



Ouabain modulates airway remodeling caused by Th2-high asthma in mice

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ABSTRACT

Objective: Ouabain, an inhibitor of Na⁺/K⁺-ATPase, is a type of endogenous hormone synthesized in the adrenal cortex and hypothalamus. Previous studies found that ouabain potentially inhibited acute inflammatory reactions such as type 2 inflammation and regulated immunological processes. In this study, we aimed to investigate ouabain effect on allergic asthma. **Methods:** BALB/c mice were submitted to chronic airway allergic inflammation induced by an ovalbumin (OVA) protocol. The animals were treated with ouabain or standard drug, budesonide. The following parameters were evaluated: cell migration, cytokine profile, IgE levels, lung histological modifications and MAPK activation. **Results:** At first, it was observed that ouabain reduced OVA-induced cell migration into the lung, observed by bronchoalveolar lavage fluid (BALF) cell counting and lung histological analysis (HE stain). Additionally, ouabain negatively modulated alarmins (IL-33 and TSLP), Th2 high cytokines levels (IL-1 β and IL-4) and tissue remodeling markers such as TNF- α and TGF- β . Treatment with ouabain also reduced OVA-specific IgE titers in BALF and serum, respectively, when compared to the OVA group. Lung histological parameters, including collagen deposition and mucus production induced by OVA were also attenuated by ouabain treatment. Finally, our results showed that p38 mitogen-activated protein kinase (MAPK) signaling pathways were suppressed by ouabain in this model. All these parameters were reduced by budesonide, a steroidal anti-inflammatory standard drug. **Conclusion:** These data together suggest that, in addition to its acute anti-inflammatory action, ouabain is also able to modulate allergic asthma.

1. Introduction

Ouabain is a steroid hormone released mainly by the adrenal gland and hypothalamus, which modulates several points of the immune system [1], including the inflammatory process. Our group has demonstrated that ouabain modulates several inflammation aspects prompted by different stimuli [2]. De Vasconcelos et al. demonstrated ouabain inhibitory effects in paw edema induced by carrageenan, compound 48/80, zymosan, prostaglandin E2, and bradykinin [3]. Additionally, ouabain was able to decrease zymosan-induced inflammation, reducing capillary permeability, cytokine release (i.e., TNF- α and IL-1 β), and neutrophil migration. These effects could be related to the reduction of NF- κ B translocation to the nucleus [4]. Ouabain also decreases cell migration and cytokine release in *Leishmania amazonensis*-triggered inflammation in mice [5]. Little is known about the mechanisms involved in cell migration inhibition induced by ouabain but could be

related to the reduction of integrin CD18 [6].

The anti-inflammatory effect of ouabain was also described in lung inflammation models. Ouabain regulates acute lung injury induced by LPS, likely through inhibition of the NF- κ B and MAPK signaling pathways [7]. In addition, our group demonstrated that ouabain reduced the recruitment of inflammatory cells in the lungs of mice sensitized by ovalbumin (OVA) and some other markers of acute allergic inflammation, namely IL-4 and IL-13 concentrations and OVA-specific IgE serum titer [8].

Airway inflammation is one of the major pathological outcomes triggered by heterogeneous conductive airway allergic disorders such as asthma and rhinitis [9]. The inflammatory responses in these cases are unusual and arise from harmful interplay of genetic susceptibility and environmental influences [10,11]. During the allergic pathophysiological process, IgE-dependent mechanisms promote mast cell activation and release of mediators, resulting in bronchoconstriction, exudation,

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edema of the airways and recruitment of inflammatory cells from the circulation [12]. A complete resolution of acute inflammatory events is the normal consequence of allergic inflammation. However, many airway inflammatory conditions are chronic and result from an exaggerated inflammatory response that leads to a reparatory process known as fibrosis [13,14].

Current treatments for asthma are not effective in controlling most airway remodeling and fibrosis characteristics [15]. The main characteristics of airway remodeling induced by this disease are epithelial damage, smooth muscle cell hyperplasia, glandular hyperplasia, and airway wall fibrosis, including a deposition of a thin layer of collagen in the reticular lamina of the airway epithelium [16]. These pathophysiological changes do not allow all patients to obtain control of the disease from a clinical point of view [17]. For these reasons, new therapeutic targets in asthma are urgently needed to allow control of acute inflammation, hyperresponsiveness and airway remodeling [18].

Although previous data show a negative modulating effect of ouabain in a model of allergic pulmonary inflammation [8], chronic features of this type of inflammation, such as tissue remodeling and fibrosis, have not yet been evaluated in the presence of this substance. Therefore, the aim of this study was to evaluate the effect of ouabain in an experimental model of allergic asthma induced by OVA.

2. Material and methods

2.1. Animals

Female BALB/c mice (7 weeks old) were obtained from Thomas George animal house of the Federal University of Paraíba (UFPB). Animals were kept under standard laboratory conditions on a constant 12-h light/dark cycle with temperature of $21 \pm 1^\circ\text{C}$, and food and water were given *ad libitum*. All procedures adopted in this study were approved by the Ethics Committee on Animal Use of UFPB (Protocol: 100/2016).

2.2. Experimental groups

To evaluate the ouabain effect on chronic airway allergic inflammation induced by OVA, four groups ($n = 7$) of mice were used. They were divided into saline group (non-OVA-sensitized animals and treated with saline), OVA group (animals sensitized with OVA), budesonide (BUD) group (OVA-sensitized animals and treated with the standard drug budesonide) and ouabain (OUA) group (OVA-sensitized animals and treated with the test drug ouabain).

2.3. Allergic asthma protocol

The OVA, BUD and OUA groups were sensitized with a suspension containing 50 $\mu\text{g/mL}$ OVA grade V and 10 mg/mL aluminum hydroxide given intraperitoneally on days 0 and 14 of the protocol. From day 21, the animals were challenged with 1% OVA grade II aerosol for 30 min, three times a week over nine weeks. In addition, on days 9^t, 18^t and 24, mice were challenged for 30 min with 10% OVA grade II aerosol [19]. After the last OVA challenge (day 85), the animals were euthanized (xylazine 20 to 30 mg/kg + ketamine 200 to 300 mg/kg), and serum and the bronchoalveolar lavage fluid (BALF) samples were obtained.

2.4. Treatments with ouabain and budesonide

One hour before each challenge, the BUD group mice were treated with budesonide (0.35 mg/kg), while the OUA group mice were treated with ouabain (0.56 mg/kg), both groups intraperitoneally. The ouabain dose and administration route were chosen based on our previous work, which evaluated the ouabain effect on airway allergic inflammation [8] and other inflammation models used by our group [3,4,5,6].

2.5. Btaining serum and bronchoalveolar lavage fluid (BALF)

Twenty-four hours after the last OVA challenge (day 85), the animals were euthanized (xylazine 20 to 30 mg/kg + ketamine 200 to 300 mg/kg) and BALF was obtained by injecting 1.5 mL of cold HBSS^{+/−} into lungs of the animals. Furthermore, brachial plexus was cut and blood (0.6–1.0 mL) was collected and stored at 4°C for 24 h to facilitate clot formation and serum collection. Finally, the BALF and the serum were stored at -20°C until the enzyme-linked immunosorbent assay (ELISA).

2.6. Total and differential cell count

BALF were analyzed for total leukocyte number by light microscopy using Turk solution (0.01% crystal violet in 3% acetic acid). For differential cell count, samples were centrifuged at 350 g for 10 min (4°C). Cytospin slides from each BALF sample were stained by the Fast Panoptic method. Differential cell count was determined for 100 cells by light microscopy using an oil-immersion objective.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The BALF supernatant and serum samples stored at -20°C were used to determine IL-4, IL-1 β and TNF- α levels and OVA-specific IgE by mouse-specific sandwich ELISA, according to the manufacturer's instructions (eBioscience). Optical density was read using a microplate spectrophotometer at 450 nm (microplate reader VersaMax, tunable, BN 2529, Molecular Devices).

2.8. Lung histology and morphometric studies

Lungs were inflated by injecting 1.0 mL of 10% buffered formalin using a catheter. Lungs were then removed, fixed in the same solution, and embedded in paraffin. Four-micron-thick sections of paraffin-embedded lungs were stained with hematoxylin-eosin (HE) according to standard protocols. For collagen studies, all lung fragments were stained by the special histological stain Masson's trichrome (for extracellular matrix compounds) according to standard protocols. For studies of goblet cells, all lung fragments were subjected to special histological staining with periodic acid-Schiff (PAS) according to standard protocols. Slides were examined under light microscopy (Motic BA 410), and digital photographs were captured with a Moticam 5.0 MP camera. For morphometric analysis, twenty random images from slides of lung tissue were used. Under an Axiolab light microscope (Zeiss) with $440\times$ resolution, twenty images were relayed to an image analysis system (Kontron Elektronik image analyzer; Carl Zeiss, Germany—KS400 software).

2.9. Flow cytometry protocols

Cell analysis by flow cytometry allows the distinction of heterogeneous cell populations based on parameters such as "forward-scattered light" (FSC) and "side-scattered light" (SSC), which will distinguish cells by size and granularity, respectively. The analysis was based on obtaining a forward-scattered light (FSC) vs. side-scattered light (SSC) in which the granulocyte population was gated excluding debris. This experimental design makes it possible to distinguish cells by size and granularity, which suggests that leukocytes that were reduced in the cell migration observed in BALF are expressing p-38 [8,61]. The evaluation of the expression of the phosphorylated portion of p-p38 MAPK was performed after BALF collection. For the analysis of the FSC and SSC parameters in the flow cytometer (FACSCanto™II) using specific antibody and fluorescent labeling. The specific protocol for labeling was performed according to the instructions of the manufacturer's kit used (BIOSCIENCE, Inc. Science Center Drive, San Diego, CA-USA).

2.10. Statistical analysis

Data were expressed as mean $\pm$ SEM and analyzed by GraphPad Prism 7.0 software using one-way analysis of variance (ANOVA) followed by Tukey's test. Results were considered statistically significant when $p < 0.05$. The flow cytometry data were analyzed using FlowJo 10 software.

3. Results

3.1. Ouabain reduces cell recruitment in allergic asthma

OVA-sensitized animals showed an increased cell number in BALF when compared to non-sensitized animals (saline group), while treatment with ouabain was able to reduce this cell recruitment by 72.4% when compared to the OVA group. Similarly, budesonide treatment inhibited cell migration by 76.3% (Fig. 1a). Additionally, ouabain treatment reduced the number of eosinophils by 37.6% (Fig. 1b), lymphocytes by 29.6% (Fig. 1d), and macrophages by 69% (Fig. 1e), without modulating neutrophil migration (Fig. 1c). Similar results were obtained with budesonide.

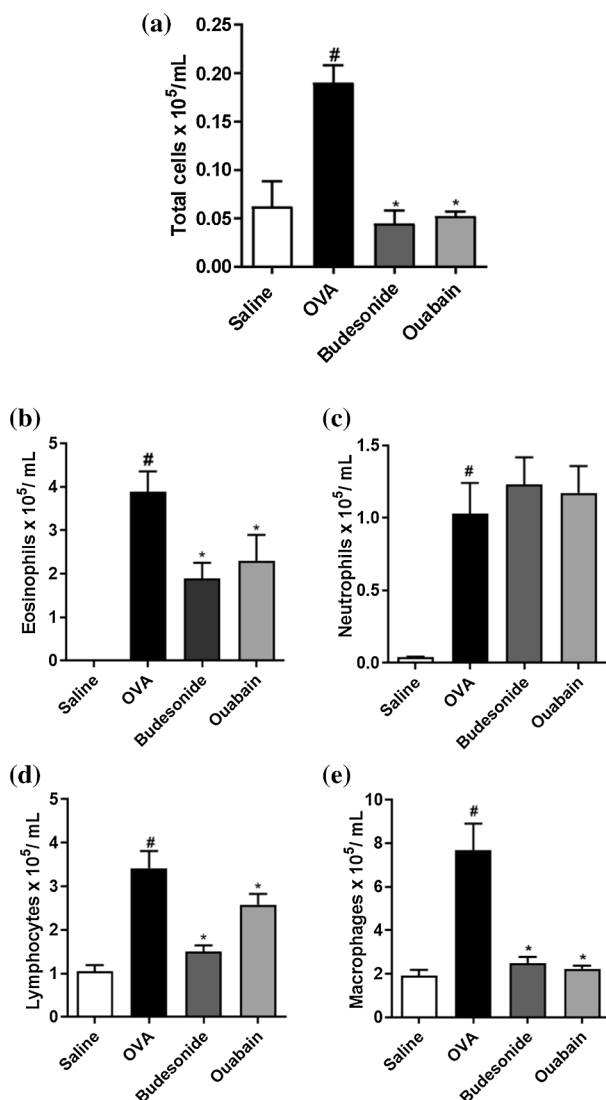


Fig. 1. Inhibition of cell migration by ouabain in the allergic asthma model. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. Data representative of four independent experiments (four groups, $n = 7$).

3.2. Ouabain inhibits cytokine production in allergic asthma

As seen in Fig. 2, OVA-induced allergic asthma triggers Th2 high (Fig. 2) cytokines production. However, when the animals were treated with ouabain, the alarmins IL-33 (Fig. 2a) and TSLP (Fig. 2b) were reduced by 38.4 and 59.3%, respectively. Additionally, IL-4 (Fig. 2c) and IL-1 β (Fig. 2b) levels were reduced significantly by 56.5 and 51.4%. The cytokines TNF- α (Fig. 2e) and TGF- β (Fig. 2f), related to chronic inflammation and tissue remodeling, was also inhibited (41.4% and 57.1%) by this steroid. As expected, the standard drug, budesonide, also lowered these cytokine levels (Fig. 2).

3.3. Ouabain decreased IgE levels induced by OVA in allergic asthma

Serum IgE levels were increased in mice challenged with OVA compared to non-sensitized mice. This increment in IgE levels was impaired when mice were treated with ouabain (49.6%) and budesonide (49.1%) (Fig. 3).

3.4. Effect of ouabain on lung tissue in allergic asthma model

As shown in Fig. 4 (A-D), the OVA group showed extensive cellular infiltration in the peribronchiolar region, while lung of animals that were treated with ouabain or budesonide displayed reduced bronchiolar cell infiltrate compared to the OVA group. Additionally, a morphometric analysis was performed to quantify the presence of inflammatory cells (Fig. 4-E). Using Masson's trichrome staining (Fig. 5), it was observed that the OVA group exhibited a large fibrotic area compared to the control group (saline), which was reduced by treatment with ouabain and budesonide. Another lung inflammatory parameter evaluated was mucus production by goblet cells. The OVA group showed (arrow) substantial mucus production, while this was reduced by ouabain and budesonide treatment (Fig. 6).

3.5. Effect of ouabain in p38 MAPK in allergic asthma model

OVA-challenge induced an increased the levels of p38 MAPK phosphorylation in cells obtained from BALF. The treatment with ouabain reduced this activation by 42.9% (Fig. 7).

4. Discussion

For a long time, ouabain's effects were attributed only to its classical mechanism of action as a cardiotonic glycoside [20], inhibiting Na⁺/K⁺-ATPase [21]. However, cardiotonic steroids such as ouabain are not only specific inhibitors of the sodium pump, they also can influence various cell signaling events in a hormone-like manner. Our group has shown over the years that ouabain treatment may trigger anti-inflammatory and anti-nociceptive effects [3,5,6]. In addition, ouabain has been shown to interfere with pulmonary inflammation induced by OVA, but this effect on tissue damage triggered by a chronic model using the same stimulus remains unknown [8]. In this work, we demonstrated that ouabain can modulate several peripheral aspects of the allergic asthma induced by ovalbumin (OVA) hypersensitivity, including cellular migration to the lungs, cytokine levels of Th2 high profiles (IL33, TSLP, IL-4, IL-1 β , TNF- α and TGF- β), immunoglobulin E levels, lung fibrosis produced during the model development and p38 MAPK activation.

Recent studies have attributed to ouabain the ability to reduce the inflammatory response in zimosan-induced peritonitis, decreasing both cell migration and vascular permeability by inhibiting activation of NF- κ B, an essential transcription factor crucial in IL-1 β and TNF- α production [4]. Furthermore, pretreatment with ouabain in a model of OVA-induced allergic inflammation is able to reduce parameters such as cell migration, Th2 cytokine profile production (i.e., IL-4 and IL-13), histopathological remodeling of pulmonary epithelial tissue, and OVA-

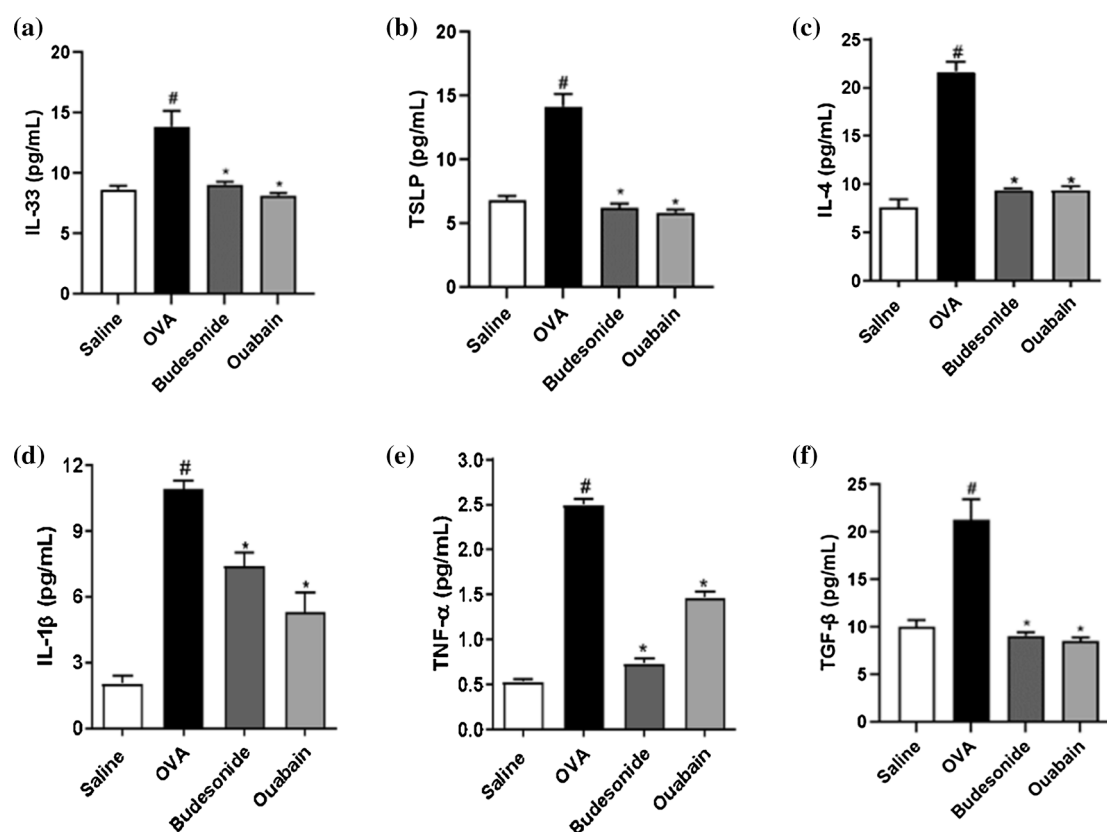


Fig. 2. Decrease in Th2-high cytokines levels by ouabain in the allergic asthma model. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. Data representative of four independent experiments (four groups, $n = 7$).

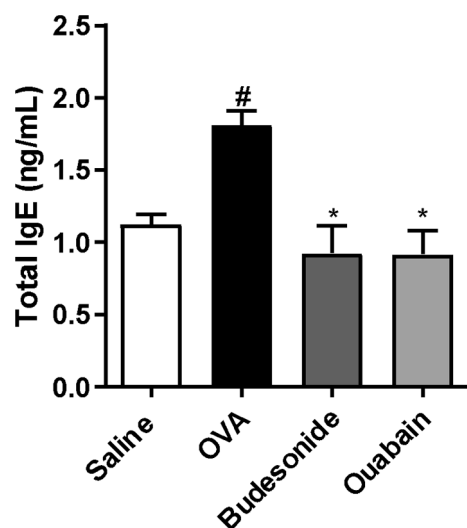


Fig. 3. Effect of ouabain on IgE levels in the allergic asthma model. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. Data representative of four independent experiments (four groups, $n = 7$).

specific IgE concentrations [8]. OVA is often used in acute and chronic allergic asthma models as a sensitizing antigen. The pathway of sensitization and the antigen choice may offer different perspectives on the pathophysiology of lower airway inflammation. For example, OVA has the advantage of being available in relatively pure forms, with low levels of endotoxin contamination, allowing the induction of a predominant Th2 phenotype response [26]. Furthermore, female mice were chosen to

run our model as they develop a more pronounced type of allergic airway inflammation than male mice after OVA challenge. This response may be associated with a reduction in the percentage of regulatory T cells in the woman's lungs. Regulatory T cells are important for the development and progression of an allergic immune response following various OVA challenges in the asthma model used in this article [57,58]. Several lines of evidence indicate that regulatory T cells and their secreted products play a central role in orchestrating the unique inflammatory response in the airways of asthmatics. Challenge with local allergens has been shown to induce an influx of CD4⁺ T cells and eosinophils into the airways of asthmatics [59]. We cannot say whether the lower number of regulatory T cells persists in female mice after OVA challenges. However, under inflammatory conditions such as allergic airway inflammation induced by OVA challenges, female mice respond with higher levels of eosinophils, CD4 T cells, B cells, cytokines and IgE when compared to male mice subjected to the same stress [60].

In the severe chronic allergic asthma model used in this study, our results showed that ouabain treatment (0.56 mg/kg) during OVA challenges reduced the number of total cells in BALF. Cell migration is a determining factor for the maintenance of inflammation [27]. The ouabain immunomodulatory actions that involve the reduction in total leukocyte migration to the inflamed sites in acute inflammatory processes are a constant in different models [6,28]. While high concentrations of ouabain inhibit ion pumping [29], non-saturating concentrations revealed that the α -subunit associates with transducing proteins and activates several intracellular signaling pathways [28,30]. In the presence of inflammatory stimuli, cell migration can be modulated by ouabain through modulation of the kinase domain of Src, which transactivates epidermal growth factor receptor (EGFR), which in turn activates the MAPK pathway [31]. In this context, our study provides evidence that ouabain is also able to modulate cellular response in chronic processes.

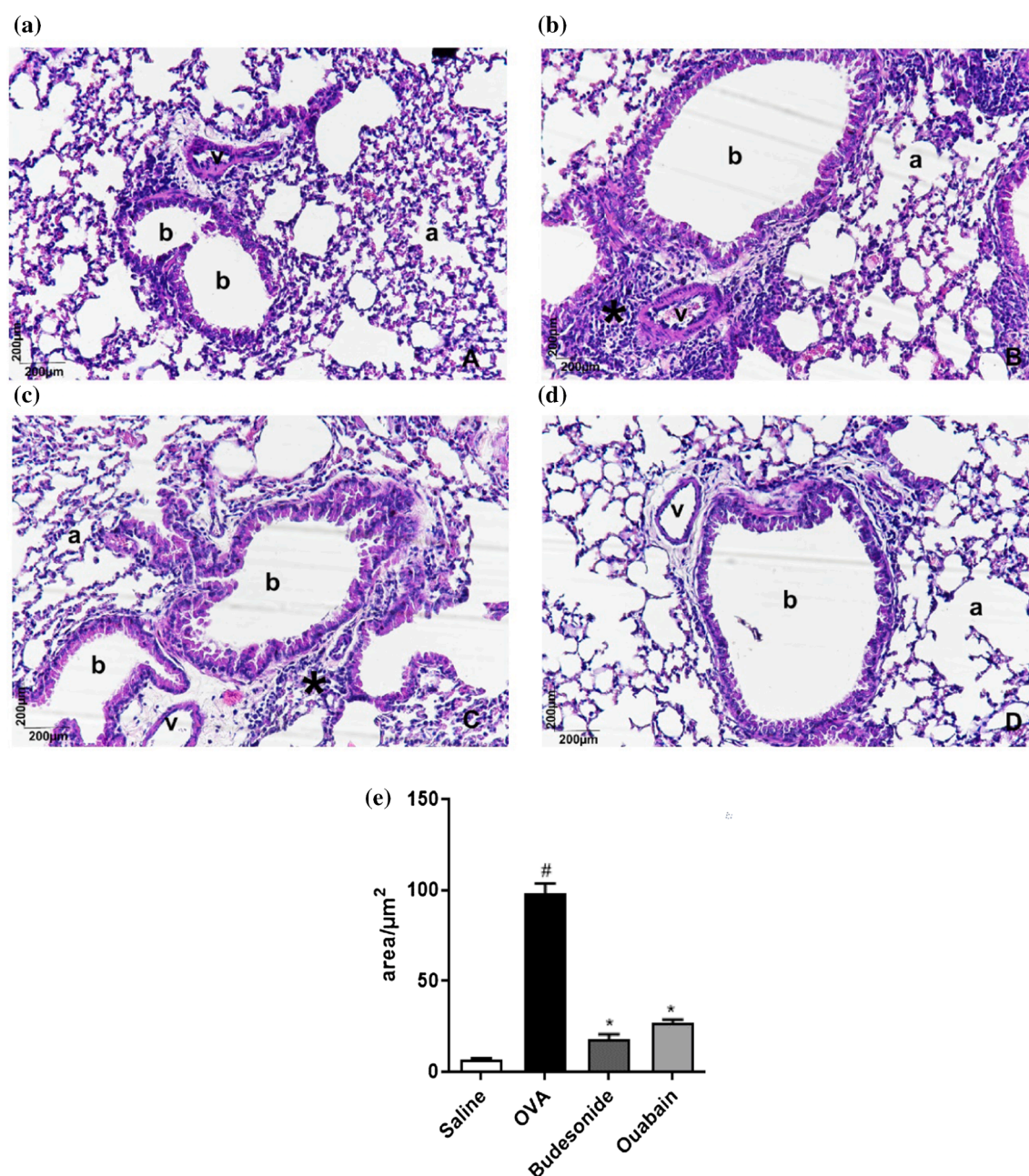


Fig. 4. Photomicrograph (A-D) of lung tissue stained with hematoxylin and eosin (HE). Sections: A-saline, B-OVA, C-ouabain, D-budesonide. *inflammatory cells. v: blood vessel. a: alveolus. b. bronchioles. E. Morphometric analysis of inflammatory lung cells stained with hematoxylin and eosin (HE). #p < 0.05 vs saline group and *p < 0.05 vs OVA group after analysis by one way-ANOVA followed by Tukey's test. Data representative of four independent experiments (four groups, n = 7).

Additionally, ouabain negatively modulates acute peritoneal inflammation in an experimental model of leishmaniasis caused by *Leishmania* (*L.*) *amazonensis*, decreasing cell migration and levels of proinflammatory cytokines, such as TNF- α and IFN- γ [5]. Ouabain is also able to modulate in vivo B cell dynamics in the peripheral blood [22], and in the mesenteric lymph node, probably due to decreased CD62L and increased CXCR5 expression [23]. By regulating signaling pathways triggered by Na⁺/K⁺-ATPase that have relevance to gene transcription, this hormone can interfere with cell differentiation and growth [24,25].

In chronic asthma, there is significant accumulation of CD4⁺ T lymphocytes in the airways [32]. This cell profile normally exhibits the cytokine Th2 profile, characterized by the production of IL-4, IL-5, and IL-13, which contribute to the recruitment of eosinophils and the development of airway hyperresponsiveness [33,34]. More recently, it

was suggested that Th17 cells, which secrete IL-17A, may also contribute to the pathogenesis of the disease [35]. Regarding the pathophysiology of chronic asthma, the Th2 phenotype (IL-4, IL-5 and IL-13) is not sufficient to explain the inflammatory response and its progression. Thus, the plasticity of cellular responses during this type of inflammation also indicates the production of cytokines from other response profiles such as Th1 (IL-1 β and TNF- α) and Th17 (IL-17) [19,36]. In this study, besides modulating IL-4 levels, a Th2 profile cytokine, ouabain also modulated IL-1 β and TNF- α levels, both Th1 profile cytokines. The ouabain inhibitory effect on IL-4 levels could be related to inhibition of mast cell activity and reduction of CD3^{high} lymphocytes [37].

Regarding the production of cytokines IL-1 β and TNF- α , the modulation of their levels may be related to the reduction of neutrophil migration in several inflammation models in vivo by ouabain [3,4,6].

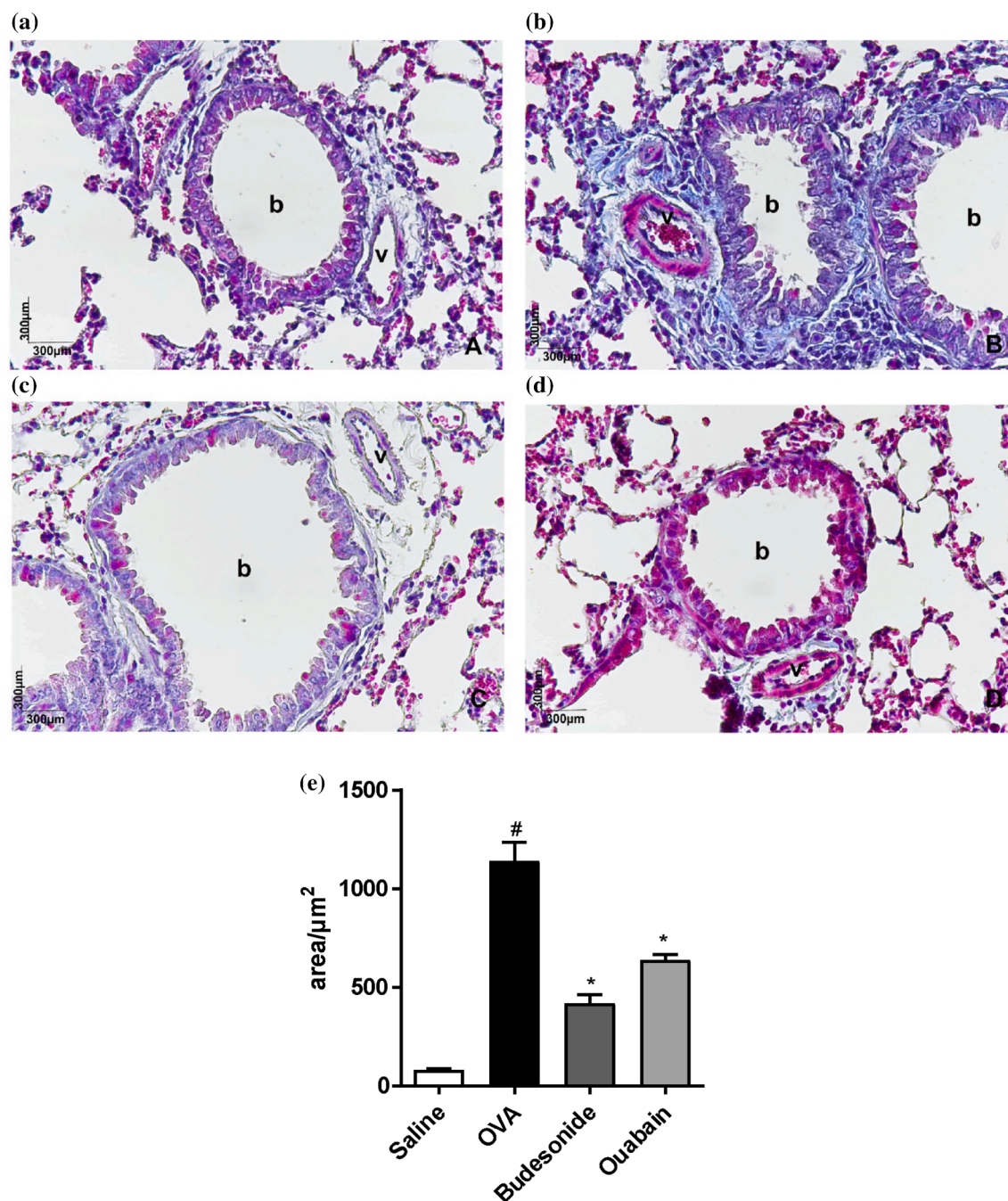


Fig. 5. Photomicrograph (A-D) and fibrosis index (E) of lung sections stained with Masson's trichrome. A-saline, B-OVA, C-ouabain, D-budesonide. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. b: bronchiole, v: blood vessel. Data representative of four independent experiments (four groups, $n = 7$).

The presence of neutrophils in substantial amounts in the mucus of asthmatic patients in the most severe and terminal phases is not yet well explained [38]. However, Nabe et al. [39] suggested that neutrophils and not eosinophils are responsible for inducing the delayed asthmatic response, serum eosinophilia is used to monitor the biochemical response to anti-IL-5 therapy. For children with asthma, peripheral eosinophilia is associated with more frequent asthma exacerbations [55]. Thus, among all asthma biomarkers, the BALF eosinophil count has the most significant data for the endotyping of this disease [56]. The reduced number of neutrophils in the asthmatic allergic model is crucial to evidence Th2 high profiles [50]. In this work, treatment with ouabain did not reduce the concentrations of this cell type.

Allergic asthma involving continuous inflammation and repair that

leads to structural airway remodeling [40]. Tissue remodeling is, by definition, the reorganization or renovation of existing tissues. This is a necessary process to maintain normal tissue homeostasis and repair damaged tissue. Remodeling occurs in all inflammatory responses, although the mechanism and severity vary depending on the disease [41]. In our model, we observed inflammatory changes in the lower airways, such as infiltration of bronchiolar cells, increased production of mucus by goblet cells and fibrosis triggered by lung fibroblast activation. These findings are characteristic of tissue remodeling caused by proliferation and differentiation of fibrotic cells that trigger collagen deposition and tissue fibrosis [42,43].

Regarding tissue remodeling, our results showed a reduction in the pulmonary fibrosis process in the groups treated with ouabain. In

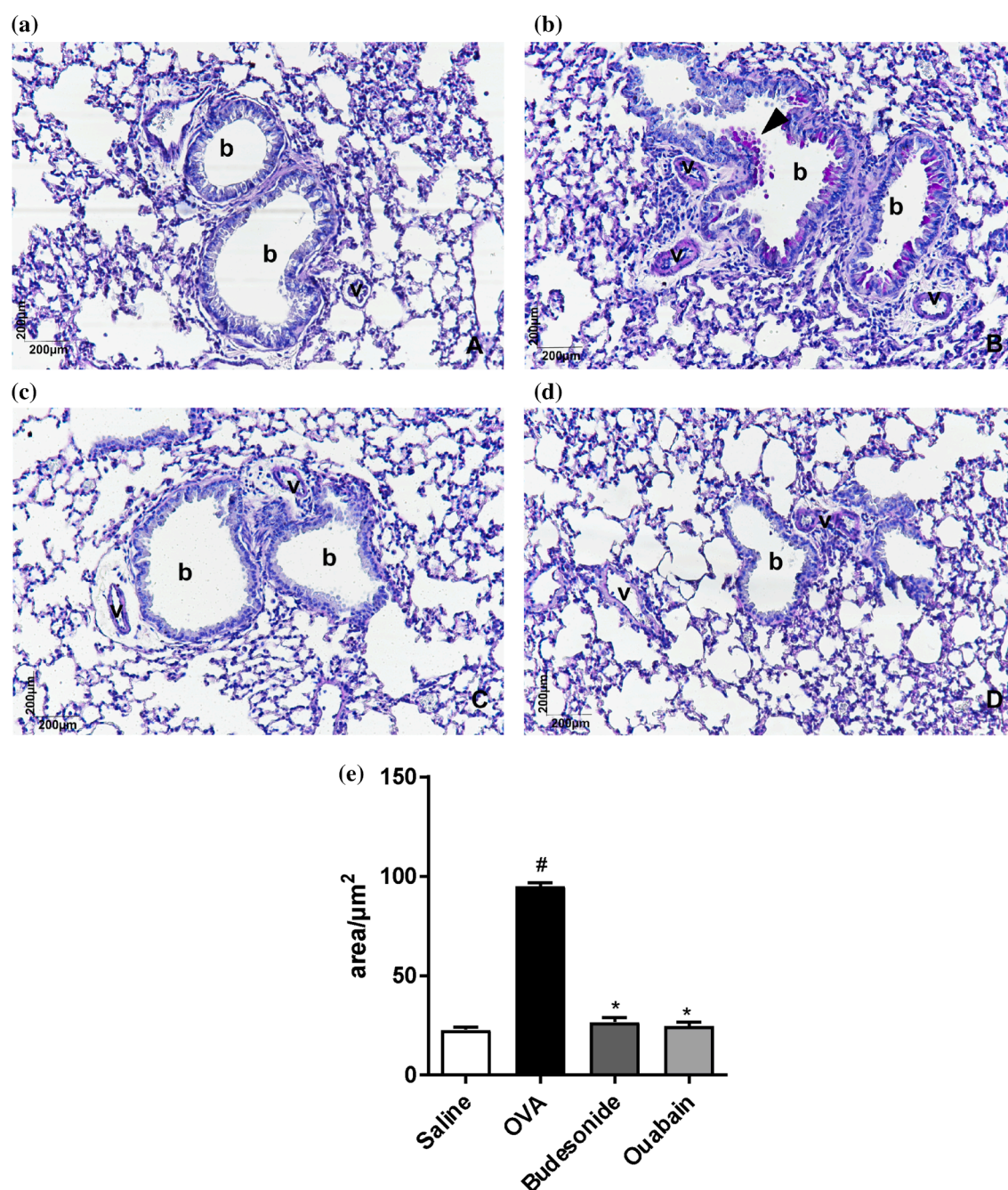


Fig. 6. Photomicrograph (A-D) and mucus index (E) of lung sections stained with PAS. A-saline, B-OVA, C-ouabain, D-budesonide. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. b: bronchiole, v: blood vessel. Data representative of four independent experiments (four groups, $n = 7$).

pulmonary inflammation, transforming growth factor- β (TGF- β) is a multi-functional cytokine implicated in the regulation of epithelial cell growth [44], epithelial-to-mesenchymal transition [45] and fibrosis [46]. Some recent research has shown that ouabain is able to inhibit in vitro the activation of pulmonary fibroblasts by blocking the TGF- β -Smad signal pathway, including downregulation of Smad2, TGF β RII transcription and Smad2 phosphorylation [47,48].

IgE has been convincingly linked to the pathophysiology of allergic asthma and other allergic conditions [49]. IgE-mediated allergic diseases involve multiple genetic and environmental components that interact to determine disease expression and lead to heterogeneous and frequently coexisting phenotypes [50]. Complex allergic diseases such as asthma and rhinitis, may be associated with allergen-specific IgE and

nonallergic mechanisms that can coexist in the same patient and share causal mechanisms [51]. In the present study, we demonstrated that ouabain significantly decreased serum IgE levels compared to the levels seen in the OVA group. These results can be related to the modulation of the maturation of B cells by ouabain [22].

MAPKs represent an important point of convergence for multiple signaling pathways that are activated during inflammation and various immunological processes. Therefore, these enzymes can impact a variety of cellular events relevant to the pathophysiology of asthma, including Th2 cell differentiation, inflammatory cell infiltration, epithelial and endothelial functions, as well as myofibroblast hyperplasia and hyper-reactivity. In fact, MAPK activation can be detected both in inflammatory cells and in bronchial epithelial cells [52].

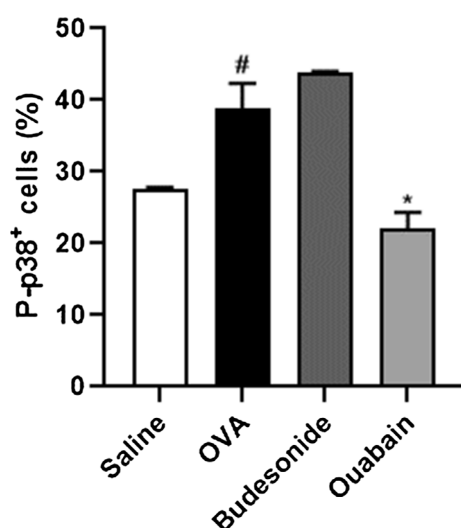


Fig. 7. Inhibition of p38 MAPK by ouabain in cells from BALF. [#] $p < 0.05$ vs saline group and ^{*} $p < 0.05$ vs OVA group after analysis by one way-ANOVA followed by Tukey's test. Data represent frequency of p38-positive cells (PE-Cy7). Data representative of four independent experiments (four groups, $n = 7$).

The secretory activity of Th2 lymphocytes is affected by p38, which by phosphorylation of the transcription factor GATA-3 promotes the synthesis of IL-4, IL-5 and IL-13. p38 is also capable of inhibiting eosinophil apoptosis and seems to be involved in the differentiation of these cells and the release of their cytokines, as well as in antigen-induced eosinopoiesis [53]. Inhibition of this MAPK may prevent allergen-induced pulmonary eosinophilia, mucus hypersecretion, and airway hyperresponsiveness, effects resulting in part from p38 activation in eosinophil apoptosis and cytokine production in airway smooth muscle cells. Our results showed that p38 expression as a pro-inflammatory feature of asthmatic lung activity was increased in animals challenged with ovalbumin. Treatment with ouabain was able to reduce the expression of this MAPK, corroborating previous data from our group, where the use of small doses of the substance inhibited p38 in different cell populations [28,54].

In conclusion, all the pathophysiological changes modulated by ouabain in this model of allergic asthma suggest that in addition to its actions in different acute inflammatory models, ouabain also acts on chronic phenotypes, suggesting a new mode of action for this substance.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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