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ORIGINAL RESEARCH ARTICLE

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Cholinergic anti-inflammatory pathway confers airway protection against oxidative damage and attenuates inflammation in an allergic asthma model

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Abstract

Asthma is characterized by the influx of inflammatory cells, especially of eosinophils as well as reactive oxygen species (ROS) production, driven by the release of the T helper 2 (Th2)-cell-associated cytokines. The cholinergic anti-inflammatory pathway (CAP) inhibit cytokines production and controls inflammation. Thus, we investigated the effects of pharmacological activation of CAP by neostigmine on oxidative stress and airway inflammation in an allergic asthma model. After the OVA challenge, mice were treated with neostigmine. We showed that CAP activation by neostigmine reduced the levels of pro-inflammatory cytokines (IL-4, IL-5, IL-13, IL-1β, and TNF-α), which resulted in a decrease of eosinophils influx. Furthermore, neostigmine also conferred airway protection against oxidative stress, attenuating ROS production through the increase of antioxidant defense, evidenced by the catalase (CAT) activity. We propose, for the first time, that pharmacological activation of the CAP can lead to new possibilities in the therapeutic management of allergic asthma.

KEYWORDS

airway inflammation, asthma, cholinergic anti-inflammatory pathway, neostigmine, oxidative stress

1 | INTRODUCTION

Allergic asthma is featured by bronchial hyperreactivity (BHR), airway remodeling, and narrowing that results in wheezing, coughing, and chest tightness. These clinical symptoms are a consequence of a chronic airway inflammatory, orchestrated by T helper 2 (Th2) cells (Deckers, De Bosscher, Lambrecht, & Hammad, 2017). Th2 lymphocytes produce cytokines, such as interleukins 4, 5, and 13 (IL-4, IL-5, and IL-13), which induces to produce immunoglobulin E (IgE), mucus overproduction, and infiltration of neutrophils, macrophages, lymphocytes, and especially eosinophils in the airway (Haspeslagh et al., 2018). Besides, in response to environmental influences, other cells profiles, such as Th1 and Th17, can secrete pro-inflammatory cytokines that contribute to the inflammation of airway (Manni et al., 2014; McKinley et al., 2008).

During asthma exacerbation, eosinophils secrete cytotoxic mediators, such as eosinophil peroxidase (EPO) that lead to reactive oxygen species (ROS) production (Dworski, 2000). The excessive formation of ROS is neutralized by the action of antioxidant defenses (Pedrazza et al., 2017). Nevertheless, allergic asthma promotes an imbalance between ROS production and the antioxidant system capability, inducing oxidative stress in the airway (da Cunha et al., 2016). Oxidative stress promote airway damage by mast cell degranulation, airway remodeling, and mucus secretion, which directly contributes to

the extent of tissue inflammation and lead to pulmonary function impairment (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Nesi et al., 2017; Zhang, Deng, Zhang, Zhang, & Bai, 2018).

The inflammation is essential for maintaining homeostasis. However, the exaggerated immune response usually results in several pathologies (Gwilt, Donnelly, & Rogers, 2007). Thus, mechanisms able to regulate the host inflammation are necessary. In this context, Borovikova et al. (2000) described a neuroimmune mechanism termed the cholinergic anti-inflammatory (CAP) pathway that inhibits cytokines production through vagus nerve stimulation and a consequent release of acetylcholine (ACh). This neuroimmune mechanism requires the interaction of ACh with the α7 subunit of the nicotinic ACh receptor (nAChR) expressed in cells, such as eosinophils (McGovern & Mazzone, 2014).

Recently, Kanashiro et al. (2016) showed that treatment with neostigmine, acetylcholinesterase (AChE) inhibitor that reinforces the cholinergic anti-inflammatory response through ACh increasing, improved the clinical symptoms of arthritis in an experimental model (Kanashiro et al., 2016). In this present study, the effects of pharmacological activation of the cholinergic anti-inflammatory pathway by neostigmine on oxidative stress and inflammation in asthmatic mice were explored.

2 | METHODS

2.1 | Animals

This study was performed with female adult BALB/cJ mice (6–8 weeks old) acquired from the Center for Experimental Biological Models (CeMBE/PUCRS). The female in this present study received a balanced chow diet and water ad libitum, were housed in cages ventilated with the temperature maintained at 21 ± 1 °C and an illumination schedule 12 hr/12 hr light–dark. All procedures with BALB/c mice were conducted according to "US National Research Council's Guide for the Care and Use of Laboratory Animals" and all study was approved by Animal Ethics Committee (7934) from the PUCRS.

2.2 | Sensitization, airway challenge, and neostigmine treatment

For induction of the allergic asthma model, on Days 0 and 7 from the experimental protocol, the BALB/cJ mice were sensitized without adjuvant by subcutaneous injection with 20 µg of ovalbumin (OVA) (Grade V, Sigma-Aldrich, St. Louis) diluted in 200 µl of Dulbecco's phosphate-buffered saline (DPBS). On days 14, 15, and 16 of the protocol, the mice were challenged via intranasal by 100 µg OVA that was diluted in 50 µl of DPBS (Silveira et al., 2019). To evaluate the pharmacological activation of the cholinergic anti-inflammatory pathway the mice received 80 µg/kg of neostigmine (Normastig, União Química, São Paulo, Brazil) intraperitoneally (Hofer et al., 2008) once a day during three consecutive days (14, 15, and 16) 30 min after of OVA challenge. The control group received only DPBS. The induction model and treatment with neostigmine are illustrated in Figure 1.

2.3 | Assessment of respiratory mechanics

Female mice were anesthetized with an injection containing ketamine (0.4 mg/g) and xylazine (0.2 mg/g). The motor nerve impulses were blocked with pancuronium bromide (1 mg/kg) intraperitoneally. Next, female mice were tracheostomized by a steel cannula and maintained in a mechanic ventilator (Flexi Vent, SCIREQ, Montreal, Canada). The technique of forced oscillation was adapted and three measurements were generated through the pause (3 s) of the respirator. A Three-parameter model was fitted data to acquire measures Newtonian resistance (Rn) tissue damping (G), and tissue elastance (H) that were evaluated in a specific software (FlexiWare, SCIREQ, Montreal, Canada) (Mori et al., 2017).

2.4 | Bronchoalveolar lavage

After the assessment of respiratory mechanics was injected two consecutive flushes in the lung with 1 ml of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) through the previously inserted tracheal cannula.

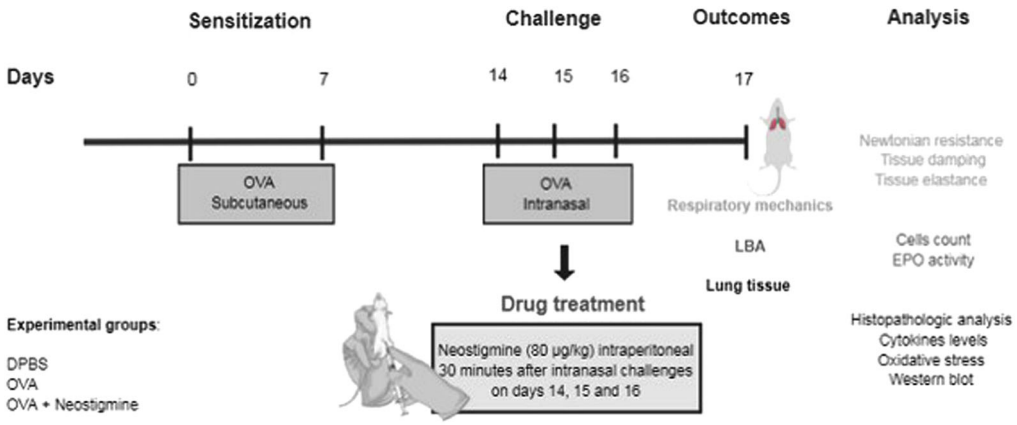

FIGURE 1 Protocol used to induce an allergic asthma model and treatment with neostigmine. DPBS, Dulbecco's phosphate-buffered saline; OVA, ovalbumin

2.5 | Cells count from mice bronchoalveolar lavage

Female mice bronchoalveolar lavage (BAL) was centrifuged at 420*g*, for 5 min, at 4°C and the pellet was resuspended in PBS (350 μl) containing 2% FBS. Total cells count (TCC) were determined in a hemocytometer (BOECO, Hamburg, Germany) through the test of Trypan blue exclusion. Slides for the differential cytology were prepared using a cytospin in a cytocentrifuge (Eppendorf, Wesseling, Germany) and stained with hematoxylin and eosin (H&E; Panótico Rápido - Laborclin, Brazil). Four hundred cells were counted through light microscopy BMX 43 (Olympus, Tokyo, Japan).

2.6 | EPO activity enzyme in BAL

We measured the EPO activity in BAL supernatant by the colorimetric assay (Strath, Warren, & Sanderson, 1985). The sample was incubated for 1 hr in working reagent (0.1 mM O-phenylenediamine (OPD), 0.05 M Tris pH 8.0, Triton X-100, and 1 mM hydrogen peroxide). We stop the reaction by sulfuric acid 1.0 M and the absorbance was evaluated at a wavelength of 492 nm.

2.7 | Lung histopathologic analysis

Lung tissues of female mice were perfused with buffered formalin (10%) on a gravity column (20 mmHg), removed and embedded in blocks of paraffin. The tissues from female mice were cut into 5 μm sections and stained with H&E (Cytological Products Soldan, Brazil). Images of the sections lung tissue were captured through BMX 43 microscope equipped with a digital camera DP73 (Olympus). To evaluate peribronchial and perivascular infiltrate were performed ten measurements in each region using imaging software (CellSens Standard Olympus). For analysis, the mean of at least five areas was assessed for each female mice. In order to identify mucus-secreting goblet, sections were stained with Alcian Blue (InLab, Brazil) and were evaluated on a subjective scale following degrees of attribution: 0 value: when no mucus-secreting goblet cell was evident, 1: when occasional mucus-secreting goblet cell detectable, the 2 value when most bronchial was surrounded by mucus-secreting goblet cell, and the 3 value was set when all bronchial was surrounded by mucus-secreting goblet cell.

2.8 | Measurement of lung cytokines

The lungs were collected from BALB/c mice and prepared in a solution of PBS. A Multiplex Assay kit (MILLIPLEX®, Millipore, Germany) was utilized to simultaneously measure interleukin 1 beta (IL-1β), tumor necrosis factor α (TNF-α), and IL-13. Similarly, was used ProcartaPlex (Thermo Fisher Scientific, Waltham, USA) for IL-5, IL-4, and IL-10 measure. All cytokines were measured by MagPix (MILLIPLEX®) and the results were analyzed through the software xPONENT® 4.2 (MILLIPLEX®). Cytokines in lung homogenate were expressed in pg of cytokines/mg of total protein.

2.9 | Oxidative stress in lung tissue

The collected lungs were homogenized (1:10, w/v) in a solution contained 20 mM sodium phosphate buffer and 140 mM potassium chloride (KCl). The homogenates of lung tissue were centrifuged at 750 g during 10 min, at 4°C. The lung tissue supernatant from BALB/c mice was used for the analysis of ROS production and antioxidant enzyme activities (SOD, CAT, and GPx).

2.10 | ROS activity

ROS quantification was on the basis of the oxidation of 2′7′-dichlorofluorescein (H2DCF), according to the method of LeBel et al. (1992). The supernatant of lung tissue was incubated in 100 μM of 2′7′-dichlorofluorescein diacetate (H2DCF-DA) solution. This reaction (dichlorofluorescein-DCF) was measured at λ_{em} = 488 nm and λ_{ex} = 525 nm. The results were plotted as nmol DCF/mg protein.

2.11 | Superoxide dismutase (SOD) activity

The SOD activity is attributed to the ability of pyrogallol to self-oxidizing, a process highly dependent on superoxide (substrate for SOD). In presence of SOD occurs the autoxidation inhibition. Thus, the supernatant of lung tissue was mixed in a solution containing 1 mM EDTA, 50 mM Tris, 80 U/ml catalase (CAT), and 0.8 mM pyrogallol and whose activity was indirectly measured at 420 nm (Greenwald, 2018). The results were plotted as SOD units/mg protein.

2.12 | CAT activity

The method used for measurement of the CAT activity is on the basis of the decrease in consumption of hydrogen peroxide at 240 nm (Aebi, 1984). Thus, the lung tissue supernatant from BALB/c mice was incubated in 20 mM hydrogen peroxide (H_2O_2), 0.1% of Triton X-100, and 10 mM potassium phosphate, pH 7.4. The unit of CAT was established as 1 μmol of H2O2 consumed each minute and the results were plotted as CAT units/mg protein.

2.13 | Glutathione peroxidase (GPx) activity

For the Glutathione peroxidase (GPx) analysis in lung tissue from female mice, we used the method according to (Wendel, 1981) that use tert-butylhydroperoxide as a substrate. The supernatant of lungs tissue from mice was incubated in a solution (0.15 U/ml glutathione reductase, 2 mM glutathione, 0.5 mM tert-butylhydroperoxide, 0.4 mM azide, and 0.1 mM nicotinic adenine dinucleotide phosphate [NADPH]). NADPH disappearance was monitored in a spectrophotometer at 340 nm. The unit of GPx was established as 1 μmol of NADPH consumed each minute. The specific activity was plotted as GPx units/mg protein.

2.14 | Immunofluorescence in lung tissue

Lung tissue slides from mice were deparaffinized and sequentially treated with 10 mM sodium citrate ($Na_3C_6H_5O_7$) for 10 min and 0.3% H2O2 in methanol (CH_3OH) for 20 min, then rinsed carefully with PBS. Sections were blocked for 30 min with 10% bovine serum albumin (BSA) in PBS. Afterward, they were incubated with anti-AChE and anti-NFκB p65 (1:500; Thermo Fisher Scientific) antibodies for 40 min, followed by incubation with secondary antibody anti-rabbit FITC (1:500) for 40 min. The cellular nuclei were stained using Hoechst (1:2,000) and images of the sections were captured by Zeiss LSM 5 Exciter microscope (Oberkohen, Germany).

2.15 | Western blot analysis in lung tissue

The lung tissue from BALB/c mice was homogenized in a solution containing CHAPS (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 1 mM EDTA pH 8.0, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 10% glycerol, and 0.5% CHAPS) and centrifuged at 17.900*g*, 4°C, during 60 min. Equal amounts of lung proteins from female mice were separated with electrophoresis (SDS-PAGE) and transfer to a nitrocellulose membrane. Afterward, for transfer verification, membranes were stained using Ponceau S solution (Sigma-Aldrich). Then, the blot was washed with Tris-HCl, pH 7.4, NaCl, and 0.05% Tween (TTBS), followed by 30 min incubation in blocking solution TTBS containing 5% BSA. After, the blot was washed again with TTBS and incubated overnight at 4°C in blocking solution containing the following primary antibodies: anti-AchE (1:200), anti-α7 nAChR (1:250) (Thermo Fisher Scientific), anti-Akt (1:500), anti-pAkt (serine-473; 1:500), and anti-β-actin (1:2,000) (Cell Signaling, Danvers). After overnight incubation, the blot was then washed and incubated again for 2 hr with horseradish peroxidase-conjugated (HRP-conjugated) anti-IgG (1:2,000; Santa Cruz Biotechnology, Santa Cruz) secondary antibody. The chemiluminescence was detected by a gel documentation system (Fujifilm, LAS-3000). Band intensities were quantified through the ImageJ software.

2.16 | Protein determination

The proteins concentration in lung tissue of BALB/cJ mice was determined in accordance with Bradford, using BSA as standard (Bradford, 1976).

2.17 | Statistical analysis

The data are presented as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey post hoc test. Differences were considered significant at $^*p < .05$. Results were analyzed using Statistical Package for the Social Sciences, version 20.0 (SPSS Inc., Chicago, IL) and graphs were made using Prism GraphPad (version 5.0, GraphPad Software Inc., San Diego, California).

3 | RESULTS

3.1 | Neostigmine treatment decreases inflammatory cells from BAL in an allergic asthma model

In the first instance, we visualized that the OVA group had a significant increase in the TCC when compared with the control group. This influx of inflammatory cells found in the OVA group was reduced in the group treated with neostigmine (Figure 2a). Also, we showed a significant increased in the count of macrophages, neutrophils, lymphocytes, and eosinophils in the OVA group as compared with the control group. However, neostigmine-treated group decreased the number of these leukocytes in BAL from mice when compared with the OVA group (Figure 2b–f). Eosinophilia in the airway is one hallmark features of allergic asthma and can potentially contribute to allergic inflammation in a number of ways by the release of various mediators. In the EPO granular protein analysis, we demonstrated that OVA significantly increased the EPO activity when compared with the control group. On the other hand, we showed that the treatment with neostigmine was able to reduce the EPO activity in BAL when compared with the OVA group (Figure 2g).

3.2 | Effects of neostigmine treatment in the inflammatory infiltrate and goblet cells hyperplasia on lung tissue in an allergic asthma model

We decided to investigate whether neostigmine treatment can reduce inflammatory infiltrate in the lung tissue. First, lung sections from BALB/c mice were stained with H&E and we observed that only induction with OVA was able to induce airway inflammation. In mice that receiving neostigmine, both peribronchial and perivascular inflammatory infiltrates were reduced (Figure 3a–c). Allergic asthma is characterized not only through inflammatory infiltrate but also by the increase of goblet cells and the mucus overproduction, as shown in Figure 3d,e. Nevertheless, the neostigmine-treated group did not have significant changes in mucus production compared with the OVA group (Figure 3d,e).

3.3 | Neostigmine increases Akt-pAkt immunocontent and improves oxidative stress on lung tissue in an allergic asthma model

The cholinergic anti-inflammatory pathway via α7 nAChR promotes a PI3K/Akt and PKC signaling that protects the cell from oxidative stress. Thereby, we investigated the Akt immunocontent by Western blot in lung tissue from BALB/c mice. Asthmatic mice had a significant decrease in Akt immunocontent in the lung when compared with the control group. Otherwise, the neostigmine-treated group had an increase in immunocontent of Akt when compared with the OVA group (Figure 4a). Moreover, the neostigmine-treated group also had an increase in pAkt when compared with the OVA group (Figure 4b). Thereby, we investigated whether

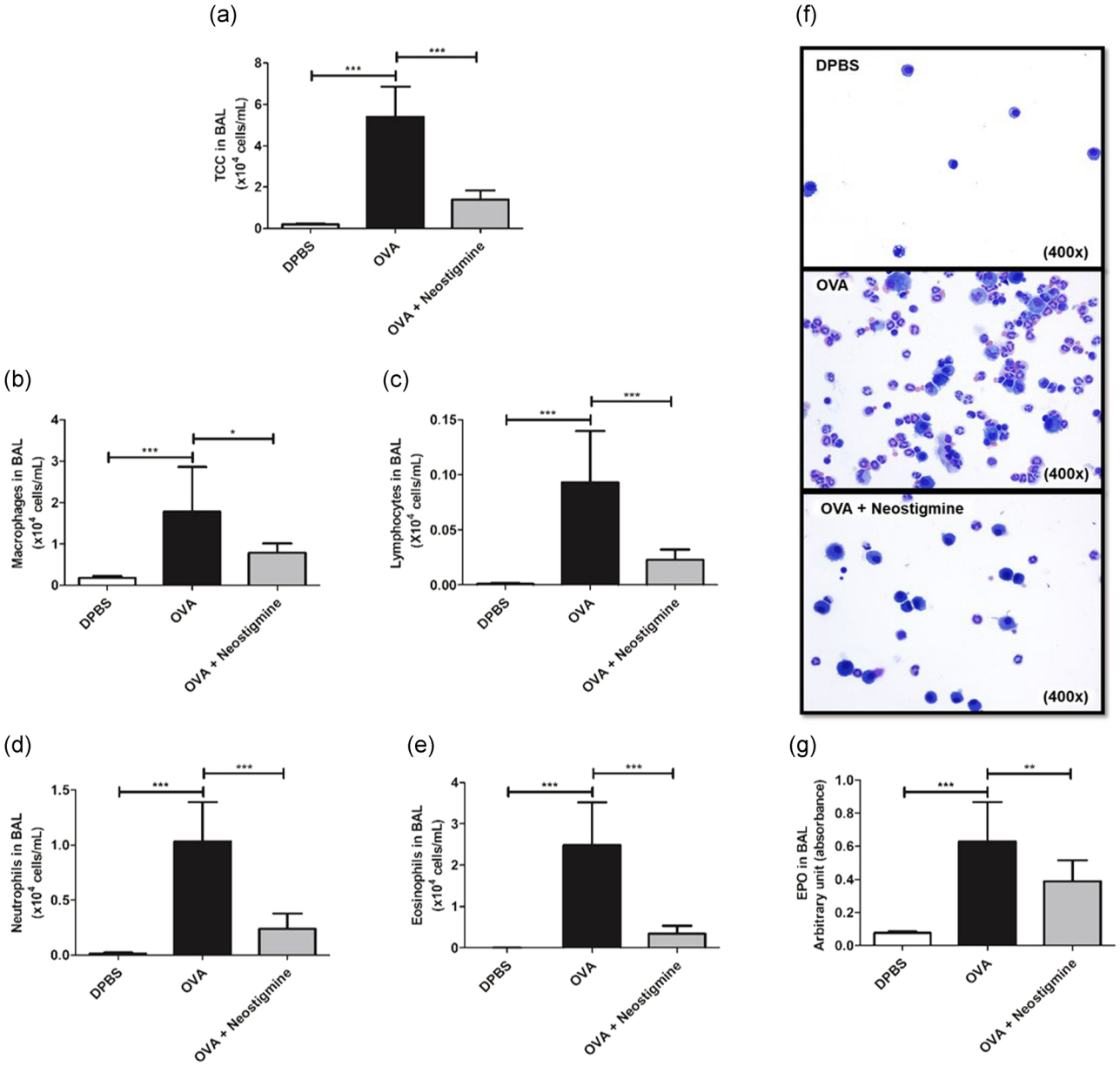

FIGURE 2 Mice treated with neostigmine decreased cells count and EPO activity in BAL. (a) Total cells count, (b) absolute macrophages count, (c) absolute lymphocytes count, (d) absolute neutrophils count, (e) absolute eosinophils count. (f) Representative image of the differential cells count (H&E, 400x magnification) and (g) EPO activity. Results are expressed as mean ± SD, for eight animals in each group. BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; OVA, ovalbumin. Different from the DPBS group ***$p < .001$, different from OVA group *$p < .05$, **$p < .01$, and ***$p < .001$ (One-way ANOVA followed by Tukey test)

neostigmine could prevent the formation of oxidative stress in female mice lungs. First, we evaluated the neostigmine effect on ROS production in the lungs. Mice from the OVA group had an increased ROS production when compared with the control mice. On the other hand, the neostigmine treatment decreased ROS formation when compared with the OVA group (Figure 4c). We also tested the effect of neostigmine treatment on the enzymatic antioxidant defenses (SOD, CAT, and GPx). As can be seen in Figure 4, we observed a significant reduction in the SOD, CAT, and GPx activity in the OVA group when compared with the control group (Figure 4d–f). We demonstrated that the neostigmine treatment did not alter SOD (Figure 4d) and GPx activity (Figure 4f) when compared with the OVA group. However, neostigmine-treated mice increased the CAT activity compared with the OVA-challenged mice (Figure 4e).

3.4 | Neostigmine treatment reduces NFκB p65 immunocontent and decreases the levels of pro-inflammatory cytokines on lung tissue in an allergic asthma model

The cholinergic anti-inflammatory system controls of inflammation inhibiting the NFkB translocation. We observe that the OVA group

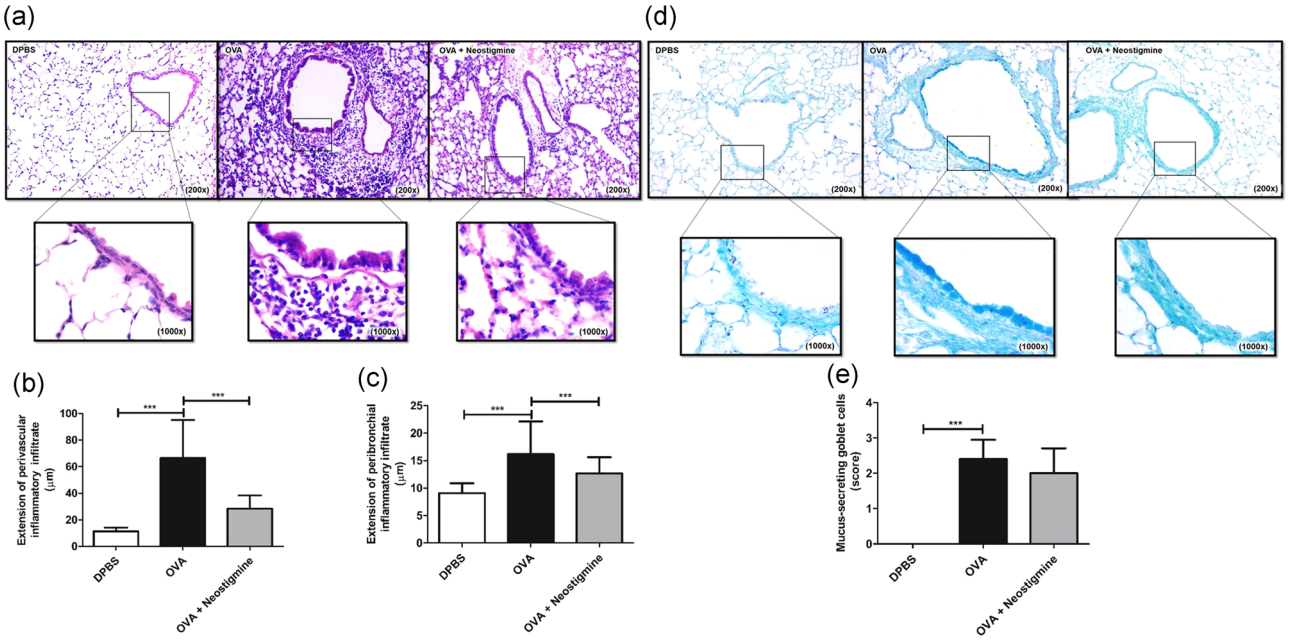

FIGURE 3 Effects of neostigmine treatment in the inflammatory infiltrate and goblet cells hyperplasia in the lung tissue. (a) Representative lung sections stained with H&E (200x and 1000x magnification). Histological quantification of (b) perivascular and (c) peribronchial infiltrate. (d) Representative lung sections stained with alcian blue (200x and 1000x magnification). (e) Histological quantification of mucus-secreting goblet cells. Results are expressed as mean ± SD, for eight animals in each group. DPBS, Dulbecco's phosphate-buffered saline; H&E, hematoxylin and eosin. Different from the DPBS group ***$p < .001$, different from OVA group ***$p < .001$ (One-way ANOVA followed by Tukey test)

mice increased NFκB p65 immunocontent whereas mice treated with neostigmine decreased NFκB p65 immunocontent in mice lung tissue (Figure 5a). After, we explored levels of IL-4, IL-5, and IL-13 from Th2 cells, which contribute to the classic symptoms of allergic asthma and levels of IL-1β and TNF-α from others profile that may also contribute to the exacerbation of the disease. In addition, we also measured regulatory cytokine IL-10 levels. IL-4, IL-5, IL-13, IL-1β, and TNF-α were significantly increased in OVA-challenged mice compared with the control group (Figure 5b–f). Surprisingly, neostigmine treatment was could decrease the levels of Th2 cytokines (Figure 5b–d) as well as IL-1β and TNF-α (Figure 5e,f). Nevertheless, there were no significant differences between experimental groups in the IL-10 levels (Figure 5g).

3.5 | Effect of neostigmine treatment in AChE and α7 nAChR immunocontent on lung tissue in an allergic asthma model

As presented in Figure 6, we observed that OVA-challenged mice increased AChE immunocontent in comparison to the DPBS group. However, the reduction in the neostigmine-treated group was not observed when compared with the OVA group (Figure 6a). We also performed immunofluorescence and observed the same profile of increase in the AChE immunocontent in OVA group when compared with the DPBS group (Figure 6b). The anti-inflammatory effects of the cholinergic pathway require the interaction of ACh with α7 nAChR. We showed that α7 nAChR immunocontent did not alter between OVA and DPBS groups. Otherwise, we demonstrated that neostigmine-treated group had a significant increase in α7 nAChR immunocontent when compared with the OVA group (Figure 6c).

3.6 | Neostigmine treatment improves the parameters of respiratory mechanics in an allergic asthma model

Finally, for the possible therapeutic potential of the pathway by neostigmine, it is important to determine whether our data has effects on respiratory mechanics. We observed no significant differences between groups in the resistance of airway (Figure 7a). However, tissue damping increased in the OVA group whereas the neostigmine-treated group had a decrease in this parameter (Figure 7b). A similar effect was seen for tissue elastance (Figure 7c).

4 | DISCUSSION

The knowledge that allergic asthma is a disorder mediated by Th2 lymphocytes encouraged drug development that inhibits cytokines release of this profile, such as Mepolizumab, a monoclonal antibody that acts directly against IL-5 which are now successfully used in the clinic (Bel et al., 2014). On the other hand, the success of the monoclonal antibody use is not observed in some patients. One explains possible is because these strategies far have focused on inhibiting one cytokine at a time. In this way, mechanisms such as the

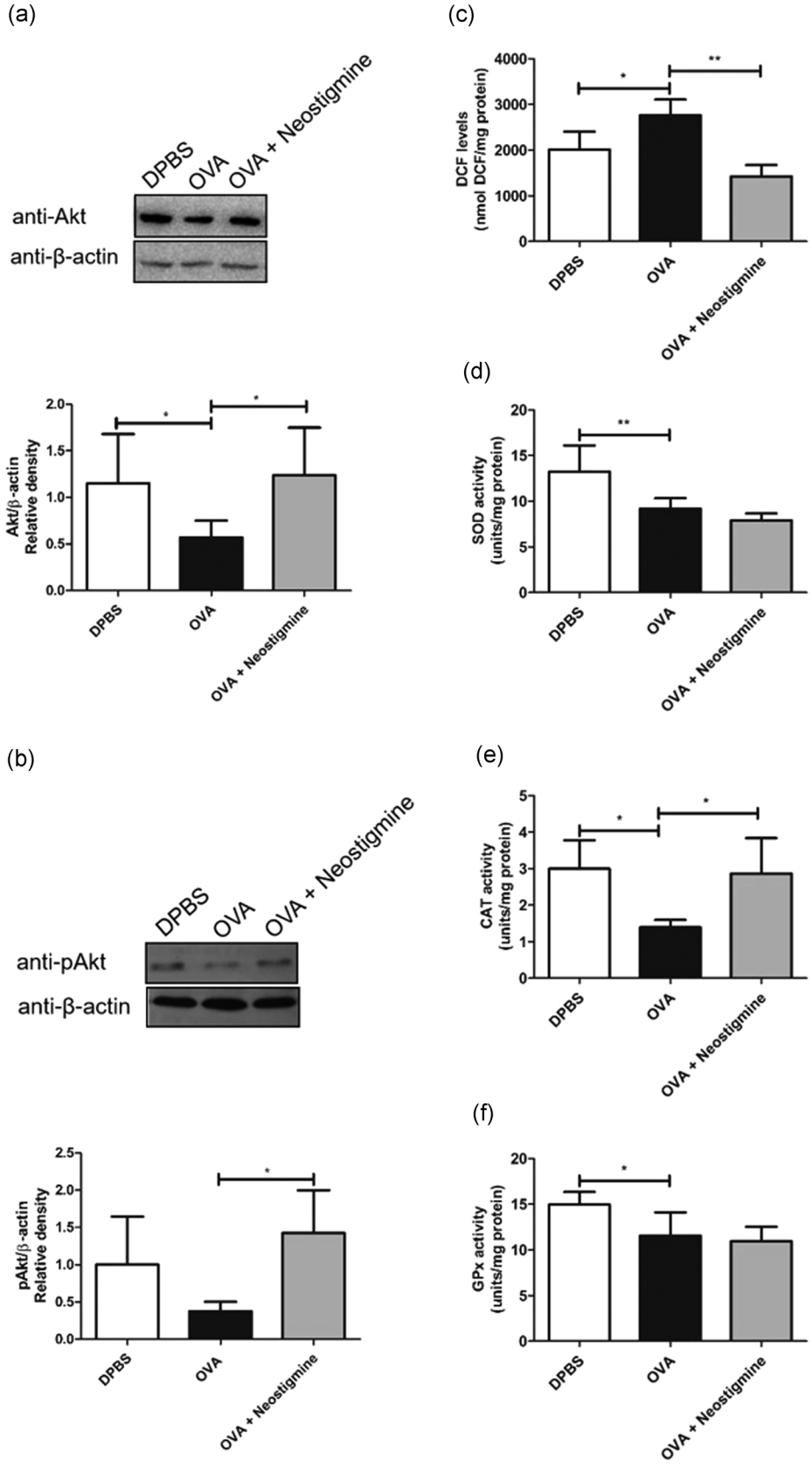

FIGURE 4 Neostigmine treatment reduced Akt-pAkt immunocontent and oxidative stress in the lung. (a) Akt analyzed by Western blot. (b) pAkt (serine 473) analyzed by Western blot. (c) Reactive oxygen species production by DCF. (d) superoxide dismutase, (e) catalase, and (f) glutathione peroxidase activity. Results are expressed as mean ± SD, for eight animals in each group. DPBS, Dulbecco's phosphate-buffered salin; OVA, ovalbumin. Different from the DPBS group $^{*}p < .05$, $^{**}p < .01$, different from the OVA group $^{*}p < .05$, $^{**}p < .01$ (One-way ANOVA followed by Tukey test)

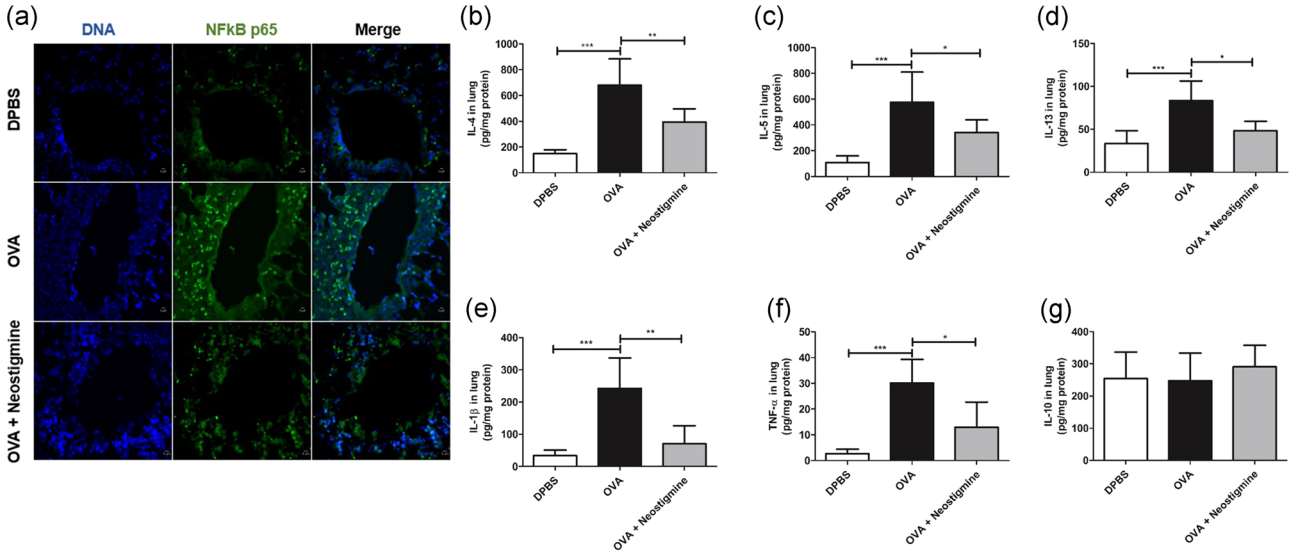

FIGURE 5 Neostigmine decreased NFκB p65 immunocontent and reduced release of pro-inflammatory cytokines in lung. (a) NFκB p65 analyzed by immunofluorescence staining. (b) IL-4. (c) IL-5. (d) IL-13. (e) IL-1β. (f) TNF-α, and (g) IL-10 levels. Results are expressed as mean ± SD, for eight animals in each group. DPBS, Dulbecco's phosphate-buffered salin; OVA, ovalbumin. Different from the DPBS group ***$p < .001$, different from the OVA group *$p < .05$, **$p < .01$ (One-way ANOVA followed by Tukey test)

cholinergic anti-inflammatory system that inhibit the release of several pro-inflammatory cytokines can provide important insights. This system act through ACh release from the vagus nerve and consequent activation of α7 nAChR (Wang et al., 2003). The protective effects of cholinergic anti-inflammatory pathway activation have been reported in several inflammatory diseases, such as acute lung injury (ALI), sepsis, colitis, pancreatitis, and rheumatoid arthritis (Ghia, Blennerhassett, El-Sharkawy, & Collins, 2007; Su et al., 2007; van Maanen, Stoof, LaRosa, Vervoordeldonk, & Tak, 2010; van Westerloo et al., 2006; Wang et al., 2004). Koopman and coworkers demonstrated positives results when extend the preclinical data to the clinic, showing that vagus nerve stimulation and a consequent inhibits of TNF lead to reducing the severity of rheumatoid arthritis (Koopman et al., 2016). In this present study, our data revealed that cholinergic anti-inflammatory pathway activation through neostigmine conferred airway protection against oxidative stress and decreased inflammation of airway in asthmatic BALB/c mice.

Initially, we observed a high influx of inflammatory cells on airways in OVA-challenged mice. Meanwhile, in BALB/c mice treated with neostigmine, it was evident the decrease cells infiltrate,

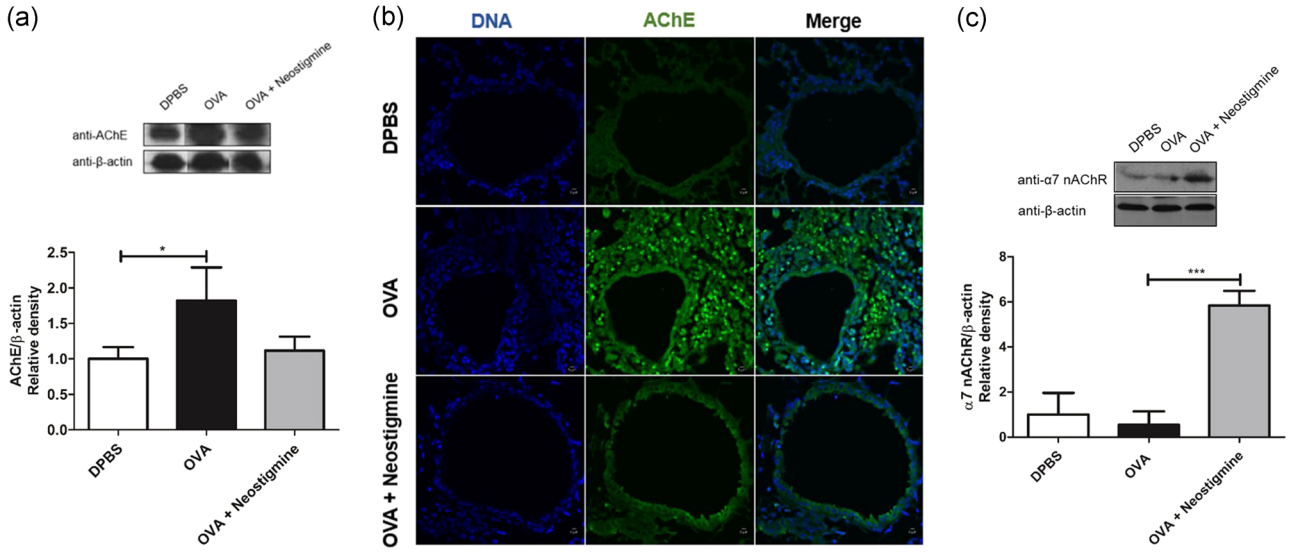

FIGURE 6 The effect of neostigmine treatment in AChE and α7 nAChR immunocontent in the lung tissue. (a) AChE analyzed by Western blot. (b) AChE analyzed by immunofluorescence staining. (c) α7 nAChR analyzed by Western blot. Results are expressed as mean ± SD, for six animals in each group. AChE, acetylcholinesterase; DPBS, Dulbecco's phosphate-buffered saline. Different from the DPBS group *$p < .05$, different from OVA group *$p < .05$, ***$p < .001$ (One-way ANOVA followed by Tukey test)

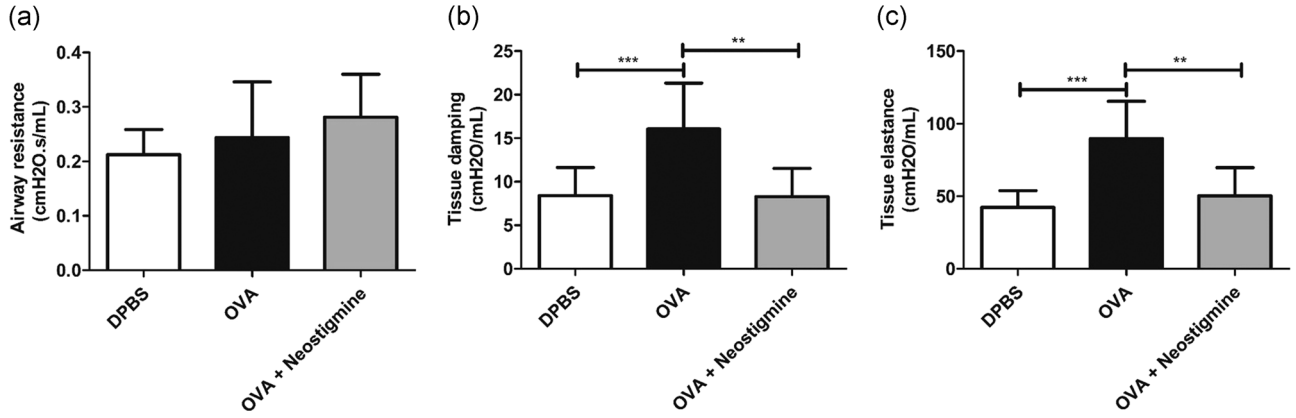

FIGURE 7 Neostigmine treatment improved parameters of respiratory mechanics. (a) Airway resistance (Raw). (b) tissue damping and (c) tissue elastance. Results are expressed as mean ± SD, for eight animals in each group. DPBS, Dulbecco's phosphate-buffered saline; OVA, ovalbumin. Different from the DPBS group ***$p < .001$, different from the OVA group **$p < 0.01$ (One-way ANOVA followed by Tukey test)

especially of eosinophils. Indeed, experimental studies also have revealed that cholinergic anti-inflammatory system activation promotes the reduction of cells recruitment on inflammatory diseases (Kanashiro et al., 2016). Our histological data corroborated with these findings. We observed an intense peribronchial and perivascular infiltrate in the asthmatic group whereas neostigmine-treated mice showed a decrease in both infiltrates. In addition, mucus hypersecretion also contributes to the disease exacerbation (Haspeslagh et al., 2018). However, in the histological analysis of lung tissue stained with Alcian blue, is not possible to observe a decreased mucus hypersecretion in the group treated with neostigmine.

A massive eosinophils infiltration in the airway and an increased EPO activity was observed in the OVA-induced asthma group. This enzyme is a potent ROS generating and the treatment with neostigmine decreased the release of granular proteins from eosinophils, suggesting that enhancement of the cholinergic anti-inflammatory system can have effects over parameters of oxidative stress. Recently, Kim et al. (2018) described a molecular mechanism whereby α7 nAChR promotes a signaling cascade that involves the activation of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3K)/Akt, promoting the translocation of nuclear factor erythroid 2-related Factor 2 (Nrf2 /NFE2L2), which drives the expression of heme oxygenase (HO)-1, protecting the kidney against ischemia. Thus, this pathway can have an important antioxidant effect. On the basis of this, we analyzed the Akt on mice lung tissue and observed that neostigmine increased immunocontent of Akt and pAkt, suggesting activation of the PI3K/Akt route and protein PKC by neostigmine treatment. Recently, Mishra, Banga, and Silveyra (2018) showed that oxidative stress could aggravate inflammation of airway by inducing pro-inflammatory mediators, mucus hypersecretion, increasing bronchial hyperresponsiveness, and of vascular permeability. We verified an increase in ROS production in the OVA group whereas neostigmine neutralized this response. During oxidative stress, the cellular homeostasis depends on protective antioxidant mechanisms. In the lung, the major enzymatic antioxidants are SOD, CAT, and GPx (Rahman, Biswas, & Kode, 2006). SOD is necessary to neutralize the O_2 formed in excess in the tissue. The neutralization of O_2 through SOD produces H_2O_2 that requires the CAT and GPx activity for removal. The activity of SOD, CAT, and GPx was diminished in the OVA group, probably because during the inflammatory response they are being highly consumed for ROS removing. Our results showed that the treatment with neostigmine did not alter the SOD and GPx activity. However, we demonstrated an increase in CAT activity in the lung tissue. We believe which DCF produced was diminished in the lungs of mice treated with neostigmine because ROS are being consumed through the antioxidant activity shown by an increase in the CAT activity in this present study. We demonstrated, for the first time, the antioxidant effect of the cholinergic anti-inflammatory pathway through pharmacological activation with neostigmine in an allergic asthma model.

Studies suggest that cholinergic anti-inflammatory system control inflammation through a direct effect on the synthesis of pro-inflammatory cytokines (Hofer et al., 2008; Kox et al., 2012). It is known which NFκB induces the expression of pro-inflammatory genes and the uncontrolled activation of NFκB can contribute to the allergic asthma exacerbation (Sun et al., 2017). In BALB/c mice submitted to allergic asthma model, we observed an NFκB increase p65 whereas the activation of the cholinergic anti-inflammatory system through neostigmine reduced the translocation. In fact, it is well known which α7 nAChR inhibits the nuclear translocation of NFκB through signaling of activator of transcription 3/Janus-activated kinase 2 signaling (STAT3/JAK-2). Thus, thereafter, we explored the generation of IL-4, IL-5, and IL-13 that strongly contributes to the exacerbation of allergic asthma. In our study, asthmatic BALB/c mice presented higher levels of this cytokines when compared to the control group. IL-4 induces production of IgE and is necessary for Th2 immunity development. Besides that, eosinophilia in lung tissue is driven by IL-5 and the IL-13 contribute to the bronchial hyperreactivity and for goblet cell metaplasia, which clogs the airway lumen (Lambrecht & Hammad, 2015). We showed

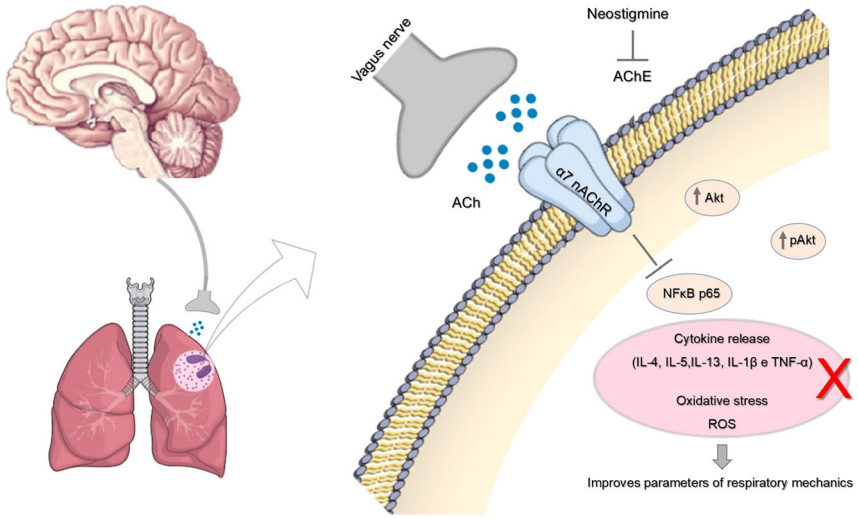

FIGURE 8 Summary of the pharmacological activation of the cholinergic anti-inflammatory pathway by neostigmine in the allergic asthma model. ACh, acetylcholine; AChE, acetylcholinesterase; AKT, protein kinase; pAkt, phosphorylated protein kinase; IL, interleukine; NFκB p65, nuclear factor kappa; ROS, reactive oxygen species; α7 nAChR, α7 nicotinic acetylcholine receptor

that neostigmine decreased these cytokines in asthmatic mice. This reduction in IL-5 supports our data in eosinophil infiltration. On the other hand, a decrease in mucus hypersecretion and IL-13 levels were not observed. Gundavarapu et al. (2012), using a similar model of allergic asthma induction, showed that the expression of dominant mucin gene (MUC5AC), does not depend on IL-13 levels and the α7 nAChRs can be required for mucus formation. Although asthma is classically associated with eosinophilia and Th2 profile, in some cases it may occur a mixed Th1 and Th17 cytokine production. In this context, we also measured IL-1β and TNF-α. The IL-1β release can cause airway epithelial cell dysfunction (Lambrecht, Hammad, & Fahy, 2019). TNF-α induces the release of inflammatory cytokines, exacerbates lung cell damage and also contributes to cell migration into allergic lung tissue in asthmatic mice (Sun et al., 2017). Our data showed that allergic asthmatic BALB/c mice that received neostigmine treatment have reduced IL-1β and TNF-α. Nevertheless, we did not visualize an increase of IL-10 in the group which received neostigmine treatment when compared with the OVA group. In accordance, other authors also observed which cholinergic anti-inflammatory system no change anti-inflammatory cytokines, corroborating with our results (Pinheiro et al., 2015).

The cholinergic anti-inflammatory system may be pharmacologically activated through neostigmine, an acetylcholinesterase inhibitor, that lead to an increase in ACh levels (Kanashiro et al., 2016). In this way, we investigated neostigmine treatment effect on AChE immunocontent in lung tissue. We verified, by immunofluorescence microscopy and Western blot, that AChE immunocontent increased in the OVA group. However, the immunocontent did not decrease in mice treated with neostigmine. We believed that this result can be because of the time of lung tissue collection. Analyzes 24 hours after the last administration of neostigmine may have hidden possible effects in the immunocontent because of the half-life of the drug, which is approximately 60 min. Interestingly, even with a short half-life, our results showed a beneficial effect by cholinergic anti-inflammatory pathway activation.

Wang et al. observed that a7 nAChR plays a critical in inflammation control through the cholinergic anti-inflammatory system. These authors demonstrated that stimulation of vagus nerve inhibits TNF production in mice, however, fails to inhibit TNF synthesis in a7 deficient C57BL mice (Wang et al., 2003). It is assumed which a7 nAChR mediates the control of inflammation by JAK-STAT3 that lead to inhibition the translocation of NFκB to the nucleus. The GTS-21, an agonist of a7 nAchR, downregulates NFκB and reduces TNF-a, IL-1ß, and IL-6 cytokines release in an experimental model of lung injury (Mei et al., 2018). In the present study, we also observe a reduced NFκB p65 translocation and showed that cholinergic anti-inflammatory system has a direct effect on pro-inflammatory cytokines in BALB/c mice submitted an allergic asthmatic model. The α7 receptor not only activate this via but also promotes the activation of the transcription factor Nrf2 by PKA and Akt, driving expression of HO-1, a pathway known to have antioxidant regulation (Kalkman & Feuerbach, 2016). The activation of the cholinergic anti-inflammatory system by nicotine reduced oxidative stress through HO-1in a mice model of liver injury (Park, Kang, & Lee, 2013) Our data also showed that activation of the cholinergic anti-inflammatory system protected the airways against oxidative damage. Thereafter, we speculated whether neostigmine could change the expression of α7 nAChR and our results showed an increase of α7 nAChR immunocontent, corroborating with our other results.

Finally, we showed that the cholinergic anti-inflammatory pathway activation through neostigmine was able to decrease the release of the pro-inflammatory cytokines and attenuates oxidative stress in an allergic asthma model. Thereafter, we explored whether our findings have effects on parameters of respiratory mechanics. We observed that airway resistance did not alter in groups studied. Mori et al. (2017) also found no changes in this parameter in a similar model of asthma. In corroboration with our data and using the same induction model, Silveira et al. (2019) also showed which this parameter does not alter between groups. We believe that this result

is possible because it is an acute model and we did not use adjuvant. On the other hand, mice that received neostigmine treatment improvement tissue damping and tissue elastance compared with mice with allergic asthma. Thereby, to summarize our results, Figure 8 shows the effects of cholinergic anti-inflammatory activation by neostigmine in an allergic asthma model.

The investigation of our study revealed that neostigmine was able to activate the cholinergic anti-inflammatory system. Taken together, our data evidenced that the mice submitted a model of allergic asthma and treated with neostigmine reduced the levels of IL-4, IL-5, IL-13, IL-1β, and TNF-α, which resulted in a decrease of eosinophils influx and reduced the secretion of cytotoxic mediator EPO. Besides, treatment with neostigmine also conferred airway protection against oxidative damage, attenuating ROS production through an increase of antioxidant defense, evidenced by the CAT activity. Our findings showed that the protective effect found occurs by NFκB inhibition and increased Akt-pAkt during α7 nAChR signaling and lead to improved parameters of lung function. In summary, this is the first study to reveal that pharmacological activation of the cholinergic anti-inflammatory pathway can lead to new strategies for inhibits the release of pro-inflammatory cytokines and provides airway protection against oxidative damage in allergic asthma.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

G. L. A. designed the study, acquired data, analysis, and interpretation of the data and wrote the manuscript. J. S. S., D. B. K., C. L, M. S. C., R. V. B., E. P. M., and F. S. F. acquired the data, revised the article, and approved the final version. R. T. S., A. T. S. W. and P. M. C. P. revised the article and approved the final version. A. A. C. supervised the study, designed the work, revised the article, and approved the final version.

DATA AVAILABILITY

The data which support the findings of our study are available from the corresponding author upon reasonable request

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