

Protease Inhibitor Reduces Airway Response and Underlying Inflammation in Cockroach Allergen-Induced Murine Model

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Abstract—Protease(s) enhances airway inflammation and allergic cascade. In the present study, effect of a serine protease inhibitor was evaluated in mouse model of airway disease. Mice were sensitized with cockroach extract (CE) or Per a 10 and treated with 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) 1 h before or after challenge to measure airway response. Mice were euthanized to collect bronchoalveolar lavage fluid (BALF), blood, and lung to evaluate inflammation. AEBSF treatment significantly reduced the AHR in allergen-challenged mice in dose-dependent manner ($p \leq 0.01$). IgE ($p \leq 0.05$) and Th2 cytokines ($p \leq 0.05$) were significantly reduced in treated mice. AEBSF treatment lowered total cell ($p \leq 0.05$), eosinophil ($p \leq 0.05$), and neutrophil ($p \leq 0.05$) in BALF and lung tissue. Oxidative stress parameters were impaired on treatment in allergen-challenged mice ($p \leq 0.05$). AEBSF had therapeutic effect in allergen-induced airway resistance and underlying inflammation and had potential for combination or as add-on therapy for respiratory diseases.

KEY WORDS: protease inhibitor; allergy; cockroach extract; oxidative stress; airway inflammation.

INTRODUCTION

Prevalence of allergic diseases has been increasing from the last few decades. Allergy is a disease of multifunctional etiology, elicited by allergen exposure. A major group of allergen extracts possess protease activity [1]. Stewart et al. demonstrated that protease activity of allergen in house dust potentiates allergic exacerbation [2]. Allergens having serine protease activity are present in

house dust mite, insects, molds, pollen, etc. [3–7]. *Periplaneta Americana* allergen extract contains 22 IgE-binding components that include a protease allergen Per a 10.

Per a 10, a major cockroach allergen, is a serine protease that augments allergic airway inflammation in mice [8, 9]. Srivastava et al. showed that immunotherapy with inactive Per a 10 suppresses Th2 response and lung inflammation in Per a 10-sensitized mice [10]. Further, proteolytically active Per a 10 biases dendritic cells toward Th2 response whereas the inactivated protease have lesser potential [11].

Presently, corticosteroids, short-, and long-acting β_2 -adrenoceptor agonists are the mainstay of asthma treatment as advocated by Disease Management Guidelines. However, their long-term use causes side effect [12–14]. Other existing therapies for airway inflammatory disease are leukotriene receptor antagonist, phosphodiesterase inhibitors, and mast cell stabilizers, aimed to control disease symptoms, mainly airway responsiveness, reduced airflow resistance, cough and disease exacerbation. However, these approaches require lifetime therapy, and its cessation leaves to recurrence of disease. Recently, much interest is gone into developing more effective treatment for airway inflammatory diseases. Drugs which are under development or licensed include new generation phosphodiesterase inhibitors, statins, anti-cytokines, kinase inhibitors,

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Abbreviations: AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; BAL, Bronchoalveolar lavage; BALF, Bronchoalveolar lavage fluid; CE, Cockroach extract; DCFH-DA, 2',7'-Dichlorofluorescein-diacetate; MCh, Methacholine; MFI, Mean fluorescence intensity; PEEP, Positive end expiratory pressure; ROS, Reactive oxygen species; RT, Room temperature; SEM, Standard error mean; TMB, 3,3',5,5'-Tetramethylbenzidine; OPD, o-Phenylenediamine; PAS, Periodic acid–Schiff; HRP, Horseradish peroxidase; NADPH, Nicotinamide adenine dinucleotide phosphate ELISA

Enzyme-linked immunosorbent assay; Ns, Not significant

anti-IgE, peroxisome proliferator-activated receptor gamma, peptide therapy, etc. Studies also show that protease inhibitor is potentially effective in reduction of allergen-induced airway response and inflammation [15, 16].

Serine protease inhibitors targeting specific or a broad range of proteases may effectively attenuate allergic airway inflammation. 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) is a serine protease inhibitor of broad specificity and high affinity with ability to reduce reactive oxygen species (ROS) production with no interference with vital parameters [17, 18]. Recently, a study showed that serine protease inhibitor attenuates airway inflammation in a food allergen-induced mouse model [19]. Protease inhibitor to use as therapy has to be achieved against multiple allergen extract containing protease. Further, it is required to study the pathway involved in giving protection against the disease. Therefore, the present study was aimed to measure the therapeutic potential of AEBSF in mouse model sensitized with cockroach allergen.

MATERIALS AND METHODS

Animal Ethics, Immunization and Treatment Protocol

Female BALB/c mice of 4–6 weeks were procured from Central Drug Research Institute, Lucknow (India). Mice were kept in experimental conditions for 15 days to get acclimatize.

Standard chow diet (*ad libitum*) and purified water were provided to mice and were kept at 12-h controlled light/dark cycle. The experimental protocol followed was approved by animal ethics committee of “CSIR-Institute of Genomics and Integrative Biology” (Delhi).

The experiments were conducted with cockroach extract (CE) and Per a 10 allergen along with a phosphate buffered saline (PBS) control group. For each set, five groups of six healthy mice were taken for the study. Mice were sensitized by 10 µg of allergen through i.p. injection on days 0, 7, and 14 and challenged with 5 µg of allergen on days 21, 23, and 25. Mice were treated with 10 or 30 µg of AEBSF (Sigma, St. Louis) 1 h before or after allergen challenge in groups. PBS control group of mice was sensitized and challenged with PBS. Similarly, group of mice were sensitized and challenged with CE or Per a 10, and no inhibitor treatment was given to these animals.

Assessment of Airway Resistance

Airway resistance of mice was evaluated using FlexiVent TM ventilator (Scireq, Montreal, Que.,

Canada). Pressure transducer was calibrated with cannula (18 gauge) at 0 and 30 cm H₂O. Mice were anesthetized by i.p injection of 10 mg/kg xylazine and 100 mg/kg pentobarbital. Calibrated cannula was firmly placed by tracheotomy and connected with controlled respirator. Ventilation was delivered at 150 breaths/min and with 3-cm H₂O positive end expiratory pressure (PEEP).

Respiratory system resistance was measured from nonparalyzed mice by keeping the chest wall intact. Airway response of mice was recorded for different dose of methacholine (MCh) (2, 4, 8, 12, 16, and 20 mM) and normalized with basal level.

Sample Collection

Mice were euthanized; blood was collected, sera separated by centrifugation at 400g for 10 min and kept at –20 °C for further analysis. Approximately 1.2 ml of bronchoalveolar lavage fluid (BALF) was collected by three-time instillation of 0.5 ml of chilled PBS into the lung. BALF was centrifuged at 400g for 10 min at 4 °C, supernatant was separated and preserved at –80 °C, and cell pellet was used for cellular infiltration and intracellular ROS. The lungs were fixed with neutral-buffered formalin for histopathology.

Cell Count and Eosinophil Peroxidase Activity in BALF

The bronchoalveolar lavage (BAL) pellet was suspended in PBS, and total cell count was determined using trypan blue staining (Sigma Aldrich Co., St. Luis, USA) under light microscope. Smear of cell pellet was prepared on glass slide, stained with Leishman's stain to count percentage of eosinophils and neutrophils.

Eosinophil peroxidase activity was measured in BALF supernatant by spectrophotometric method. Briefly, 100 µl of each sample was taken in duplicate in microtiter plate (Nuncimmuno, Denmark), and 100 µl of substrate solution (0.1 mM o-phenylene-diaminedihydrochloride, 0.1 % Triton X-100, and 1 mM hydrogen peroxide in 0.05 M Tris-HCl) was added in each well. The plate was incubated at 37 °C, reaction was stopped by adding 50 µl of 4 M sulfuric acid, and absorbance was measured at 492 nm using spectrophotometer (Bio-Rad Laboratories Ltd., UK).

Measurement of Specific Antibodies

Relative concentration of specific IgE and IgG1 was measured in serum samples of mice by enzyme-linked

immunosorbent assay (ELISA). Briefly, 100 μ l of 0.1-M carbonate buffer (pH 9.6) containing 100 ng allergen was dispensed in each well of microtiter plate (Nunc-Immuno, Denmark) and incubated overnight at 4 °C. Plate was washed with PBS and blocked with 3 % defatted milk for 3 h at 37 °C. One hundred-microliter sample (serum 1:10 v/v dilution in PBS) per well was taken, and plate was incubated overnight at 4 °C. The plate was washed with PBST (0.05 % Tween-20 in PBS). IgG1 was estimated using anti-mouse IgG1HRP (1:1,000, BD Pharmingen). IgE estimation was done by incubating the plate with biotinylated anti-mouse IgE (1:1,000, BD Pharmingen) at room temperature (RT) for 90 min followed by streptavidin-peroxidase (1:1,000; BD Pharmingen) for 30 min. Plates were developed using o-phenylenediamine (OPD), and absorbance was read at 492 nm.

Estimation of Cytokines in BALF

IL-4, IL-5 (BD Pharmingen CA, USA), and IL-13 (eBioscience CA, USA) were determined in BALF following manufacturer's instructions. One hundred microliters of provided capture antibody (1:250 v/v) for each cytokine was coated separately in microtiter plates in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plates were washed with PBST, blocked with assay diluent for 1 h at RT. Standards of different dilutions and BALF samples (100 μ l) were added and incubated at RT for 2 h. The plates were washed and incubated with biotinylated detector antibody labeled with avidin-horseradish peroxidase (HRP) at RT for 1 h. Tetramethylbenzidine (TMB) (Sigma Aldrich) substrate solution was added in each well and developed by incubating at 37 °C in dark. Reaction was stopped by addition of 50- μ l 4 N H₂SO₄. Absorbance was measured at 450 nm with wavelength correction at 570 nm.

Measurement of Intracellular ROS

ROS was measured in BAL cell pellets from mice. Pellets were washed with PBS, and cells were incubated with 3.3 μ M 2,7-dichlorofluorescein diacetate (DCFH-DA) (Sigma) in PBS for 10 min at RT in dark. DCFH-DA was allowed to react with intracellular hydrogen peroxide and peroxidases for 10 min; cells were washed with PBS and examined under fluorescence-activated cell sorter (Guava Technologies, CA, USA).

Measurement of 8-Isoprostane in BALF

To estimate the oxidative stress, 8-isoprostane level in BALF was measured following the manufacturer's

instructions of enzyme immunoassay kit (Cayman Chemical, MI, USA). Briefly, standards and BALF samples were mixed (50 μ l/well) with 50 μ l of 8-isoprostane tracer, and subsequently, 50 μ l of 8-isoprostane antiserum was added. Plate was incubated for 1 h at RT and washed five times with buffer, 200 μ l of pNPP. The plate was incubated in dark at RT for 60 min with gentle shaking. Absorbance was taken at 420 nm.

Histopathology

The lungs fixed on formalin were embedded in paraffin, and sections of 4- μ m thickness were cut, taken on slide, and stained with hematoxylin and eosin [20]. Slides were observed under light microscope (10 $\times$), and inflammation score was determined on basis of cellular infiltration by experimentally blinded person. To evaluate the role of AEBSF in attenuation of allergen-induced mucus production, lung section was stained with periodic acid-Schiff (PAS). Mucus production in bronchial epithelium was scored based on number of PAS-stained cells as grade 1 < 20 % cells stained, grade 2 20–40 % stained cells, grade 3 40–60 %, grade 4 60–80 %, and grade 5 > 80 % airways affected [21]. The lung sections were also stained with metachromatic dye toluidine blue to depict the mast cells and observed under microscope in $\times$ 20 magnification. Mast cell accumulation in the lung was scored on the basis of cells per microscopic field [22].

Statistical Analysis

The numerical value of each parameter were fed into the software GraphPad Prism and GraphPadInstat (GraphPad Software, San Diego, CA, USA) for statistical analysis in groupwise manner. The statistically significant difference of nonparametric data was determined using one-way ANOVA followed by Dunnett's multiple comparison tests between allergen-challenged (not treated with protease inhibitor) and AEBSF-treated mice groups. Graph of each parameter with standard error mean (SEM) was plotted in Microsoft excel.

RESULTS

Airway Response in Allergen-Challenged Mice and AEBSF-Treated Mice

Airway resistance was evaluated by increasing dose of MCh stimulation with PBS as basal level response. At 20 mM of MCh, the normalized airway resistance was

increased from 1.3 in the control group to 2.4 in CE-challenged mice (Fig. 1a). AEBSF treatment of 10 or 30 μ g before CE challenge to mice reduced the airway resistance to 1.5 or 1.3, respectively ($p \leq 0.01$ or $p \leq 0.001$). The mice treated with 10 or 30 μ g of AEBSF after CE challenge showed reduced airway response to 1.8 or 1.3, respectively ($p \leq 0.01$ or $p \leq 0.001$).

In Per a 10-challenged mice, airway resistance was increased to 2.6 as compared to basal airway resistance of 1.3 (Fig. 1b). AEBSF treatment of 10 or 30 μ g before Per a 10 challenge to mice lowered the airway resistance to 1.7 or 1.3, respectively ($p \leq 0.01$ or $p \leq 0.001$). Same doses of AEBSF given to mice after Per a 10 challenge had reduced the airway response to 1.7 or 1.4, respectively ($p \leq 0.01$ or $p \leq 0.001$). The data indicated dose-dependent reduction in the airway response. Lower dose of MCh stimulation showed that AEBSF treatment given before allergen challenge has better effect in reduction of airway response in comparison to the treatment given after challenge. Also, the treatment was more effective in reduction of CE-induced airway resistance in comparison to Per a 10.

AEBSF Reduced Cellular Infiltration in the Lungs

CE-challenged mice had higher number of total cells, eosinophil, and neutrophil count in BALF. Mice treated with 10 or 30 μ g of AEBSF before CE challenge showed

significant reduction in total cell and eosinophil and neutrophil count ($p \leq 0.01$ or $p \leq 0.01$; $p \leq 0.001$ or $p \leq 0.001$; $p \leq 0.01$ or $p \leq 0.01$) in BALF (Fig. 2a–c). Same doses of AEBSF given after challenge also reduced the total cell, eosinophil, and neutrophil significantly ($p \leq 0.05$ or $p \leq 0.01$; $p \leq 0.05$ or $p \leq 0.01$; $p \leq 0.05$ or $p \leq 0.05$).

AEBSF treatment given before Per a 10 challenge to mice in 10- or 30- μ g doses significantly reduced the total cell and eosinophil and neutrophil count ($p \leq 0.01$ or $p \leq 0.001$; not significant (ns) or $p \leq 0.01$; $p \leq 0.01$ or $p \leq 0.001$) (Fig. 2a–c). Same doses of AEBSF given after challenge also reduced the total cell, eosinophil, and neutrophil count significantly ($p \leq 0.05$ or $p \leq 0.01$; $p \leq 0.05$ or $p \leq 0.05$; $p \leq 0.05$ or $p \leq 0.01$).

CE challenge had increased EPO activity in BALF. CE-challenged mice given 10 or 30 μ g of AEBSF either before or after challenge showed reduced EPO activity ($p \leq 0.001$ or $p \leq 0.001$) (Fig. 2d).

Per a 10-challenged mice had increased EPO activity in BALF. AEBSF treatment of 10 or 30 μ g given before challenge reduced the EPO activity (ns or $p \leq 0.001$); however, treatment given after challenge reduced EPO activity significantly ($p \leq 0.01$ or $p \leq 0.01$) (Fig. 2d).

Data indicate that AEBSF treatment before challenge of Per a 10 is more effective in reduction of total cell count in BALF, while the effect of AEBSF treatment was more

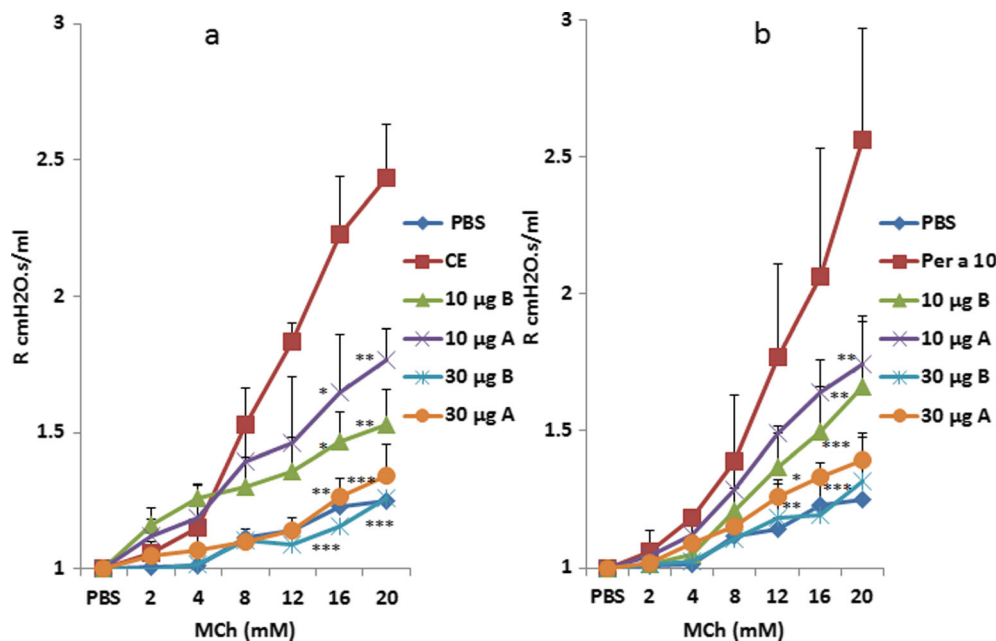


Fig. 1. AEBSF treatment reduces AHR. On day 26, AHR of **a** CE- and **b** Per a 10-sensitized and AEBSF-treated mice in response to methacholine (2–20 mM) was taken in FlexiVent. Mice were anesthetized by i.p. injection of 10 mg/kg ketamine and 100 mg/kg pentobarbital, and respiratory system resistance was measured. The data represents the airway resistance $\pm$ SEM of six mice each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

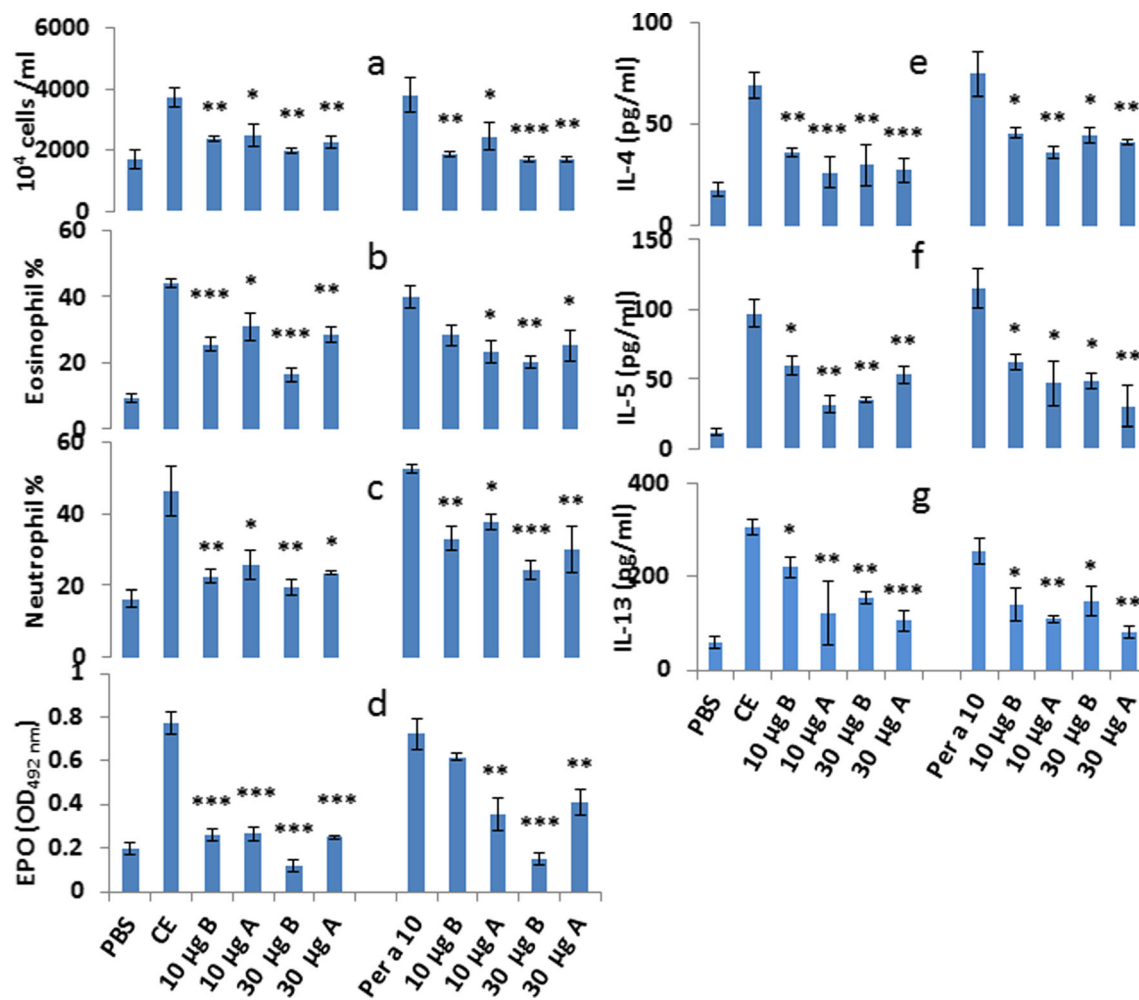


Fig. 2. Cell count in BALF of mice. BAL cell pellet was resuspended in PBS, and total cells were counted using trypan blue under the microscope and presented as **a** total cell count in terms of cells/ml of BALF. **b** Eosinophil percentage and **c** neutrophil percentage were evaluated by counting minimum of 150 cells. **d** EPO activity was evaluated in BALF of mice. Th2 cytokines **e** IL-4, **f** IL-5, and **g** IL-13 were estimated in BALF supernatant. The data represents the airway resistance $\pm$ SEM of six mice each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

pronounced in reduction of eosinophil in BALF of CE-challenged mice.

AEBSF Treatment Reduced Cytokine

IL-4 and IL-13 level in BALF of CE-challenged mice was increased (Fig. 2e, g). AEBSF treatment of 10 or 30 µg before CE challenge to mice was less effective in reduction of the IL-4 and IL-13 ($p \leq 0.01$ or $p \leq 0.01$ and $p \leq 0.05$ or $p \leq 0.01$) in comparison to treatment given after CE challenge ($p \leq 0.001$ or $p \leq 0.001$ and $p \leq 0.01$ or $p \leq 0.001$). AEBSF treatment of 10 or 30 µg before Per a 10 challenge to mice was less effective in reduction of IL-4 and IL-13 ($p \leq 0.05$ or $p \leq 0.05$ and $p \leq 0.05$ or $p \leq 0.05$) in comparison to

treatment given after Per a 10 challenge ($p \leq 0.01$ or $p \leq 0.01$ and $p \leq 0.01$ or $p \leq 0.01$).

Mice challenged with CE had 97 ± 11 pg/ml of IL-5 in BALF while control group had 12 ± 3 pg/ml (Fig. 2f). Treatment of 10 or 30 µg AEBSF before ($p \leq 0.05$ or $p \leq 0.01$) and after ($p \leq 0.01$ or $p \leq 0.01$) CE challenge significantly reduced the IL-5. In BALF of Per a 10-challenged mice, IL-5 level was 115 ± 14 pg/ml, which was significantly reduced by AEBSF treatment of 10 or 30 µg before challenge ($p \leq 0.05$ or $p \leq 0.05$). Same doses of AEBSF treatment given after Per a 10 challenge to mice also lowered IL-5 ($p \leq 0.05$ or $p \leq 0.01$).

AEBSF treatment after challenge was more efficacious in reduction of IL-4 and IL-13 in BALF of CE- and

Per a 10-challenged mice. Reduction of cytokines was more effective in CE-challenged mice in comparison to Per a 10-challenged mice.

AEBSF Treatment Lowers Immunoglobulin in Mice

CE sensitization increased the specific serum IgE and IgG1 levels in mice (Fig. 3a, b). AEBSF treatment of 10 or 30 μ g before CE challenge to mice significantly reduced IgE and IgG1 ($p \leq 0.05$ or $p \leq 0.01$ and ns or $p \leq 0.05$). Same doses of AEBSF treatment given after CE challenge to mice reduced the IgE and IgG1 ($p \leq 0.01$ or $p \leq 0.01$ and $p \leq 0.05$ or $p \leq 0.01$). In Per a 10-sensitized mice, AEBSF treatment of 10 or 30 μ g given before challenge reduced IgE and IgG1 ($p \leq 0.05$ or $p \leq 0.05$ and $p \leq 0.01$ or $p \leq 0.01$). AEBSF treatment of 10 or 30 μ g given after Per a 10 challenge to mice lowered IgE ($p \leq 0.05$ or $p \leq 0.01$ and $p \leq 0.01$ or $p \leq 0.01$).

Allergen-Induced Oxidative Stress Reduced by AEBSF

CE challenge to mice increased the 8-isoprostane in BALF from basal level of 37 ± 2 to 118 ± 4 pg/ml (Fig. 3c). AEBSF treatment of 10 or 30 μ g given before CE challenge significantly reduced 8-isoprostane ($p \leq 0.05$ or $p \leq 0.001$). Same doses of AEBSF given after CE challenge also lowered the 8-isoprostane ($p \leq 0.001$ or $p \leq 0.001$). Per a 10-challenged mice had increased level of 8-isoprostane. AEBSF treatment of 10 or 30 μ g before Per a 10 challenge significantly reduced the 8-isoprostane ($p \leq 0.05$ or $p \leq 0.05$). Same doses of AEBSF given after Per a 10 challenge to mice also lowered the 8-isoprostane ($p \leq 0.001$ or $p \leq 0.01$).

CE challenge to mice induced the intracellular ROS production. AEBSF treatment of 10 or 30 μ g before CE challenge significantly reduced the ROS ($p \leq 0.05$ or $p \leq 0.001$) (Fig. 3d) in the BAL cell pellets. Same doses of AEBSF given after CE challenge to mice lowered the ROS

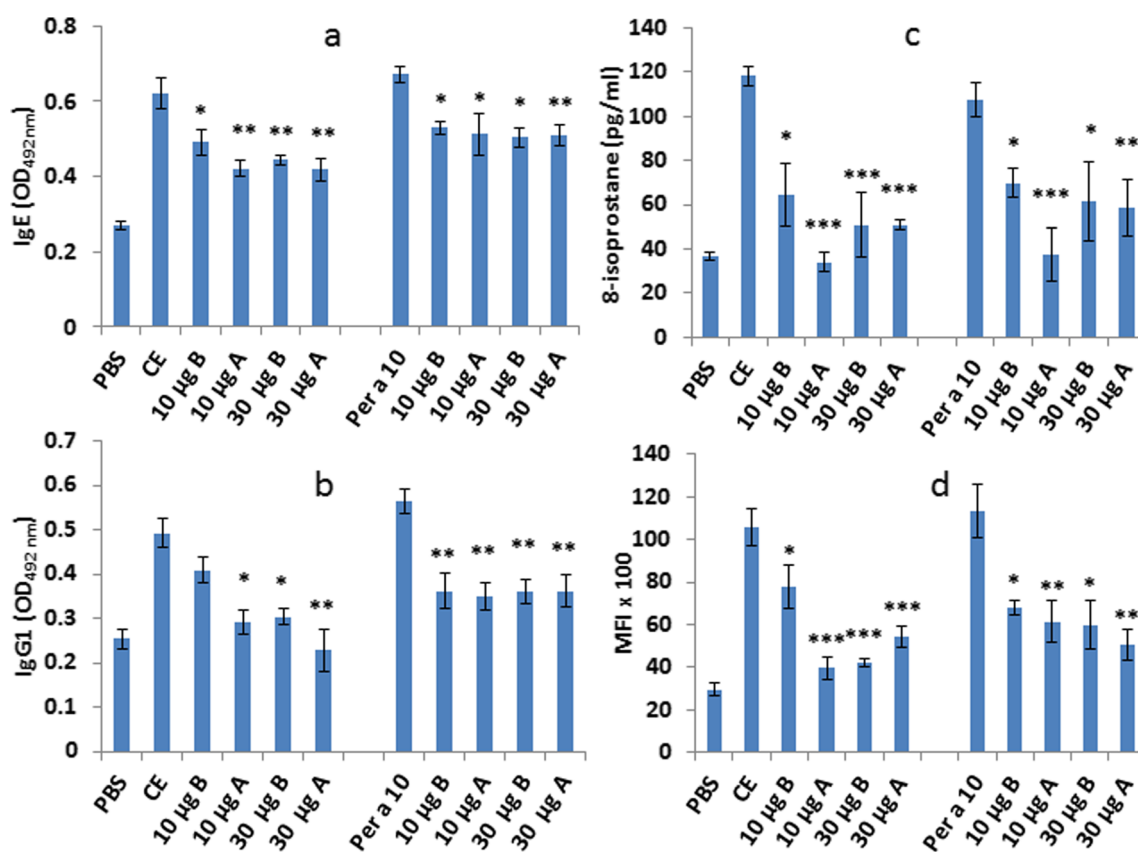


Fig. 3. Reduction in immunoglobulin and oxidative stress due to AEBSF treatment. Specific **a** IgE and **b** IgG1 were estimated using ELISA. **c** The oxidative stress in lung tissue was measured in terms of 8-isoprostane. **d** Cells were incubated with DCFH-DA, and the fluorescence intensity was measured. Data are presented as mean $\pm$ SEM of six mice each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

level ($p \leq 0.001$ or $p \leq 0.001$). AEBSF treatment of same doses of given before ($p \leq 0.05$ or $p \leq 0.05$) and after ($p \leq 0.01$ or $p \leq 0.01$) Per a 10 challenge significantly lowered the ROS level.

Protease Inhibitor treatment Prevents Allergen-Induced Changes in Airway

H&E-stained lung sections revealed marked increase in peribronchial infiltration of cells (Fig. 4l) in CE-challenged mice (Fig. 4b) as compared to control mice (Fig. 4a). AEBSF treatment of 10 or 30 μg given to mice before CE challenge markedly reduced the cellular infiltration (Fig. 4d, f) ($p \leq 0.05$ or $p \leq 0.001$). Reduction in cellular infiltration was also observed in mice, those were treated with same doses of AEBSF after CE challenge (Fig. 4e, g) ($p \leq 0.001$

or $p \leq 0.01$). Per a 10-challenged mice had increased cellular infiltration in the lungs (Fig. 4c). AEBSF treatment of 10 or 30 μg given to mice before Per a 10 challenge lowered the cellular infiltration (Fig. 4h, j) ($p \leq 0.05$ or $p \leq 0.001$). AEBSF treatment of same doses given to the mice after Per a 10 challenge had reduced cellular infiltration (Fig. 4i, k) ($p \leq 0.01$ or $p \leq 0.01$).

CE challenge to mice induced mucus production (Fig. 5b). Mice treated with 10 or 30 μg of AEBSF before CE challenge had markedly reduced the mucus production (Fig. 5d, f) ($p \leq 0.05$ or $p \leq 0.01$). AEBSF treatment of same doses given to mice after CE challenge reduced the mucus production (Fig. 5e, g) ($p \leq 0.05$ or $p \leq 0.05$). Mice treated with 10 or 30 μg of AEBSF before Per a 10 challenge had reduced mucus in the lungs (Fig. 5h, j) ($p \leq 0.05$ or $p \leq 0.05$). Significant reduction in mucus production was also

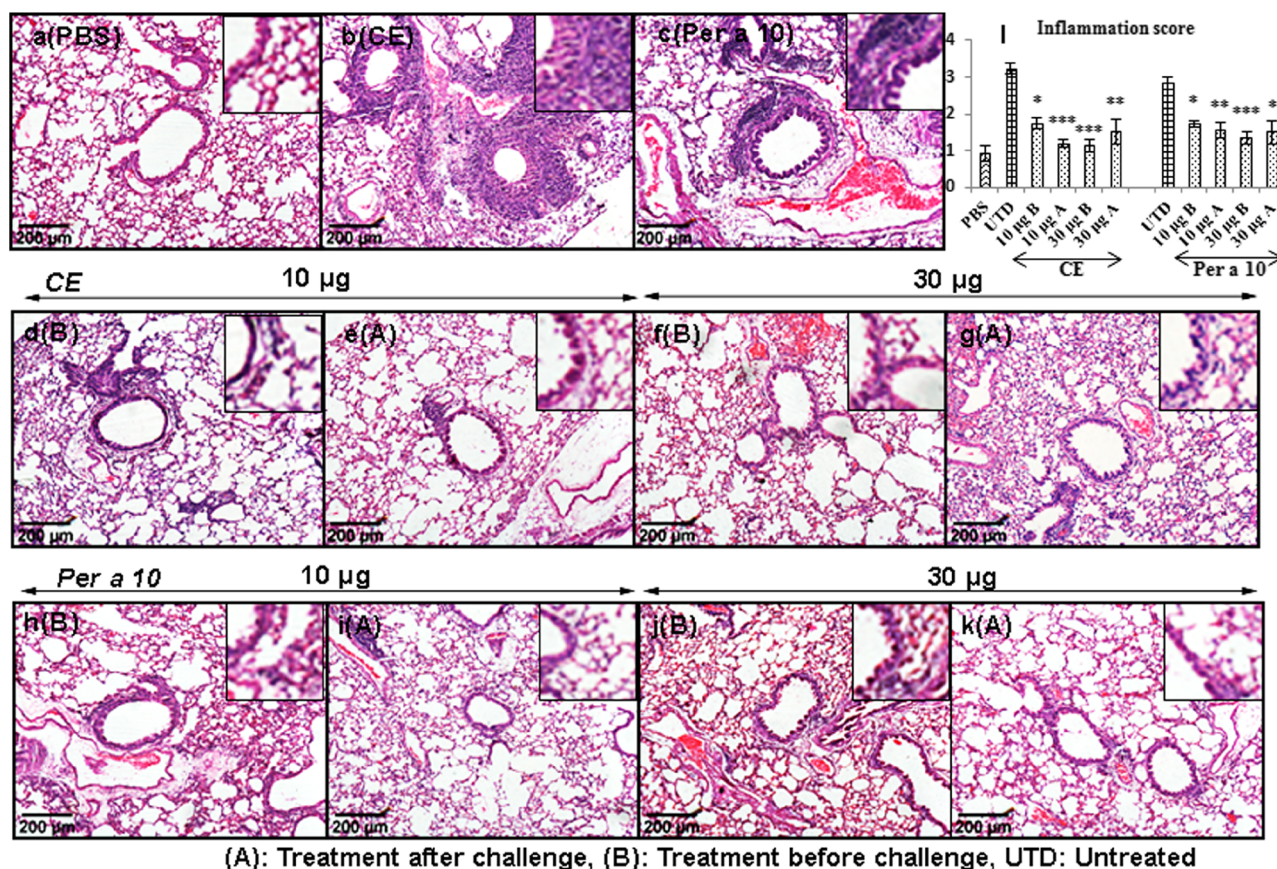


Fig. 4. H&E-stained lung sections showing the reduction in cellular infiltration due to AEBSF treatment. **a** PBS control, **b** CE challenged, **c** Per a 10 challenged, **d** 10 μg AEBSF treated before challenge in CE-sensitized mice, **e** 10 μg AEBSF treated after challenge in CE-sensitized mice, **f** 30 μg AEBSF treated before challenge in CE-sensitized mice, **g** 30 μg AEBSF treated after challenge in CE-sensitized mice, **h** 10 μg AEBSF treated before challenge in Per a 10-sensitized mice, **i** 10 μg AEBSF treated after challenge in Per a 10-sensitized mice, **j** 30 μg AEBSF treated before challenge in Per a 10-sensitized mice, **k** 30 μg AEBSF treated after challenge in Per a 10-sensitized mice, **l** inflammation score. The data represents the airway resistance $\pm$ SEM of six mice each group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

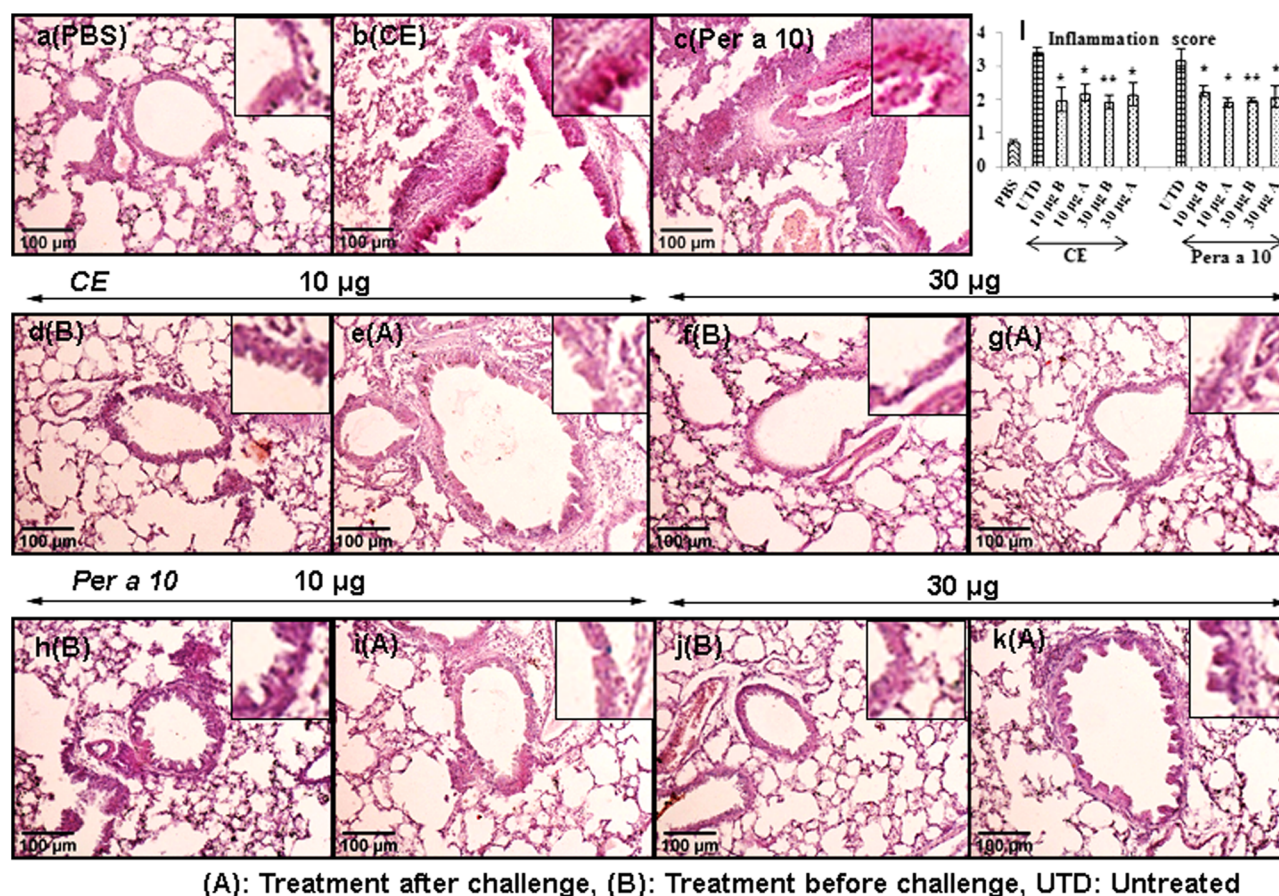


Fig. 5. PAS-stained lung sections indicating the reduction in mucus production after AEBSF treatment. **a** PBS control, **b** CE challenged, **c** Per a 10 challenged, **d** 10 µg AEBSF treated before challenge in CE-sensitized mice, **e** 10 µg AEBSF treated after challenge in CE-sensitized mice, **f** 30 µg AEBSF treated before challenge in CE-sensitized mice, **g** 30 µg AEBSF treated after challenge in CE-sensitized mice, **h** 10 µg AEBSF treated before challenge in Per a 10-sensitized mice, **i** 10 µg AEBSF treated after challenge in Per a 10-sensitized mice, **j** 30 µg AEBSF treated before challenge in Per a 10-sensitized mice, **k** 30 µg AEBSF treated after challenge in Per a 10-sensitized mice, **l** inflammation score. The data represents the airway resistance $\pm$ SEM of six mice each group. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

observed in mice treated with same doses of AEBSF after Per a 10 challenge (Fig. 5i, k) ($p \leq 0.05$ or $p \leq 0.05$).

CE challenge to mice induced mast cell accumulation in the lung (Fig. 6l, b) compared to control group (Fig. 6a). AEBSF treatment of 10 or 30 µg given to mice before CE challenge markedly reduced the number of mast cell in the lungs (Fig. 6d, f) ($p \leq 0.05$ or $p \leq 0.01$). Lesser number of mast cells was also observed in the lungs of mice treated with same doses of AEBSF after CE challenge ($p \leq 0.05$ or $p \leq 0.01$) (Fig. 6e, g). Per a 10-challenged mice had increased number of mast cells in the lungs (Fig. 6c). Same doses of AEBSF given before (Fig. 6h, j) ($p \leq 0.05$ or $p \leq 0.05$) and after (Fig. 6h, j) Per a 10 challenge ($p \leq 0.05$ or $p \leq 0.05$) significantly reduced the mast cell accumulation.

AEBSF treatment to mice reduced peribronchial infiltration of cells with best effect on 30 µg given to mice before allergen challenge. Similarly, 30 µg of AEBSF treatment given to the mice before allergen challenge was found to be most effective in reduction of mucus production and mast cell accumulation.

DISCUSSION

Asthma is characterized by reversible airflow obstruction, bronchial hyperresponsiveness, and underlying inflammation of the airways. Most asthma cases are allergic in origin and elicited due to allergen exposure. Allergens having serine protease activity are common in the nature and may enhance the allergic cascade. Presence of

proteolytic activity in natural sources of allergen may increase the accessibility of immune system to the antigens by disrupting epithelial barrier [23]. Furthermore, proteases may provide an adjuvant effect and aggravate the allergic immune response of self and other potential allergens present in the microenvironment [9]. Therefore, proteolytic activity in airways plays a pivotal role in aggravation of allergic response. The present study was aimed to elucidate the effect of protease inhibition in allergic airway disease in mouse model derived from allergen having proteolytic activity. The prophylactic and therapeutic effects of a serine protease inhibitor AEBSF were evaluated in different doses.

Studies have shown that serine protease inhibitors are effective in reduction of airway resistance [24]. Specific protease inhibitor may not have

significant effect in reduction of histamine/MCh-induced bronchial hyperresponse [25, 26]. Therefore, a protease inhibitor with broad specificity was selected in the present study and showed that its treatment reduced the airway resistance in CE-challenged mice. Similar result in airway response was obtained with Per a 10-challenged mice. Protease inhibitor reduces the airway response by stabilizing mast cell [27], reducing the kininogen to bradykinin conversion [28], and inhibiting hydrolysis of vasoactive intestinal peptide and peptide histidine-methionine [29].

Allergen sensitization directs DC to modify T cells for induction of Th2 immuneresponse. More importantly, protease activity biases the immune response toward Th2 through modulating DCs by reducing IL-12 secretions [11]. Our data indicate that

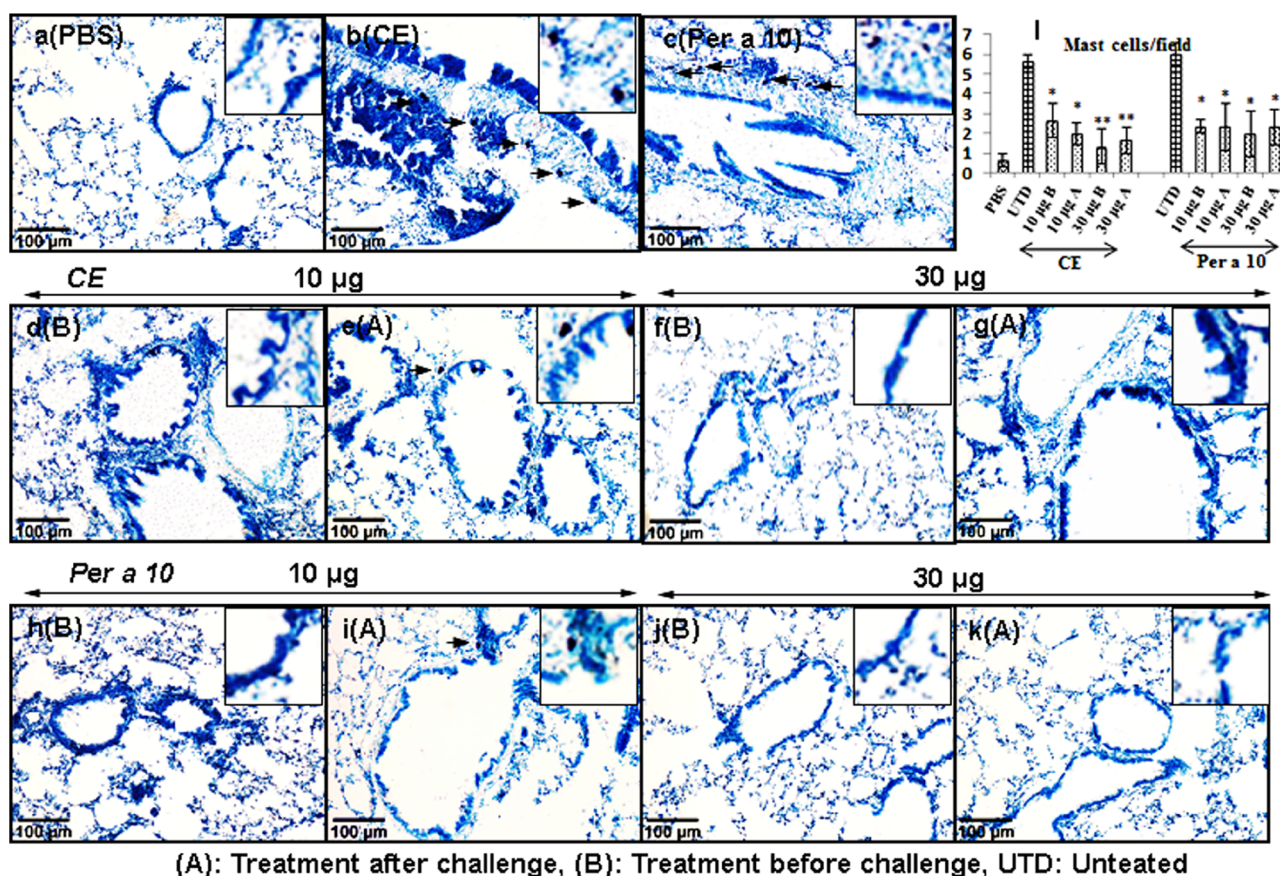


Fig. 6. Toluidine blue stained lung sections indicating the reduction in mast cell accumulation (mast cells were indicated by arrows). **a** PBS control, **b** CE challenged, **c** Per a 10 challenged, **d** 10 µg AEBSF treated before challenge in CE-sensitized mice, **e** 10 µg AEBSF treated after challenge in CE-sensitized mice, **f** 30 µg AEBSF treated before challenge in CE-sensitized mice, **g** 30 µg AEBSF treated after challenge in CE-sensitized mice, **h** 10 µg AEBSF treated before challenge in Per a 10-sensitized mice, **i** 10 µg AEBSF treated after challenge in Per a 10-sensitized mice, **j** 30 µg AEBSF treated before challenge in Per a 10-sensitized mice, **k** 30 µg AEBSF treated after challenge in Per a 10-sensitized mice, **l** mast cell/microscopic field. The data represents the airway resistance±SEM of six mice each group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

sensitization and challenge to mice with CE/Per a 10 induce IL-4, IL-5, and IL-13 cytokine. IL-4 and IL-13 cytokines are involved in lung diseases including AHR, airway inflammation, goblet cell metaplasia, and mucus hypersecretion [30]. IL-5 is a major participant for the eosinophil infiltration in allergic lung [31]. The present study showed that AEBSF treatment to mice effectively reduced airway inflammation, Th2 cytokines, and concomitantly mucus production in allergen-challenged mice. Similar result was obtained by ovalbumin-sensitized mice treated with nefamostatate mesilate [32].

Allergen sensitization in mice leads to marked elevation of serum IgE levels. The results indicate that immunization of mice with CE elevated IgE level in serum and mast cell in the lungs. AEBSF treatment to the mice reduced serum IgE level and mast cell, indicating antiallergic effect of AEBSF. In a previous study, Na-Tosyl-Phe chloromethyl ketone has reduced the mast cell activation and degranulation [33].

Oxidative stress is a common feature of inflamed airway accumulating excessive ROS. NADPH oxidase is a primary enzyme for ROS production in epithelial and inflammatory cells [34]. An NADPH oxidase inhibitor apocynin was effective in attenuation of ovalbumin-induced AHR and inflammation [35]. Present study showed that AEBSF treatment reduced the oxidative stress in CE/Per a-10 challenged mice.

Protease inhibitor treatment before allergen challenge could reduce the airway resistance more effectively than treatment given after challenge; possible reason could be that the protease inhibitor reduces activation of bronchodilators like kininogen to bradykinin conversion [28] and hydrolysis of vasoactive intestinal peptide and peptide histidine-methionine [29]. The treatment of protease inhibitor is effective in lowering of airway resistance and inflammation when given to the mice challenged with crude extract in comparison to mice that are challenged with purified protease allergen. Moreover, the therapeutic effect of AEBSF treatment in mice indicated that the effect of treatment is independent of protease activity of allergen. The therapeutic effect of serine protease inhibitor targets on endogenous proteases. However, the study is not able to define the target proteases and bioavailability in mice. A delivery system to enhance the persistence of protease inhibitor may improve the therapeutic effect. Moreover, the utility of AEBSF requires therapeutic evaluation in human.

CONCLUSION

In summary, AEBSF reduced the protease allergen-induced airway hyperresponsiveness, inflammation, and oxidative stress effectively in mouse model. The present study demonstrated that AEBSF will be useful in combination or as add-on therapy for allergic airway inflammatory diseases.

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