

ORIGINAL RESEARCH ARTICLE

Reactive oxygen species are involved in eosinophil extracellular traps release and in airway inflammation in asthma

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Abstract

In asthma, there are high levels of inflammatory mediators, reactive oxygen species (ROS), and eosinophil extracellular traps (EETs) formation in airway. Here, we attempted to investigate the ROS involvement in EETs release and airway inflammation in OVA-challenged mice. Before the intranasal challenge with ovalbumin (OVA), animals were treated with two ROS inhibitors, *N*-acetylcysteine (NAC) or diphenyleneiodonium (DPI). We showed that NAC treatment reduced inflammatory cells in lung. DPI and NAC treatments reduced eosinophil peroxidase (EPO), goblet cells hyperplasia, proinflammatory cytokines, NF- κ B p65 immunopositive, and oxidative stress in lung. However, only the NAC treatment improved mitochondrial energy metabolism. Moreover, the treatments with DPI and NAC reduced EETs release in airway. This is the first study to show that ROS are needed for EETs formation in asthma. Based on our results, NAC and DPI treatments can be an interesting alternative for reducing airway inflammation, mitochondrial damage, and EETs release in asthma.

KEYWORDS

diphenyleneiodonium, DNA extracellular traps, experimental asthma, *N*-acetylcysteine, oxidative stress

Abbreviations: AHR, Airway hyperresponsiveness; ATP, Adenosine 5-triphosphate; BALF, Bronchoalveolar lavage fluid; CAT, Catalase; DCIP, 2,6-Dichloroindophenol; DPBS, Dulbecco's phosphate buffered saline; DPI, Diphenyleneiodonium; ECP, Eosinophil cationic protein; EDN, Eosinophil-derived neurotoxin; EETs, Eosinophil extracellular traps; EPO, Eosinophil peroxidase; G, Tissue damping; GPx, Glutathione peroxidase; H, Tissue elastance; H&E, Hematoxylin and eosin; H2DCF-DA, 2',7'-Dichlorofluorescein diacetate; IFN, Interferon; IgE, Immunoglobulin E; IL, Interleukin; MBP, Major basic protein; NAC, *N*-acetylcysteine; NADPH, Nicotinamide adenine dinucleotide phosphate; OVA, Ovalbumin; Pi, Phosphate inorganic; PI, Propidium iodide; Rn, Resistance; ROS, Reactive oxygen species; SDH, Succinate dehydrogenase; SOD, Superoxide dismutase; TNF- α , Tumor necrosis factor- α .

1 | INTRODUCTION

In asthma, there is elevated levels of proinflammatory cytokines, immunoglobulin E (IgE), and reactive oxygen species (ROS) leading to impairment in lung function, mucus overproduction, airway wall remodeling, and airway narrowing (Comhair & Erzurum, 2010; Elias et al., 2003; Renauld, 2001). In asthma, eosinophils migrate to the airway to form ROS such as superoxide, hydrogen peroxide, and hydroxyl radical which may increase airway injury (Comhair & Erzurum, 2010; Cunha et al., 2016; Henricks & Nijkamp, 2001). Under physiological status, ROS are kept in a balance due, in large part, the neutralization capacity of the enzymatic antioxidant defense system, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). However, the exposure of lung to oxidative stress can increase injury to mitochondria and inactivate the electron transport chain (ETC) and consequently increasing ROS formation (Zmijewski et al., 2008; Zmijewski, Lorne, Banerjee, & Abraham, 2009).

Extracellular DNA traps released by eosinophils have been described as an important mechanism of the innate immune response in allergic diseases (Morshed, Yousefi, Stockle, Simon, & Simon, 2012; Muniz et al., 2017; Yousefi et al., 2008). Stimulated eosinophils rapidly release extracellular DNA fibers, decorated with intact eosinophil granules forming eosinophil extracellular traps (EETs), that trapping and killing various microorganisms (Yousefi et al., 2008). Moreover, EETs contains granule proteins with antimicrobial activity such as eosinophil peroxidase (EPO; Morshed et al., 2012; Muniz et al., 2017; Yousefi et al., 2008). Dworski, Simon, Hoskins, and Yousefi (2011) reported the first evidence that EETs are present in the lungs of asthmatics patients. Besides that, Cunha et al. (2014) demonstrated that in OVA-challenged mice, there was an enhancement in EETs release in airway. EETs can be pathogenic when they are produced in excess (Cunha et al., 2016; Ueki et al., 2016). Nevertheless, the persistence of EETs can be proinflammatory, but their role in immune responses in respiratory disease is unclear.

ROS participation in extracellular DNA traps formation depends on stimulus. Evidence has reported that ROS production is essential for extracellular DNA traps formation; other studies have observed that they can also be released independently of ROS (Barth et al., 2016; Morshed et al., 2012; Muniz et al., 2017). However, to date, the mechanism of EETs formation and the pathophysiologic function in allergic asthma has not been elucidated. Thus, the aim of this study was to analyze the effect of a precursor of glutathione, N-acetylcysteine (NAC), and an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), diphenyleneiodonium (DPI), in the EETs formation and in airway inflammation in OVA-challenged mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Female BALB/cJ (6–8 weeks of age), specific pathogen-free from the Center for Experimental Biological Models (CeMBE, Pontificia Universidade Católica do Rio Grande do Sul - PUCRS) were used

for all experiments. The animals were fed standard ration for rodents and access to water ad libitum, housed in cages and kept in a 12/12 hr light/dark cycle. All animal experiments were performed according to "US National Research Council's Guide for the Care and Use of Laboratory Animals". All study was conducted with the approval of the PUCRS Animal Ethics Committee (7910).

2.2 | Sensitization and challenge

Mice were sensitized with subcutaneous injections of 20 µg of ovalbumin (OVA, Grade V, Sigma, MO) without adjuvant, diluted in 200 µl of Dulbecco phosphate buffered saline (DPBS, Gibco, MA), on Days 0 and 7 of the protocol. Followed by three intranasal challenges with 100 µg of OVA without adjuvant, diluted in 50 µl of DPBS, on Days 14, 15, and 16 (Cunha et al., 2016). Control animals received DPBS (Figure 1a).

2.3 | Treatment protocol

To investigate ROS participation in EETs release and asthma physiopathology, we utilized two ROS inhibitors, namely: NAC (União Química, São Paulo, Brazil) and DPI (Sigma, MO). Besides NAC being an antioxidant thiol and a glutathione precursor, it is a mucolytic and anti-inflammatory drug. DPI is an NADPH oxidase inhibitor which reduces superoxide production. Mice were treated intranasally with DPI 1 mg/kg or NAC 15 mg/100 g of body weight, 45 min before the three intranasal challenges (Barth et al., 2016). On days 17 of the protocol, the animals were anesthetized (ketamine 0.4 mg/g and xylazine 0.2 mg/g) and pulmonary function test was performed. Also, on days 17 of the protocol, animals were euthanized, bronchoalveolar lavage fluid (BALF) was collected and lung tissue was removed for the experimental analyses. Figure 1a shows the timeline of the experimental protocol.

2.4 | Total and differential cell counts in BALF

The trachea was cannulated and 1 ml of phosphate buffered saline (PBS) 2% fetal bovine serum (FBS) was instilled for the BALF collection. BALF was centrifuged and the pellet was diluted in PBS 2% FBS to perform total and differential cell counts. The total cell counts were determined in the Neubauer chamber (BOECO, Hamburg, Germany). For the differential cell counts, the slides were performed through cytopspin in a cytocentrifuge (Eppendorf, Wesseling, Germany) and stained with hematoxylin and eosin (H&E; Newprov, Paraná, Brazil). Four hundred cells were counted in an Olympus BX43 Microscope (Olympus, Tokyo, Japan).

2.5 | Analysis of the EPO activity in BALF

EPO activity was measured by the colorimetric assay based in the oxidation of O-phenylenediamine (OPD, Sigma, MO) in the presence of hydrogen peroxide. BALF supernatant was transferred to a 96-well plate and working reagent was added (0.1 mM

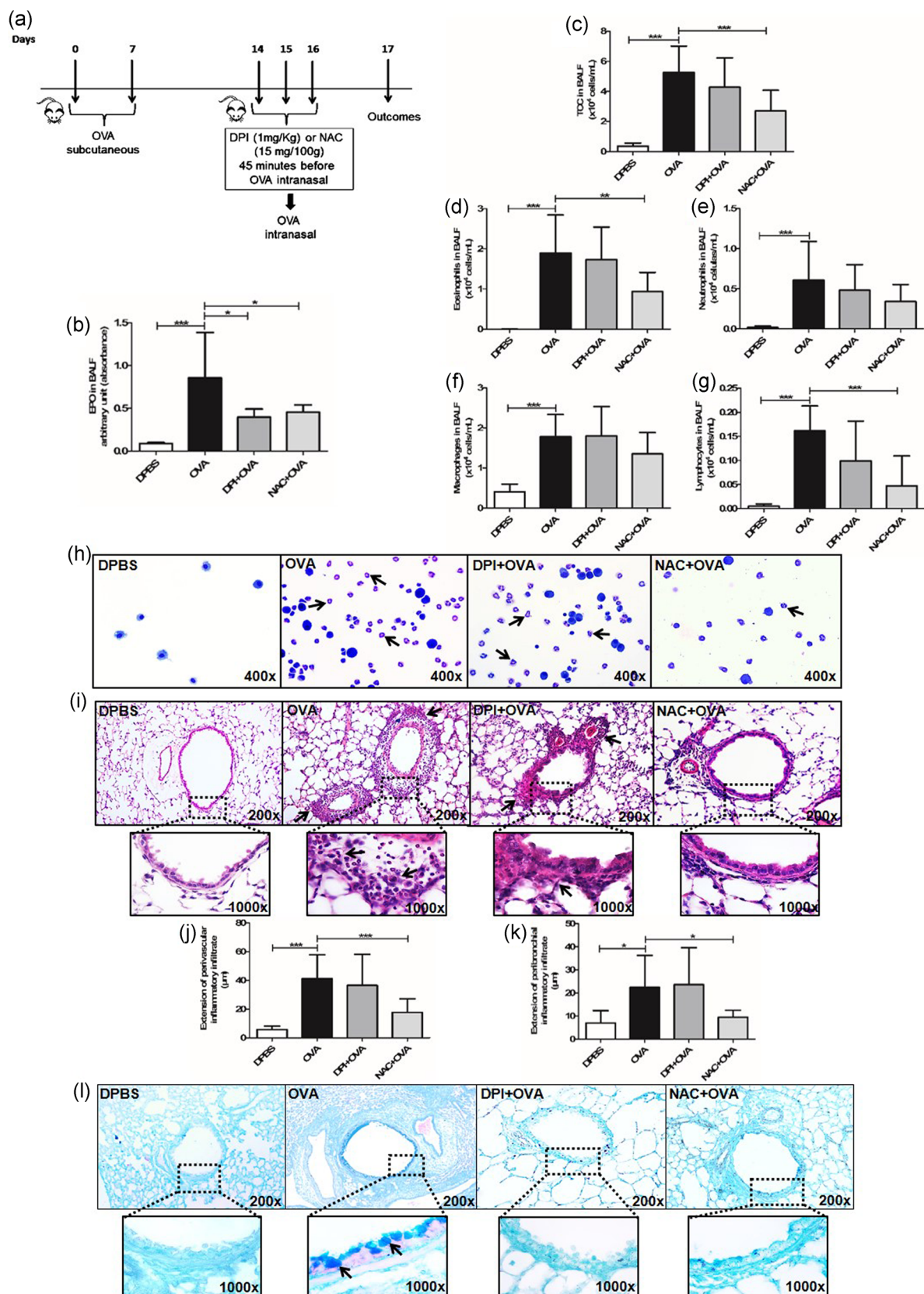


FIGURE 1 Continued.

OPD, 0.05 M Tris pH 8.0, Triton X -100, and 1 mM hydrogen peroxide). The plate was incubated protected from light for 1 hr. After this period the reaction was stopped with 50 μ l of sulfuric acid 1.0 M and absorbance was measured in a spectrophotometer at 492 nm (Strath, Warren, & Sanderson, 1985).

2.6 | Lung histopathologic analysis

After euthanasia, lungs were fixed in 10% buffered formalin. Tissues were embedded in paraffin and histological sections of 5 μ m were performed. For evaluation the inflammatory infiltrate the sections were stained with H&E. For evaluation of the extent of peribronchial and perivascular inflammatory infiltrate in micrometers, 10 measurements were performed starting from the end of the bronchial or vessel epithelium to the end of the inflammatory infiltrate using Olympus CellSens Standart software (Olympus, Tokyo, Japan). At least five fields were evaluated to obtain the average for each mouse. For evaluation of goblet cells hyperplasia and mucus production, the sections were stained with alcian blue. Images were taken in an Olympus BX43 Microscope (Olympus, Tokyo, Japan).

2.7 | Assessment of lung function

The animals were anesthetized with ketamine (0.4 mg/g) and xylazine (0.2 mg/g). Pulmonary function test was performed after cannulation of the trachea. The animal was maintained in the flexiVent system (flexiVent, SCIREQ, Montreal, Canada) for 5 min before start the test and after was administered pancuronium (1 mg/kg) intraperitoneally. Three measurements of forced oscillation technique (FOT) were performed during a pause of the respirator (3 s). A four-parameter model with constant phase tissue impedance was fitted to the Zrs data to obtain Resistance (Newtonian resistance - Rn) measurements, the which equates to airway resistance in mouse due to complacency of the chest wall, G (tissue damping), which represents the resistance of small airways where air movement occurs mainly by diffusion and H (tissue elastance). The data were analyzed in a specific software (flexiWare, SCIREQ, Montreal, Canada), where airway resistance and elastic properties (viscosity and elasticity) of the lung were measured through a pulmonary impedance (Rn, G, and H; Hantos, Daróczy, Suki, Nagy, & Fredberg, 1992).

2.8 | Cytokine levels in lung

Lungs were prepared by homogenization in PBS 1X and the levels of interleukin (IL)-5, IL-13, interferon (IFN)- γ , IL-1 β , tumor necrosis factor (TNF)- α , and IL-10 were measured by multiplex assay using magnetic beads using a Milliplex MAP mouse kit (MILLIPLEX, Millipore, Germany) and ProcartaPlex Multiplex immunoassay (Thermo-Life Technology, MA) according to the manufacturer's recommendations. Analyzes were performed on the Magpix equipment (Millipore, Germany). Results were analyzed using xPONENT Solutions Software (Luminex Corporation, Texas, EUA).

2.9 | Analysis of NF κ B p65 by immunofluorescence in lung

Analysis of the NF κ B p65 immunocontent by immunofluorescence was performed in formalin-fixed lungs. First, lungs were embedded in paraffin and histological sections of 5 μ m were performed. Slices were dewaxed in xylol, rehydrated in water, and incubated with 9 mM citrate pH 9.0 for 10 min. After the slices were incubated with 0.3% hydrogen peroxide and methanol for 20 min. Afterward, slices were blocked with 10% bovine serum albumin (BSA) for 30 min. The sections were incubated for 40 min with anti-NF κ B p65 (1:500, Thermo-Life Technology, MA), followed for fluorescein isothiocyanate (FITC) antirabbit (1:250, Thermo-Life Technology, MA) for 30 min. The slices were incubated with Hoechst 33342 (1:2,000; Invitrogen, Carlsbad) for 4 min. The images were taken in a Zeiss LSM5 Exciter confocal microscope (Zeiss LSM5, Oberkochen, Germany).

2.10 | Lung preparation

For evaluation of ROS formation and antioxidant enzyme activities, the lungs were homogenized (1:10, w/v) in 140 mM potassium chloride and 20 mM sodium phosphate buffer. For mitochondrial energy metabolism analyses, lungs were frozen and thawed three times to disrupt the mitochondrial membranes. Next, samples were homogenized (1:20, w/v) in buffer containing 10 mM Trizma base, 250 mM sucrose, 50 UI/ml heparin, and 2 mM EDTA. For Na⁺,K⁺-ATPase activity analysis, lungs were homogenized (1:10, w/v) in medium buffer (0.32 mM sucrose buffer, 5.0 mM HEPES, and 1.0 mM EDTA, pH 7.5). Finally, the lung samples were centrifuged and the supernatants were collected.

FIGURE 1 NAC and DPI decrease EPO activity and goblet cells hyperplasia while NAC reduces inflammatory cells in airway. (a) Protocol used to induce a murine asthma model in BALB/cJ mice and treatments with DPI and NAC. (b) Analysis of the EPO activity in lung. (c) Total cell counts in BALF. (d–g) Differential cell counts in BALF (eosinophils, neutrophils, macrophages, and lymphocytes). (h) A representative illustration of BALF cells (x400 magnification, arrows indicate eosinophils). (i) Histopathological analysis in lung. Representative photomicrographs of sections stained with H&E (x200 and x1,000 magnification, arrow indicate inflammatory infiltrate). (j,k) Morphometric analysis perivascular and peribronchial with H&E staining. (l) Representative photomicrographs of sections stained with alcian blue (x200 and x1,000 magnification, arrows indicate globet cells). Data represent the mean $\pm$ SD, n = 20 animals per group. * p < .05, ** p < .01, and *** p < .001 (one-way ANOVA followed by Tukey test). ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; DPBS, Dulbecco phosphate buffered saline; DPI, diphenyleneiodonium; EPO, eosinophil peroxidase; NAC, N-acetylcysteine; OVA, ovalbumin; TCC, total cell counts [Color figure can be viewed at wileyonlinelibrary.com]

2.11 | ROS levels in lung

ROS production in lung was determined by the technique based on the oxidation of the 2'7' dichlorofluorescein diacetate (H2DCF-DA) described by LeBel, Ischiropoulos, and Bondy (1992). The lung samples were incubated in medium containing 100 μ M H2DCF-DA. Then the reaction produces a fluorescent compound, dichlorofluorescein (DCF), which was determined at 488 nm of excitation and 525 nm of emission. The data were plotted as nmol DCF/mg protein.

2.12 | SOD activity in lung

The SOD analysis in lung was based on the technique of the self-oxidizing ability of pyrogallol, a highly superoxide dependent process which is a substrate for SOD (Marklund, 1985). Inhibition of the self-oxidation of pyrogallol happens in the presence of SOD and its activity can be indirectly measured at 420 nm. The samples were mixed with 1 mM EDTA, 50 mM Tris, 80 U/ml CAT, and 0.8 mM pyrogallol. To calculate the SOD activity present in the samples, a calibration curve was performed with purified SOD. The SOD activity was plotted as units of SOD/mg protein.

2.13 | CAT activity in lung

The CAT analysis in the lung was based on the measure of the decrease in hydrogen peroxide consumption at 240 nm (Aebi, 1984). The samples were incubated in medium containing 20 mM hydrogen peroxide, 0.1% of Triton X-100, and 10 mM potassium phosphate, pH 7.4. One CAT unit was defined as hydrogen peroxide 1 μ M consumed per minute. The CAT activity was plotted as units of CAT/mg protein.

2.14 | GPx activity in lung

The GPx analysis was determined in lung according to Wendel (1981) and tert-butylhydroperoxide was utilized as substrate. The samples were mixed in medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM tert-butylhydroperoxide, and 0.1 mM NADPH. One GPx unit was defined as 1 μ M of NADPH consumed per minute. The decay of the NADPH was monitored at 340 nm in a spectrophotometer. The GPx activity was plotted in GPx units/mg of protein.

2.15 | Complex II, SDH, and complex IV activities in lung

The analysis of complex II was measured after to decrease the absorbance by reduction of 2,6-dichloroindophenol (DCIP) at 600 nm (Fischer et al., 1985). Lung samples were mixed in buffer containing 40 mM potassium phosphate, 16 mM sodium succinate, and 8 mM DCIP. Afterward, 4 mM sodium azide, 7 mM rotenone, and 40 mM DCIP were added. The activity of SDH was measured after reduction of the absorbance by reduction of the DCIP in the presence of phenazine methosulfate at 600 nm (Fischer et al., 1985). The data

were plotted as nmol/minute/mg of protein. The activity of complex IV was determined by a decrease in absorbance due to oxidation of reduced cytochrome c to 550 nm (Rustin et al., 1994). Lung homogenate was mixed in buffer containing 10 mM potassium phosphate, 0.6 mM n-dodecyl- α -D-maltoside, and 7 μ g of reduced cytochrome c. The data were plotted as nmol/minute/mg of protein.

2.16 | Na⁺,K⁺-ATPase activity in lung

Na⁺,K⁺-ATPase activity was evaluated according to Wyse et al. (2000). Tissue homogenate was added the reaction buffer containing 5.0 mM magnesium chloride, 80.0 mM sodium chloride, 20.0 mM potassium chloride, 40.0 mM Tris-Tris-hydrochloric acid, and 3.0 mM adenosine 5-triphosphate (ATP), pH 7.4. Controls were added ouabain 1.0 mM. The data were plotted as nmol of phosphate inorganic (P_i) released per minute per mg of protein.

2.17 | Quantification of extracellular DNA traps and confocal microscopy in BALF eosinophils

Extracellular DNA was quantified in BALF supernatant using the Quant-iT dsDNA HS kit (Invitrogen, Carlsbad) and measured in the Qubit 2.0 fluorimeter (Invitrogen), according to the manufacturer's recommendations. To visualize the EETs formation, cells obtained in BALF (2×10^5 /ml) were plated in 8-chamber culture slides (BD Falcon, NJ) and incubated for 1 hr at 37°C with 5% carbon dioxide. Afterward, the BALF eosinophils were fixed with 4% paraformaldehyde (PFA) for 45 min and incubated with anti-EPO and anti-histone H2B (1:250, Santa Cruz Biotechnology, Dallas) for 45 min. After this time, BALF eosinophils were incubated for 30 min with FITC anti-goat (1:100, Santa Cruz Biotechnology, Dallas) and alexa fluor 633 anti-goat (1:100, Invitrogen). Next, the BALF eosinophils were incubated with Hoechst 33342 DNA dye (1:2,000, Invitrogen) for 4 min. Images were performed on a Zeiss LSM5 Exciter confocal microscope (Zeiss LSM5, Oberkochen, Germany).

2.18 | Scanning electron microscopy of BALF eosinophils

BALF eosinophils (2×10^5 /ml) were placed in a 6-well plate with coverslips pretreated with 0.0005% poly-L-lysine (Sigma, MO) and incubated for 1 hr at 37°C with 5% carbon dioxide. After that, BALF eosinophils were fixed with 2.5% glutaraldehyde. After three washes in PBS, the samples were postfixed with osmium tetroxide solution 2% for 45 min. Afterward, BALF eosinophils were dehydrated in graded acetone: 30%, 50%, 70%, $2 \times 95\%$, and $2 \times 100\%$ for 10 min each. The critical point technique with carbon dioxide was performed, followed by mounting on metallic support with carbon tape. The samples were covered with a thin layer of gold (metallization). The images were performed in a scanning electron microscope Inspect F50 (FEI, Oregon, EUA).

2.19 | Analysis of cell death in BALF eosinophils

BALF eosinophils were evaluated for apoptosis by Annexin V and necrosis by propidium iodide (PI), according to the manufacturer's instructions (BD Pharmingen). BALF eosinophils were incubated with Annexin V-FITC and PI for 15 min and analyzed by flow cytometry (FACS Canto II, BD Bioscience). Results were analyzed using FlowJo software version X.0.7 (TreeStar, OR). This analysis allows the discrimination between the necrotic cells (Annexin V⁺/PI⁺), late apoptotic or necrotic cells (Annexin V⁺/PI⁺), and early apoptotic cells (Annexin V⁺/PI⁻).

2.20 | Protein determination

The proteins content in lungs were determined using the Qubit Protein Assay Kit (Invitrogen) for cytokines analyses. In accordance to Lowry et al. (1951) for proteins from oxidative stress and mitochondrial energy metabolism. In accordance to Bradford (1976) for proteins from Na⁺,K⁺-ATPase.

2.21 | Statistical analysis

Data are expressed as mean ± standard deviation (SD). We used one-way analysis of variance (ANOVA) followed by Tukey post hoc and significance level adopted was $p < .05$. Statistical analyses and graphs were performed using the GraphPad Prism Software, version 5 (GraphPad Software, San Diego).

3 | RESULTS

3.1 | NAC and DPI decrease EPO activity and goblet cells hyperplasia while NAC reduces inflammatory cells in airway

In EPO granular protein analysis, we demonstrated that OVA significantly increased EPO activity, whereas NAC and DPI significantly decreased EPO in BALF (Figure 1b). We observed that the OVA group had a significant increase in the total cell counts, as well as an increase in eosinophils, neutrophils, macrophages, and lymphocytes counts compared with the control group. After that, we showed that the NAC-treated OVA group decreased total cell counts as well as eosinophils and lymphocytes counts in BALF compared with the OVA group. In contrast, the DPI-treated OVA group did not have an alteration in BALF inflammation (Figure 1c–h). In histopathological examination, we showed an increase in inflammatory cells located in the peribronchial and perivascular areas in OVA-challenged mice, whereas the NAC-treated OVA group had a decrease in the pulmonary influx of cells (Figure 1i–k). The DPI treatment did not change the influx of cells for the airway (Figure 1i–k). In addition, we demonstrated that OVA-challenged mice had an increase in goblet cells compared with the control group, whereas NAC and DPI reduced goblet cells hyperplasia compared with the OVA group (Figure 1l).

3.2 | NAC and DPI improve lung function

Tissue damping and tissue elastance were significantly increased in the OVA-challenged mice compared with the DPBS group. In the DPI-treated OVA group, we showed a decrease in tissue damping and tissue elastance when compared with the OVA group. Moreover, we showed in the NAC-treated OVA group a decrease only in tissue elastance. On the other hand, we did not observe differences between the groups in airway resistance (Figure 2a–c).

3.3 | NAC and DPI decrease inflammatory cytokines and NFκB p65 immunocontent in lung

In cytokines analyses in lung, we showed that OVA-challenged mice increased IL-5, IL-13, IFN-γ, TNF-α, and IL-1β when compared with the control group. On the other hand, NAC and DPI treatment reduced IL-5, IFN-γ, and IL-1β. Meanwhile, only DPI reduced TNF-α. We did not observe differences between the groups in IL-10 concentration (Figure 2d–i). We observe that OVA-challenged mice increased NFκB p65 immunocontent, whereas that NAC and DPI treatments decreased NFκB p65 immunocontent in lung (Figure 2j).

3.4 | NAC and DPI decrease ROS production while that only NAC improves antioxidant defenses in lung

We demonstrate that OVA significantly increased the levels of ROS in lung when compared with the control group. In addition, we showed that NAC and DPI decreased ROS production in lung when compared with the OVA group (Figure 3a). We did not observe any significant difference in SOD activity between the groups (Figure 3b). We observed a significant decrease in CAT and GPx activity in the OVA group compared with the control group (Figure 3c,d). Moreover, we verified that only NAC treatment significantly increased the CAT activity compared with the OVA group (Figure 3c). We also showed that OVA group significantly increased SOD/CAT ratio when compared with the control group. On the other hand, we verified that only NAC decreased significantly SOD/CAT ratio compared with the OVA group (Figure 3e).

3.5 | NAC improves mitochondrial energy metabolism in lung

We observed that OVA group significantly decreased complex II, SDH, and complex IV activities when compared with the control group (Figure 3f–h). In addition, we demonstrated that the only NAC-treated OVA group increased SDH and complex IV activities in lung when compared with the OVA group (Figure 3f,h). In Na⁺,K⁺-ATPase analysis, we showed that the OVA group significantly decreased Na⁺,K⁺-ATPase activity when compared with the control group. On the other hand, DPI and NAC treatments did not alter this enzyme (Figure 3i).

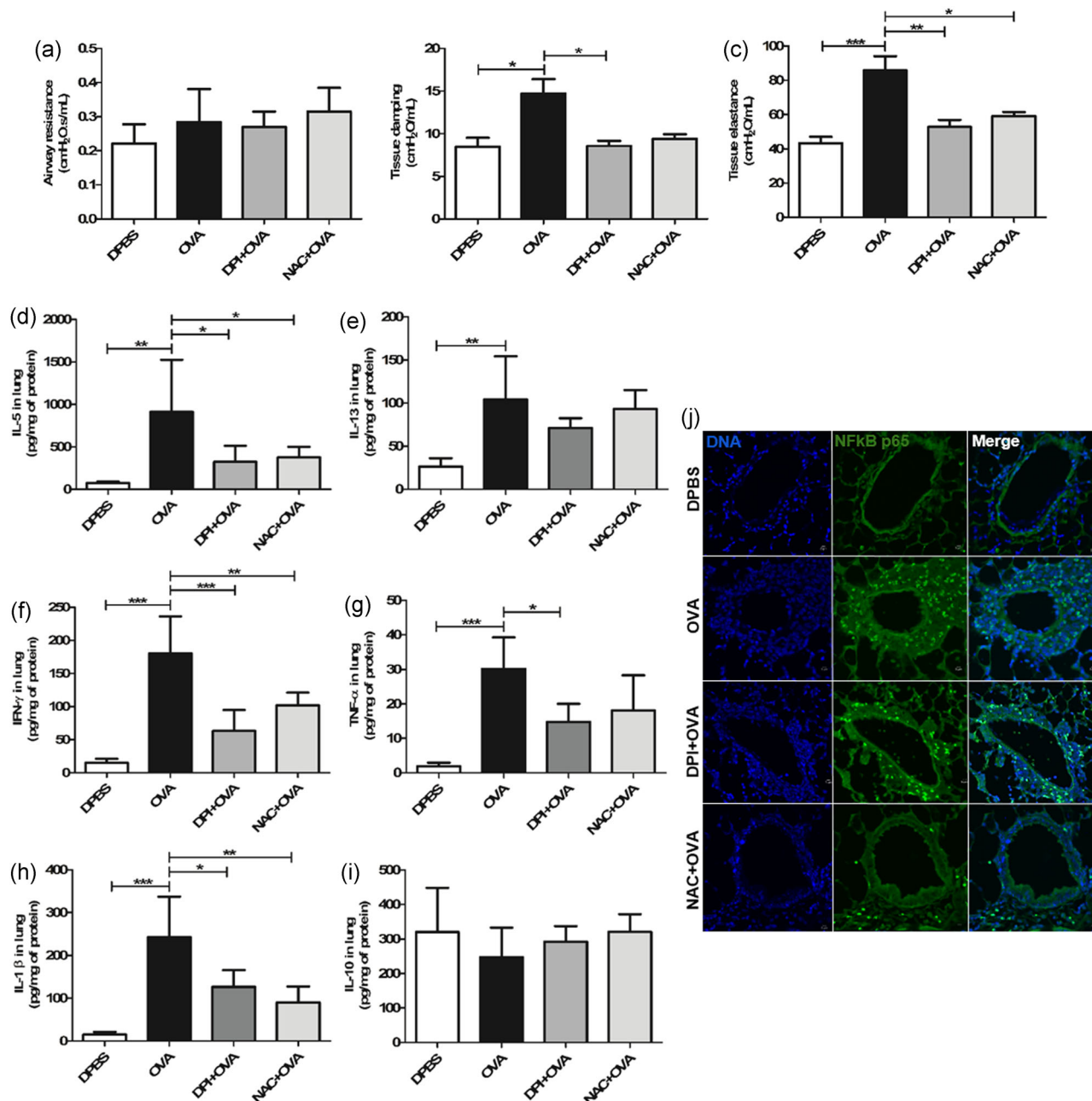


FIGURE 2 NAC and DPI improve lung function, decrease inflammatory cytokines, and NF- κ B p65 immunocontent. (a-c) Assessment of lung function: resistance (Rn), tissue damping (G), and tissue elastance (H). (d-i) Cytokine levels in lung (IL-5, IL-13, IFN- γ , IL-1- β , TNF- α , and IL-10). Cytokine levels were measured by multiplex assay using magnetic beads. (j) Analysis of the NF- κ B p65 protein by immunofluorescence in lung sections. The sections were incubated with the primary antibody, anti-NF- κ B p65 (1:500), followed for secondary antibody, FITC antirabbit (1:250). The sections were incubated with Hoechst 33342 (1:2,000). Data represent the mean $\pm$ SD, $n = 10$ animals per group. * $p < .05$, ** $p < .01$, and *** $p < .001$ (one-way ANOVA followed by Tukey test). ANOVA, analysis of variance; DPBS, dulbecco phosphate buffered saline; DPI, diphenyleneiodonium; IFN, interferon; IL, interleukin; NAC, N-acetylcysteine; OVA, ovalbumin; TNF, tumor necrosis factor [Color figure can be viewed at wileyonlinelibrary.com]

3.6 | EETs release depends on ROS production in an active process without cell death

In immunofluorescence microscopy and scanning electron microscopy, we observed that eosinophils from asthmatic mice released EETs colocalized with EPO, but not colocalized with histone H2B (Figure 4a,b). Moreover, OVA-challenged mice had an increase in extracellular DNA concentration in BALF when compared with the

DPBS group (Figure 4c). After that, we investigated whether ROS would be necessary for EETs formation in the airway in OVA-challenged mice. In immunofluorescence microscopy and scanning electron microscopy, NAC and DPI abrogated OVA-induced EETs formation (Figure 4a,b). Similarly, NAC and DPI treatments decreased extracellular DNA concentrations in the BALF from OVA-challenged mice compared with the OVA group, confirming our findings (Figure 4c). In addition, EETs release was not due

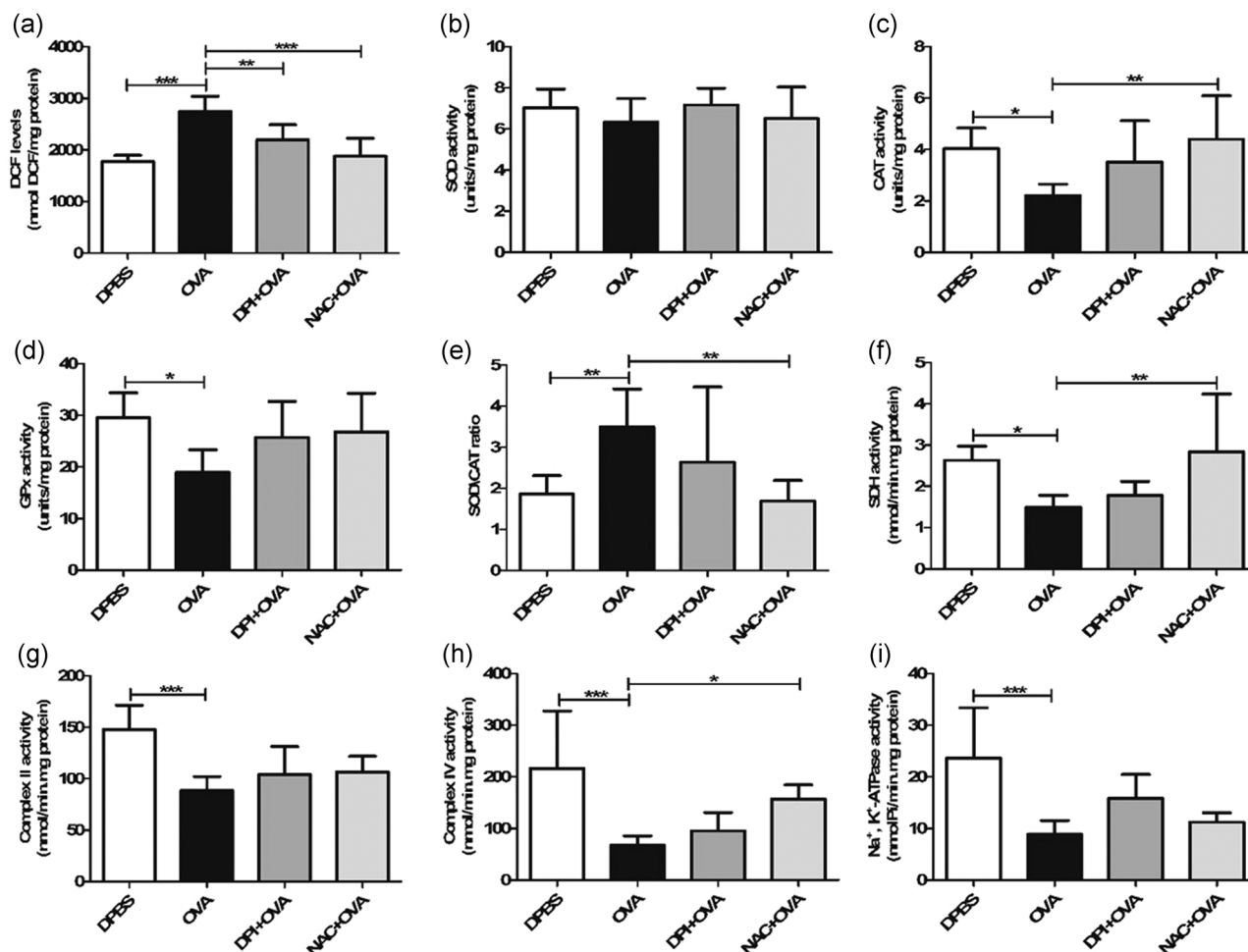


FIGURE 3 NAC and DPI decrease oxidative stress and improve mitochondrial energy metabolism in lung. (a) ROS production in lung. (b)–(e) Activities of antioxidants enzymes (SOD, CAT, and GPx) in lung. (f)–(h) Mitochondrial energy metabolism (complex II, SDH, and complex IV) in lung. (i) Na^+ , K^+ -ATPase activity in lung. Data represent the mean $\pm$ SD, $n = 10$ animals per group. * $p < .05$, ** $p < .01$, and *** $p < .001$ (one-way ANOVA followed by Tukey test). ANOVA, analysis of variance; CAT, catalase; DCF, dichlorofluorescein; DPBS, dulbecco phosphate buffered saline; DPI, diphenyleneiodonium; GPx, glutathione peroxidase; NAC, N-acetylcysteine; OVA, ovalbumin; SDH, succinate dehydrogenase; SOD, superoxide dismutase

to apoptosis or necrosis as shown in BALF, as cell viability remained high between the groups, showing that most cells present negative staining for annexin V and PI (Figure 4d).

4 | DISCUSSION

Infiltration of eosinophils to airway is one of the hallmark characteristics of asthma and plays a critical role in lung damage. In lungs, eosinophils can produce ROS, cytokines, chemokines, lipid mediators, cytotoxic granules, and EETs (Comhair & Erzurum, 2010; Cunha et al., 2016). This study shows the first evidence that ROS are necessary for EETs formation in BALF of asthmatic mice. We observed that ROS inhibition improves airway inflammation, lung function, oxidative stress, and reduces EETs release in OVA-challenged mice.

Evidence has reported that oxidative stress plays a significant role in the immunopathogenesis of asthma, associated with damage to biological molecules, increase in inflammation, and impairment in lung function

(Andreadis, Hazen, Comhair, & Erzurum, 2003). We showed that in asthmatic mice there was an increase in inflammatory cells with a high influx of eosinophils in airway as well as an increase in goblet cells hyperplasia, a typical pathological feature of asthma. More than 70% of cells found in BALF after OVA challenge were eosinophils. We observed that only NAC-treated OVA group decreased inflammatory cells mainly in eosinophils number. Moreover, we verified that DPI and NAC decreased goblet cells hyperplasia. After that, we decided to evaluate the EPO activity in BALF, which is a granular protein of the eosinophils. EPO is the most abundant protein of the secondary granules and generates potentially damaging ROS, including hypochlorous acid (Wang et al., 2006). We observed that OVA increased EPO release in airway, whereas DPI and NAC significantly decreased the EPO activity. In accordance with our result, evidence has shown that ROS plays an essential role in the release of granular proteins from eosinophils (Y. C. Lee et al., 2004; Nesi et al., 2017). Although DPI did not reduce eosinophil number, it decreased the EPO activity in BALF. DPI inhibits NADPH oxidase decreasing ROS production and, consequently, it impairs the

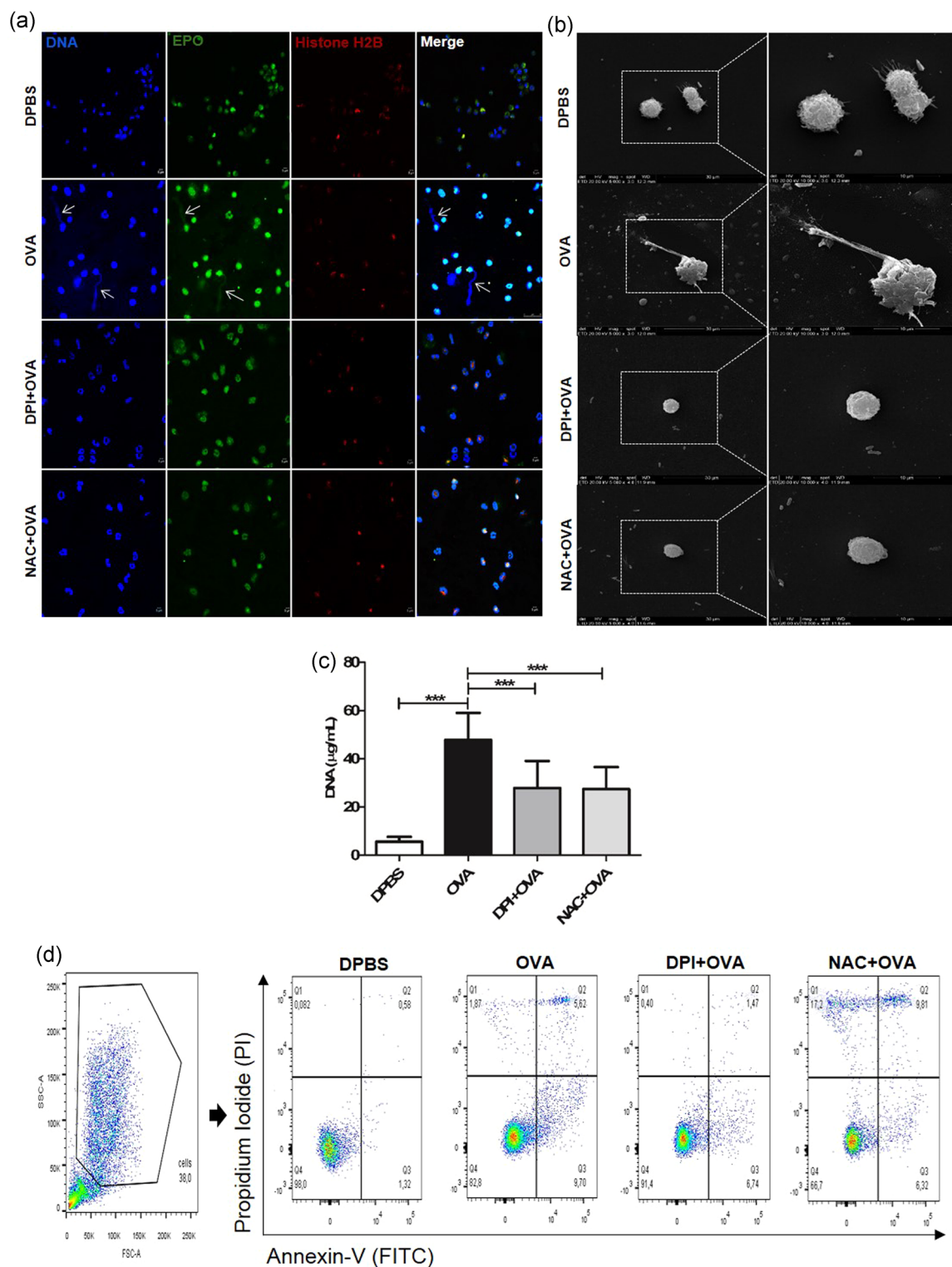


FIGURE 4 Continued.

release of granular proteins from eosinophils (Nesi et al., 2017). Later, in lung function evaluation, we observed an increase in tissue damping and tissue elastance in OVA-challenged mice. We did not observe any difference between the groups in airway resistance. In agreement to our result, Mori et al. (2017) also demonstrated that for resistance, there are no significant differences between an acute asthma model and the control group. NAC and DPI improved lung function in OVA-induced asthma model and this occurred probably because of a reduction in mucus viscosity and airway inflammation. NAC is an anti-inflammatory and a thiol antioxidant that breaks mucus, by substituting sulfhydryl groups by disulfide bonds, reducing mucus viscosity and improving lung function (K. S. Lee et al., 2007). In contrast, DPI is a NADPH oxidase inhibitor which is an enzyme responsible for ROS production. NADPH oxidase activation increases proinflammatory mediators overproduction such as cytokines (I. T. Lee & Yang, 2012), which may increase pathological modifications in airway of asthmatic mice.

In asthma, there is a production of high levels of inflammatory mediators such as proinflammatory cytokines (Kudo, Ishigatsubo, & Aoki, 2013). In the OVA group, we showed an increase of IL-5, IL-13, IFN- γ , TNF- α , and IL-1 β levels in lung tissue which contribute to airway inflammation, airway hyperresponsiveness (AHR), mucus hypersecretion, and airway remodeling. On the other hand, DPI and NAC decreased IL-5, IFN- γ , and IL-1 β levels in lung without modifying the concentration of IL-10. Therefore, we hypothesized that ROS inhibition could reduce inflammatory cytokines in lung via NF κ B p65 inhibition. In active NF κ B, subunits p50 and p65 translocate to the nucleus and increase the generation of proinflammatory cytokines but can also regulate ROS production (Oka, Kamata, Kamata, Yagisawa, & Hirata, 2000). We observe that OVA-challenged mice increased NF κ B p65 immunocent content whereas DPI and NAC significantly decreased NF κ B p65 in lung, demonstrating that ROS inhibition modulates the NF κ B p65 activity. In this context, NAC is a precursor of glutathione and evidence has shown that glutathione repletion suppresses transcription factors such as NF κ B which is involved in lung inflammation (Dodd, Dean, Copolov, Malhi, & Berk, 2008). Furthermore, proinflammatory stimuli induce ROS via activation of NADPH oxidase, and consequently, increase activation of NF κ B (Dodd et al., 2008). ROS may result from inflammation and, it may also initiate and augment inflammatory response.

It is well known that oxidative stress is involved in the immunopathogenesis of asthma (Henricks & Nijkamp, 2001). We observed that in the OVA group there was an increase in ROS production in lungs analyzed by the technique based on the oxidation of H2DCF-DA. Furthermore, the OVA group had a decrease in CAT and GPx, and SOD was not altered in lung, suggesting an unbalance in oxidant-antioxidant status. Unbalanced SOD/CAT ratio results in increase of hydrogen peroxide production and accumulation (Oliveira, 2015). The antioxidant enzyme SOD promotes the dismutation of superoxide in hydrogen peroxide, whereas CAT and GPx catalyze the reduction of hydrogen peroxide (da Cunha et al., 2011). We showed that an increase in ROS production did not enhance the consumption of SOD in lung. Thus, normal levels of SOD increase hydrogen peroxide formation and a decrease in CAT and GPx increase hydrogen peroxide accumulation in lungs, increasing oxidative stress. Moreover, we showed that DPI and NAC decreased ROS production in lung. However, we verified that only NAC increased the CAT activity and significantly improved SOD/CAT ratio compared to the OVA group. NAC is a scavenger of ROS and is metabolically converted to a glutathione precursor that is crucial for the antioxidant defenses and it may be used as a therapeutic alternative (Mokhtari, Afsharian, Shahhoseini, Kalantar, & Moioni, 2017). NAC decreased the consumption of CAT and reduced hydrogen peroxide accumulation in OVA-challenged mice, decreasing oxidative stress in airway. In contrast, DPI inhibits NADPH oxidase and consequently decreases superoxide formation. NADPH oxidase catalyzes the formation of superoxide by the one-electron reduction of oxygen, using NADPH as a reducing agent (Babior, 1999).

Studies have reported an association between mitochondrial injury and lung inflammatory diseases (Aguilera-Aguirre et al., 2009). Cells exposition to ROS can inactivate the mitochondrial ETC and, consequently, to decrease ATP production and increase ROS formation (Zmijewski et al., 2008; Zmijewski et al., 2009). Furthermore, the high flux of electrons through the mitochondrial ETC predisposes superoxide generation. The most important site of mitochondrial superoxide formation is in complex I, complex II, and complex III (Quinlan et al., 2012). In our study, we verified that in OVA-challenged mice ROS production increased and, consequently, decreased SDH, complex II, and complex IV. Moreover, we demonstrated that only the NAC-treated OVA group increased SDH and complex IV activities which significantly

FIGURE 4 EETs release depends on ROS production in an active process without cell death. (a) Immunofluorescence microscopy of EETs formation in BALF eosinophils (x630 magnification, scale bars = 5 μ m, arrows indicate EETs formation). Eosinophils obtained in BALF (2×10^5 /ml) were plated and incubated at 37°C with 5% carbon dioxide for 1 hr. After that, the cells were fixed with 4% PFA and incubated with primary antibodies, anti-EPO and anti-histone H2B (1:250). After this time, cells were incubated with the secondary antibodies, FITC anti-goat (1:100) and alexa fluor 633 anti-goat (1:100). Next, the cells were incubated with Hoechst 33342. (1:2,000). (b) Scanning electron microscopy of EETs formation in BALF eosinophils (scale bars = 10 and 30 μ m). BALF eosinophils (2×10^5 /ml) were placed and incubated for 1 hr at 37°C with 5% carbon dioxide. After that, cells were fixed with 2.5% glutaraldehyde. The samples were postfixed with osmium tetroxide solution 2%, followed by dehydration in graded acetone. The critical point technique with carbon dioxide was performed, followed by mounting on metallic support with carbon tape. The samples were covered with a thin layer of gold (metallization). (c) Quantification of extracellular DNA traps in BALF. Extracellular DNA was quantified in BALF supernatant using the Quant-iT dsDNA HS kit and measured in the Quibit 2.0 fluorimeter. (d) Analysis of cell death in BALF cells by Annexin V (x axis) and PI (y axis). BALF cells were incubated with Annexin V and PI for 15 min and analyzed by flow cytometry. Data represent the mean $\pm$ SD, $n = 20$ animals per group. *** $p < .001$ (one-way ANOVA followed by Tukey test). ANOVA, analysis of variance; BALF, bronchoalveolar lavage fluid; DPBS, dulbecco phosphate buffered saline; DPI, diphenyleneiodonium; EPO, eosinophil peroxidase; NAC, N-acetylcysteine; OVA, ovalbumin; PFA, paraformaldehyde; PI, propidium iodide [Color figure can be viewed at wileyonlinelibrary.com]

improved mitochondrial energy metabolism in lung. In this context, NAC protects mitochondrial function by promoting cysteine donation and maintaining glutathione levels. Glutathione is a crucial antioxidant which prevents or repairs oxidative damage generated in aerobic metabolism (Marí et al., 2013).

Na^+/K^+ -ATPase controls cellular ionic gradient, cellular membrane potential and its activity is vulnerable to ROS and to ATP depletion (Schweinberger et al., 2014). In consequence of ETC injury and oxidative damage, we observed that in the OVA group there is a decrease in the Na^+/K^+ -ATPase activity. On the other hand, DPI and NAC were not effective in restoring the levels of this enzyme. Reduction in Na^+/K^+ -ATPase activity enhances activation of leukocytes and causes downregulation of responses to β -agonists in β -adrenergic receptors (Agrawal et al., 2005; Lichtstein & Samuelov, 1982; Scheid, Honeyman, & Fay, 1979).

EETs formation is related to various pathologies, including allergic diseases (Dworski et al., 2011). In this context, studies have shown that EETs releases are present in lungs of asthmatic patients and in experimental asthma models (Cunha et al., 2014; Dworski et al., 2011). We showed in immunofluorescence microscopy analysis that BALF eosinophils from asthmatic mice released EETs colocalized with EPO. Increase in EETs production in asthma may contribute to lung obstruction by increasing the viscosity of mucus and it, consequently, may decrease lung function in asthma. Moreover, EETs have cytotoxic proteins as EPO that significantly contribute to the airway injury in asthma. Studies have shown that ROS production is essential for extracellular DNA traps release (Morshed et al., 2012; Yousefi et al., 2008). We showed that NAC and DPI treatments reduced EETs formation in BALF eosinophils in an experimental asthma model. Thus, our result demonstrates that ROS are required for EETs release in airway in an experimental asthma model. Scavenger NAC is a glutathione precursor and decreased ROS formation in lung and, consequently, decreased EETs formation. In contrast, DPI inhibits

NADPH oxidase and decreases ROS formation and, consequently, abrogated EETs formation suggesting that NADPH oxidase is required to EETs formation. In contrast to our results, Muniz et al. (2017) demonstrated that EETs release, after *Aspergillus fumigatus* fungus stimulation, is ROS independent. However, it is not known how ROS participates in EETs formation. In neutrophil extracellular traps (NETs) formation, ROS are required for elastase and myeloperoxidase release from azurophilic granules into the cytoplasm, a prerequisite for its translocation to the nucleus, contributing to chromatin decondensation (Galluzzi et al., 2012; Papayannopoulos, Metzler, Hakim, & Zychlinsky, 2010). We believe that in EETs formation, ROS increases eosinophils granular proteins release such as EPO, and this protein could contribute to EETs release in asthma. However, more studies are needed to investigate this mechanism. In EETs formation it is not clear whether DNA is of mitochondrial or nuclear origin. In our study, we showed that EETs released were not colocalized with histone H2B, which suggests that the DNA released by EETs is of mitochondrial origin. Histones compact and protect nuclear DNA, on the other hand, in mitochondrial DNA there are no histones (Alexeyev, Shokolenko, Wilson, & LeDoux, 2010). In this context, Yousefi et al. (2008) showed that EETs contained sequences of mitochondrial DNA. Brinkmann et al. (2004) showed that NETs formation is a process that involves cell death. Subsequent evidence has shown that DNA extracellular traps are not formed by cell death or lysis (Morshed et al., 2012; Yousefi et al., 2008). We observed that EETs release was not due to apoptosis or necrosis in BALF, as cell viability remained high in all groups, showing negative staining for annexin V and PI. In agreement to our result, previous studies of our group demonstrated that EETs release in asthma model was not due to apoptosis or necrosis either (Cunha et al., 2014, 2016).

Although DPI and NAC are ROS inhibitors, both caused different effects in an asthma model. DPI was not able to reduce eosinophils infiltrate in airway, increase antioxidant enzymes, and improve mitochondrial energy metabolism in lung. In contrast, NAC was

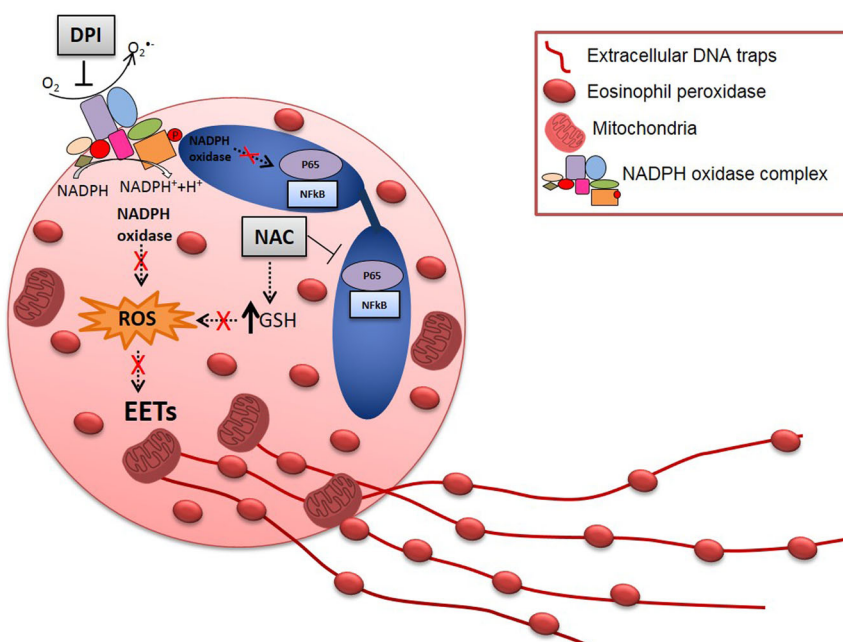


FIGURE 5 Summary of the effect of DPI and NAC in EETs formation in OVA-challenged mice. DPI, diphenyleneiodonium; NAC, N-acetylcysteine; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; EETs, eosinophil extracellular traps; GSH, glutathione [Color figure can be viewed at wileyonlinelibrary.com]

more effective to reduce pathological changes in experimental asthma model. NAC was able to reduce eosinophils infiltrate, increase antioxidant enzymes, and improve mitochondrial energy metabolism. However, both treatments were able to reduce EPO, globet cells, ROS, and EETs in lung. In our study, we evaluated the only EPO activity, which is an important eosinophil granular protein. However, other proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) may be important in EETs release; other studies should investigate these proteins in asthma. Thus, to exemplify our results, Figure 5 shows the effects of DPI and NAC in EETs formation in OVA-challenged mice.

In summary, this is the first study to report that viable eosinophils release EETs dependent on ROS in an experimental asthma model. In our study, DPI and NAC treatments improved airway inflammation, lung function, oxidative stress, and reduced EETs formation in asthmatic mice. Therefore, a decrease in ROS formation due to DPI or NAC treatments reduced EETs release in BALF, showing that ROS regulate the activation process of EETs release in asthma.

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CONFLICT OF INTERESTS

The authors declare that they have no commercial or financial conflict of interests.

AUTHOR CONTRIBUTIONS

J. S. S. and A. A. C. designed the studies. J. S. S., A. A. C., G. L. A., D. B. K., M. S., E. P. M., F. F., R. B. G., and R. V. B. contributed to the data collection. J. S. S., A. A. C., P. M. P., and A. T. W. analyzed and interpreted the work. J. S. S. and A. A. C. wrote the manuscript. All authors have read, revised and approved the final version of this manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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