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J Appl Physiol 109:855-863, 2010. First published 15 July 2010; doi:10.1152/japplphysiol.00333.2010

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Sex-specific lung remodeling and inflammation changes in experimental allergic asthma

Mariana A. Antunes,¹ Soraia C. Abreu,¹ Adriana L. Silva,¹ Edwin R. Parra-Cuentas,² Alexandre M. Ab'Saber,² Vera L. Capelozzi,² Tatiana P. T. Ferreira,³ Marco A. Martins,³ Patricia M. R. Silva,³ and Patricia R. M. Rocco¹

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Submitted 29 March 2010; accepted in final form 12 July 2010

Antunes MA, Abreu SC, Silva AL, Parra-Cuentas ER, Ab'Saber AM, Capelozzi VL, Ferreira TP, Martins MA, Silva PM, Rocco PR. Sex-specific lung remodeling and inflammation changes in experimental allergic asthma. *J Appl Physiol* 109: 855–863, 2010. First published July 15, 2010; doi:10.1152/japplphysiol.00333.2010.—There is evidence that sex and sex hormones influence the severity of asthma. Airway and lung parenchyma remodeling and the relationship of ultrastructural changes to airway responsiveness and inflammation in male, female, and oophorectomized mice (OVX) were analyzed in experimental chronic allergic asthma. Seventy-two BALB/c mice were randomly divided into three groups ($n = 24$ /each): male, female, and OVX mice, whose ovaries were removed 7 days before the start of sensitization. Each group was further randomized to be sensitized and challenged with ovalbumin (OVA) or saline. Twenty-four hours after the last challenge, collagen fiber content in airways and lung parenchyma, the volume proportion of smooth muscle-specific actin in alveolar ducts and terminal bronchiole, the amount of matrix metalloproteinase (MMP)-2 and MMP-9, and the number of eosinophils and interleukin (IL)-4, IL-5, and transforming growth factor (TGF)- β levels in bronchoalveolar lavage fluid were higher in female than male OVA mice. The response of OVX mice was similar to that of males, except that IL-5 remained higher. Nevertheless, after OVA provocation, airway responsiveness to methacholine was higher in males compared with females and OVX mice. In conclusion, sex influenced the remodeling process, but the mechanisms responsible for airway hyperresponsiveness seemed to differ from those related to remodeling.

airway hyperresponsiveness; collagen; estrogen; actin

IMPORTANT SEX DIFFERENCES in the natural history of asthma have been repeatedly pointed out in the literature (1). In males asthma is more common during prepuberty, whereas in females it occurs more often after puberty (9). A role for sex hormones in modulating asthma and its exacerbation can be deduced from these sex differences (13), particularly in situations of hormone replacement therapy (36) and postmenopause (28).

Experimental studies have produced contradictory results concerning the effects of sex hormones on lung inflammation and airway hyperresponsiveness in asthma (6, 8, 19, 22). One study reported less inflammation and more sensitivity to methacholine in male than female mice (6). Another study concluded that lung inflammation and hyperactivity are in-

dependent of sex in mice (8). However, the same study (8) observed a sex-specific impact on a third process involving structural airway changes in asthma, known as remodeling. The quantitative relationship between remodeling, inflammation, and airway responsiveness is not clear (15, 37). In that study (8), sex slightly influenced the potency of inhaled corticosteroid to reverse bronchoconstriction (8), suggesting that an estrogen-dependent remodeling process might explain the different reversion pattern in females compared with males. Thus the role of sex in inflammation, hyperresponsiveness, and remodeling as well as the relationship between these processes in asthma still needs to be clarified.

The aim of the present study was to investigate the effect of sex on the remodeling process in asthma and the relationship of these ultrastructural changes to responsiveness and inflammation in male, female, and oophorectomized female (OVX) mice in an experimental model of chronic allergic asthma.

METHODS

Animals. This study was approved by the Ethics Committee of the Carlos Chagas Filho Institute of Biophysics, Health Sciences Center, Federal University of Rio de Janeiro. All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the National Research Council.

Protocol. The experiments were performed on 72 adult BALB/c mice (age 8–9 wk) with an average body weight between 25 and 28 g, receiving water and food ad libitum. A total of 24 male (M) and 48 female (F) mice was used. Twenty-four of these 48 females were oophorectomized (OVX) 1 wk before the beginning of the experimental protocol as previously described (29). These animals were further randomized to be sensitized and challenged with sterile ovalbumin (OVA, albumin from chicken egg white; A5503, Sigma-Aldrich, St. Louis, MO) or saline. In the allergic inflammation groups, mice were immunized by intraperitoneal injection of 10 μ g of sterile OVA in 0.1 ml of saline on each of seven alternate days. Forty days after the beginning of sensitization, intratracheal challenge was performed with the following protocol. Mice were treated with sevoflurane anesthesia. A 0.5-cm-long midline cervical incision was made to expose the trachea, and 20 μ g of OVA in 20 μ l of warm (37°C) sterile saline (0.9% NaCl) was instilled. The cervical incision was closed with 5.0 silk suture, and the mice were returned to their cage. The animals recovered rapidly after surgery. This procedure was performed three times, with a 3-day interval between instillations. No adjuvants were used in the present protocol (2, 40). The control group (SAL) received saline instead of OVA during both sensitization and challenge. Five mice from each group ($n = 30$) were used to compute airway hyperresponsiveness, evaluate the cytokine and cell count in

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bronchoalveolar lavage fluid (BALF), and analyze metalloproteinases in pulmonary tissue. Lung histology was analyzed in seven mice per group ($n = 42$). Mice were anesthetized and euthanized by sectioning of the abdominal aorta and vena cava, yielding a massive hemorrhage that quickly killed the animals.

Airway responsiveness. Twenty-four hours after the last challenge, the animals were sedated (Compaz, Cristália, Itapira, São Paulo, Brazil; 1 mg ip) and anesthetized (Thiopentax, Cristália; 20 mg/kg ip). They were then tracheotomized and ventilated with a constant-flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) using the following parameters: frequency of 100 breaths/min, tidal volume of 0.2 ml, and fraction of inspired oxygen of 0.21. A positive end-expiratory pressure (PEEP) of 2 cmH₂O was applied. At the beginning of the protocol, two deep inhalations ($3 \times$ tidal volume) were performed to standardize the volume history. One dose of saline (baseline) followed by increasing doses of methacholine (Sigma Chemical, St. Louis, MO; 300, 1,000, 3,000, and 10,000 μ g/kg) was administered via a Silastic catheter tied into the jugular vein to measure airway responsiveness. Data were stored 30 s and 1, 3, and 5 min after agonist injection. Airflow and tracheal pressure (Ptr) were measured after the maximal increase in Ptr was achieved after each intravenous infusion of methacholine (31, 40). Respiratory system resistance (R) was obtained with the equation of motion of the respiratory system: $\text{Ptr}(t) = E \cdot V(t) + R \cdot V'(t)$, where t is time, E is respiratory system elastance, V is volume, and V' is airflow; the result is expressed as a percentage of baseline measurements. The methacholine dose-response curve lasted 30 min.

Light microscopy. Animals were initially sedated, anesthetized, and tracheotomized as previously described. A laparotomy was done, and heparin (1,000 IU) was intravenously injected in the vena cava. The trachea was clamped at end expiration (PEEP = 2 cmH₂O) and, after the animal's death, lungs were removed en bloc at the same end-expiratory pressure in all groups to avoid distortion of lung morphometry. The right lungs were quick-frozen by immersion in liquid nitrogen, fixed with Carnoy solution, and embedded in paraffin (30). Four-micrometer-thick slices were cut and subjected to hematoxylin and eosin staining. Morphometric analysis was performed with an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The volume fraction of the lung occupied by collapsed alveoli (alveoli with rough or plicate walls) or normal pulmonary areas were determined by the point-counting technique (38), at a magnification of $\times 200$ across 10 random noncoincident microscopic fields. Briefly, points falling on collapsed or normal pulmonary areas were counted and divided by the total number of points in each microscopic field. The airway bronchoconstriction index of the central and peripheral airways was determined by counting the points falling on the airway lumen and those falling on airway smooth muscle and on the epithelium, at a magnification of $\times 400$. The perimeter of the airways was estimated by counting the intercepts of the lines of the integrating eyepiece with the epithelial basal membrane. The areas of smooth muscle and airway epithelium were corrected in terms of airway perimeter by dividing their values by the number of intercepts of the line system with the epithelial basal membrane of the corresponding airway. Because the number of intercepts (NI) of the lines with the epithelial basal membrane is proportional to the airway perimeter and the number of points (NP) falling on airway lumen is proportional to airway area, the magnitude of bronchoconstriction (contraction index, CI) was computed by the relationship $\text{CI} = \text{NI} \sqrt{\text{NP}}$. Measurements were performed in five airways from each animal at $\times 400$ magnification (31, 40).

Collagen (picrosirius-polarization method) (26) and elastic fibers (Weigert resorcin-fuchsin method with oxidation) (16) were quantified in the alveolar septa and in the airways. The alveolar septa quantification was carried out with the aid of a digital analysis system and specific software (Image-Pro Plus 5.1 for Windows; Media Cybernetics, Silver Spring, MD) under $\times 200$ magnification. The

images were generated by a microscope (Axioplan, Zeiss) connected to a camera (Sony Trinitron CCD, Sony, Tokyo, Japan), fed into a computer through a frame grabber (Oculus TCX, Coreco, St Laurent, PQ, Canada) for off-line processing. The thresholds for collagen and elastic fibers were established after enhancement of contrast up to the point where the fiber was easily identified as either birefringent (collagen) or black (elastic) bands. Bronchi and blood vessels were carefully avoided during the measurements. The area occupied by fibers was determined by digital densitometric recognition. To avoid any bias due to septal edema or alveolar collapse, the areas occupied by the elastic and collagen fibers measured in each alveolar septum were divided by the length of each studied septum. The results are expressed as the amount of elastic and collagen fibers per unit of septum length ($\mu\text{m}^2/\mu\text{m}$). Collagen and elastic fiber content were quantified in the whole circumference of the two largest, transversally cut airways present in the sections. Transversally cut airways are defined when the short-to-long diameter ratio is larger than 0.6. Results were expressed as the area of collagen or elastic fibers divided by the perimeter of the basement membrane ($\mu\text{m}^2/\mu\text{m}$).

Immunohistochemistry. Strips ($2 \times 2 \times 10$ mm) from the right lung were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry using monoclonal antibody against α -smooth muscle actin (Dako, Carpinteria, CA) at a 1:500 dilution. Sections were then rinsed with Tris-buffered saline and sequentially incubated with biotinylated rabbit anti-mouse IgG (Dako, Cambridge, UK) at a dilution of 1:400, followed by streptavidin combined in vitro with biotinylated horseradish peroxidase at a dilution of 1:1,000 (Dako). The reaction product was developed with diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin for 1 min, dehydrated through graded alcohols, and mounted in resinous medium. Known positive controls were included with each run, and negative controls had the primary antibody omitted (12). The analysis was performed on the slides stained for α -smooth muscle actin with application of the point-counting technique (38). Using a 121-point grid, we calculated the volume proportion of smooth muscle-specific actin in terminal bronchioles and alveolar ducts as the relation between the number of points falling on actin-stained and nonstained tissue. Measurements were done at $\times 400$ in each slide.

Electron microscopy. To obtain a stratified random sample, three $2 \times 2 \times 2$ -mm slices were cut from different segments of the left lung and then fixed with glutaraldehyde 2.5% and 0.1 M phosphate buffer (pH 7.4) for 60 min at -4°C . Ultrathin sections from selected areas were examined and micrographed in a JEOL electron microscope (JSM-6100F; Tokyo, Japan). Submicroscopic analysis of lung tissue showed that the extension and distribution of the parenchymal alterations were inhomogeneous along the bronchiole and alveolar tissue (alveolar ducts and alveoli). Thus electron micrographs representative of the lung specimen (Sal and OVA groups) were enlarged to a convenient size to visualize the following inflammatory and remodeling structural defects: 1) epithelial detachment, 2) eosinophil infiltration, 3) neutrophil infiltration, 4) degenerative changes of ciliated airway epithelial cells, 5) subepithelial fibrosis, 6) elastic fiber fragmentation, 7) smooth muscle hypertrophy, 8) myofibroblast hyperplasia, and 9) mucous cell hyperplasia (20). Pathological findings were graded according to a 5-point semi-quantitative severity-based scoring system: 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = changes in 26–50%, 3 = changes in 51–75%, and 4 = changes in 76–100% of examined tissue. Fifteen electron microscopy images were analyzed per animal.

Confocal microscopy. The epithelial/basement membrane components of bronchioli and alveoli were studied by confocal microscopy of cells stained with anti-cytokeratin 7 (anti-Ck7) and anti-collagen V fluorescence immunohistochemistry. Cells were incubated with anti-Ck7 (monoclonal antibody, Dako, 1:600) and anti-collagen V (polyclonal antihuman type V collagen antibody, 1:50) (35), followed by double staining with fluorescein and rhodamine (rhodamine-conjugated goat anti-mouse IgG-R, dilution 1:40; Santa Cruz Biotechnology, Santa Cruz, CA). Images were obtained with a Zeiss LSM-410

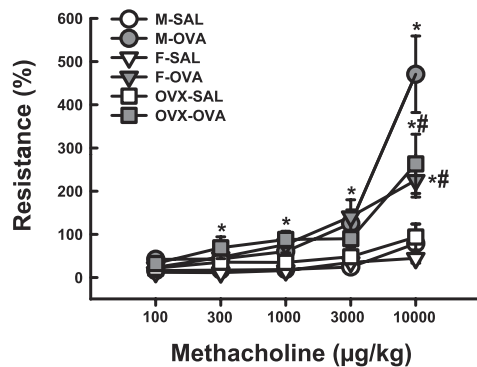


Fig. 1. Airway hyperresponsiveness induced by increasing methacholine doses (100, 300, 1,000, 3,000, and 10,000 $\mu\text{g}/\text{kg}$). M, male mice; F, female mice; OVX, oophorectomized mice; Sal, mice sensitized and challenged with saline; OVA, mice sensitized and challenged with ovalbumin. Values are means $\pm$ SE of 7 mice in each group. *Significantly different from the respective Sal group ($P < 0.05$); #significantly different from M-OVA ($P < 0.05$).

laser-scanning confocal microscope. Serial optical sections were performed with Simple 32 C-imaging computer software (LSM Image Browser software, Carl Zeiss). Z-series sections were collected at 0.6 μm with a $\times 60$ Plan Apo lens and a scan zoom of $\times 2$. All images were collected at the same photomultiplier tube settings and processed and reconstructed with NIH Image software. Contrast and color levels were adjusted with Adobe Photoshop 7.0.

Zymography. Matrix metalloproteinase (MMP)-2 and MMP-9 were measured in whole lung tissue with a zymography technique. The samples were subjected to electrophoresis on a 10% acrylamide gel containing 1% of gelatin in the presence of SDS under nonreducing conditions. The gels were stained with Coomassie brilliant blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activity appeared as a clear band against a blue background. Molecular

weights of gelatinolytic bands were estimated with recombinant protein molecular weight markers. Enzyme amount was quantified by measuring the intensity of the negative bands with a densitometric analyzer.

Evaluation of bronchoalveolar lavage fluid. To evaluate BALF, a polyethylene cannula was inserted into the trachea and a total volume of 1.0 ml of phosphate-buffered saline (PBS) containing 10 mM EDTA was instilled and aspirated. Samples were centrifuged at 300 g for 10 min. The supernatant was used for inflammatory cytokine analysis, and the pellet was resuspended in 0.25 ml of PBS. Total leukocyte counts in the BALF were performed in Neubauer chambers under light microscopy after dilution of the samples in Türk solution (2% acetic acid). Differential leukocyte counts were performed in cytocentrifuged smears stained by the May-Grünwald-Giemsa method. The amount of interleukin (IL)-4, IL-5, and transforming growth factor (TGF)- β in the cell-free BALF was evaluated by ELISA in accordance with manufacturer's instructions (Duo Set, R&D Systems, Minneapolis, MN).

Statistical analysis. The normality of the data was tested by Kolmogorov-Smirnov's test with Lilliefors' correction, while Levene's median test was used to evaluate the homogeneity of variances. If both conditions were satisfied, two-way ANOVA followed by Tukey's test was used. To compare nonparametric data, two-way ANOVA on ranks followed by Dunn's post hoc test was selected. The significance level was set at 5%. Parametric data are expressed as means $\pm$ SE, while nonparametric data are expressed as median (interquartile range). All tests were performed with SigmaStat 3.1 (Jandel, San Raphael, CA).

RESULTS

Airway responsiveness. OVA groups presented increased methacholine-induced airway responsiveness compared with the respective Sal groups (Fig. 1). The M-OVA group exhibited significant increase in airway resistance after the highest methacholine dose exposure (10,000 $\mu\text{g}/\text{kg}$) compared with the F-OVA and OVX-OVA groups (Fig. 1, $P < 0.05$).

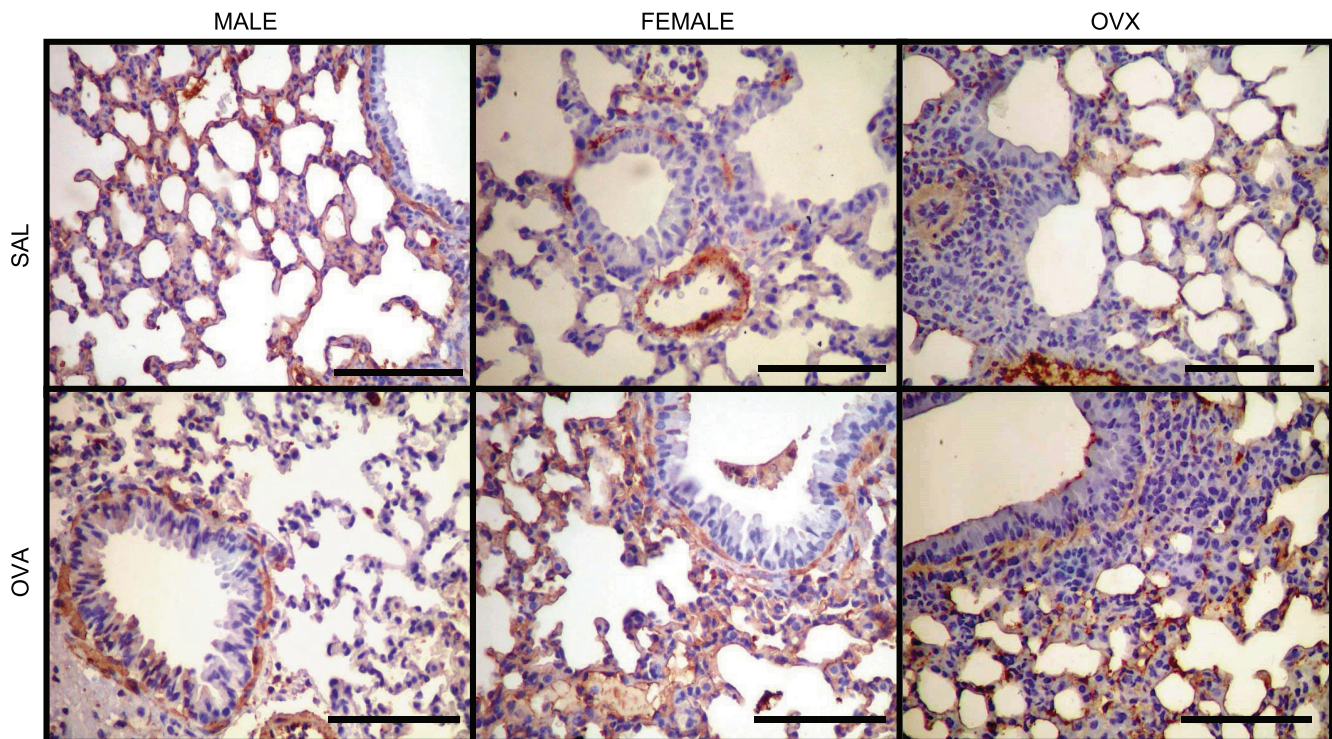


Fig. 2. Immunohistochemical staining for smooth muscle-specific actin in male, female, and OVX BALB/c mice. Note positive brown-reddish staining for α -actin in bronchiole and alveoli. Scale bars, 400 μm .

Table 1. Lung morphometry

	Male		Female		Oophorectomy	
	Sal	OVA	Sal	OVA	Sal	OVA
Normal, %	97.12 ± 0.87	88.78 ± 1.69*	95.04 ± 0.75	75.12 ± 3.41*†	97.09 ± 0.95	85.95 ± 2.05*‡
Collapse, %	2.88 ± 0.87	11.22 ± 1.69*	4.96 ± 0.75	24.88 ± 3.41*†	2.91 ± 0.95	14.05 ± 2.05*‡
Contraction index	2.83 ± 0.11	3.82 ± 0.18*	2.98 ± 0.08	3.76 ± 0.19*	3.00 ± 0.10	3.66 ± 0.08*
Collagen fiber, $\mu\text{m}^2/\mu\text{m}$						
Airways	15.73 ± 1.35	26.49 ± 1.98*	15.53 ± 1.88	35.11 ± 2.74*†	16.75 ± 2.35	22.09 ± 3.48*‡
Alveolar septa	0.007 ± 0.001	0.009 ± 0.001*	0.007 ± 0.001	0.016 ± 0.001*†	0.007 ± 0.001	0.013 ± 0.002*‡
Elastic fiber, $\mu\text{m}^2/\mu\text{m}$						
Airways	2.39 ± 0.26	2.63 ± 0.50	2.38 ± 0.18	2.13 ± 0.28	2.18 ± 0.23	2.39 ± 0.29
Alveolar septa	0.021 ± 0.001	0.021 ± 0.002	0.022 ± 0.002	0.022 ± 0.001	0.020 ± 0.003	0.020 ± 0.003
Actin, %						
Terminal bronchiole	7.93 ± 0.60	9.97 ± 0.57*	7.36 ± 1.24	12.57 ± 0.67*†	6.88 ± 0.63	8.88 ± 0.32*‡
Alveolar duct	2.64 ± 0.10	3.39 ± 0.24*	2.74 ± 0.36	4.49 ± 0.40*†	2.69 ± 0.25	3.33 ± 0.15*‡

Values are mean ± SE fraction area of normal and collapsed alveoli, contraction index, collagen and elastic fibers in airways and alveolar septa, and volume proportion of smooth-muscle specific actin in terminal bronchiole and alveolar duct of 7 male, female, or oophorectomized female mice in each group. All values were computed in 10 random, noncoincident fields per animal. Sal, mice sensitized and challenged with saline; OVA, mice sensitized and challenged with ovalbumin. *Significantly different from Sal ($P < 0.05$); †significantly different from Male-OVA ($P < 0.05$); ‡significantly different from Female-OVA ($P < 0.05$).

Light microscopy. Morphometric analysis of lungs revealed a significant increase in the fraction area of alveolar collapse, collagen fiber content in airways and alveolar septa, and the volume proportion of smooth muscle-specific actin in terminal bronchioles and alveolar ducts (Fig. 2, $P < 0.01$) in F-OVA compared with M-OVA and OVX-OVA, but the reduction in central airway diameter was similar in all OVA groups (Table 1).

Elastic fiber content in the airway and alveolar septa was similar in all groups.

Ultrastructural analysis. Electron microscopy analysis revealed several characteristics of asthma in OVA groups: intracellular edema, thickness of basement membrane, airway epithelial cell detachment from basement membrane, loss of the cilia in ciliated cells, mucous cell hypertrophy, and preserva-

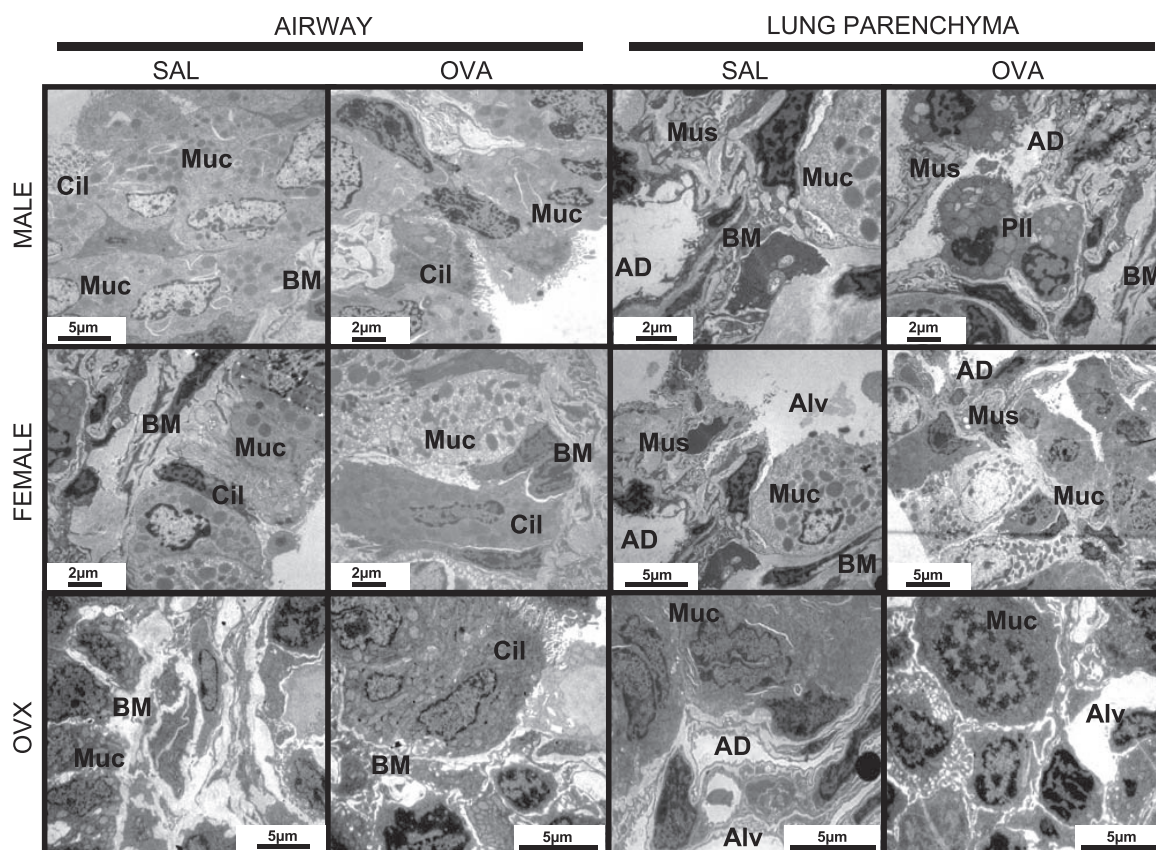


Fig. 3. Electron microscopy of airway and lung parenchyma in male, female, and OVX BALB/c mice. BM, basal membrane; Muc, mucous cell; Cil, ciliated cell; AD, alveolar duct; Alv, alveoli; PII, pneumocyte type II; Mus, muscle cell.

Table 2. *Electron microscopy semiquantitative analysis*

	Male		Female		Oophorectomy	
	Sal	OVA	Sal	OVA	Sal	OVA
Epithelial detachment	0 (0–0)	2.5 (2–3)*	0 (0–0)	4 (3–4)*†	0 (0–0)	2.5 (2–3)*‡
Eosinophil infiltration	0 (0–0)	2 (2–3)*	0 (0–0)	4 (3–4)*†	0 (0–0)	2 (2–3)*‡
Neutrophil infiltration	0 (0–0)	2.5 (2–3)*	0 (0–0)	4 (3–4)*†	0 (0–0)	2 (2–3)*‡
Ciliated airway epithelial cell degeneration	0 (0–0)	3 (2–3)*	0 (0–0)	3.5 (3–4)*	0 (0–0)	2 (2–3)*‡
Subepithelial fibrosis	0 (0–0)	2.5 (2–3)*	0 (0–0)	4 (4–4)*†	0 (0–0)	2 (2–2)*‡
Elastic fiber fragmentation	0 (0–0)	3 (2–3)*	0 (0–0)	3.5 (3–4)*†	0 (0–0)	2 (2–3)*‡
Smooth muscle hypertrophy	0 (0–0)	2 (2–3)*	0 (0–0)	4 (3–4)*†	0 (0–0)	2 (2–2)*‡
Myofibroblast hyperplasia	0 (0–0)	2 (1–3)*	0 (0–0)	3.5 (3–4)*†	0 (0–0)	2 (2–2)*‡
Mucous cell hyperplasia	0 (0–0)	2 (1–3)*	0 (0–0)	3.5 (3–4)*†	0 (0–0)	2 (2–2)*‡

Values are medians (25th percentile–75th percentile) of 5 animals/group. Pathological findings were graded according to a 5-point semiquantitative severity-based scoring system: 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% of the examined tissue in male, female, and oophorectomized mice ($n = 5/\text{group}$). *Significantly different from Sal ($P < 0.05$); †significantly different from Male-OVA ($P < 0.05$); ‡Significantly different from Female-OVA ($P < 0.05$).

tion of alveolar ducts and alveoli (Fig. 3). All OVA groups had increased qualitative scores of airway epithelial cell detachment from the basement membrane, degenerative changes in ciliated airway epithelial cells, eosinophil and neutrophil infiltration, myofibroblast and mucous cell hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy (Table 2). These ultrastructural changes were more frequent in F-OVA compared with M-OVA and OVX-OVA ($P > 0.05$). Confocal microscopy showed basement membrane rupture and increased collagen V deposition in F-OVA; however, these alterations were less intense in M-OVA and OVX-OVA (Fig. 4).

Zymography. The increase in MMP-2 and MMP-9 activity above control values in pulmonary tissue of the F-OVA group was higher than in the M-OVA group (Fig. 5, $P < 0.01$). Oophorectomy (OVX-OVA) reestablished MMP-9 activity to a level that was similar to that of the M-OVA group (Fig. 5).

Bronchoalveolar lavage fluid analysis. The total number of cells, eosinophils, neutrophils, and mononuclear cells (Fig. 6) and IL-4, IL-5, and TGF- β levels in BALF was higher in F-OVA compared with M-OVA. IL-4 and TGF- β levels were similar in M-OVA and OVX-OVA, while the total number of cells, eosinophils, neutrophils, and mononuclear cells (Fig. 6)

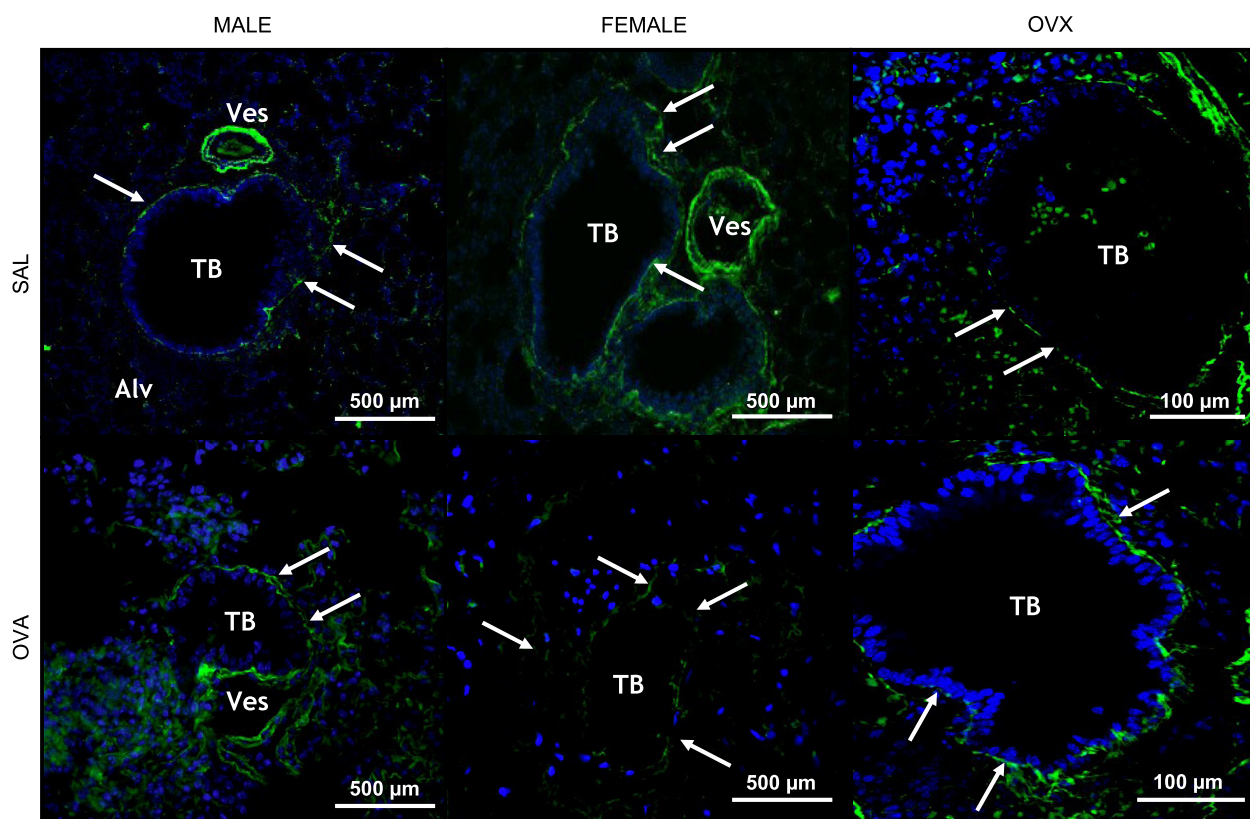


Fig. 4. Confocal microscopy evidenced 3-dimensional perspective of morphological aspects of pulmonary tissue in male, female, and OVX BALB/c mice. Note the intense positive green immunofluorescent staining for collagen V in both bronchiolar areas and lung parenchyma (white arrows). Ves, pulmonary vessel; Alv, alveoli; TB, terminal bronchiole.

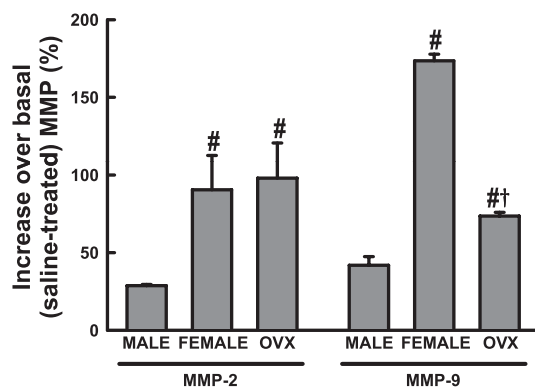


Fig. 5. Percentage increase in matrix metalloproteinase (MMP)-2 and MMP-9 levels of the OVA groups over the respective Sal groups in pulmonary tissue of male, female, and OVX BALB/c mice. Data are presented as means $\pm$ SE. #Significantly different from male-OVA group ($P < 0.05$); †significantly different from female-OVA group ($P < 0.05$).

as well as IL-5 level dropped after oophorectomy but remained higher than in M-OVA (Fig. 7).

DISCUSSION

The present study found a dissociative effect of sex on airway hyperresponsiveness and lung parenchyma remodeling in a murine model of chronic allergic asthma (40). IL-4 and IL-5 levels, as well as total and differential cell counts in BALF, were higher in female than male OVA mice and were reduced after oophorectomy. TGF- β level in BALF, collagen fiber and α -smooth muscle actin contents, and ultrastructural airway changes (such as subepithelial fibrosis, elastic fiber fragmentation, smooth muscle hypertrophy, myofibroblast hyperplasia, and mucous cell hyperplasia) were also more prominent in F-OVA compared with M-OVA and OVX-OVA, suggesting an important role of female sex hormones in the modulation of pulmonary remodeling in asthma. Lung inflam-

mation and remodeling in F-OVA were not followed by changes in airway hyperresponsiveness.

BALB/c mice were chosen for their ability to develop airway hyperresponsiveness and BALF eosinophilia after chronic sensitizations and challenges with OVA (2). Although it has been previously described that chronic challenges in mice can induce allergen tolerance, the present protocol was able to reproduce some aspects of chronic human asthma such as airway hyperresponsiveness, BALF eosinophilia, smooth muscle hypertrophy, basement membrane thickness, and mucous gland hyperplasia (40). The protocol began with animals aged 8–9 wk in order to rule out the influence of the pubertal period (27). The oophorectomized group was included in the study to facilitate the identification of differences between sexes that might be dependent on female sex hormones. A 1-wk interval was established between the oophorectomy and the beginning of sensitization to allow reduction of circulating estrogen levels and at the same time avoid a delay in beginning the study (29), since that would affect protocol duration and the aging process.

The impact of sex on inflammatory process, pulmonary function, and airway responsiveness remains contradictory (4, 14, 22). Remodeling was thought to result from chronic repetitive injury to the airway wall caused by inflammation; nevertheless, evidence suggests that there is not necessarily a quantitative relationship between inflammation and remodeling (21). The present results show that inflammation and remodeling were increased in females after chronic exposure to allergens, even though these changes did not lead to an increase in methacholine-induced airway resistance.

The dissociation between inflammation, remodeling, and airway hyperresponsiveness (15, 37) seems to be influenced by sex through distinct pathways. Card et al. (6) have shown that airway hyperresponsiveness in males is mediated mainly by vagal reflex pathways, and not by differences in innate responsiveness of airway smooth muscle. In addition, evidence also

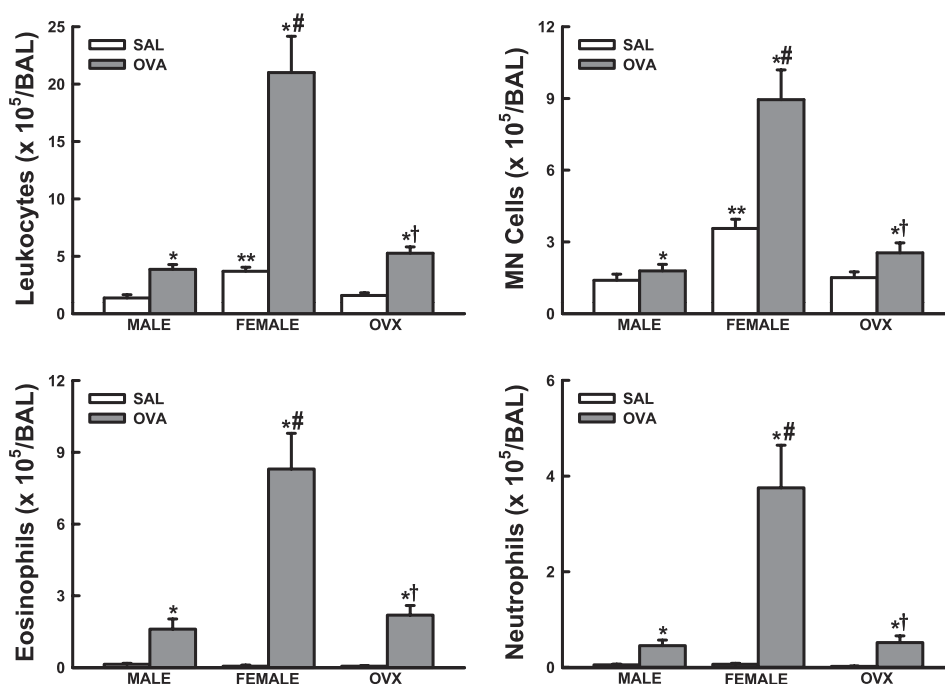


Fig. 6. Total and differential cellularity in bronchoalveolar lavage fluid (BALF) in male, female, and OVX BALB/c mice. MN cells, mononuclear cells, lymphocytes, and macrophages. Data are presented as means $\pm$ SE. *Significantly different from the respective Sal group ($P < 0.05$); **significantly different from male-Sal group ($P < 0.05$); ##significantly different from male-OVA group ($P < 0.05$); †significantly different from female-OVA group ($P < 0.05$).

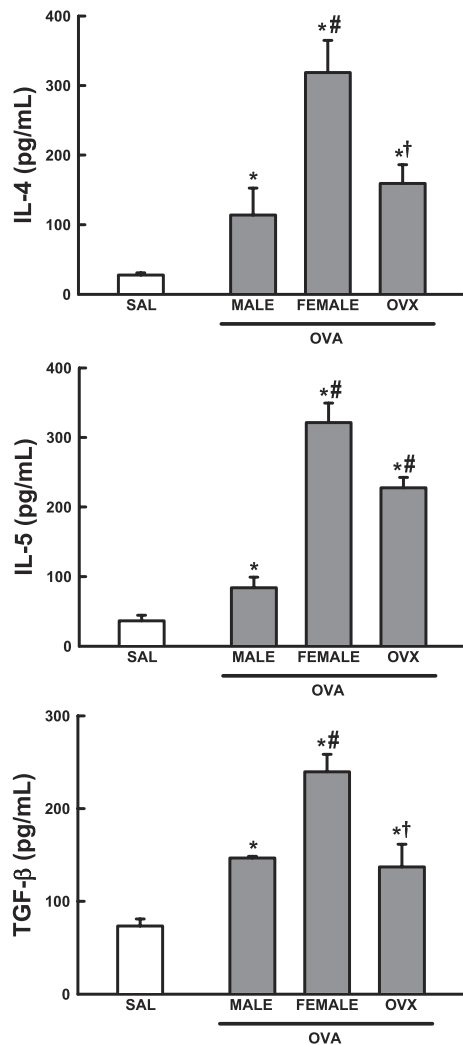


Fig. 7. Interleukin (IL)-4, IL-5, and transforming growth factor (TGF)- β levels in BALF in male, female, and OVX BALB/c mice. Since no significant differences were observed among saline-treated animals, 1 Sal group was used. Data are presented as means $\pm$ SE. *Significantly different from Sal group ($P < 0.05$); #significantly different from male-OVA group ($P < 0.05$); †significantly different from female-OVA group ($P < 0.05$).

demonstrates that estrogen may reduce airway hyperresponsiveness by increasing epithelial acetylcholinesterase activity (10). In short, airway hyperresponsiveness is directly influenced by additional mechanisms, not necessarily related to inflammation and/or remodeling.

The present study revealed greater extracellular matrix remodeling in female mice with asthma, characterized by increased collagen deposition, α -smooth muscle actin content, and ultrastructural degeneration (epithelial detachment, subepithelial fibrosis, elastic fiber fragmentation, smooth muscle hypertrophy, myofibroblast hyperplasia, mucous cell hyperplasia). These phenomena were ameliorated by ovary removal 1 wk before the beginning of allergen exposure, suggesting a key role of estrogen in pulmonary remodeling. In this context, estrogen stimulates collagen deposition in postmenopausal women under hormonal replacement therapy (3) and acts on the tissue repair process (17, 32). Conversely, two recent experimental studies have suggested a protective role of estro-

gen against airway remodeling in murine asthma (11, 19). This could be explained by the use of both estrogen replacement in male mice, without an oophorectomized control, and high doses of estrogen (11, 19). Therefore, there may be a circulating estrogen threshold required for production of either beneficial or deleterious remodeling effects.

Our findings underscore previous observations of increased inflammation in female mice compared with males (7). This may also be related to estrogen circulating levels, since ovary removal reduced IL-4, but not IL-5, in BALF of OVX mice. IL-4 and 5 play a role in the regulation of collagen synthesis by stimulating transforming growth factors (TGF- β) and increasing the expression of type I collagen through activation of transcription factors (34, 39). There is no precise information in the literature concerning the mechanisms by which female sex hormones influence IL-5 levels in asthma models (5, 24, 29). Therefore, we may speculate, based on the present experimental protocol of allergic asthma, that the role of female sex hormones in the worsening of lung remodeling is mediated mainly by the increase of IL-4.

Injury to pulmonary epithelium stimulates the adjacent mesenchyma to participate in the remodeling process through release of several growth factors. In this line, TGF- β induces both airway smooth muscle cell proliferation and synthesis and secretion of extracellular matrix proteins by fibroblasts, which is in accordance with the positive correlation previously described between TGF- β levels and degree of subepithelial fibrosis in asthma (25). In addition, in vitro studies using wound repair models have evidenced that estrogen is associated with migration and proliferation of dermal fibroblasts and increased TGF- β production by these cells (32). These data are in agreement with our study, which showed increased TGF- β in BALF in females leading to greater fibrogenesis.

MMP-2 and MMP-9, produced by inflammatory and structural cells, are the major metalloproteinases involved in asthma. Estrogen increases the enzymatic activity of MMP-2 in vascular smooth muscle cells (18) and stimulates MMP-9 expression in corneal epithelial cells (33). In our study, female mice presented higher expression of MMP-2 and MMP-9 in pulmonary tissue compared with males after a chronic allergic stimulus; however, removal of ovaries prevented MMP-9 increase, while MMP-2 remained unaltered. Furthermore, increased MMP-9 levels in sputum have also been related to asthma severity, mainly in nonreversible airway obstruction cases (23).

To the best of our knowledge, no other study has analyzed experimental models of chronic allergic asthma in male, female, and oophorectomized female mice, not only evaluating the inflammatory and remodeling processes but also investigating the interaction between them. However, our study presented limitations, such as the estrus cycle phase not being analyzed in the female mouse group. However, no significant interanimal differences were observed in females despite possible differing cycle phases and the absence of oophorectomized or male groups submitted to sex hormone replacement therapy in order to evaluate whether the approach would restore the analyzed parameters similar to those of female groups.

In conclusion, in the present experimental model of chronic allergic asthma female sex induced greater lung inflammation and remodeling, which were minimized after removal of the

ovaries, suggesting a role of sex hormones in these processes. Nevertheless, after an OVA challenge, airway responsiveness with methacholine was higher in male compared with female mice. Thus the mechanisms responsible for airway hyperresponsiveness appeared to be different from those involved in remodeling. Our experimental study helps us to better understand the mechanisms by which female sex hormones may influence asthma progression, and to highlight new investigation points for clinical studies.

ACKNOWLEDGMENTS

The authors express their gratitude to Andre Benedito da Silva for animal care, Jaqueline Lima do Nascimento, Cynthia Samary, Johnatas Dutra Silva, and Patrícia Barbosa Jurgilas for their skillful technical assistance during the experiments, Alba Silva for her help with oophorectomy surgery, Ana Lucia Neves da Silva for her help with microscopy, and Claudia Buchweitz for assistance in editing the manuscript.

GRANTS

This research was funded by grants from The Centers of Excellence Program (PRONEX-FAPERJ), The Brazilian Council for Scientific and Technological Development (MCT/CNPq), The Carlos Chagas Filho Rio de Janeiro State Research Supporting Foundation (FAPERJ), and The São Paulo State Research Support Foundation (FAPESP).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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