, augmenting collagen production.

Considering the collagen content in lung vessels, surprisingly the 1400W treatment also reduced the collagen content in saline-treated animals. Although in other studies we have not observed any statistically difference between saline- and saline-1400W-treated animals in collagen deposition in airways [14] and in lung tissue [15], there was some tendency in this reduction in saline- and 1400W-treated animals. One possible explanation was related to the fact that constitutive expression and production of iNOS-derived nitric oxide can be responsible for the homeostasis maintenance. It has been shown that iNOS is expressed in constitutively form in almost all brain regions and in some peripheral tissues of normal human [41]. In addition, Guo *et al.* [42], studying the expression of NOS in the human lung, had shown, through molecular cloning, that NO synthesis in normal human airways was due to the continuous expression of the inducible NOS (iNOS) isoform in airway epithelial cells. In normal human lung, the expression of iNOS is strikingly decreased by inhaled corticosteroids and β -adrenergic agonists, medications commonly used in treatment of inflammatory airway diseases [42]. Considering that corticosteroids are potent iNOS inhibitors, these findings were clearly in agreement with our results.

The effects of nitric oxide on elastic fibers deposition are very defectively studied. Although our group had previously shown that nitric oxide inhibition reduced the elastic fibers content in airways and lung tissue [14, 15], we have not evaluated the possible mechanisms involved. In the current study, the iNOS inhibition reduced the MMP-9, TIMP-1, and TGF- β . These mediators act not only in the production of collagen fibers, but also in some different extracellular matrix components, including elastic fibers.

The present study has some important limitations, since the results obtained in animal models have to be carefully transposed to humans. However, it is

important to remember that the guinea pig model is one of the best animal models to study asthma, since its lung anatomy is similar to that of humans, and it mimics some of the characteristics of human asthma as eosinophilic inflammatory and remodeling process [16].

In conclusion, the specific blockage of iNOS-derived NO attenuates antigen-induced airway vascular remodeling, reducing both collagen and elastic fiber deposition as well as the vascular wall area. These effects could be attributed to effects of 1400W in reducing both MMP-9 and TIMP-1 expression and also TGF- β expression in peribronchial vascular wall.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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