

Role of adenosine in airway inflammation in an allergic mouse model of asthma

Ming Fan, S. Jamal Mustafa *

Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA

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Abstract

In the present study, we examined dynamic changes in cellular profile of bronchoalveolar lavage (BAL) fluid after adenosine challenge in ragweed sensitized and challenged mice. Mice systemically sensitized and airway challenged with ragweed showed marked airway inflammation manifesting increased eosinophils, lymphocytes, neutrophils and activated macrophages in BAL. Adenosine challenge further enhanced influx of inflammatory cells into BAL, notably neutrophils from 1 to 72 h and eosinophils from 1 to 48 h time-points ($p < 0.05$), which sharply rose at 6-h time-point following adenosine challenge. Greater infiltration of lymphocytes into BAL was observed at 1 and 72 h and macrophages from 6 to 72 h ($p < 0.05$) after adenosine challenge. Accordingly, markers of eosinophils, neutrophils and mast cells were analyzed at 6-h time-point after adenosine challenge. Adenosine challenge significantly increased the levels of eosinophil peroxidase, neutrophil myeloperoxidase and β -hexosaminidase in BAL. There were more significant effects of adenosine challenge on the degranulation of mast cells in the lung than that in blood. The chemoattractant, eotaxin, was detected in BAL, which increased after adenosine challenge. Theophylline, a non-specific adenosine receptor antagonist, prevented adenosine-enhanced infiltration of inflammatory cells and their respective markers. Our findings suggest that adenosine plays an important role in airway inflammation in an allergic mouse model.

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1. Introduction

Adenosine, an endogenous signaling nucleoside that modulates many physiological processes has been implicated in playing an ever increasingly important role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD) [1,2]. There is evidence suggesting that adenosine is produced in the asthmatic airway [3]. In patients with asthma, both

* Corresponding author. Current Address: Department of Physiology and Pharmacology and Center for Interdisciplinary Research in Cardiovascular Sciences, PO Box 9105, West Virginia University, Robert C. Byrd Health Sciences Center, Morgantown, WV 26506. Tel.: +1 252 744 2740; fax: +1 252 744 3203.

E-mail address: mustafas@mail.ecu.edu (S. Jamal Mustafa).

allergen- and exercise-induced airway obstructions were related to elevation of adenosine concentration in plasma [4,5]. Adenosine-induced bronchoconstriction has been studied extensively [6–8].

There is now increasing evidence showing that adenosine plays an active role in airway inflammatory responses in asthma, in addition to mediating bronchospasm. Inhaled adenosine causes the release of serum neutrophil chemotactic factor in asthmatics [9] and airway microvascular leakage in sensitized but not in control animals [10]. Mice with elevated levels of adenosine develop eosinophilic lung inflammation and mucus hypersecretion, which were reversed by lowering adenosine levels in the lung [11]. Further confirmation by recent clinical studies demonstrate that inhalation of AMP induces significant increases in sputum eosinophils and neutrophils in subjects with asthma, but not in healthy subjects [12,13]. Also, supporting data from our previous studies have shown that adenosine challenge amplifies the inflammatory response including increases in influx of inflammatory cells into bronchoalveolar lavage (BAL) fluid in our allergic mouse model [14] 1 h after an adenosine aerosol challenge.

Many cell types such as mast cells [15], lymphocytes [16], eosinophils [17], neutrophils [18], macrophages [19], and airway epithelial cells [20] play important roles in the exacerbation of asthma involving adenosine signaling. Recently, our laboratory has shown the involvement of mast cells in adenosine-mediated airway hyperresponsiveness in this allergic mouse model [21].

Mast cells can release mediators that have both immediate and chronic effects on airway constriction and inflammation [22]. A series of *in vitro* studies indicated that adenosine markedly enhances the release of histamine and other preformed mediators from immunologically primed mast cells [23,24]. Recently, it has been reported that stimulation of adenosine A_{2B} receptors in a human mast cell line increases the production of Th₂ cytokines [16]. Most of these studies are done in mast cells either obtained from the mechanical dispersion or enzymatic digestion of whole lung, or mast cell lines. It may be difficult to reproduce the milieu of mediators and other factors present in asthmatic lungs in these *in vitro* studies.

Eosinophil granulocytes are important effector cells in asthma. They contain large amounts of cytotoxic

and basic proteins. Eosinophil peroxidase (EPO) is one of the most abundant proteins in eosinophils, and used as a cell specific marker for these cells [25]. It has been shown that allergen-challenged patients with allergic rhinitis show an increase in the levels of EPO-stained mucosal eosinophils and free EPO-stained granules in nasal biopsies [26]. The mechanism underlying eosinophil migration to the airway remains intriguing. Chemokines are small inducible cytokines involved in trafficking and activation of leukocytes. Among them, a more selective recruitment of eosinophils is likely to occur in response to the members of the CC chemokine subfamily of which eotaxin is the most important mediator [27]. However, there is no current data in the murine model of asthma that reflects the effects of adenosine on the status of eosinophils and their trafficking in asthmatic airways.

While the eosinophil is classically associated with mild to moderate asthma, neutrophils have been reported in the airways of severe, steroid-dependent asthmatics and are a prominent feature of patients dying from sudden-onset fatal asthma [28,29]. Neutrophils contain and produce several inflammatory mediators and destructive proteases that are capable of damaging the airways and surrounding tissues [30]. The role of neutrophils in asthma remains unclear. Our previous studies showed a robust increase in eosinophils and neutrophils 1 h after adenosine exposure in ragweed sensitized and challenged mice [14]. In the present study, the dynamic changes in airway inflammatory responses to adenosine and functional status of mast cells, neutrophils, and eosinophils in an allergic mouse model were investigated.

2. Materials and methods

2.1. Mice sensitization and challenge

Male BALB/c mice, 6 to 8 weeks of age, free of specific pathogens, were obtained from Harlan Laboratories (Indianapolis, IN). The animals were maintained on a ragweed-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of East Carolina University.

Sensitization was performed according to the protocol described earlier from this laboratory [14]. Mice were sensitized on days 1 and 6 with *i.p.* injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 µg per dose with

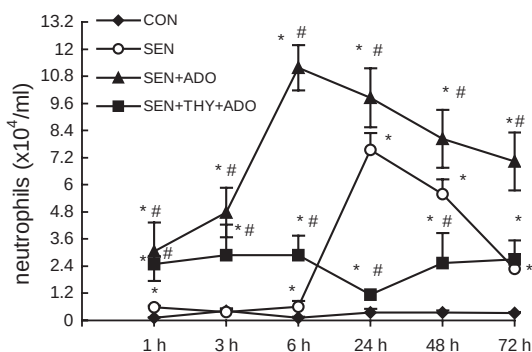


Fig. 1. Level of neutrophils in BAL from 1 to 72 h time-points. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=3$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN.

200 μ l Imject® Alum (Pierce Laboratories, Rockford, IL). Non-sensitized control animals only received the Imject® Alum with the same volumes. Ten days after sensitization, the mice were placed in a Plexiglas chamber and challenged with 0.5% aerosolized ragweed or with 0.9% saline as a control, using an ultrasonic nebulizer (DeVilbiss Somerset, PA) for 20 min both in the morning and afternoon for three days. The aerosolization of allergen was performed at a flow rate of 2 ml/min, and the aerosol particles have a median aerodynamic diameter of less 4 μ m (De Vibiss).

Mice were divided into following groups: (1) *sensitization group (SEN)*: mice were sensitized and challenged with ragweed using the same protocol described above. Twenty-four hours after the last challenge with aerosolized ragweed, mice were aerosolized with 0.9% saline for 2 min; (2) *sensitization + adenosine group (SEN+ADO)*: mice were treated similar to the SEN group, plus aerosolized with 6 mg/ml of adenosine instead of 0.9% saline, for 2 min; (3) *sensitization + theophylline + adenosine group (SEN+THY+ADO)*: 24 h after the last challenge with aerosolized ragweed, mice received nebulized theophylline (12 mg/ml) for 3 min, and 15 min later, aerosolized adenosine (6 mg/ml) for 2 min; (4) *control group (CON)*: mice received only vehicles for sensitization and challenge, and 24 h after the last challenge with aerosolized 0.9% saline, mice received nebulized 0.9% saline for 2 min.

2.2. Studies of dynamic changes in cellular profiles in BAL

At various time-points (1 to 72 h) after the last treatment, mice were sacrificed by i.p. injection (0.1 ml pentobarbitone sodium 200 mg/ml). The trachea was cannulated to perform BAL; 0.8 ml phosphate-buffered saline (PBS) was intro-

duced into the lungs via the tracheal cannula and carefully withdrawn. This was repeated three additional times to collect remaining cells. The lavage fluid was placed into polystyrene tubes on ice. The BAL was centrifuged at 1500 rpm for 6 min at 4 °C (Beckman®, T.J Model-6 Centrifuge). After removing the supernatant, BAL cells were resuspended in 1 ml of PBS. The total cells were counted manually in a hemocytometer chamber (Fisher). $1\sim5\times10^3$ cells were spun onto glass slides (Cytospin 3, Cytospin, Shandon, UK), air dried, fixed with methanol and stained with Diff-Quik stain set (DADE). A differential count of at least 300 cells was made according to standard morphologic criteria. The number of cells recovered per mouse was calculated and expressed as mean $\pm$ SEM per ml for each group.

2.3. An evaluation of inflammatory cell markers at 6-h time-point following adenosine challenge

Based on the studies of dynamic changes in cellular profiles in BAL (Figs. 1–4), the time-point of 6 h after the last treatment was chosen to further evaluate inflammatory cell markers. Mice were sacrificed by i.p. injection 6 h after the above treatment. Blood was collected by cardiac puncture and heparinized. Plasma was promptly separated by centrifugation and stored at -20 °C until the time of analysis. Lungs were lavaged four times with 0.8 ml PBS, the recovered solution was pooled (average recovery 3.04 ± 0.1 ml), and the total volume of recovered fluid was adjusted to 3.2 ml by adding PBS. The supernatant was collected and stored at -20 °C for further analysis. BAL cells were prepared and counted following the same protocol previously described.

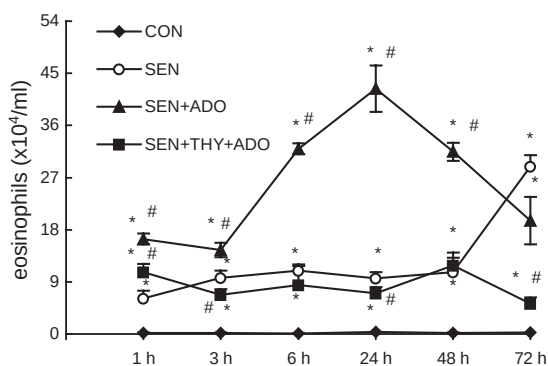


Fig. 2. Level of eosinophils in BAL from 1 to 72 h time-points. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=3$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN.

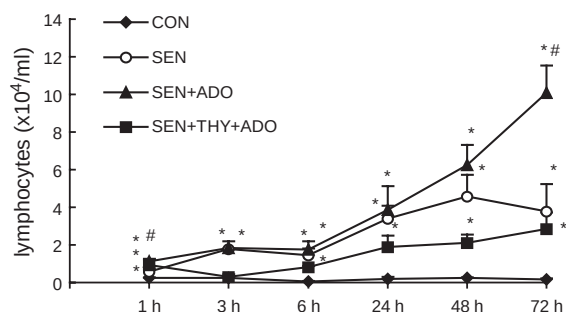


Fig. 3. Level of lymphocytes in BAL from 1 to 72 h time-points. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=3$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN.

2.3.1. Mast cell β -hexosaminidase activity in plasma and in BALF supernatant

A mast cell mediator-release assay was performed by measurement of β -hexosaminidase release [31]. To determine the enzymatic activity of released β -hexosaminidase, 10 μ l of plasma or 30 μ l of BAL supernatant samples were transferred to a well containing 50 μ l of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in citrate buffer (0.2 M citric acid, 0.2 M sodium citrate, pH 4.5). After 6 h at 37 °C, the reaction was terminated by addition of 80 μ l of 1 M Tris solution, pH 10.7, and the absorbance was read at 405 nm to measure β -hexosaminidase activity (Automated Microplate Reader, ELx800, BIO-TEK INSTRUMENTS®, INC). 50 μ l of reagent with 10 and 30 μ l of PBS were used as blanks for both plasma and supernatant samples, respectively. In addition, 10 μ l of plasma sample mixed with 50 μ l of PBS was used as control in an assay of β -hexosaminidase activity in plasma due to the plasma color. Reading for each plasma sample was subtracted from the control reading. Levels of β -hexosaminidase activities were normalized with protein level in plasma and in BAL, separately. The protein was measured using a Bio-Rad Protein Assay (BIO-RAD, NY).

2.3.2. Neutrophil myeloperoxidase activity in plasma and in BAL supernatant

Myeloperoxidase (MPO) activity of plasma or BAL was determined as previously described [32]. Briefly, 100 μ l of plasma or 300 μ l of BAL supernatant was mixed with 300 μ l of Hank's BSS containing 0.25% bovine serum albumin, 250 μ l of 0.1 M dibasic potassium phosphate (pH 7.0), 50 μ l of 1.25 mg/ml of *O*-dianisidine, and 50 μ l of hydrogen peroxide and incubated for 10 min at 25 °C. The reaction was halted with the addition of 50 μ l of sodium azide, and absorbance at 460 nm was measured

(BECKMAN DU-600). 100 μ l or 300 μ l of Hank's BSS instead of plasma or BAL supernatant was mixed with the reagent for blanks. 100 μ l of each plasma sample was mixed with 650 μ l of Hank's BSS and used as control to correct the plasma color. Reading for each plasma sample was subtracted from control reading. Levels of MPO activities were normalized with proteins in plasma and in BAL, separately.

2.3.3. Eosinophil peroxidase (EPO) activity in BAL supernatant

Substrate solution was prepared according to the method described by Strath et al. [33]. Substrate solution contained the following components at the final concentrations indicated in parentheses: *O*-phenylenediamine OPD, (0.1 mM), Triton X-100 (1 ml/l), Hydrogen peroxide (1 mM) and Tris (0.05 M); the pH was adjusted to 8.0 with 1 M HCl. The substrate solution was kept in the dark at -20 °C.

Lyophilized BAL samples were dissolved in 80 μ l PBS. 50 μ l of the BAL and 100 μ l of substrate solution were mixed together and incubated in a water bath at 37 °C. Thirty minutes later, the reaction was terminated by the addition of 60 μ l of 4 M sulphuric acid, and the absorbance was measured at a wavelength of 492 nm by an Automated Microplate Reader (ELx800, BIO-TEK INSTRUMENTS®, INC). The substrate solution was used as blank. Levels of EPO activity in BAL were normalized for BAL proteins.

2.3.4. Eotaxin in BAL supernatant

Level of eotaxin in the BAL was measured using a sensitive commercially available mouse Eotaxin Quantikine®

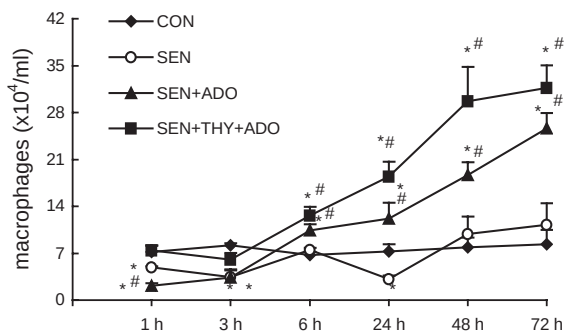


Fig. 4. Level of macrophages in BAL from 1 to 72 h time-points. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=3$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN.

ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

2.4. Statistical analysis

All values are expressed as mean $\pm$ SEM. Analysis of variance was used to determine the levels of significance between groups. Single pairs of groups were compared by two-tailed Student's *t* test using Microsoft® Excel 97 SR-2 provided by Micron Electronics, Inc. A *p* value of <0.05 was considered statistically significant.

2.5. Chemicals and drugs

Ragweed pollen extract was purchased from Greer Laboratories (Lenoir, NC). Inject® Alum was purchased from Pierce Laboratories (Rockford, IL). Adenosine, theophylline, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, bovine serum albumin, *O*-dianisidine, *O*-phenylenediamine, sodium azide, Triton X-100, and hydrogen peroxide were all purchased from Sigma Chemical Co. (St. Louis, MO). Diff-Quik stain set was purchased from Dade Behring Inc. (Newark, DE). Mouse Eotaxin Quantikine® ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN).

3. Results

3.1. Time-course of cell recruitment into BAL after adenosine challenge

Mice systemically sensitized and airway challenged with ragweed exhibited marked airway inflammation manifesting a large number of neutrophils, lymphocytes, eosinophils, and activated macrophages in BAL. Twenty-four hours after the last ragweed challenge, mice were aerosolized with adenosine, and groups of mice were examined at various time-points (1 to 72 h). BAL showed amplified recruitment of neutrophils into the airways after aerosol of adenosine (SEN+ADO group) at every time-point compared with ragweed sensitized mice receiving vehicle challenge (SEN group, $p < 0.05$). The recruitment of neutrophils into BAL reached a peak at the 6-h time-point after adenosine challenge (Fig. 1). Similarly, infiltration of eosinophils into BAL increased after adenosine challenge from 1 to 48 h compared with SEN group ($p < 0.05$). The number of eosinophils peaked at 24 h in SEN+ADO group, which was different from a peak at 72 h in SEN group (Fig. 2). Viewing the various time-points for neutrophils (peaked at 24 h) and eosinophils (peaked at 72 h) in SEN group, adenosine challenge not only increased the magnitude of neutrophil and eosinophil infiltrations into BAL, but it also shifted their

peak time-point (6 h for neutrophils and 24 h for eosinophils). Pretreatment with theophylline, a non-selective adenosine receptor antagonist, inhibited adenosine-enhanced recruitment of both neutrophils and eosinophils.

Lymphocytes showed greater influx into BAL after adenosine challenge at 1 and 72 h time-points compared to the SEN group ($p < 0.05$, Fig. 3). Aerosolization of adenosine (SEN+ADO) increased infiltration of macrophages into BAL from 6 to 72 h compared with SEN group ($p < 0.05$, Fig. 4). Theophylline reduced the adenosine-induced increase in the recruitment of lymphocytes into airways. Interestingly, theophylline increased the macrophages in BAL compared with SEN group.

3.2. Inflammatory cells in BAL at the 6-h time-point

As shown in Figs. 1 and 2, the number of neutrophils and eosinophils rose sharply at the 6 h after adenosine challenge, this time-point was chosen to further study inflammatory cell count and their markers in BAL.

The SEN group showed an increase in total and differential cell numbers in the BAL compared to the CON group of mice (Table 1). Total cell count in ragweed sensitized and challenged mice increased by approximately 3 folds compared to control mice. Differential cell numbers were also higher in allergen sensitized and challenged mice. In the CON group, greater than 96% of the total lavage cells were macrophages, whereas eosinophils were less than 1%. Ragweed immunization significantly increased lymphocytes (7.51% of total cells), neutrophils (3.78% of total cells) and eosinophils (51.63% of total cells) (Table 1). Eosinophils exhibited a robust increase in BAL after allergen challenge. Lymphocytes and neutrophils increased by 25 and 7 folds, respectively, compared to control mice. The morphological signs of activation of macrophages e.g. enlargement, cytoplasmic projections and multiple vesicles were observed in allergen sensitized and challenged mice.

Adenosine aerosolization to ragweed sensitized and challenged mice further increased the infiltrations of inflammatory cells into the airways. Total cell numbers increased by 3 folds in adenosine group (SEN+ADO group) compared to SEN group (Table 1). Infiltration of eosinophils into BAL was significantly increased by adenosine challenge, which was ~ 3 fold greater in SEN+ADO group compared with SEN group. Also, neutrophils significantly increased from $0.74 \pm 0.26 \times 10^4/\text{ml}$ in SEN group to $12.85 \pm 1.68 \times 10^4/\text{ml}$ (17 fold higher) after adenosine challenge ($p < 0.05$). Lymphocytes in SEN+ADO group showed a trend towards an increase after adenosine challenge although there was no statistical difference. Activated macrophages were still observed. There was no effect of

Table 1

Cell numbers ($\times 10^4/\text{ml}$) in BAL at the 6-h time-point

Total		Differential			
		Macrophages	Lymphocytes	Neutrophils	Eosinophils
CON	6.78 ± 1.55	6.56 ± 1.51	0.06 ± 0.03	0.11 ± 0.06	0.05 ± 0.02
SEN	$19.58 \pm 4.09^*$	7.26 ± 1.92	$1.47 \pm 0.30^*$	$0.74 \pm 0.26^*$	$10.11 \pm 2.29^*$
SENS+ADO	$58.78 \pm 3.32^{*,\#}$	$14.01 \pm 3.24^{*,\#}$	$1.75 \pm 0.33^*$	$12.85 \pm 1.68^{*,\#}$	$30.17 \pm 3.44^{*,\#}$
SEN+THY+ADO	$27.78 \pm 5.09^{*,\#,\&}$	$14.94 \pm 2.02^{*,\#}$	$0.74 \pm 0.39^{*,\&}$	$2.39 \pm 0.58^{*,\#,\&}$	$9.71 \pm 3.08^{*,\&}$

CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=8-10$ for each group. * $p<0.05$ compared with CON; # $p<0.05$ compared with SEN; & $p<0.05$ compared with SEN+ADO.

adenosine challenge on total and differential cell numbers observed in CON mice (data not shown).

Theophylline, a non-selective adenosine receptor antagonist, was given before adenosine challenge and it was found that the increase in the infiltration of inflammatory cells into BAL enhanced by adenosine was prevented by pretreatment with theophylline. Eosinophils decreased from $30.17 \pm 3.44 \times 10^4/\text{ml}$ in adenosine challenged mice to $9.70 \pm 3.08 \times 10^4/\text{ml}$ (decreased by 67.8%) in theophylline pretreated mice ($p<0.05$). Neutrophils and lymphocytes decreased by 81.4% and 57.7%, respectively, after theophylline treatment. There were no significant differences between pretreatment and post treatment with theophylline in the CON groups (data not shown).

3.3. Inflammatory cell markers at the 6-h time-point

As inflammatory cells migrated into the airways at the 6-h time-point, elevated inflammatory cell markers were detected in BAL after mice were sensitized and challenged with ragweed. Levels of the mast cell marker β -hexosaminidase increased 2.3 fold in allergen sensitized and chal-

lenged mice compared to control mice. The highest level of β -hexosaminidase activity in BAL was detected in adenosine challenged mice (SEN+ADO group) (Fig. 5A). β -hexosaminidase activity in BAL increased by about 1.59 fold in SEN+ADO group over SEN group of mice ($p<0.05$). Similar changes in β -hexosaminidase activity in plasma were also observed (Fig. 5B). Airway adenosine challenge had a more significant effect on β -hexosaminidase release from mast cells in BAL than that in blood. BAL had 2-fold greater β -hexosaminidase than plasma in the SEN+ADO group. Theophylline treatment attenuated β -hexosaminidase release both in BAL and plasma in SEN+ADO group. β -hexosaminidase activities decreased by 49.1% and 47% in BAL and plasma after theophylline treatment, respectively.

Neutrophil MPO activity as a marker for the activation of neutrophils showed increases both in BAL and plasma in allergen sensitized and challenged mice. Adenosine challenge further increased the levels of MPO activities both in BAL and plasma. Levels of MPO activity increased by 76% and 81% after adenosine challenge in BAL and plasma, respectively (Fig. 6). The increased MPO in SEN+ADO

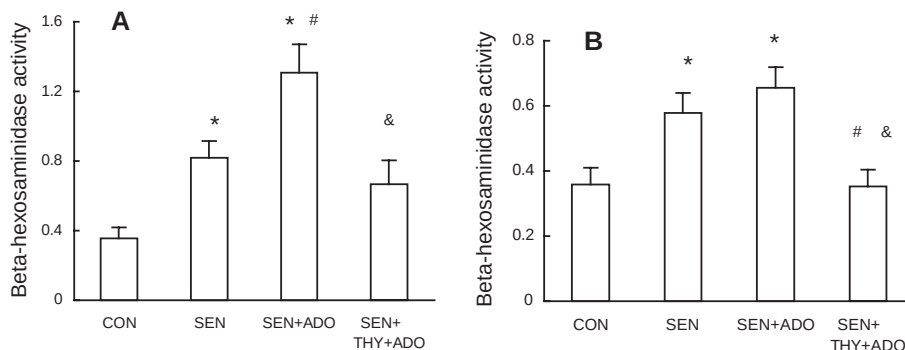


Fig. 5. Level of beta-hexosaminidase activities at the 6-h time-point in BAL (A) and in plasma (B). CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=8-10$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN; & $p<0.05$, compared with SEN+ADO.

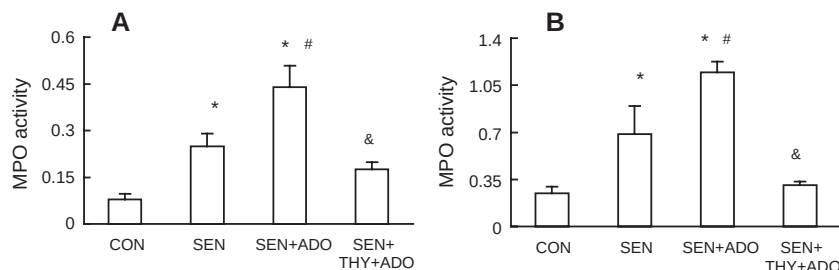


Fig. 6. Level of MPO activities at the 6-h time-point in BAL (A) and in plasma (B). CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=8-10$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN; & $p<0.05$, compared with SEN+ADO.

animals was inhibited by theophylline pretreatment. MPO activities decreased by 60.1% and 73.3% in BAL and plasma after theophylline treatment, respectively.

EPO activity in BAL increased from 0.13 ± 0.03 to 0.57 ± 0.06 after allergen sensitization and challenge ($p<0.05$). EPO activity in SEN animals was further elevated by adenosine challenge (Fig. 7). After adenosine challenge, levels of EPO activity increased by about 50% compared to SEN mice, which was attenuated by pretreatment with theophylline. EPO activity decreased by 38% in BAL after theophylline treatment.

3.4. Levels of eotaxin in BAL

Level of eotaxin in control mice was 34.38 ± 0.75 pg/ μ l. Mice sensitized and challenged with ragweed showed increased levels of eotaxin in BAL over CON group (Fig. 8, $p<0.05$). The highest level of eotaxin was observed in adenosine challenged mice (SEN+ADO group) (Fig. 8). Theophylline again blocked elevation of eotaxin in SEN+ADO

group. The eotaxin levels decreased by 26.9% in BAL after theophylline treatment.

4. Discussion

Asthma is a common inflammatory disease of the airways. Allergen-induced asthma is an important form of this disease. Upon exposure to an allergen, inflammatory cells including eosinophils, neutrophils, lymphocytes, macrophages and mast cells infiltrate the airways. In this study, the eosinophil counts progressively increased in the lung lavage and reached a maximum at 72 h in ragweed sensitized and challenged mice (SEN group). The allergen sensitization and challenge also increased neutrophils in BAL. The maximum level of neutrophils in BAL occurred at 24

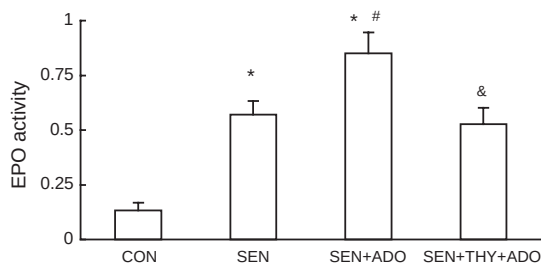


Fig. 7. Level of EPO activity at the 6-h time-point in BAL. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=8-10$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN; & $p<0.05$, compared with SEN+ADO.

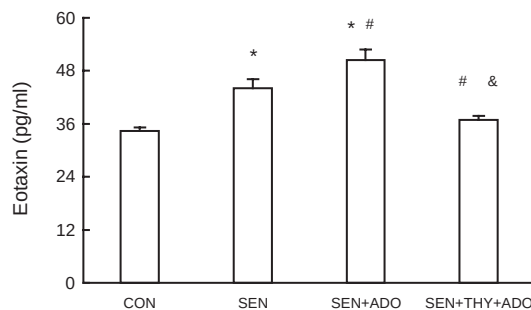


Fig. 8. Eotaxin levels at the 6-h time-point in BAL. CON: control group; SEN: sensitized group; SEN+ADO: sensitization+adenosine (6 mg/ml) group; SEN+THY+ADO: sensitization+theophylline (12 mg/ml)+adenosine (6 mg/ml) group, see Materials and methods for details. $n=8-10$ for each group. * $p<0.05$, compared with CON; # $p<0.05$, compared with SEN; & $p<0.05$, compared with SEN+ADO.

h, which was earlier than eosinophils. Previous studies have shown similar patterns of inflammatory reactions after allergen exposure within the lungs [34] and airway lumen [35]. Interestingly, adenosine challenge further enhanced the recruitment of eosinophils and neutrophils (Figs. 1 and 2). Also, adenosine challenge shifted the maximum level of neutrophils leftward to 6 from 24 h and eosinophils from 72 to 24 h time-point in SEN mice, respectively.

With regard to the effect of adenosine on allergic airway inflammation, Spruntulis and Broadley [36] demonstrated that inhalation of 5'-AMP by sensitized guinea-pigs caused a rapid influx of inflammatory cells (within 1 h) into the airways but not in non-sensitized animals. Further supportive data from very recent clinical investigations have shown that AMP challenge had marked effects on airway migration of eosinophils [12], and increased sputum neutrophils in asthmatic patients [13]. Our study here demonstrated for the first time that adenosine challenge not only amplified influx of inflammatory cells into airways but also shifted leftward the peak time-point of recruiting inflammatory cells to the airways of allergic mice.

Eosinophils have emerged as a major inflammatory cell type in asthma, and an increase in eosinophils is often observed in the lungs of asthmatics [35]. In our study, the number of eosinophils was significantly increased in ragweed sensitized and challenged mice. These numbers were further potentiated by aerosolized adenosine rising sharply at 6 h with a peak at 24 h (Fig. 2). Adenosine challenge resulted in elevation of EPO activity. This may be due to the larger number of eosinophils present in the SEN+ADO group. Theophylline, a non-specific adenosine receptor antagonist, blocked adenosine-enhanced infiltration of eosinophils and elevation of EPO activity in BAL. It has been reported that adenosine A₃ receptors are coupled to G α i, and their activation on eosinophils can elevate intracellular Ca²⁺ levels [37] that regulate eosinophil degranulation [38]. Consistent with our finding, recent studies in adenosine deaminase deficient mice have shown a large increase in eosinophils in the interstitium and BAL throughout the lung, which were associated with elevated adenosine levels [11]. Treatment with the A₃ adenosine receptor antagonist MRS1523, almost completely abolished eosinophil infiltration into the airways of ADA-deficient mice [17]. However, our data contrast

with some observations that adenosine challenge had no effect on eosinophil activation as measured by H₂O₂ generation in vitro [39] or eosinophilic cationic protein in serum and sputum [12] from asthmatic patients. This discrepancy could be due to differences attributed to the in vitro nature of cell activation experiments performed or to differences in mouse and human eosinophils, or the time-points studied.

The recruitment of eosinophils into the airways of sensitized mice suggests the presence of eosinophil chemoattractants in the lung during allergic airway inflammation. Eotaxin, a CC chemokine, is the hallmark eosinophil chemoattractant released in the lung in many animal models of eosinophilic airway inflammation [27,40]. In the present study, eotaxin was detected in the lung lavage and was higher in the SEN group when compared to controls. The highest level of eotaxin was measured in adenosine challenged mice (SEN+ADO group), which is likely due to a greater number of eotaxin-producing cells in BAL. The possibility of an elevated eotaxin expression in SEN+ADO mice could not be excluded since a higher level of eotaxin mRNA expression has been demonstrated in ADA-deficient mice due to elevated adenosine level in the lung over ADA control mice [17].

Eotaxin was initially found to be selective for eosinophils [27], but has now been shown to be chemotactic for basophils [41], lymphocytes [42], and mast cells [43]. Indeed, in parallel with the levels of eotaxin, higher concentrations of β -hexosaminidase were also found in BAL and blood samples from allergen sensitized mice (SEN group). Adenosine challenge significantly increased β -hexosaminidase in BAL. It is important to note that there was a 2 fold greater level of β -hexosaminidase in BAL than in plasma from SEN+ADO group, and there was no significant increase in β -hexosaminidase in plasma following adenosine challenge, indicating that adenosine challenge had more significant effect on mast cells in lungs than in blood. The possible explanation for this finding is that delivery of adenosine to the lung would be expected to affect lung mast cell the most. Increased β -hexosaminidase release was prevented by theophylline pretreatment. In support of these findings, it has been reported that in the ADA-deficient mice, there is extensive lung mast cell degranulation, and treatment of ADA-deficient mice with theophylline prevented 30–40% of the mast cell degranulation [15].

The degranulation of mast cells and release of pro-inflammatory mediators, cytokines and chemokines [16,23,24] are also attributed to the influx of leukocytes in BAL since mast cell tryptase could stimulate IL-8 production and intercellular adhesion molecule-1 expression. Both of these factors are known to play a key role in the recruitment of neutrophils and eosinophils to the lung [44]. This was supported by our finding that adenosine challenge sharply increased influx of neutrophils at 6 h, and also supported by other studies that revealed extensive mast cells degranulation and neutrophils accumulation in a mast cell-dependent manner 4 h after adenosine challenge [45]. Meanwhile, adenosine-induced release of serum neutrophil chemotactic factor [9] may be another chemotactic factor for neutrophils. In addition, the present study showed that adenosine challenge also increased neutrophil degranulation, since higher MPO levels were measured in BAL and plasma. In contrast to the observation with β -hexosaminidase, the level of MPO was 2.6-times higher in plasma than in BAL from the SEN+ADO group. The increased level of MPO activity was inhibited by theophylline. Activation of adenosine receptors on neutrophils elicits both pro- and anti-inflammatory events. Binding of adenosine at A_1 receptors increases neutrophil chemotaxis and phagocytosis [46]. Activation of the A_{2A} receptors by adenosine exerts anti-inflammatory effects [46]. In the present study, pro-inflammatory effects of adenosine on neutrophils seemed to overshadow its potential anti-inflammatory effect since adenosine challenge sharply increases the infiltration of neutrophils and the increased level of MPO at the 6-h time-point in ragweed sensitized animals.

In summary, our studies showed that adenosine challenge enhanced the influx of inflammatory cells, notably eosinophils and neutrophils, and also shifted leftward the peak time-point of recruiting inflammatory cells into the airways in ragweed sensitized and challenged mice. Meanwhile, the markers of mast cells, eosinophils and neutrophils were significantly increased by adenosine challenge. All of these adenosine effects were prevented by theophylline. These data, therefore, indicate an active role for adenosine in the exacerbation of airway inflammation in this allergic mouse model.

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