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ORIGINAL PAPER

Postnatal overnutrition in mice leads to impaired pulmonary mechanics in response to salbutamol

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Abstract Obesity increases the risk of respiratory disease, which is associated with airway hyperresponsiveness. Although the molecular underpinnings of this phenomenon are not well established, lung remodeling is known as an important factor in this process and could potentially explain compromised lung functions. In the present study, the obesity was induced by postnatal overnutrition in Swiss mice and we investigated the pulmonary mechanics after aerosolization of saline, methacholine, and salbutamol. The lungs were prepared for morphometric analysis. Obese animals showed bronchoconstriction in response to methacholine, as evidenced by airway and tissue resistance, tissue elastance, and hysteresivity. Salbutamol was effective at recovering the response only for airway resistance but not for tissue mechanics. We suggest that this impaired response in obese mice is related to collapsed alveolar, to inflammatory cells, and to elevated deposition collagen fibers in parenchymal tissue.

Keywords Overnutrition · Tissue resistance · Bronchoconstrictor · Bronchodilator · Extracellular matrix

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Introduction

Obesity is associated with many pulmonary diseases, including asthma, chronic obstructive disease, sleep disorders, and other ailments. Many times, obesity can adversely impact lung function by stiffening of the total respiratory system [1] and/or many biologically active substances [2]. Recently, several studies have related obesity and asthma because the asthma is more severe in obese subjects, but is adiposity a driver of many diseases [3]. The adipose tissue secretes many biologically active substances that play an important role in stimulating airway hyperresponsiveness (AHR) and inflammatory processes are a cause of excessive narrowing of the airways [2, 4]. The obesity and respiratory system interaction has been under investigation over the last five decades, however various questions remain unclear. Moreover, further investigation of obesity itself is necessary to explore the consequences and mechanisms of the body weight increases in the lung tissue. In this context, the obesity induced in neonatal mice overnutrition could be more representative of natural animal life because the obesity was induced without genetic deficient, drugs or subjected hypercaloric diets. Postnatal overnutrition has substantial influences on the long-term regulation of body mass and has an effective importance in the development of obesity in adulthood as described by Ye et al. [5]. Also, theses authors confirmed that neonatal overfeeding enhances the lung inflammatory markers released from lung macrophages and develops the AHR.

Persistent inflammation is considered a driving force behind airway injury and repair in asthmatic subjects. These constant structural changes of bronchial wall and airway remodeling have been implicated as playing a role in persistent AHR [6]. Changes in the extracellular matrix

(ECM) are characterized by an increased deposition of type I, III, and V collagen fibers, fibronectin, tenascin, perlecan, and laminin beta 2. In addition, ECM changes can involve a decrease in collagen IV, laminin alpha1, and chondroitin sulfate content [7]. The interconnectivity between intracellular proteins, plasma membrane proteins, and the ECM relies on dynamic processes that are activated by bronchospasm-inducing mediators [2, 6].

Therefore, we hypothesized that later response to postnatal overnutrition mice included deposition of ECM elements that can distort the pulmonary mechanics, especially to bronchoconstriction and bronchodilation responses. The central aim of our study was to evaluate the pulmonary mechanics in obese and control mice following the administration of an aerosolized bronchoconstrictor (MCh) and bronchodilator (salbutamol).

Materials and methods

Animals and tissue collection

All experiments involving animals were approved by the Ethics Committee of the Federal University of Alfenas (protocol number 422/2012) and were conducted in accordance with the Declaration of Helsinki for the welfare of experimental animals. All animals were housed at 22 ± 2 °C and maintained in a 12:12 h light–dark cycle and they were fed with normal chow and water ad libitum. Pregnant mice were monitored closely for day of birth. On postnatal day 1, the newborn male Swiss mice were randomly distributed into three pups per dam and six dams were used, thereby inducing postnatal overnutrition by increasing milk availability [8] through reduced litter size (RL) and twelve (12) per dam and two dams were used, normal litter size (NL). Each litter represented pups from two to five different dams, which increases genetic variability within the litter. The body mass of the offspring was monitored every 3 days until weaning (day 21 after birth) and weekly thereafter until the age of 10 weeks (or approximately 70 days of life). After being weaned, all male pups were fed with normal chow. The RL were assigned as the obese group ($n = 18$) and NL as the control group ($n = 20$). After 10 weeks, the control and obese mice were handle for pulmonary mechanics or tissue parameters (adipose and lung). All animals were anesthetized (pentobarbital sodium, 68 mg/kg, i.p. and xylazine, 12 mg/kg, i.p.) for pulmonary mechanics or tissue collection. Adipose tissue was collected by excision and weighing of the periepididymal and retroperitoneal adipose tissues was carried out. The tissue mass weights are expressed in grams per 100 g body weight. Lung was removed and prepared for morphometric analyses.

Pulmonary mechanics

Animals (obese and control) were tracheostomized (18-gauge metal IV adaptor) and mechanically ventilated with a tidal volume of 10 ml/kg, a breathing frequency of 120 breaths/min, and 3 cm of H_2O-positive end expiratory pressure applied using a small animal ventilator (flexiVent, SCIREQ, Montreal, Quebec, Canada). The animals were paralyzed with an injection of pancuronium bromide (0.5 ml/kg, i.p.) and Tramal (50 mg/kg, i.m.) and kept warm using a heated nest. The pulmonary system input impedance (Zrs) was measured by applying 3 s of oscillatory volume perturbation to the tracheal cannula, which was connected to the airway opening. By fitting the constant phase model [9] to the obtained data, the airway resistance (R_{aw}), tissue damping (Gtis), tissue elastance (Htis), and hysteresivity (η) mechanical parameters were estimated. This technique was especially designed to measure the input Zrs in small animals [10] and has been described in detail previously [9, 11]. The experiments were conducted with the chest opened up and with the lungs exposed to minimize the mechanical effects of the chest wall. Thoracotomy was performed on anesthetized mice under mechanical ventilation and was completed within 15 min.

The bronchoconstriction and bronchodilation was tested. The animals sequentially received saline solution (0.9 %; vehicle), MCh (100 mg/ml; MCh, acetyl-beta-methylcholine chloride, St. Louis, USA) and salbutamol (1.5 mg/ml, Aerolin, GlaxoSmithKline, Barnard Castle, England). All compounds were aerosolized over a period of 10 s using an ultrasonic device (Aeroneb, Aerogen, Ireland). Then, for each substance, all aforementioned pulmonary parameters were recorded every 15 s over the 5 min following administration. A 10-min interval separated the administration of each solution. To standardize lung volume histories, the lungs were inflated twice to a 30-cm H_2O pressure (recruitment maneuver) before each molecule administration. For each mouse, pulmonary mechanics parameters were obtained from the highest values of the coefficient of determination (i.e., a control parameter measuring the fitness of the model). Animals were killed by rapid exsanguination via the abdominal aorta while under anesthesia.

Morphometric analyses

The right lung was fixed in 10 % buffered paraformaldehyde (0.1 M; pH 7.4) and embedded in paraffin. Four-micron-thick sections were cut and stained with hematoxylin and eosin (H&E). Lung morphology was analyzed from ten random areas in non-adjacent microscopic fields on a conventional light microscope (Nikon, Tokyo, Japan)

equipped with an integrating eyepiece with a coherent system composed of a 100-point grid consisting of 50 lines of known length. The collapsed and normal pulmonary areas volume fractions were determined using the point-counting technique [12, 13] at a magnification of 200×. Neutrophils, mononuclear (MN) cells, and lung tissue were evaluated at a 1000× magnification. In each microscopic field, points falling on neutrophils and MN cells were counted and divided by the total number of points falling on tissue area.

Collagen III and total collagen fibers (picrosirius-polarization method, collagens exhibit different interference colors and intensities of birefringence in tissue sections; blazing colors) were quantified in alveolar septa and airway walls [14]. Alveolar septa measurements were made under a 200× magnification, and airway measurements were made under a 400× magnification on a conventional light microscope with polarized light (Nikon, Japan). Elastic fibers (orcein stain method, brown) were quantified in alveolar septa and airway walls [15]. Alveolar septa and airway measurements were made under a 400× magnification using a conventional light microscope (Nikon, Tokyo, Japan). Collagen and elastic fibers were analyzed in ten non-adjacent fields using the images obtained by the Image Pro-Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance was assessed with parametric methods. Adipose tissue mass, histology parameters, and inflammatory cell data were analyzed using Student's t test. Two-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test was used to analyze pre- and post-weaning body mass and pulmonary mechanics data. For each analysis, a value of $p < 0.05$ was considered statistically significant. The statistical analyses and graphs were generated using GraphPad Prism software (version 6.0, San Diego, CA, USA).

Results

Postnatal overnutrition by increasing milk availability induced a significant elevation in the body (3–10 weeks; $p < 0.005$) and in periepididymal and retroperitoneal adipose tissue ($p < 0.05$) mass compared with the control group (Fig. 1).

All pulmonary mechanics parameters analyzed (R_{aw}, Gtis, Htis, η) after aerosolized saline administration were similar in both the control and obese group and were designated as basal values. However, after aerosolized MCh

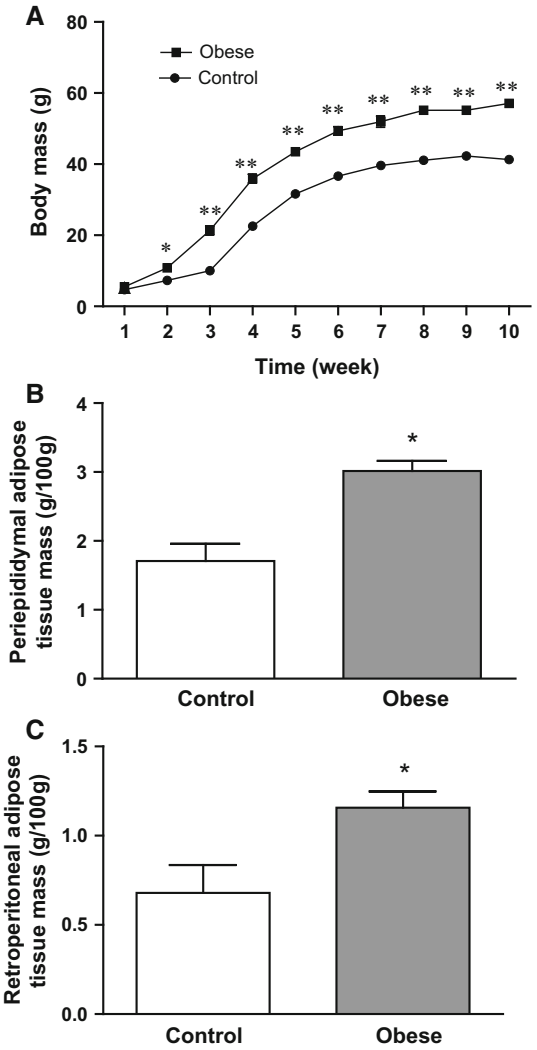

Fig. 1 Body mass (**a**), periepididymal adipose tissue mass (**b**), and retroperitoneal adipose tissue mass (**c**) of control and obese mice. The body mass was measured from the first to tenth weeks in control and obese mice ($n = 10$). *$p < 0.05$ and **$p < 0.005$, compared with the control group

administration, there was a significant increase in R_{aw} in both groups compared with the basal values. As expected, the obese group showed an accentuated bronchoconstriction response to MCh (1.23 ± 0.23 cm H_2O s/ml; $p < 0.0001$) when compared both with basal values (0.23 ± 0.02 cm H_2O s/ml) and with control group MCh values (0.68 ± 0.08 cm H_2O s/ml; $p < 0.01$) (Fig. 2a). The obese group displayed a significant difference ($p < 0.0001$) after salbutamol aerosolization (0.45 ± 0.07 cm H_2O s/ml) in comparison to MCh (1.23 ± 0.23 cm H_2O s/ml), showing their ability to recover after an induced bronchoconstriction, which was also observed in the control group. The Gtis values exhibited a similar behavior of response to aerosolized MCh as that observed for the R_{aw}, but after aerosolized salbutamol, the Gtis values in the obese group

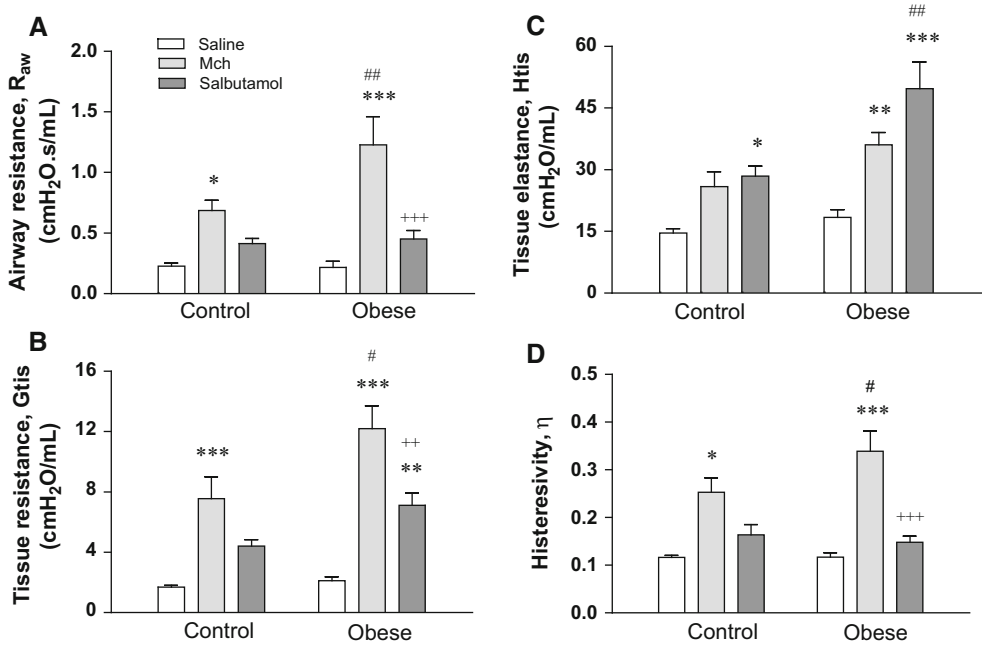

Fig. 2 Pulmonary mechanic values. **a** R_{aw}, **b** Gtis, **c** Htis, and **d** η values in control and obese mice after aerosolization with saline, methacholine (MCh) and salbutamol. Values are presented as the mean ± SEM. *$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$ for MCh or Salbutamol vs. saline. $^{+}p < 0.05$, $^{++}p < 0.001$, and $^{+++}p < 0.0001$ for the comparison between Salbutamol and MCh within the same group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$ for the comparison between the groups; control: $n = 10$ and obese: $n = 8$

(7.12 ± 0.80 cm H_2O/ml) were significantly higher than with saline (2.13 ± 0.24 cm H_2O/ml; $p < 0.001$) (Fig. 2b). Thus, for the Gtis values in the obese mice, the recovery was not reached and we did not confirm the expected bronchodilation effect of beta 2 agonists. The Htis values increased in the obese groups after MCh administration (36.10 ± 3.01 cm H_2O/ml) when compared with basal values (18.36 ± 1.91 cm H_2O/ml) (Fig. 2c; $p < 0.05$). After salbutamol aerosolization, Htis values in the control and obese groups are different (28.43 ± 2.45 and 49.75 ± 6.47 cm H_2O/ml, respectively and $p < 0.001$). The Htis values show that the salbutamol effect was not efficient enough to reverse the MCh effects in control and obese group. The η values increased significantly in both groups after administration of aerosolized MCh (0.34 ± 0.04, obese vs. 0.25 ± 0.03 cm H_2O/ml, control) in comparison to basal values (0.11 ± 0.004 cm H_2O/ml) (Fig. 2d). After salbutamol administration, the η values returned to basal in both control groups, but the obese group, values were statistically different from the MCh values ($p < 0.0001$).

Morphometric analysis of the lung parenchyma showed a significant reduction in the normal alveolar fraction and an elevated proportion of collapsed alveoli in the obese animals compared with the control group. The number of inflammatory cells (total cells, neutrophil cells and MN cells) increased significantly in the obese group when compared with the control groups (Table 1). An increased density of type III collagen fibers was detected in the airway ($p < 0.5$) and parenchymal tissue ($p < 0.001$) of the obese mice compared with the control group (Table 2 and Fig. 3). No difference in the elastic fibers fraction was observed between the studied groups.

Discussion

Our results showed that the bronchodilator salbutamol exerted a protective effect against the bronchoconstrictor MCh on airway resistance but failed to protect the tissue resistance and elastance parameters in mice rendered obese by postnatal overnutrition. Lung morphometric analyses

Table 1 Lung histology

Groups	Control	Obese
Normal area (%)	84.20 ± 2.41	45.50 ± 4.70*
Alveolar collapse (%)	15.80 ± 2.40	54.50 ± 4.81*
Total cells (%)	17.74 ± 1.10	37.80 ± 1.40***
Neutrophils (%)	2.50 ± 0.43	7.00 ± 0.52***
Mononuclear cells (MN, %)	15.70 ± 0.88	30.74 ± 1.53***

Values are mean ± SEM of six animals in each group. All values were computed in ten random, non-coincident fields per mice

* ($p < 0.05$) significantly different from control group

*** ($p < 0.0001$) significantly different from control group

Table 2 Collagen and elastic fibers content in airway and lung parenchyma

	Collagen fibers (%)		Elastic fibers (%)	
	Airway Col III	Parenchyma Col tot	Airway	Parenchyma
Control	12.70 ± 1.20	17 ± 1.87	50.48 ± 3.27	17.71 ± 2.89
Obese	17.08 ± 1.47*	30.37 ± 3.16**	50.11 ± 2.89	22.19 ± 0.09

Values are mean ± SEM of 6–7 animals in each group

Col III collagen type III, *Col tot* collagen fiber total

* ($p < 0.05$) significantly different from control group

** ($p < 0.001$) significantly different from control group

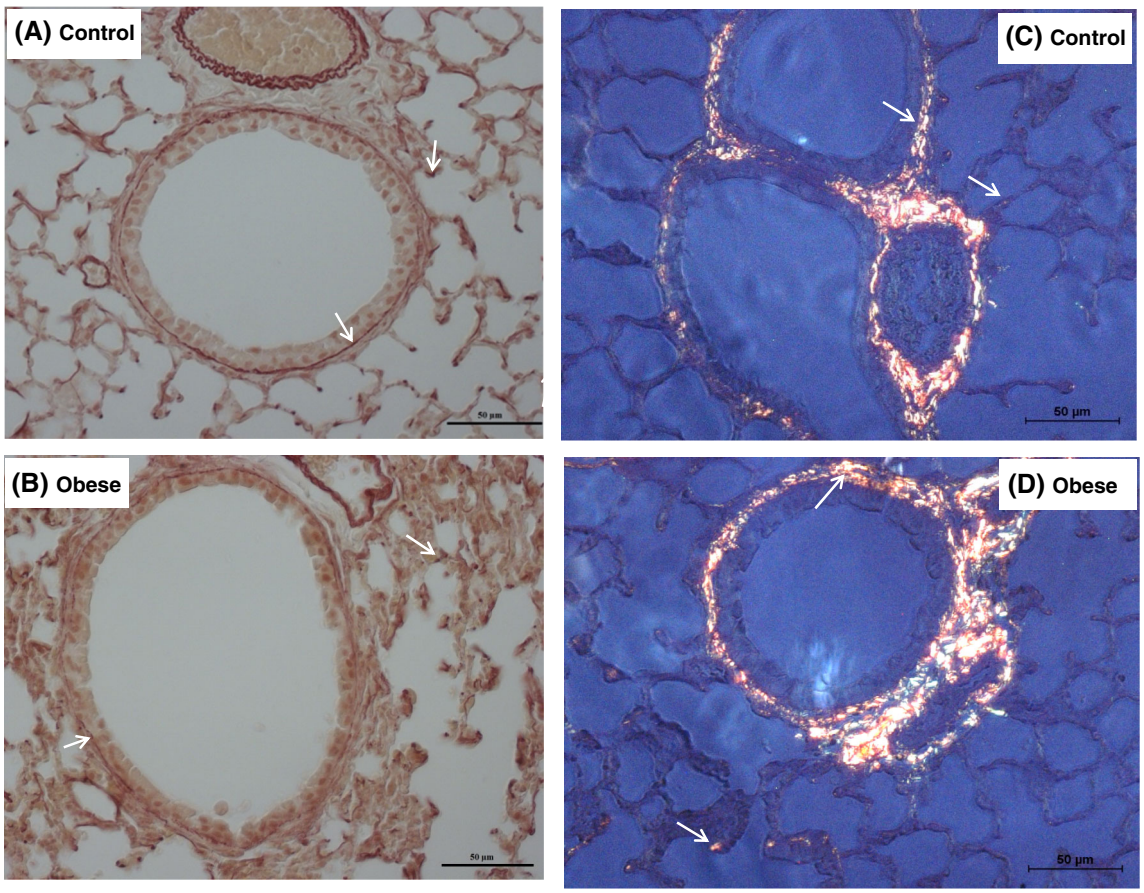

Fig. 3 Representative lung airway and parenchyma photomicrographs stained by the orcein method (elastic fibers, brown; **a** and **b**) and the picrosirius-polarization method (collagen fibers, different blazing colors; **c** and **d**). *Arrows* indicate collagen or elastic fibers in the lung parenchyma and airways. Magnification: × 200. *Scale bars* 50 µm

showed an increase in the fraction of collapsed area size, the number of inflammatory cells, and an elevation of collagen III content in the obese mice.

The present study has some connected points observed by Ye et al. [5]. These authors used the same obesity model induced by overnutrition and they reported that in a reduced litter there was an increase in body mass, which was sustained until adulthood, as described before [8, 16, 17]. In particular, the increased abdominal adipose tissue in the obese group, as seen in the present study, demonstrated that this model can efficiently induce obesity as demonstrated by Glavas et al. [18] and Liu et al. [19]. These authors have shown neonatal overnutrition-induced obesity in Swiss mice by measuring body composition and fat and lean mass percentage using quantitative magnetic resonance and dual-energy X-ray absorptiometry scan, respectively. So, this obesity model is effective and could be more representative of natural conditions, with a

potential relevance for human pathologies because it could mimic obese young people with early AHR development.

Lu et al. [20] reported that *db/db* mice (8–12 weeks of age) have innate, nonspecific AHR. Conversely, Johnston et al. [21] studied mice with diet-induced obesity (20–22 weeks of age) and observed no effect on basal airway parameters and parenchymal oscillation mechanics, including R_{aw}, Gtis, and Htis, when compared with controls. In our study, the obese mice did not express innate bronchoconstriction increase but it was evidenced after aerosolized MCh administration. Shore [2] congregated many studies and provided very promising explanations for the innate AHR observed in obese animals in addition to other points linked with asthma and inflammation in humans. However, this association has not been consistently observed [22]. In fact, the detailed mechanistic basis for AHR in obese animals remains unknown. A probable general interpretation involves mechanical factors related to the reductions in lung volume [2], considering that AHR is known to appear when the subject is breathing at low lung volume [23]. Lu et al. [20] reported a reduced lung volume at end expiration in *db/db* mice than wild-type mice. Some observations argue against the explanation proposed by Johnston et al. [24] in obese Cpe^{fat} mice, in which normal lung volumes were observed. Another explanation for innate AHR, although unclear, involves the multiple stimuli that promote airway inflammation, which would consequently induce AHR. Pro-inflammatory cytokines produced by adipose tissue macrophages can contribute to the occurrence of inappropriate physiological control in the body. In the lungs, many of these adipose tissue-derived molecules have the capacity to promote AHR and increase the occurrence of asthma in obese subjects [2]. The adipose tissue secretes many biologically active adipokines with diverse functions. Some of these molecules are hormones, such as leptin, adiponectin, resistin, and ghrelin, which play a role in the regulation of glucose metabolism and are involved in the development of obesity, diabetes, inflammation, autoimmunity, and metabolic syndromes [25]. Recent studies emphasized that adipocytokines, such as interleukin-6 and 8 (IL-6 and 8), tumor necrosis factor α (TNF-α), plasminogen activator inhibitor-1 or chemokine and monocyte chemoattractant protein-1 (MCP-1) [25], are expressed in the adipose tissue [2]. In our study, the inflammatory cells (total cells, neutrophil cells, and MN cells) were increased in the obese mice, although they did not exhibit innate increase of airway resistance. However, Ye et al. [5] investigated the short- and long-term effects of obesity induced by the same model used in the present study and demonstrated that total inflammatory cells in the lung was evident in long-term (150 day of life). The mice age, in days of life, investigated in the present study was 70 days.

The usual bronchoconstriction agonist MCh has been widely used in airway narrowing and AHR diagnosis [26]. MCh induces muscle contraction by stimulating the muscarinic cholinergic receptors that are found in the airways and the lung parenchyma [26–28]. The airway smooth muscle relaxation can be induced by beta2-agonist drugs, which are frequently defined as effective bronchodilators in clinical practice [29]. In our study, we assessed the recovery response to inhaled salbutamol (beta2-agonist) and observed an impairment in terms of the Htis and Gtis parameters in the obese group, indicating an uncoordinated pulmonary ability to tissue relaxation. Clinical studies reported a worsening of asthma and chronic obstructive pulmonary disease with the chronic use of beta2-agonists in conventional doses [30, 31]. The reduced protective effect against MCh relates to smooth muscle beta2-receptors [30], which may be associated with subsensibility to salbutamol [32]. Also, the failure of the salbutamol dose to revert the high degree of receptor occupancy induced [32]. The consequence of bronchoconstrictive response to inhaled MCh is pronounced, and it is associated with dose-dependent increases in functional residual capacity [33].

The Gtis/Htis ratio, which is referred to as η, increased in obese mice after bronchoconstriction. This was related to a modification of the collagen–elastin fiber network implying a loss of accumulated energy due to altered lung morphology, which caused heterogeneity [34]. The heterogeneity of the lung parenchyma is also demonstrated by the elevated fraction of collapsed alveoli in the obese mice. Remarkably, our pulmonary mechanics analyses were performed on an experimental mouse model of obesity that is induced by postnatal overnutrition, which means that our model takes into consideration the possible interactions between airway smooth muscle contraction, recovery response, inflammation, and morphological remodeling of the lung tissue.

Although we did not measure the levels of pro-inflammatory cytokines, it is possible to suppose that the increased tissue cellularity could be associated with the elevated ECM inflammation and collagen deposition observed only after bronchoconstriction and bronchodilation, with evident lung tissue damage demonstrated by the pulmonary mechanics parameters. Lung tissue remodeling following airway inflammation in asthma [4, 35–37] and emphysema [38] was previously reported. Specifically, in asthmatic humans, the epithelial and mesenchymal cells cause a persistent inflammatory infiltration and induce histological changes in the airway wall, characterized by an increased thickness of the basement membrane, collagen deposition, and smooth muscle hypertrophy and hyperplasia [4, 37]. Subepithelial collagens cause a thickening of and increase the density of the basement membrane [37]. We observed an elevation in the collagen fiber content in

the airway and parenchymal tissue in the obese mice, which was also observed by Saraiva et al. [36]. They demonstrated an increased collagen deposition, smooth muscle actin content, and ultrastructural degeneration of airways in obese mice with asthma. Recently, Ye et al. [5] studied the relation of the obesity induced by neonatal overfeeding and evidenced the relation between obesity, inflammation, and remodeling. However, this relation needs to be deepened to really understand the interactions between inflammation and the remodeling process in obesity. Eosinophils have a role in stimulating airway matrix remodeling in asthmatic subjects, directly or indirectly, by producing mediators and cytokines [6]. Without considering any peculiarities of specific inflammatory cell types, the presence of inflammation has been directly related to the degree of alveolar destruction and disease severity in humans with emphysema. It is known that the elastic load provided by the lung parenchyma is transmitted to the airways through the alveolar attachments, resulting in a mechanical interdependence between airways and the parenchyma [38]. Collagen deposits can alter the lungs' mechanical properties. This interconnectivity needs to be explored further, especially because in our differential study, this phenomenon has only been highlighted in obese Swiss mice generated by postnatal overnutrition.

In conclusion, the obese mice induced by maternal overnutrition exhibit an accentuated response to aerosolized bronchoconstrictor MCh registered by R_{aw}, tissue resistance, and hysteresivity. The responses to the aerosolized bronchodilator salbutamol against the MCh effect in the obese mice are imparied in regarding tissue mechanics parameters. The differential response to bronchodilators is reported as beta2 receptor downregulation or receptor occupancy and can be associated with morphological changes (increase in the collapsed alveoli area, inflammatory cells number, and collagen fiber fraction), as seen in the present study. Our data support the evidence that lung architecture is no longer uniform and lung function is no longer coordinated in obese mice.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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