

Activation of angiotensin-converting enzyme 2 (ACE2) attenuates allergic airway inflammation in rat asthma model

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ABSTRACT

Angiotensin-I converting enzyme (ACE) is positively correlated to asthma, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS) and is highly expressed in lungs. ACE2, the counteracting enzyme of ACE, was proven to be protective in pulmonary, cardiovascular diseases. In the present study we checked the effect of ACE2 activation in animal model of asthma. Asthma was induced in male wistar rats by sensitization and challenge with ovalbumin and then treated with ACE2 activator, diminazene aceturate (DIZE) for 2 weeks. 48 h after last allergen challenge, animals were anesthetized, blood, BALF, femoral bone marrow lavage were collected for leucocyte count; trachea for measuring airway responsiveness to carbachol; lungs and heart were isolated for histological studies and western blotting. In our animal model, the characteristic features of asthma such as altered airway responsiveness to carbachol, eosinophilia and neutrophilia were observed. Western blotting revealed the increased pulmonary expression of ACE1, IL-1 β , IL-4, NF- κ B, BCL2, p-AKT, p-p38 and decreased expression of ACE2 and I κ B. DIZE treatment prevented these alterations. Intraalveolar interstitial thickening, inflammatory cell infiltration, interstitial fibrosis, oxidative stress and right ventricular hypertrophy in asthma control animals were also reversed by DIZE treatment. Activation of ACE2 by DIZE conferred protection against asthma as evident from biochemical, functional, histological and molecular parameters. To the best of our knowledge, we report for the first time that activation of ACE2 by DIZE prevents asthma progression by altering AKT, p38, NF- κ B and other inflammatory markers.

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1. Introduction

Asthma is a chronic inflammatory disorder of the airways in which different cell types, in particular mast cells, eosinophils, T lymphocytes, neutrophils play a critical role (Nicklas, 1997; Patel, 2014). Asthma is a major cause of chronic morbidity and mortality throughout the world and its prevalence increased considerably over the past 20 years, especially in children (Stevenson and Birrell, 2011; Nagai, 2012; Ezeamuzie et al., 2014). Asthma is a condition characterized by persistent or recurrent airway inflammation, airway hyper-responsiveness (AHR), chronic airway remodeling and various degrees of reversible airflow limitation (Bousquet et al., 2000; Nials and Uddin, 2008; Blume and Davies, 2013). Airway remodeling means persistent changes in airway structure including sub-basement fibrosis, mucus hypersecretion, injury to epithelial cells, smooth muscle hypertrophy and angiogenesis (Global

Initiative for asthma Guidelines (GINA), National Asthma Education and Prevention Program (NAEPP), 2007) (DiGiovanni et al., 2009).

The renin angiotensin system (RAS) has been widely implicated in the pathophysiology of cardiovascular disorders. RAS operates via two counteracting arms - ACE1/Ang II/AT $_1$ axis and ACE2/Ang-(1-7)/Mas axis. ACE1 produces Ang II which causes vasoconstriction, proliferation, fibrosis, and inflammation through the activation of the AT $_1$ receptor (Shenoy et al., 2013). ACE2 is major Ang-(1-7)-forming enzyme as it converts Ang II to Ang-(1-7) with 500 folds greater affinity. Ang (1-7) is endogenous ligand for the G protein-coupled receptor Mas and promotes vasodilatation, anti-proliferation, baroreflex sensitivity, potentiation of bradykinin activity and AT $_1$ receptor antagonism (Warner et al., 2007). RAS plays a critical role in pathogenesis of pulmonary hypertension, pulmonary fibrosis in chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS) (Kuba et al., 2006; Kaparianos and Argyropoulou, 2011). An increased expression of ACE2 and Ang-(1-7) with ACE1 inhibitors (ACEi) or Angiotensin receptor blockers (ARBs) indicates their beneficial role in degrading Ang II locally (Ferreira et al., 2010). ACE2 knockout worsened bleomycin – induced acute lung injury and rhACE2 abrogated

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it (Rey-Parra et al., 2012). Taken together, ACE2 might be a potential target for the treatment of asthma.

Diminazene aceturate (DIZE) is the most commonly used therapeutic agent for trypanosomiasis in domestic livestock. It is thought to inhibit DNA replication and mitochondrial type II topoisomerases, but as an off-target effect, it increases the activity of the ACE2-mediated angiotensin conversion and this is considered the actual mechanism behind its beneficial effects (Kulemina and Ostrov, 2011). DIZE treatment has been shown to prevent pulmonary hypertension (Shenoy et al., 2013). Here we have hypothesized that the activation of the protective arm of the RAS by novel ACE2 activator – DIZE may attenuate various characteristics of asthma in rat asthma model.

2. Materials and methods

2.1. Animals

All the experiments were performed on male adult Wistar rats (200–260 g; 8–9 weeks old). The animals were procured from Central Animal Facility, National Institute of Pharmaceutical Education and Research (NIPER), S.A.S. Nagar, India. The housing of the animals was done as three animals per cage in a room with controlled ambient temperature ($22 \pm 2^\circ\text{C}$), humidity $50 \pm 10\%$ and 12 h light/dark cycle. Feed (Pranav Agro Industries, New Delhi) and water were provided *ad libitum*. All experimental protocols were approved by Institutional Animal Ethics Committee (IAEC no: 13/26) and were performed in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines.

2.2. Experimental protocol

Till 27 days, two groups of animals – normal control and asthma control were there. From the 28th day, i.e. the day from which treatment was started, animals were divided into following five groups ($n = 6$) (Fig. 1): 1) NC – Normal control rats sensitized and challenged with phosphate buffer saline and treated with 0.9% NaCl, i.p. 1 h before ovalbumin challenge, 2) AC – Asthma control rats sensitized and challenged with ovalbumin and treated with 0.9% NaCl, i.p. 1 h before ovalbumin challenge. 3) LD, 4) MD, 5) HD – Asthmatic rats treated with Diminazene Aceturate at 7.5 (low), 15 (medium) and 25 (high) $\text{mg}\cdot\text{kg}^{-1}$ doses respectively. Diminazene aceturate (DIZE) was dissolved in water and administered at 7.5, 15 and 25 $\text{mg}\cdot\text{kg}^{-1}$ dose i.p., 1 h before ovalbumin challenge from 28 to 42 day daily. In airway responsiveness and histopathology study, we got promising results at 15 $\text{mg}\cdot\text{kg}^{-1}$ dose of DIZE. Hence for the subsequent western blotting

and oxidative stress measurements, only 15 $\text{mg}\cdot\text{kg}^{-1}$ dose group was used.

2.3. Animal sensitisation and challenge

Male wistar rats (200–260 g at the beginning of sensitization) were sensitized on experiment days 0, 7, 14 and 21 with ovalbumin + aluminium hydroxide adjuvant (1 + 20 mg i.p.). The aluminium hydroxide acts as adjuvant to promote IgE production (Belvisi et al., 2005). Ovalbumin challenge was performed from 21 to 42 days thrice a week (Fig. 1). In this study, animals were exposed through flow-directed Nose Only Exposure chamber (CH technologies, USA). BANG (bio aerosol nebulizer generator) was used for generating ovalbumin (1% w/v solution) (grade V; Sigma-Aldrich, MO, USA; diluted in phosphate buffer saline) aerosol by plunging air at high pressure of 170 kPa through nozzle and primary airflow rate of 600 L h^{-1} was drawn through chamber, which was regularly measured and controlled by air flow meter (Alicat Scientific, AZ, USA). The aerosol was moved forward by a continuous air flow of 2000 L h^{-1} . Each animal was placed in a special restrainer to guarantee nose only exposure for 1 h, resulting in an aerosolized ovalbumin volume of about 20 mL (Bundschuh et al., 2001).

2.4. Bronchoalveolar and femoral marrow lavage

Forty-eight hours following the last allergen challenge, animals were anesthetized with a mixture of ketamine and xylazine ($60\text{ mg}\cdot\text{kg}^{-1}$ and $6\text{ mg}\cdot\text{kg}^{-1}$, respectively; i.p.), the trachea was exposed and the rats were placed with the head elevated 30° from the horizontal plane. Next, a 16-gauge cannula was inserted into the trachea and lungs were gently rinsed and aspirated twice with 0.5 mL of saline. Fluid collected was centrifuged (1000 rpm for 8 min at 4°C) and supernatant was frozen and stored at -80°C until assayed. Pellet was resuspended in 100 μL PBS and this bronchoalveolar lavage fluid (BALF) was used for total and differential cell count by automatic hematoanalyser. Femoral marrow lavage (FML) and peripheral blood analysis for total WBC count and differential cell count were also performed.

2.5. Blood collection

Forty-eight hours after last allergen challenge, about 400 μL blood was collected from tail vein. 100 μL of whole blood was used for total & differential cell count by automatic hematoanalyser (Melet Schloesing, France). Remaining blood was immediately centrifuged ($2500 \times g$; 5 min, 4°C) and plasma was stored at -80°C until further assayed.

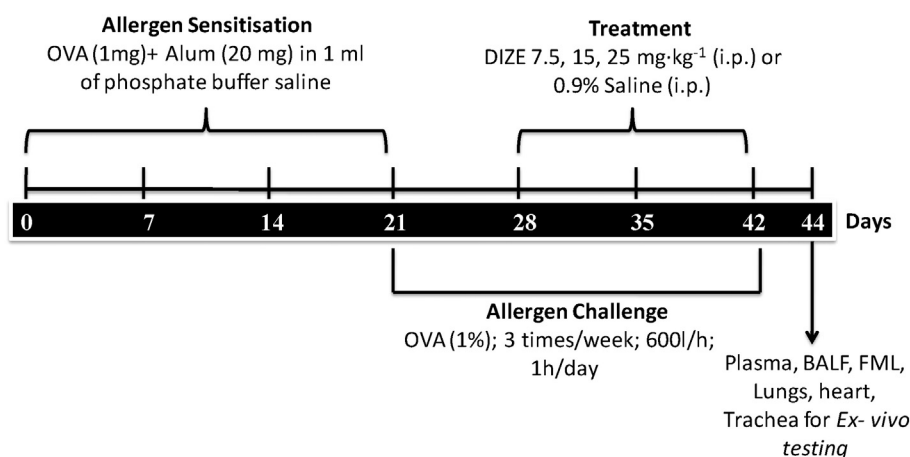


Fig. 1. Experimental protocol. Rats sensitized on experiment days 0, 7, 14 and 21 with ovalbumin (1 mg i.p.) administered with aluminium hydroxide adjuvant (20 mg i.p.). Ovalbumin challenge was performed from 21 to 42 days after the beginning of sensitization three times a week. The animals were killed 48 h after the last challenge.

2.6. Lung and heart isolation

Forty-eight hours following the last inhalation, part of the animals were anesthetized (i.p.) with the mixture of ketamine (60 mg kg⁻¹) and xylazine (6 mg kg⁻¹). A midline neck incision was made and the trachea was exposed. The trachea was then clamped and the lungs were collected in functional residual capacity. In some animals, the left lung and heart were collected for histological analysis and the right lung was removed, snap froze in liquid nitrogen and kept at -80 °C for western blotting. The tracheas of the animals were used to evaluate airway responsiveness.

2.7. Airway responsiveness test

Forty eight hours following the last challenge with ovalbumin, animals were sacrificed under ketamine-xylazine anaesthesia. Trachea of each animal was removed and placed in ice-cold oxygenated Krebs-Henseleit solution (KHS). Trachea was properly freed from adhering adventitious tissue. Care was taken not to stretch the trachea or damage the C-shaped rings while cleaning. Intact tracheal rings (3–4 mm in length) were cut from the cleaned trachea. KHS comprised of (in mmol·L⁻¹): NaCl-118; KCl-4.7; MgSO₄·7H₂O-1.2; CaCl₂·2H₂O-2.6; KH₂PO₄-1.2; NaHCO₃-25; Glucose-5.5; pH 7.4. Tracheal rings were carefully mounted with help of stainless steel hooks on the isometric force transducer in water-jacketed organ bath chamber. A resting tension of 1 g was applied to tracheal ring. Each ring was allowed to equilibrate in vertical chambers (25 mL) (Harvard Apparatus) filled with KHS (37 °C) bubbled with carbogen (5% CO₂ + 95% O₂) for 40 min. KHS was changed every 15 min during this equilibration period. At the beginning of each experiment, tracheal rings were primed with 80 μmol L⁻¹ KCl (depolarizing solution) to check the viability of tissue. Responses to graded doses of carbachol (10⁻¹⁰ to 10⁻⁵ mol L⁻¹ of KHS) were recorded using an isometric force transducer (ADInstruments, Australia), which was fed to an amplifier-data acquisition system (PowerLab 8/35, ADInstruments, Australia) and visualized on a personal computer.

At the end of experiment, tracheal rings were blotted dry and their lengths and weights were measured to calculate tension normalized to cross-sectional area (mg mm⁻²) using following formula:

$$\text{Cross-sectional area (mm}^2\text{)} = \frac{\text{Weight (mg)}}{\text{Length (mm)} * \text{Density (mg} \cdot \text{mm}^{-3}\text{)}}$$

(Density of vascular smooth muscles of trachea wall: 2.46 mg mm⁻³) Carbachol (Sigma-Aldrich, MO, USA) responses to different concentration were expressed in terms of mg·mm⁻².

2.8. Measurement of cytokine

The concentration of cytokine, interleukin IL-4 in BALF (Jarajapu et al., 2013) and plasma were measured using commercially available ELISA kit as per the manufacturer's instructions (Sigma-Aldrich, MO, USA).

2.9. Nitrite, GSH and MDA determination

All animals were sacrificed at the end of study after BALF collection and lung artery was perfused with PBS to remove blood. Tissue homogenates were prepared with 0.1 M Tris-HCl-EDTA buffer (pH 7.4), and supernatant of homogenates was employed to estimate nitric oxide in the form of nitrite (μM g⁻¹ of protein) (Bitar et al., 2005), total protein, MDA (μM mg⁻¹ protein) (Tikoo et al., 2011) and GSH (nM mg⁻¹ of protein).

2.10. Airway and heart histology

Lungs and heart from all animals were removed and immersed in 10% formalin and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin (H & E) and examined for pathological changes under light microscopy (El-Hashim et al., 2012). Another set of lung sections were also stained with picosirius red to evaluate fibrillar collagen and connective tissue matrix depositions. Three sections from each rat were assessed. A semiquantitative four-level lung pathology score was used to grade the extent of abnormalities in each microscopic field at 400×. The grading scale is as follows: 0-normal histology, 1-minimal changes, 2-moderate changes, 3-severe changes. Heart hypertrophy was evaluated by counting no. of nuclei in each HPF (high power focus).

2.11. Western blotting

Protein isolation and western blotting was performed as previously described (Karpe et al., 2012). Briefly, frozen lung tissues were thawed, minced and homogenized in lysis buffer containing surfactants, protease and phosphatase inhibitors. Protein samples were resolved using 8% and 10%; sodium dodecyl sulphate-polyacrylamide gels depending on the molecular weight of desired proteins. These were then transferred to nitrocellulose membranes and were analysed with below mentioned antibodies (SantaCruz Biotechnology, Inc., CA, USA). Blotting was divided into three sections: (a) RAS Components: Angiotensin-I converting enzyme I (ACE1), Angiotensin Converting Enzyme II (ACE2) (b) Inflammatory components: NF-κB and its phosphorylated form, IκB and IL-1β. (c) MAP Kinase signalling: p38 mitogen activated protein kinase (p38) and its phosphorylated form (p-p38); phosphorylated protein kinase B (AKT). The antigen-primary antibody complexes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using enhanced chemiluminescence substrate (Invitrogen, CA, USA) and ECL hyperfilm (GE Healthcare Pvt. Ltd., UK). Blots were scanned and analysed using ImageJ software (NIH, USA). β-actin, α-tubulin or corresponding total protein served as internal standards to normalize the results.

3. Statistical analysis

All the data were expressed as mean ± SEM. Means of all groups were compared by one-way analysis of variance (ANOVA) followed by the Tukey's post-test, unless otherwise specified, using GraphPad Prism (v5.01) (GraphPad Software, Inc., CA, USA). The level of significance was set to *P* < 0.05.

4. Results

4.1. Diminazene aceturate improves morphometry

As compared with control rats, significant loss in body weight was observed in ovalbumin sensitized and challenged (asthmatic) rats. This loss in body weight was prevented by treatment with diminazene aceturate (DIZE). As compared to control rats, asthmatic rats showed significant increase in lungs to body weight ratio and treatment of asthmatic rats with DIZE restored lungs to body weight ratio and prevented airway remodelling. Though absolute heart weight decreased in asthma rats, relative heart weight remained unaffected in both asthmatic and the treated rats (Table 1).

4.2. Restoration of airway responsiveness by diminazene aceturate

Parasympathetic activation leads to contraction of smooth muscles of airway through muscarinic receptors. So we generated cumulative concentration-response curves with carbachol (10⁻¹⁰ to 10⁻⁵ M), which is a muscarinic receptor agonist to get an insight about function

Table 1
Morphometry.

Parameter	NC	AC	DIZE
BW change (%)	15.78 ± 1.304	−9.5 ± 2.8***	0.033 ± 2.101 [#]
Absolute lung weight (g)	2.23 ± 0.10	2.59 ± 0.11	2.28 ± 0.11
Lung weight/BW (mg g ^{−1})	7.63 ± 0.4	11.3 ± 0.49***	8.95 ± 0.049 ^{##}
Absolute heart weight (g)	0.94 ± 0.03	0.79 ± 0.02*	0.83 ± 0.03
Heart weight/BW (mg g ^{−1})	3.2 ± 0.10	3.47 ± 0.10	3.27 ± 0.10

NC, normal control; AC, asthma control; DIZE, ovalbumin-challenged, DIZE (15 mg kg^{−1}; i.p.) – treated rats. BW, body weight. One-way ANOVA followed by Tukey's test. Each data point is represented as mean ± SEM, n = 6 rats/group.

* $P < 0.05$.

*** $P < 0.001$ vs. NC.

[#] $P < 0.05$.

^{##} $P < 0.01$ vs. AC.

and structural alterations of airways (Rodrigues-Machado et al., 2013). There was a significant attenuation of contraction in asthma rats (Emax: 1.388 ± 0.22 mg mm^{−2} vs. Emax: 5.535 ± 0.43 mg mm^{−2} in NC). Asthmatic animals treated with ACE2 activator, diminazene aceturate (DIZE) mitigated this aberrant response. Asthmatic animals treated with low dose (7.5 mg kg^{−1}; i.p.) of DIZE showed slight increase (Emax: 2.967 ± 0.49 mg mm^{−2}) as compared to asthmatic rats in contractile response while medium (15 mg kg^{−1}; i.p.) and high dose

(25 mg kg^{−1}; i.p.) of DIZE led to significant increase in contractile response (Emax: 4.429 ± 0.39 mg mm^{−2} & 4.786 ± 0.47 mg mm^{−2} respectively) (Fig. 2B).

pD₂ value is the negative logarithm of concentration of drug required to occupy and activate 50% of receptors required for response. pD₂ value remained unchanged in all the groups indicating that there is no alteration in affinity for the receptors (Fig. 2C).

4.3. Restoration of cell count in BALF, FML and blood by treatment with diminazene aceturate

Asthmatic rats on one hand showed significant surge in total WBC, eosinophils, neutrophils, monocytes counts and on the other hand a significant fall was observed in lymphocyte count. Treatment of asthmatic rats with DIZE restored cell counts in BALF. Similar results, in many but not in all cell types, were observed in case of femoral marrow lavage (FML) and peripheral blood (Suppl. Tables 1–3).

4.4. Diminazene aceturate restores nitric oxide availability in lungs

Nitric oxide (NO) is an autocrine and paracrine signalling molecule that exerts both beneficial and detrimental effects on airway smooth muscle tone and inflammation/remodelling (Silkoff et al., 2000; Keller

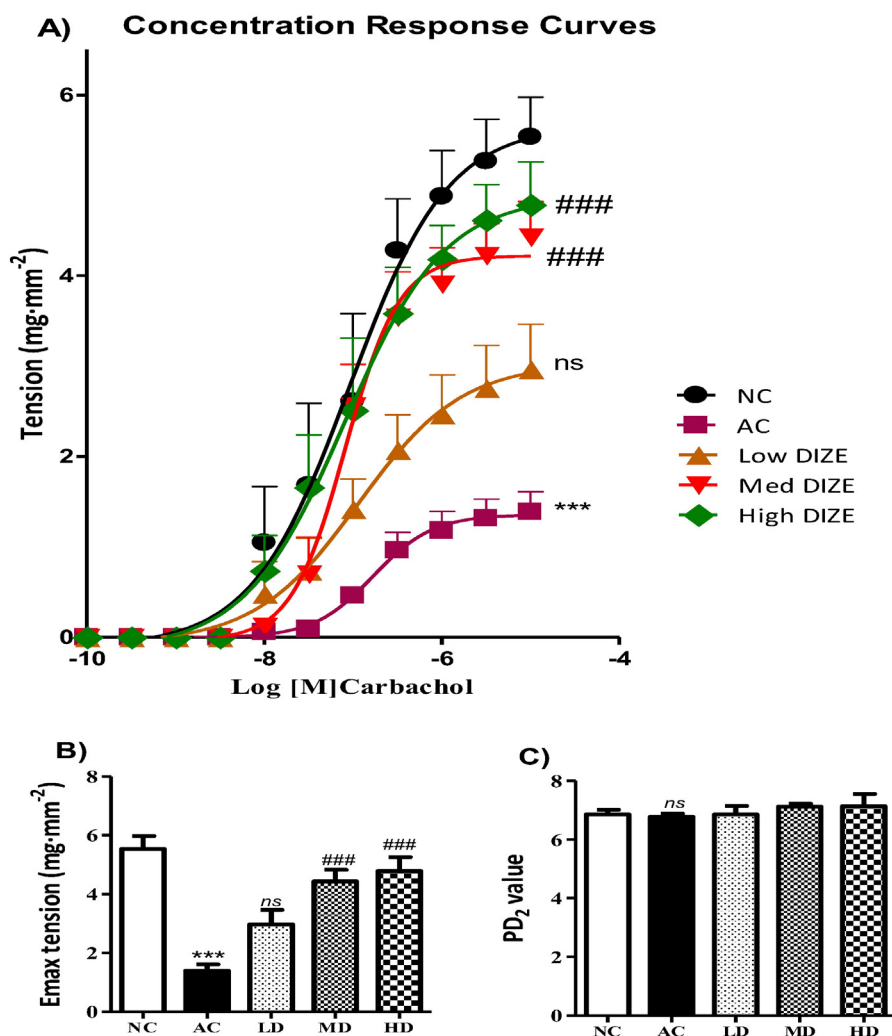


Fig. 2. Airway responsiveness with carbachol ex vivo. (A) Cumulative concentration response curves (CRC) of carbachol (10^{-10} to 10^{-5} M) in rings (3–4 mm) isolated from trachea of rats. One-way ANOVA followed by Bonferroni post-test. (B) Emax (mg mm^{−2}) of the CRCs. (C) pD₂ values. One-way ANOVA followed by Tukey's post-test. NC, normal control; AC, asthma control; LD, MD, HD are ovalbumin-challenged, DIZE (7.5, 15, 25 mg kg^{−1} respectively; i.p.) – treated rats. Each data point is represented as mean ± SEM, n = 6 rats/group. *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. AC; ns, non-significant.

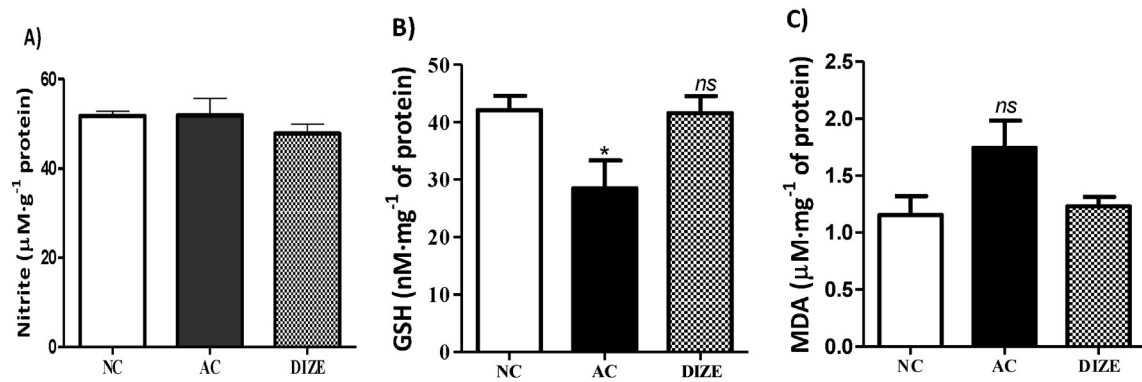


Fig. 3. Oxidative stress markers. (A) Nitrite ($\mu\text{M}\cdot\text{g}^{-1}$ protein), (B) GSH ($\text{nM}\cdot\text{mg}^{-1}$ protein), (C) MDA ($\mu\text{M}\cdot\text{mg}^{-1}$ protein) in lung homogenate of NC, normal control; AC, asthma control; DIZE, ovalbumin-challenged, DIZE (15 mg kg^{-1} ; i.p.) – treated. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, $n = 6$ rats/group. * $P < 0.05$ vs. NC; ns, non-significant.

et al., 2005). So, nitrite, the stable oxidation product of NO, was estimated to quantify the nitric oxide produced by lungs (Hussain et al., 2012). We failed to observe any change in the level of nitrite ruling out the role of nitric oxide in airway responsiveness and remodelling in our setting (Fig. 3A).

4.5. Oxidative stress is reduced by diminazene aceturate treatment

MDA and GSH were determined to quantify pulmonary oxidative stress. There was a significant decrease in GSH ($32.56 \pm 8.31\text{ nM mg}^{-1}$ protein) (Fig. 3B) and a small increase in MDA ($1.664 \pm 0.21\text{ }\mu\text{M mg}^{-1}$ protein) (Fig. 3C) in asthmatic rats as compared to control rats ($42.14 \pm 2.22\text{ nM mg}^{-1}$ protein and $1.241 \pm 0.15\text{ }\mu\text{M mg}^{-1}$ protein respectively). Treatment with DIZE restored MDA ($1.588 \pm 0.361\text{ }\mu\text{M mg}^{-1}$ protein) and GSH ($39.73 \pm 2.78\text{ nM mg}^{-1}$ protein). Oxidative stress leads to inflammation and apoptosis in asthma (Li and Nel, 2006).

4.6. Diminazene aceturate treatment palliates airway and vascular remodelling

Lung sections from normal control rats consistently showed normal histology with hematoxylin & eosin and picosirius red stains. As seen in Fig. 4A, the normal control group exhibited lung parenchyma with normal alveolar septa and bronchiolar wall thickness as well as aerated alveoli. The intra-acinar vessels exhibited a normal morphology. In contrast, the ovalbumin – sensitized and challenged (asthmatic group) group exhibited large interalveolar interstitial thickening, resulting mainly from the accumulation of inflammatory cells. Bronchiolar wall thickening was observed, mainly due to hypertrophy and hyperplasia of the bronchial epithelium and smooth muscle cell layer. Moreover, a reduction in the alveolar lumen was also observed (Fig. 4B). Fig. 5B shows severe perivascular and peribronchial fibrosis suggesting airway remodelling in asthmatic group. In DIZE-low dose treated group, the lung parenchyma exhibited reduced inflammation in the interalveolar

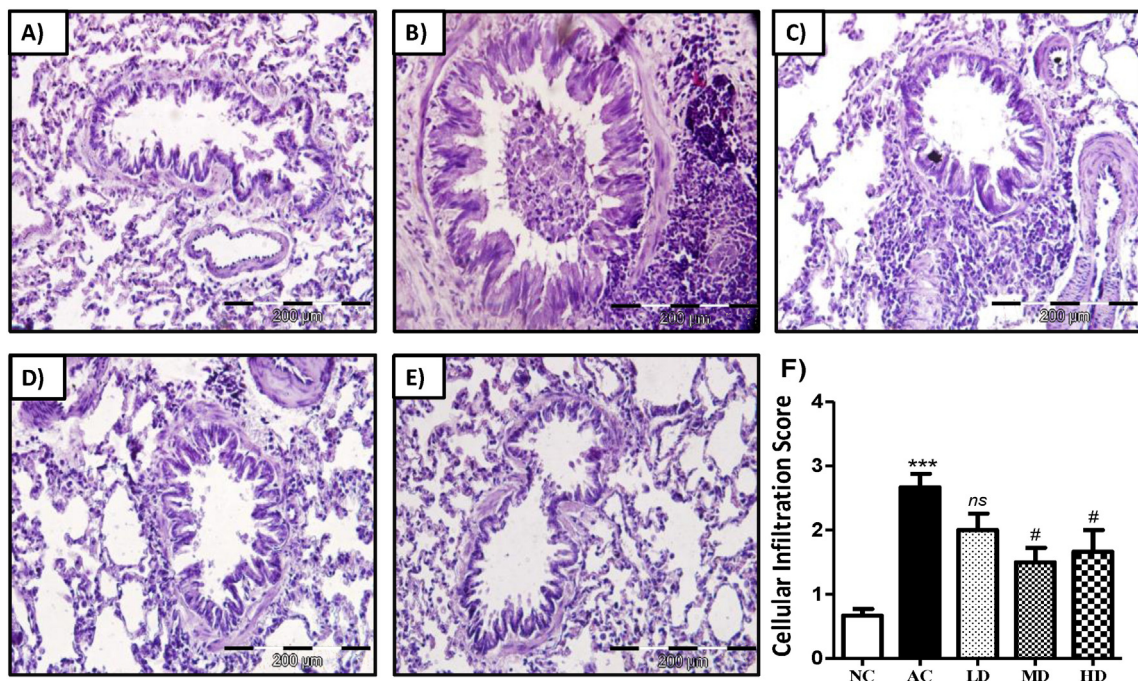


Fig. 4. Representative low-magnification light photomicrographs display H&E staining of whole lung. (A) Normal Control, (B) Asthma control, (C) ovalbumin-challenged, DIZE (7.5 mg kg^{-1} ; i.p.) treated, (D) ovalbumin-challenged, DIZE (15 mg kg^{-1} ; i.p.) treated (E) ovalbumin-challenged, DIZE (25 mg kg^{-1} ; i.p.) treated rats. Quantification of histological cellular infiltration was done by cellular infiltration score (F). One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, $n = 6$ rats/group. *** $P < 0.001$ vs. NC; * $P < 0.05$, ns, non-significant vs. AC.

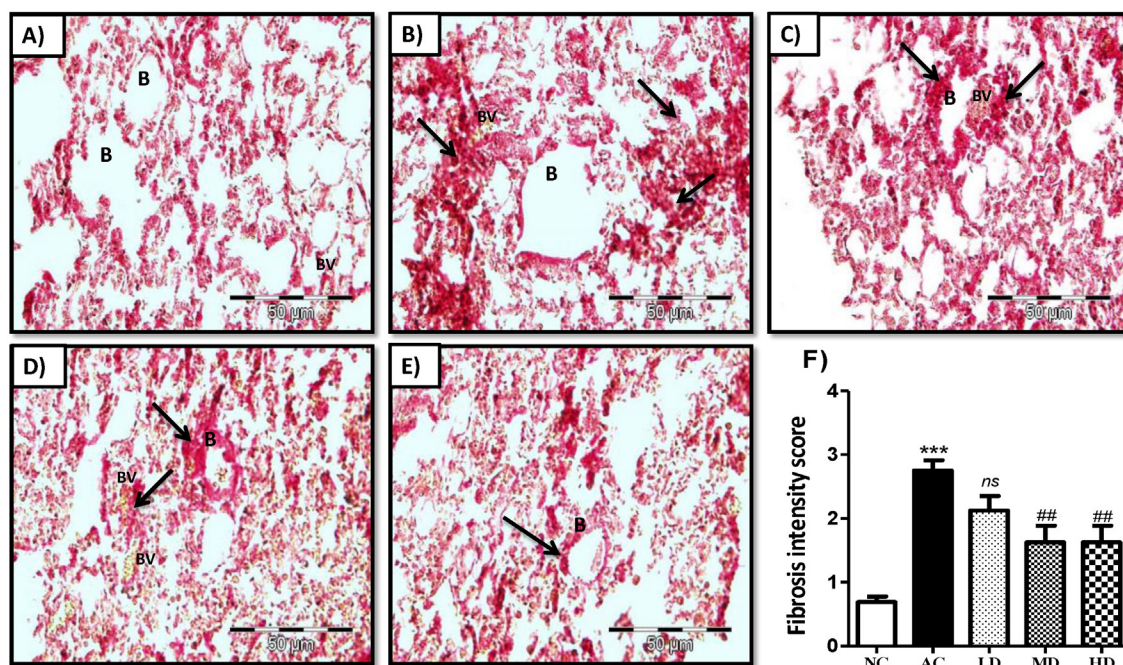


Fig. 5. Representative low-magnification light photomicrographs displayed picosirius red staining lung. (A) Normal Control, (B) Asthma control, (C) ovalbumin-challenged, DIZE (7.5 mg kg^{-1} ; i.p.) treated, (D) ovalbumin-challenged, DIZE (15 mg kg^{-1} ; i.p.) treated (E) ovalbumin-challenged, DIZE (25 mg kg^{-1} ; i.p.) treated rats. (F) Quantification of histological perivascular and peribronchial fibrosis was done by fibrosis intensity score. B, bronchus; BV, blood vessel. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, $n = 6$ rats/group. *** $P < 0.001$ vs. NC; # $P < 0.05$, ns, non-significant vs. AC.

space, with the normal appearance of alveolar lumen and bronchioles (Fig. 4C). Morphometric analyses revealed that treatment with medium and high dose of DIZE completely reversed airway and vessel

remodelling in asthmatic rats (Fig. 4D–E). DIZE treatment ameliorated perivascular and peribronchial inflammatory cell infiltration and perivascular and peribronchial fibrosis significantly (Fig. 5D–E).

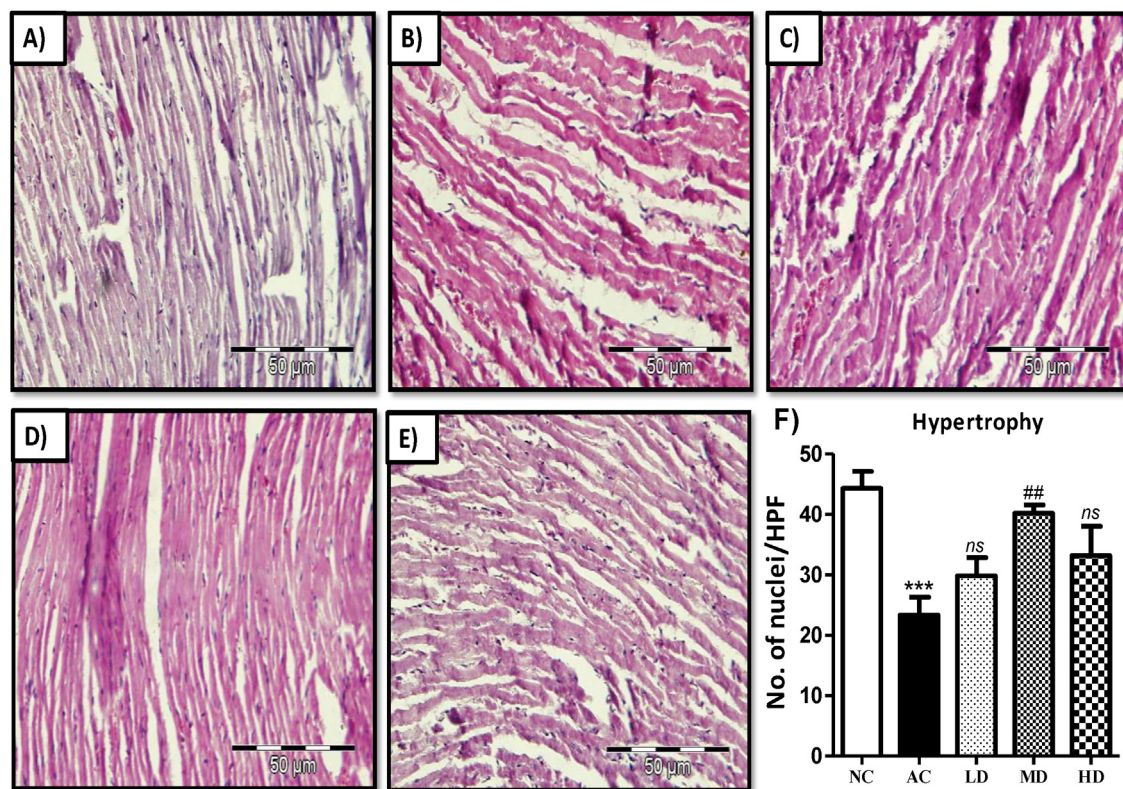


Fig. 6. Representative photomicrographs of Hematoxylin & Eosin (H&E) stained heart. (A) Normal Control, (B) Asthma control, (C) ovalbumin-challenged, DIZE (7.5 mg kg^{-1} ; i.p.) treated, (D) ovalbumin-challenged, DIZE (15 mg kg^{-1} ; i.p.) treated (E) ovalbumin-challenged, DIZE (25 mg kg^{-1} ; i.p.) treated rats. (F) Left ventricular hypertrophy was quantified by counting no. of nuclei per high power focus (HPF) and cardiomyocyte diameter. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, $n = 6$ rats/group. *** $P < 0.001$ vs. NC; ## $P < 0.01$ vs. AC; ns, non-significant.

4.7. Ventricular hypertrophy is assuaged by diminazene aceturate

Asthma is likely to be associated with vascular remodelling, which increases resistance to the blood flow from heart thereby posing burden on the right ventricle (Said et al., 2010). So, we thought of checking the architecture of right ventricle of asthmatic rats. Asthmatic rats exhibited a significant enhancement in myocyte diameter and drop in nucleus count of the right ventricle in comparison to control rats suggesting that structural remodelling in airways led to an increase in pulmonary vascular resistance and subsequent right ventricular hypertrophy (Fig. 6A and B). Asthmatic animals treated with DIZE mitigated the aggrandized cardiomyocyte diameter and cratered nucleus count as compared to control asthmatic rats (Fig. 6D and E).

4.8. Altered ACE1 and ACE2 expression is restored by diminazene aceturate

Ang II/AT1R and Ang-1-7/Mas receptor are involved in the airway inflammation and remodelling associated with chronic asthma (Wang et al., 2008; El-Hashim et al., 2012). Hence, immunoblotting was performed to check any alteration in the protein levels of various RAS components like AT₁, AT₂, ACE1 and ACE2. There was no change in the expression of AT₁ and AT₂ receptors (Data not shown). However, ACE1 expression was found to be upsurged and ACE2 expression decreased significantly in asthmatic rats as compared to that of normal control rats. This increased expression in asthmatic condition clearly shows that ACE1 aggravates the asthmatic condition. Treatment of asthmatic rats with DIZE restored the expression of ACE1 and ACE2 (Fig. 7).

4.9. Diminazene aceturate treatment alleviates inflammation and apoptosis

As asthma is an inflammatory condition in which various inflammatory mediators like IL-4, IL-1 β , IL-6, TGF- β , I κ B and NF- κ B etc. play a crucial role, we measured certain inflammatory components (IL-1 β , NF- κ B, I κ B). In line with the level of IL-4 in plasma and BALF of asthmatic rats (Suppl. Table 4), inflammatory cytokine IL-1 β , inflammatory transcription factor NF- κ B escalated and I κ B, the constitutive inhibitor of NF- κ B, decreased significantly in lungs of asthma and DIZE treatment normalized all of them both at tissue level (Fig. 8) and biochemical level in BALF and plasma (Suppl. Table 4). Anti-apoptotic BCL₂ diminished in asthma and DIZE treatments restituted its level in lungs (Fig. 8).

4.10. Diminazene aceturate represses cytosolic signal transducers

It has been reported that various pathological events in case of allergic inflammation are triggered by autacoids (e.g., leukotrienes), cytokines, chemokines and growth factors whose production and biological effects depend at least in part on activation of MAPKs. It is also reported that protective arm of RAS system i.e. ACE2/ANG (1-7)/Mas receptor axis produces its beneficial effects by decreasing phosphorylation of various MAP kinases like AKT, C-Jun N-terminal kinase (JNK) and p38. So, we carried out immunoblotting of various MAP kinases to find out their basal expression in asthmatic condition. We found that there was significant increase in expression p-AKT and p-p38, but not p-JNK (Data not shown) in asthmatic rats. This increased expression was prevented by treatment with DIZE (Fig. 9B–C).

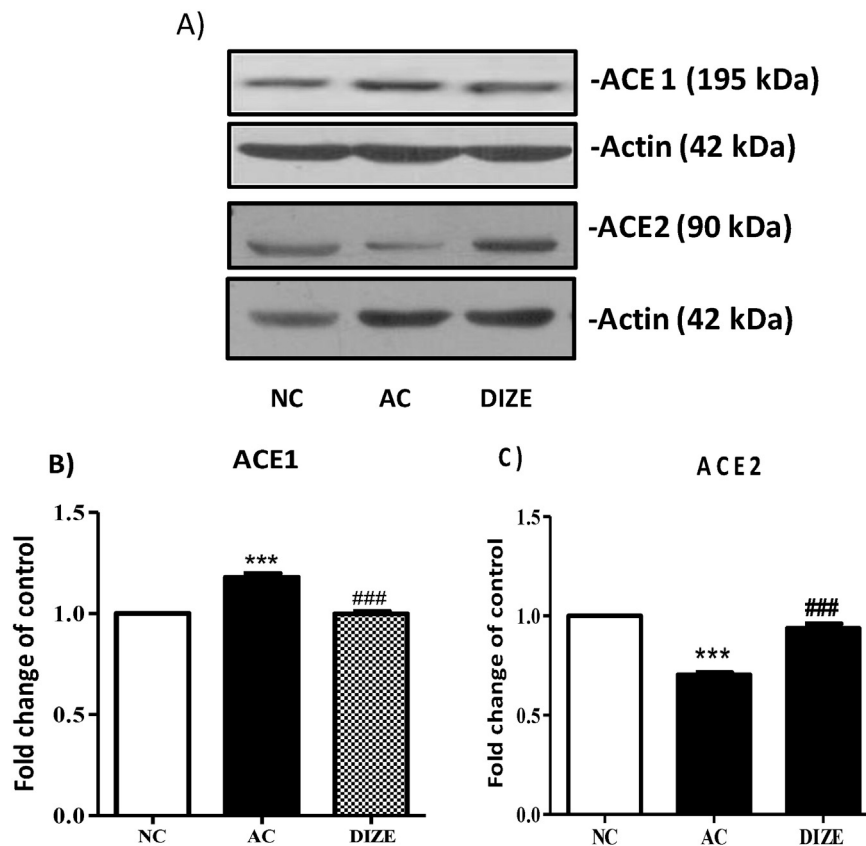


Fig. 7. Pulmonary RAS components — ACE1 and ACE2. Representative blots (A) and densitometric quantification of at least three independent experiments ($n = 3$) showing relative levels of ACE1 (B) (normalized to β -actin) and ACE2 (C) (normalized to β -actin). NC, normal control; AC, asthma control; DIZE, ovalbumin-challenged, DIZE (15 mg kg⁻¹, i.p.) treated rats. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, $n = 3$. *** $P < 0.001$ vs. NC; ### $P < 0.0001$ vs. AC.

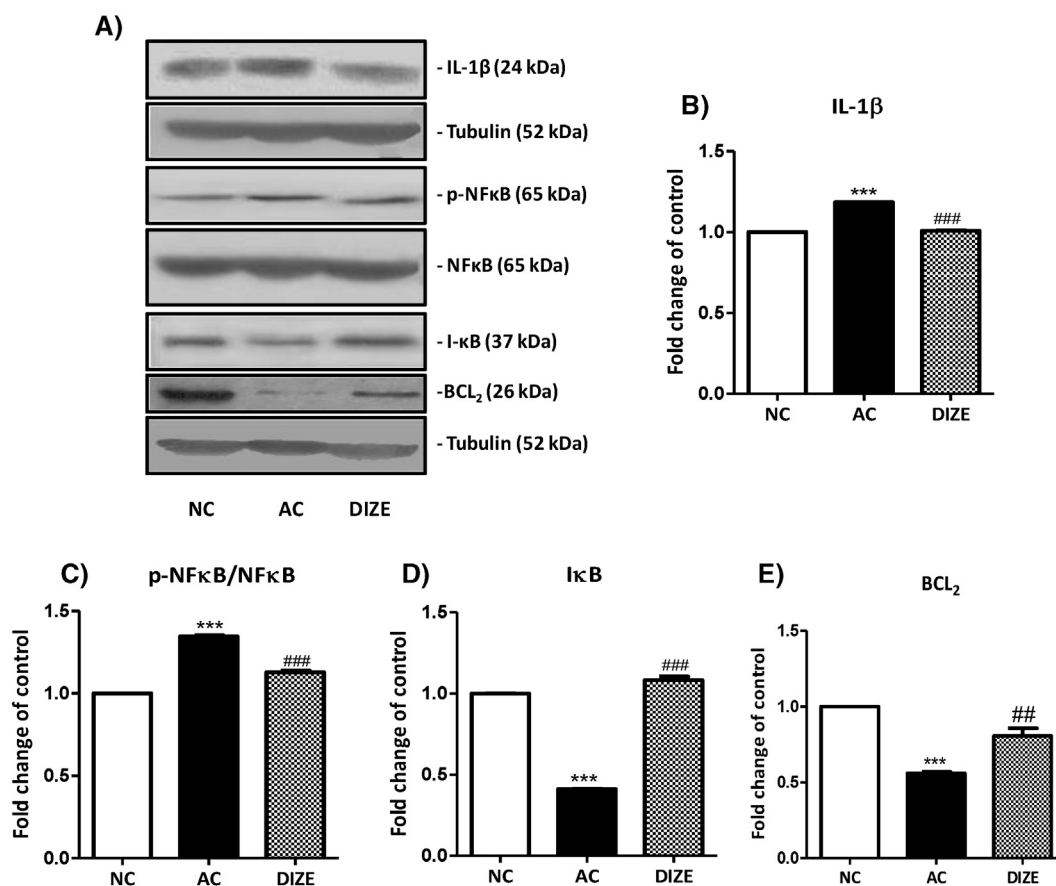


Fig. 8. Effect of DIZE on inflammatory and apoptotic mediators- IL-1 β , I κ B, NF- κ B and BCL $_2$ protein levels in lungs. Representative blots (A) and densitometric quantification of at least three independent experiments ($n = 3$) showing relative levels of IL-1 β (normalized to α -tubulin) (B), p-NF- κ B (normalized to total NF- κ B) (C), I κ B (normalized to α -tubulin) (D) and BCL $_2$ (normalized to α -tubulin) (E). NC, normal control; AC, asthma control; DIZE, ovalbumin-challenged, DIZE (15 mg kg $^{-1}$; i.p.) treated rats. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM ($n = 3$). *** $P < 0.001$ vs. NC; ## $P < 0.01$, ### $P < 0.001$ vs. AC.

5. Discussion

In the present study, we provide evidence that diminazene aceturate (DIZE), an ACE2 activator, corrects the airway inflammation, remodeling, obstruction and responsiveness associated with asthma. The characteristic features of asthma are airway hyperresponsiveness (AHR), eosinophilia, edema, increased mucus production, bronchial inflammation and increased immunoglobulin E levels (Tipnis et al., 2000). Along with the eosinophils and mast cells, T helper type-2 (TH2) cells also contribute to initiate and develop allergic asthma. Despite advances in the recognition of key cellular and molecular mechanisms involved in asthma, we still lack definitions regarding prevention, treatment or the best time to initiate asthma treatment (Durrani et al., 2011).

It has been already reported that the immune inflammatory disorder like asthma is associated with the decreased body weight of ovalbumin-induced hyperresponsive rats (Kandhare et al., 2013). In the present study, rats challenged with ovalbumin also showed drastic fall in body weight. Treatment with DIZE showed significant attenuation of ovalbumin-induced reduction in body weight of rats.

RAS which operates via two counteracting axes — ACE1/Ang II/AT $_1$ R and ACE2/Ang-(1-7)/MasR are involved in the pathophysiology of COPD, pulmonary hypertension and acute respiratory distress syndrome (ARDS), with the former mediating pathogenesis and latter having the protective role (Kaparianos and Argyropoulou, 2011). ACE2 is downregulated in experimental and clinical lung fibrosis and limits the concentration of local Ang II (Li et al., 2008). In our model of asthma, pulmonary ACE got upregulated and ACE2 got downregulated. Treatment with DIZE normalized their levels. It has also been demonstrated that Ang-(1-7), via the receptor Mas, acts as an anti-inflammatory

agent in an acute murine model of asthma (El-Hashim et al., 2012). Combining both these statements, it can be inferred that the protective effect of DIZE was due to the increased synthesis of Ang-(1-7). DIZE-activated ACE2 was associated with significant dose-dependent decrease in the perivascular and peribronchial inflammation, fibrosis and goblet cell hyper/metaplasia induced by ovalbumin.

In the present study, ovalbumin challenged group exhibited a significant right ventricle hypertrophy and treatment with DIZE reversed this response. Increased vascular resistance in airway in asthma poses chronic pressure overload i.e., increased afterload on the right ventricle leading to its adaptive hypertrophy. As the right ventricle hypertrophy is reversed by DIZE, it is likely that the cardioprotective effect of DIZE is related to decrease in pulmonary vascular resistance.

Allergic airway inflammation and AHR development involve wide array of inflammatory cells and mediators. Inflammatory mediators play a key role in the asthma. In present study also, there was significant increase in the inflammatory agents like IL-4, total WBC count, eosinophils, neutrophils, monocytes etc. and treatment with DIZE, which is angiotensin converting enzyme 2 activator capable of mimicking the anti-fibrotic, anti-proliferative and anti-inflammatory effects of Ang-(1-7) (Wiemer et al., 2002; Santos and Ferreira, 2006), prevented the development of airway and pulmonary vascular remodelling, pulmonary inflammation. It also improved airway responsiveness in rat with chronic allergic lung inflammation.

Most reports showed that the airway responsiveness to methacholine, a muscarinic agonist analogous to carbachol, increased in asthmatic rats (Cho et al., 2004; Deng et al., 2013) whereas in our study, asthmatic rats showed a drastic fall in the airway responsiveness to carbachol similar to that reported by Rodrigues-Machado et al.

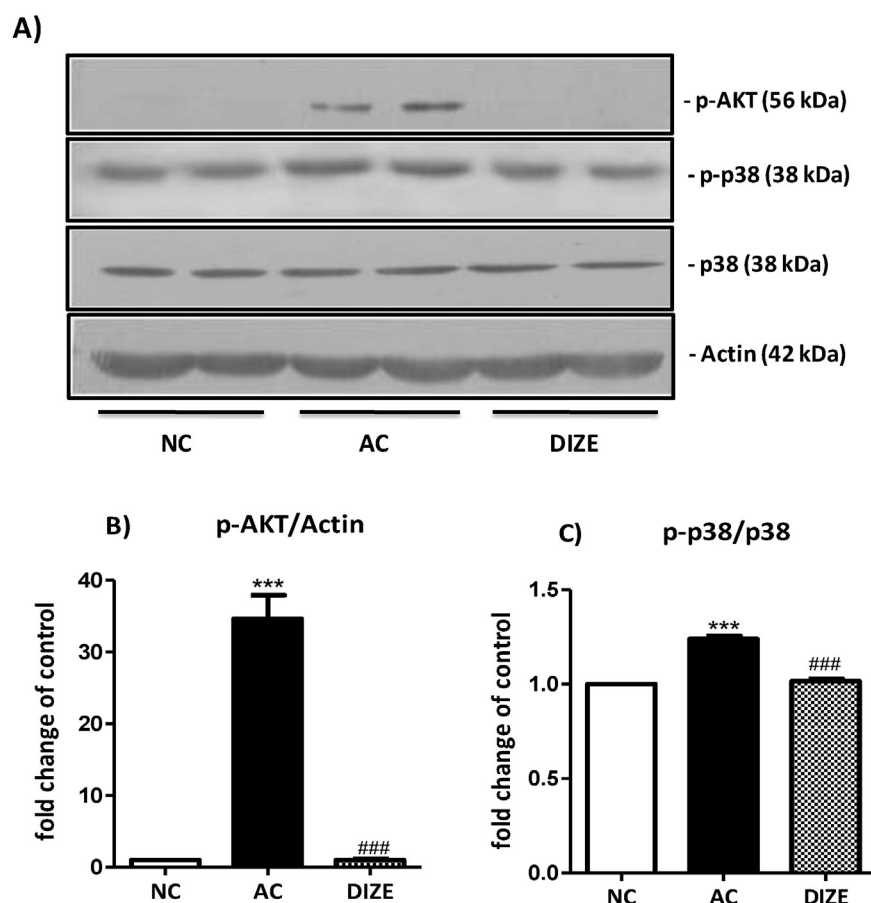


Fig. 9. Effect of DIZE on MAPK signalling. Representative blots (A) and densitometric quantification of at least three independent experiments ($n = 3$) showing relative levels of p-AKT (B) (normalized to β -actin) and p-p38 (C) (normalized to p38). NC, normal control; AC, asthma control; DIZE, ovalbumin-challenged, DIZE (15 mg kg^{-1} ; i.p.) treated rats. One-way ANOVA followed by Tukey's post-test. Each data point is represented as mean $\pm$ SEM, ($n = 3$). *** $P < 0.001$ vs. NC; ### $P < 0.001$ vs. AC.

(Rodrigues-Machado et al., 2013), who provided a plausible explanation. Attenuated tracheal ring contractions suggest that the airways in these rats underwent an extensive remodelling, that prevented the contraction in response to muscarinic receptor stimulation. Duration of allergen exposure, thereby the extent of airway remodelling determines the direction (increased or decreased) of airway responsiveness to parasympathetic activation (Palman et al., 2000). Excessive extracellular matrix (ECM) deposition and reticular basement membrane thickening (RBM) resist the muscle shortening i.e., muscle contraction in response to electric field stimulation or pharmacological agents (Bramley et al., 1995; Milanese et al., 2001). We also speculate that carbachol might be acting in a way different from that of methacholine. In our study, DIZE treatment improved the contractile response, suggesting that DIZE attenuates airway remodelling, induced by allergic inflammation. Unchanged pD_2 value in all the groups indicates that there is no alteration in affinity for the receptors. Thus, change in the number of receptors or downstream signalling molecules may be attributed for the change in contractile response of the tracheal muscles. Majority of the reports suggested the harmful role of NO in promoting bronchoconstriction and remodelling (Benetti et al., 2013; Abdel-Fattah et al., 2015). But we failed to observe any change in NO may be because they measured nitrogen oxides (NOx) (both nitrite and nitrate) in BALF whereas we measured only nitrites in lung homogenates.

It has been reported that MAP kinases are involved in the inflammatory responses induced by cigarette smoke, endotoxin, and oxidative stress through activation and release of proinflammatory cytokines/chemokines and activation of inflammatory cell migration. In signalling

pathways of airway inflammation, some of the MAPKs and pro-inflammatory cytokines such as IL-1 β , transcription factors, such as nuclear factor- κ B (NF- κ B) have been shown to play an important role (Li et al., 2014; Yan and Choi, 2014). IL-1 β was shown to activate MAPKs and AKT (Chen et al., 2007). Concurrent with this, IL-1 β , AKT, MAPKs were increased in our study also. Activation of p38 MAPK leads to allergen-induced pulmonary eosinophilia, mucus hypersecretion, and airway hyperresponsiveness. We also found that there was increased phosphorylation of AKT, p38 and NF- κ B. This suggests that DIZE acts by downregulation of various MAP Kinases. Improved airway response, airway remodelling, molecular markers of inflammation, apoptosis correlated with the expression of ACE2 activated by DIZE.

In conclusion, our data show that activation of ACE2 by DIZE significantly reduced the ovalbumin-induced perivascular and peribronchial fibrosis, inflammatory cell infiltration and improved airway hyporesponsiveness. Thus activation of protective arm of RAS i.e. ACE2/ANG-(1-7)/Mas receptor axis may prove to be an excellent target for various inflammatory disorders like asthma.

Conflict of interest

The authors declare no conflicts of interest.

Disclosure statement

The authors have nothing to disclose

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2016.06.026>.

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