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Effects of Repeated Stress on Distal Airway Inflammation, Remodeling and Mechanics in an Animal Model of Chronic Airway Inflammation

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Key Words
Stress · Experimental asthma model · Extracellular matrix remodeling · Eosinophils

Abstract
Background/Aims: Epidemiological studies suggest that stress has an impact on asthmatic exacerbations. We evaluated if repeated stress, induced by forced swimming, modulates lung mechanics, distal airway inflammation and extracellular matrix remodeling in guinea pigs with chronic allergic inflammation. ***Methods:*** Guinea pigs were submitted to 7 ovalbumin or saline aerosols (1–5 mg/ml during 4 weeks; OVA and SAL groups). Twenty-four hours after the 4th inhalation, guinea pigs were submitted to the stress protocol 5 times a week during 2 weeks (SAL-S and OVA-S groups). Seventy-two hours after the 7th inhalation, guinea pigs were anesthetized and mechanically ventilated. Resistance and elastance of the respiratory system were obtained at baseline and after ovalbumin challenge. Lungs were removed, and inflammatory and extracellular matrix remodeling of distal airways was assessed by morphometry. Adrenals were removed and weighed. ***Results:*** The relative adrenal weight was greater in stressed guinea pigs compared to non-stressed animals ($p < 0.001$). Repeated stress increased the percent elastance of the respiratory system after antigen challenge and eosinophils and lymphocytes in the OVA-S compared to the OVA group ($p < 0.001$, $p = 0.003$ and $p < 0.001$). Neither collagen nor elastic fiber contents were modified by stress in sensitized animals. ***Conclusions:*** In this animal model, repeated stress amplified bronchoconstriction and inflammatory response in distal airways without interfering with extracellular matrix remodeling.

Introduction

Asthma is a chronic airway disease, and many factors are associated with asthmatic exacerbations such as air pollution, infections and environmental background. In addition, stress remains a clinically relevant factor for asthmatics. Several epidemiological studies showed that 20–35% of asthmatics experiencing exacerbations occur during periods of stress [1–4]. In addition, the mental health of asthmatic children has been linked to morbidity and mortality of these patients [4–6]. Ritz et al. [4] showed a tendency of bronchoconstriction in asthmatics under negative emotional conditions.

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Considering a simplistic analysis of mutual interactions among stress, immune, neuroendocrine and behavioral systems, the stress response activates the hypothalamic-pituitary-adrenal axis and adrenomedullary system, leading to a release of glucocorticoids and catecholamines which, in turn, could influence the immune system and the course of diseases [4, 6, 7].

However, it is important to consider that stressors are not single entities, and different stressors, such as physical and psychological ones, associated or not, may influence these systems in different ways. In this regard, several cytokines, growth factors and lipid mediators of inflammation can have an influence on the hypothalamic-pituitary-adrenal axis response, particularly acting on the locus ceruleus. There was a sequential release of tumor necrosis factor, interleukin (IL)-1 and IL-6. In addition, both IL-1 and interferon modulate central and peripheral parts of the sympathetic nervous system. It is well known that interferon can inhibit corticotropin-releasing hormone on the anterior pituitary [8].

It is important to notice that stress associated with an immune challenge is a combination of sickness and classic stress syndromes. The sickness syndrome includes anorexia, nausea, fatigue, sleep disturbances, fever and increased basal metabolic rate. These responses modify pain or afferent neural systems and the acute-phase reaction, cell adhesion molecules or fibrinogen. Psychological stressors influence the central nervous system leading to mental illness. On the other hand, toxins, pathogens and physical stressors induce endocrine responses that interact with immune and inflammatory systems [8–11].

There are several animal models of stress induction [12–17]. The forced swim stress is a type of unavoidable stress that induces an effort to survive with an escape deficit [16, 17]. Furthermore, this method has been used as an animal model of behavioral despair/depression associated with the modulation of inflammatory cells and cytokines [16–19]. It is important to emphasize that this stress animal model includes both psychological and physical stressors, which in turn influence allergic responses induced by repeated ovalbumin exposures.

In this study, our primary aim was to assess if repeated forced swim stressors modulate respiratory system mechanics, airway inflammatory response and extracellular matrix remodeling in guinea pigs with chronic pulmonary allergic inflammation.

Methods

All guinea pigs received humane care in compliance with the Guide for Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985), and all experiments described in this study were approved by the institutional review board of the University of São Paulo (São Paulo, Brazil; number of the process: 267/06).

Experimental Groups

Guinea pigs were divided into 4 different groups: (1) animals that received aerosolized saline (SAL, n = 8); (2) animals that received aerosolized ovalbumin (OVA, n = 8); (3) animals that performed forced swimming and received aerosolized saline (SAL-S, n = 8), (4) animals that performed forced swimming and received aerosolized ovalbumin (OVA-S, n = 8).

Induction of Chronic Pulmonary Allergic Inflammation

Male Hartley guinea pigs, weighing 300–400 g, were placed in a plexiglass box (30 × 15 × 20 cm) coupled to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). A solution of ovalbumin (grade V, Sigma Chemical Co., Saint Louis, Mo., USA) diluted in 0.9% NaCl (saline) was prepared. The animals received 7 inhalations during 4 weeks with increasing concentrations of ovalbumin (1–5 mg/ml) in order to neutralize tolerance (fig. 1). Control animals received aerosolized saline at the same time points. The solution was continuously aerosolized into the environment until respiratory distress (sneezing, cough or retraction of the thoracic wall) occurred, or until 15 min had elapsed, as previously described [20].

Forced Swim Stress

Twenty-four hours after the 4th inhalation, animals were submitted to the forced swim protocol, 5 days a week for 2 consecutive weeks, to induce behavioral stress (fig. 1). Each guinea pig was placed alone into a transparent box (length 50 cm, width 40 cm, depth 30 cm), containing clean potable water at 25°C. The performance of each animal was observed and monitored. The animals swam up to 10 min or until they presented signs of fatigue or danger of drowning. It is important to remember that guinea pigs only breathe through their noses, minimizing the risk of water aspiration [16, 17]. It is important to emphasize that in this protocol of stress induction, there are both psychological and physical stressors, and it is not possible to separate the role of each component in the functional and inflammatory responses. In addition, it is important to highlight that the functional pulmonary evaluations were performed 24 h after the last exposure to the forced swim stress protocol to exclude any effects of the water temperature and exercise on pulmonary mechanical measurements.

Respiratory System Mechanical Evaluation

Seventy-two hours after the 7th inhalation, animals were anesthetized with pentobarbital sodium (50 $mg \cdot kg^{-1}$, i.p.), tracheostomized and mechanically ventilated at 60 breaths/min with a tidal volume of 8 $ml \cdot kg^{-1}$ using a Harvard 683 ventilator (Harvard Apparatus, South Natick, Mass., USA).

Tracheal pressure (Ptr) was measured with a 142PC05D differential pressure transducer (Honeywell, Freeport, Ill., USA) connected to a side tap in the tracheal cannula. Airflow (V') was determined using a pneumotachograph (Fleisch 4-0, Richmond, Va., USA) connected to the tracheal cannula and to a Honeywell

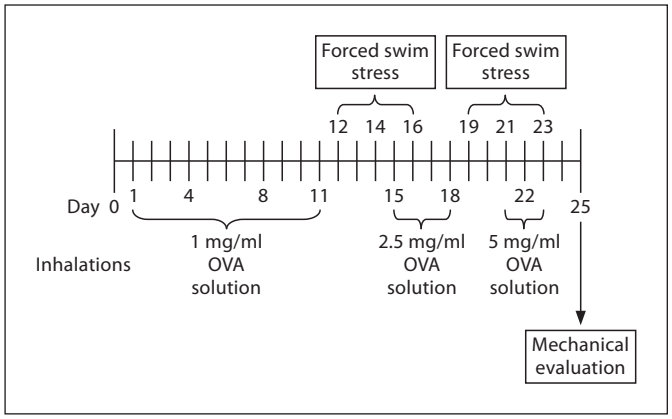

Fig. 1. Time line of the experimental protocol. The guinea pigs were submitted to 7 inhalations (2 per week, with 2- to 3-day intervals, during 4 weeks) with aerosols of saline or ovalbumin (OVA) solution with increasing doses of antigen. From the 1st to the 4th inhalation, the dose used was 1 mg/ml of ovalbumin (2 weeks). For the 5th and 6th inhalations (3rd week), animals were inhaled with 2.5 mg/ml of ovalbumin, and for the 7th inhalation (beginning of week 4), the dose of 5 mg/ml of antigen was used. The forced swim stress started 24 h after the 4th inhalation during 5 days a week for 2 weeks. The solution of ovalbumin or 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 the 7th inhalation, all guinea pigs were anesthetized, exsanguinated and lungs were removed and submitted to the experimental protocol of respiratory system mechanics and airway inflammatory and remodeling evaluation.

163PC01D36 differential pressure transducer, as previously described [21–23]. Changes in lung volume (V) were determined by digital integration of the airflow signal. Nine to 10 respiratory cycles were averaged to provide 1 data point. Respiratory system elastance (Ers) and resistance (Rrs) were obtained using the equation of motion of the respiratory system:

$$Ptr(t) = Ers \cdot V(t) + Rrs \cdot V'(t),$$

where t is time.

After baseline measurements of Ptr and V', we performed ovalbumin (30 $mg \cdot ml^{-1}$) or normal saline challenges aerosolized into the breathing circuit for 1 min 2 times. Measurements of Ptr and V' were taken 1, 3 and 5 min after the beginning of the first challenge (fig. 1).

After the lung mechanic measurements, the anterior chest wall was removed and lungs were washed with heparinized saline solution (1:40). Guinea pigs were exsanguinated, a positive end-expiratory pressure of 5 cm H_2O was applied to the respiratory system, the airways were occluded at the end of expiration, and the lungs were removed en bloc.

Adrenal Weight

The adrenal glands were also dissected, removed and weighed after exsanguinations. The body and adrenal weight ratios were expressed as relative adrenal weight, which was calculated by dividing adrenal weight by total body weight.

Morphometric Studies

The left lung was fixed with 4% buffered formaldehyde. Sections representing peripheral areas of the lung were cut and processed for paraffin embedding. Histological sections (5 μm thick) were cut and stained with hematoxylin and eosin and were evaluated by researchers blinded to the protocol design. We evaluated the number of eosinophils and lymphocytes on distal airway walls (the area between the bronchial epithelium and the adventitia) employing an integrating eyepiece (10^4 μm^2 of the total area) [21, 22]. Ten to 20 fields were analyzed per lung at a magnification of ×1,000.

Distal Airway Extracellular Matrix Remodeling

Histological sections were stained for collagen fibers by picrosirius (Direct Red 80, CI 35780, Aldrich, Milwaukee, Wisc., USA) and for elastic fibers by Weigert's resorcin-fuchsin. We measured the total area of the distal airway wall and collagen or elastic fibers (μm^2) in 10 distal airways, using polarized light for collagen evaluation, at a magnification of ×200, in an image analysis system (Image J, version 1.30) [23]. The collagen or elastic content (%) was expressed as the relation between the quantity of collagen or elastic fibers in a specific frame and the total area of this frame.

Statistical Analysis

Statistical analysis was performed using SigmaStat 10.0 software (Jandel, Calif., USA). Comparison among groups was performed by two-way analysis of variance, followed by the Holm-Sidak method. A p value <0.05 was considered significant.

Results

Respiratory System Mechanical Evaluation

There were no differences in Rrs baseline values among the 4 experimental groups. The OVA (99.37 ± 32.57%) and OVA-S (129.47 ± 33.79%) groups had an increase in %Rrs after antigen challenge compared to the control groups (SAL 15.29 ± 4.37 and SAL-S 13.33 ± 4.33%; $p < 0.001$; d.f. = 1; F = 18.35; fig. 2a).

In regard to Ers baseline values, there were no differences among the 4 experimental groups. The sensitized animals (OVA 383.39 ± 72.09 and OVA-S 643.53 ± 111.702%) had an increase in %Ers after antigen challenge compared to the control groups (SAL 34.34 ± 13.05 and SAL-S 17.79 ± 3.20%; $p < 0.001$; d.f. = 1; F = 43.60). In addition, sensitized-stressed animals (OVA-S group) had an increase in %Ers compared to only sensitized animals (OVA group; $p < 0.05$; d.f. = 1; F = 3.21; fig. 2b).

Inflammatory Cell Infiltration

Figure 3a shows the number of lymphocytes in distal airways of the 4 experimental groups. There was an increase in the OVA (7.26 ± 0.38 cells/10^4 μm^2; $p < 0.05$) and OVA-S (8.91 ± 0.33 cells/10^4 μm^2) groups compared to normal saline-exposed animals (SAL 4.94 ± 0.33 and

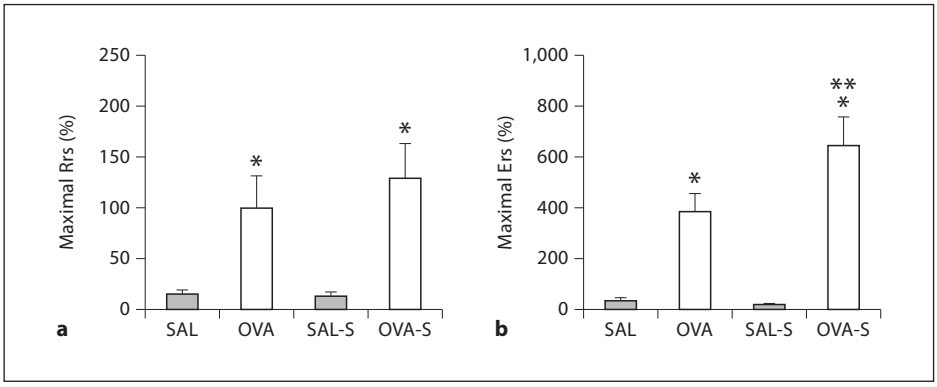

Fig. 2. a Mean ± SE of the maximal response of Rrs after antigen challenge of the 4 experimental groups. OVA and OVA-S groups had greater values than those observed in control groups (* $p < 0.001$, compared to SAL and SAL-S groups). **b** Mean ± SE of the maximal response of Ers after antigen challenge of the 4 experimental groups. OVA and OVA-S groups had greater values than those observed in control groups (* $p < 0.001$, compared to SAL and SAL-S groups). OVA-S animals had a significant increase in the %Rrs compared to the OVA group (** $p < 0.05$).

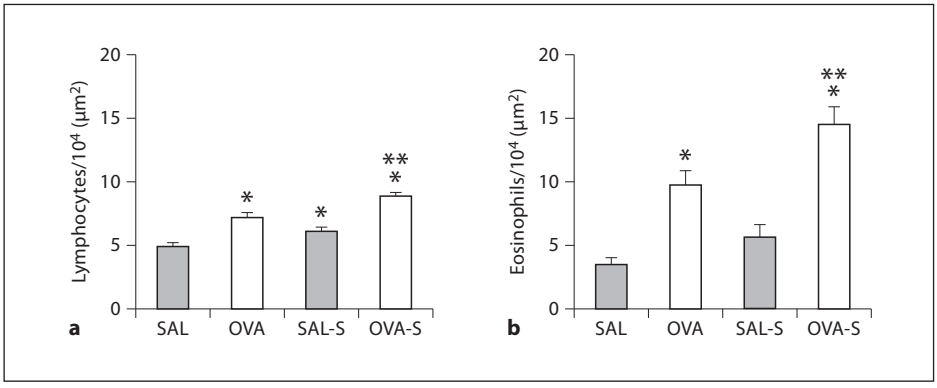

Fig. 3. a Mean ± SE of the number of lymphocytes in the airway walls of the 4 experimental groups. OVA and OVA-S groups had greater values than those observed in the control groups (SAL and SAL-S groups, * $p < 0.001$). Stressed groups (SAL-S and OVA-S groups) had a significant increase in the number of lymphocytes compared to non-stressed controls (SAL and OVA groups, respectively; * $p < 0.001$). **b** Mean ± SE of the number of eosinophils in the airway walls of the 4 experimental groups. OVA and OVA-S groups had greater values than those observed in the control groups (SAL and SAL-S groups, * $p < 0.001$). There was a significant difference between OVA and OVA-S groups (** $p = 0.003$).

SAL-S 6.20 ± 0.28 cells/10^4 μm^2; $p < 0.001$; d.f. = 1; F = 57.92). The number of lymphocytes in the OVA-S group was greater compared to the OVA group ($p < 0.001$; d.f. = 1; F = 19.27). There were no differences between SAL and SAL-S groups.

Figure 3b shows the number of eosinophils in distal airways of the 4 experimental groups. Ovalbumin-exposed animals (OVA 9.75 ± 1.12 and OVA-S 14.49 ± 1.38 cells/10^4 μm^2) had an increase in the number of eosinophils compared to saline-exposed animals (SAL 3.44 ± 0.58 and SAL-S 5.64 ± 0.96 cells/10^4 μm^2; $p < 0.001$; d.f. = 1; F = 42.95). In addition, there was an increase in the number of eosinophils in the OVA-S group compared to the OVA group ($p = 0.003$; d.f. = 1; F = 9.03).

Adrenal Weight

The relative adrenal weight of the animals submitted to the stress protocol (0.53 ± 0.03 × 10^{-3} mg) was greater than that observed in non-stressed groups (0.37 ± 0.01 × 10^{-3} mg; $p < 0.001$; d.f. = 1; F = 19.15).

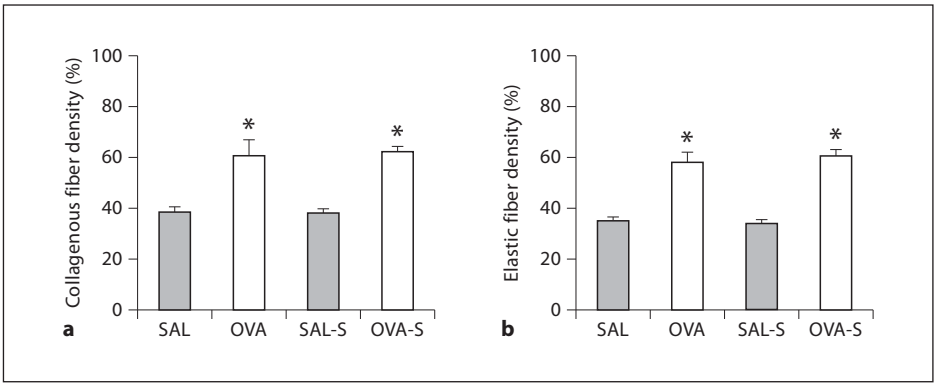

Fig. 4. a Mean ± SE of values of collagen content in the airway walls of the 4 experimental groups. The OVA group had greater values than those observed in the OVA-S and control groups (SAL and SAL-S groups, * $p < 0.001$). **b** Mean ± SE of values of elastic fiber content in the airway walls of the 4 experimental groups. OVA and OVA-S groups had lower values than those observed in the control groups (SAL and SAL-S groups, * $p < 0.001$).

Extracellular Matrix Remodeling of Distal Airways

Regarding collagen fibers (fig. 4a), there was an increase in collagen content in distal airways in ovalbumin-exposed animals (OVA 60.54 ± 6.35 and OVA-S 62.10 ± 2.14%) compared to saline-exposed groups (SAL 38.48 ± 1.92 and SAL-S 38.07 ± 1.61%; $p < 0.001$; d.f. = 1; F = 29.54). There was no difference between OVA and OVA-S groups.

Considering elastic airway fibers (fig. 4b), there was an increase in elastic content in distal airways in ovalbumin-exposed animals (OVA 58.15 ± 4.12 and OVA-S 60.85 ± 2.42%) compared to saline-exposed groups (SAL 34.86 ± 1.75 and SAL-S 33.96 ± 1.63%; $p < 0.001$; d.f. = 1; F = 49.53). There was no difference between OVA and OVA-S groups. Figure 5 shows the representative photomicrographs of airway walls stained with picrosirius (a–d) and Weigert's resorcin-fuchsin (e–h) from guinea pigs inhaled with saline (a, e) or those chronically exposed to ovalbumin (b, f). The guinea pigs submitted to repeated exposures to forced swim stressor and inhaled with saline (c, g) or ovalbumin (d, h) are also represented.

Discussion

Asthma is considered a chronic airway inflammatory disease in which clinical features may be worsened by several factors including stress and emotions [24]. Although epidemiological data corroborate this assumption, experimental information is scarce, and few studies have been dedicated to analyze the effects of emotions and stress on distal airway inflammation and remodeling. The current study shows that repeated stress, induced by repeated forced swim, amplified distal airway responsiveness to antigen challenge, which was associated with an increase in eosinophils and lymphocytes in distal airways. However, airway extracellular matrix remodeling was not modified by stress in this animal model.

There was an increase in relative adrenal weight of stressed animals compared to non-stressed guinea pigs, showing that this protocol was able to induce a stress reaction. This result corroborates the study by Ulrich-Lai et al. [25], where the authors showed that chronic stress induced an increase in adrenal weight due to hyperplasia in the outer zona fasciculata and hypertrophy in the inner zona fasciculata and medulla in rats. In addition, Almeida-Reis et al. [26] applied the same stress protocol used in the present study and showed that animals subjected to the repeated forced swim stress had greater values of serum cortisol and adrenal weight compared to non-stressed groups, and catecholamine levels were unaltered in all groups.

Capelozzi et al. [16] showed that swimming-induced stress amplified mononuclear cell recruitment to the lungs in guinea pigs that performed 31 days of the stress protocol. These authors also showed that the amount of these cells was reduced when animals were under fluoxetine treatment. The studies mentioned above and other authors suggest that the stress protocol performed in the present study can be considered a model of chronic stress [24, 27, 28].

As we mentioned previously, it is not possible to distinguish between psychological and physical stress performing the forced swim stress protocol, which is considered a limitation of this study. A further important issue

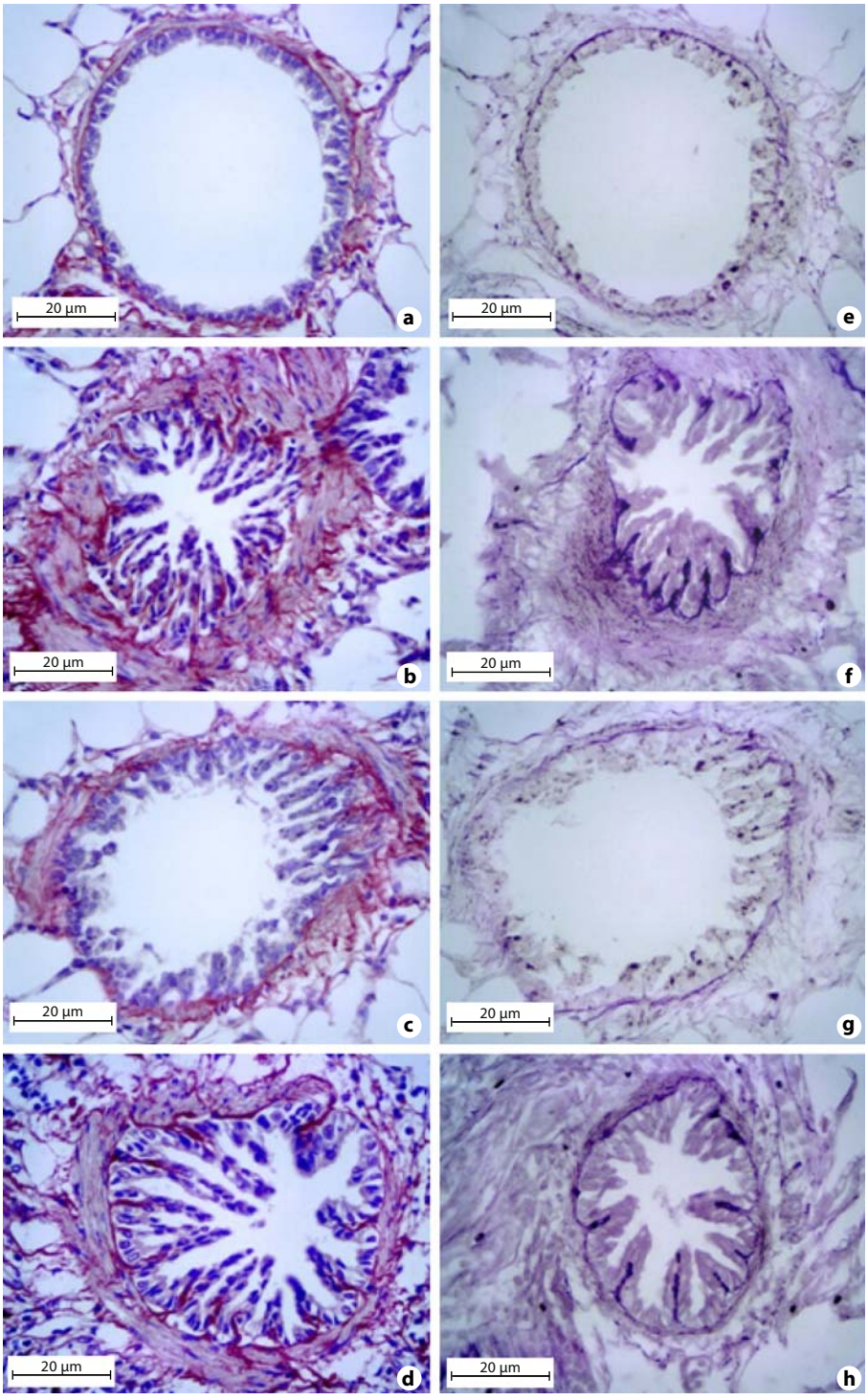

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Fig. 5. Photomicrographs representative of airway walls stained with picrosirius (**a–d**) and Weigert's resorcin-fuchsin (**e–h**) from guinea pigs inhaled with saline (**a**, **e**) or those chronically exposed to ovalbumin (**b**, **f**). The guinea pigs submitted to repeated exposures to forced swim stressor and inhaled with saline (**c**, **g**) or ovalbumin (**d**, **h**) are also represented. Although sensitized animals had an increase in the total collagen and elastic fiber density compared to saline-exposed guinea pigs, there were no differences in these extracellular matrix components concerning either stressed or non-stressed animals that were repeatedly exposed to ovalbumin inhalations (OVA and OVA-S groups).

is that there is a lack of 2 days in the stress protocol to avoid lactic acid buildup, pain and fatigue of animals [26]. We did not observe any difference in the swim time (data not shown) among the 4 experimental groups, which in turn could be evidence of physical training.

Considering asthmatic functional repercussions induced by stress, several authors observed negative associations between mood states and a reduction in forced expiratory volume in 1 s [4, 29]. Höglund et al. [2] studied 41 undergraduate students, 22 with allergy, 16 asthmatics

and 19 controls, in a low stress period and in association with a major exam, and the forced expiratory volume in 1 s of the control group only differed significantly from the group of asthmatics during the stress phase.

In this experimental model, the airway responsiveness was evaluated as the maximal responses of the Rrs and Ers after ovalbumin challenge. It is important to point out that some authors consider that the relation between baseline and maximal functional responses may represent airway responsiveness after antigen challenge. In the present study, this effect was observed in ovalbumin-exposed animals.

We demonstrated that repeated exposures to physical stress have a functional detrimental effect on distal airway responses since the Ers was increased in sensitized-stressed compared to only sensitized animals after ovalbumin challenge.

Many mechanisms may be involved in the modulation of these pulmonary mechanical alterations. Ers may be altered due to distal airway or distal lung (alveolar) alterations. Although asthma is defined as a chronic airway disease, the importance of distal lung tissue in functional asthma impairment has also been addressed [30]. In this regard, Reis et al. [2009, unpubl. obs.] showed that in the same animal model, a repeated forced swimming stressor increased lung distal constriction. These responses were associated with an increase in actin content, inducible nitric oxide synthase expression and oxidative stress pathway activation, suggesting that nitric oxide contributes to pulmonary stress-induced structural and functional alterations.

Another aspect to be highlighted in order to understand the increase in airway hyperresponsiveness after antigen challenge is the importance of airway eosinophilic and lymphocytic recruitment [31]. In order to test if the functional responses were associated with an increase in these inflammatory responses, we quantified the number of eosinophils and lymphocytes that infiltrated the distal airway wall. Ovalbumin-exposed animals had an increase in both eosinophils and lymphocytes. We have previously shown in this animal model of chronic pulmonary allergic inflammation that these lymphocytes were mainly represented by CD4+ Th2 lymphocytes [20, 26] and that both the eosinophils and lymphocytes in this animal model of chronic allergic inflammation have an increased expression of inducible nitric oxide synthase and neuronal nitric oxide synthase on the parenchyma and airways [23, 32, 33].

Both eosinophilic and lymphocytic responses were amplified by repeated stress in ovalbumin-exposed animals. Our hypothesis to explain these findings may be related to the imbalance of Th1/Th2, with a shift toward Th2, induced by repeated stress exposures. Corroborating this idea, Almeida-Reis et al. [26] applied the same stress protocol used in the present study and showed that repeated swim stress exposure reduces mucociliary clearance due to mucus rheological property alterations which was associated with IL-4 activation. Reis et al. [2009, unpubl. obs.] also evaluated IL-4-, IL-5- and IL-13-positive cells on distal lung parenchyma of guinea pigs repeatedly exposed to ovalbumin and submitted to the swim stress protocol. The authors observed an amplification of this Th2 response in sensitized and stressed animals.

The biological properties of eosinophils include the release of toxic granule proteins, oxygen-free radicals, eicosanoids, Th2-like cytokines and growth factors. Once activated, products from eosinophils contract human bronchial smooth muscle, increase vascular permeability, induce airway hyperresponsiveness and induce the shedding of the surface epithelium in keeping with the hypothesis of eosinophil-induced damage of the bronchi [34].

Several opposing results have been described concerning the effects of stress in eosinophil recruitment [35, 36]. Portela et al. [37] observed that sensitized and stressed rats had an enhancement of airway edema and lymphocytic infiltration. However, the number of eosinophils was unaffected. Capelozzi et al. [16] found an enhancement of eosinophilic density on the alveolar wall in stressed guinea pigs. Portela et al. [37] observed that the number of polymorphonuclear cells was not altered by stress or diazepam treatment. In humans, Liu et al. [3] analyzed the effects of low stress or a stress phase in college students with mild asthma. They found that the number of sputum eosinophils was increased during the stress phase.

Many substances produced during the stress responses modulated eosinophilic recruitment and apoptosis [36, 38, 39]. Eosinophilic apoptosis was mediated by the presence of survival-enhancing asthmatic cytokines such as IL-3, IL-5 and granulocyte macrophage colony-stimulating factor. Higher concentrations of these proinflammatory cytokines could inhibit the proapoptotic effects of glucocorticoids [40]. Chida et al. [41] showed that early psychological and physical stresses aggravated methacholine responsiveness and airway inflammation. Evaluating these studies, we consider the need to more profoundly evaluate the determinants of stress-induced eosinophilic recruitment.

Although in the present study we found an increase in the total collagen and elastic fiber content in the distal airway wall in ovalbumin-exposed animals compared to saline-exposed guinea pigs, there were no differences between OVA and OVA-S groups in spite of the amplification of the inflammatory response in stressed and sensitized animals.

Extracellular matrix remodeling has important clinical significance contributing to the irreversibility of lung function alterations observed in asthmatic patients [42, 43]. Many mediators were involved in these responses including Th2 cytokines, transforming growth factor-β_1 and nitric oxide. In this regard, Prado et al. [32] also demonstrated that the inhibition of nitric oxide by 1400W treatment reduced extracellular collagen in the airways. Recent evidence has shown the importance of metalloproteinase (MMP) expression in asthma pathogenesis [44]. These substances are involved in the degradation of extracellular matrix components, inflammatory cell trafficking, host defenses and tissue repair [45].

Some studies showed that catecholamines act as modulators of the expression of MMPs [46, 47]. Yang et al. [47] showed that norepinephrine treatment increased MMP-2, MMP-9 and vascular endothelial growth factor levels. Briest et al. [46] studied the significance of MMPs in noradrenalin-induced remodeling of rat hearts. After 3 days of continuous infusion of noradrenalin, MMP-2 activity was elevated in both ventricles. Miller et al. [48] showed that corticosteroids inhibit the expression of transforming growth factor-β_1 in eosinophils and macrophages. However, other studies do not corroborate the role of corticosteroids in reducing extracellular matrix remodeling in asthma [49].

Our hypothesis is that the apparent absence of stress-related amplification of the extracellular matrix remodeling may in fact represent a counteracting effect between pro- and anti-fibrotic stimuli induced by the chronic inflammatory milieu and the release of stress-related mediators.

In conclusion, repeated stress amplified pulmonary hyperresponsiveness, which was associated with an increase in eosinophils and lymphocytes around these airways without affecting extracellular matrix remodeling.

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